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Kursad Turksen *Editor*

Cell Biology and Translational Medicine, Volume 20

Organ Function, Maintenance, Repair in Health and Disease

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Editor

Cell Biology and Translational Medicine, Volume 20

Organ Function, Maintenance, Repair in Health and Disease

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Preface

In this next volume in the Cell Biology and Translational Medicine series, we continue to explore how insights from state-of-the-art cell biological studies can impact translational medicine. Amongst topics explored here are recent developments that provide important new understanding of stem cell-based reprograming, and therapeutics that underscore where we stand with respect to translational medicine and novel cell- and/or drug-based therapeutic options for a variety of diseases and conditions . As with each of the volumes in this series, we continue to highlight timely, often emerging, topics and novel approaches with potential to accelerate our understanding of various diseases with the ultimate goal of improving therapeutic options.

I remain very grateful to Gonzalo Cordova, the Publishing Editor of the series, and wish to acknowledge his continued support.

Finally, sincere thanks to the contributors not only for their support of the series, but also for their willingness to share their insights and all their efforts to capture both the advances and the remaining obstacles in their areas of research. I trust readers will find their contributions as interesting and helpful as I have.

Ottawa, ON, Canada Kursad Turksen

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Direct Cardiac Reprogramming: Current Status and Future Prospects

Krishna Kumar Haridhasapavalan, Atreyee Borthakur, and Rajkumar P. Thummer

Abstract

Advances in cellular reprogramming articulated the path for direct cardiac lineage conversion, bypassing the pluripotent state. Direct cardiac reprogramming attracts major attention because of the low or nil regenerative ability of cardiomyocytes, resulting in permanent cell loss in various heart diseases. In the field of cardiology, balancing this loss of cardiomyocytes was highly challenging, even in the modern medical world. Soon after the discovery of cell reprogramming, direct cardiac reprogramming also became a promising alternative for heart regeneration. This review mainly focused on the various direct cardiac reprogramming approaches (integrative and non-integrative) for the derivation of induced autologous cardiomyocytes. It also explains the advancements in cardiac reprogramming over the decade with the pros and cons of each approach. Further, the review highlights the importance of clinically relevant (non-integrative) approaches and their challenges for the prospective applications for personalized medicine. Apart from direct cardiac reprogramming, it also discusses the other strategies for generating cardiomyocytes from different sources. The understanding of these strategies could pave the way for the efficient generation of integration-free functional autologous cardiomyocytes through direct cardiac reprogramming for various biomedical applications.

Keywords

Cardiomyocytes · Cardiovascular diseases · Cell therapy · Direct cardiac reprogramming · Integrative and non-integrative approaches

Abbreviations

9C	9 small molecules
BMP	Bone morphogenetic protein
c-Kit	Tyrosine kinase receptors
CVDs	Cardiovascular diseases
Dmap1	DNA methyltransferase 1-associated protein 1
ESCs	Embryonic stem cells
G	GATA4
iPSCs	Induced pluripotent stem cells
islet-1	Homeodomain transcription factor
Lin^-	Lineage-negative
M	MEF2C

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PDGFRα	Platelet-derived growth factor receptor-alpha
Sca-1	Stem cell antigen-1
T	TBX5
WHO	World Health Organization

1 Introduction

In today's world of advancing health research, cardiovascular diseases (CVDs) still remain one of the leading causes of mortality and morbidity. The World Health Organization (WHO) in 2019 reported nearly 32% of all deceased (i.e., 17.7 million) to have succumbed to CVDs annually, which is expected to surpass 23.6 million by the next decade (Kaptoge et al. 2019). The primary CVD of concern is coronary heart disease (also known as ischemic heart disease), which accounts for 14.4% of these cases, closely followed by cerebrovascular disease, accounting for 11.2%. The major causes of CVDs are the use of tobacco, alcohol consumption, stress, poor/unhealthy diet, sedentary lifestyle, etc., in our daily life. Although ~90% of CVDs are preventable with medications, exercise, healthy diet, avoidance of tobacco, and alcohol (McGill Jr et al. 2008), an increase in mortality is observed in the recent times, which might be due to inadequate preventive measures taken against the same (Mendis et al. 2011).

Out of the variety of CVDs, the ones like ischemic heart disease, cardiomyopathies and arrhythmias mostly affect the functionality of cardiomyocytes (Mendis et al. 2011), mainly due to apoptosis and necrosis in the cardiac tissue. Cardiomyocytes are the functional unit of the heart that is majorly responsible for the conduction system. In a healthy human heart, the average left ventricle has roughly 4 billion cardiomyocytes, while a post-infarct heart has a myocyte shortage of about 1 billion (Murry et al. 2006). Most of the diseases, namely, ischemic heart disease and myocardial infarction, are due to loss of regenerative capacity of the host tissue by the remaining myocytes and the consequent weakening of the diseased heart over time. The loss of cardiomyocytes leads to the formation of scar tissue by the spontaneous division and migration of fibroblasts over the damaged area, which, in turn, results in improper contraction. This myocardial growth transition gives rise to terminally differentiated cardiomyocytes (adult) that are characterized by binucleated cells with arrested cell cycle. Naturally, human heart has a limited capacity to regenerate cardiomyocytes as indicated by lasting scar tissue following myocardial infarction and ultimately culminates in chronic heart failure in the long run.

A common therapeutic approach includes pharmacotherapy, which is mainly focused on limiting disease progression instead of repairing and restoring healthy tissue and function. Therefore, the limited efficacy of this current treatment has generated interest in considering other viable and long-lasting therapeutic strategies. The search for effective ways to treat infarcted hearts has increased remarkably. In this regard, cell-based therapy for cardiac regeneration appears to be a promising alternative to achieve cardiac repair. In the near future, cell therapy could be a possible solution to control current epidemic rates of heart failure by transplanting autologous functional cardiomyocytes to support regeneration of the heart in diseased patients. Various strategies have been developed to date to make an attempt to generate cardiomyocytes from different cell types (Fig. 1), which are explained below.

2 Strategies to Generate Cardiomyocytes from Different Cells

2.1 Cardiomyocytes Derived from Cardiac Progenitor Cells

Cardiac progenitor cells (also sometimes called cardiac stem cells) are a heterogeneous group of endogenous multipotent progenitor cells in the heart and are identified by the expression of various markers such as tyrosine kinase receptors (c-Kit) and/or stem cell antigen-1 (Sca-1), the homeodomain transcription factor (islet-1), platelet-derived growth factor receptor-alpha (PDGFRα), or the ability to grow into cardiospheres (Beltrami et al. 2003; Oh et al.

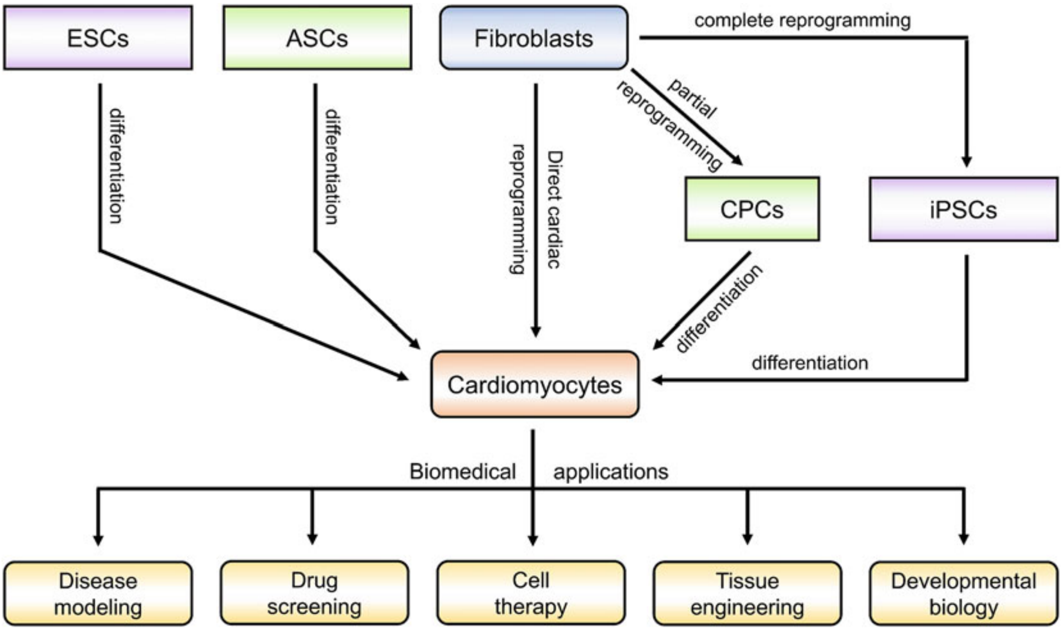

Fig. 1 Schematic of different strategies to generate cardiomyocytes from different sources and the biomedical application of the generated cells

2003; Messina et al. 2004; Laugwitz et al. 2005; Chong et al. 2011, 2014; Amini et al. 2017). In the year 2003, Beltrami et al. first demonstrated the differentiation ability of lineage-negative (Lin$^-$) c-kit$^+$ cells, isolated from the adult rat heart, toward cardiomyocytes, smooth muscle, and endothelial cells (Beltrami et al. 2003). Soon after, Schneider and colleagues established the isolation of the Sca-1$^+$ subpopulation from adult mouse hearts and showed in vitro differentiation of these cells into cardiomyocytes in the presence of 5-azacytidine, a DNA demethylating agent (Oh et al. 2003). Notably, the authors also demonstrated the in vivo differentiation of Sca-1$^+$ cells to cardiomyocytes after intravenous injection in mice (Oh et al. 2003). Further investigation of these cells led to the isolation of Sca-1$^+$ and c-kit$^+$ subpopulation of cardiosphere-forming cells from both mouse and human hearts (Messina et al. 2004). Interestingly, after cardiosphere formation, spontaneously beating mouse cells were reported without co-culturing with neonatal rat cardiomyocytes, unlike the human cardiospheres, which require co-culturing of rat neonatal cells (Messina et al. 2004). Another subpopulation expressing Isl1 was also discovered in rat, mouse, and human hearts as clusters in atria and single cells in ventricles (Laugwitz et al. 2005). These cells lack Sca-1 and c-kit expression and, when co-cultured with rat neonatal cardiomyocytes, differentiated into cardiomyocytes. Furthermore, studies have also demonstrated the derivation of cardiomyocytes from the PDGFRα$^+$ subpopulation of cells from mouse and human hearts (Chong et al. 2011, 2014; Le and Chong 2016). Interestingly, Raghunathan et al. demonstrated the conversion of human adipogenic mesenchymal stem cells-derived cardiac progenitor cells into pacemaker-like cells, a specialized cardiomyocyte, through the ectopic expression of SHOX2, HCN2, and TBX5 transcription factors (Raghunathan et al. 2020). In general, these cardiac progenitor cells are in an inactivated or quiescent state under normal physiological conditions. In this state, cardiac progenitor cells do not contribute to the regeneration of cardiomyocytes; however, upon cardiac injury, these progenitor cells can get activated and subsequently differentiate into cardiomyocytes (Le and Chong 2016).

Despite the identification of these different cardiac progenitor cell populations, their physiological and pathophysiological functions are not entirely understood (Amini et al. 2017). Notably, these cells induce unfavorable or regenerative effects upon exogenously delivered within the injured heart (Le and Chong 2016). Moreover, the molecular mechanisms behind these effects still remains unclear (Le and Chong 2016). Thus, the applications of these progenitor cells are limited to the regeneration of cardiac tissue after injury.

2.2 Cardiomyocytes Derived from Adult Stem Cells

Adult stem cells such as mesenchymal stem cells are another cell source that can be used to differentiate them into functional cardiomyocytes. Several studies on hematopoietic stem cells were inconclusive with respect to cardiac fate determination (Orlic et al. 2001; Balsam et al. 2004; Kawada et al. 2004; Murry et al. 2004). Therefore, researchers focused on non-hematopoietic stem cells, such as mesenchymal stem cells, as a primary source to obtain cardiomyocytes. In this regard, adult mouse bone marrow-derived non-hematopoietic stem cells developed features of cardiomyocytes when being treated with 5-azacytidine (Makino et al. 1999). Similarly, studies reported the formation of cardiomyocytes from mouse mesenchymal stem cells in the presence of 5-azacytidine (Hattan et al. 2005; Antonitsis et al. 2007) and also by injecting into the mouse embryos (Jiang et al. 2002). However, 5-azacytidine has been reported to induce carcinogenicity by introducing mutations in the somatic cell genome (Alagesan and Griffin 2014), thus serving as a roadblock to the therapeutic applications of differentiated cells. Therefore, Shen et al. focused on the downstream targets of 5-azacytidine and found the significant upregulation of miR-1-2 during differentiation (Shen et al. 2017). The authors demonstrated that mimics of miR-1-2 promoted the differentiation of bone marrow-derived mesenchymal stem cells into functional cardiomyocytes by activating the Wnt/β-catenin signaling pathway (Shen et al. 2017). Similarly, miR-1 has been reported to induce the differentiation of mesenchymal stem cells into myocardial cells only in a specific medium, i.e., serum-free cardiomyogenic medium containing 10 nM 5-azacytidine (Zhao et al. 2016).

Alternatively, Shim et al. obtained human cardiomyocyte-like cells from adult bone marrow stem cells by treating them with a low concentration (10^{-9} M) of dexamethasone (corticosteroid) (Shim et al. 2004). On the other hand, differentiation of bone marrow-derived clonal subpopulation by co-culturing method showed phenotypes of a heterogeneous populations of cells comprising cardiomyocytes, endothelial cells, and smooth muscle cells (Yoon et al. 2005). Likewise, Cai et al. employed the same co-culturing method with minor modifications (1:10 instead of 1:4 ratio) to differentiate bone marrow-derived mesenchymal stem cells into cardiomyocytes (Cai et al. 2012). Apart from 5-azacytidine and miRNAs, several growth factors/cytokines, microenvironment, caveolin-1, vanilloid receptor 1, and histone deacetylase 1 were reported to induce cardiac differentiation of mesenchymal stem cells (Guo et al. 2018). Moreover, these mesenchymal stem cells (along with fresh bone marrow) promoted the activation of angiogenesis, inhibition of fibrosis, and decrease in apoptosis to restore heart function in the infarcted swine model (Pak et al. 2003). Among the various sources of mesenchymal stem cells like the umbilical cord, adipose tissue, placenta, hair follicle, skeletal muscle, etc., adipose tissue serves as an easily obtainable source compared to the invasive process of bone marrow aspiration. Kakkar et al. underscore the merits associated with the induction of adipose tissue-derived stem cells with TGF-β1, which is nontoxic and a more efficient cardiac inducer compared to 5-azacytidine (Kakkar et al. 2019). Another study focused on human amniotic fluid-derived mesenchymal stem cells, which were effectively differentiated into the cardiomyogenic lineage upon treatment with 10 μM 5-azacytidine and 20% human platelet lysate (Markmee et al. 2020).

Ramesh et al. summarized the use of various biological and chemical inducers that enable the cardiac differentiation of adult stem cells into cardiomyocytes (Ramesh et al. 2021). However, direct transplantation of these cells is limited due to low differentiation or success rate in vivo, and these do not entirely reciprocate the functional and morphological characteristics of cardiomyocytes (Guo et al. 2018).

2.3 Cardiomyocytes Derived from Pluripotent Stem Cells

In 1999, Guan et al. reported differentiation of undifferentiated embryonic stem cells (ESCs) into cardiomyocytes, neuronal, skeletal muscle, and epithelial and vascular smooth muscle cells. In this study, Guan et al. concluded that the differentiation of ESCs toward cardiomyocytes was influenced by cell density, medium, and its supplements, type of cells, and time of seeding cells (Guan et al. 1999). Contrastingly, Kehat et al. demonstrated cell density independent differentiation of human ESCs toward cardiomyocytes with similar structural and functional properties of early stage cardiomyocytes (Kehat et al. 2001). In this study, the authors performed differentiation by forming embryoid bodies and then seeded these embryoid bodies in 0.1% gelatin-coated petri dishes. They observed the first embryonic bodies with rhythmically contracting areas on day 4 (Kehat et al. 2001), which is 2 days earlier than the previous study (Guan et al. 1999). However, most of these studies used two different culture conditions for ESCs maintenance and differentiation. Interestingly, Denning and colleagues developed a common culture condition for the maintenance of ESCs and subsequently demonstrated efficient differentiation of these cells toward cardiomyocytes (Denning et al. 2003).

Soon after the astounding discovery of induced pluripotent stem cells (iPSCs) from mouse fibroblasts (Takahashi and Yamanaka 2006), the same group generated human iPSCs from adult human fibroblasts and differentiated into cardiac and other cells as an evidence of pluripotency (Takahashi et al. 2007). For the directed differentiation, Yamanaka et al. followed the previously reported protocol (Laflamme et al. 2007) in which human ESCs were treated with Activin A and bone morphogenetic protein (BMP) 4 to form beating cardiomyocytes. Further, induction with Activin A and BMP4 enhanced the generation of cardiomyocytes; however, there was a lot of variability between cell lines and experiments (Paige et al. 2010). Using small molecules, Lian et al. fine-tuned the Wnt/β-catenin signaling pathway to generate cardiomyocytes robustly from multiple pluripotent cells. In this study, the authors showed that Wnt signaling activation is crucial for mesoderm formation from pluripotent cells, whereas its inhibition was crucial for the sequential differentiation of these cells into cardiomyocytes (Lian et al. 2012). This was further fine-tuned by Kadari et al. which reported the formation of three different phases, namely, cardiovascular induction, cardiac specification, and cardiomyocyte enrichment (Kadari et al. 2015). In the first phase, authors used CHIR99021 and BMP4 to stimulate cardiac induction and then used Wnt inhibitor (XAV939) to induce cardiac specification (Kadari et al. 2015). In order to reduce line-to-line variability, they performed lactate enrichment to obtain pure populations of cardiomyocytes with a very high efficiency (Kadari et al. 2015).

Ou et al. described a protocol wherein co-culturing of iPSCs with neonatal cardiomyocytes resulted in cardiomyocytes expressing cardiac-specific genes like Mef2c, cTnT, and MLC-2 V (Ou et al. 2016), indicating efficient differentiation and enhanced proliferation ability upon co-culture. Studies also suggest the administration of electrical stimulations in human iPSC or cardiosphere-derived cells to achieve functionally mature cardiomyocytes (Ma et al. 2018; Nazari et al. 2020). These stimulations could mimic the native property of synchronous contractions of the heart and aid in attaining a functionally mature state in shorter time duration (Ma et al. 2018; Nazari et al. 2020). Funakoshi et al. provided insights into the advantage of generating human PSC-derived mature cardiomyocytes that have enhanced contraction force, mitochondrial oxidative property, and improved sarcomere structure (Funakoshi et al.

2021). Transplantation of these mature cells compared to the functionally immature ones generated better grafts, suggesting the prior manipulation of the cells before the transplant could help mitigate arrhythmias and serve as a safer therapy (Funakoshi et al. 2021). Exciting opportunities exist in the field of PSC-derived cardiomyocytes, but not without a series of hurdles preventing its use in the clinical setting. Some of them include the fetal-like immature phenotype of generated cardiomyocytes, variability in the cardiac subtype, the risk of arrhythmogenesis upon transplant, teratoma formation, and immune rejection, to name a few.

2.4 Direct Cardiac Reprogramming Strategies to Derive Cardiomyocytes

2.4.1 Direct Cardiac Reprogramming of Mouse Fibroblasts

Several studies have generated induced cardiomyocytes by directly reprogramming somatic cells using different integrative and non-integrative (Fig. 2; Table 1) approaches (Ieda et al. 2010; Fu et al. 2013; Nam et al. 2013; Wada et al. 2013; Muraoka et al. 2014; Wang et al. 2014, 2015 Lee et al. 2015; Cao et al. 2016; Miyamoto et al. 2018; Paoletti et al. 2020; Yamakawa and Ieda 2021). The very first study to generate fibroblast-derived induced cardiomyocytes was reported in 2010 (Ieda et al. 2010). The authors selected 13 potential cardiogenic factors critical for survival and cardiogenesis in the embryo from the previously reported microarray analysis data of variable expression patterns observed between myocytes and non-myocytes cells (Ieda et al. 2009). Additionally, mesoderm-specific transcription factor-1 (Mesp1) was also included due to its cardiac reprogramming ability in *Xenopus laevis* (David et al. 2008). Of these 14 factors, this study identified a combination of three transcription factors, namely, GATA4, MEF2C, and TBX5 (referred to as GMT), sufficient to reprogram mouse cardiac/dermal fibroblasts to induced cardiomyocytes (Ieda et al. 2010), bypassing the pluripotent stem cell state. These induced cells exhibited almost similar gene expression pattern and electrophysiology and contracted spontaneously as native cardiomyocytes. However, the

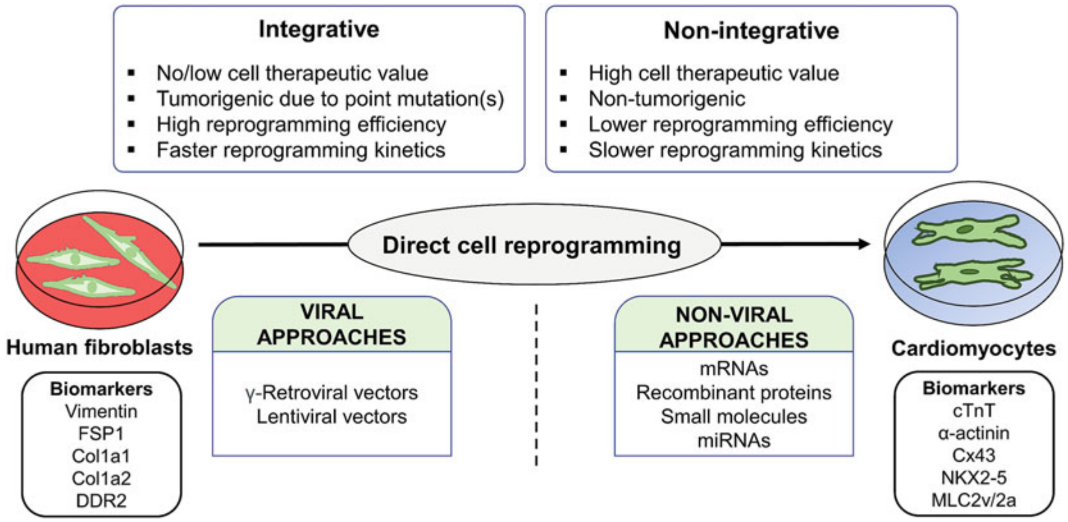

Fig. 2 Schematic illustration of different direct cardiac reprogramming approaches. FSP1, fibroblast specific protein 1; Col1a1, collagen alpha-1(I) chain; Col1a2, collagen alpha-2(I) chain; DDR2, discoidin domain receptor 2; cTnT, cardiac Troponin T; Cx43, connexin 43; NKX2.5, NK2 Homeobox 5; MLC2v/2a, myosin light chain-2-cardiac ventricular/atrial isoforms

Table 1 Overview of reprogramming approaches (mouse)

Reprogramming approaches	Reprogramming factors	Source cell	Cardiac gene expression	In vivo	References
Retroviral vectors	GMT	Postnatal cardiac/ dermal fibroblasts	30% GFP$^+$ cells, beating CMs	Yes	Ieda et al. (2010)
Retroviral vectors	OSKM	MEF	40% cTnT$^+$ cells, spontaneously contracting, beating CMs	No	Efe et al. (2011)
Lentiviral vectors	TM + MyoCD	MEF	2% MYH6: tdTomato$^+$ cells, 12% cTnT$^+$ cells	No	Protze et al. (2012)
Retroviral vectors	GMTH	TTF, CF	9.5% more CMs than GMT	Yes	Song et al. (2012)
Lentiviral vectors	miR-1, 133, 208, 499 + JAK inhibitor I	Noncardiac myocytes	Tenfold increased efficiency of reprogramming	Yes	Jayawardena et al. (2012)
Lentiviral vectors	HNGMT	MEF	>50-fold efficient than GMT alone	Yes	Addis et al. (2013)
Retroviral vectors	GHMT	TTF	~2% cTnT$^+$ cells, increased to ~17% with MyoCD	No	Nam et al. (2013)
Retroviral vectors	GMT + miR-133a	MEF	Sevenfold increased efficiency than GMT	No	Muraoka et al. (2014)
Lentiviral vectors	HNGMT + SB431542	MEF, ACF	Fivefold increased efficiency of iCM generation compared to GMT (2% vs. 0.25%)	Yes	Ifkovits et al. (2014)
Lentiviral vectors	OCT4 + Parnate, Forskolin, CHIR99021, SB431542	MEF, TTF	Spontaneously contracting iCMs	No	Wang et al. (2014)
Retroviral vectors	OSKM + vitamin C	MEF	Enhanced expression and beating cardiomyocytes (day 11)	No	Talkhabi et al. (2015)
Retroviral vectors	GHMT + miR-1, miR-133 + Y-27632, A83–01	MEF, AF	60% enhanced efficiency, contracting iCMs in <2 weeks	No	Zhao et al. (2015)
mRNA	GMT	NCF	iCM like cells, cardiomyocyte marker genes expressed at 2 weeks	No	Lee et al. (2015)
Retroviral and lentiviral vectors	GMT + BMI1 deletion	TTF, CF, MEF	cTnT tenfold increase, MT + BMI deletion resulted in beating iCMs	No	Zhou et al. (2016)
Lentiviral vectors	GMT	RCF	GATA4 decreases post-infarct cardiac fibrosis	Yes	Mathison et al. (2017)
Adenoviral vectors	GMT	RCF	6% cTnT $^+$ cells, MYH7$^+$ iCMs (50%)	Yes	Mathison et al. (2017)
Retroviral vectors	GMT + SB431542, XAV939	MCF	Eightfold increased efficiency, beating iCMs at 1 week	Yes	Mohamed et al. (2017)
Retroviral vectors	GHMT + AKT, ZNF281	TTF	~45% cTnT$^+$ after day 7; twofold increase in beating cells >4 weeks	No	Zhou et al. (2017)
Lentiviral vectors	GMTH	TTF	72.4% α-MHC$^+$ iCMs, cardiac markers (α-MHC, β-MHC, ANF, N, and cTnT)	No	Tian et al. (2018)
Adeno-associated virus (AAV)	AAV-GMT and AAV-Tβ4 (chimeric)	MEF, MCF	cTnT expression highest in the GMTTβ4 group compared to GMT (32% vs. 22%)	Yes	Yoo et al. (2018)
Lentiviral vectors	GMT/GMTHMyoCD	p63 knockout MEF	20-fold cTnT expression, three–fivefold GMT expression, spontaneous contractions in co-culture	No	Patel et al. (2018)

(continued)

Table 1 (continued)

Reprogramming approaches	Reprogramming factors	Source cell	Cardiac gene expression	In vivo	References
Sendai viral vectors	GMT	MEF, TTF	Sendai viral-GMT vectors more efficient than retroviral-GMT vectors, ∼20% cTnT^{+} cells were matured during in vivo cardiac reprogramming	Yes	Miyamoto et al. (2018)
Retroviral vectors	GMTH	MEF	iCMs with enhanced contractile cardiac structures	No	Zhang et al. (2019)
Retroviral vectors (quad-cistronic)	GMTH	MEF	Splicing order of M-G-T-H-enhanced reprogramming	No	Zhang et al. (2019)
Retroviral vectors	GMT + IGF-1, MM589, A83-01, PTC-209	MEF, NCF	Three–fourfold increase in cTnT and α-actinin (MEF); eight–ninefold increase in spontaneous beating of iCMs (NCF)	No	Guo et al. (2019)
Retroviral vectors	GT + M isoform (Mi2/Mi4)	MEF	Efficiency of Mi2 > Mi4 isoform	No	Wang et al. (2020)
Lentiviral vectors	GMT + sodium butyrate, ICG-001, retinoic acid	RCF	4-fold cTnT expression, spontaneous contractions >4 week	No	Singh et al. (2020)
Small molecules	CHIR99021, RepSox, Forskolin, valproic acid, Parnate, TTNPB + PTC-209	MEF, CF	α-MHC^{+} cTnT^{+}, spontaneously beating iCMs	No	Testa et al. (2020)
Sendai viral vectors	GMT	MCF	iCMs (tdTomato and cTnT^{+}) > > 4 weeks	Yes	Isomi et al. (2021)
Adenoviral vectors	OSKM	Myocardial infarction model	Partial in vivo reprogramming, CDH1^{+}, FUT4^{+} cells	Yes	Kisby et al. (2021)
Retroviral and lentiviral vectors	GMTMyoCD, Sall4	MICF	Beating iCMs >28 day, 22.5% cTnT^{+} cells	No	Zhao et al. (2021)
Retroviral vectors	GMT + SB431542	TTF, MEF	~twofold more iCMs compared to control	No	Bektik et al. (2021)
Retroviral vectors	GHMT + DMSO	MEF	~threefold cTnT^{+} cells, ~sixfold increase in MYH6-mCherry^{+} cells	No	Lim et al. (2021)

G-GATA4; *M*, *MEF2C*; *T*, *TBX5*; *H*, *HAND2*; *N*, NKX 2.5; *HNGMT*, Hand2; Nkx 2.5 + GMT; *OSKM*, O*ct3/4,Sox2,Klf4, Myc*; *MyoCD*, myocardin; *miRNA*, microRNA; *JAK*, *janus kinase*; *CM*, cardiomyocyte; *iCM*, induced CM; *GFP*, *green fluorescent protein; MEF*, mouse embryonic *fibroblasts*; CF, cardiac fibroblasts; *TTF*, tail tip fibroblasts; *ACF*, adult cardiac fibroblasts; *AF*, adult fibroblasts; *NCF*, neonatal cardiac fibroblasts; *RCF*, rat cardiac fibroblasts; *MICF*, cultured cardiac fibroblasts isolated from adult mice with myocardial infarction; *cTnT*, cardiac troponin T; *MYH*/MHC, myosin heavy chain; *ANF*, natriuretic peptide A; *CDH1*, cadherin-1; *FUT4*, fucosyltransferase 4; *AAV-Tβ4*, adeno-associated virus carrying thymosin B$_4$; *BMI1*, polycomb complex protein; *AKT*, protein kinase B; *ZNF281*, zinc finger protein 281; *TGFβ*, transforming growth factor beta; *DMSO*, dimethyl sulfoxide

efficiency of the beating cells was very low, and the majority of the cell population was only partially reprogrammed. The reason behind this inefficient reprogramming is that the likelihood of transducing a single cell with all three factors is low and the imbalanced stoichiometric expression of these factors (Lee et al. 2015; Wang et al. 2015). Relatively high levels of MEF2C protein expression compared to GATA4 and TBX5 improved reprogramming efficiency and the quality of induced cardiomyocytes (Wang et al. 2015). Of the two isomeric forms of MEF2C, viz., Mi2 and Mi4, the former, in combination with GATA4 and TBX5, reprogrammed mouse embryonic fibroblasts more efficiently than the latter (Wang et al. 2015). This might be the possible reason for the discrepancy in reprogramming efficiency reported by different groups (Yamakawa and Ieda 2021). Further, the addition of HAND2 to this reprogramming cocktail enhanced the efficiency, irrespective of the stoichiometry using either cocktail of retroviral-GMT vectors or retroviral single polycistronic-MGT vector (Song et al. 2012; Nam et al. 2013; Zhang et al. 2019; Wang et al. 2020b). These studies thus demonstrated the requirement of HAND2 in the GMT cocktail (GMT + HAND2) in the direct cardiac reprogramming of mouse fibroblasts into functional cardiomyocytes.

Using the Yamanaka factors (OCT4, SOX2, KLF4, and c-MYC), Efe et al. partially reprogrammed mouse fibroblasts, instead of inducing full pluripotency, and then derived cardiomyocytes by diverting them toward cardiac lineage with specific media conditions (Efe et al. 2011). The first spontaneous beating was observed after 11 days in this approach (Efe et al. 2011), compared to 4–5 weeks in the first study (Ieda et al. 2010). The addition of ascorbic acid (Vitamin C) to the Yamanaka factors enhances the derivation of cardiomyocytes from mouse fibroblasts through a partial pluripotent reprogramming strategy (Talkhabi et al. 2015). With a different set of transcription factors (TBX5, MEF2C, and MYOCD), cardiomyocyte-like cells were obtained by time-dependent conversion of mouse embryonic fibroblasts through the lentiviral expression of these three factors, upregulating a broader spectrum of cardiac genes (Protze et al. 2012), compared to the combination used by Ieda et al. (2010). Similarly, screening a combination of transcription factors to establish a minimally efficient reprogramming cocktail, Hirai et al. reported the use of MYOCD with MEF2C and GATA6 to generate smooth muscle resembling cells with characteristic cardiac marker genes and reduced fibroblast-specific gene expression (Hirai et al. 2018).

Delivering miRNAs in an integration-free manner, Jayawardena et al. established the derivation of cells having characteristics of cardiomyocytes via transient expression of muscle-specific miR-1, miR-133, miR-208, and miR-499 in mouse cardiac fibroblasts by a single transfection (Jayawardena et al. 2012). The authors also reported further enhancement of cardiomyocyte-like cells by the addition of JAK inhibitor I, which is believed to induce expression of cardiac ion channels as well as enhance α-MHC (Jayawardena et al. 2012). Following this, Muraoka et al. included miR-133 in the GMT reprogramming cocktail and demonstrated a sevenfold increase in the reprogramming efficiency and improved kinetics of <12 days from 30 days, compared to GMT alone in mouse embryonic fibroblasts (Muraoka et al. 2014). Apart from mouse embryonic fibroblasts, Muraoka et al. showed enhanced cardiac reprogramming in adult mouse and human cardiac fibroblasts with the inclusion of miR-133 in GMT or GMT with MESP1 and MYOCD reprogramming factors. Notably, miR-133 promoted cardiac reprogramming by suppressing Snai1, a key molecular roadblock of cardiac reprogramming and a master regulator of epithelial-to-mesenchymal transition, that facilitated the repression of fibroblast gene expression (Muraoka et al. 2014). Similarly, the addition of miR-1 and miR-133 to the GMT + HAND2 and A83-01 (a TGF-β signaling inhibitor) cocktail resulted in a maximum number of spontaneously contracting cells from mouse embryonic fibroblasts (Zhao et al. 2015).

Using a calcium indicator GCaMP, Addis et al. constructed a reporter system driven by cardiac-specific troponin-T promoter and demonstrated that NKX2.5 in combination with GMT + HAND2 (GMT + HAND2 + NKX2.5) is the most effective cocktail in cardiac reprogramming, resulting in 50-fold increase in cardiomyocyte formation efficiency than with GMT factors (Addis et al. 2013). This GMT + HAND2 + NKX2.5 combination in the presence of SB432542, a potent small molecule that inhibits TGF-β signaling pathway, further improved the reprogramming of mouse embryonic and adult fibroblasts up to fivefold (Ifkovits et al. 2014). Similarly, Zhao et al. showed that the suppression of pro-fibrotic signaling by TGF-β or Rho-associated kinase inhibitors remarkably enhanced the efficiency by up to 60% and dramatically enhanced the kinetics of cardiac reprogramming of mouse embryonic fibroblasts (Zhao et al. 2015).

Although the conventional methods using retroviral and lentiviral vectors are efficient and robust, they result in random viral integration in the host cell genome, leading to insertional mutagenesis and tumorigenicity (Zhao et al. 2015; Borgohain et al. 2019; Haridhasapavalan et al. 2019; Dey et al. 2021). These issues can be circumvented employing integration-free approaches of reprogramming, which have minimal or no genetic modifications (Zhao et al. 2015; Borgohain et al. 2019; Haridhasapavalan et al. 2019; Dey et al. 2021). In an effort to develop clinically suitable direct reprogramming strategies, Wang et al. established a defined cocktail of small molecules (SB431542, CHIR99021, Parnate, and Forskolin) that, along with a single pluripotency factor, OCT4, reprogrammed mouse fibroblasts to ventricular-like cardiomyocytes through a cardiac progenitor state (Wang et al. 2014). Following this, two different groups reported the generation of mouse cardiomyocytes using different combinations of small molecules to generate these cells in an integration-free manner (Fu et al. 2015; Park et al. 2015). Remarkably, Fu et al. reprogrammed mouse fibroblasts using only a cocktail of small molecules (without any transcription factors), namely, CHIR99021, RepSox, Forskolin, valproic acid, Parnate and TTNPB, into chemical-induced cardiomyocyte-like cells pass through a cardiac progenitor state and bypassing pluripotent state (Fu et al. 2015). Interestingly, the same cocktail has been used previously by the same group to generate chemical-iPSCs (Hou et al. 2013); however, the culture condition appears to play a crucial role in generating desired cells. Similarly, Park et al., with a different combination of small molecules (Forskolin, A-8301, SC1, CHIR99021, and BayK 8,644), converted mouse fibroblasts into cardiomyocyte-like cells (Park et al. 2015), passing through a progenitor state. In both these small molecule-based studies, the reprogramming efficiency of mouse tail tip fibroblasts was very low compared to embryonic fibroblasts (Fu et al. 2015; Park et al. 2015). Alternatively, a novel integration-free approach employing multiple mRNA transfections of GMT for just 2 weeks reprogrammed mouse cardiac fibroblasts directly to cardiomyocytes (Lee et al. 2015). This study demonstrated that the expression of cardiomyocyte-specific marker genes is dependent on the stoichiometric ratio of GMT mRNAs (Lee et al. 2015). However, in all these studies, reprogramming efficiency was compromised significantly compared to integrating viral vectors.

Considering the risk factors associated with integrating vectors as well as the requirement of transgene expression for a limited duration to bring about reprogramming, the focus now shifted toward finding a more clinically relevant set of non-integrating vectors for direct cardiac reprogramming. Keeping this in mind, several studies reported the use of integration-free adenoviral or adeno-associated viral or Sendai viral vectors to safely and effectively generate induced cardiomyocytes from mouse fibroblasts (Mathison et al. 2017; Miyamoto et al. 2018; Yoo et al. 2018; Isomi et al. 2021). One such approach involved the administration of adenoviral-GMT vectors in post-infarct rats, successfully bringing about the transdifferentiation of fibroblasts into cardiomyocytes as well as ameliorating the cardiac condition (Mathison et al. 2017). This strategy proved as effective as the use of lentiviral-GMT vectors, generating ~6% cardiac troponin-T expressing cells with an elevated ejection fraction compared to the control, further suggesting that the transient expression of reprogramming factors using adenoviral vectors could be sufficient in bringing about cardiac reprogramming (Mathison et al. 2017). In an effort to accomplish in vivo direct cardiac reprogramming, Kisby et al. utilized direct adenoviral vector injection to transiently induce the expression of pluripotency factors (Yamanaka factors) in healthy and post-infarct mouse hearts (Kisby et al. 2021). This resulted in partial reprogramming and therefore was insufficient to regenerate the post-infarct heart. Remarkably, Yoo et al. demonstrated an in vivo chimeric approach using an adeno-associated viral vectors encoding the combination of GMT along with thymosin β4 (an antifibrotic angiogenic protein) that facilitated regeneration in the injured heart by promoting the upregulation of cardiac-specific genes as well as a gradual downregulation of fibrosis-specific genes (Yoo et al. 2018). In the near future, the use of thymosin β4 will serve as a probable clinical aid in cardiovascular regeneration (Yoo et al. 2018).

In another effort to bring about reprogramming using Sendai viral-GMT vectors, Ieda and colleagues reported the efficient generation of cardiomyocytes with a 100-fold enhanced beating compared to the use of retroviral-GMT from mouse cardiac fibroblasts in both in vitro and in vivo (Miyamoto et al. 2018). As early as 7 days post-transduction, Sendai virus-mediated in vivo reprogramming resulted in the generation of cardiomyocytes, which enhanced cardiac function in the post-infarct heart after 4 weeks of transplantation in immunodeficient mice (Miyamoto et al. 2018). The same group reproduced Sendai virus-mediated in vivo reprogramming in immunocompetent mice and showed that the generated cardiomyocytes not only managed to exist in post-infarct heart conditions for up to 4 weeks but also reduced fibrosis by collagen suppression, thereby improving cardiac function for at least 12 weeks (Isomi et al. 2021). This study reported a reprogramming efficiency of 2.5% after 4 weeks of viral transduction (Isomi et al. 2021), whereas the previous study showed an efficiency of 1.5% after 1 week of viral transduction (Miyamoto et al. 2018).

2.4.2 Direct Cardiac Reprogramming of Human Fibroblasts

In contrast to the murine cardiac reprogramming, several studies have demonstrated that GMT cocktail is insufficient to reprogram human fibroblasts to cardiomyocytes (Fu et al. 2013; Nam et al. 2013; Wada et al. 2013; Muraoka et al. 2014; Singh et al. 2016; Yamakawa and Yamakawa and Ieda 2021) and requires additional factors (Table 2). These results concluded that additional factors may be required to induce cardiac reprogramming in human somatic cells. Therefore, Nam et al. included MYOCD and muscle-specific miRNAs (miR-1 and miR-133) in combination with GMT to reprogram human fibroblasts into cardiomyocytes (Nam et al. 2013). The authors reported a heterogenous population of reprogrammed cells with varying levels of cardiac gene expression and only a small subset of cells showing spontaneous contractility. Interestingly, another study reported that MESP1, along with the ETS2 transcription factor, reprogrammed human dermal fibroblasts to cardiac progenitor cells via lentiviral-based approach; however, the recombinant protein forms of the same transcription factors when co-expressed in human dermal fibroblasts resulted in the derivation of immature cardiomyocytes (Islas et al. 2012). Subsequently, the inclusion of MESP1 and ESRRG/NR3B3 (a nuclear receptor that plays a critical role in mitochondrial biogenesis) to GMT cocktail reprogrammed human fibroblasts derived from different sources such as ESCs, fetal heart, and neonatal skin (Fu et al. 2013). This study showed phenotypic shift and induced global cardiac gene expression in the starting cell type, albeit at a low frequency (Fu et al. 2013). Fu et al. further showed enhanced reprogramming with the addition of MYOCD and ZFPM2 (FOG2), but the efficiency still remained low (Fu et al. 2013). Notably, they also reported the importance of TGF-β signaling in the cardiac reprogramming of human cells. Similarly, by adding MESP1 and MYOCD to the reprogramming cocktail (GMT + MESP1 + MYOCD), Wada et al. reported the successful generation of cardiomyocyte-like cells from human fibroblasts (Wada et al. 2013). In order to further understand the time course of induction, Wada et al. constructed doxycycline-inducible lentiviral vectors and found that the induction of GMT + MESP1 + MYOCD expression for 2 weeks is sufficient for the stable conversion of human fibroblasts to cardiomyocytes (Wada et al. 2013). These studies demonstrated that, unlike mouse cells, reprogramming human cells is time-consuming and inefficient (Fu et al. 2013; Nam et al. 2013; Wada et al. 2013). Further addition of miR-133 to the human cardiac fibroblasts overexpressing GMT + MESP1 + MYOCD improved cardiac reprogramming efficiency as well as the kinetics by directly suppressing Snai1 (Muraoka et al. 2014). This can be correlated with the Nam et al.'s study (Nam et al. 2013), which collectively suggests that suppressing Snail is essential for efficient cardiac reprogramming of human cells.

Table 2 Overview of reprogramming approaches (human)

Reprogramming approaches	Reprogramming factors	Source cell	Cardiac gene expression	In vivo	References
Retroviral vectors	G, MyoCD, T, H + miR-1, miR-133	HFF	~17% cTnT$^+$ cells, spontaneously contracting cells	No	Nam et al. (2013)
Retroviral vectors	GMT, ESRRG, MESP1	HF	13 ± 9.3% αMHC-mCherry$^+$ cTNT$^+$ cells at 2 weeks	No	Fu et al. (2013)
Retroviral vectors	GMTMyoCD, MESP1	HCF, HDF	~5.60% α-actinin$^+$ cTnT$^+$ cells, synchronous contraction in co-culture	No	Wada et al. (2013)
Retroviral vectors	GMT + miR-133a	MEF	Sevenfold enhanced beating iCMs than GMT	No	Muraoka et al. (2014)
Lentiviral vectors	GMTHMyoCD + miR-590	HCF	~5% cTnT$^+$ cells, suppression of specificity protein 1 (Sp1)	No	Singh et al. (2016)
Small molecules	CHIR99021, A83-01, BIX01294, AS8351, SC1, Y27632, OAC2, SU16F, JNJ018409	HFF	6.6% cTnT expression	No	Cao et al. (2016)
Lentiviral vectors	GMTNMyoCD + miR-1, miR-133a	HDF	Cardiac markers TNNT2$^+$, ACTN2$^+$, intracellular Ca2$^+$ transients, iCM-like cells	No	Christoforou et al. (2017)
Retroviral vectors	GMT, ESRRG, MESP1, MyoCD, ZFPM2 + H/miR-1	H9-differentiated fibroblasts	Early progress of iCM reprogramming, 16.7% CM-like cells	No	Bektik et al. (2017)
Sendai viral vectors	GMTMyoCD, MESP1 + miR-133	HCF	Inclusion of miR-133 resulted in 15% cTnT$^+$ cells, 10% cells contracted synchronously with neonatal rat CMs	No	Miyamoto et al. (2018)
microRNAs	miR-1, 133, 208, 499	HCF	Upregulation of GMTH genes, ~11% cTnT$^+$ cells, Spontaneous calcium transients at 30 days (~38% cells)	No	Paoletti et al. (2020)
Lentiviral vectors	GMTHMyoCD + miR-590 + sodium butyrate, ICG-001, retinoic acid	HCF	~ 23% cTnT$^+$ cells, ~5% cells spontaneous beating in co-culture	No	Singh et al. (2020)
Lentiviral vectors	GM+ Tead1	HCF	~5% cTnT$^+$ cells, fourfold increase in α-sarcomeric actinin (4 weeks)	No	Singh et al. (2021)

G, *GATA4*; *M*, *MEF2C*; *T*, *TBX5*; *H*, *HAND2*; *N*, NKX 2.5; *MyoCD*, myocardin; *ESRRG*, estrogen-related receptor gamma; *MESP1*, mesoderm-specific transcription factor 1; *ZFPM2*, zinc-finger protein; *ZNF281*, zinc finger protein 281; *miRNA*, microRNA; *JAK*, *janus kinase*; *CM*, cardiomyocytes; *iCM*, induced CMs; CF, cardiac fibroblasts; *HF*, human fibroblasts; *HFF*, human foreskin fibroblasts; *HDF*, human dermal fibroblasts; *HCF*, human cardiac fibroblasts; *cTnT*, cardiac troponin T; *ACTN2*, actinin alpha 2; *TNNT2*, troponin T type 2; *αMHC*, alpha myosin heavy chain

Using a different combination of transcription factors and miRNA, Singh et al. demonstrated that human cardiac fibroblasts, upon treatment with GMT, HAND2, and MYOCD along with miR-590, resulted in cardiac troponin-T expressing cells that exhibited spontaneous contractions. Particularly, the addition of miR-590 serves as a suitable alternative to enhance the reprogramming efficiency by suppression of specificity protein 1 (Singh et al. 2016), a zinc finger protein that regulates fibrosis genes (Verrecchia et al. 2001). Another study demonstrated transdifferentiation of human dermal fibroblasts toward cardiac cell lineage using the same Nam et al. cocktail along with NKX2.5 transcription factor (Christoforou et al. 2017).

Further, it has been shown that the efficiency can be increased either by the inhibition of Janus kinase 1 or glycogen synthase kinase 3 or by the addition of NRG1, a protein that promotes cardiomyocyte proliferation and myocardial regeneration (Christoforou et al. 2017).

Remarkably, in an effort to generate integration-free cardiomyocytes, Ding and colleagues reported chemically induced cardiomyocytes that exhibited uniform contractility as a result of treating human fibroblasts with a defined cocktail of 9 small molecules (9C), including those molecules that have been reported to downregulate fibroblasts-specific genes (Cao et al. 2016). These 9C-treated fibroblasts have the ability to repair infarcted mouse hearts upon transplantation. Using a combinatorial approach with a defined set of small molecules and GMT + HAND2, Singh et al. demonstrated the reprogramming of human fibroblasts toward cardiomyocytes, exhibiting calcium transients and spontaneous beating in co-culture conditions (Singh et al. 2020). Using another non-integrative approach that included miR-1, miR-133, miR-208, and miR-499 (miR-combo), the authors were able to generate immature cells expressing cardiac troponin-T and exhibiting spontaneous calcium oscillation after 15 and 30 days post-transfection, respectively (Paoletti et al. 2020). Similarly, another group used non-integrative Sendai viral vectors to deliver GMT + MESP1 + MYOCD (as mentioned in Wada et al. 2013) or GMT + MESP1 + MYOCD/miR-133 (as mentioned in Muraoka et al. 2014) in human cardiac fibroblasts, resulting in the generation of cardiac troponin-T$^+$ cells (Miyamoto et al. 2018). Interestingly, threefold higher cardiac troponin-T$^+$ cells were reported with Sendai viral vectors having GMT + MESP1 + MYOCD in combination with miR-133 compared to the one without miR-133 (Miyamoto et al. 2018). Further, the authors demonstrated synchronous beating of cardiac troponin-T$^+$ cells from human cardiac fibroblasts transduced with Sendai viral vectors-GMTMM/miR-133 when co-cultured with neonatal rat cardiomyocytes. To summarize, human cardiac reprogramming is generally less efficient and has slower kinetics than murine cardiac reprogramming (Chen et al. 2017). Therefore, further refinements are required to enhance the efficiency and kinetics with a robust, clinically applicable strategy.

Alternatively, Yu et al. employed a transgene-free CRISPR-based approach to knockout Dmap1 (DNA methyltransferase 1-associated protein 1) and revealed its significance in regulating the reprogramming process (Yu et al. 2019). This novel therapeutic intervention modulates the process by reducing promoter methylation and elevating the expression of Nkx2-5 by up to 11% (Yu et al. 2019). Another study demonstrates the use of lineage reprogramming to generate induced cardiac progenitor cells via CRISPR/Cas9-mediated transcription activation (Wang et al. 2020a). Targeting endogenous cardiac factor combination of GMT + HAND2, Wang et al. successfully reprogrammed human fibroblasts into cardiac progenitor cells that have the potential to differentiate further into induced cardiomyocytes (Wang et al. 2020a). The use of such novel technologies, apart from the conventional reprogramming methods, may serve as a potential approach to patient-specific cardiac cell therapy.

These studies clearly demonstrate that fibroblasts can be directly reprogrammed toward the cardiac lineage (Table 2) and lay the foundation for future refinements with respect to the inclusion of other essential factors in the reprogramming cocktails, improving reprogramming efficiency and understanding the underlying mechanisms of the cardiac reprogramming process. Further extensive studies are required to explore their in vivo reprogramming potential for regenerative therapies.

3 Prospects and Challenges

The search for an effective cure to cardiovascular diseases has resulted in extensive research in different strategies of cardiomyocyte regeneration, including direct cardiac reprogramming. In spite of the vast majority of studies reporting the use of different integrative (γ-retroviral and lentiviral vectors) and non-integrative (adenoviral vectors, adeno-associated vectors, Sendai viral vectors, recombinant proteins, mRNA, miRNA, small molecules, etc.) reprogramming approaches, the failure of these technologies in translating in the clinical setting still remains a major setback and necessitates the need for further research in the same.

Direct cardiac reprogramming holds multiple advantages in various aspects: (i) the process is fast and can be further enhanced by optimizing the reprogramming cocktail or by other genetic/chemical modifications and (ii) bypassing the pluripotent stage and thereby overcoming the risks of insertional mutagenesis or malignant transformation; (iii) the autologous and immune-compatible cardiomyocytes generated are patient-specific, rendering the process a perfect fit for cell therapy and (iv) allows for precise control over time and dosage of application of reprogramming factors, further providing a better understanding of the stage-specific role of the individual factors; (v) a well-optimized protocol of direct cardiac reprogramming is easily reproducible in a laboratory setting. Owing to the numerous advantages of direct cardiac reprogramming over other approaches, it may serve as an attractive strategy in the generation of functional cardiomyocytes.

Direct cardiac reprogramming may be an appealing prospect in cardiac regeneration but not without a plethora of unique challenges yet to be overcome in the near future. Owing to the complex epigenetic mechanisms tightly regulating the reprogramming process in human fibroblasts, they are more resistant to forming fully functional (beating) and mature cardiomyocytes compared to their murine counterparts. A major concern here is enhancing the reprogramming efficiency in order to generate cells, sufficient enough to ameliorate the condition of the post-infarct heart, for which approximately 50% of the cells must be reprogrammed (Chen et al. 2014). However, existing literature shows only about 5–10% cells giving rise to induced cardiomyocytes and as low as 0.01–0.1% cells exhibiting functional characteristics (Ghiroldi et al. 2017). Enhancing the reprogramming efficiency is critical in order to bring direct cardiac reprogramming closer to clinic, thereby throwing light on the importance of other combination of transcription factors and/or small molecules, miRNA, etc. Increasing number of studies has focused on the use of small molecule inhibitors along with the existing reprogramming cocktails and has successfully increased the yield of contractile-induced cardiomyocytes. The importance of altering certain signaling pathways relevant to the mechanism of fibrosis, viz., TGF-β and Wnt signaling, that act as barriers in the reprogramming process needs to be further investigated (Ifkovits et al. 2014; Mohamed et al. 2017). In addition to this, studies on inhibition of key epigenetic barriers like Bmi1 and the consequent enhancement of induced cardiomyocyte generation signifies the role of epigenetic mechanisms in the reprogramming process. Although the exact molecular mechanisms still remain elusive, targeting these pathways can aid in the enhancement of efficiency of direct cardiac reprogramming.

The quest for an integration-free cardiac reprogramming method has also thrown light on the possibility of using reprogramming transcription factors in the form of recombinant proteins. Toward this end, so far, researchers have successfully expressed and purified transcription factors like ETS2 (Haridhasapavalan et al. 2020), MESP1 (Haridhasapavalan et al. 2021a), GATA4 (Haridhasapavalan et al. 2021b), TBX5 (Haridhasapavalan et al. 2022a), HAND2 (Haridhasapavalan et al. 2022b), and MEF2C (Haridhasapavalan et al. 2022c) as transducible versions of bioactive recombinant proteins from the heterologous system (*E. coli*). These reprogramming proteins have a significant role in the cardiac milieu and may serve as a safer strategy for cardiac transdifferentiation and improve the prospects of these cells for biomedical applications.

4 Conclusion

Direct cardiac reprogramming has enormously progressed in the last decade and has immense potential to become one of the leading cardiovascular regenerative therapies in the coming years. Overcoming critical barriers of reprogramming in mouse fibroblasts, the challenges associated with the same in case of human fibroblasts and in vivo models are yet to be tackled. In addition to unravelling the complex molecular mechanisms associated with cardiac regeneration, it is essential to look into the gene delivery methods, safety concerns, as well as the long-term survival of the regenerated induced cardiomyocytes in vivo. With numerous challenges ahead, the potential benefits and opportunities still outweigh the cons associated with direct cardiac reprogramming. Further research and establishment of optimized protocols of direct cardiac reprogramming will open new avenues in generating cardiomyocytes for benefit of cardiovascular ailments.

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References

Addis RC et al (2013) Optimization of direct fibroblast reprogramming to cardiomyocytes using calcium activity as a functional measure of success. J Mol Cell Cardiol 60:97–106

Amini H et al (2017) Cardiac progenitor cells application in cardiovascular disease. J Cardiovasc Thorac Res 9:127

Antonitsis P et al (2007) In vitro cardiomyogenic differentiation of adult human bone marrow mesenchymal stem cells. The role of 5-azacytidine. Interact Cardiovasc Thorac Surg 6:593–597

Balsam LB et al (2004) Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium. Nature 428:668–673

Bektik E et al (2017) Single cell qPCR reveals that additional HAND2 and microRNA-1 facilitate the early reprogramming progress of seven-factor-induced human myocytes. PLoS One 12:e0183000

Bektik E et al (2021) Inhibition of CREB-CBP signaling improves fibroblast plasticity for direct cardiac reprogramming. Cell 10:1572

Beltrami AP et al (2003) Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell 114:763–776

Borgohain MP et al (2019) An insight into DNA-free reprogramming approaches to generate integration-free induced pluripotent stem cells for prospective biomedical applications. Stem Cell Rev Rep 15:286–313

Cai B et al (2012) microRNA-124 regulates cardiomyocyte differentiation of bone marrow-derived mesenchymal stem cells via targeting STAT3 signaling. Stem Cells 30:1746–1755

Cao N et al (2016) Conversion of human fibroblasts into functional cardiomyocytes by small molecules. Science 352:1216–1220

Chen JX et al (2014) Somatic cell reprogramming into cardiovascular lineages. J Cardiovasc Pharmacol Ther 19:340–349

Chen Y et al (2017) Direct reprogramming of fibroblasts into cardiomyocytes. Stem Cell Res Ther 8:1–8

Le T, Chong J (2016) Cardiac progenitor cells for heart repair. Cell Death Discov 2:1–4

Chong JJ et al (2011) Adult cardiac-resident MSC-like stem cells with a proepicardial origin. Cell Stem Cell 9:527–540

Chong JJ et al (2014) Developmental origins and lineage descendants of endogenous adult cardiac progenitor cells. Stem Cell Res 13:592–614

Christoforou N et al (2017) Core transcription factors, microRNAs, and small molecules drive transdifferentiation of human fibroblasts towards the cardiac cell lineage. Sci Rep 7:1–15

David R et al (2008) MesP1 drives vertebrate cardiovascular differentiation through Dkk-1-mediated blockade of Wnt-signalling. Nat Cell Biol 10:338–345

Denning C et al (2003) Common culture conditions for maintenance and cardiomyocyte differentiation of the human embryonic stem cell lines, BG01 and HUES-7. Int J Dev Biol 50:27–37

Dey C et al (2021) An overview of reprogramming approaches to derive integration-free induced pluripotent stem cells for prospective biomedical applications. Recent Adv iPSC Technol:231–287

Efe JA et al (2011) Conversion of mouse fibroblasts into cardiomyocytes using a direct reprogramming strategy. Nat Cell Biol 13:215–222

Fu J-D et al (2013) Direct reprogramming of human fibroblasts toward a cardiomyocyte-like state. Stem Cell Rep 1:235–247

Fu Y et al (2015) Direct reprogramming of mouse fibroblasts into cardiomyocytes with chemical cocktails. Cell Res 25:1013–1024

Funakoshi S et al (2021) Generation of mature compact ventricular cardiomyocytes from human pluripotent stem cells. Nat Commun 12:1–23

Ghiroldi A et al (2017) Regenerating the human heart: direct reprogramming strategies and their current limitations. Basic Res Cardiol 112:1–14

Alagesan S, Griffin MD (2014) Autologous and allogeneic mesenchymal stem cells in organ transplantation: what do we know about their safety and efficacy? Curr Opin Organ Transplant 19:65–72

Guan K et al (1999) Embryonic stem cell differentiation models: cardiogenesis, myogenesis, neurogenesis, epithelial and vascular smooth muscle cell differentiation in vitro. Cytotechnology 30:211–226

Guo X et al (2018) Cardiomyocyte differentiation of mesenchymal stem cells from bone marrow: new regulators and its implications. Stem Cell Res Ther 9: 1–12

Guo Y et al (2019) Enhancing cardiac reprogramming by suppressing specific CC chemokine signaling pathways. bioRxiv 522995

Haridhasapavalan KK et al (2019) An insight into non-integrative gene delivery approaches to generate transgene-free induced pluripotent stem cells. Gene 686:146–159

Haridhasapavalan KK et al (2020) Codon optimization, cloning, expression, purification, and secondary structure determination of human ETS2 transcription factor. Mol Biotechnol 62:485–494

Haridhasapavalan KK et al (2021a) Soluble expression, purification, and secondary structure determination of human MESP1 transcription factor. Appl Microbiol Biotechnol 105:2363–2376

Haridhasapavalan KK et al (2021b) Generation of cell-permeant recombinant human transcription factor GATA4 from *E. coli*. Bioprocess Biosyst Eng 44: 1131–1146

Haridhasapavalan KK et al (2022a) Generation of a transducible version of a bioactive recombinant human TBX5 transcription factor from *E. coli*. Curr Res Biotechnol 4:66–77

Haridhasapavalan KK et al (2022b) Generation of a recombinant version of a biologically active cell-permeant human HAND2 transcription factor from *E. coli*. Scientific Reports

Haridhasapavalan KK et al (2022c) Generation of recombinant version of a bioactive human MEF2C transcription factor from *E. coli*. Healthcare Research and Related Technologies – Proceedings of NERC 2022 (Springer Nature)

Hattan N et al (2005) Purified cardiomyocytes from bone marrow mesenchymal stem cells produce stable intracardiac grafts in mice. Cardiovasc Res 65:334–344

Hirai H et al (2018) Direct reprogramming of fibroblasts into smooth muscle-like cells with defined transcription factors – brief report. Arterioscler Thromb Vasc Biol 38:2191–2197

Hou P et al (2013) Pluripotent stem cells induced from mouse somatic cells by small-molecule compounds. Science 341:651–654

Ieda M et al (2009) Cardiac fibroblasts regulate myocardial proliferation through β1 integrin signaling. Dev Cell 16:233–244

Ieda M et al (2010) Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell 142:375–386

Ifkovits JL et al (2014) Inhibition of TGFβ signaling increases direct conversion of fibroblasts to induced cardiomyocytes. PLoS One 9:e89678

Islas JF et al (2012) Transcription factors ETS2 and MESP1 transdifferentiate human dermal fibroblasts into cardiac progenitors. Proc Natl Acad Sci 109: 13016–13021

Isomi M et al (2021) Direct reprogramming with Sendai virus vectors repaired infarct hearts at the chronic stage. Biochem Biophys Res Commun 560:87–92

Jayawardena TM et al (2012) MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibroblasts to cardiomyocytes. Circ Res 110:1465–1473

Jiang Y et al (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature 418:41–49

Kadari A et al (2015) Robust generation of cardiomyocytes from human iPS cells requires precise modulation of BMP and WNT signaling. Stem Cell Rev Rep 11:560–569

Kakkar A et al (2019) Adipose tissue derived mesenchymal stem cells are better respondents to TGFβ1 for in vitro generation of cardiomyocyte-like cells. Mol Cell Biochem 460:53–66

Kaptoge S et al (2019) World Health Organization cardiovascular disease risk charts: revised models to estimate risk in 21 global regions. Lancet Glob Health 7:e1332–e1345

Kawada H et al (2004) Nonhematopoietic mesenchymal stem cells can be mobilized and differentiate into cardiomyocytes after myocardial infarction. Blood 104:3581–3587

Kehat I et al (2001) Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest 108: 407–414

Kisby T et al (2021) Adenoviral mediated delivery of OSKM factors induces partial reprogramming of mouse cardiac cells in vivo. Adv Ther 4:2000141

Laflamme MA et al (2007) Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. Nat Biotechnol 25:1015–1024

Laugwitz K-L et al (2005) Postnatal isl1+ cardioblasts enter fully differentiated cardiomyocyte lineages. Nature 433:647–653

Lee K et al (2015) Peptide-enhanced mRNA transfection in cultured mouse cardiac fibroblasts and direct reprogramming towards cardiomyocyte-like cells. Int J Nanomedicine 10:1841

Lian X et al (2012) Robust cardiomyocyte differentiation from human pluripotent stem cells via temporal modulation of canonical Wnt signaling. Proc Natl Acad Sci 109:E1848–E1857

Lim CK et al (2021) Dimethyl sulfoxide (DMSO) enhances direct cardiac reprogramming by inhibiting the bromodomain of coactivators CBP/p300. J Mol Cell Cardiol 160:15–26

Ma R et al (2018) Electrical stimulation enhances cardiac differentiation of human induced pluripotent stem cells for myocardial infarction therapy. Antioxid Redox Signal 28:371–384

Makino S et al (1999) Cardiomyocytes can be generated from marrow stromal cells in vitro. J Clin Invest 103: 697–705

Markmee R et al (2020) Differentiation of cardiomyocyte-like cells from human amniotic fluid mesenchymal stem cells by combined induction with human platelet lysate and 5-azacytidine. Heliyon 6:e04844

Mathison M et al (2017) In situ reprogramming to transdifferentiate fibroblasts into cardiomyocytes using adenoviral vectors: implications for clinical myocardial regeneration. J Thorac Cardiovasc Surg 153:329–339

McGill HC Jr et al (2008) Preventing heart disease in the 21st century: implications of the Pathobiological determinants of atherosclerosis in youth (PDAY) study. Circulation 117:1216–1227

Mendis S et al (2011) Global atlas on cardiovascular disease prevention and control. World Health Organization

Messina E et al (2004) Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res 95:911–921

Miyamoto K et al (2018) Direct in vivo reprogramming with Sendai virus vectors improves cardiac function after myocardial infarction. Cell Stem Cell 22(91–103):e5

Mohamed TM et al (2017) Chemical enhancement of in vitro and in vivo direct cardiac reprogramming. Circulation 135:978–995

Muraoka N et al (2014) MiR-133 promotes cardiac reprogramming by directly repressing Snai1 and silencing fibroblast signatures. EMBO J 33:1565–1581

Murry CE et al (2004) Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. Nature 428:664–668

Murry CE et al (2006) Regeneration gaps: observations on stem cells and cardiac repair. J Am Coll Cardiol 47: 1777–1785

Nam Y-J et al (2013) Reprogramming of human fibroblasts toward a cardiac fate. Proc Natl Acad Sci 110:5588–5593

Nazari H et al (2020) Electrical stimulation induces differentiation of human cardiosphere-derived cells (hCDCs) to committed cardiomyocyte. Mol Cell Biochem 470:29–39

Oh H et al (2003) Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. Proc Natl Acad Sci 100:12313–12318

Orlic D et al (2001) Mobilized bone marrow cells repair the infarcted heart, improving function and survival. Proc Natl Acad Sci 98:10344–10349

Ou D et al (2016) Co-culture with neonatal cardiomyocytes enhances the proliferation of iPSC-derived cardiomyocytes via FAK/JNK signaling. BMC Dev Biol 16:1–12

Paige SL et al (2010) Endogenous Wnt/β-catenin signaling is required for cardiac differentiation in human embryonic stem cells. PLoS One 5:e11134

Pak HN et al (2003) Mesenchymal stem cell injection induces cardiac nerve sprouting and increased tenascin expression in a swine model of myocardial infarction. J Cardiovasc Electrophysiol 14:841–848

Paoletti C et al (2020) MicroRNA-mediated direct reprogramming of human adult fibroblasts toward cardiac phenotype. Front Bioeng Biotechnol 8:529

Park G et al (2015) Conversion of mouse fibroblasts into cardiomyocyte-like cells using small molecule treatments. Biomaterials 54:201–212

Patel V et al (2018) p63 silencing induces reprogramming of cardiac fibroblasts into cardiomyocyte-like cells. J Thorac Cardiovasc Surg 156(556–565):e1

Protze S et al (2012) A new approach to transcription factor screening for reprogramming of fibroblasts to cardiomyocyte-like cells. J Mol Cell Cardiol 53:323–332

Raghunathan S et al (2020) Conversion of human cardiac progenitor cells into cardiac pacemaker-like cells. J Mol Cell Cardiol 138:12–22

Ramesh S et al (2021) Cardiac differentiation of mesenchymal stem cells: impact of biological and chemical inducers. Stem Cell Rev Rep 17:1343–1361

Shen X et al (2017) Differentiation of mesenchymal stem cells into cardiomyocytes is regulated by miRNA-1-2 via WNT signaling pathway. J Biomed Sci 24:1–8

Shim WS et al (2004) Ex vivo differentiation of human adult bone marrow stem cells into cardiomyocyte-like cells. Bioch Biophys Res Commun 324:481–488

Singh VP et al (2016) MiR-590 promotes transdifferentiation of porcine and human fibroblasts toward a cardiomyocyte-like fate by directly repressing specificity protein 1. J Am Heart Assoc 5:e003922

Singh VP et al (2020) Enhanced generation of induced cardiomyocytes using a small-molecule cocktail to overcome barriers to cardiac cellular reprogramming. J Am Heart Assoc 9:e015686

Singh VP et al (2021) Hippo pathway effector Tead1 induces cardiac fibroblast to cardiomyocyte reprogramming. J Am Heart Assoc 10:e022659

Song K et al (2012) Heart repair by reprogramming non-myocytes with cardiac transcription factors. Nature 485:599–604

Takahashi K et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872

Talkhabi M et al (2015) Ascorbic acid promotes the direct conversion of mouse fibroblasts into beating cardiomyocytes. Biochem Biophys Res Commun 463:699–705

Testa G et al (2020) Bmi1 inhibitor PTC-209 promotes chemically-induced direct cardiac reprogramming of cardiac fibroblasts into cardiomyocytes. Sci Rep 10:1–16

Tian J et al (2018) Optimization and enrichment of induced cardiomyocytes derived from mouse fibroblasts by reprogramming with cardiac transcription factors. Mol Med Rep 17:3912–3920

Verrecchia F et al (2001) Blocking sp1 transcription factor broadly inhibits extracellular matrix gene expression in vitro and in vivo: implications for the treatment of tissue fibrosis. J Investig Dermatol 116:755–763

Wada R et al (2013) Induction of human cardiomyocyte-like cells from fibroblasts by defined factors. Proc Natl Acad Sci 110:12667–12672

Wang H et al (2014) Small molecules enable cardiac reprogramming of mouse fibroblasts with a single factor, Oct4. Cell Rep 6:951–960

Wang L et al (2015) Stoichiometry of Gata4, Mef2c, and Tbx5 influences the efficiency and quality of induced cardiac myocyte reprogramming. Circ Res 116:237–244

Wang J et al (2020a) Lineage reprogramming of fibroblasts into induced cardiac progenitor cells by CRISPR/Cas9-based transcriptional activators. Acta Pharm Sin B 10:313–326

Wang L et al (2020b) Isoform specific effects of Mef2C during direct cardiac reprogramming. Cell 9:268

Yamakawa H, Ieda M (2021) Cardiac regeneration by direct reprogramming in this decade and beyond. Inflamm Regen 41:1–10

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676

Yoo SY et al (2018) Chimeric adeno-associated virus-mediated cardiovascular reprogramming for ischemic heart disease. ACS Omega 3:5918–5925

Yoon Y-s et al (2005) Clonally expanded novel multipotent stem cells from human bone marrow regenerate myocardium after myocardial infarction. J Clin Invest 115:326–338

Yu JS et al (2019) CRISPR-knockout screen identifies Dmap1 as a regulator of chemically induced reprogramming and differentiation of cardiac progenitors. Stem Cells 37:958–972

Zhang Z et al (2019) Stoichiometric optimization of Gata4, Hand2, Mef2c, and Tbx5 expression for contractile cardiomyocyte reprogramming. Sci Rep 9:1–10

Zhao Y et al (2015) High-efficiency reprogramming of fibroblasts into cardiomyocytes requires suppression of pro-fibrotic signalling. Nat Commun 6:1–15

Zhao X-L et al (2016) MicroRNA-1 effectively induces differentiation of myocardial cells from mouse bone marrow mesenchymal stem cells. Artif Cells Nanomed Biotechnol 44:1665–1670

Zhao H et al (2021) Sall4 and Myocd empower direct cardiac reprogramming from adult cardiac fibroblasts after injury. Front Cell Dev Biol 9:608367

Zhou Y et al (2016) Bmi1 is a key epigenetic barrier to direct cardiac reprogramming. Cell Stem Cell 18:382–395

Zhou H et al (2017) ZNF281 enhances cardiac reprogramming by modulating cardiac and inflammatory gene expression. Genes Dev 31:1770–1783

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Stem Cell-Based Therapeutic Approaches in Genetic Diseases

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Abstract

Stem cells, which can self-renew and differentiate into different cell types, have become the keystone of regenerative medicine due to these properties. With the achievement of superior clinical results in the therapeutic approaches of different diseases, the applications of these cells in the treatment of genetic diseases have also come to the fore. Foremost, conventional approaches of stem cells to genetic diseases are the first approaches in this manner, and they have brought safety issues due to immune reactions caused by allogeneic transplantation. To eliminate these safety issues and phenotypic abnormalities caused by genetic defects, firstly, basic genetic engineering practices such as vectors or RNA modulators were combined with stem cell-based therapeutic approaches. However, due to challenges such as immune reactions and inability to target cells effectively in these applications, advanced molecular methods have been adopted in ZFN, TALEN, and CRISPR/Cas genome editing nucleases, which allow modular designs in stem cell-based genetic diseases' therapeutic approaches. Current studies in genetic diseases are in the direction of creating permanent treatment regimens by genomic manipulation of stem cells with differentiation potential through genome editing tools. In this chapter, the stem cell-based therapeutic approaches of various vital genetic diseases were addressed wide range from conventional applications to genome editing tools.

Keywords

Conventional stem cell therapies · CRISPR · Genetic disease · Genome editing tools · TALEN · ZFN

Abbreviations

AAV9	Adeno-associated virus serotype-9
ALS	Amyotrophic lateral sclerosis
ASCs	Adult stem cells
BDNF	Brain-derived neurotrophic factor
BM-MSCs	Bone marrow-derived MSCs
Cas9	Associated protein 9
CF	Cystic fibrosis
CFTR	Cystic fibrous transmembrane conductivity regulator
CFU-F	Colony-forming unit-fibroblast
CFZF	CFTR Zinc-Finger Protein Fusion

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CK	Creatine kinase
CRISPR	Clustered regularly interspaced short palindromic repeat
DMD	Duchenne muscular dystrophy
DPR	Dipeptide repeat
DSB	Double-strand break
DUX4	Double homeobox protein 4
ECFCs	Endothelial colony-forming cells
ESCs	Embryonic stem cells
FBN1	Fibrillin-1
FSHD	Facioscapulohumeral muscular dystrophy
FUS	Fused in sarcoma/translocated in liposarcoma
FVIII	Factor VIII
HA	Hemophilia A
HBB	Hemoglobin subunit β
HbF	Hemoglobin F
HBs	Mutant hemoglobin
HD	Huntington's disease
HDR	Homology-directed repair
HGPRT	Hypoxanthine-guanine phosphoribosyltransferase
HLA	Human leukocyte antigen
HSCs	Hematopoietic stem cells
HTT	Huntingtin
HuBECs	Human bronchial epithelial cells
iPSCs	Induced pluripotent stem cells
ISCT	International Society for Cell & Gene Therapy
LCR	Locus control region
LGMD	Limb-girdle muscular dystrophy
LNT	Lesch-Nyhan disease
MMR	Mismatch repair
MSCs	Mesenchymal stem cells
MuSCs	Muscle stem cells
MyoD1	Myogenic differentiation 1
NHEJ	Non-homologous end joining
NPCs	Neural progenitor cells
PAM	Protospacer adjacent motif
PAX7	Paired box 7
PBMCs	Peripheral blood mononuclear cells
Pitx2c	c-Isoform of the pituitary homeobox 2 transcription factor
P-MSCs	Placenta-derived MSCs
PNA	Peptide nucleic acid
RNAi	RNA interference
RVD	Repeat variable di-residues
SCD	Sickle cell disease
SCNT	Somatic cell nuclear transfer
sgRNA	Single-guide RNA
shRNA	Short hairpin RNA
SMA	Spinal muscular atrophy
SMN	Survival motor neuron
SMN-FL	Survival motor neuron-full length
SMN-Δ7	Survival motor neuron-lacking exon 7
SREs	Splicing-regulatory elements
ssODNs	Single-stranded oligodeoxynucleotides
TA	Tibialis anterior
TALENs	Transcription activator-like effector nucleases
TALEs	Transcription activator-like effectors
X-CGD	X-linked chronic granulomatous disease
ZFNs	Zinc-finger nucleases
μDys	Microdystrophin

1 Introduction

Exploring the underlying mechanisms of genetic diseases and the effect of genotype on phenotype is the primary step in genetic disease treatment approaches. Studies on the underlying mechanisms of human diseases and the development of therapeutic options have been carried out through in vitro or in vivo studies. Although in vitro studies are far from physiological and anatomical consistencies, they are crucial to reveal the findings in explaining the basic mechanisms of diseases. On the other hand, because the human genome contains regions conserved with other mammalian genomes, it has been possible to explain the mechanisms of human diseases using animals such as rats, mice, or non-human primates. The lack of the systemic point of view of in vitro studies has been revealed by in vivo studies of mammals with high homology to humans. For example, mice, which are a notable example in the study of human diseases, have only 1% non-homologous genome (Mouse

Genome Sequencing Consortium 2002), contributing to the advances of studies on human diseases.

Genetic disease modeling and treatment studies focus on cells carrying the genetic defect that constitutes its phenotype. Although the molecular mechanisms of genetic diseases at the cellular level are understood in in vitro approaches, it is not possible to observe systemic effects. In contrast, in vivo animal models allow the systemic effects of phenotype change to be demonstrated. However, although mammalian species with high homology are utilized to study genetic diseases, it is not possible to conduct in vivo disease models and therapy studies in all genetic disease types due to the change in the species-specific effects of genetic defects, and especially animal models are inapplicable for late-onset genetic diseases (Halevy and Urbach 2014). For this reason, the use of stem cells of human origin, which have a high capacity to transform into different cell types, provides great advancements for understanding human genetic diseases.

Cells derived from disease-carrying or healthy individuals can be used to develop genetic disease models or therapeutic options (Avior et al. 2016). Developing a model from a healthy individual or patient may vary according to the characteristics of the disease. For example, if a model is to be developed on a genetic disease with a complex genotypic structure, strategies can be followed to confirm the exact mechanism of the disease based on the cells of the patient or to model a known disease phenotype through cells obtained from a healthy individual (Avior et al. 2016; Sterneckert et al. 2014). In both cases, stem cells emerge as unique tools for understanding the molecular mechanisms in genetic diseases and their effect on phenotype, as they have the potential to express the genetic defect in particular cell types.

In stem cell-based disease recapitulating or genetic correction methods, induced stem cells derived from stem cells or somatic cells of a healthy or disease-carrying individual can be used in two approaches (Choi et al. 2013; Jang and Ye 2016; Park et al. 2017; Xia et al. 2018; Pratumkaew et al. 2021): (i) elucidating genotype-phenotype relationships, understanding disease pathogenesis, or developing drug screening platforms by inducing disease-causing mutations in stem cells and (ii) development of direct therapeutic options by manipulation of cells with genetic correction in stem cells carrying disease-causing mutation (Fig. 1).

This chapter focuses on current stem cell-based approaches in genetic diseases. In this context, conventional stem cell-based approaches

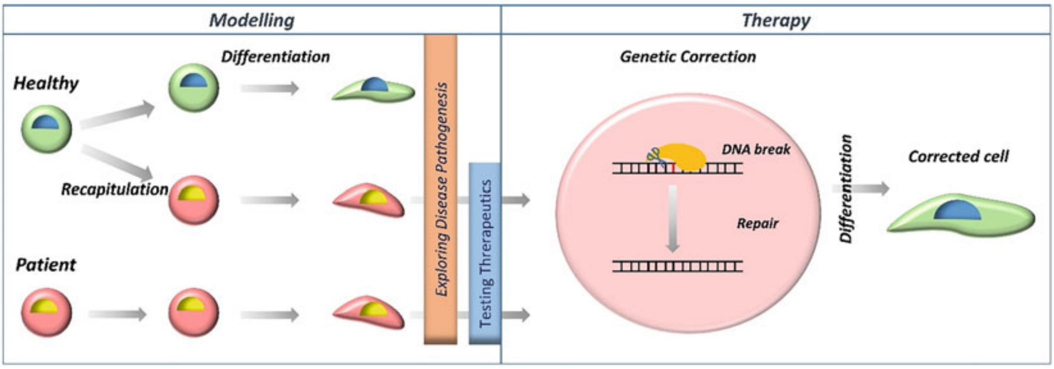

Fig. 1 Stem cell technologies for modeling and therapy of genetic diseases. Understanding the genetic and molecular mechanisms for the expression of the abnormal phenotype in genetic diseases, the disease genotype can be recapitulated in stem cells obtained from healthy individuals, by using genome editing tools. By monitoring phenotypic abnormalities, disease pathogenesis can be elucidated, and platforms can be developed for testing potential therapeutics. Genetically corrected stem cells can be used for therapeutic purposes by designing genome editing tools

and state-of-the-art systems of genome manipulation techniques in stem cell-based methods in genetic diseases are mentioned.

2 Conventional Stem Cell Therapies in Genetic Disease

Stem cells are non-differentiated cells with the ability to self-renew and differentiate into a specific cell type (Zakrzewski et al. 2019). These cells, which offer attractive options in the treatment of various diseases due to these properties, have become one of the most crucial elements of regenerative medicine and have revolutionized medicine.

Stem cell-based therapies are treatments that mainly involve the use of embryonic stem cells (ESCs), adult stem cells (ASCs), or induced pluripotent stem cells (iPSCs) in an autologous or allogeneic manner (Zhang et al. 2020). This therapeutic approach has also been frequently used in the research for treatments for genetic diseases, which are defined as abnormalities in an individual's genetic makeup. In the preclinical studies, the selection of the stem cell type to be used in the treatment, the safety of the use of these cells in in vivo applications, the effects to be created in animal models, and various pre-treatments (induction, coculture, or treatment with amino acids, growth factor, etc.) that may be efficient in increasing these effects were explored (Table 1). After obtaining preliminary results, which can reverse the course of the disease and promise its safety, clinical studies have been introduced (Table 2). In these studies, stem cells are grown in culture for the treatment of various genetic diseases and then transplanted to the patient in various methods and at different dosages. In addition to the limitations of stem cells (isolation, ethical issues, teratoma formation, etc.), the greatest dilemma encountered in transplantation studies is that the response to treatment can vary according to the phenotype of the patient. Besides, the procedure of administration of cells to patients and the dosage regimen differ in the studies. One of the other complications encountered in clinical studies on genetic diseases is the use of allogeneic stem cells in order to transplant healthy stem cells carrying unmutated genes, and this causes an immune response in the host. All these findings revealed the essentials of studies examining the long-term safety of stem cell therapy, focusing on autologous cell transplantation studies and optimizing transplantation protocols.

3 New Generation of Stem Cell-Based Genetic Therapy

The main significative of the success of stem cell-based treatment approaches for genetic diseases is not carrying disease-specific genomic defects and not forming immune response. For this reason, studies focused on modifications that create the gene products that ensure normal function in the cells with genetic defect. Early approaches to achieving normal gene expression in patients involved manipulation of gene expression with custom vectors. The most common methods have been based on vectors of viral origin capable of carrying human gene sequences. Viruses can infect human cells and transfer the genes that will enable the expression of gene products that cannot be expressed by the patient. Lentiviruses, retroviruses, herpes simplex, rhabdoviruses, adenoviruses, and especially adeno-associated viruses have been frequently used (Lundstrom 2018; Robbins and Ghivizzani 1998). Although viral vectors provide advanced methods for gene therapy, they have limitations such as immunogenicity, DNA packaging capacity, undesired genome integration, and carcinogenesis (Lundstrom 2018; Yin et al. 2014). To eliminate these limitations, vector platforms based on inorganic particulate, lipid nanoparticles, peptide-based vectors, or natural/synthetic polymers have been developed with advanced materials science techniques (Ramamoorth and Narvekar 2015). Although safety and higher efficiency of carrying genetic material are achieved with these approaches, challenges have arisen such as the ability of vectors to escape from the immune system, targeting, and entry of mammalian cells (Yin et al. 2014; Ramamoorth and Narvekar 2015;

Table 1 Preclinical studies on conventional stem cell therapies in genetic diseases

Cell type	Genetic disease	Method	Outcome	References
MuSCs	DMD	Transplantation of MuSCs into dystrophic dog	MuSC systemic transplantation resulted in long-term dystrophin expression, muscle damage course limitation with an increased regeneration efficiency, and persisting stabilization of the dog's clinical status	Rouger et al. (2011)
MuSCs	DMD	Treatment with the NAD$^+$ precursor NR	NR has a favorable effect on MuSC function and regeneration in mdx mice. NR prevents MuSC senescence	Zhang et al. (2016)
MuSCs	DMD	Treatment of mdx mice with AA	AA treatment stimulated MuSC activation and proliferation during muscle regeneration	Zou et al. (2016)
MuSCs	DMD	Treatment of mdx mice with PDGF-BB	Analysis of muscles from the mice treated with PDGF-BB showed an increased population of MuSCs and an increase in the number of regenerative fibers, with a reduction in inflammatory infiltrates	Piñol-Jurado et al. (2017)
MuSCs	DMD	Treatment of mdx mice with EGF	EGF treatment activated asymmetric divisions of dystrophin-deficient MuSCs in mdx mice, increasing progenitor numbers, enhancing regeneration, and restoring muscle strength	Wang Feige et al. (2019a, b, c)
MuSCs	DMD	Treatment of mdx mice with PMO in glycine	PMO co-administered with glycine in mdx mice produced up to a 50-fold increase in abdominal muscle dystrophin compared to PMO in saline Glycine enhanced satellite cell proliferation and muscle regeneration by increasing activation of the mTORC1 Glycine also increased the transplantation efficiency of exogenous satellite cells and primary myoblasts in mdx mice	Lin et al. (2020)
MuSCs	DMD	Treatment of DMD model rats with MCT-KD	MCT-KD promoted the proliferation of MuSCs, suggesting enhanced muscle regeneration The muscle strength of DMD model rats fed with MCT-KD was significantly improved even at the age of 9 months	Fujikura et al. (2021)

(continued)

Table 1 (continued)

Cell type	Genetic disease	Method	Outcome	References
MuSCs	DMD	Treatment of MuSCs and mdx mice with antagomir-92a	Treatment with antagomir-92a increased capillary density and tissue perfusion, which was accompanied by an increase in MuSCs However, antagomir-92a suppressed myogenic differentiation in MuSC culture	Verma et al. (2019)
MuSCs	DMD	Ablation of miR-133b in the mdx mice	Ablation of miR-133b exacerbated the dystrophic phenotype of DMD-afflicted skeletal muscle by dysregulating MuSCs involved in muscle biogenesis, in addition to affecting signaling pathways related to inflammation and fibrosis	Taetzsch et al. (2021)
MuSCs	DMD	Depletion of dystrophic MuSCs in mdx mice	Depletion of MuSCs at 2 months of age in dystrophic mice resulted in histological improvements (less damaged fibers, less fibrosis, increased sarcolemma integrity, and increased the size of the remaining myofibers)	Boyer et al. (2019)
MSCs	DMD	Treatment of BM-MSCs with Wnt3a and transplantation into mdx mice	Wnt3a-treated BM-MSCs in mdx mice resulted in long-term amelioration of the dystrophic phenotype and restore dystrophin expression in the muscle	Shang et al. (2016)
MSCs	DMD	Transplantation of cell fusions of allogeneic human MB and human BM-MSC into mdx mice	At 90 days post-transplant of DEC increases in dystrophin expression correlated with improved muscle strength and function in mdx mice The differentiated human MB/ BM-MSC DEC cell line introduced novel therapeutic approach combining myogenic and immunomodulatory properties of MB and MSC	Siemionow et al. (2021)
MSCs	DMD	Transplantation of DP-MSCs into mdx mice and CXMD$_j$ dog	DP-MSC-treated mdx mice and CXMD$_j$ dog did not show serious adverse effects DP-MSC treatment downregulated severe inflammation in DMD muscles and increased recovery in grip-hand strength and improved tetanic force and home-cage activity	Nitahara-Kasahara et al. (2021)

(continued)

Table 1 (continued)

Cell type	Genetic disease	Method	Outcome	References
MSCs	SMA	Treatment of UC-MSCs with VPA	In response to VPA, increased SMN mRNA and protein expression was observed in UC-MSC and FBs isolated from patients with SMA	Iwatani et al. (2017)
MSCs	SMA	Treatment of AD-MSCs with PNA	After 2 weeks of treatment, cultures supplemented with PNA showed a longer survival and an increase in the expression of SMN2	Mohseni et al. (2019)
MSCs	SMA	Transplantation of human A-MSCs into SMA model mice	Human A-MSC transplantation in the uterus of mice showed better performance in all motor function tests and improved cellular and behavioral outcomes	Shaw et al. (2021)
MSCs	ALS	Neural induction of human BM-MSCs and transplantation into SOD1 mice	A single treatment with neurogenin 1-induced BM-MSCs was sufficient to improve motor function during the symptomatic period, whereas non-induced BM-MSCs required repeated transplantation to achieve similar levels of motor function improvement	Chan-Il et al. (2013)
MSCs	ALS	Transplantation of BM-MSCs into SOD1 mice	After transplantation of BM-MSCs, the survival rate of SOD1 mice increased and motor dysfunction reduced Transplanted BM-MSCs inhibited inflammatory response in SOD1 mice	Forostyak et al. (2011), Uccelli et al. (2012), Zhou et al. (2013), Boido et al. (2014), Forostyak et al. (2014)
MSCs	ALS	Transplantation of AD-MSCs into SOD1 mice	AD-MSCs transplanted to SOD1 mice, delayed motor dysfunction, and disease progression	Marconi et al. (2013), Kim et al. (2014b)
MSCs	ALS	Transplantation of UC-MSCs into SOD1 mice	Evaluating UC-MSC distribution after administration showed that the transplantation in brain ventricles permits a longer permanence of UC-MSCs near the injured tissues UC-MSC transplantation delayed motor neuron death but did not prevent disease progression in SOD1 mice	Violatto et al. (2015), Sironi et al. (2017)
MSCs	ALS	Transplantation of human A-MSCs into SOD1 mice	Compared with the control group, multiple A-MSC transplantations significantly retarded disease progression, extended survival, improved motor function, prevented motor neuron loss, and decreased neuroinflammation in SOD1 mice	Sun et al. (2014)

(continued)

Table 1 (continued)

Cell type	Genetic disease	Method	Outcome	References
MSCs	CF	Human A-MSCs coculture with CF airway epithelial cells	When human A-MSCs cocultured with respiratory epithelial cells, human A-MSCs acquired a detectable CFTR expression Gap junction-mediated intercellular communication was involved in the correction of CFTR chloride channel activity upon the acquisition of an epithelial phenotype by hAMSCs in coculture with CF cells	Paracchini et al. (2012), Carbone et al. (2014, 2018)
MSCs	CF	Transplantation of human MSCs into CF model mice	Human MSCs decreased bacterial burden in CF model mice infected with *Pseudomonas aeruginosa* Inflammatory action of MSCs was through regulating the inflammatory transcription regulator PPARγ as well as the production of chemokines ultimately impacting the phenotype of the inflammatory response	Bonfield et al. (2013), Sutton et al. (2016, 2017)
MSCs	CF	EVs derived from L-MSCs	EVs derived from L-MSCs could be used to control the hyper-inflamed condition in CF mediated by upregulation of the PPARγ axis	Zulueta et al. (2018)
MSCs	HD	Transplantation of BM-MSCs into QA-lesioned HD model rat	Transplantation of BM-MSCs significantly reduced motor dysfunction and ventriculomegaly in the lesioned brain In the striatum of the BM-MSC-treated group, the expression levels of NGF, BDNF, GDNF, and CNTF were significantly elevated when compared with those of the control group	Jiang et al. (2011), Moraes et al. (2012), Lin et al. (2011)
MSCs	HD	Transplantation of BM-MSCs into 3NP HD model rat	Increased BDNF, collagen type I, and fibronectin (but not GDNF or CNTF) were observed in the brains of BM-MSC-transplanted rats In addition, none of the transplanted BM-MSCs expressed neural phenotypes	Rossignol et al. (2011)

(continued)

Table 1 (continued)

Cell type	Genetic disease	Method	Outcome	References
MSCs	HD	Transplantation of BM-MSCs into R6/2 mice	Treated R6/2 mice displayed significantly less neuropathological deficits, more significant behavioral sparing relative to untreated R6/2 mice Compared to the control group, the treated R6/2 mice showed an increased survival rate	Rossignol et al. (2015), Yu-Taeger et al. (2019)
MSCs	HD	Combined treatment with BM-MSCs and LER into 3NP HD model rat	The combined therapy improved motor and behavior performance and increased BDNF, Wnt, and β-catenin protein expression Amelioration of striatum tissue injury was observed	Elbaz et al. (2019)
MSCs	HD	Transplantation of UC-MSCs into R6/2 mice	R6/2 mice-transplanted UC-MSCs displayed significantly less neuropathological deficits, relative to untreated R6/2 mice	Fink et al. (2013)
HSCs	SCD thalassemia	Transplantation of HSCs in utero	This approach takes advantage of the immunological immaturity and normal developmental characteristics of the fetus to achieve donor-specific tolerance	Peranteau et al. (2015)
ESCs	DMD	Transplantation of murine ESCs into mdx mice	Transplanted ESCs differentiated into Pax7$^+$ cells with MuSC characteristics. Dystrophin$^+$ fibers were observed in the muscles of mdx mice	Chal et al. (2015)
ESCs	DMD	Transplantation of murine ESCs into mdx mice	After transplantation of ESCs, teratomas formed in adult skeletal muscle differentiated in vivo to produce a large number of myogenic progenitors Teratoma-derived myogenic progenitors contributed MuSCs and could be used as the source of potent transplantable MuSCs	Chan et al. (2018)
ESCs	HD	Neural differentiation of human ESCs and transplantation into QA-lesioned HD model rat	Neuronal induction directed ESCs to enriched populations of DARPP32-expressing forebrain GABA neurons Transplantation of these human forebrain GABA neurons and their progenitors into the striatum of QA-lesioned mice resulted in the generation of large populations of DARPP32$^+$ GABA neurons	Ma et al. (2012)

(continued)

Table 1 (continued)

Cell type	Genetic disease	Method	Outcome	References
ESCs/ iPSCs	HD	Neural differentiation of human PSCs and transplantation into QA-lesioned HD model rat	Human PSCs were induced to differentiate into medium-sized spiny neurons Transplanted into the striatum of QA-lesioned rats, hPSC-derived neurons survived and differentiated into DARPP-32 neurons, leading to a restoration of apomorphine-induced rotation behavior	Delli Carri et al. (2013)
ESCs/ iPSCs	HD	Neural differentiation of human PSCs and transplantation into HD model rat	Activin A induced lateral ganglionic eminence characteristics in nascent neural progenitors derived from hPSCs Human lateral ganglionic eminence progenitors readily differentiated into post-mitotic neurons expressing the DARPP32, following transplantation in the adult striatum in a rat model of HD	Arber et al. (2015)
iPSCs	HD	Neural differentiation of HD-iPSCs and transplantation into HD model rat	Results indicated that, although HD-iPSC carrying 72 CAG repeats can form GABAergic neurons and give rise to significant behavioral recovery in a rat model of HD, developed HD pathology at later stages of transplantation	Jeon et al. (2012)
iPSCs	HD	Differentiation into NSCs and transplantation into HD model mice	NSCs survived and differentiated into region-specific neurons in HD mice Amelioration of locomotor deficits was shown in HD mice that received NSCs	Al-Gharaibeh et al. (2017)
iPSCs	HD	Differentiation into NPCs and transplantation into QA-lesioned HD model rat	After transplantation, animals showed significant behavioral improvements Transplanted NPCs partially replaced the lost neurons, enhanced endogenous neurogenesis, reduced inflammatory responses, and reconstituted the damaged neuronal connections	Yoon et al. (2020)
iPSCs	DMD	Treatment with Wnt activator and BMP receptor inhibitor	This method induced the differentiation of myogenic cells. hiPSC-derived myogenic cells differentiated into myofibers in muscles of mdx mice after transplantation	Sakai-Takemura et al. (2018)

(continued)

Table 1 (continued)

Cell type	Genetic disease	Method	Outcome	References
iPSCs	ALS	Differentiation into NSCs and transplantation into SOD1 mice	NSCs derived from iPSCs migrated into the central nervous system Compared with control, treated SOD1 mice exhibited improved neuromuscular function and motor unit pathology and increased survival rate	Nizzardo et al. (2014)
iPSCs	CF	Differentiation of CF-iPSCs into lung progenitor cells	CF-specific lung progenitor cells produced from human CF iPSCs formed respiratory epithelium when subcutaneously transplanted into immunodeficient mice	Mou et al. (2012)

AA acetoacetate, *A-MSCs* amniotic mesenchymal stem cells, *AD-MSCs* adipose-derived mesenchymal stem cells, *ALS* amyotrophic lateral sclerosis, *BDNF* brain-derived neurotrophic factor, *BM-MSCs* bone marrow-derived mesenchymal stem cells, *BMM* bone marrow-derived macrophages, *BMP* bone morphogenetic protein, *CF* cystic fibrosis, *CFTR* cystic fibrosis transmembrane conductance regulator, *CNTF* ciliary neurotrophic factor, *DARPP32* dopamine- and cAMP-regulated phosphoprotein 32 kDa, *DEC* dystrophin expressing chimeric, *DMD* Duchenne muscular dystrophy, *DP-MSCs* dental pulp-derived mesenchymal stem cells, *EGF* epidermal growth factor, *ESCs* embryonic stem cells, *EVs* extracellular vesicles, *GABA* γ-aminobutyric acid, *GDNF* glial cell line-derived neurotrophic factor, *HD* Huntington's disease, *hfMSCs* human fetal mesenchymal stem cells, *IL* interleukin, *iPSCs* induced pluripotent stem cells, *L-MSCs* lung mesenchymal stem cells, *MB* myoblasts, *MCT-KD* ketogenic diet with medium-chain triglycerides, *MSCs* mesenchymal stem cells, *LER* lercanidipine, *mTORC1* mammalian target of rapamycin complex 1, *MuSCs* muscle stem cells, *NAD* nicotinamide adenine dinucleotide, *NGF* nerve growth factor, *NPCs* neural precursor cells, *NR* nicotinamide riboside, *NSCs* neural stem cells, *PDGF-BB* platelet-derived growth factor BB, *PMO* phosphorodiamidate morpholino oligomer, *PNA* ponasterone A, *PPARγ* peroxisome proliferator activator receptor gamma, *PSCs* pluripotent stem cells, *QA* quinolinic acid, *SMA* spinal muscular dystrophy, *SMN* survival motor neuron, *UC-MSCs* umbilical cord-derived mesenchymal stem cells, *VEGF* vascular endothelial growth factor, *VPA* valproic acid, *3NP* 3-nitropropionic acid

Hardee et al. 2017). In parallel with the developments in these methods, which are still under effort, the developments in genome editing techniques for genetic manipulation have started a new era in stem cell manipulations.

Genome editing techniques incorporate targeted breaks in specific genomic locus of custom-designed nucleases followed by strand repair. Zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas-associated nucleases are utilized in genome editing.

ZFNs, which emerge with the complex of the DNA-binding region and the catalytic (FokI) enzyme region, commit the double-strand break (DSB) in the relevant DNA region (Urnov et al. 2010). Cys2-His2 fingers, consisting of antiparallel β-sheet opposing α-helix structures of about 30 amino acids, each form a DNA-binding (zinc-finger protein) region that regulates 3 bp of DNA. Although ZFNs provide advanced approaches for genome editing, they also have limitations due to the high affinity of the zinc-finger domains to G-rich sequences or off-target binding (Brookhouser et al. 2017). Therefore, current studies have focused on the designs of ZFNs that provide target-site binding with high specificity.

Transcription activator-like effectors (TALEs) containing highly conserved repeat sequences provide DNA binding via their 33 to 35 aa conserved motifs in their central region. TALENs ensure DNA-binding specificity by virtue of their hypervariable amino acids (also called repeat variable di-residues, RVD) at locations 12 and 13 in the conserved region (Becker and Boch 2021). Unlike ZFNs, TALENs do not have a limitation as having the same target-site sequences of DNA

Table 2 Clinical studies on conventional stem cell therapies in genetic diseases

Cell types		Genetic disorder	Donor	Number of patients	Phase	Clinical trial ID/ref.
MSCs	BM-MSCs	DMD	Autologous	20	Phase I and II	NCT03067831
	BM-MSCs	DMD	Autologous	25	Phase I and II	NCT01834066
	UC-MSCs	DMD	Allogeneic	15	Phase I and II	NCT01610440
	UC-MSCs	DMD	Allogeneic	10	Phase I and II	NCT02285673
	UC-MSCs	DMD	Allogeneic	1	Phase I	NCT02235844/Patel and Riordan (2015)
	UC-MSCs	DMD	Allogeneic	10	Phase I	NCT02484560/Dai et al. (2018)
	BM-MSCs	IRD	Allogeneic	14	Phase I	NCT01531348/Tuekprakhon et al. (2021)
	BM-MSCs	IRD	Autologous	500	–	NCT03011541/Weiss and Levy (2018)
	UC-MSCs	IRD	Allogeneic	18	Phase I and II	NCT04315025/Mangunsong et al. (2019)
	UC-MSCs	IRD	Allogeneic	20	Phase I	NCT05147701
	UC-MSCs	IRD	Allogeneic	50	Phase II	NCT04763369
	WJ-MSCs	IRD	Allogeneic	32	Phase III	NCT04224207/Özmert and Arslan (2020)
	AD-MSCs	SMA	Allogeneic	10	Phase I and II	NCT02855112/Mohseni et al. (2020)
	MSCs	ALS	Autologous	3	Phase I	NCT02987413
	BM-MSCs	ALS	Autologous	30	Phase I	NCT02881489
	BM-MSCs	ALS	Autologous	20	Phase I and II	NCT04821479/Petrou et al. (2021)
	BM-MSCs	ALS	Autologous	6 8	Phase I Phase I	NCT01759797/Nabavi et al. (2019) NCT01771640/Nabavi et al. (2019)
	BM-MSCs	ALS	Autologous	28	Phase I and II	NCT02917681
	BM-MSCs	ALS	Autologous	12 14 48 263	Phase I and II Phase II Phase II Phase III	NCT01051882/Petrou et al. (2016) NCT01777646/Petrou et al. (2016) NCT02017912/Berry et al. (2019) NCT03280056
	BM-MSCs	ALS	Autologous	72 115	Phase I and II Phase III	NCT01363401 NCT04745299
	BM-MSCs	ALS	Autologous	26	Phase I and II	NCT03828123/Sykova et al. (2017)
	UC-MSCs	ALS	Allogeneic	20	Phase I	NCT05003921
	UC-MSCs	ALS	Allogeneic	30	Phase II	NCT01494480
	WJ-MSCs	ALS	Allogeneic	20	Phase I and II	NCT04651855
	WJ-MSCs	ALS	Allogeneic	30	Phase I	NCT02881476/Barczewska et al. (2019)
	AD-MSCs	ALS	Autologous	1 27 60	Phase I Phase I Phase II	NCT01142856 NCT01609283/Staff (2016) NCT03268603
	AD-MSCs	ALS	Allogeneic	19	Phase I	NCT02492516

(continued)

Table 2 (continued)

Cell types		Genetic disorder	Donor	Number of patients	Phase	Clinical trial ID/ref.
	AD-MSCs	ALS	Autologous	52	Phase I and II	NCT02290886
	AD-MSCs	ALS	Autologous	30	Phase I	NCT03296501/Kuzma-Kozakiewicz et al. (2018)
	DP-MSCs	HD	Allogeneic	6 35 35	Phase I Phase II Phase II and III	NCT02728115 NCT03252535 NCT04219241
	BM-MSCs	CF	Allogeneic	14	Phase I	NCT02866721
	BM-MSCs	Hemophilia	Allogeneic	20	Phase I	NCT02108132
HSCs		Thalassemia	Allogeneic (haploidentical)	30 800	Phase IV Phase IV	NCT03171831 NCT04009525
		Thalassemia	Semi-allogeneic	10	Phase I	NCT02986698/MacKenzie et al. (2020)
		Thalassemia	Allogeneic	150	–	NCT02307786
		SCD thalassemia	Allogeneic (haploidentical)	6	Phase I	NCT03249831
		SCD thalassemia	Allogeneic (haploidentical)	20	Phase II	NCT03367546
		SCD thalassemia	Allogeneic	25	Phase I and II	NCT00730314
		SCD thalassemia	Allogeneic	29	Phase I and II	NCT02435901
		SCD thalassemia	Allogeneic	40	Early phase I	NCT04776850
		SCD	Allogeneic	25	Phase I and II	NCT02065596
		SCD	Allogeneic	38	–	NCT04207320
		SCD	Allogeneic	78	Phase III	NCT04046705
		SCD	Allogeneic (haploidentical)	15	Phase I	NCT03279094
		SCD	Allogeneic	58	Phase II	NCT04018937
		ALS	Autologous	14	Phase II and III	NCT01933321
NSCs		ALS	Allogeneic	18 18	Phase I Phase II	NCT01348451 NCT01730716
		ALS	Allogeneic	18	Phase I	NCT01640067/Gelati et al. (2022)
ESCs		IRD	Allogeneic	10	Phase I	NCT03944239
		IRD	Allogeneic	12	Phase I and II	NCT03963154
		ALS	Allogeneic	16	Phase I and II	NCT03482050
iPSCs		HD SMA IRD ALS	Autologous	120	–	NCT00874783
		Thalassemia	Autologous	2	–	NCT03222453

Clinical trials that are recruiting, not yet recruiting, enrolling by invitation, active but not recruiting, completed, or unknown: https://clinicaltrials.gov. Accessed 25 January 2022

AD-MSCs adipose-derived mesenchymal stem cells, *ALS* amyotrophic lateral sclerosis, *BM-MSCs* bone marrow-derived mesenchymal stem cells, *CF* cystic fibrosis, *DMD* Duchenne muscular dystrophy, *ESCs* embryonic stem cells, *HD* Huntington's disease, *HSCs* hematopoietic stem cells, *IRD* inherited retinal dystrophy, *NSCs* neural stem cells, *SCD* sickle cell disease, *SMA* spinal muscular dystrophy, *UC-MSCs* umbilical cord-derived mesenchymal stem cells, *WJ-MSCs* Wharton's jelly-derived mesenchymal stem cells locus, but TALENs have some challenges in their implementation due to the fact that the 5′ base of the TALEN target region must be thymine, and they are negatively affected by DNA methylation (Cox et al. 2015; Bultmann et al. 2012).

The CRISPR/Cas system, which was discovered as a mechanism in the adaptive immune system of prokaryotes, is the most recent genome editing tool. This RNA-guided system is generally classified according to the structural variation in the Cas gene and their organization. Class II CRISPR-associated protein 9 (Cas9) is the most used type that consists of a single CRISPR/Cas9 endonuclease and a single-guide RNA (sgRNA) particularly designed for the target DNA region (Brookhouser et al. 2017). Guide RNA comprises a complementary 20 bp region specific to the target DNA region and a protospacer adjacent motif (PAM) that guides the Cas9 protein used for catalytic activity. The part that determines the specificity in the CRISPR/Cas9 system is the sgRNA component, which yields high modularity (Wiedenheft et al. 2012; Li et al. 2020a). Although CRISPR/Cas-based technologies are the most appropriate approaches for high specificity, intense efforts are still being made to overcome the limitations caused by off-target effects.

Double-strand breaks (DSB) eventuated custom-designed nuclease activity in eukaryotic cells enter into non-homologous end joining (NHEJ) or homology-directed repair (HDR) with donor DNA template. In the homology-directed repair mechanism, mutation correction or new sequence insertion can be accomplished by reconstructing the broken DNA sequences with the exogenous DNA template (Verma and Greenberg 2016). Alternatively, by NHEJ mechanisms, the DSB site can be edited by small insertion or deletion mutations without a donor DNA (Chang et al. 2017). These advanced gene editing tools are quite remarkable in stem cell-based modeling and treatment approaches of genetic diseases (Byrne et al. 2014). Current approaches to these promising methods for correcting disease-causing mutations and providing a healthy phenotype are discussed according to stem cell types.

3.1 Muscle Stem Cells (MuSCs)

Muscle stem cells (MuSCs), also known as satellite cells, are cells involved in the growth and repair of skeletal muscles (Yin et al. 2013; Snijders et al. 2015). These cells are named as satellite cells because of their anatomical location between the sarcolemma and the basal lamina of the muscle fibers. These cells, which are dormant in adult muscle, can differentiate into myoblasts that will take part in repair following muscle injury (Snijders et al. 2015; Siegel et al. 2011). They also have the ability to self-renew to maintain their populations. Given their contribution to muscle regeneration, these cells have frequently been studied for stem cell-based therapy of Duchenne muscular dystrophy (DMD).

DMD is a neuromuscular disorder that results from frameshift mutations in the dystrophin gene, resulting in the loss of physiological activity of skeletal muscle (Ervasti 2007; Emery et al. 2015). Current studies on the treatment of DMD have aimed to repair the mutated dystrophin gene of MuSCs and restore their functional loss by using different methods. For this purpose, Nik-Ahd et al. used single-stranded oligodeoxynucleotides (ssODNs) to repair the dystrophin gene in MuSCs (Nik-Ahd and Bertoni 2014). First, MuSCs were isolated from hind limb muscles of DMD model mdx mice, and these cells were transfected with fluorescently labeled peptide nucleic acid (PNA)-ssODNs and then transplanted back into mdx mice. It was observed that the number of dystrophin^{+} fibers increased over time. Many studies have used adeno-associated virus serotype-9 (AAV9) and CRISPR technology together for the excision of intervening DNA that causes mutations in MuSCs (Tabebordbar et al. 2016; Nance et al. 2019; Kwon et al. 2020). Tabebordbar et al., in their study, found that about 4% of MuSCs they isolated from mdx mice treated with in vivo AAV9 CRISPR were regulated by mutant exon 23 gene (Tabebordbar et al. 2016). Nance et al. removed the tibialis anterior (TA) muscle instead of isolating cells ~17 months after injection from mice in which AAV9.Cas9 and AAV9.gRNA were co-injected to avoid events such as missing and/or mislabeling or cell loss during the isolation of MuSCs (Nance et al. 2019). A portion of the muscle was used for dystrophin expression analysis, and analysis showed 22.4% ± 13.0% dystrophin$^+$ fibers in AAV9 CRISPR-treated mice. The other half of the TA muscle was grafted into immunodeficient NSG.mdx4cv mice to investigate whether the mutant exon 23 gene was recovered in MuSCs. At 6 weeks post-graftment, mdx mice treated with AAV9 CRISPR had a higher proportion of dystrophin$^+$ fibers than the control group. All these studies showed that transduction of MuSCs by AAV9 is possible during the active myogenesis stage in rodents. Unlike these studies, Vallejo et al. showed that miR-31 was suppressed in cells after the c-isoform of the pituitary homeobox 2 transcription factor (Pitx2c), which is known to play the role in myogenesis, was transfected into MuSCs isolated from mdx mice with a lentiviral bicistronic vector (pLVX-Pitx2c-IRES-ZsGreen) (Vallejo et al. 2018). Accordingly, an increase in the amount of dystrophin mRNA transcripts lacking mutant exons was observed. Also in the study, injection of cells into the TA muscle of immunocompromised mdx mice resulted in overexpression of Pitx2c in cells and an increase in regenerated fiber size. Further analysis revealed that Pitx2c activates the miR-106b/miR-503/miR-23b/miR-15b pathway, stimulating the proliferation of MuSCs and increasing their regenerative potential. Increased cell and transcript numbers resulted in improved physical performance in mice.

Considering its contribution to muscle regeneration, MuSCs have a profoundly high potential for use in stem cell therapy in muscular diseases. However, studies have revealed that there are some limitations that prevent the use of these cells in stem cell therapy. One of these is that a limited number of cells can be isolated from dystrophic muscle. This limitation prevents working with cells obtained from sick individuals and thus prevents autologous studies. In addition, allogeneic studies revealed that immunosuppression should definitely be used. In addition, the low survival rate of these cells after transplantation and their negligible migration rate from the area where they are transplanted prevent long-term gene expression and/or an expected level of improved physical performance.

3.2 Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells (MSCs) were defined as plastic-adherent, fibroblast-like, and colony-forming unit-fibroblast (CFU-F) cells, and it was stated that these cells are a subpopulation of bone marrow cells (Friedenstein et al. 1966; Friedenstein et al. 1970). These cells are also called mesenchymal stromal cells since they also form the basis of the stroma cells, known as the supporting part of the tissues (Horwitz et al. 2005). In order to classify these cells, which do not have unique cell surface markers, the International Society for Cell & Gene Therapy (ISCT) has defined the following criteria (Dominici et al. 2006; Aslan et al. 2018):

1. Cells must be able to adhere to plastic under basic culture conditions.
2. Cells should express only CD73, CD90, and CD105, lacking expression of the hematopoietic surface markers CD14, CD34, CD45 or CD11b, CD19 or CD79a, and HLA-DR surface molecules.
3. Cells must be able to differentiate into adipocytes, chondrocytes, and osteoblasts in vitro.

MSCs, whose paracrine effect has been proven in recent years thanks to the extracellular vesicles they secrete, can be easily isolated from many different tissues and organs, expanded ex vivo, and used in an autologous manner (Aslan et al. 2018). Besides, MSCs weakly express human leukocyte antigen (HLA) class I and do not express HLA class II (Toma et al. 2002). This property ensures that the immune response to these cells is less after transplantation.

Considering all their properties, the potential therapeutic efficacy of MSCs for various genetic diseases has been investigated. Many studies have been conducted to repair the dystrophin gene in MSCs for the treatment of DMD disease (Xiong et al. 2010; Feng et al. 2012; Wang et al.

2019a; Li et al. 2012). In these studies, MSCs were transduced with various vectors (adenovirus, retrovirus, and baculovirus) containing the microdystrophin (μDys) gene to genetically engineer MSCs. After transplantation of these cells into mdx mice, dystrophin expression was observed in the muscles of the mice (Xiong et al. 2010; Feng et al. 2012; Wang et al. 2019a), and creatine kinase (CK) levels were decreased (Wang et al. 2019a; Xiong et al. 2010). In addition, transplanted MSCs were shown to differentiate into MuSCs in one of these studies (Feng et al. 2012). In a recent study using recombinant AAV9-μDys and MSCs, MSCs were not transduced, and their immunomodulatory properties were used to reduce the host immune response against recombinant AAV9-μDys (Hayashita-Kinoh et al. 2021). In this study, firstly, AAV9-μDys and bone marrow-derived MSCs (BM-MSCs) were injected intravascularly together in dystrophic dogs. One week after this injection, BM-MSC and AAV9-μDys injections were repeated 1 day apart. The analyses showed that pre-treatment of MSCs and injection of recombinant AAV9 provided immune tolerance in dystrophic dogs as well as increased expression of the transgene, resulting in improvement in dystrophic phenotypes. The findings suggested that the vector dose required to produce a therapeutic effect can be reduced with this method.

Cystic fibrosis (CF) is a lethal autosomal recessive disease caused by a mutation in the cystic fibrous transmembrane conductivity regulator (CFTR) gene, which encodes a protein expressed in respiratory epithelial cells, pancreas, biliary tract, sweat glands, intestines, and genitourinary system (Cantin et al. 2015). The most common mutation observed is the deletion of phenylalanine 508 (p.F508del). Exosomes, which are secreted from MSCs and play the main role in intercellular cargo transfer, have been used in mutation correction (Villamizar et al. 2021). For this purpose, human BM-MSCs were first transfected with a plasmid containing a novel CFTR Zinc-Finger Protein Fusion (CFZF) with transcriptional activation domains of VP64, P65, and Rta (VPR) to target the CFTR promoter and transcriptionally activate its expression. The exosomes of MSCs were then isolated, and their effect on human bronchial epithelial cells (HuBECs) carrying the F508del mutation obtained from CF patients was studied. The results showed that CFZF-VPR can be packaged into exosomes of MSCs and delivered to HuBECs for strong expression of CFTR.

Another genetic disease in which genetically modified MSCs are used is Huntington's disease (HD). HD is an autosomal dominant neurodegenerative disease that causes neuronal death in which a toxic form of the protein of huntingtin (HTT) gene is formed as a result of CAG repeat expansions in the HTT gene (O'Donovan 1993). One of the approaches attempted for the treatment of this disease is the design of BM-MSCs overexpressing brain-derived neurotrophic factor (BDNF), which has been shown to stimulate the growth and migration of new neurons and intrastriatal transplanting into HD model mice (Dey et al. 2010; Pollock et al. 2016). Findings as a result of this stem cell-based neurotherapeutic approach showed that mice treated with BM-MSCs/BDNF had an increase in neurogenesis-like activities and slowed neurodegenerative processes. Another approach is RNA interference (RNAi) technology. In the study, BM-MSCs expressing short hairpin RNA (shRNA) antisense against HTT gene were designed, and these cells were cocultured with U87 and SH-SY5Y target cells, resulting in decreased levels of mutant protein of HTT gene expressed in target cells (Olson et al. 2012). Kolli et al. used two CRISPR/Cas9 plasmids that nick the DNA in the untranslated region upstream of the open reading frame, and the other nicks the DNA at the exon 1-intron border to prevent toxic protein of HTT gene formation in BM-MSCs from YAC128 mice with HD transgene (Kolli et al. 2017). The results showed that the in vitro transcription of the HTT gene was affected, and gene silencing processing was accomplished successfully.

Hemophilia A (HA) is an X-linked recessive disease that causes insufficient blood clotting as a result of a mutation in factor VIII (FVIII) gene (Bolton-Maggs and Pasi 2003). For a functional protein synthesis, Kumar et al. transduced placenta-derived MSCs (P-MSCs) with a lentiviral vector encoding the FVIII gene (Kumar et al.

2018). In vitro analyses showed that these gene-modified cells retain their multipotency and express high levels of FVIII. P-MSCs were then injected into fetuses of mice at 14.5 embryonic day, and varying rates of expression were observed in offspring after birth. In another study by the same researchers, co-transplantation of modified endothelial colony-forming cells (ECFCs) and P-MSCs into F8 knockout HA mice reduced the volume of blood loss from 562.13 ± 19.84 to 155.78 ± 44.93 μl in a tail clip assay (Gao et al. 2019). Wang et al. studied an alternative gene therapy that could lead to sustained FVIII expression in HA mice. In this study, the vector encoding the FVIII gene was first microinjected into single-cell embryos of wild-type mice, and then BM-MSCs were isolated from these transgenic mice (Wang et al. 2013a). BM-MSCs were then transplanted into HA mice. Analyses showed that the FVIII gene was expressed in HA mice during 5 weeks of observation. In addition, plasma FVIII activities of HA mice increased from 0% to 32% after transplantation, and the activated partial thromboplastin time value decreased compared to untreated HA mice (45.5 s vs. 91.3 s). Apart from studies in animal models, autologous MSCs transduced with a lentiviral vector encoding the FVIII gene are being investigated for the treatment of HA disease in a clinical study (NCT03217032).

Considering its properties such as isolation from many tissues and organs, paracrine effect, and immunomodulatory properties, MSCs are the most favor stem cell type in stem cell therapy studies for various diseases. However, there are some uncertainties that prevent the use of these cells for clinical therapy. The study using human BM-MSCs revealed liver fibrosis induced by these cells (Russo et al. 2006). To date, there have been no reports of in vivo ectopic tissue formation in clinical studies, although preclinical studies have shown this to be a possibility (Bernardo and Fibbe 2012; Thakker and Yang 2014). In addition, because these cells are multipotent, they have the tumorigenic capacity, and genetic instabilities that may occur after their manipulation are not yet clear (Barkholt et al. 2013; Singh et al. 2016). For all these reasons, studies showing that these cells are safe in the long term, apart from temporary improvements, need to be increased.

3.3 Hematopoietic Stem Cells (HSCs)

Hematopoietic stem cells (HSCs) are multipotent cells capable of differentiating into any type of blood cell, including myeloid-lineage and lymphoid-lineage cells (Till and Mc 1961). These long-lived cells, which are found in many organs such as peripheral blood, bone marrow, and umbilical cord blood, can be genetically manipulated and then easily administered to the patient by intravenous infusion (Kondo et al. 2003). All these properties have increased the interest in the use of these cells, especially in the modeling and treatment methods of hematology-related genetic diseases.

Sickle cell disease (SCD) and β-thalassemia are the two most common inherited hemoglobinopathies in the world (Cavazzana et al. 2017). SCD was first described by James Herrick in 1910 as the presence of “odd, elongated, sickle-shaped red blood cells” (Herrick 1910). It is one of the most common genetic diseases caused by a point mutation (Glu6-Val) that occurs as a result of the conversion of hemoglobin in red blood cells to thymine of adenine nucleotide at position 20 of the hemoglobin subunit β (HBB) gene, which encodes the β-globin chain. In these patients, under hypoxic conditions, mutant hemoglobin (HB) polymerizes and causes red blood cells to form a sickle shape when they assemble in parallel. This disease, also known as hemolytic anemia, results in vaso-occlusive crises, systemic inflammation, and multi-organ damage and significantly shortens the patient’s lifespan (Eaton and Hofrichter 1987). Current treatments for SCD include blood transfusions and allogeneic transplantation of HSCs from a healthy donor (Klaver-Flores et al. 2020). Besides, there are drugs (hydroxyurea, glutamine, crizanlizumab, and voxelotor) that have been shown to be effective in the treatment of mild SCD patients and approved by the FDA (Ataga et al. 2017; Eaton and Bunn 2017). However, these drugs cannot cure severe SCD patients. The best option for these patients is still allogeneic HSC transplantation. For this treatment, there is still a risk of immunodeficiency and mortality, leading to reduced quality of life, in view of inadequate donor matching and chemotherapeutic conditioning (Klaver-Flores et al. 2020). In order to eliminate these risks, current studies have focused on collecting HSCs from patients' own bone marrow, genetically modifying these cells, and then returning these cells to the patient.

β-Thalassemia, on the other hand, is a genetic disease similar to SCD, resulting from the absence or reduction of β-globin expression due to single nucleotide substitution or oligonucleotide addition or deletion (Lockwood et al. 2022; Rachmilewitz and Giardina 2011). This disease does not cause vaso-occlusive crises like SCD, but a severe anemia is observed in the patient (Germino-Watnick et al. 2022). Therapeutic globin vectors used in HSC gene therapy studies may be applicable for both β-thalassemia and SCD.

In this area, the first studies started with the use of lentiviral vectors for gene addition. Pawliuk et al. designed a β^A globin gene variant that inhibits HB polymerization and incorporated this variant into a lentiviral vector that they optimized for transfer to HSCs and gene expression in the adult red blood cell line (Pawliuk et al. 2001). They observed that hematological parameters were improved after transplantation into mouse SCD models, and inhibition of red blood cell dehydration and sickling was achieved. Perumbeti et al. showed that lentiviral transmission of the human γ-globin gene as the β-globin regulatory control element in HSCs resulted in sufficient postnatal fetal hemoglobin expression to treat SCD in the Berkeley "humanized" sickle mouse (Perumbeti et al. 2009). In a recent study, the potential of the lentiviral vector (CCL-βAS3-FB) encoding the human HBB gene to inhibit hemoglobin polymerization to prevent the sickling of red blood cells was studied (Romero et al. 2013). The results showed that the lentiviral vector could efficiently transfer the anti-sickling β-globin gene and improve the physiological parameters of the resulting red blood cells. In the vectors used in all these studies, an anti-sickling HBB transgene was placed below the 16-kb human β-locus control region (LCR) (transcriptional control of HBB promoter and key regulatory elements) required for high and regulated expression of the endogenous HBB gene family. Since lentiviral vectors do not contain the entire LCR, the three transcriptionally strongest regions (HS2, HS3, and HS4) were selected from the DNase I hypersensitive region (HS) and reduced in size to fit the vector. In this context, a study examining the effect of the combination of different HS was also conducted (Weber et al. 2018). In this study, HSCs from SCD patients were transduced with lentiviral vectors containing HS2 and HS3 (β-AS3) or HS2, HS3, and HS4 (β-AS3 HS4). Incorporation of the HS4 region showed negligible improvement in transgene expression and greatly reduced vector titer and infectivity in HSCs. On the other hand, lentiviral vectors containing only HS2 and HS3 could be efficiently transferred into HSCs, and it was observed that approximately 60% of the total HBB chains were anti-sickling HBB.

The first clinical trial involving lentiviral β-globin gene transfer was conducted in France (Cavazzana-Calvo et al. 2010). An adult patient with monthly transfusion-dependent severe β-thalassemia since early childhood has been shown to become transfusion-independent approximately 1 year after gene transfer. The hemoglobin level in the blood was kept between 9 and 10 g/dl, and it was observed that one-third of this hemoglobin level contained vector-encoded β-globin. Two studies were then introduced using the BB305 lentiviral vector for transfer of the anti-sickling β-globin ($\beta^{A\text{-}T87Q}$) variant to CD^{34+} cells isolated from patients with β-thalassemia and SCD (NCT01745120 and NCT02151526, respectively) (Thompson et al. 2018; Ribeil et al. 2017). In the study of patients with β-thalassemia, 12 of 13 patients with severe β-thalassemia and 6 of 9 patients with mild β-thalassemia became transfusion-independent approximately 26 months after infusion of gene-modified autologous cells. In the remaining patients, the need for transfusion decreased (Thompson et al. 2018). In the study of the SCD patient, the therapeutic anti-sickling β-globin level remained high even after 15 months of treatment (Ribeil et al. 2017). Clinical trials of NCT02633943 and NCT04628585 were initiated to observe the long-term results of these studies (Magrin et al. 2022).

In addition to the lentiviral vector BB305, the TNS9.3.55 vector containing larger LCR fragments and a longer β-globin promoter sequence was also used in a clinical study (NCT01639690) (Boulad et al. 2013). This study was performed on patients with β-thalassemia, and it was reported that none of the patients were transfusion-independent. This was attributed to the low vector copy number and the severity of the disease. In a clinical study conducted in Italy (NCT02453477), the GLOBE vector containing only the HS2 and HS3 regions of the LCR was used (Marktel et al. 2019). In the study conducted on a total of nine β-thalassemia patients, three adults and six children, the need for transfusion in adults decreased, while three of four evaluable pediatric patients became transfusion-independent. The researchers reported that a higher proportion of vector copy numbers was associated with a better outcome when younger and in the presence of proliferating HSC cells. Early results of a study (NCT02186418) performed with a lentiviral vector encoding the γ-globin gene instead of β-globin gene transfer were described (Grimley et al. 2020). Significant improvement in vaso-occlusive crises was observed in all three SCD patients who participated in the study, and it was reported that this treatment method showed promise in obtaining permanent responses.

Preclinical studies involving gene editing technologies have been conducted in the treatment of SCD and β-thalassemia diseases (Hoban et al. 2015, 2016; Dever et al. 2016; DeWitt et al. 2016). In a study by Hoban et al., they used ZFN technology to correct the mutation in the β-globin gene in CD^{34+} cells isolated from the bone marrow of SCD patients (Hoban et al. 2015). They observed that ZFNs efficiently affected gene correction at the β-globin locus in HSCs when administered with the lentiviral vector or an oligonucleotide donor. The modified cells were then engrafted in immunodeficient mice and shown to be able to differentiate into erythroid, myeloid, and lymphoid cell types. The same researchers also conducted a study comparing CRISPR/Cas9 and TALEN technologies to correct the mutation in the β-globin gene in addition to ZFN (Hoban et al. 2016). As a result of this study, the researchers reported that they observed the highest gene correction rate in the cells they obtained using CRISPR/Cas9.

In the preclinical studies, especially with the use of CRISPR/Cas9 technology, promising results have been obtained, and clinical studies including gene editing technologies have been started since 2018. In some of these studies, it was aimed to correct the β-globin gene (NCT04774536, NCT04819841, NCT04205435), while in others, it was aimed to increase the expression of hemoglobin F (HbF), which does not contain the mutated β-globin gene, through the modification of regulatory elements like BCL11A (NCT04853576, NCT05145062, NCT04443907, NCT03653247, NCT04208529, NCT03745287, NCT04211480, NCT03655678, NCT03432364).

One of the studies whose results are described in these studies is a study aiming to increase HbF production in erythroid as a result of ZFN-mediated degradation of the GATA binding region of BCL11A in autologous CD^{34+} cells (NCT03432364) (Walters et al. 2021). In this study, which was performed on five β-thalassemia patients, although HbF levels increased to approximately 23.5%, these levels could not be maintained, and this prevented the patients from becoming transfusion-independent. On the other hand, in a study conducted on SCD patients for the same purpose, percentage (%) HbF levels were 1–11% in the first analysis, while this level increased to 15–29% at week 13 in four patients and then increased to 14–39% at week 26 in three patients. At 65 weeks of follow-up, the level was still observed at 35% HbF in one patient (NCT03653247) (Alavi et al. 2021). In studies NCT03655678 and NCT03745287, a total of two patients, one with β-thalassemia and the other with SCD, were administered CRISPR/Cas9-edited autologous CD^{34+} cells targeting BCL11A by intravenous infusion (Frangoul et al. 2021). More than a year later, increases in HbF levels and transfusion independence were observed in two of the patients. It has also been reported that patient with SCD has a decrease in vaso-occlusive crises.

Considering the limitations of allogeneic HSC transplantation (donor finding, immune response, etc.) used in the current treatment of inherited hemoglobinopathy diseases, treatment methods that include gene addition or gene editing technologies to autologous cells reveal promising results. However, it is clear that there are many matters (isolation of cells from patients, conditioning, vector design, gene delivery, gene editing methods, dose/frequency of administration of cells to patients after modification, etc.) that are not yet known and need to be optimized in order for these technologies to be safe, effective, and low-cost, and more research needs to be performed.

3.4 Embryonic Stem Cells (ESCs)

ESCs, which can be isolated in the early stages of embryo development and have the ability to differentiate into all cells in the three germ layers, have the potential to be used for modeling genetic diseases or for therapeutic purposes (Halevy and Urbach 2014; Zakrzewski et al. 2019). ESCs have the potential to differentiate into any cell type, making them superior modeling platform for many diseases (Song et al. 2010). For example, using lentiviruses containing shRNAs targeting the human survival motor neuron (SMN)-full length (SMN-FL) and SMN-Δ7 (lacking exon 7) isoforms, knockdown of two isoforms whose expression varies depending on developmental periods in human ESCs has been studied (Wang et al. 2013b). Researchers examining isoform-dependent neuronal differentiation from ESCs have designed a model that may explain the efficacy of SMN-FL and SMN-Δ7 gene isoforms on the spinal muscular atrophy (SMA) phenotype. Another ESC-based approach has been introduced for facioscapulohumeral muscular dystrophy (FSHD), which is characterized by degradation in signaling pathways that affect myogenesis as a result of loss of function in the transcriptional repression of the double homeobox protein 4 (DUX4) gene (Chaudhari et al. 2020). With DUX4 activation in human ESCs transfected with the pCMV-DUX4 vector, a novel disease model has been developed in which disease potential therapeutic compounds can be evaluated.

After the discovery of the genome editing efficiency of programmable nucleases, it has also come to the fore in engineering of ESCs, especially since it allows manipulation on a scale ranging from one to several kilobases of nucleotides (Park and Telugu 2013; Andrey and Spielmann 2017; Dolan et al. 2019). In a study about the effect of TDP43 protein on the pathogenesis of amyotrophic lateral sclerosis (ALS), TALEN-mediated G298S TDP43 mutation was induced in H9 ESCs (Sun et al. 2018). They have reported that differentiated cells exhibited significant TDP43 expression similar to patient-derived iPSCs compared to control groups, after insertion of mutant cDNA into ECS via HDR, and this may contribute to the understanding of the effect of this mutation on ALS, a fatal neurodegenerative disease caused by loss of motoneurons, progression. TALEN-mediated isogenic HD human ESCs were created to understand the contribution of HTT gene CAG repeat number and differentiated cells in the pathogenesis of HD (Ooi et al. 2019). The relationship of CAG repeats with tissue-specific HD pathogenesis has been revealed by the differentiation of the mutated ESCs into neural progenitor cells (NPCs), neurons, hepatocytes, and skeletal muscle myotubes, following the modification of the HTT gene exon 1 of the H9 ESCs with varying numbers of CAG repeats.

The CRISPR/Cas9 system on ESCs has been used to develop models of differentiation profiles of neurodegenerative diseases into different cell types. CRISPR/Cas9 editing has been performed in the DARPP32 gene region of ESCs to use dopamine receptor expression as a display for the characterization of medium spiny neurons (Hunt et al. 2017). Thus, by explaining the neural developmental pathways, they suggested a method that can be used in models of neurodegenerative diseases such as HD. Another genome editing approach has been performed to model Wilson's disease, a rare genetic disease characterized by Cu accumulation with the loss of function as a result of mutation of ATP7B gene (Lalioti et al. 2010). In a study in which human ESCs were differentiated into hepatocyte-like cells following the induction of mutation in the ATP7B gene using the CRISPR/Cas9 tool, it was reported that a model that expressed Wilson's disease phenotype could be developed, consistently (Kim et al. 2020).

Due to ethical concerns in isolating and using human-derived ESCs, apart from the limited number of human ESC-genome manipulation studies, its use comprises the development of disease models in animals by methods such as knock-out/knock-in studies in one-cell embryo or ESC lines. Disease modeling methods based on various techniques have been suggested in one-cell embryo, using deoxynucleotides that carry the disease-causing mutation and nucleases such as CRISPR or TALEN or using somatic cell nuclear transfer methods (SCNT) following genetic modification (Wefers et al. 2013; Yasue et al. 2014; Chen et al. 2015; Wettstein et al. 2015; Li et al. 2016; Ukai et al. 2017; Lee et al. 2020). It has been considered that these approaches hold a great promise in the future, especially for understanding the pathological features of genetic diseases and for a deep understanding of their systemic effects.

Genetic disease therapeutic approaches based on genome manipulation cannot be developed in patient ESCs, because of ethical concerns. Therefore, ESC-based therapeutic approaches consist of few studies on patient animal-derived ESCs. It has been reported that myogenic differentiation can be programmed with dox-inducible lentiviral vectors that encode paired box 7 (PAX7) transcription factor of normal ESCs and iPSCs (Darabi et al. 2012). In vivo studies in NOD/SCID mice have shown regenerative activity and dystrophin expression, which may be significant for DMD. The genetic correction has been performed with engineered *Streptococcus pyogenes* Cas9 nuclease variants that recognize NGN PAMs (SpCas9-NG) on HD mouse ESCs (Oura et al. 2021). Correction of prolonged CAG repeats in the HTT gene of mouse HD-ESCs through SpCas9-NG has shown that it can reverse the HD abnormal phenotype in vitro differentiated cells and in animal model. Similar to HD, fragile X-related disorders occur as a result of elongation of CGG repeats located in the exon of the FMR1 gene (Lozano et al. 2014). It has been reported that mutations in the mismatch repair (MMR) protein, which controls repeats in the gene exon region, have a critical role in the disease (Zhao et al. 2018). As a result of D1185N point mutation induced in MLH3 genes in mouse ESCs via CRISPR/Cas9, repeat prolongation was observed in control groups, while MLH3 mutations were reported to significantly inhibit repeat prolongation.

Although ESCs have the ability to differentiate into cell types in each germ layer, they are favor to be used from animals rather than humans because they bring many ethical problems together. On the other hand, animal models with high homology to humans shed a significant light for elucidating disease pathogenesis or for the development of therapeutics, and it still comprises challenges due to its onset profile and species-specific pathogenesis.

3.5 Induced Pluripotent Stem Cells (iPSCs)

IPSCs derived by reprogramming of somatic cells stand out as excellent tools for modeling diseases and developing therapeutic approaches. Although patient-specific iPSC studies are crucial for a deep understanding of diseases, isogenic iPSC disease models and approaches come into prominence. In a study that can be adaptable in X-chromosomal disorders, the potential of viral vector integration and integration-free iPSC induction methods to create a disease model has been studied, comprehensively (Hinz et al. 2019). The episomal reprogramming method has been shown to be more effective for X-linked disorders than iPSCs using lentivirus. For HD, piggyBac vectors were used to ensure shRNA expression to create disease models from human and mouse iPSCs through silencing the HTT gene (Szlachcic et al. 2017). It has been reported that as a result of HTT gene knock-down, a novel model has been suggested that allows the analysis of impaired signaling pathways in HDs.

As with other stem cells, current studies have focused on highly specific genetic manipulation with genome editing tools in engineering of iPSCs. Early studies have incorporated ZFN-mediated stem cell manipulations, the first efficient nuclease genome editing methods. In order to eliminate its off-target effects, high specificity active dimers of ZFNs could be formed. A group that proposes the design of a nuclease pool by recombination of ZFN subunits (α-helix), followed by the selection of the most specific ZFN structure via the reporter gene, performed ZFN HDR in iPSCs derived by using lentiviral reprogramming vectors in dermal fibroblast cells of SCD patients (Maeder et al. 2009; Sebastiano et al. 2011). After selecting the ZFN designs that most specifically target the human β-globin gene from the nuclease pool, they have transfected iPSC cells with ZFN expression plasmids. It has been also revealed that disease-causing modification in the β-globin loci can be effectively caused by appropriate donor design for HDR. iPCS-mediated model studies have also been conducted for ALS. The effect of mutations of Fused in sarcoma/translocated in liposarcoma (FUS), which is one of the ALS-causing genes, on motoneurons have been studied (Lenzi et al. 2015). It has been reported that rigorous ALS phenotype was achieved with P525L mutation in TALEN-mediated heterozygous or homozygous mutagenesis, due to the absence of P525L mutation in iPSCs derived from fibroblasts obtained from patients. Similarly, there are many reports that models can be developed to explain the pathological features of ALS by creating different mutations in the FUS gene with the TALEN-mediated genome editing (Ichiyanagi et al. 2016; Akiyama et al. 2019).

On the other hand, TALEN-mediated genome editing techniques are also promising in modeling rare diseases with genetic modifications. The rare Lesch-Nyhan disease (LND) caused by mutations in the gene encoding hypoxanthine-guanine phosphoribosyltransferase (HGPRT) has been modeled by using TALENs (Jinnah 2009). It has been demonstrated that it is possible to design isogenic mutated pluripotent cells by TALEN-mediated homozygous disruption of the HGPRT1 gene without any off-target effects in healthy human pluripotent stem cells (Frank et al. 2013). As a result of the differentiation of the mutation-carrying stem cells, it has been observed that the phenotype exhibited by patient-specific iPSCs is recapitulated.

These methods assure powerful tools not only for model construction but also for the further analysis of molecular functions of specific gene mutations identified in diseases. CRISPR has started to be utilized frequently in the most trending studies in stem cell-mediated disease modeling approaches. For the study of HD disease, iPCSs containing the HTT gene with different numbers of CAG repeats have been designed (Tousley and Kegel-Gleason 2016). In one of the iPSC approaches that can be used in modeling HD disease, the manipulation technique that enables the induction of multiple allelic mutations with high specificity with CRISPR/Cas9 (HDR) has been revealed (An et al. 2014).

In autism spectrum disorders, the CRISPR/Cas9-mediated genome editing technique has been utilized iPSCs derived from fibroblasts to explore the effect of the mutations on the CHD8 gene which is a member of the CHD family of ATP-dependent chromatin remodeling factors (Wang et al. 2017). With the transcriptome analysis, its role in the mechanisms of interneuron differentiation has been explained. Autosomal dominant Marfan syndrome is characterized by abnormalities in the skeletal and cardiovascular tissues due to mutations in the fibrillin-1 (FBN1) gene (Robinson et al. 2006). Since the late-onset profile of Marfan syndrome cannot be modeled by animal models, TALEN-mediated genetic correction has been performed on iPSCs derived from patients' fibroblasts, as well as CRISPR/Cas9-mediated FBN1 knock-out in human ESCs (Park et al. 2017). Researchers have suggested that the phenotypic effects of the FBN1 gene can be clearly demonstrated by these models constructed with FBN1 loss- and gain-of-function model in ESCs. Clinical studies are needed in iPSC modeling of genetic diseases. For example, in a study whose results have not been reported yet

(NCT036112310), clinical research has been initiated to model genetic diseases in iPSC cells to be derived from human urine, skin, and blood samples.

In the treatment of genetic diseases, conventional methods have started to be replaced by next-generation approaches comprising genetic manipulations. The approaches frequently used in gene therapy techniques are the use of integrative and non-integrative vectors (Pan et al. 2019; Ghosh et al. 2020; Poletti and Mavilio 2021). In this context, DMD is a disease in which significant progress has been suggested in the genome manipulation techniques (Béroud et al. 2007). The effect of myogenic differentiation 1 (MyoD1) gene in iPSCs of DMD patients was studied with the dox-inducible lentiviral system carrying the MyoD1 gene (Abujarour et al. 2014). The findings of successful differentiation from iPSCs to muscle cells have shown the potential therapeutic use of genome-manipulated stem cells. In order to eliminate the risk of undesired genomic integration of lentiviral vectors, it has been reported that stem cells can differentiate into myogenic precursors as a result of transferring the full-length dystrophin gene in mice iPSCs via Bxb1 integrase-plasmid (Zhao et al. 2014). One of the first approaches in SMA abnormalities with genome manipulation tools involves the use of non-viral and integrated episomal vectors. It has been reported that the abnormal phenotype of the disease in the SMA mouse model was suppressed in the approach that enables the expression of the SMN1-like gene as a result of manipulation of the SMN2 gene in patient iPSCs (Corti et al. 2012).

The use of vectors of viral origin (adenoviral, adeno-associated viral, herpesviral, or retroviral) depends on many factors such as target cell type, package capacity, inflammation risk, or integration capacity, so, the risks should be well evaluated in effective vector-based gene therapy approaches (Ghosh et al. 2020). However, with the discovery of nuclease tools that allow programmable and modular designs, a new era has begun in the treatment of genetic diseases (Fig. 2).

ZFN-mediated genetic correction methods are frequently used in SCD, in which abnormal hemoglobin formation is observed as a result of mutation in codon 6 of β-globin and conversion of glutamine to valine (Kim et al. 2014a; Lonergan et al. 2001). In the study, in which derived iPSCs were used by reprogramming BM-MSCs isolated from adult SCD patients, HDR-mediated genetic correction method was applied using ZFN tool on mutant β-globin alleles (Zou et al. 2011a). They have shown that disease-causing mutations can be confirmed with vector designs that target specific genes. Researchers emphasized that the correct selection of the site-specific recombinase approach is critical in order not to observe off-target effects in ZFN-mediated HDR techniques. Genetic correction methods based on manipulation of stem cells with nucleases are also promising for rare genetic diseases. ZFN-mediated genetic correction has been performed on iPSCs derived from BM-MSCs of X-linked chronic granulomatous disease (X-CGD) patients, characterized with CYBB mutation affecting reactive oxygen production pathways by neutrophils and monocytes (Zou et al. 2011b). They reported that exposing a single copy of the defected gp91phox to ZFN-mediated gene correction resulted in normal gene expression without any off-target effect.

TALENs have been used in human iPSCs to elucidate the genetic background of various metabolic diseases. In a study, it has been emphasized that TALEN-mediated genome editing techniques are promising in understanding the metabolic effects of different mutations in diseases (Ding et al. 2013). By recapitulating the p.F508del mutation in the CFTR gene via the TALEN-mediated HDR method, a multi-step human intestinal organoid protocol has been suggested to analyze therapeutics in physiological conditions of CF disease (Cutting 2015; Fleischer et al. 2020). It has been shown that the CFTR protein fully acquires normal expression and activation on iPSC-derived CFTR-corrected organoids. Applications of TALENs for correction of genetic diseases have also been applied to iPSCs derived from SCD patients (Ramalingam et al. 2014). Mutations in the HBB β-globin alleles were found to be significantly expressed in cells that differentiated into erythroids,

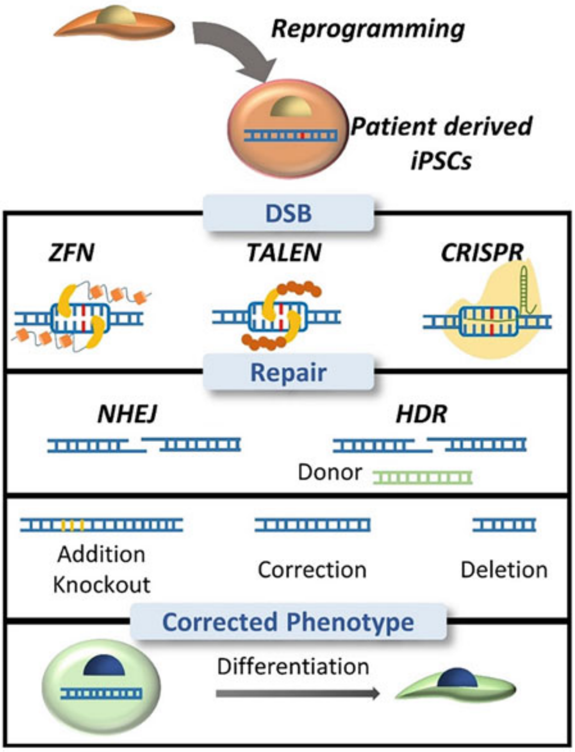

Fig. 2 Basic principle in genome editing of patient-derived iPSCs for therapy. Somatic cells with abnormal phenotype from patients are derived into iPSC through reprogramming. Gene regions with disease-causing mutations are recognized by programmable nucleases, and double-strand break (DSB) is achieved. According to the design of the genome editing method, DSB is repaired by non-homologous end joining (NHEJ) or homology-directed repair (HDR) methods. Depending on the therapeutic purpose of the disease, gene addition, knock-out, or deletion are possible, as well as correction of a specific mutation. The genome-edited iPSC is expected to exhibit the confirmed healthy phenotype after differentiation

compared to uncorrected cells, following gene correction.

CRISPR-mediated genome editing techniques are promising for the treatment of β-thalassemia, one of the most common genetic diseases in the world, caused by mutations in the human HBB gene. It has been observed that normal HBB gene expression of cells that differentiated into erythroblasts could be achieved without off-target, when genetic correction of the HBB gene has been performed on patient-derived iPSCs using CRISPR/Cas9 and piggyBac transposon (Xie et al. 2014). It has been reported that functional HBB gene expression has been revealed in cells that differentiated into erythrocytes, when CRISPR gene correction, insertion in exon 1 of the HBB cDNA, with two guide RNAs has been applied to iPSCs derived from peripheral blood mononuclear cells of two β-thalassemia patients (Cai et al. 2018).

The fact that the CRISPR genome editing technique is a highly effective method and the ability of iPSCs to differentiate into various cell types has made CRISPR-mediated iPSCs manipulation methods also used in neurodegenerative diseases. In order to evaluate the off-target effect, correction has been performed with exon skipping, frameshifting, and exon knock-in methods mediated by TALEN and CRISPR homologous recombination using unique regions in the dystrophin gene (Li et al. 2015). The researchers have reported that the dystrophin gene could be corrected in pluripotent cells derived from exon 44-deficient fibroblasts obtained from DMD patients, and myogenic cells achieved dystrophin expression after differentiation into skeletal muscle cells. In another study targeting the deletion of mutant exon 23 of the dystrophin gene, it has been reported that more effective genome editing can be performed in the HDR-mediated CRISPR/Cas9 system, thanks to the donor containing selective genes (Jin et al. 2020). It has been also emphasized that it accomplished corrected dystrophin expression in iPSCs and muscles of mdx mice transplanted with iPSC-derived progenitor cells. Another approach has been developed by using another DSB repairing method, non-homologous end joining (NHEJ). The researchers have performed CRISPR/Cas9-mediated excision of the approximately 725-kb region between exon 45 and 55 for the reframe of the dystrophin gene in iPSCs obtained from DMD patients. They have found that the stem cells were able to differentiate into cardiac and skeletal muscle cells, and they were able to express the functional dystrophin gene despite the deleted excision (Young et al. 2016). TALEN and CRISPR-mediated genetic correction methods have been applied on iPSCs derived from patient blood cells for another type of muscular dystrophy, limb-girdle muscular dystrophy (LGMD) (Turan et al. 2016). As a result of the study, it has been reported that as a result of dysferlin nonsense (p.R1905X) and α-sarcoglycan missense (p.R77C) mutation correction, cells that differentiated into muscle progenitors exhibited functional protein expression, and the proteins showed correct localization in the cell.

The main purpose of genome manipulations for HD is to replace elongated CAG repeats with normal-length CAG repeats, as mentioned before. In this regard, a study based on manipulation of patient iPSCs with BAC vectors containing the whole HTT gene containing the normal-length CAG region has shown that normal HTT gene expression can be achieved (An et al. 2012). Genetic correction of iPSCs using CRISPR/Cas9 and piggyBac transposon has shown that stem cells differentiated into snaptically active neurons with the change in gene expression (Xu et al. 2017). Similarly, in a study in which the plasmid-based CRISPR/Cas9 genome editing module was designed, both genetic corrections through the manipulation of HTT gene CAG repeats and HTT gene knock-out were performed in human HD-iPSCs (Dabrowska et al. 2020).

If the pathological features of genetic diseases are well defined, it may be possible to develop modular approaches with genome editing techniques. When the SOD1 point mutation was corrected using the CRISPR/Cas9 system on iPSCs derived from ALS patients, abnormalities in signaling pathways that cause motor neuron differentiation-specific disease character have been explained (Bhinge et al. 2017). A study of ALS patients with peripheral blood mononuclear cell (PBMC)-derived iPSC aimed to reduce overexpression of dipeptide repeat (DPR) proteins resulting from the hexanucleotide repeat in the C9ORF72 gene (Cheng et al. 2019). Researchers performing CRISPR/Cas9 genome-wide knock-out screen for mechanisms that can control overexpression of DPR have shown that DPR translation can be suppressed with the help of DDX3X RNA helicase.

SMA disease is characterized by degeneration of spinal motor neurons mediated by decreased SMN protein as a result of deletion or mutations in the SMN gene (Ahmad et al. 2016). It has been reported that using CRISPR/Cpf1 and ssODN in SMA patient-derived iPSCs, the SMN2 840 T to C correction performed with approximately 10% efficiency and achieved SMN1-like gene expression (Zhou et al. 2018). In particular, due to the low efficiency of homologous recombination techniques that require donor DNA, the researchers restored SMN expression via NHEJ with genome manipulation. For example, instead of the SMN gene, intronic splicing-regulatory elements (SRE), which are key modifiers in SMA, have been addressed (Li et al. 2020b; Lin et al. 2020). It has been observed that the SMA phenotype in iPSCs was eliminated as a result of manipulation of two SREs with the CRISPR/Cas9-mediated NHEJ method in iPSCs derived from the skin fibroblast of an SMA patient. They also reported successful CRISPR-mediated correction in mice germline cells. In another approach based on manipulation of patient iPSCs, the SMN2-GFP reporter region is integrated via CRISPR/Cas9 to create reporter cells that do not affect the splicing mechanisms of the SMN2 gene (Wang et al. 2019b). With this method, which was developed especially for testing potential compounds, possible drug candidates have been also evaluated.

Each of the approaches based on iPSC-mediated genome manipulation techniques has advances and challenges. For example, viral vectors allow highly efficient genome manipulation but can cause mutagenesis as a result of undesired integrations, while non-viral vectors provide stable and specific gene expression and but cause cytotoxicity (Paolini Sguazzi et al. 2021). On the other hand, while the most recent genome editing tools can provide programmable, targeted, and selective approaches that eliminate the risk of tumorigenesis, challenges still need to be overcome, such as the continuous control of gene products emerging from iPSCs.

4 Conclusion and Future Perspectives

Genetic diseases comprise many various types of diseases that need to be faced with a lot of challenges to overcome the adverse systemic effects. Comprehensive solutions are needed to treat these vital diseases. Stem cell-based therapy emerged as the first platform for the treatment of genetic diseases. One of the greatest challenges encountered in studies is the use of mostly allogeneic stem cells to transplant healthy stem cells carrying unmutated genes, resulting in the formation of an immune response in the host. To avoid this problem, gene therapy aimed at correcting the gene causing the disease has been combined with stem cell therapy. In this context, firstly, basic genetic engineering approaches based on vectors were used in the patient's own stem cells. Although vectors are designed with specific gene sequences to ensure normal gene expression, viral vectors fail such to insufficient packaging capacity or undesired genome integration, while non-viral ones lack such as targeting and entry of patient cells as well as immune system issues.

Advanced state-of-the-art approaches have been proposed in stem cell-based genetic disease treatment methods, thanks to the advancements in molecular sciences. The use of stem cells that are be able to differentiate into various cell types with these approaches, which incorporate the eliminate of genetic defects in specific genome regions via nuclease-based genome editing tools, has uncovered promising results in the last few decades. The primary results of these methods, which comprise genome editing nucleases performing DSB only in specific genome regions, followed by directed repair in this region, have highlighted new options for the treatment of genetic diseases. Although promising findings were suggested by in vitro and in vivo studies, future studies are needed to eliminate off-target effects and increase effectiveness of these approaches in order to perform clinical application of genome editing tools in stem cell-based treatments for genetic diseases.

References

Abujarour R et al (2014) Myogenic differentiation of muscular dystrophy-specific induced pluripotent stem cells for use in drug discovery. Stem Cells Transl Med 3:149–160. https://doi.org/10.5966/sctm.2013-0095

Ahmad S, Bhatia K, Kannan A, Gangwani L (2016) Molecular mechanisms of neurodegeneration in spinal muscular atrophy. J Exp Neurosci 10:39–49. https://doi.org/10.4137/JEN.S33122

Akiyama T et al (2019) Aberrant axon branching via Fos-B dysregulation in FUS-ALS motor neurons. EBioMedicine 45:362–378. https://doi.org/10.1016/j.ebiom.2019.06.013

Al-Gharaibeh A et al (2017) Induced pluripotent stem cell-derived neural stem cell transplantations reduced behavioral deficits and ameliorated neuropathological changes in YAC128 mouse model of huntington's disease. Front Neurosci 11:628. https://doi.org/10.3389/fnins.2017.00628

Alavi A et al (2021) Preliminary safety and efficacy results from precizn-1: an ongoing phase 1/2 study on zinc finger nuclease-modified autologous CD34+HSPCs for sickle cell disease (SCD). Blood 138:2930. https://doi.org/10.1182/blood-2021-151650

An MC, O'Brien RN, Zhang N, Patra BN, De La Cruz M, Ray A, Ellerby LM (2014) Polyglutamine disease modeling: epitope based screen for homologous recombination using CRISPR/Cas9 system. PLoS

Curr 6. https://doi.org/10.1371/currents.hd.0242d2e7ad72225efa72f6964589369a

An MC et al (2012) Genetic correction of Huntington's disease phenotypes in induced pluripotent stem cells. Cell Stem Cell 11:253–263. https://doi.org/10.1016/j.stem.2012.04.026

Andrey G, Spielmann M (2017) CRISPR/Cas9 genome editing in embryonic stem cells. In: Enhancer RNAs. Springer. https://doi.org/10.1007/978-1-4939-4035-6_15

Arber C et al (2015) Activin a directs striatal projection neuron differentiation of human pluripotent stem cells. Development 142:1375–1386. https://doi.org/10.1242/dev.117093

Aslan A, Allahverdiyev AM, Bagirova M, Abamor ES (2018) Problems in stem cell therapy for cardiac repair and tissue engineering approaches based on graphene and its derivatives. Curr Stem Cell Res Ther 13:447–457. https://doi.org/10.2174/1574888X13666180510110055

Ataga KI et al (2017) Crizanlizumab for the prevention of pain crises in sickle cell disease. N Engl J Med 376: 429–439. https://doi.org/10.1056/NEJMoa1611770

Avior Y, Sagi I, Benvenisty N (2016) Pluripotent stem cells in disease modelling and drug discovery. Nat Rev Mol Cell Biol 17:170–182. https://doi.org/10.1038/nrm.2015.27

Barczewska M et al (2019) Safety of intrathecal injection of Wharton's jelly-derived mesenchymal stem cells in amyotrophic lateral sclerosis therapy. Neural Regen Res 14:313–318. https://doi.org/10.4103/1673-5374.243723

Barkholt L et al (2013) Risk of tumorigenicity in mesenchymal stromal cell-based therapies–bridging scientific observations and regulatory viewpoints. Cytotherapy 15:753–759. https://doi.org/10.1016/j.jcyt.2013.03.005

Becker S, Boch J (2021) TALE and TALEN genome editing technologies. Gene Genome Editing 2: 100007. https://doi.org/10.1016/j.ggedit.2021.100007

Bernardo ME, Fibbe WE (2012) Safety and efficacy of mesenchymal stromal cell therapy in autoimmune disorders. Ann NY Acad Sci 1266:107–117. https://doi.org/10.1111/j.1749-6632.2012.06667.x

Béroud C et al (2007) Multiexon skipping leading to an artificial DMD protein lacking amino acids from exons 45 through 55 could rescue up to 63% of patients with Duchenne muscular dystrophy. Hum Mutat 28:196–202

Berry JD et al (2019) NurOwn, phase 2, randomized, clinical trial in patients with ALS: safety, clinical, and biomarker results. Neurology 93:e2294–ee305. https://doi.org/10.1212/WNL.0000000000008620

Bhinge A, Namboori SC, Zhang X, VanDongen AMJ, Stanton LW (2017) Genetic correction of SOD1 mutant iPSCs reveals ERK and JNK activated AP1 as a driver of neurodegeneration in amyotrophic lateral sclerosis. Stem Cell Rep 8:856–869. https://doi.org/10.1016/j.stemcr.2017.02.019

Boido M et al (2014) Human mesenchymal stromal cell transplantation modulates neuroinflammatory milieu in a mouse model of amyotrophic lateral sclerosis. Cytotherapy 16:1059–1072. https://doi.org/10.1016/j.jcyt.2014.02.003

Bolton-Maggs PH, Pasi KJ (2003) Haemophilias A and B. Lancet 361:1801–1809. https://doi.org/10.1016/S0140-6736(03)13405-8

Bonfield TL, Lennon D, Ghosh SK, DiMarino AM, Weinberg A, Caplan AI (2013) Cell based therapy aides in infection and inflammation resolution in the murine model of cystic fibrosis lung disease. https://doi.org/10.4236/scd.2013.32019

Boulad F et al (2013) First US phase I clinical trial of globin gene transfer for the treatment of beta-thalassemia major. Blood 122:716. https://doi.org/10.1182/blood.V122.21.716.716

Boyer JG, Han S, Prasad V, Khalil H, Vagnozzi RJ, Molkentin JD (2019) Satellite cell depletion in early adulthood attenuates muscular dystrophy pathogenesis. BioRxiv 857433. https://doi.org/10.1101/857433

Brookhouser N, Raman S, Potts C, Brafman DA (2017) May I cut in? Gene editing approaches in human induced pluripotent stem cells. Cell 6:5. https://doi.org/10.3390/cells6010005

Bultmann S et al (2012) Targeted transcriptional activation of silent oct4 pluripotency gene by combining designer TALEs and inhibition of epigenetic modifiers. Nucleic Acids Res 40:5368–5377. https://doi.org/10.1093/nar/gks199

Byrne SM, Mali P, Church GM (2014) Genome editing in human stem cells. In: Methods in enzymology. Elsevier. https://doi.org/10.1016/B978-0-12-801185-0.00006-4

Cai L et al (2018) A universal approach to correct various HBB gene mutations in human stem cells for gene therapy of beta-thalassemia and sickle cell disease. Stem Cells Transl Med 7:87–97. https://doi.org/10.1002/sctm.17-0066

Cantin AM, Hartl D, Konstan MW, Chmiel JF (2015) Inflammation in cystic fibrosis lung disease: pathogenesis and therapy. J Cyst Fibros 14:419–430. https://doi.org/10.1016/j.jcf.2015.03.003

Carbone A et al (2014) Correction of defective CFTR/ENaC function and tightness of cystic fibrosis airway epithelium by amniotic mesenchymal stromal (stem) cells. J Cell Mol Med 18:1631–1643. https://doi.org/10.1111/jcmm.12303

Carbone A et al (2018) Gap junctions are involved in the rescue of CFTR-dependent chloride efflux by amniotic mesenchymal stem cells in coculture with cystic fibrosis CFBE41o- cells. Stem Cells Int 2018:1203717. https://doi.org/10.1155/2018/1203717

Cavazzana-Calvo M et al (2010) Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. Nature 467:318–322. https://doi.org/10.1038/nature09328

Cavazzana M, Antoniani C, Miccio A (2017) Gene therapy for beta-hemoglobinopathies. Mol Ther 25:1142–1154. https://doi.org/10.1016/j.ymthe.2017.03.024

Chal J et al (2015) Differentiation of pluripotent stem cells to muscle fiber to model Duchenne muscular dystrophy. Nat Biotechnol 33:962–969. https://doi.org/10.1038/nbt.3297

Chan-Il C, Young-Don L, Heejaung K, Kim SH, Suh-Kim H, Kim SS (2013) Neural induction with neurogenin 1 enhances the therapeutic potential of mesenchymal stem cells in an amyotrophic lateral sclerosis mouse model. Cell Transplant 22:855–870. https://doi.org/10.3727/096368912X637019

Chan SS et al (2018) Skeletal muscle stem cells from PSC-derived teratomas have functional regenerative capacity. Cell Stem Cell 23(74–85):e6. https://doi.org/10.1016/j.stem.2018.06.010

Chang HHY, Pannunzio NR, Adachi N, Lieber MR (2017) Non-homologous DNA end joining and alternative pathways to double-strand break repair. Nat Rev Mol Cell Biol 18:495–506. https://doi.org/10.1038/nrm.2017.48

Chaudhari N, Rickard AM, Roy S, Droge P, Makhija H (2020) A non-viral genome editing platform for site-specific insertion of large transgenes. Stem Cell Res Ther 11:380. https://doi.org/10.1186/s13287-020-01890-6

Chen Y et al (2015) Functional disruption of the dystrophin gene in rhesus monkey using CRISPR/Cas9. Hum Mol Genet 24:3764–3774. https://doi.org/10.1093/hmg/ddv120

Cheng W et al (2019) CRISPR-Cas9 screens identify the RNA helicase DDX3X as a repressor of C9ORF72 (GGGGCC)n repeat-associated non-AUG translation. Neuron. 104: 885–898 e8. https://doi.org/10.1016/j.neuron.2019.09.003

Choi SM et al (2013) Efficient drug screening and gene correction for treating liver disease using patient-specific stem cells. Hepatology 57:2458–2468. https://doi.org/10.1002/hep.26237

Corti S et al (2012) Genetic correction of human induced pluripotent stem cells from patients with spinal muscular atrophy. Sci Transl Med 4:165ra62. https://doi.org/10.1126/scitranslmed.3004108

Cox DB, Platt RJ, Zhang F (2015) Therapeutic genome editing: prospects and challenges. Nat Med 21:121–131. https://doi.org/10.1038/nm.3793

Cutting GR (2015) Cystic fibrosis genetics: from molecular understanding to clinical application. Nat Rev Genet 16:45–56. https://doi.org/10.1038/nrg3849

Dabrowska M, Ciolak A, Kozlowska E, Fiszer A, Olejniczak M (2020) Generation of new isogenic models of huntington's disease using CRISPR-Cas9 technology. Int J Mol Sci 21:1854. https://doi.org/10.3390/ijms21051854

Dai A et al (2018) Efficacy of stem cell therapy in ambulatory and nonambulatory children with Duchenne muscular dystrophy – phase I-II. Degener Neurol Neuromuscul Dis 8:63–77. https://doi.org/10.2147/DNND.S170087

Darabi R, Arpke RW, Irion S, Dimos JT, Grskovic M, Kyba M, Perlingeiro RC (2012) Human ES- and iPS-derived myogenic progenitors restore DYSTROPHIN and improve contractility upon transplantation in dystrophic mice. Cell Stem Cell 10:610–619. https://doi.org/10.1016/j.stem.2012.02.015

Delli Carri A et al (2013) Developmentally coordinated extrinsic signals drive human pluripotent stem cell differentiation toward authentic DARPP-32+ medium-sized spiny neurons. Development 140:301–312. https://doi.org/10.1242/dev.084608

Dever DP et al (2016) CRISPR/Cas9 beta-globin gene targeting in human haematopoietic stem cells. Nature 539:384–389. https://doi.org/10.1038/nature20134

DeWitt MA et al (2016) Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells. Sci Transl Med 8:360ra134. https://doi.org/10.1126/scitranslmed.aaf9336

Dey ND et al (2010) Genetically engineered mesenchymal stem cells reduce behavioral deficits in the YAC 128 mouse model of Huntington's disease. Behav Brain Res 214:193–200. https://doi.org/10.1016/j.bbr.2010.05.023

Ding Q et al (2013) A TALEN genome-editing system for generating human stem cell-based disease models. Cell Stem Cell 12:238–251. https://doi.org/10.1016/j.stem.2012.11.011

Dolan AE et al (2019) Introducing a spectrum of long-range genomic deletions in human embryonic stem cells using type I CRISPR-Cas. Mol Cell 74(936–50):e5. https://doi.org/10.1016/j.molcel.2019.03.014

Dominici M et al (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317

Eaton WA, Bunn HF (2017) Treating sickle cell disease by targeting HbS polymerization. Blood 129:2719–2726. https://doi.org/10.1182/blood-2017-02-765891

Eaton WA, Hofrichter J (1987) Hemoglobin S gelation and sickle cell disease. Blood 70:1245–1266

Elbaz EM, Helmy HS, El-Sahar AE, Saad MA, Sayed RH (2019) Lercanidipine boosts the efficacy of mesenchymal stem cell therapy in 3-NP-induced Huntington's disease model rats via modulation of the calcium/calcineurin/NFATc4 and Wnt/β-catenin signalling pathways. Neurochem Int 131:104548. https://doi.org/10.1016/j.neuint.2019.104548

Emery AE, Muntoni F, Quinlivan RC (2015) Duchenne muscular dystrophy. Oxford University Press, Oxford. https://doi.org/10.1093/med/9780199681488.001.0001

Ervasti JM (2007) Dystrophin, its interactions with other proteins, and implications for muscular dystrophy. Biochim Biophys Acta 1772:108–117. https://doi.org/10.1016/j.bbadis.2006.05.010

Feng SW, Chen F, Cao J, Yu MJ, Liang YY, Song XM, Zhang C (2012) Restoration of muscle fibers and satellite cells after isogenic MSC transplantation with microdystrophin gene delivery. Biochem Biophys Res Commun 419:1–6. https://doi.org/10.1016/j.bbrc.2012.01.029

Fink KD et al (2013) Transplantation of umbilical cord-derived mesenchymal stem cells into the striata of R6/2 mice: behavioral and neuropathological analysis. Stem Cell Res Ther 4:130. https://doi.org/10.1186/scrt341

Fleischer A et al (2020) iPSC-derived intestinal organoids from cystic fibrosis patients acquire CFTR activity upon TALEN-mediated repair of the p.F508del mutation. Mol Ther Methods Clin Dev 17:858–870. https://doi.org/10.1016/j.omtm.2020.04.005

Forostyak S, Homola A, Turnovcova K, Svitil P, Jendelova P, Sykova E (2014) Intrathecal delivery of mesenchymal stromal cells protects the structure of altered perineuronal nets in SOD1 rats and amends the course of ALS. Stem Cells 32:3163–3172. https://doi.org/10.1002/stem.1812

Forostyak S, Jendelova P, Kapcalova M, Arboleda D, Sykova E (2011) Mesenchymal stromal cells prolong the lifespan in a rat model of amyotrophic lateral sclerosis. Cytotherapy 13:1036–1046. https://doi.org/10.3109/14653249.2011.592521

Frangoul H et al (2021) CRISPR-Cas9 gene editing for sickle cell disease and β-thalassemia. N Engl J Med 384:252–260. https://doi.org/10.1056/NEJMoa2031054

Frank S, Skryabin BV, Greber B (2013) A modified TALEN-based system for robust generation of knockout human pluripotent stem cell lines and disease models. BMC Genomics 14:773. https://doi.org/10.1186/1471-2164-14-773

Friedenstein A, Piatetzky-Shapiro I, Petrakova K (1966) Osteogenesis in transplants of bone marrow cells. Development 16:381–390

Friedenstein A, Chailakhjan R, Lalykina K (1970) The development of fibroblast colonies in monolayer cultures of Guinea-pig bone marrow and spleen cells. Cell Prolif 3:393–403. https://doi.org/10.1111/j.1365-2184.1970.tb00347.x

Fujikura Y, Sugihara H, Hatakeyama M, Oishi K, Yamanouchi K (2021) Ketogenic diet with medium-chain triglycerides restores skeletal muscle function and pathology in a rat model of Duchenne muscular dystrophy. FASEB J 35:e21861. https://doi.org/10.1096/fj.202100629R

Gao K et al (2019) Potential long-term treatment of hemophilia A by neonatal co-transplantation of cord blood-derived endothelial colony-forming cells and placental mesenchymal stromal cells. Stem Cell Res Ther 10:34. https://doi.org/10.1186/s13287-019-1138-8

Gelati M, Profico DC, Ferrari D, Vescovi AL (2022) Culturing and expansion of "clinical grade" neural stem cells from the fetal human central nervous system. In: Neural progenitor cells. Springer. https://doi.org/10.1007/978-1-0716-1783-0_5

Germino-Watnick P, Hinds M, Le A, Chu R, Liu X, Uchida N (2022) Hematopoietic stem cell gene-addition/editing therapy in sickle cell disease. Cell 11:1843. https://doi.org/10.3390/cells11111843

Ghosh S, Brown AM, Jenkins C, Campbell K (2020) Viral vector systems for gene therapy: a comprehensive literature review of progress and biosafety challenges. Appl Biosaf 25:7–18. https://doi.org/10.1177/1535676019899502

Grimley M et al (2020) Early results from a phase 1/2 study of Aru-1801 gene therapy for sickle cell disease (SCD): manufacturing process enhancements improve efficacy of a modified gamma globin lentivirus vector and reduced intensity conditioning transplant. Blood 136:20–21. https://doi.org/10.1182/blood-2020-140963

Halevy T, Urbach A (2014) Comparing ESC and iPSC-based models for human genetic disorders. J Clin Med 3:1146–1162. https://doi.org/10.3390/jcm3041146

Hardee C, Arévalo-Soliz L, Hornstein B, Zechiedrich L (2017) Advances in non-viral DNA vectors for gene therapy. Genes (Basel) 8(2): 65. Published. https://doi.org/10.3390/genes8020065

Hayashita-Kinoh H et al (2021) Improved transduction of canine X-linked muscular dystrophy with rAAV9-microdystrophin via multipotent MSC pretreatment. Mol Ther Methods Clin Dev 20:133–141. https://doi.org/10.1016/j.omtm.2020.11.003

Herrick JB (1910) Peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anemia. Arch Intern Med 6:517–521

Hinz L, Hoekstra SD, Watanabe K, Posthuma D, Heine VM (2019) Generation of isogenic controls for in vitro disease modelling of X-chromosomal disorders. Stem Cell Rev Rep 15:276–285. https://doi.org/10.1007/s12015-018-9851-8

Hoban MD et al (2015) Correction of the sickle cell disease mutation in human hematopoietic stem/progenitor cells. Blood 125:2597–2604. https://doi.org/10.1182/blood-2014-12-615948

Hoban MD et al (2016) CRISPR/Cas9-mediated correction of the sickle mutation in human CD34+ cells. Mol Ther 24:1561–1569. https://doi.org/10.1038/mt.2016.148

Horwitz E et al (2005) Clarification of the nomenclature for MSC: the International Society for cellular therapy position statement. Cytotherapy 7:393–395. https://doi.org/10.1080/14653240500319234

Hunt C, Pouton C, Haynes J (2017) Characterising the developmental profile of human embryonic stem cell-derived medium spiny neuron progenitors and assessing mature neuron function using a CRISPR-generated human DARPP-32WT/eGFP-AMP reporter line. Neurochem Int 106:3–13. https://doi.org/10.1016/j.neuint.2017.01.003

Ichiyanagi N et al (2016) Establishment of in vitro FUS-associated familial amyotrophic lateral sclerosis model using human induced pluripotent stem cells. Stem cell reports 6:496–510. https://doi.org/10.1016/j.stemcr.2016.02.011

Iwatani S et al (2017) Gestational age-dependent increase of survival motor neuron protein in umbilical cord-derived mesenchymal stem cells. Front Pediatr 5:194. https://doi.org/10.3389/fped.2017.00194

Jang YY, Ye Z (2016) Gene correction in patient-specific iPSCs for therapy development and disease modeling. Hum Genet 135:1041–1058. https://doi.org/10.1007/s00439-016-1691-5

Jeon I et al (2012) Neuronal properties, in vivo effects, and pathology of a Huntington's disease patient-derived induced pluripotent stem cells. Stem Cells 30:2054–2062. https://doi.org/10.1002/stem.1135

Jiang Y, Lv H, Huang S, Tan H, Zhang Y, Li H (2011) Bone marrow mesenchymal stem cells can improve the motor function of a Huntington's disease rat model. Neurol Res 33:331–337. https://doi.org/10.1179/016164110X12816242542571

Jin Y, Shen Y, Su X, Weintraub NL, Tang Y (2020) Effective restoration of dystrophin expression in iPSC (Mdx)-derived muscle progenitor cells using the CRISPR/Cas9 system and homology-directed repair technology. Comput Struct Biotechnol J 18:765–773. https://doi.org/10.1016/j.csbj.2020.03.012

Jinnah HA (2009) Lesch-Nyhan disease: from mechanism to model and back again. Dis Model Mech 2:116–121. https://doi.org/10.1242/dmm.002543

Kim D et al (2020) Human embryonic stem cell-derived wilson's disease model for screening drug efficacy. Cell 9:872. https://doi.org/10.3390/cells9040872

Kim HS, Bernitz JM, Lee DF, Lemischka IR (2014a) Genomic editing tools to model human diseases with isogenic pluripotent stem cells. Stem Cells Dev 23: 2673–2686. https://doi.org/10.1089/scd.2014.0167

Kim KS, Lee HJ, An J, Kim YB, Ra JC, Lim I, Kim SU (2014b) Transplantation of human adipose tissue-derived stem cells delays clinical onset and prolongs life span in ALS mouse model. Cell Transplant 23:1585–1597. https://doi.org/10.3727/096368913X673450

Klaver-Flores S et al (2020) Genomic engineering in human hematopoietic stem cells: hype or hope? Front Genome Ed 2:615619. https://doi.org/10.3389/fgeed.2020.615619

Kolli N, Lu M, Maiti P, Rossignol J, Dunbar GL (2017) CRISPR-Cas9 mediated gene-silencing of the mutant huntingtin gene in an in vitro model of huntington's disease. Int J Mol Sci 18:754. https://doi.org/10.3390/ijms18040754

Kondo M et al (2003) Biology of hematopoietic stem cells and progenitors: implications for clinical application. Annu Rev Immunol 21:759–806. https://doi.org/10.1146/annurev.immunol.21.120601.141007

Kumar P, Gao K, Wang C, Pivetti C, Lankford L, Farmer D, Wang A (2018) In utero transplantation of placenta-derived mesenchymal stromal cells for potential fetal treatment of hemophilia a. Cell Transplant 27: 130–139. https://doi.org/10.1177/0963689717728937

Kuzma-Kozakiewicz M et al (2018) Intraspinal transplantation of the adipose tissue-derived regenerative cells in amyotrophic lateral sclerosis in accordance with the current experts' recommendations: choosing optimal monitoring tools. Stem Cells Int 2018:4392017. https://doi.org/10.1155/2018/4392017

Kwon JB et al (2020) In vivo gene editing of muscle stem cells with adeno-associated viral vectors in a mouse model of duchenne muscular dystrophy. Mol Ther Methods Clin Dev 19:320–329. https://doi.org/10.1016/j.omtm.2020.09.016

Lalioti V, Sandoval I, Cassio D, Duclos-Vallee JC (2010) Molecular pathology of Wilson's disease: a brief. J Hepatol 53:1151–1153. https://doi.org/10.1016/j.jhep.2010.07.008

Lee K, Uh K, Farrell K (2020) Current progress of genome editing in livestock. Theriogenology 150:229–235. https://doi.org/10.1016/j.theriogenology.2020.01.036

Lenzi J et al (2015) ALS mutant FUS proteins are recruited into stress granules in induced pluripotent stem cell-derived motoneurons. Dis Model Mech 8:755–766. https://doi.org/10.1242/dmm.020099

Li HL et al (2015) Precise correction of the dystrophin gene in duchenne muscular dystrophy patient induced pluripotent stem cells by TALEN and CRISPR-Cas9. Stem Cell Rep 4:143–154. https://doi.org/10.1016/j.stemcr.2014.10.013

Li H et al (2016) Generation of biallelic knock-out sheep via gene-editing and somatic cell nuclear transfer. Sci Rep 6:33675. https://doi.org/10.1038/srep33675

Li Q, Zhai Q, Geng J, Zheng H, Chen F, Kong J, Zhang C (2012) Transgene expression and differentiation of baculovirus-transduced adipose-derived stem cells from dystrophin-utrophin double knock-out mouse. Neural Regen Res 7:1695–1702. https://doi.org/10.3969/j.issn.1673-5374.2012.22.003

Li H, Yang Y, Hong W, Huang M, Wu M, Zhao X (2020a) Applications of genome editing technology in the targeted therapy of human diseases: mechanisms, advances and prospects. Signal Transduct Target Ther 5:1. https://doi.org/10.1038/s41392-019-0089-y

Li JJ et al (2020b) Disruption of splicing-regulatory elements using CRISPR/Cas9 to rescue spinal muscular atrophy in human iPSCs and mice. Natl Sci Rev 7: 92–101. https://doi.org/10.1093/nsr/nwz131

Lin YT et al (2011) Human mesenchymal stem cells prolong survival and ameliorate motor deficit through trophic support in Huntington's disease mouse models. PLoS One 6:e22924. https://doi.org/10.1371/journal.pone.0022924

Lin C et al (2020) Glycine enhances satellite cell proliferation, cell transplantation, and oligonucleotide efficacy in dystrophic muscle. Mol Ther 28:1339–1358. https://doi.org/10.1016/j.ymthe.2020.03.003

Lockwood CJ, Moore T, Copel J, Silver RM, Resnik R, Dugoff L, Louis J (2022) Creasy and Resnik's maternal-fetal medicine-E-book: principles and practice. Elsevier Health Sciences

Lonergan GJ, Cline DB, Abbondanzo SL (2001) Sickle cell anemia. Radiographics 21:971–994. https://doi.org/10.1148/radiographics.21.4.g01jl23971

Lozano R, Rosero CA, Hagerman RJ (2014) Fragile X spectrum disorders. Intractable Rare Dis Res 3:134–146. https://doi.org/10.5582/irdr.2014.01022

Lundstrom K (2018) Viral vectors in gene therapy. Diseases 6:42. https://doi.org/10.3390/diseases6020042

Ma L et al (2012) Human embryonic stem cell-derived GABA neurons correct locomotion deficits in quinolinic acid-lesioned mice. Cell Stem Cell 10: 455–464. https://doi.org/10.1016/j.stem.2012.01.021

MacKenzie TC et al (2020) In utero stem cell transplantation in patients with alpha thalassemia major: interim results of a phase 1 clinical trial. Blood 136:1. https://doi.org/10.1182/blood-2020-142698

Maeder ML, Thibodeau-Beganny S, Sander JD, Voytas DF, Joung JK (2009) Oligomerized pool engineering (OPEN): an 'open-source' protocol for making customized zinc-finger arrays. Nat Protoc 4:1471–1501. https://doi.org/10.1038/nprot.2009.98

Magrin E et al (2022) Long-term outcomes of lentiviral gene therapy for the beta-hemoglobinopathies: the HGB-205 trial. Nat Med 28:81–88. https://doi.org/10.1038/s41591-021-01650-w

Mangunsong C, Putera B, Haifa R, Suwandjaja M, Sharina A, Sasongko MB, Wirohadidjojo YW (2019) Safety issues of peribulbar injection of umbilical cord mesenchymal stem cell (Uc-Msc) in patients with retinitis pigmentosa. Cytotherapy 21:S83–S83. https://doi.org/10.1016/j.jcyt.2019.03.500

Marconi S et al (2013) Systemic treatment with adipose-derived mesenchymal stem cells ameliorates clinical and pathological features in the amyotrophic lateral sclerosis murine model. Neuroscience 248:333–343. https://doi.org/10.1016/j.neuroscience.2013.05.034

Marktel S et al (2019) Intrabone hematopoietic stem cell gene therapy for adult and pediatric patients affected by transfusion-dependent ß-thalassemia. Nat Med 25:234–241. https://doi.org/10.1038/s41591-018-0301-6

Mohseni R et al (2019) Overexpression of SMN2 gene in motoneuron-like cells differentiated from adipose-derived mesenchymal stem cells by Ponasterone A. J Mol Neurosci 67:247–257. https://doi.org/10.1007/s12031-018-1232-x

Mohseni R et al (2020) Clinical outcome of the Intrathecal administration of the allogeneic side population adipose derived mesenchymal stem cells in werdnig hoffman patients: an open-label phase I clinical trial. https://doi.org/10.21203/rs.3.rs-122983/v1

Moraes L et al (2012) Neuroprotective effects and magnetic resonance imaging of mesenchymal stem cells labeled with SPION in a rat model of Huntington's disease. Stem Cell Res 9:143–155. https://doi.org/10.1016/j.scr.2012.05.005

Mou H et al (2012) Generation of multipotent lung and airway progenitors from mouse ESCs and patient-specific cystic fibrosis iPSCs. Cell Stem Cell 10:385–397. https://doi.org/10.1016/j.stem.2012.01.018

Mouse Genome Sequencing Consortium (2002) Initial sequencing and comparative analysis of the mouse genome. Nature 420:520–562. https://doi.org/10.1038/nature01262

Nabavi SM et al (2019) Safety, feasibility of intravenous and intrathecal injection of autologous bone marrow derived mesenchymal stromal cells in patients with amyotrophic lateral sclerosis: an open label phase I clinical trial. Cell J 20:592–598. https://doi.org/10.22074/cellj.2019.5370

Nance ME et al (2019) AAV9 edits muscle stem cells in normal and dystrophic adult mice. Mol Ther 27:1568–1585. https://doi.org/10.1016/j.ymthe.2019.06.012

Nik-Ahd F, Bertoni C (2014) Ex vivo gene editing of the dystrophin gene in muscle stem cells mediated by peptide nucleic acid single stranded oligodeoxynucleotides induces stable expression of dystrophin in a mouse model for Duchenne muscular dystrophy. Stem Cells 32:1817–1830. https://doi.org/10.1002/stem.1668

Nitahara-Kasahara Y et al (2021) Dental pulp stem cells can improve muscle dysfunction in animal models of Duchenne muscular dystrophy. Stem Cell Res Ther 12:78. https://doi.org/10.1186/s13287-020-02099-3

Nizzardo M et al (2014) Minimally invasive transplantation of iPSC-derived ALDHhiSSCloVLA4+ neural stem cells effectively improves the phenotype of an amyotrophic lateral sclerosis model. Hum Mol Genet 23:342–354. https://doi.org/10.1093/hmg/ddt425

O'Donovan MC (1993) A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. Cell 72:971–983

Olson SD et al (2012) Examination of mesenchymal stem cell-mediated RNAi transfer to Huntington's disease affected neuronal cells for reduction of huntingtin. Mol Cell Neurosci 49:271–281. https://doi.org/10.1016/j.mcn.2011.12.001

Ooi J et al (2019) Unbiased profiling of isogenic huntington disease hPSC-Derived CNS and peripheral cells reveals strong cell-type specificity of CAG length effects. Cell Rep 26: 2494–508e7. https://doi.org/10.1016/j.celrep.2019.02.008

Oura S et al (2021) Precise CAG repeat contraction in a Huntington's disease mouse model is enabled by gene editing with SpCas9-NG. Commun Biol 4:771. https://doi.org/10.1038/s42003-021-02304-w

Özmert E, Arslan U (2020) Management of retinitis pigmentosa by Wharton's jelly derived mesenchymal stem cells: preliminary clinical results. Stem Cell Res Ther 11:1–16. https://doi.org/10.1186/s13287-020-1549-6

Pan D, Buning H, Ling C (2019) Rational design of gene therapy vectors. Mol Ther Methods Clin Dev. 12:246–247. https://doi.org/10.1016/j.omtm.2019.01.009

Paolini Sguazzi G, Muto V, Tartaglia M, Bertini E, Compagnucci C (2021) Induced pluripotent stem cells (iPSCs) and gene therapy: a new era for the treatment of neurological diseases. Int J Mol Sci 22:13674. https://doi.org/10.3390/ijms222413674

Paracchini V et al (2012) Amniotic mesenchymal stem cells: a new source for hepatocyte-like cells and induction of CFTR expression by coculture with cystic fibrosis airway epithelial cells. J Biomed Biotechnol 2012:575471. https://doi.org/10.1155/2012/575471

Park JW et al (2017) Recapitulating and correcting marfan syndrome in a cellular model. Int J Biol Sci 13:588–603. https://doi.org/10.7150/ijbs.19517

Park KE, Telugu BP (2013) Role of stem cells in large animal genetic engineering in the TALENs-CRISPR era. Reprod Fertil Dev 26:65–73. https://doi.org/10.1071/RD13258

Patel A, Riordan N (2015) Allogeneic transplantation of human umbilical cord mesenchymal stem cells (Uc-Msc) for a single male patient with duchenne muscular dystrophy (Dmd). Cytotherapy 17:S46–S46. https://doi.org/10.1016/j.jcyt.2015.03.469

Pawliuk R et al (2001) Correction of sickle cell disease in transgenic mouse models by gene therapy. Science 294:2368–2371. https://doi.org/10.1126/science.1065806

Peranteau WH, Hayashi S, Abdulmalik O, Chen Q, Merchant A, Asakura T, Flake AW (2015) Correction of murine hemoglobinopathies by prenatal tolerance induction and postnatal nonmyeloablative allogeneic BM transplants. Blood 126:1245–1254. https://doi.org/10.1182/blood-2015-03-636803

Perumbeti A, Higashimoto T, Urbinati F, Franco R, Meiselman HJ, Witte D, Malik P (2009) A novel human gamma-globin gene vector for genetic correction of sickle cell anemia in a humanized sickle mouse model: critical determinants for successful correction. Blood 114:1174–1185. https://doi.org/10.1182/blood-2009-01-201863

Petrou P et al (2016) Safety and clinical effects of mesenchymal stem cells secreting neurotrophic factor transplantation in patients with amyotrophic lateral sclerosis: results of phase 1/2 and 2a clinical trials. JAMA Neurol 73:337–344. https://doi.org/10.1001/jamaneurol.2015.4321

Petrou P, Kassis I, Yaghmour NE, Ginzberg A, Karussis D (2021) A phase II clinical trial with repeated intrathecal injections of autologous mesenchymal stem cells in patients with amyotrophic lateral sclerosis. Front Biosci (Landmark Ed) 26:693–706. https://doi.org/10.52586/4980

Piñol-Jurado P et al (2017) Platelet-derived growth factor BB influences muscle regeneration in Duchenne muscle dystrophy. Am J Pathol 187:1814–1827. https://doi.org/10.1016/j.ajpath.2017.04.011

Poletti V, Mavilio F (2021) Designing lentiviral vectors for gene therapy of genetic diseases. Viruses 13:1526. https://doi.org/10.3390/v13081526

Pollock K et al (2016) Human mesenchymal stem cells genetically engineered to overexpress brain-derived neurotrophic factor improve outcomes in huntington's disease mouse models. Mol Ther 24:965–977. https://doi.org/10.1038/mt.2016.12

Pratumkaew P, Issaragrisil S, Luanpitpong S (2021) Induced pluripotent stem cells as a tool for modeling hematologic disorders and as a potential source for cell-based therapies. Cell 10:3250. https://doi.org/10.3390/cells10113250

Rachmilewitz EA, Giardina PJ (2011) How I treat thalassemia. Blood 118(13):3479–3488. https://doi.org/10.1182/blood-2010-08-300335

Ramalingam S, Annaluru N, Kandavelou K, Chandrasegaran S (2014) TALEN-mediated generation and genetic correction of disease-specific human induced pluripotent stem cells. Curr Gene Ther 14:461–472. https://doi.org/10.2174/1566523214666140918101725

Ramamoorth M, Narvekar A (2015) Non viral vectors in gene therapy-an overview. J Clin Diagn Res 9:GE01–6. https://doi.org/10.7860/JCDR/2015/10443.5394

Ribeil JA et al (2017) Gene therapy in a patient with sickle cell disease. N Engl J Med 376:848–855. https://doi.org/10.1056/NEJMoa1609677

Robbins PD, Ghivizzani SC (1998) Viral vectors for gene therapy. Pharmacol Ther 80:35–47

Robinson PN et al (2006) The molecular genetics of Marfan syndrome and related disorders. J Med Genet 43:769–787. https://doi.org/10.1136/jmg.2005.039669

Romero Z et al (2013) Beta-globin gene transfer to human bone marrow for sickle cell disease. J Clin Invest 123:3317–3330. https://doi.org/10.1172/JCI67930

Rossignol J et al (2011) Mesenchymal stem cell transplantation and DMEM administration in a 3NP rat model of Huntington's disease: morphological and behavioral outcomes. Behav Brain Res 217:369–378. https://doi.org/10.1016/j.bbr.2010.11.006

Rossignol J et al (2015) Reductions in behavioral deficits and neuropathology in the R6/2 mouse model of Huntington's disease following transplantation of bone-marrow-derived mesenchymal stem cells is dependent on passage number. Stem Cell Res Ther 6:9. https://doi.org/10.1186/scrt545

Rouger K et al (2011) Systemic delivery of allogenic muscle stem cells induces long-term muscle repair and clinical efficacy in duchenne muscular dystrophy dogs. Am J Pathol 179:2501–2518. https://doi.org/10.1016/j.ajpath.2011.07.022

Russo FP et al (2006) The bone marrow functionally contributes to liver fibrosis. Gastroenterology 130:1807–1821. https://doi.org/10.1053/j.gastro.2006.01.036

Sakai-Takemura F et al (2018) Premyogenic progenitors derived from human pluripotent stem cells expand in floating culture and differentiate into transplantable myogenic progenitors. Sci Rep 8:6555. https://doi.org/10.1038/s41598-018-24959-y

Sebastiano V et al (2011) In situ genetic correction of the sickle cell anemia mutation in human induced pluripotent stem cells using engineered zinc finger nucleases. Stem Cells 29:1717–1726. https://doi.org/10.1002/stem.718

Shang YC, Wang SH, Xiong F, Peng FN, Liu ZS, Geng J, Zhang C (2016) Activation of Wnt3a signaling promotes myogenic differentiation of mesenchymal stem cells in mdx mice. Acta Pharmacol Sin 37:873–881. https://doi.org/10.1038/aps.2016.38

Shaw SW et al (2021) Prenatal transplantation of human amniotic fluid stem cell could improve clinical outcome of type III spinal muscular atrophy in mice. Sci Rep 11:9158. https://doi.org/10.1038/s41598-021-88559-z

Siegel AL, Kuhlmann PK, Cornelison DD (2011) Muscle satellite cell proliferation and association: new insights from myofiber time-lapse imaging. Skelet Muscle 1:7. https://doi.org/10.1186/2044-5040-1-7

Siemionow M, Szilagyi E, Cwykiel J, Domaszewska-Szostek A, Heydemann A, Garcia-Martinez J, Siemionow K (2021) Transplantation of dystrophin expressing chimeric human cells of myoblast/mesenchymal stem cell origin improves function in duchenne muscular dystrophy model. Stem Cells Dev 30:190–202. https://doi.org/10.1089/scd.2020.0161

Singh A, Singh A, Sen D (2016) Mesenchymal stem cells in cardiac regeneration: a detailed progress report of the last 6 years (2010-2015). Stem Cell Res Ther 7:82. https://doi.org/10.1186/s13287-016-0341-0

Sironi F et al (2017) Multiple intracerebroventricular injections of human umbilical cord mesenchymal stem cells delay motor neurons loss but not disease progression of SOD1G93A mice. Stem Cell Res 25: 166–178. https://doi.org/10.1016/j.scr.2017.11.005

Snijders T, Nederveen JP, McKay BR, Joanisse S, Verdijk LB, van Loon LJ, Parise G (2015) Satellite cells in human skeletal muscle plasticity. Front Physiol 6:283. https://doi.org/10.3389/fphys.2015.00283

Song H, Chung SK, Xu Y (2010) Modeling disease in human ESCs using an efficient BAC-based homologous recombination system. Cell Stem Cell 6:80–89. https://doi.org/10.1016/j.stem.2009.11.016

Staff NP et al (2016) Safety of intrathecal autologous adipose-derived mesenchymal stromal cells in patients with ALS. Neurology 87:2230–2234. https://doi.org/10.1212/WNL.0000000000003359

Sterneckert JL, Reinhardt P, Scholer HR (2014) Investigating human disease using stem cell models. Nat Rev Genet 15: 625–639. https://doi.org/10.1038/nrg3764

Sun H et al (2014) Multiple systemic transplantations of human amniotic mesenchymal stem cells exert therapeutic effects in an ALS mouse model. Cell Tissue Res 357:571–582. https://doi.org/10.1007/s00441-014-1903-z

Sun X, Song J, Huang H, Chen H, Qian K (2018) Modeling hallmark pathology using motor neurons derived from the family and sporadic amyotrophic lateral sclerosis patient-specific iPS cells. Stem Cell Res Ther 9:315. https://doi.org/10.1186/s13287-018-1048-1

Sutton MT et al (2017) Mesenchymal stem cell soluble mediators and cystic fibrosis. J Stem Cell Res Ther 7. https://doi.org/10.4172/2157-7633.1000400

Sutton MT et al (2016) Antimicrobial properties of mesenchymal stem cells: therapeutic potential for cystic fibrosis infection, and treatment. Stem Cells Int 2016: 5303048. https://doi.org/10.1155/2016/5303048

Sykova E et al (2017) Transplantation of mesenchymal stromal cells in patients with amyotrophic lateral sclerosis: results of phase I/IIa clinical trial. Cell Transplant 26:647–658. https://doi.org/10.3727/096368916X693716

Szlachcic WJ, Wiatr K, Trzeciak M, Figlerowicz M, Figiel M (2017) The generation of mouse and human Huntington disease iPS cells suitable for in vitro studies on huntingtin function. Front Mol Neurosci 10:253. https://doi.org/10.3389/fnmol.2017.00253

Tabebordbar M et al (2016) In vivo gene editing in dystrophic mouse muscle and muscle stem cells. Science 351:407–411. https://doi.org/10.1126/science.aad5177

Taetzsch T, Shapiro D, Eldosougi R, Myers T, Settlage RE, Valdez G (2021) The microRNA miR-133b functions to slow Duchenne muscular dystrophy pathogenesis. J Physiol 599:171–192. https://doi.org/10.1113/JP280405

Thakker R, Yang P (2014) Mesenchymal stem cell therapy for cardiac repair. Curr Treat Options Cardiovasc Med 16:323. https://doi.org/10.1007/s11936-014-0323-4

Thompson AA et al (2018) Gene therapy in patients with transfusion-dependent beta-thalassemia. N Engl J Med 378:1479–1493. https://doi.org/10.1056/NEJMoa1705342

Till JE, Mc CE (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 14:213–222

Toma C, Pittenger MF, Cahill KS, Byrne BJ, Kessler PD (2002) Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. Circulation 105:93–98. https://doi.org/10.1161/hc0102.101442

Tousley A, Kegel-Gleason KB (2016) Induced pluripotent stem cells in huntington's disease research: progress and opportunity. J Huntingtons Dis 5:99–131. https://doi.org/10.3233/JHD-160199

Tuekprakhon A et al (2021) Intravitreal autologous mesenchymal stem cell transplantation: a non-randomized phase I clinical trial in patients with retinitis pigmentosa. Stem Cell Res Ther 12:52. https://doi.org/10.1186/s13287-020-02122-7

Turan S, Farruggio AP, Srifa W, Day JW, Calos MP (2016) Precise correction of disease mutations in induced pluripotent stem cells derived from patients with limb girdle muscular dystrophy. Mol Ther 24: 685–696. https://doi.org/10.1038/mt.2016.40

Uccelli A et al (2012) Intravenous mesenchymal stem cells improve survival and motor function in experimental amyotrophic lateral sclerosis. Mol Med 18:794–804. https://doi.org/10.2119/molmed.2011.00498

Ukai H, Kiyonari H, Ueda HR (2017) Production of knock-in mice in a single generation from embryonic stem cells. Nat Protoc 12:2513–2530. https://doi.org/10.1038/nprot.2017.110

Urnov F, Rebar E, Holmes M, Zhang HS, Gregory PD (2010) Nature Rev Genet 11:636–646

Vallejo D et al (2018) PITX2 enhances the regenerative potential of dystrophic skeletal muscle stem cells. Stem Cell Rep 10:1398–1411. https://doi.org/10.1016/j.stemcr.2018.03.009

Verma P, Greenberg RA (2016) Noncanonical views of homology-directed DNA repair. Genes Dev 30:1138–1154. https://doi.org/10.1101/gad.280545.116

Verma M, Asakura Y, Asakura A (2019) Inhibition of microRNA-92a increases blood vessels and satellite cells in skeletal muscle but does not improve duchenne muscular dystrophy–related phenotype in mdx mice. Muscle Nerve 59:594–602. https://doi.org/10.1002/mus.26433

Villamizar O, Waters SA, Scott T, Grepo N, Jaffe A, Morris KV (2021) Mesenchymal stem cell exosome delivered zinc finger protein activation of cystic fibrosis transmembrane conductance regulator. J Extracell Vesicles 10:e12053. https://doi.org/10.1002/jev2.12053

Violatto MB et al (2015) Longitudinal tracking of triple labeled umbilical cord derived mesenchymal stromal cells in a mouse model of amyotrophic lateral sclerosis. Stem Cell Res 15:243–253. https://doi.org/10.1016/j.scr.2015.06.010

Walters MC et al (2021) Updated results of a phase 1/2 clinical study of zinc finger nuclease-mediated editing of BCL11A in autologous hematopoietic stem cells for transfusion-dependent beta thalassemia. Blood 138: 3974. https://doi.org/10.1182/blood-2021-147907

Wang Q, Gong X, Gong Z, Ren X, Ren Z, Huang S, Zeng Y (2013a) The mesenchymal stem cells derived from transgenic mice carrying human coagulation factor VIII can correct phenotype in hemophilia a mice. J Genet Genomics 40:617–628. https://doi.org/10.1016/j.jgg.2013.11.002

Wang ZB, Zhang X, Li XJ (2013b) Recapitulation of spinal motor neuron-specific disease phenotypes in a human cell model of spinal muscular atrophy. Cell Res 23:378–393. https://doi.org/10.1038/cr.2012.166

Wang P, Mokhtari R, Pedrosa E, Kirschenbaum M, Bayrak C, Zheng D, Lachman HM (2017) CRISPR/Cas9-mediated heterozygous knockout of the autism gene CHD8 and characterization of its transcriptional networks in cerebral organoids derived from iPS cells. Mol Autism 8:11. https://doi.org/10.1186/s13229-017-0124-1

Wang SH, Shang YC, Yu MJ, Xiong F, Zhang C (2019a) Genetically engineered mesenchymal stem cells with human micro-dystrophin improve skeletal muscle histopathology in mdx mice. Journal of Biomaterials and Tissue Engineering 9:50–61. https://doi.org/10.1166/jbt.2019.1941

Wang Y et al (2019b) Drug screening with human SMN2 reporter identifies SMN protein stabilizers to correct SMA pathology. Life Sci Alliance 2:10.26508/lsa.201800268

Wang YX et al (2019c) EGFR-Aurka signaling rescues polarity and regeneration defects in dystrophin-deficient muscle stem cells by increasing asymmetric divisions. Cell Stem Cell 24: 419–32 e6. https://doi.org/10.1016/j.stem.2019.01.002

Weber L et al (2018) An optimized lentiviral vector efficiently corrects the human sickle cell disease phenotype. Mol Ther Methods Clin Dev 10:268–280. https://doi.org/10.1016/j.omtm.2018.07.012

Wefers B, Meyer M, Ortiz O, Hrabe de Angelis M, Hansen J, Wurst W, Kuhn R (2013) Direct production of mouse disease models by embryo microinjection of TALENs and oligodeoxynucleotides. Proc Natl Acad Sci USA 110:3782–3787. https://doi.org/10.1073/pnas.1218721110

Weiss JN, Levy S (2018) Stem cell ophthalmology treatment study: bone marrow derived stem cells in the treatment of retinitis Pigmentosa. Stem Cell Investig 5:18. https://doi.org/10.21037/sci.2018.04.02

Wettstein R, Bodak M, Ciaudo C. (2015) Generation of a knockout mouse embryonic stem cell line using a paired CRISPR/Cas9 genome engineering tool. In: Embryonic stem cell protocols. Springer. https://doi.org/10.1007/7651_2015_213

Wiedenheft B, Sternberg SH, Doudna JA (2012) RNA-guided genetic silencing systems in bacteria and archaea. Nature 482:331–338. https://doi.org/10.1038/nature10886

Xia G, Terada N, Ashizawa T (2018) Human iPSC models to study orphan diseases: muscular dystrophies. Curr Stem Cell Rep 4:299–309. https://doi.org/10.1007/s40778-018-0145-5

Xie F, Ye L, Chang JC, Beyer AI, Wang J, Muench MO, Kan YW (2014) Seamless gene correction of beta-thalassemia mutations in patient-specific iPSCs using CRISPR/Cas9 and piggyBac. Genome Res 24:1526–1533. https://doi.org/10.1101/gr.173427.114

Xiong F et al (2010) Microdystrophin delivery in dystrophin-deficient (mdx) mice by genetically-corrected syngeneic MSCs transplantation. In Transplantation proceedings. Elsevier, pp 2731–2739

Xu X et al (2017) Reversal of phenotypic abnormalities by CRISPR/Cas9-mediated gene correction in Huntington disease patient-derived induced pluripotent stem cells. Stem Cell Rep 8:619–633. https://doi.org/10.1016/j.stemcr.2017.01.022

Yasue A et al (2014) Highly efficient targeted mutagenesis in one-cell mouse embryos mediated by the TALEN and CRISPR/Cas systems. Sci Rep 4:5705. https://doi.org/10.1038/srep05705

Yin H, Price F, Rudnicki MA (2013) Satellite cells and the muscle stem cell niche. Physiol Rev 93:23–67. https://doi.org/10.1152/physrev.00043.2011

Yin H, Kanasty R, Eltoukhy AA, Vegas AJ, Dorkin JR, Anderson DG (2014) Non-viral vectors for gene-based therapy. Nat Rev Genet 15:541–555. https://doi.org/10.1038/nrg3763

Yoon Y et al (2020) Neural transplants from human induced pluripotent stem cells rescue the pathology and behavioral defects in a rodent model of huntington's disease. Front Neurosci 14:947. https://doi.org/10.3389/fnins.2020.558204

Young CS et al (2016) A single CRISPR-Cas9 deletion strategy that targets the majority of DMD patients restores dystrophin function in hiPSC-derived muscle cells. Cell Stem Cell 18:533–540. https://doi.org/10.1016/j.stem.2016.01.021

Yu-Taeger L et al (2019) Intranasal administration of mesenchymal stem cells ameliorates the abnormal dopamine transmission system and inflammatory reaction in the R6/2 mouse model of Huntington disease. Cell 8:595. https://doi.org/10.3390/cells8060595

Zakrzewski W, Dobrzynski M, Szymonowicz M, Rybak Z (2019) Stem cells: past, present, and future. Stem Cell Res Ther 10:68. https://doi.org/10.1186/s13287-019-1165-5

Zhang FQ, Jiang JL, Zhang JT, Niu H, Fu XQ, Zeng LL (2020) Current status and future prospects of stem cell therapy in Alzheimer's disease. Neural Regen Res 15: 242–250. https://doi.org/10.4103/1673-5374.265544

Zhang H et al (2016) NAD(+) repletion improves mitochondrial and stem cell function and enhances life span in mice. Science 352:1436–1443. https://doi.org/10.1126/science.aaf2693

Zhao C et al (2014) Recombinase-mediated reprogramming and dystrophin gene addition in mdx mouse induced pluripotent stem cells. PLoS One 9: e96279. https://doi.org/10.1371/journal.pone.0096279

Zhao X, Zhang Y, Wilkins K, Edelmann W, Usdin K (2018) MutLγ promotes repeat expansion in a fragile X mouse model while EXO1 is protective. PLoS Genet 14:e1007719. https://doi.org/10.1371/journal.pgen.1007719

Zhou C, Zhang C, Zhao R, Chi S, Ge P, Zhang C (2013) Human marrow stromal cells reduce microglial activation to protect motor neurons in a transgenic mouse model of amyotrophic lateral sclerosis. J Neuroinflammation 10:52. https://doi.org/10.1186/1742-2094-10-52

Zhou M et al (2018) Seamless genetic conversion of SMN2 to SMN1 via CRISPR/Cpf1 and single-stranded oligodeoxynucleotides in spinal muscular atrophy patient-specific induced pluripotent stem cells. Hum Gene Ther 29:1252–1263. https://doi.org/10.1089/hum.2017.255

Zou J, Mali P, Huang X, Dowey SN, Cheng L (2011a) Site-specific gene correction of a point mutation in human iPS cells derived from an adult patient with sickle cell disease. Blood 118:4599–4608. https://doi.org/10.1182/blood-2011-02-335554

Zou JZ et al (2011b) Oxidase-deficient neutrophils from X-linked chronic granulomatous disease iPS cells: functional correction by zinc finger nuclease-mediated safe harbor targeting. Blood 117:5561–5572. https://doi.org/10.1182/blood-2010-12-328161

Zou X et al (2016) Acetoacetate accelerates muscle regeneration and ameliorates muscular dystrophy in mice. J Biol Chem 291:2181–2195. https://doi.org/10.1074/jbc.M115.676510

Zulueta A et al (2018) Lung mesenchymal stem cells-derived extracellular vesicles attenuate the inflammatory profile of cystic fibrosis epithelial cells. Cell Signal 51:110–118. https://doi.org/10.1016/j.cellsig.2018.07.015

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Gene Therapeutic Delivery to the Salivary Glands

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Abstract

The salivary glands, exocrine glands in our body producing saliva, can be easily damaged by various factors. Radiation therapy and Sjogren's syndrome (a systemic autoimmune disease) are the two main causes of salivary gland damage, leading to a severe reduction in patients' quality of life. Gene transfer to the salivary glands has been considered a promising approach to treating the dysfunction. Gene therapy has long been applied to cure multiple diseases, including cancers, and hereditary and infectious diseases, which are proven to be safe and effective for the well-being of patients. The application of this treatment on salivary gland injuries has been studied for decades, yet its clinical progress is delayed. This chapter provides a coup d'oeil into gene transfer methods and various gene/vector types for salivary glands to help the new scientists and update established scientists on the progress that has been made during the past decades for the treatment of salivary gland disorders.

Keywords

Gene therapy · Gene transfer · Radiation damage · Salivary gland regeneration · Sjogren's syndrome

Abbreviations

5-AZA	5-aza-20-deoxycytidine
AAV	Adeno-associated virus
Ad5 vector	Adenoviral vector type 5
AdCMV-hEPO	Adenoviral vector (Ad) cytomegalovirus (CMV) and human erythropoietin (hEPO)
AdEF1α-hEPO	Adenoviral vector (Ad) with human elongation factor-1α (EF1α) and human erythropoietin (hEPO)
AdhAQP1	Adenoviral human aquaporin-1
AdLTR2EF1α-hEPO	Adenoviral vector (Ad), 251-bp envelope sequence plus 607-bp long-terminal repeat (LTR), paired with human elongation factor-

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	1α (EF1α) and human erythropoietin (hEPO)
AIDS	Acquired immune deficiency syndrome
AQP1/5	Aquaporin 1/5
BDNF	Brain derived neurotrophic factor
CAS 9	CRISPR-associated protein 9
CFTR	Cystic fibrosis transmembrane conductance regulator
COX1	Cyclooxygenase 1
CSP	Constitutive secretory pathway
DNA	Deoxyribonucleic acid
Fc	Fragment of crystallization
FGF	Fibroblast growth factor
GFP	Green fluorescent protein
Gli1	Glioma-associated oncogen 1
hEPO	Human erythropoietin
hNTR	Human neurturin
IL17	Interleukin 17
KGF	Keratinocyte growth factor
MHC	Major histocompatibility complex
NKCC1 co-transporter	Na-K-Cl co-transporter
PGIS	Prostacyclin synthase
RNA	Ribonucleic acid
RSP	Regulatory secretory pathways
sgRNA	Single guide RNA
SHH	Sonic Hedgehog
UAGT	Ultrasound-assisted gene transfer

1 Introduction to Gene Delivery Methods

Gene therapeutic delivery is the strategy of genetic modification or transferring genes into the patient's cells to treat diseases. The idea originated from the search for cancer treatment and has been extensively studied for decades and theoretically can be applied as potential management for multiple defective organs in the body, hereditary disorders, and infections in humans. Currently, this therapeutic approach consists of two primary types: germline therapy and somatic cell gene therapy.

1.1 Germline Genetic Approach

The germline gene delivery is performed in the pre-embryo phase, in which permanent genetic changes will be made and passed down to the next generations. One of the advantages of modifying genes on reproductive cells is the feasibility of eradicating some genetic diseases passing down through families or improving the good genes of human beings. However, this approach has also raised myriads of ethical concerns among scientists and leaders worldwide, as they worry that the irreversible changes in the human genome could be detrimental and may lead to unforeseeable consequences. According to the Oviedo Convention (2017), human rights and dignity should be prioritized over scientific ambition. Therefore, the establishment of pregnancy with modified germ cells has been prohibited in all the member countries for the concerns about its safety and efficacy (Baylis and Ikemoto 2017). However, if this approach were to be banned altogether, the utilization of somatic cell therapy would also be tightened due to the provision that the edited genes in somatic cells may unintentionally affect the reproductive cells and pass down to the next generation. Some pre-clinical experiments have been conducted on the embryos of animals, including rhesus monkeys (Chen et al. 2015), rabbits (Yuan et al. 2016), and mice (Huai et al. 2017). These studies have demonstrated successful the feasibility of editing germline cells in mammals. Yet, so far, rarely any clinical trials have been performed on genetic delivery on human reproductive cell lines, owing to its complexity and violation of morality. Hence, its possibility and practicality still need to be clarified (Doxzen and Halpern 2020; Fox 2002).

1.2 Somatic Cell Genetic Approach

Somatic cell therapy involves introducing genetic material, usually, RNA or DNA, into a human's targeted diploid cells. This approach is more conventional and benign as theoretically it does not pass the genetic changes onto descendants. Somatic gene therapy often consists of two basic techniques: in vivo and ex vivo. Concerning the in vivo technique, the genetically altered material will be injected directly into the patient's body as viral or plasmid vectors. Once inside the body, the therapeutic gene will incorporate with the targeted cells to fabricate beneficial productions, which aim to fix damaged tissues or restore an organ's function (Hutt et al. 2022). In the ex vivo method, cells with damaged genes will be taken from the patients, then genetically modified in the laboratory. After that, the modified cells will be reinjected into the body. Compared to in vivo therapy, the ex vivo counterpart is less risky and often generates fewer adverse effects on the body's immune system; however, this technique can only be utilized on certain types of cells or tissues. Currently, hematopoietic cells are among the most popular cells used for ex vivo gene therapy (Zubler 2007). Somatic cell gene therapy has been extensively researched and applied in multiple diseases such as renal diseases (Blum et al. 2017; Maruyama 2006; Herold et al. 2002), sickle cell disease (Stone et al. 2021; Hsieh et al. 2020; Brendel et al. 2020), cystic fibrosis (Kim et al. 2022; Reyne et al. 2021; Marquez Loza et al. 2021; Yang et al. 2019; Xia et al. 2019), cancer (Chen et al. 2022; Oishi et al. 2022; Liu et al. 2022; Dong et al. 2022; Iacobas and Iacobas 2022), and acquired immune deficiency syndrome (AIDS) (Zhang et al. 2022; Zaidane et al. 2020; Xiao et al. 2019; Qadir and Zafar 2017; Bovolenta et al. 2013).

Lately, gene therapy has also been considered potential prevention and management for Covid-19. Regarding the prevention of Covid-19, many RNA and adenoviral vector vaccines have been briefly approved and widely administrated globally. Even though these vaccines are considered effective in preventing the disease, their long-term safety and efficiency should be further explored and continuously followed up (Harford et al. 2022; Nakagami and Morishita 2022; Flotte and Gao 2021; Abu Abed 2021; Nakagami 2021). One of the disadvantages of this treatment is its short-term effect since the edited somatic cells will soon come to their apoptosis phage and be cleared out from the body. Hence, to maintain the therapy's effectiveness, the patients are recommended to retake the treatment after some time and in some circumstances, over their lifetime (Cornel et al. 2019).

2 Types of Genes/Vectors in Salivary Gland Gene Transfer

Gene therapy, in essence, is an alteration in the expression of a gene, either its upregulation, downregulation, addition, or deletion in the desired cells or tissues. Conventionally, it involved the introduction of a vector (carrier) with an open gene sequence of the target gene and a poly-A tail. More recently, this area has evolved to include different approaches, extending to the silencing of genes and sophisticated gene editing technologies. Delivery of the genes into the cytoplasm after crossing the cell membrane and the endosome is the most challenging part of transduction (Fig. 1) (Passineau 2017). Salivary gland epithelial cells are convenient targets and offer relatively easy permissibility. So, various methods have been experimented with over the years, as discussed below.

2.1 Viral Vector-Based Transmission

Viruses were naturally one of the best candidates for transgenic approaches, given their ability to enter the cells and manipulate the cellular machinery for their own transcription and multiplication. Their attenuated forms can be used to deliver the gene of interest.

Adenoviruses Adenoviruses are non-enveloped double-stranded DNA viruses. They have been most abundantly employed for salivary gland research, particularly the serotypes Ad5 and

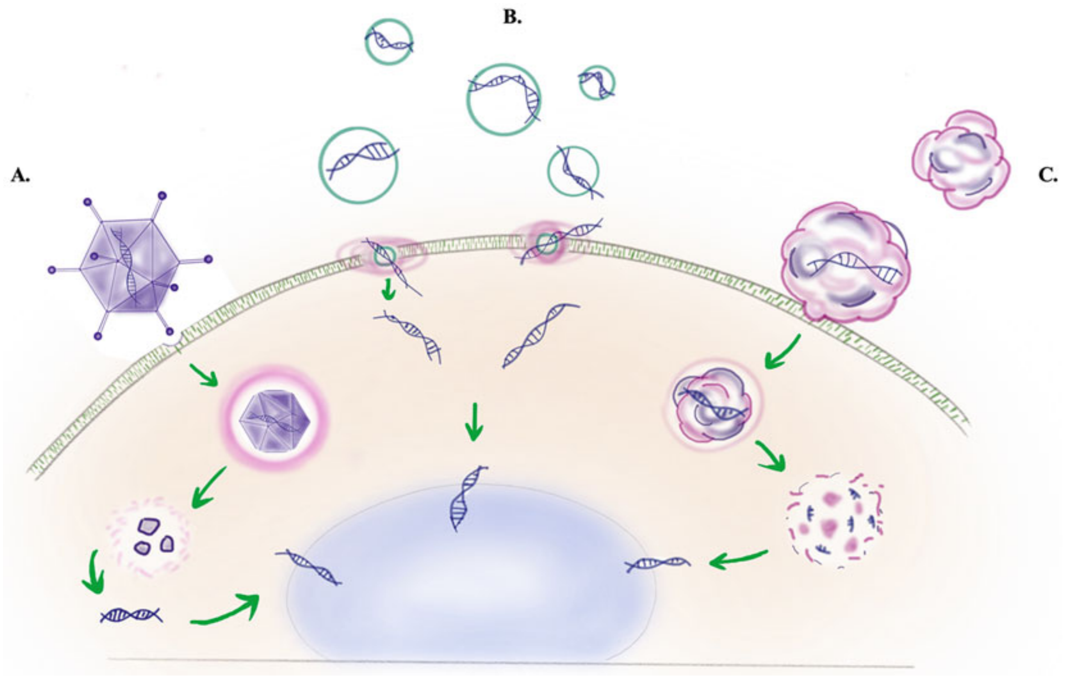

Fig. 1 Basic biology of exogenous gene transfer to a target cell: (**a**) Viral vectors enter through particular receptor-ligand interactions. Upon entering, they have the mechanisms to escape the endosome, delivering their genetic material for further processing. On the downside, the viral residues invariably get recognized by MHC receptors. (**b**) The sonoporation technique allows direct entry of the transgene into the cells by creating transient pores. (**c**) Nanoparticles/micelles can enter the cells, move through the endosome by biochemical interactions, and have reduced MHC recognition, yet, the precise mechanisms remain challenging to counter the dynamic cell microenvironment. (Image adapted from Passineau 2017)

serotype-2 adeno-associated viral (AAV2). Ad5 vector is readily accepted by the salivary epithelial cells (Samuni and Baum 2011), while there are concerns over the acinar cell transfection with AAV2 serotype (Di Pasquale et al. 2020). Ad5 is associated with high immunogenicity, while AAV2 is less associated with the MHC recognition (Voutetakis et al. 2004). Dr. Baum and their team have made various efforts to modify these vectors to increase the duration of transgenic expression, increase acinar cell transfection, and reduce the immunogenicity (Zheng et al. 2012; Rocha et al. 2013). Adding retroviral elements increased the expression duration to 2–3 months (Rocha et al. 2013; Zheng et al. 2008b), while deletion of the replication E gene further increased it to 6 months (Zheng et al. 2012).

Recently, Di Pasquale et al. (2020) identified the most efficient AAV44.9 serotype to transduce acinar cells. Nevertheless, they concluded with the species-specific ability of each serotype, probably due to cellular, molecular, and genetic differences (Di Pasquale et al. 2020). Furthermore, there are some speculations about the neutralization of AAV vectors due to the presence of already present antibodies to its serotypes in disease-free populations as well as patients with Sjogren's syndrome and other autoimmune conditions. Neutralization can potentially decrease the transduction and the expression of the intended gene. However, the evidence so far is unclear, if this effect is clinically relevant. The lowest concentration of antobodies has been found to be toward the 4, 5, and 12 serotypes, while the highest to AAV5 (Corden et al. 2017). A successful clinical trial using AAV2 (Alevizos et al. 2017) can be used as a reference and is thus considered safe for future studies. Adeno Simian Virus 40 (SV 40) is another common vector from this class of virus; its use is more common in the in vitro cell transfections (Azuma et al. 1993).

Retroviruses/Lentiviruses Retroviruses are enveloped single-stranded RNA viruses with high pathogenic potential. Their attenuated forms can be readily used for transfections. However, due to their requirement for the cells to be actively dividing, they fall short for salivary gland cells. Salivary gland cells are mostly considered post-mitotic with limited division physiologically. It can be possible to transfect these cells by pre-treatment with mitogenic agents such as adrenergic agents, without which the transduction rates can be as low as null (Baum and O'Connell 1999; Barka and van der Noen 1996). Lentiviruses do not require cell division but are not as efficient (Shai et al. 2002). Herpes simplex and vaccinia viruses are more efficient than lentiviruses, yet they may induce a lymphocytic response (Barka and van der Noen 1996).

2.2 Non-viral-Based Transmission

Various methods of non-viral transgenic modification are, namely, gene guns, electroporation, sonoporation, and laser irradiation (Samuni and Baum 2011). Plasmids, polymers, and liposomes can be used to employ these methods. The most of recent and common methods are described below. Plasmids alone have shown limited success in transduction in the glands (Baum and O'Connell 1999). A systemic comparison of viral and non-viral methods (Zufferey and Aebischer 2004; Baum et al. 2015) can be referred to for more information.

Nanoparticles Gene delivery into the cytoplasm usually requires a carrier to transport or increase the pores of the cell membrane using microtubules (Passineau et al. 2010). Due to this, these methods have limited transfection rates. As the name suggests, nanoparticles are small biochemical constructs specially modified to have enhanced potential to enter the cells with the least immune reaction. Arany et al. utilized such a polymer to transduce NKCC1 (an ion co-transporter in acinar cells) and anti-apoptotic genes for ameliorating the radiation effect (Arany et al. 2013). These co-polymers consist of a siRNA binding site, positively-negatively charged, and hydrophobic residues, giving it a neutral charge at physiological pH. Upon contact with the acidic endosomal environment, protonation of the hydrophobic residue allows the polymer to interact with its membrane and thus deliver the siRNA into the cytoplasm (Convertine et al. 2010). More recently, Dr. Ovitt and their group tested serum-containing and serum-free conditions for delivering these nanoparticles. Serum proteins caused aggregation and reduced the transfection efficiency (Malcolm et al. 2018). This result highlights the importance of choosing the proper mode of delivery for such therapies, majorly parenteral or local routes. Their work indicates that the local route, here salivary glands, must be the most efficient in reducing the interaction with serum proteins, although this route might lead to a reduction in the toxicity tolerance of these substances.

Sonoporation Ultrasound-assisted gene transfer (UAGT) technology was explored to avoid administering viral-like particles into the glands, which was expected to reduce the host immune response (Passineau et al. 2010). Briefly, the process involves the introduction of a carrier solution containing plasmid vectors and lipid-shelled microtubules through bloodless cannulation of submandibular ducts. The microtubules are destroyed by high ultrasound energy, which creates cavitation in the cell membranes to allow the plasmids to enter the cells. It is believed to be less damaging to cell homeostasis as well as in maintaining a long-term transgenic expression (Wang et al. 2017a).

Artificial Complex and Epigenetic Editing The conventional transduction methods invariably have used non-human sources, majorly viral vectors and genetic sequences of bacterial origin. Wang et al. (2017b) introduced two novel technologies. First was the addition of artificial transcriptional complexes using the dCAS9 system to activate the native genes

(Wang et al. 2017b). The second was to activate the gene using an epigenetic modification of the gene site. The first method was carried out by transfection of three plasmids, namely, single-guide (sgRNA)-expressing plasmid, MS2-effector plasmid, and dCAS9 effector plasmidin, 1:1:1 ratio in the presence of polyethyleneimine (for permissive cells) and lipofectamine (for low permissive cells). Global demethylation for initiating epigenetic modification was carried out in the presence of 5-aza-20-deoxycytidine (5-AZA) and trichostatin. Both methods successfully activated the native AQP1 gene, achieving comparable expression levels to adenoviral-based transfection (Wang et al. 2017b) (Table 1).

Table 1 Various in vitro and in vivo methods of gene delivery and transgenic modification in the salivary glands

References	Clinical condition/ target	Type of transmission mode	Gene insertion/ modification	Comments/efficiency/period of transmission and expression
Vector based				
Ferreira et al. (2018) and Lombaert et al. (2020)	Radiation injury in murine and porcine models	AAV2	Human neurturin (hNTR) gene	Transduction 10 days prior to radiation gave more consistent results than 60 days' post-radiation
Hu et al. (2018) and Hai et al. (2016)	Radiation injury in murine and porcine models	Ad5	Sonic Hedgehog (Shh), Gli1 genes	
Alevizos et al. (2017)	Radiation injury in patients	AAV2	AQP1	5 out of 11 patients had improved salivary gland function (71–500% increase in parotid flow 3–4.7 years after treatment, with improved symptoms for ~2–3 years)
Zeng et al. (2017)	Sjogren's syndrome	AD5-GFP	Cystic fibrosis transmembrane conductance regulator	Treatment was done after 6–7 days of transduction and observed over 6 months after transduction
Lai et al. (2016)	Sjogren's syndrome	AAV2	AQP1	AQP1 expression was increased, 4 and 6 weeks' post cannulation in the salivary and lacrimal glands, respectively
Non-vector based				
Wang et al. (2017a)	Pulmonary arterial hypertension	UAGT	Fusion protein embodying cyclooxygenase-1 (Cox1) and prostacyclin synthase (PGIS) domains	
Wang et al. (2017b)	Human salivary ductal cell line and primary salivary human stem/ progenitor cells	dCAS9-SAM activator, global demethylation	AQP-1	AQP-1 expression in the cells was comparable to adenoviral-based transfection
Wu et al. (2015)	Sjogren's syndrome in Aec1/Aec2 mice	UAGT	Extracellular portion of the IL-17 receptor fused to fragment of crystallization (Fc)	Transfection efficiency was more than 10^7 but less than 10^9 adenovirus vectors administered, 48 h post-transfection

3 Gene Therapies on Targeted Salivary Glands

While gene therapy has been studied since the early 1980s, its application to the treatment of salivary gland dysfunctions only started more than a decade later, when the first report was published in 1994 by Mastrangeli et al. (1994). Since then, more studies have been conducted using both viral and non-viral vectors carrying corrected genes delivered into salivary glands through a ductal system in the oral cavity or systemically (Geguchadze et al. 2012, 2014; Zheng et al. 2008a, 2015). The technique called retroductal cannulation, which has long been applied in contrast to radiography for sialography, is now employed in therapeutic gene delivery. This procedure, considered a minimally invasive technique, can easily be accomplished without any anesthesia in patients (Samuni and Baum 2011). Clinically, the technique of gene delivery is mainly performed on major salivary glands such as submandibular (through Wharton's ducts) (Geguchadze et al. 2014; Geguchadze et al. 2012) and parotid glands (through Stensen's ducts) (Stenman et al. 2022; Fukumura et al. 2022).

Regarding the submandibular glands, a study by Zheng et al. has been conducted on the in vitro and in vivo effects of new retroviral factors in a serotype 5 adenoviral vector, AdLTR2EF1-α-hEPO on rats (Zheng et al. 2008a). The authors compared the expression of the new vector with two traditional Ad5 vectors, AdEF1α-hEPO and AdCMV-hEPO. They figured out that in 3 months the AdLTR2EF1α-hEPO vector helped to increase significantly the levels of human erythropoietin (hEPO) and hematocrits. The novel vector was considered potential not only for salivary gland gene therapy but also for many other clinical applications, where prolonged expression of the gene transferred is beneficial (Zheng et al. 2008a). Other studies have employed ultrasound-assisted techniques and the pseudo-typed adeno-associated–viral vector-mediated gene for salivary gland engineering. Research by Geguchadze et al. aimed to utilize the adeno-associated virus (AAV) with the canonical AAV2 gene in the mouse submandibular glands (Geguchadze et al. 2012). The findings demonstrated that a high dose of AAV increased the expression of salivary gland proteome by 32%, and a lower dose of 100-fold would decrease the result to 7%, as proved by whole-proteome profiling. They also suggested that among three serotypes 5, 8, and 9, the AAV2 vector with serotype 5 was the most propitious vector system for salivary gland gene delivery (Geguchadze et al. 2012). A more recent study from the same team attempted to compare the host responses and the efficiency of two techniques: a novel ultrasonic nonviral gene delivery and a traditional plasmid vector in mice submandibular glands (Geguchadze et al. 2014). The authors have successfully involved the fundamentals of ultrasound-assisted gene transfer (UAGT) into the salivary gland, based on the sonoporation method, which allows the biophysical transition of a vector across the membrane of salivary gland epithelial cells. Moreover, the impact of experimental nonviral gene transfer was also explored while utilizing the hallmark of salivary gland gene therapy as a model. They realized that the sole sonoporation method, without any gene transfer vector, can only have minimum effects (around 6%) on the proteome of the salivary gland. When combined with a plasmid vector, the effects of sonoporation were much more potent, causing a significant disruption of the salivary gland proteomic profile (Geguchadze et al. 2014).

In the same year, another Phase II pioneering UAGT trial of AdhAQP1 has also been conducted by Wang et al. (2015a). But this time, the experiment was performed on swine parotid glands. After being irradiated unilaterally in a 10Gy electron beam targeted, all the subjects were treated with ultrasonic AdhAQP1 gene transfer, which resulted in an intermediate re-establishment of salivary flow after 2 days and 7 days post-treatment. From these preclinical results, this present research provided convincing evidence that UAGT can be a promising method of gene delivery, which is expected to replace the adenovirus vector in transferring the hAQP1 gene as well as further progress to the Phase I human clinical trial (Wang et al. 2015a). Furthermore, another recent research on the parotid gland has also been performed by Alevizos et al. (2017) to evaluate the extended in vivo response of adenoviral human aquaporin-1 (AdhAQP1) gene delivery in five head and neck irradiated patients (Alevizos et al. 2017). All the patients were treated with therapeutic gene transfer in one side of the parotid gland and then were followed up for 3–4 years after that. The researchers collected the salivary flow rate and performed other assays in the follow-ups, which concluded that there were considerable 71–500% improvements in parotid flow rate after 4 years of treatment. Despite some minor alterations in [Na^+] and [Cl^-] ion levels in stimulated salivary flow, no adverse effects were observed in both preclinical and clinical settings (Alevizos et al. 2017). These studies have provided concrete evidence about the feasibility of therapeutic gene transfer to restore proper salivary gland functions and reduce hyposalivation in patients with Sjogren's syndrome and irradiation-damaged glands. They also open a new path for more advanced diagnosis, prognosis, and development of specified gene therapies. Although the course of salivary gland gene therapy is quite optimistic in many years to come, much further investigation, especially Phase I clinical trials, must be carried out before this method can be widely employed in the clinics.

4 The Application of Genetic Therapeutic Delivery in Salivary Gland Regeneration

4.1 Radiation Injury

Radiotherapy in head and neck cancer patients causes iatrogenic damage to the surrounding healthy tissues, including the salivary glands. Reduction in salivary secretions and change in composition leads to difficulty in eating, swallowing, loss of taste, oral and systemic infections, and thus a considerable drop in the survivors' quality of life. Since there is a lack of a gold standard treatment for this condition, constant efforts have been made to restore salivary function, particularly utilizing gene therapy and its latest advances. For such studies, retro ductal injections are most commonly performed for salivary glands; recently, a detailed procedure in non-human primates was published by El Helou et al. (2021).

One of the earliest pieces of evidence of the use of genetic manipulation for the treatment of radiation-induced xerostomia can be attributed to Dr. Bruce Baum and their group where they introduced the AQP1 receptor in salivary glands (Vitolo and Baum 2002), reaching phase 1/2 clinical trials (Alevizos et al. 2017) over 16–18 years (Baum et al. 2017). Further, at the cellular level, increased expression of AQP1 and enhanced reduction of acinar cell size was observed after adenoviral AQP1 transduction, giving crucial insight into the mechanism of action of this treatment (Teos et al. 2016). Later, human keratinocyte growth factor (hKGF) (Baba et al. 2007; Zheng et al. 2009) and fibroblast growth factor (FGF2) (Guo et al. 2014) transcribing viral vectors for ameliorating the radiation effects were also explored. The hybrid serotype 5 adenoviral vectors were administered a day before radiation exposure. Also, both single-dose and fractionated-dose murine models were tested. Oral mucositis, one of the common sequelae of radiation-induced injury, was considerably reduced in the hKGF transgenic mice. KGF, also known as FGF7, is secreted in the serum, which raises concerns over its carcinogenic potential in FGFR2 receptor-positive tumors. So, though their SCC model showed safety in their studies, prudent speculations have limited its clinic entry (Baba et al. 2007; Zheng et al. 2009).

Using nanoparticle-driven silencing RNAs, Ovitt and their group reported that the transgenic knockdown of pro-apoptotic genes prevented radiation-induced injury in salivary gland cells in vitro and murine salivary cell glands in vivo (Arany et al. 2012, 2013). Tousled-like kinase 1, involved in DNA replication and repair, was exogenously expressed by Ad5 and AAV9 viral vector transduction, giving protection from radiation-induced injury (Timiri Shanmugam et al. 2013).

Dr. Hoffman and their group initially used adenovirus serotype 5 to deliver neurturin (NTR) in murine models but then moved to the AAV2 vector due to its prolonged expression and less immunogenicity. When introduced 24 h before radiation injury, NTR restored the salivary gland function. This therapeutic effect was attributed to the protection of parasympathetic innervation, which maintained the salivary gland's function through stimulation (Ferreira et al. 2018; Lombaert et al. 2020). The AAV2-based vector encoding human NTRN, used previously for Parkinson's disease clinical trials (ClinicalTrials.gov: NCT00252850 and NCT00400634), showed improved salivary functions when administered prior to radiation (Ferreira et al. 2018). Their group further moved to the porcine model due to their pathophysiological and anatomical similarity to humans. Also, this model allowed differential treatment on contralateral parotid glands due to their size and accessibility. Reduced immunoglobulin and complement-related genes were observed in the pre-radiated treated groups, which could indicate reduced humoral and innate immune response, respectively (Lombaert et al. 2020). A similar approach, restoration of neural pathways for salivary gland protection, was followed by Hai and group, where they emphasized the Hedgehog pathway and its role in radiation injury. After working with murine models, they more recently focused on porcine samples (Hai et al. 2014, 2016, 2018; Hu et al. 2018). Introduction of adenovirus carrying Sonic Hedgehog gene (Ad-Shh) 4 weeks after radiation improved vascular (CD31, VEGF) as well as neural (acetylcholinesterase, BDNF, neurturin) function. Apoptosis (p21/CDKN1A) and radiation-induced inhibition of autophagy (AMPK-ULK1 pathway) were also reduced (Hu et al. 2018).

4.2 Sjogren's Syndrome

Sjogren's syndrome (SS), an autoimmune disease, is another major clinical condition that reduces salivary function, leading to a decrease in the quality of life of the patients suffering from it. While its pathophysiology is attributed to multiple genetic changes and variations, efforts have been made to target one or more of such pathways to relieve the patient from this condition if not completely cure them.

Sequestration of IL17, a proinflammatory cytokine associated with SS, was achieved by inducing the expression of its receptor-like protein using UAGT. This result reduced kallikrein-related peptidases, including the putative autoantigen Klk1b22 (Wu et al. 2015). Previously, similar attempts were made using viral vectors (Nguyen et al. 2011). BMP6 is invariably associated with the loss of salivary function in SS patients, which can be attributed to BMP6 regulating the expression of AQP5, a water channel essential for the function of salivary glands (Lai et al. 2016). Lai et al. (2016) treated non-obese diabetic (NOD) mice with AAV2-AQP1 viral vectors in order to restore the fluid movement in the acinar cells. Along with the restoration of acinar cell function, inflammation was also decreased after transfection (Lai et al. 2016). Citing the alteration of the function of the channel, cystic fibrosis transmembrane conductance regulator (CFTR), in the SS lacrimal and salivary ductal cells, Zeng et al. hypothesized its correction for the improvement of ductal and, in turn, acinar function in the glands. Along with the CFTR corrector C18 and VX770, transgenic overexpression of CFTR was induced using the AD5 vector. Inflammation and tissue damage were reduced along with the restoration of acinar cell function (Zeng et al. 2017).

4.3 Systemic and Endocrinal Applications

Over the years, the salivary glands have emerged as relatively easy targets for transgenic alterations given their accessibility, permissibility, and ability to secrete in an endocrinal as well as exocrine manner. However, the ability to secrete into the blood steam (endocrinal) or the ducts (exocrine) is a double-edged sword, since, in some particular instances, limitation to either one of them might be preferable or even necessary. Nonetheless, various attempts have been made to convert them into bioreactors for clinically relevant conditions and challenges. Exocrine secretions are expected from the acinar cell's apical membrane into the ducts (regulated secretory pathway also known as RSP), while endocrinal would be beneficial from their basolateral membranes into the bloodstream (constitutive secretory pathway, also known as CSP) (Racz et al. 2015; Rowzee et al. 2013).

One of the challenging and prevalent conditions of the current times is diabetes. Thus, exploration of salivary glands as endocrinal bioreactors for its treatment was expected and much welcomed. Since insulin requirements are in the serum; it is beneficial to have transgenic therapies that induce basolateral secretions from the acinar cells. Thus, a modified insulin recombinant was transduced using an AD5 vector by Rowzee et al. in a murine diabetic model (Rowzee et al. 2013). Also, due to the longer bioavailability of pro-insulin over insulin, and its relatively low efficacy, the authors hypothesized that pro-insulin was a better candidate to maintain sufficient insulin levels without the chances of hypoglycemia (Rowzee et al. 2013). Using another strategy, Wang et al. (2015b) introduced Exendin-4 with an AAV vector to induce insulin production (Wang et al. 2014, 2015b). One of the most interesting works in salivary gland bioreactor studies is the production of Peptide YY, a satiation signalling hormone. Its production in the saliva can potentially be used to curb the urge to eat in patients suffering from obesity (Acosta et al. 2011).

Similar to the radiation studies in salivary gland damage, Rocha et al. studied lacrimal glands post-radiation. They used a modified E1 replication-deficient Ad5 vector with the erythropoietin encoding gene, utilizing its anabolic effect in epithelial cells, apart from its usual role observed in red blood cell formation. Restoration of tear secretions and corneal protection were the most significant findings. Nonetheless, the role of erythropoietin in neoplasm growth limits its translation further (Rocha et al. 2013). Erythropoietin naturally gets secreted into the circulation from the salivary glands through CSP, while growth hormone enters the saliva through RSP (Racz et al. 2015). Since the secretion of growth hormone in the saliva is undesirable, their group, over the years, explored various mutations of the protein in order to convert its secretion from RSP to CSP (Racz et al. 2015; Wang et al. 2005; Samuni et al. 2008). While the efforts made in this direction were commendable, bringing the salivary gland research leaps forward, the functional limitation of these mutated proteins remained a challenge, which can be explored further.

Pulmonary arterial hypertension (PAH) is a heart condition that requires consistent administration of prostacyclin (PGI2) or its derivatives in order for the patient to survive. Since its administration through parenteral infusions requires medical sophistication and adds to the patient's discomfort, an endogenous production site could provide a certain amount of relief. Zhimin Wang et al. (2017a) introduced COX1-PGI2 using UAGT. The fusion of PGI2 with COX1 provided structural stability, and the circulating levels of PGI2 were approximately 30% of that seen with intravenous perfusions (Wang et al. 2017a).

5 Conclusion

Gene therapeutics to the salivary gland is an exciting arena for the scientific community, both for the treatment of salivary gland disorders as well as some systemic conditions. Gene therapeutics have become a reality with the advent of the latest transfection and transduction techniques, leading to increased efficacy, more precision, and reduced side effects. While viral-based approaches have been used conventionally, non-viral techniques are gaining popularity. Each technique has a unique advantage to it, so the selection highly depends on the intended target and aim of the therapy. Understandably, the exact mechanism of each gene might be challenging, but in-depth knowledge of the target gene and its transcribed protein should be used to base the type of therapies applied. The challenges with the germline approach remain more ethical, while for somatic it's the non-permanence of the effect of the therapy. Immune reaction to foreign agents also limits its applications to an extent. Nonetheless these challenges can be overcome as the benefits are outpowered by the risks involved.

References

Nakagami H, Morishita R (2022) COVID-19 vaccine utilizing the technology of gene therapy. Nihon Ronen Igakkai Zasshi 59(1):34–38

Abu Abed OS (2021) Gene therapy avenues and COVID-19 vaccines. Genes Immun 22(2):120–124

Acosta A, Hurtado MD, Gorbatyuk O, La Sala M, Duncan D, Aslanidi G et al (2011) Salivary PYY: a putative bypass to satiety. PLoS One 6(10):e26137

Alevizos I, Zheng C, Cotrim AP, Liu S, McCullagh L, Billings ME et al (2017) Late responses to adenoviral-mediated transfer of the aquaporin-1 gene for radiation-induced salivary hypofunction. Gene Ther 24(3):176–186

Arany S, Xu Q, Hernady E, Benoit DSW, Dewhurst S, Ovitt CE (2012) Pro-apoptotic gene knockdown mediated by nanocomplexed siRNA reduces radiation damage in primary salivary gland cultures. J Cell Biochem 113(6):1955–1965

Arany S, Benoit DSW, Dewhurst S, Ovitt CE (2013) Nanoparticle-mediated gene silencing confers radioprotection to salivary glands in vivo. Mol Ther 21(6): 1182–1194

Azuma M, Tamatani T, Kasai Y, Sato M (1993) Immortalization of normal human salivary gland cells with duct-, myoepithelial-, acinar-, or squamous phenotype by transfection with SV40 ori- mutant deoxyribonucleic acid. Lab Investig 69(1):24–42

Baba Y, Yazawa T, Kanegae Y, Sakamoto S, Saito I, Morimura N et al (2007) Keratinocyte growth factor gene transduction ameliorates acute lung injury and mortality in mice. Hum Gene Ther 18(2):130–141

Barka T, van der Noen HM (1996) Retrovirus-mediated gene transfer into salivary glands in vivo. Hum Gene Ther 7(5):613–618

Baum BJ, O'Connell BC (1999) In vivo gene transfer to salivary glands. Crit Rev Oral Biol Med 10(3): 276–283

Baum BJ, Alevizos I, Chiorini JA, Cotrim AP, Zheng C (2015) Advances in salivary gland gene therapy – oral and systemic implications. Expert Opin Biol Ther 15(10):1443–1454

Baum BJ, Afione S, Chiorini JA, Cotrim AP, Goldsmith CM, Zheng C (2017) Gene therapy of salivary diseases. In: Seymour GJ, Cullinan MP, Heng NC (eds) Oral biology: molecular techniques and applications. Springer, New York, pp 107–123

Baylis F, Ikemoto L (2017) The Council of Europe and the prohibition on human germline genome editing. EMBO Rep 18(12):2084–2085

Blum S, Shapir N, Miari R, Lerner B, Koren B, Doenyas-Barak K et al (2017) TARGT gene therapy platform for correction of anemia in end-stage renal disease. N Engl J Med 376(2):189–191

Bovolenta C, Porcellini S, Alberici L (2013) Therapeutic genes for anti-HIV/AIDS gene therapy. Curr Pharm Biotechnol 14(5):488–500

Brendel C, Negre O, Rothe M, Guda S, Parsons G, Harris C et al (2020) Preclinical evaluation of a novel Lentiviral vector driving lineage-specific BCL11A knockdown for sickle cell gene therapy. Mol Ther Methods Clin Dev 17:589–600

Chen Y, Zheng Y, Kang Y, Yang W, Niu Y, Guo X et al (2015) Functional disruption of the dystrophin gene in rhesus monkey using CRISPR/Cas9. Hum Mol Genet 24(13):3764–3774

Chen XZ, Guo R, Zhao C, Xu J, Song H, Yu H et al (2022) A novel anti-cancer therapy: CRISPR/Cas9 gene editing. Front Pharmacol 13:939090

Convertine AJ, Diab C, Prieve M, Paschal A, Hoffman AS, Johnson PH et al (2010) pH-responsive polymeric micelle carriers for siRNA drugs. Biomacromolecules 11(11):2904–2911

Corden A, Handelman B, Yin H, Cotrim A, Alevizos I, Chiorini JA (2017) Neutralizing antibodies against adeno-associated viruses in Sjögren's patients: implications for gene therapy. Gene Ther 24(4): 241–244

Cornel MC, Howard HC, Lim D, Bonham VL, Wartiovaara K (2019) Moving towards a cure in genetics: what is needed to bring somatic gene therapy to the clinic? Eur J Hum Genet 27(3):484–487

Di Pasquale G, Perez Riveros P, Tora M, Sheikh T, Son A, Teos L et al (2020) Transduction of salivary gland acinar cells with a novel AAV vector 44.9. Mol Ther – Methods Clin Dev 19:459–466

Dong C, Xiong J, Ni J, Fang X, Zhang J, Zhu D et al (2022) Intracellular miRNA-triggered surface-enhanced Raman scattering imaging and dual gene-silencing therapy of cancer cell. Anal Chem 94(26): 9336–9344

Doxzen K, Halpern J (2020) Focusing on Human Rights: a framework for CRISPR germline genome editing ethics and regulation. Perspect Biol Med 63(1):44–53

El Helou G, Goodman JF, Blevins M, Caudell DL, Ponzio TA, Sanders JW (2021) Retrograde parotid gland infusion through Stensen's duct in a non-human primate for vectored gene delivery. JoVE 174:e62645

Ferreira JNA, Zheng C, Lombaert IMA, Goldsmith CM, Cotrim AP, Symonds JM et al (2018) Neurturin gene therapy protects parasympathetic function to prevent irradiation-induced murine salivary gland hypofunction. Mol Ther Methods Clin Dev 9:172–180

Flotte TR, Gao G (2021) Immune responses to recombinant adenoviruses as gene therapy vectors and COVID-19 vaccines: a two-edged sword. Hum Gene Ther 32(13–14):645–646

Fox JL (2002) Eugenics concerns rekindle with application of gene therapy and genetic counseling. Nat Biotechnol 20(6):531–532

Fukumura M, Ishibashi K, Nakaguro M, Nagao T, Saida K, Urano M et al (2022) Salivary gland polymorphous adenocarcinoma: Clinicopathological features and gene alterations in 36 Japanese patients. J Oral Pathol Med 51(8):710–720

Geguchadze RN, Machen L, Zourelias L, Gallo PH, Passineau MJ (2012) An AAV2/5 vector enhances safety of gene transfer to the mouse salivary gland. J Dent Res 91(4):382–386

Geguchadze R, Wang Z, Zourelias L, Perez-Riveros P, Edwards PC, Machen L et al (2014) Proteomic profiling of salivary gland after nonviral gene transfer mediated by conventional plasmids and minicircles. Mol Ther Methods Clin Dev 1:14007

Guo L, Gao R, Xu J, Jin L, Cotrim AP, Yan X et al (2014) AdLTR2EF1α-FGF2-mediated prevention of fractionated irradiation-induced salivary hypofunction in swine. Gene Ther 21(10):866–873

Hai B, Qin L, Yang Z, Zhao Q, Shangguan L, Ti X et al (2014) Transient activation of hedgehog pathway rescued irradiation-induced hyposalivation by preserving salivary stem/progenitor cells and parasympathetic innervation. Clin Cancer Res 20(1):140–150

Hai B, Zhao Q, Qin L, Rangaraj D, Gutti VR, Liu F (2016) Rescue effects and underlying mechanisms of Intragland Shh gene delivery on irradiation-induced hyposalivation. Hum Gene Ther 27(5):390–399

Hai B, Zhao Q, Deveau MA, Liu F (2018) Delivery of sonic hedgehog gene repressed irradiation-induced cellular senescence in salivary glands by promoting DNA repair and reducing oxidative stress. Theranostics 8(4): 1159–1167

Harford JB, Kim SS, Pirollo KF, Chang EH (2022) TP53 gene therapy as a potential treatment for patients with COVID-19. Viruses 14(4):739

Herold BC, Marcellino D, Marcelin G, Wilson P, Burrow C, Satlin LM (2002) Herpes simplex virus as a model vector system for gene therapy in renal disease. Kidney Int 61(Suppl 1):S3–S8

Hsieh MM, Bonner M, Pierciey FJ, Uchida N, Rottman J, Demopoulos L et al (2020) Myelodysplastic syndrome unrelated to lentiviral vector in a patient treated with gene therapy for sickle cell disease. Blood Adv 4(9): 2058–2063

Hu L, Zhu Z, Hai B, Chang S, Ma L, Xu Y et al (2018) Intragland Shh gene delivery mitigated irradiation-induced hyposalivation in a miniature pig model. Theranostics 8(16):4321–4331

Huai C, Jia C, Sun R, Xu P, Min T, Wang Q et al (2017) CRISPR/Cas9-mediated somatic and germline gene correction to restore hemostasis in hemophilia B mice. Hum Genet 136(7):875–883

Hutt JA, Assaf BT, Bolon B, Cavagnaro J, Galbreath E, Grubor B et al (2022) Scientific and regulatory policy committee points to consider: nonclinical research and development of in vivo gene therapy products, emphasizing adeno-associated virus vectors. Toxicol Pathol 50(1):118–146

Iacobas S, Iacobas DA (2022) Personalized 3-gene panel for prostate cancer target therapy. Curr Issues Mol Biol 44(1):360–382

Kim YJ, Nomakuchi T, Papaleonidopoulou F, Yang L, Zhang Q, Krainer AR (2022) Gene-specific nonsense-mediated mRNA decay targeting for cystic fibrosis therapy. Nat Commun 13(1):2978

Lai Z, Yin H, Cabrera-Pérez J, Guimaro MC, Afione S, Michael DG et al (2016) Aquaporin gene therapy corrects Sjögren's syndrome phenotype in mice. Proc Natl Acad Sci U S A 113(20):5694–5699

Liu XY, Zhang X, Yang JB, Wu CY, Wang Q, Lu ZL et al (2022) Multifunctional amphiphilic peptide dendrimer as nonviral gene vectors for effective cancer therapy via combined gene/photodynamic therapies. Colloids Surf B Biointerfaces 217:112651

Lombaert IMA, Patel VN, Jones CE, Villier DC, Canada AE, Moore MR et al (2020) CERE-120 prevents irradiation-induced Hypofunction and restores immune homeostasis in porcine salivary glands. Mol Ther Methods Clin Dev 18:839–855

Malcolm DW, Varghese JJ, Sorrells JE, Ovitt CE, Benoit DSW (2018) The effects of biological fluids on colloidal stability and siRNA delivery of a pH-responsive micellar nanoparticle delivery system. ACS Nano 12(1):187–197

Marquez Loza LI, Cooney AL, Dong Q, Randak CO, Rivella S, Sinn PL et al (2021) Increased CFTR expression and function from an optimized lentiviral vector for cystic fibrosis gene therapy. Mol Ther Methods Clin Dev 21:94–106

Maruyama S (2006) Gene therapy for interstitial renal disease. Nihon Rinsho 64(Suppl 2):662–666

Mastrangeli A, O'Connell B, Aladib W, Fox PC, Baum BJ, Crystal RG (1994) Direct in vivo adenovirus-mediated gene transfer to salivary glands. Am J Phys 266(6 Pt 1):G1146–G1155

Nakagami H (2021) Development of COVID-19 vaccines utilizing gene therapy technology. Int Immunol 33(10): 521–527

Nguyen CQ, Yin H, Lee BH, Chiorini JA, Peck AB (2011) IL17: potential therapeutic target in Sjögren's syndrome using adenovirus-mediated gene transfer. Lab Investig 91(1):54–62

Oishi T, Ito M, Koizumi S, Horikawa M, Yamamoto T, Yamagishi S et al (2022) Efficacy of HSV-TK/GCV system suicide gene therapy using SHED expressing modified HSV-TK against lung cancer brain metastases. Mol Ther Methods Clin Dev 26:253–265

Passineau M (2017) Salivary gland gene therapy in experimental and clinical trials. In: Cha S (ed) Salivary gland development and regeneration: advances in research and clinical approaches to functional restoration. Springer International Publishing, Cham, pp 217–228

Passineau MJ, Zourelias L, Machen L, Edwards PC, Benza RL (2010) Ultrasound-assisted non-viral gene transfer to the salivary glands. Gene Ther 17(11):1318–1324

Qadir MI, Zafar M (2017) TSG101: tumor susceptibility gene 101 (tsg101) product-role in therapy against HIV/AIDS. Crit Rev Eukaryot Gene Expr 27(3): 237–246

Racz G, Zheng C, Goldsmith C, Baum B, Cawley N (2015) Toward gene therapy for growth hormone deficiency via salivary gland expression of growth hormone. Oral Dis 21(2):149–155

Reyne N, Cmielewski P, McCarron A, Delhove J, Parsons D, Donnelley M (2021) Single-dose Lentiviral mediated gene therapy recovers CFTR function in cystic fibrosis knockout rats. Front Pharmacol 12: 682299

Rocha EM, Cotrim AP, Zheng C, Riveros PP, Baum BJ, Chiorini JA (2013) Recovery of radiation-induced dry eye and corneal damage by Pretreatment with adenoviral vector-mediated transfer of erythropoietin to the salivary glands in mice. Hum Gene Ther 24(4): 417–423

Rowzee AM, Perez-Riveros PJ, Zheng C, Krygowski S, Baum BJ, Cawley NX (2013) Expression and secretion of human proinsulin-B10 from mouse salivary glands: implications for the treatment of type I diabetes mellitus. PLoS One 8(3):e59222

Samuni Y, Baum BJ (2011) Gene delivery in salivary glands: from the bench to the clinic. Biochim Biophys Acta (BBA) – Mol Basis Dis 1812(11):1515–1521

Samuni Y, Zheng C, Cawley NX, Cotrim AP, Loh YP, Baum BJ (2008) Sorting of growth hormone-erythropoietin fusion proteins in rat salivary glands. Biochem Biophys Res Commun 373(1):136–139

Shai E, Falk H, Honigman A, Panet A, Palmon A (2002) Gene transfer mediated by different viral vectors following direct cannulation of mouse submandibular salivary glands. Eur J Oral Sci 110(3):254–260

Stenman G, Fehr A, Skalova A, Vander Poorten V, Hellquist H, Mikkelsen LH et al (2022) Chromosome translocations, gene fusions, and their molecular consequences in pleomorphic salivary gland adenomas. Biomedicine 10(8):1970

Stone EF, Avecilla ST, Wuest DL, Lomas-Francis C, Westhoff CM, Diuguid DL et al (2021) Severe delayed hemolytic transfusion reaction due to anti-Fy3 in a patient with sickle cell disease undergoing red cell exchange prior to hematopoietic progenitor cell collection for gene therapy. Haematologica 106(1):310–312

Teos LY, Zheng CY, Liu X, Swaim WD, Goldsmith CM, Cotrim AP et al (2016) Adenovirus-mediated hAQP1 expression in irradiated mouse salivary glands causes recovery of saliva secretion by enhancing acinar cell volume decrease. Gene Ther 23(7):572–579

Timiri Shanmugam PS, Dayton RD, Palaniyandi S, Abreo F, Caldito G, Klein RL et al (2013) Recombinant AAV9-TLK1B administration ameliorates fractionated radiation-induced xerostomia. Hum Gene Ther 24(6):604–612

Vitolo J, Baum B (2002) The use of gene transfer for the protection and repair of salivary glands. Oral Dis 8(4): 183–191

Voutetakis A, Kok MR, Zheng C, Bossis I, Wang J, Cotrim AP et al (2004) Reengineered salivary glands are stable endogenous bioreactors for systemic gene therapeutics. Proc Natl Acad Sci USA 101(9): 3053–3058

Wang J, Cawley NX, Voutetakis A, Rodriguez YM, Goldsmith CM, Nieman LK et al (2005) Partial redirection of transgenic human growth hormone secretion from rat salivary glands. Hum Gene Ther 16(5):571–583

Wang J, Wang F, Xu J, Ding S, Guo Y (2014) Double-strand adeno-associated virus-mediated exendin-4 expression in salivary glands is efficient in a diabetic rat model. Diabetes Res Clin Pract 103(3):466–473

Wang Z, Zourelias L, Wu C, Edwards PC, Trombetta M, Passineau MJ (2015a) Ultrasound-assisted nonviral gene transfer of AQP1 to the irradiated minipig parotid gland restores fluid secretion. Gene Ther 22(9): 739–749

Wang J, Wen J, Bai D, Guo Y (2015b) Injection of submandibular gland with recombinant Exendin-4 and adeno-associated virus for the treatment of diabetic rats. Zhong Nan Da Xue Xue Bao Yi Xue Ban 40(11): 1179–1185

Wang Z, Benza RL, Zourelias L, Sanguino A, Geguchadze R, Shields KJ et al (2017a) In vivo endocrine secretion of prostacyclin following expression of a Cyclooxygenase-1/prostacyclin fusion protein in the salivary glands of rats via nonviral gene therapy. Hum Gene Ther 28(8):681–689

Wang Z, Pradhan-Bhatt S, Farach-Carson MC, Passineau MJ (2017b) Artificial induction of native Aquaporin-1 expression in human salivary cells. J Dent Res 96(4): 444–449

Wu C, Wang Z, Zourelias L, Thakker H, Passineau MJ (2015) IL-17 sequestration via salivary gland gene therapy in a mouse model of Sjogren's syndrome suppresses disease-associated expression of the putative autoantigen Klk1b22. Arthritis Res Ther 17(1):198

Xia E, Zhang Y, Cao H, Li J, Duan R, Hu J (2019) TALEN-mediated gene targeting for cystic fibrosis-gene therapy. Genes (Basel) 10(1):39

Xiao Q, Guo D, Chen S (2019) Application of CRISPR/Cas9-based gene editing in HIV-1/AIDS therapy. Front Cell Infect Microbiol 9:69

Yang Q, Soltis AR, Sukumar G, Zhang X, Caohuy H, Freedy J et al (2019) Gene therapy-emulating small molecule treatments in cystic fibrosis airway epithelial cells and patients. Respir Res 20(1):290

Yuan L, Sui T, Chen M, Deng J, Huang Y, Zeng J et al (2016) CRISPR/Cas9-mediated GJA8 knockout in rabbits recapitulates human congenital cataracts. Sci Rep 6:22024

Zaidane I, Ouladlahsen A, Bensghir R, Chihab H, Jadid FZ, Fihry RE et al (2020) An assessment of toll-like receptor 7 and 8 gene polymorphisms with susceptibility to HIV-1 infection, AIDS development and response to antiretroviral therapy. Immunol Lett 227: 88–95

Zeng M, Szymczak M, Ahuja M, Zheng C, Yin H, Swaim W et al (2017) Correction of ductal CFTR activity rescues acinar cell and pancreatic and salivary gland functions in mouse models of autoimmune disease. Gastroenterology 153(4):1148–1159

Zhang Z, Hou W, Chen S (2022) Updates on CRISPR-based gene editing in HIV-1/AIDS therapy. Virol Sin 37(1):1–10

Zheng C, Vitolo JM, Zhang W, Mineshiba F, Chiorini JA, Baum BJ (2008a) Extended transgene expression from a nonintegrating adenoviral vector containing retroviral elements. Mol Ther 16(6):1089–1097

Zheng C, Vitolo JM, Zhang W, Mineshiba F, Chiorini JA, Baum BJ (2008b) Extended transgene expression from a nonintegrating adenoviral vector containing retroviral elements. Mol Thery 16(6):1089–1097

Zheng C, Cotrim AP, Sunshine AN, Sugito T, Liu L, Sowers A et al (2009) Prevention of radiation-induced oral mucositis after adenoviral vector-mediated transfer of the keratinocyte growth factor cDNA to mouse submandibular glands. Clin Cancer Res 15(14):4641–4648

Zheng C, Cotrim AP, Nikolov N, Mineshiba F, Swaim W, Baum BJ (2012) A novel hybrid Adenoretroviral vector with more extensive E3 deletion extends transgene expression in submandibular glands. Hum Gene Ther Methods 23(3):169–181

Zheng C, Baum BJ, Liu X, Goldsmith CM, Perez P, Jang SI et al (2015) Persistence of hAQP1 expression in human salivary gland cells following AdhAQP1 transduction is associated with a lack of methylation of hCMV promoter. Gene Ther 22(9):758–766

Zubler RH (2007) Ex vivo expansion of haematopoietic stem cells and gene therapy development. Swiss Med Wkly 137(Suppl 155):31S–35S

Zufferey R, Aebischer P (2004) Salivary glands and gene therapy: the mouth waters. Gene Ther 11(19): 1425–1426

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Cancer Stem Cells and Their Therapeutic Usage

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Abstract

Cancer stem cells (CSC) have unique characteristics which include self-renewal, multi-directional differentiation capacity, quiescence/dormancy, and tumor-forming capability. These characteristics are referred to as the "stemness" properties. Tumor microenvironment contributes to CSC survival, function, and remaining them in an undifferentiated state. CSCs can form malignant tumors with heterogeneous phenotypes mediated by the tumor microenvironment. Therefore, the crosstalk between CSCs and tumor microenvironment can modulate tumor heterogeneity. CSCs play a crucial role in several biological processes, epithelial–mesenchymal transition (EMT), autophagy, and cellular stress response. In this chapter, we focused characteristics of cancer stem cells, reprogramming strategies cells into CSCs, and then we highlighted the contribution of CSCs to therapy resistance and cancer relapse and their potential of therapeutic targeting of CSCs.

Keywords

Cancer stem cells · Therapeutic targeting · Treatment

Abbreviations

5-FU	5-Fluorouracil
ABC	ATP binding cassette
ADC	Antibody-drug conjugate
AML	Acute myeloid leukemia
AuNM	Gold nanomaterial
AuNP	Gold nanoparticle
bFGF	Basic fibroblast growth factor
BMP4	Bone morphogenetic protein 4
CAF	Cancer-associated fibroblast
CAR-T	Chimeric antigen receptor T
CCSC	Colorectal stem cell
CD44v	CD44 variant
CEMP	CSC epigenomic monitoring platform
CESP	Anti-EGFR and Anti-CD133
CHK1/CHK2	Checkpoint kinase 1/2
CNS	Central nervous system
CRC	Colorectal cancer
CSC	Cancer stem cell
CTL	Cytotoxic T lymphocytes
E/M	Epithelial–mesenchymal
ECM	Extracellular matrix
EGFR	Epidermal growth factor receptors

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EMT	Epithelial–mesenchymal transition
ErbB-2	Erb-B2 receptor tyrosine kinase 2
ERK 1/2	Extracellular signal-regulated kinase 1/2
EV	Extracellular vesicle
EZH2	Enhancer of zeste homolog 2
GLUT1	Glucose transporter 1
GNP	Gold nanoprobes
GR	Glucocorticoid receptor
H_2O_2	Hydrogen peroxide
HER2	ErbB-2 tyrosine-protein kinase receptor
Hh	Hedgehog
HIF	Hypoxia-inducible factor
hiNeuroS	Human-induced neurosphere
hiNSC	Human-induced NSC
HSC	Hematopoietic stem cells
IL	Interleukin
iNSC	Induced NSC
JAK-STAT	Janus kinase and signal transducer and activator of transcription
JNK	C-Jun N-terminal kinase
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5
LncRNA	Long non-coding RNA
LP	Lipid-polymer
LPP	Lipid peroxidation product
MAPK	Mitogen-activated protein kinase
MDR	Multidrug-resistant
MDSC	Myeloid-derived suppressor cell
MET	Mesenchymal–epithelial transition
MHC	Major histocompatibility complex
MIF	Macrophage migration inhibitory factor
MiRNA	MicroRNA
MKP-1	MAPK phosphatase 1
MSC	Mesenchymal stem cell
NDV	Newcastle disease virus
NF-Kβ	Nuclear factor kappa B
NK cell	Natural killer cell
NP	Nanoparticle
NSC	Neural stem cells
OV	Oncolytic virus
OXPHOS	Oxidative phosphorylation
PcG	Polycomb group
PDGF	Platelet-derived growth factor
PD-L1	Programmed death ligand 1
PEG	Polyethylene glycol
PGK1	Phosphoglycerate kinase 1
PI3K	Phosphoinositide 3-kinase
PLGA	Poly lactic-co glycolic acid
PPAR	Peroxisome proliferator-activated receptor
PTX	Paclitaxel
RB	Retinoblastoma
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SC	Stem cell
SDF	Stromal-derived factor
SERS	Surface-enhanced Raman spectroscopy
SFRP4	Secreted frizzled-related protein 4
SLM	Salinomycin
SLRP	Small leucine-rich proteoglycan
SMAD	Suppressor of mothers against decapentaplegic
SOX2	Sex determining region Y-box 2
SP	Side population
STAT	Signal transducer and activator of transcription
TAA	Tumor-associated antigen
TAM	Tumor-associated macrophage
TAN	Tumor-associated neutrophil
TGF- β	Transforming growth factor-beta
TKI	Tyrosine kinase inhibitor
TNBC	Triple-negative breast cancer
TNC	Tenascin-C
TNF-alpha	Tumor necrosis factor alpha
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand
Treg	Regulatory T cell
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor

1 Introduction

Over the past decade, increasing evidence suggests that cancer cells develop hierarchically within tumors, in which a subpopulation of cancer stem cells (CSCs) supports tumor growth properties (Yu et al. 2012; Hung et al. 2019). CSCs have a "cancer-initiating ability" and "tumor-initiating cell" or "tumorigenic cell" definitions used to describe putative CSCs (Clarke et al. 2006). Despite their tumor-initiating abilities, the immune-evading properties of CSCs are claimed to be more important for tumor regrowth (Tsuchiya and Shiota 2021). In 1994, the first evidence of CSCs was described in acute myeloid leukemia (AML), using flow cytometry to detect cells with CD34+ CD38– cell surface markers (Al-Hajj et al. 2003). Then, it was identified in other types of tumors including breast cancer, colon cancer, glioblastoma, and head and neck squamous cell carcinoma (Hung et al. 2019).

CSCs have unique characteristics which include self-renewal, multi-directional differentiation capacity, quiescence/dormancy, and tumor-forming capability. In general, these characteristics are referred to as the "stemness" properties (Yu et al. 2012; Zhu and Fan 2018). As a result, CSCs show several similar properties to normal stem cells (Beck and Blanpain 2013; Ayob and Ramasamy 2018). Stemness properties are regulated through several pathways that include wingless-related integration site (Wnt), Hedgehog (Hh), Notch, transforming growth factor-beta (TGF-β), signal transducer and activator of transcription (STAT), and Hippo-YAP/TAZ, non-coding RNAs, chromatin remodeling complexes, and these pathways are highly deregulated in CSCs (Wang et al. 2010; Zhu and Fan 2018; Maroufi et al. 2020). It is suggested that tumor microenvironment, tumor heterogeneity, origin of CSCs, and genetic factors can also reorganize the biological properties of CSCs (Maccalli and De Maria 2015; Zhu and Fan 2018). Particularly, tumor microenvironment contributes to CSC survival, function, and remaining them in an undifferentiated state (Moharil et al. 2017). CSCs can form malignant tumors with heterogeneous phenotypes mediated by the tumor microenvironment (Clarke et al. 2006). Therefore, the crosstalk between CSCs and tumor microenvironment can modulate tumor heterogeneity.

CSCs have specific markers, like CD133, CD44 (Hung et al. 2019), α2β1-integrin, p63, CK5/14, and CK8/18 (Yokoyama et al. 2019; Martincorena et al. 2015; Medema 2013). These markers are preferred to identification and targeting of CSC populations (Zhou et al. 2021). CSCs express different specific markers depending on the origin of tumor or tumor type. Therefore, there are not any universal markers of CSCs (Xiao et al. 2017; Moharil et al. 2017). The specific CSC markers are generally preferred as a prognostic and overall survival indicator in many cancers (Maroufi et al. 2020). The presence of multiple CSC markers in tumors has indicated that CSCs can form heterogeneous populations (Zhu and Fan 2018). It was suggested that combined markers can correctly indicate CSC phenotype (Zhou et al. 2021). However, some of these markers can also be expressed in both normal stem cells and non-stem cells (Clarke et al. 2006). Therefore, CSC marker targeted therapies can cause the disruption of tissue homeostasis. Targeting multiple markers may provide to accurately eliminate CSCS. Particularly, CD44 variant (CD44v) isoforms such as CD44v8-10 were found to have high tumor initiation capacity in gastric cancer. This finding suggests important of further research to identify and target CSC marker variant isoforms (Lau et al. 2014). The ideal CSC markers were suggested to maintain stemness properties of CSCs such as Oct4, Sox2, and Nanog and not expressed in normal stem cells (Zhou et al. 2021). Single-cell RNA sequencing was shown as an ideal method to investigate novel CSC-specific markers (Zhu and Fan 2018).

CSCs play a crucial role in several biological processes, epithelial–mesenchymal transition (EMT), autophagy, and cellular stress response. CSCs have capacities to induce invasion, metastasis, treatment resistance, and tumor relapse (Moharil et al. 2017). They can lead to treatment failure and cancer relapse by self-renewing and causing the heterogeneous lineages of cancer cells. CSCs can also undergo a quiescent state to become resistant to conventional therapies (Clarke et al. 2006; Phi et al. 2018). In light of this information, in this chapter the origination of CSCs, mechanisms involved in CSC-mediated therapy resistance and cancer recurrence, and therapeutic targeting of CSCs will be handled in detail.

2 Reprogramming into CSCs

Several hypotheses have been suggested for reprogramming of related cells into CSCs. It is assumed that epigenetic and genetic mutations may transform normal stem cells/progenitor cells into CSCs by rendering them independent of growth signals or resistant to anti-growth signals. On the other hand, it was also suggested that differentiated cells/cancer cells can convert to CSCs by obtaining the self-renewal capacity through mutations. Furthermore, CSCs can arise from the transformation of other tissue stem cells (Bomken et al. 2010; Ghaffari 2011; Clarke et al. 2006; Sell 2010; Zhu and Fan 2018). However, it is claimed that the stem cells/progenitor cells are most likely the basic cells to create CSCs (Zamur et al. 2020). Transcription factor SOX2 expression that increase in early stage of tumorigenesis can contribute to CSCs formation by increasing stemness properties (Boumahdi et al. 2014). Furthermore, cell–cell fusion or horizontal gene transfer was assumed to result in the acquisition of stemness properties (Bjerkvig et al. 2005). For example, CSCs can arise from the fusion of stem cells and other mutant cells (Eun et al. 2017).

Tumor growth alters the tumor microenvironment, contributing to CSC formation. Particularly, the interaction between CSCs with the tumor microenvironment may lead to the expansion of CSCs (Clarke et al. 2006). On the other hand, EMT in differentiated cells was declared to induce a stem cell-like phenotype (Moharil et al. 2017). The cellular responses to stress, hypoxic environment, high level of replication stress induce the stem-like phenotype (Bae et al. 2016; Lida et al. 2012). Oxidative stress is another cellular stress which triggers cancer cells to enhanced stem-like phenotype. Several studies demonstrated that a low dose of H_2O_2 treatment in culture induced oxidative stress and stem-like phenotypes in breast and lung cancer cell lines (Gopal et al. 2016; Saijo et al. 2016). It was suggested that prolonged exposure of stem cells' DNA to inflammation-induced factors such as reactive oxygen species (ROS), reactive nitrogen species (RNS), and lipid peroxidation products (LPPs) may cause the conversion stem cells into CSCs (Ohnishi et al. 2013). High-level replication stress is linked with altered DNA replication and cell cycle. Studies demonstrated a direct relationship between CSC and high-level replicative stress in glioma and colorectal cancer. It has been reported that CSCs show high tolerance to replication stress due to robust DNA damage response (Hung et al. 2019).

3 The Contribution of CSCs to Therapy Resistance and Cancer Relapse

The mechanisms of therapy resistance in cancer are divided into two categories, intrinsic and acquired. Intrinsic mechanisms cover factors that can render therapy resistance prior to any treatment. Acquired mechanisms include initiation of resistance after treatment (Zhou et al. 2021). As intrinsic or acquired CSCs show resistance to chemotherapy, radiotherapy, and immunotherapy, comprehensive identification of biological and immunological properties of CSCs is required to develop CSC-targeted therapeutic interventions providing complete tumor eradication (Maccalli and De Maria 2015). The CSC mechanisms contributing to therapy resistance and cancer relapse include dormancy/quiescence, high efficacy of DNA damage repair and anti-apoptotic mechanism, metabolic alteration, EMT, multidrug-resistant (MDR), tumor environment, epigenetics, and signaling pathways. By understanding of these mechanisms, effective treatments can be developed to improve the clinical outcomes of cancer patients (Abdullah and Chow 2013; Phi et al. 2018).

CSCs have ability to divide symmetrically or asymmetrically in different condition (Zamur et al. 2020). It was indicated that different proliferation conditions and therapeutic stress could alter the mitotic division pattern of CSCs. It has been reported that asymmetric division exerts a tumor suppressor effect, while symmetric division may contribute to tumor development. Especially, symmetric cell division is shown to be necessary during tumor transformation (Batlle and Clevers 2017). However, it has been asserted that asymmetric division of CSCs can help the survival of CSCs under different therapeutic conditions, contributing to tumor recurrence (Huang et al. 2021). Quiescence or dormant is a state of reversible cell-cycle arrest at the G0/G1 stage that is activated in normal stem cells to protect them against cytotoxic stress-induced exhaustion. The quiescence is controlled by various molecules and signaling pathways including p53 tumor suppressor, retinoblastoma protein (Rb), p21, p27, and p57 cyclin-dependent protein kinase inhibitors, several miRNAs (Cheung and Rando 2013). Many cancer exhibit a dormancy state, leading to metastasis, immune system evasion, and therapy resistance. It has been suggested that cancer cells may acquire a dormant state to metastasize to distant regions through the activation of p38 MAPK, TGFβ family members, EMT process, etc. Association between quiescence and worse prognosis has been reported in colorectal cancer (CRC) patients (Marzagalli et al. 2021). Dormant CSCs have high DNA repair activity, and anti-apoptosis properties, resulting in resistance of CSCs to several therapeutic drugs (Murayama and Gotoh 2019; Huang et al. 2021). Particularly, the dormant leukemia stem cells have been indicated to show resistance to all tyrosine kinase inhibitors (TKIs). The niche of leukemia stem cells has been reported to contribute to quiescence or cell survival, leading to resistance of leukemia stem cells to TKIs (Claude Chomel et al. 2012).

CSCs display resistance to cell death due to the high efficacy of DNA damage repair and anti-apoptotic mechanisms. High activation of checkpoint kinase 1 (CHK1) and CHK2 was indicated to cause resistance of CSCs to radiotherapy (Bao et al. 2006). The c-MYC-CHK1/CHK2 complex was demonstrated to induce radiotherapy resistance by modulating DNA damage-checkpoint response and CSC properties (Wang et al. 2013). CSCs can utilize anti-apoptotic mechanisms to escape from the immune system and show therapeutic resistance. It was declared that overexpression of anti-apoptotic proteins such as BCL-2 and BCL-XL in CSCs may cause resistance of CSCs against T- or NK cell-induced apoptosis (Tsuchiya and Shiota 2021).

Deregulated energy metabolism has been implicated as an important hallmark of cancer cells and CSCs (Hanahan and Weinberg 2000). Metabolic change plays a crucial role in the decision of cell fate. Thus, stemness characteristics are regulated by genetic, epigenetic mechanisms, and cellular metabolic reprogramming in a complex manner. It has been stated that metabolic shifts may make the cells sensitive to genetic and epigenetic changes during the formation of CSCs (Nimmakayala et al. 2021). Tumor cells often alter their energy metabolism from oxidative phosphorylation (OXPHOS) to aerobic glycolysis even in the presence of oxygen to satisfy their metabolic needs due to their rapid proliferation. CSCs were indicated to convert their metabolism toward aerobic glycolysis by increasing GLUT1 transporters (Palorini et al. 2014). Hypoxic condition was indicated to induce a metabolic switch toward aerobic glycolysis by induced glycolytic gene expression, decreasing mitochondrial function and ROS production in the CSC population (Gammon et al. 2013). It was also suggested other metabolic stress conditions can induce a shift from aerobic respiration to aerobic glycolysis in CSCs, supporting the survival of CSCs (Loureiro et al. 2017). However, tumors may exhibit different metabolic patterns simultaneously. Various studies have revealed distinct CSC subpopulations with different metabolic activities in tumors. The existence of two CSC subpopulations was reported in breast cancer in which EMT CSCs and MET CSCs exhibit a high rate of glycolysis and oxidative phosphorylation, respectively (Nimmakayala et al. 2021). It has been claimed that components of the tumor microenvironment can induce metabolic heterogeneity in the CSC populations. Consequently, this may lead to therapeutic challenges in targeting the CSC metabolic pathways (Ertas et al. 2021; Huang et al. 2021). However, it has been reported that CSCs have intact mitochondrial function and the stemness property of CSCs can be regulated by mitochondria, regardless of metabolic phenotype (Nimmakayala et al. 2021).

EMT is a critical process that transforms adherent epithelial cells into migratory cells by disrupting cell–cell interactions. Several signaling pathways including TGFβ/Smad, PI3K/AKT, ERK1/2, NF-kβ, Wnt/β-catenin, and Notch pathways participate in EMT process by regulating mainly transcription factors Snail1, Twist, Slug, and Zeb (Ayob and Ramasamy 2018). Therefore, CSCs may acquit a metastatic property through an EMT process (Moharil et al. 2017). It has been implicated that EMT can induce a stem-like state in non-CSCs, rendering them drug resistance (Ayob and Ramasamy 2018). However, several studies suggested that EMT induction in cancer cells may not form stemness properties. Overexpression of Snail1 (EMT transcription factor) was found to inhibit self-renewal and metastatic characteristics. It was also reported that downregulation of Prrx1 (EMT inducer) induces metastasis of breast stem-like cancer cells (Celià-Terrassa et al. 2012; Ocaña et al. 2012). However, a hybrid epithelial/mesenchymal (E/M) state in which mesenchymal inducers and epithelial inducers are upregulated was demonstrated to contribute to CSC stemness features, and metastatic potential (Marzagalli et al. 2021).

ATP binding cassette (ABC) transporters classified into seven gene subfamilies (ABCA-G) are involved in therapy resistance of CSC population by exporting toxic substrates. High expression of ABCG2 and ABCB1 in CSCs results in higher resistance to therapeutic agents and disease relapse (Phi et al. 2018). Hh, Wnt/β-catenin, Notch, and EGFR pathways are involved in regulation of drug resistance gene expression in CSC population (Begicevic and Falasca 2017). The overexpression of Wnt signaling was reported to lead to transcription of multidrug resistance genes such as ABCB1/MDR-1 by stabilizing β-catenin in CSCs (Moharil et al. 2017). Stemness-related transcription factor Oct-4 was declared to be involved in regulating the expression of ABC family members (Murayama and Gotoh 2019). Side population (Sp) cells exhibiting low Hoechst 33342 staining pattern were identified in hematopoietic stem cells (HSCs), brain, lung, and ovarian cancer. SP cells were found to exhibit high expression of ABC transporters (ABCB1 and ABCG2) and some stemness properties (Patrawala et al. 2005). The presence of multiple activated ABC transporters, limited knowledge about SP-related pathways that modulate ABC transporter expression, and restricted research on characteristics of the SP cells were suggested to be prominent reasons for therapeutic resistance (Zhou et al. 2021).

Tumor microenvironment plays a crucial role in CSC maintenance, invasion, metastasis, therapeutic resistance, and cancer recurrence. Cancer cells are capable of dysregulating the microenvironment to initiate protumorigenic microenvironment. Dysregulated microenvironment can mainly contribute to survival of cancer cells with more resistant and aggressive phenotypes and the activation of stem cell-related signals, thus enhancing therapy resistance and cancer recurrence (Ayob and Ramasamy 2018). Therefore, there is a mutual interaction between components of tumor microenvironment and cancer cells, and CSCs. Cancer cells and CSCs mediate the immunosuppressive state by directing immune cells such as tumor-associate macrophages (TAM), tumor-associated neutrophils (TANs), and myeloid-derived suppressor cells (MDSCs) within the tumor microenvironment (Kitamura et al. 2015; Lau et al. 2017; Marzagalli et al. 2021). Glioblastoma CSCs were reported to secrete immunomodulatory factors such as TGF-β to induce Treg differentiation, thus evading immune system (Tsuchiya and Shiota 2021). CSCs were indicated to have high expression of immune inhibitory cell surface signals such as programmed death ligand 1 (PD-L1) to evade adaptive immunity (Lau et al. 2017). Extracellular vesicles (EVs) mediate the transport of biomolecules and cell signaling between cells via the exocytosis process. EVs conduce to tumor progression and therapy resistance by transporting several tumorigenic molecules between cancer cells. Especially, cancer cells and CSCs were indicated to secrete a high amount of EVs to provide an immunosuppressive microenvironment (Ertas et al. 2021). Thus, CSCs can evade immune attacks by recruiting immune suppression cells and increasing expression of immune suppressive factors. Especially, activation of Wnt/β-catenin, Notch, and Hh pathways was suggested to induce immune escape of CSCs, generating an immunosuppressive state in cancers (Codony-Servat and Rosell 2015). It was suggested that immune evasion can promote CSCs propagation to distinct sites. CSCs have been reported to have defects in antigen presentation and antigen processing due to the downregulation of MHC molecules and antigen processing molecules, respectively. Due to heterogeneous tumor populations, it is considered that the mechanisms that regulate antigen presentation and antigen processing molecules of CSCs may be varied. It has also been reported that T cell-mediated targeting of CSCs may be abolished due to downregulation of tumor-associated antigens (TAAs). Natural killer cells (NK) could eliminate cells with a low or lack of MHC-I expression. As CSCs have low or lack MHC-I expression, it has been claimed that NK cells may eliminate them. Considering the immunosuppressive tumor microenvironment, it was suggested that noncancerous cells within the microenvironment may eliminate NK cell-mediated CSC lysis. Furthermore, it has been put forward that CSCs may be protected from NK cell-mediated lysis due to decreased NK cell-activating receptor ligands and increased MHC molecules. Therefore, CSCs can evade immune system through different way, leading to regrowth of tumors (Tsuchiya and Shiota 2021). Stroma within the microenvironment covers the extracellular matrix (ECM), fibroblasts, endothelial cells, adipocytes, neurons, and stellate cells. Disruption of stromal signaling can initiate tumorigenesis by upregulating stem cell signals. Stromal ablation was reported to expand the stemness-like cells. Single-cell sequencing technique has exhibited the heterogeneity of stromal cells. Therefore, identification of specific stromal subpopulations supporting stemness-like properties seems necessary (Ferguson et al. 2021). During tumor progression, fibroblasts transform into cancer-associated fibroblasts (CAFs) via activation of cancer cells-derived secretory molecules such as basic fibroblast growth factor (bFGF), transforming growth factor-beta (TGF-B), platelet-derived growth factor (PDGF), and interleukin (IL-)6. A bidirectional interaction between CAFs and CSCs has been demonstrated (Ayob and Ramasamy 2018). CAFs were reported to mediate cancer stemness by activating the Wnt/β-catenin signaling pathway in colon cancer (Vermeulen et al. 2010). CAFs promote self-renewal and invasion of CSCs by secreting specific cytokines and chemokines to create a chemoresistant microenvironment (Phi et al. 2018; Zhu and Fan 2018). Mesenchymal stem cells (MSCs) crosstalk with CSCs to promote proliferation, angiogenesis, invasion, metastasis, drug resistance, dormant/quiescent state, and generate an immunosuppressive microenvironment, through the secretion of extracellular vesicles (EV) or exosomes (Bliss et al. 2016). MSCs were shown to produce CXCL12, IL-6, and IL-8 to induce the stemness property of CSCs (Cabarcas et al. 2011). MSCs were also indicated to maintain the undifferentiated state of CSCs via secreting Germli 1 (Davis et al. 2014). Vascular endothelial cells interact with cancer cells to promote the therapy resistance. They have been indicated to enhance leukemia stem cell quiescence and self-renewal by activating the mitogen-activated protein kinase (MAPK) pathway (Ferguson et al. 2021). The ECM is a microenvironment component composed of collagens, glycoproteins, proteoglycans, and integrins that mediate crosstalk between the tumor microenvironment and tumor cells (Lu et al. 2012). It was suggested that overexpression of ECM components can alter the effectiveness of therapeutic drugs. Tenascin-C (TNC) is one of the ECM components and deregulated its expression in stem cell niche was associated with cancer progression and drug resistance (Fukunaga-Kalabis et al. 2010). Leucine-rich proteoglycans (SLRPs) were demonstrated to promote stem-like phenotype, quiescent phenotype, and drug resistance (Farace et al. 2015). Matrix metalloproteinases (ECM enzymes) were demonstrated to gain metastatic properties to dormant CSCs (Zamur et al. 2020). It was shown that hyaluronic acid as a component of ECM can modulate CSC stemness and response to differentiation therapy (Lee et al. 2019). Hypoxia or low oxygen condition develops due to abnormal tumor vascularization, a distance of supporting blood vessels, and uncontrollable proliferation rate of cancer cells. The hypoxic condition was associated with self-renewal, EMT, quiescence, and drug resistance (Carnero and Lleonart 2016). It has been suggested that hypoxia in tumor microenvironment may keep CSCs in undifferentiated forms by upregulating stemness-related genes (Murayama and Gotoh 2019). Furthermore, the hypoxic condition was also reported to increase ROS levels that activate stress signaling in CSCs to keep CSCs in an undifferentiated state (Pavlides et al. 2010). Hypoxia-inducible factors (HIFs), including HIF1α and HIF2α, indued in hypoxic condition participate in diverse cellular processes such as survival, metabolism, proliferation, EMT, angiogenesis, and metastasis (Semenza 2014). HIF-1α which is a mediator of angiogenesis was particularly associated with worse patient survival (Moharil et al., 2018). It was declared that HIF-1α takes part in CSC self-renewal, CSC quiescence and protects them against DNA damage (Phi et al. 2018; Zhu and Fan 2018).

Epigenetic mechanisms contribute to the maintenance of CSCs. Polycomb group (PcG) proteins are developmental regulators that modify chromatin into a transcriptionally repressive state. Two polycomb repressive complexes, PRC1 and PRC2, were indicated to contribute to the self-renewal of stem cells. PRC2 is known in mammals as an Enhancer of Zeste homolog 2 (EZH2) and generates the histone mark H3K27me3. As EZH2 was demonstrated to be related to poorly differentiated malignancies, it was suggested that PRC1 and PRC2 may protect the undifferentiated state in tumors (Raaphorst et al. 2003; Sparmann and van Lohuizen 2006). Non-coding RNAs including miRNAs and long non-coding RNAs (lncRNA) participate in the regulation of CSCs properties such as self-renewal, differentiation, drug resistance, tumor initiation, and disease recurrence (Phi et al. 2018; Huang et al. 2021). MiR-200b and let-7c were reported to block Hg signaling in CSCs, diminishing therapeutic resistance (Ahmad et al. 2013). It was declared that MiR-708-5p could eliminate lung cancer stem cell-like phenotypes by inhibiting the Wnt/β-catenin signaling pathway (Liu et al. 2018). It was indicated that the epigenomics of CSCs vary prominently from cancer cells (Wainwright and Scaffidi 2017) and are involved in many processes such as tumorigenesis, metastasis, EMT, and tumor heterogeneity (Sharma et al. 2009). As a result, CSC's epigenetics play a critical role in therapy resistance and cancer relapse.

Dysregulation of signal pathways including Wnt, Notch, TGF-β, NF-kβ, Hh, JAK-STAT, PPAR, and PI3K-AKT leads to invasion, metastasis, tumor recurrence. Particularly, defects in Wnt, Hh, and Notch signaling activities were found in different human cancers including breast, pancreatic, prostate, gastric, cervical, colon cancer, etc. (Siziopikou et al. 2003; Dontu et al. 2004; Karhadkar et al. 2004). Wnt/β-catenin signaling pathway regulates the self-renewal capacity of CSCs via activating target genes, including c-MYC, Axin2, SOX4, TCF7, and LGR5 (Zhu and Fan 2018; Huang et al. 2021). Abnormal activation of Wnt was indicated to transform dormant CSCs into active CSCs (Giancotti 2013). Notch, Wnt/β -catenin, Hh, TGFβ, PI3K/Akt were found to promote the regrowth of tumor mass after cancer treatment (Marzagalli et al. 2021). The NF-κB pathway mediates inflammation and CSC-like phenotype in tumors. The positive feedback loop between EMT and NF-κB pathway was reported to increase CSC-like properties (Wu et al. 2017). Obviously, many signal pathways play the main role in the regulation of the properties of CSCs. Therefore, inhibition of these signal pathways can be a logical strategy to improve prognosis by eliminating CSCs. However, CSCs are regulated by complex mutual interaction between these signaling pathways. Especially, the interaction of Wnt/β-catenin and NF-kβ signaling pathways was indicated to contribute survival and proliferation of CSCs. Wnt/β-catenin signaling was reported to mediate an increase in Notch-Gli1 expression and transcriptional activity, promoting the survival and proliferation of colorectal CSCs

(Noubissi et al. 2009). Furthermore, it has been reported that some therapeutic agents could activate some signaling pathways associated with tumorigenesis. It was suggested that targeting CSC-related signal pathways is challenging as it is not fully known whether there is synergism or antagonism between different signaling pathways (Huang et al. 2021).

4 Therapeutic Targeting of CSCs

CSCs form a small percentage of tumors and can cause tumor recurrence following conventional therapy. This is normally because of the targeting of the rapidly dividing cells by conventional therapies. CSCs show resistance to conventional therapies due to their slow division capacity. Consequently, the improvement of therapies targeting CSCs is necessary without affecting normal stem cells (Clarke et al. 2006; Prasetyanti and Medema 2017; Moharil et al. 2017; Yang et al. 2020). Several CSC-targeting therapies were shown to have limited activity and cause off-target effects in clinical trials (Marzagalli et al. 2021).

As CSCs play a crucial role in tumor progression, metastasis, and tumor recurrence, specifically targeting of CSCs is claimed to be the ultimate way to inhibit tumors and thus provide better patient survival. It was proposed that cancer immunotherapy may be a crucial strategy for targeting CSCs. It was suggested that natural killer (NK) cells, antibodies, CSC-based dendritic cells, and CSC-primed cytotoxic T lymphocytes (CTLs) may increase the effectiveness of cancer treatment by targeting CSCs. Different tumors express distinct CSC surface markers. Most significant markers of CSCs include CD133, CD44, IL3R, and immunoglobulin mucin TIM3. Monoclonal antibodies have been indicated to be the best choice to target CSC markers due to their specificity. For example, anti-human CD44 monoclonal antibody was shown to eliminate human acute myeloid leukemic stem cells in patient-derived AML blasts. However, most surface markers of CSCs can also be expressed in normal cells, and thus targeting these markers can induce toxicity and disrupt tissue homeostasis. Consequently, the determination of specific antigens and genetic alterations in CSCs has a crucial role in the identification of immunotherapeutic targets (Phi et al. 2018; Huang et al. 2021; Nimmakayala et al. 2021). It was proposed that cancer and immune cells may contain bacterial cells which can affect tumor progression and response to immunotherapy. Consequently, this finding supports that bacterial cells may be a target for the development of novel cancer immunotherapies (Nejman et al. 2020). Recently, chimeric antigen receptor (CAR)-T cell therapy has been developed in which engineered T cell express cytotoxic cytokines such as perforin and granzyme to eliminate tumors by recognizing tumor-associated antigens (TAAs) independent of a major histocompatibility complex (MHC) manner. However, this therapy includes some challenges. CAR-T cells only target cell surface antigens. CSC-specific surface antigens were declared to be required to only express on the surface of CSCs, not on normal cells. Furthermore, it was indicated that a lack of expression of some CSC cell surface antigens in heterogenous CSC populations can make them hard to be specifically targeted by CAR-T cells. Moreover, persistence and insufficient trafficking of CAR-T cells to tumors, and the presence of immunosuppressors are other hindrances. Some strategies have been proposed to overcome these challenges. One of these strategies is to use of bispecific CAR-T cell therapy targeting two distinct antigens expressed on tumor cells. Another strategy is the utilization of antigen-specific inhibitory CAR-T cells expressing an inhibitory receptor for antigens of normal cells, eventually preventing cytotoxicity in normal cells. The last strategy is the utilization of low-affinity CAR-T cells that can target overexpressed TAAs in tumor cells with ignoring TAAs in normal cells. Particularly, persistence was associated with the protection of CAR-T cells from exhaustion and senescence. It has been suggested that the local/intratumoral delivery of CAR-T cells can effectively kill tumor cells. Different tumors have distinct cell surface antigen profiles. Therefore, it has been suggested that determination of highly specific CSC antigens expressed on various tumors can ensure to development of universal CSC-targeted CAR-T cell therapies. Particularly, CD271, LGR5, TIM-3, CD105 were indicated to be possible targets for CAR-T cell therapy in the future (Masoumi et al. 2021).

Tumor vaccine is based on the activation of the human immune system following an injection of artificial immunogenic tumor antigen to eliminate tumor cells. The development of vaccines targeting CSCs has been aimed recently. MUC1 is a regulator of colorectal cancer stem cells (CCSCs) properties and MUC1 CSC vaccine was indicated to induce humoral immunity to eliminate CD133+ CCSCs effectively (Guo et al. 2020). However, it was proposed that targeting CSCs with a CSC vaccine is difficult due to the heterogeneity of CSC populations. On the other hand, the oncolytic virus (OV)-based therapy intends to lyse tumor cells through virus proliferation and low toxicity of virus-related proteins. It has been declared that the OV could effectively initiate CSC lysis as they infect both quiescent and dividing cells. Furthermore, several factors including quiescent state, anti-apoptotic proteins, ROS level, and DNA repair were shown to not have any effect on OV therapy (Chaurasiya et al. 2018; Huang et al. 2021). Mesenchymal stem cells (MSCs) are the most common stem cells detected in various tissues from fetal tissues to adult tissues (Pham and Vu 2019). They have several characteristics, including adhesion to the plastic surfaces, expression of CD44, CF73, CD90, and CD105 markers, lack of CD14, CD11a, CD34, CD45, CD19, CD79 alpha, and/or HLA-DR markers, and differentiation into adipocytes, osteoblasts, and chondrocytes (Dominici et al. 2006). MSCs are mostly preferred in clinical applications due to their low immunogenic properties. The migration property of MSCs toward tumors was indicated to be observed in several cancers. It was indicated that several factors secreted from tumor cells including TNF-alpha, interleukins, macrophage migration inhibitory factor (MIF), and stromal-derived factor (SDF) mediate the migration of MSCs toward the tumor environment by interacting with different surface receptors of MSCs. The migration and low immunogenicity of MSCs have made them advantageous as suitable vehicles for the delivery of various factors or agents to cancer cells. Over the past decade, MSCs modified with antitumor agents/factors have been developed as a new cancer treatment strategy for the effective delivery of antitumor agents/factors to tumor cells (Pham and Vu 2019). It was suggested that MSCs can be effective vehicles to transport OVs to tumors with decreasing their side effects on non-cancer tissues. Newcastle disease virus (NDV) infected MSCS was indicated to increase the sensitivity of glioma stem cells to radiation (Kazimirsky et al. 2016). Clinical trials have been performed using MSCs infected with OVs and have achieved excellent results. Autologous MSCs infected with oncolytic adenovirus ICOVIR-5 were used to treat children with metastatic neuroblastoma and effective result to be obtained (Garcia-Castro et al. 2010).

As dedifferentiation of cancer cells has been associated with a poorer prognosis and chemoresistance, cell differentiation could be suitable therapy. It was indicated that dual-frequency ultrasound inducing CSC differentiation can impair the stemness property of liver CSCs. It has been reported that BMP4 can increase the sensitivity of CSCs to chemotherapy by inducing CSC differentiation in glioblastoma and colorectal cancer (Piccirillo et al. 2006; Maccalli and De Maria 2015; Huang et al. 2021). It was implicated that targeting the metabolic enzyme PGK1 can promote CSC differentiation in gastric cancers (Pecqueur et al. 2013). Dexamethasone is a synthetic glucocorticoid used to treat cancer- and treatment-related symptoms. A study has identified that dexamethasone can have anti-CSC activities. In this way, the identification of therapeutic efficacy against the cancer of existing drugs with known safety profiles has been shown to have more advantages in regarding saving both time and money. It was indicated that dexamethasone promotes the expression of MKP-1 (MAPK phosphatase) in a glucocorticoid receptor (GR)-dependent manner, eventually inactivating JNK proinflammatory kinase, and thus driving pancreatic CSC differentiation, and decreasing CSC stemness properties, in vitro CSC model, and in vivo pancreatic cancer xenograft model. It was also indicated that dexamethasone likely increases chemosensitivity of pancreatic CSCs by decreasing the expression of survivin, an anti-apoptotic protein involved in multidrug resistance in pancreatic CSCs (Suzuki et al. 2020). According to these finding, differentiation therapy can be an effective therapeutic strategy due to eliminating the tumorigenic activity of CSCs.

Treatment of triple-negative breast cancer (TNBC) central nervous system (CNS) metastasis was suggested to be seriously difficult as these metastatic tumor cells lack targetable surface markers. Transdifferentiation as a cell reprogramming technology directly converts somatic fibroblasts into NSCs and this technology contributed to the generation of induced NSCs (iNSCs). It has been suggested that iNSCs have more advantageous as tumor-homing drug carriers due to not needed a pluripotent stage, ease of isolation, and opportunity of autologous therapy. It has been reported that a neurosphere-based culture system generates human-induced neural stem cell (hiNSC) variant or human-induced neurosphere (hiNeuroS) possessing high tumor-homing, antitumor capabilities, and in vivo persistence by using a SOX2 single-cell reprogramming factor. hiNeuroS-TRAIL was found to inhibit tumor and prolong survival in TNBC parenchymal metastasis models and TNBC leptomeningeal carcinomatosis. It is claimed that hiNeuroS therapy may be an effective strategy for the treatment of metastatic brain cancer (Jiang et al. 2021).

As CSCs exhibit altered metabolic activity, targeting their metabolism has been shown as a potential strategy to eliminate CSCs. It was put forward that metformin and phenformin could eliminate CSCs by blocking mitochondrial function in many cancer. It was indicated that sFRP4 (Wnt inhibitor) can eliminate CSCs by targeting their metabolism in prostate and breast CSCs. Because of the metabolic heterogeneity of CSCs, it is claimed that a combinational strategy composed of glycolysis and mitochondrial inhibitors, and conventional therapy, can efficiently eliminate CSCs. On the other hand, targeting of CSC niches within tumor microenvironment can be another way. Exclusively, it was shown that targeting of HIFs may disrupt CSC niches. Another way of disrupting of CSC niches was to target CAFs to block the interaction between CAFs and CSCs. Inhibition of angiogenesis in CSC niches was shown as another effective strategy for inhibiting CSCs in many aspects. As vascular endothelial growth factor (VEGF) contributes to angiogenesis, it has been indicated that blocking of VEGR or VEGFR could disrupt CSCs. As CSC niches regulate CSCs in a complex way, it has been mentioned that more specific targets of CSC niches are needed to be determined in future studies (Huang et al. 2021).

Melatonin is a hormone secreted by the pineal gland which regulates several processes including sleep, circadian rhythms, immune system, and reproduction system. Furthermore, it was shown that melatonin can induce apoptosis and inhibit tumor metastasis and angiogenesis in malignancy (Maroufi et al. 2020). A study indicated that melatonin inhibits the viability and invasiveness of the breast CSC population by increasing the expression of E-cadherin and reducing the expression of OCT4, N-cadherin, and vimentin (Gonçalves et al. 2016). On the other hand, melatonin has been shown to induce autophagy on breast CSCs with CD44 + CD24−/low of a breast cancer cell line (MCF-7) (Dönmez et al. 2017). A recent study indicated that melatonin increases glioblastoma CSCs sensitivity to paclitaxel by inhibiting Nestin and c-Myc (Lee et al. 2018a, 2018b). A combination of 5-FU and melatonin was suggested to induce apoptosis and autophagy in colon CSCs by regulating OCT-4-PrPC axis (Lee et al. 2018a, 2018b).

Since most therapeutic drugs do not accurately target cancer cells and CSCs, they can cause off-target effects. Therefore, novel delivery systems seem necessary to specifically direct cancer drugs to cancer cells and CSCs. Especially, nanomedicine is an innovative approach that develops nano-vehicles to facilitate the delivery and cellular uptake of various therapeutic drugs to effectively eradicate cancer cells and CSCs (Qin et al. 2017). Recently, various nanoparticles (NPs), including self-assembled polymeric NPs, inorganic NPs, natural NPs based on proteins and exosomes, and antibody-drug conjugates have been developed as a delivery tool to specifically target CSCs (Ertas et al. 2021). NPs have several advantages, including that they preserve drugs from degradation, provide delivery of drugs to the target tissue, boost the drug's pharmacokinetics and stability, and bioavailability, and mediate the timed release of drugs in the target tissue. The drug's effectiveness, toxicity, and bioavailability were suggested to be associated with the properties of NPs, including size, distribution type, surface charge, drug release rate, pharmacokinetics (Singh et al. 2003). However, NPs have some disadvantages, including quickly clearance from the circulation, causing inflammation, infiltration into other tissues, and formation of aggregates (Prince et al. 2007). Poly(lactide-co-glycolide) (PLGA) is a biodegradable polymer used as a carrier for drug-loaded NPs. Salinomycin (SLM)-loaded PLGA NPs conjugated with antibody against ErbB-2 tyrosine-protein kinase receptor (HER2) were demonstrated to decrease HER2- positive CSCs, thus inhibiting tumor growth in a mouse breast cancer model in vivo and in vitro (Li et al. 2017). Lipid-polymer (LP) NPs are composed of a polymeric core and lipid shell. Salinomycin (SLM) loaded LP NPs conjugated with both anti-EGFR and anti-CD133 aptamers (CESP) were shown to eliminate tumor cells and CSC with high effectiveness, eventually inhibiting tumor growth in an osteosarcoma mouse model (Chen et al. 2018). Liposomes are amphiphilic phospholipid vesicles used for targeting hydrophilic and hydrophobic drugs to tumor bulks. Liposomes have been shown as effective tools that can increase the bioavailability and stability of drugs. Liposomes can be loaded with CSC-specific inhibitors to deliver targeted therapy against CSCs. These liposomes can be modified to provide specifically targeted therapy (Colson and Grinstaff 2012; Arabi et al. 2015). PTX and survivin siRNA-loaded dual-targeting cationic liposomes targeting CD133 CSCs were reported to promote the differentiation of glioma CSCs and cancer cell death in a U251-CD33+ glioma xenograft mouse model (Sun et al. 2018). Inorganic NPs with few nanometers in size have limited usage due to their rapid elimination. However, it was suggested that this limited utilization can be overcome by altering their shape, size, and surface properties (McNeil 2016; Sharma et al. 2018). Gold nanoparticles (AuNPs) are non-toxic and non-immunogenic, and their easy surface modification has revealed them as suitable vehicles for drug delivery (Ghosh et al. 2008). It has been reported that thiol-terminated PEG-coated Au NPs have high stability and biocompatibility. SLM-PEGylated Au NPs were indicated to enhance drug-induced CD24-/CD44+ breast CSCs death (Zhao et al. 2019). Antibody–drug conjugates (ADCs) are designed to target specific receptors on tumor cells and CSCs (Walko and West 2019). It has been reported that the selection of antigens binding with high specificity to the antibody, highly expressed on the surface of CSCs and minimally released into the circulatory system may limit ACDs' off-target toxic effects on normal cells. On the other hand, the selection of antibodies with high specific binding affinity to antigens on the surface of CSCs and a long half-life in circulation has also been considered important in this process (Hafeez et al. 2020). ADCs were determined as more advantageous than other targeted delivery approaches due to their high tumor selectivity and cytotoxicity (Villela-Martinez et al. 2017). Anti-CD33-targeting ADC was reported to target a DNA-alkylating antitumor agent to patient-derived acute myeloid leukemia (AML) cells, leading to DNA damage, cell-cycle arrest, apoptosis in vitro. CD33-targeting ADC provided tumor retardation and long-term survival in an AML xenograft model (Kovtun et al. 2018). The linkers stability that connects the drug to an antibody was shown to be crucial for designing ADCs. As most cancer drugs have high toxicity, it was reported that linkers should have sufficient stability to eliminate premature release before delivery to target tissues (Villela-Martinez et al. 2017).

As the epigenomics of CSCs vary prominently from cancer cells (Wainwright and Scaffidi 2017) and play a crucial role in therapy resistance and cancer relapse (Sharma et al. 2009), monitoring the epigenomic alterations of CSCs will ensure to determine a novel therapeutic and diagnostic target for CSCs (Ayob and Ramasamy 2018; Yang et al. 2020). Current epigenomic monitoring methods were indicated to not provide accurate results regarding the epigenomic properties of CSCs due to data loss during isolation, damage to genomic DNA, and non-cellular specificity. A CSC epigenomic monitoring platform (CEMP) was developed to monitor epigenomic alterations of CSCs effectively. It has been stated that CEMP should provide several criteria, including that does not need any isolation process, easily transfer within the nucleus, is stable in the nuclear environment, and produce stable, reproducible signals for real-time monitoring (Verma et al. 2022). Nanomaterial-based surface-enhanced Raman spectroscopy (SERS) was indicated as an ideal method (Langer et al. 2020). Gold nanomaterials (Au NMs) were shown as the ideal SERS-based nanomaterial due to their in vivo biocompatibility, biodistribution, high permeability and retention effect in cells, and multi-functionalization surface chemistry (Singh et al. 2018). However, Au NMs produced according to conventional methods have been reported to reduce nuclear internalization efficiency and induce DNA damage-mediated cell death in cancer cells (Kang et al. 2010). Self-functional Gold Nanoprobes (GNP) have been designed with several advantages including high effectiveness nuclear internalization and allowing to track epigenomes of CSC without any conformational change in genomic DNA. A unique monitoring platform using GNP provided opportunity to study structural, functional, molecular epigenetics, and nuclear proteomics in preclinical models of breast and lung CSCs. This technique was reported to distinguish between CSC from different tissues with >99% accuracy and specificity. In the future, it was claimed that this will provide an opportunity to develop advanced real-time epigenomics monitoring technologies such as nucleus-targeted drug monitoring and epigenomic prognosis and diagnostics (Verma et al. 2022).

5 Conclusion

CSCs play a crucial role in invasion, metastasis, therapeutic resistance, and cancer relapse. Therefore, nowadays, the identification and targeting of various mechanisms regulating CSC properties have begun to attract more attention. Most anti-CSC therapeutic strategies target stemness-related factors and pathways shared between CSCs and normal SCs, leading to off-target effects. Therefore, the development of novel therapeutic strategies specifically targeting CSCs is essential. It was suggested that novel therapeutic strategies for elimination of CSC populations should contain multiple drugs targeting ABC transporters, DNA damage repair mechanisms, tumor microenvironment, EMT, antitumor immunity, and stemness-related pathways. Considering CD44 variant (CD44v) isoform was found to have high tumor initiation capacity, and CSC heterogeneity, the identification, and targeting of multiple CSC-specific antigens expressed on various tumors will be an effective strategy to eliminate CSCs. Nanoparticles have been proposed as important tools for targeting resistance mechanisms in CSCs due to their ability to target chemotherapeutic drugs at higher concentrations to target sites and CSCs. Choosing the least toxic and immunogenic nanoparticles is crucial for effective cancer treatment. Due to tumor migration and low immunogenicity of MSCs, they can also be used as a delivery vehicle to provide effective cancer treatment. Consequently, the use of drug or factor-loaded NPs or MSCs modified with multiple antibodies/factors targeting concurrently multiple antigens/factors on CSCs can significantly decrease drug toxicity and side effects and increase the effectiveness of cancer treatment. Therefore, it is thought that focusing on studies in this area will provide great advantages in terms of cancer treatment in the future.

References

Abdullah LN, Chow EK-H (2013) Mechanisms of chemoresistance in cancer stem cells. Clin Transl Med 2:3–3

Ahmad A et al (2013) Inhibition of hedgehog signaling sensitizes NSCLC cells to standard therapies through modulation of EMT-regulating miRNAs. J Hematol Oncol 6:77. (2013)

Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci U S A 100:3983–3988

Arabi L, Badiee A, Mosaffa F, Jaafari MR (2015) Targeting CD44 expressing cancer cells with anti-CD44 monoclonal antibody improves cellular uptake and antitumor efficacy of liposomal doxorubicin. J Control Release 220:275–286

Ayob AZ, Ramasamy TS (2018) Cancer stem cells as key drivers of tumour progression. J Biomed Sci 25(1):20. https://doi.org/10.1186/s12929-018-0426-4

Bae KM, Dai Y, Vieweg J, Siemann DW (2016) Hypoxia regulates SOX2 expression to promote prostate cancer cell invasion and sphere formation. Am J Cancer Res 6:1078–1088. 47

Bao S et al (2006) Glioma stem cells promote radioresistance by preferential activation of the DNA damage response. Nature 444:756–760

Batlle E, Clevers H (2017) Cancer stem cells revisited. Nat Med 23:1124–1134

Beck B, Blanpain C (2013) Unravelling cancer stem cell potential. Nat Rev Cancer 13:727–738

Begicevic RR, Falasca M (2017) ABC transporters in cancer stem cells: beyond chemoresistance. Int J Mol Sci 18:2362

Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJ (2005) Opinion: the origin of the cancer stem cell: current controversies and new insights. Nat Rev Cancer 5:899–904

Bliss SA, Sinha G, Sandiford OA, Williams LM, Engelberth DJ, Guiro K, Isenalumhe LL, Greco SJ, Ayer S, Bryan M, Kumar R, Ponzio NM, Rameshwar P (2016) Mesenchymal stem cell-derived exosomes stimulate cycling quiescence and early breast cancer dormancy in bone marrow. Cancer Res 76:5832–5844

Bomken S, Fiser K, Heidenreich O, Vormoor J (2010) Understanding the cancer stem cell. Br J Cancer 103(4):439–445. https://doi.org/10.1038/sj.bjc.6605821

Boumahdi S, Driessens G, Lapouge G, Rorive S, Nassar D, Le Mercier M, Delatte B, Caauwe A, Lenglez S, Nkusi E (2014) SOX2 controls tumour initiation and cancer stem-cell functions in squamous-cell carcinoma. Nature 511:246–250

Cabarcas SM, Mathews LA, Farrar WL (2011) The cancer stem cell niche—there goes the neighborhood? Int J Cancer 129:2315–2327

Carnero A, Lleonart M (2016) The hypoxic microenvironment: a determinant of cancer stem cell evolution. Inside Cell 1:96–105

Celià-Terrassa T, Meca-Cortés O, Mateo F, Martínez de Paz A, Rubio N, Arnal-Estapé A et al (2012) Epithelial-mesenchymal transition can suppress major attributes of human epithelial tumor-initiating cells. J Clin Invest 122:1849–1868

Chaurasiya S, Chen NG, Warner SG (2018) Oncolytic Viro therapy versus cancer stem cells: a review of approaches and mechanisms. Cancers 10:124

Chen F, Zeng Y, Qi X, Chen Y, Ge Z, Jiang Z, Zhang X, Dong Y, Chen H, Yu Z (2018) Targeted salinomycin delivery with EGFR and CD133 aptamers based dual-ligand lipid-polymer nanoparticles to both osteosarcoma cells and cancer stem cells. Nanomed Nanotechnol Biol Med 14:2115–2127

Cheung TH, Rando TA (2013) Molecular regulation of stem cell quiescence. Nat Rev Mol Cell Biol 14:329–340

Chomel J-C, Sorel N, Turhan AG (2012) ABL-kinase mutations in progenitors and stem cells from chronic myeloid leukemia patients. In: Stem cells and cancer stem cells, vol 8, pp 305–315

Clarke MF, Dick JE, Dirks PB, Eaves CJ, Jamieson CH, Jones DL, Visvader J, Weissman IL, Wahl GM (2006) Cancer stem cells--perspectives on current status and future directions: AACR workshop on cancer stem cells. Cancer Res 66(19):9339–9344. https://doi.org/10.1158/0008-5472.CAN-06-3126

Codony-Servat J, Rosell R (2015) Cancer stem cells and immunoresistance: clinical implications and solutions. Transl Lung Cancer Res 4:689–703

Colson YL, Grinstaff MW (2012) Biologically responsive polymeric nanoparticles for drug delivery. Adv Mater 24:3878–3886

Davis H, Irshad S, Bansal M, Rafferty H, Boitsova T, Bardella C, Jaeger E, Lewis A, Freeman-Mills L, Giner FC, Rodenas-Cuadrado P, Mallappa S, Clark S, Thomas H, Jeffery R, Poulsom R, Rodriguez-Justo M, Novelli M, Chetty R, Silver A, Sansom OJ, Greten FR, Wang LM, East JE, Tomlinson I, Leedham SJ (2014) Aberrant epithelial GREM1 expression initiates colonic tumorigenesis from cells outside the stem cell niche. Nat Med 21:62–70

Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317

Dönmez H, Kocak N, Yildirim IH (2017) Investigation of autophagic effects of melatonin on breast cancer stem cells. Biomed. Res 28:5048–5053

Dontu G, Jackson KW, McNicholas E, Kawamura MJ, Abdallah WM, Wicha MS (2004) Role of notch signaling in cell-fate determination of human mammary stem/progenitor cells. Breast Cancer Res 6:605–615

Ertas YN, Abedi Dorcheh K, Akbari A, Jabbari E (2021) Nanoparticles for targeted drug delivery to cancer stem cells: a review of recent advances. Nanomaterials (Basel, Switzerland) *11*(7):1755. https://doi.org/10.3390/nano11107175

Eun K, Ham SW, Kim H (2017) Cancer stem cell heterogeneity: origin and new perspectives on CSC targeting. BMB Rep 50:117–125

Farace C, Oliver JA, Melguizo C, Alvarez P, Bandiera P, Rama AR, Malaguarnera G, Ortiz R, Madeddu R, Prados J (2015) Microenvironmental modulation of decorin and lumican in temozolomide-resistant glioblastoma and neuroblastoma cancer stem-like cells. PLoS One 10:e0134111

Ferguson LP, Diaz E, Reya T (2021) The role of the microenvironment and immune system in regulating stem cell fate in cancer. Trends Cancer 7(7):624–634. https://doi.org/10.1016/j.trecan.2020.12.014

Fukunaga-Kalabis M, Martinez G, Nguyen T-TK, Kim D, Santiago-Walker A, Roesch A, Herlyn M (2010) Tenascin-C promotes melanoma progression by maintaining the ABCB5-positive side population. Oncogene 29:6115

Gammon L, Biddle A, Heywood HK, Johannessen AC, Mackenzie IC (2013) Sub-sets of cancer stem cells differ intrinsically in their patterns of oxygen metabolism. PLoS One 8:e62493

Garcia-Castro J, Alemany R, Cascallo M, Martinez-Quintanilla J, del Mar Arriero M, Lassaletta A, Madero L, Ramirez M (2010) Treatment of metastatic neuroblastoma with systemic oncolytic virotherapy delivered by autologous mesenchymal stem cells: an exploratory study. Cancer Gene Ther 17:476–483

Ghaffari S (2011) Cancer, stem cells and cancer stem cells: old ideas, new developments. F1000 Med Rep 3:23. https://doi.org/10.3410/M3-23

Ghosh P, Han G, De M, Kim CK, Rotello VM (2008) Gold nanoparticles in delivery applications. Adv Drug Deliv Rev 60(11):1307–1315. https://doi.org/10.1016/j.addr.2008.03.016

Giancotti FG (2013) Mechanisms governing metastatic dormancy and reactivation. Cell 155:750–764

Gonçalves NN, Colombo J, Lopes JR, Gelaleti GB, Moschetta MG et al (2016) Effect of melatonin in epithelial mesenchymal transition markers and invasive properties of breast cancer stem cells of canine and human cell lines. PLoS One 11(3):e0150407. https://doi.org/10.1371/journal.pone.0150407

Gopal K, Gupta N, Zhang H, Alshareef A, Alqahtani H, Bigras G et al (2016) Oxidative stress induces the acquisition of cancer stem-like phenotype in breast cancer detectable by using a Sox2 regulatory region-2 (SRR2) reporter. Oncotarget 7:3111–3127

Guo M, Luo B, Pan M, Li M, Zhao F, Dou J (2020) MUC1plays an essential role in tumor immunity of colorectal cancer stem cell vaccine. Int Immunopharmacol 85:106631

Hafeez U, Parakh S, Gan HK, Scott AM (2020) Antibody drug conjugates for cancer therapy. Molecules 25:4764

Hanahan D, Weinberg RA (2000) The hallmarks of cancer. Cell 100:57–70

Huang B, Yan X, Li Y (2021) Cancer stem cell for tumor therapy. Cancers 13(19):4814. https://doi.org/10.3390/cancers13194814

Hung KF, Yang T, Kao SY (2019) Cancer stem cell theory: are we moving past the mist? J Chin Med Assoc JCMA 82(11):814–818. https://doi.org/10.1097/JCMA.0000000000000186

Jiang W, Yang Y, Mercer-Smith AR, Valdivia A, Bago JR, Woodell AS, Buckley AA, Marand MH, Qian L, Anders CK, Hingtgen SD (2021) Development of next-generation tumor-homing induced neural stem cells to enhance treatment of metastatic cancers. Sci Adv 7(24):eabf1526. https://doi.org/10.1126/sciadv.abf1526

Kang B, Mackey MA, El-Sayed MA (2010) Nuclear targeting of gold nanoparticles in cancer cells induces DNA damage, causing cytokinesis arrest and apoptosis. J Am Chem Soc 132:1517. https://doi.org/10.1021/ja9102698

Karhadkar SS, Bova GS, Abdallah N et al (2004) Hedgehog signalling in prostate regeneration, neoplasia and metastasis. Nature 431:707–712

Kazimirsky G, Jiang W, Slavin S, Ziv-Av A, Brodie C (2016) Mesenchymal stem cells enhance the oncolytic effect of Newcastle disease virus in glioma cells and glioma stem cells via the secretion of TRAIL. Stem Cell Res Ther 7:149

Kitamura T, Qian BZ, Pollard JW (2015) Immune cell promotion of metastasis. Nat Rev Immunol 15:73–86

Kovtun Y, Noordhuis P, Whiteman KR, Watkins K, Jones GE, Harvey L, Lai KC, Portwood S, Adams S, Sloss CM et al (2018) IMGN779, a novel CD33-targeting antibody-drug conjugate with DNA-alkylating activity, exhibits potent antitumor activity in models of AML. Mol Cancer Ther 17:1271–1279

Langer J, Jimenez de Aberasturi D, Aizpurua J, Alvarez-Puebla RA, Auguié B, Baumberg JJ, Bazan GC, Bell S, Boisen A, Brolo AG, Choo J, Cialla-May D, Deckert V, Fabris L, Faulds K, García de Abajo FJ, Goodacre R, Graham D, Haes AJ, Haynes CL et al (2020) Present and future of surface-enhanced Raman scattering. ACS Nano 14(1):28–117. https://doi.org/10.1021/acsnano.9b04224

Lau WM et al (2014) CD44v8-10 is a cancer-specific marker for gastric cancer stem cells. Cancer Res 74:2630–2641

Lau EY-T, Ho NP-Y, Lee TK-W (2017) Cancer stem cells and their microenvironment: biology and therapeutic implications. Stem Cells Int 2017:3714190

Lee H, Lee HJ, Jung JH, Shin EA, Kim SH (2018a) Melatonin disturbs SUMOylation-mediated crosstalk between c-Myc and nestin via MT1 activation and promotes the sensitivity of paclitaxel in brain cancer stem cells. J Pineal Res 65. https://doi.org/10.1111/jpi.12496

Lee JH, Yun CW, Han YS, Kim S, Jeong D, Kwon HY, Kim H, Baek MJ, Lee SH (2018b) Melatonin and 5-fluorouracil co-suppress colon cancer stem cells by regulating cellular prion protein-Oct4 axis. J Pineal Res 65(4):e12519. https://doi.org/10.1111/jpi.12519

Lee IC, Fadera S, Liu H-L (2019) Strategy of differentiation therapy: effect of dual-frequency ultrasound on the induction of liver cancer stem-like cells on a HA-based multilayer film system. J Mater Chem B 7:5401–5411

Li J, Xu W, Yuan X, Chen H, Song H, Wang B, Han J (2017) Polymer–lipid hybrid anti-HER2 nanoparticles for targeted salinomycin delivery to HER2-positive breast cancer stem cells and cancer cells. Int J Nanomedicine 12:6909

Lida H, Suzuki M, Goitsuka R, Ueno H (2012) Hypoxia induces CD133 expression in human lung cancer cells by up-regulation of OCT3/4 and SOX2. Int J Oncol 40: 71–79

Liu T et al (2018) Downregulation of DNMT3A by miR-708-5p inhibits lung cancer stem cell-like phenotypes through repressing Wnt/β-catenin signaling. Clin Cancer Res 24:1748–1760

Loureiro R, Mesquita KA, Magalhães-Novais S, Oliveira PJ, Vega-Naredo I (2017) Mitochondrial biology in cancer stem cells. Semin Cancer Biol 47:18–28

Lu P, Weaver VM, Werb Z (2012) The extracellular matrix: a dynamic niche in cancer progression. J Cell Biol 196:395–406

Maccalli C, De Maria R (2015) Cancer stem cells: perspectives for therapeutic targeting. Cancer Immunol Immunother CII 64(1):91–97. https://doi.org/10.1007/s00262-014-1592-1

Maroufi NF, Vahedian V, Hemati S, Rashidi MR, Akbarzadeh M, Zahedi M, Pouremamali F, Isazadeh A, Taefehshokr S, Hajazimian S, Seraji N, Nouri M (2020) Targeting cancer stem cells by melatonin: effective therapy for cancer treatment. Pathol Res Pract 216(5):152919. https://doi.org/10.1016/j.prp.2020.152919

Martincorena I, Roshan A, Gerstung M, Ellis P, Van Loo P, McLaren S et al (2015) Tumor evolution. High burden and pervasive positive selection of somatic mutations in normal human skin. Science 348:880–886

Marzagalli M, Fontana F, Raimondi M, Limonta P (2021) Cancer stem cells-key players in tumor relapse. Cancers 13(3):376. https://doi.org/10.3390/cancers13030376

Masoumi J, Jafarzadeh A, Abdolalizadeh J, Khan H, Philippe J, Mirzaei H, Mirzaei HR (2021) Cancer stem cell-targeted chimeric antigen receptor (CAR)-T cell therapy: challenges and prospects. Acta Pharm Sin B 11(7):1721–1739. https://doi.org/10.1016/j.apsb.2020.12.015

McNeil SE (2016) Evaluation of nanomedicines: stick to the basics. Nat Rev Mater 1:1–2

Medema JP (2013) Cancer stem cells: the challenges ahead. Nat Cell Biol 15:338–344

Moharil RB, Dive A, Khandekar S, Bodhade A (2017) Cancer stem cells: an insight. J Oral Maxillofac PatholJOMFP 21(3):463. https://doi.org/10.4103/jomfp.JOMFP_132_16

Murayama T, Gotoh N (2019) Drug resistance mechanisms of cancer stem-like cells and their therapeutic potential as drug targets. Cancer Drug Resist (Alhambra, Calif.) 2(3):457–470. https://doi.org/10.20517/cdr.2019.36

Nejman D, Livyatan I, Fuks G, Gavert N, Zwang Y, Geller LT et al (2020) The human tumor microbiome is composed of tumor type specific intracellular bacteria. Science 368:973e80

Nimmakayala RK, Karmakar S, Kaushik G, Rauth S, Barkeer S, Marimuthu S, Ponnusamy MP (2021) Targeted therapy for cancer stem cells. In: Batra SK, Ponnusamy MP (eds) Gene rgulation and therapeutics for cancer. CRC Press, Baco Raton

Noubissi FK et al (2009) Wnt signaling stimulates transcriptional outcome of the hedgehog pathway by stabilizing GLI1 mRNA. Cancer Res 69:8572–8578

Ocaña OH, Córcoles R, Fabra A, Moreno-Bueno G, Acloque H, Vega S et al (2012) Metastatic colonization requires the repression of the epithelial-mesenchymal transition inducer prrx1. Cancer Cell 22:709–724

Ohnishi S, Ma N, Thanan R, Pinlaor S, Hammam O, Murata M, Kawanishi S (2013) DNA damage in inflammation-related carcinogenesis and cancer stem cells. Oxidative Med Cell Longev 2013:9

Palorini R, Votta G, Balestrieri C, Monestiroli A, Olivieri S, Vento R, Chiaradonna F (2014) Energy metabolism characterization of a novel cancer stem cell-like line 3AB-OS. J Cell Biochem 115:368–379

Patrawala L et al (2005) Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic. Cancer Res 65:6207–6219

Pavlides S, Tsirigos A, Vera I, Flomenberg N, Frank PG, Casimiro MC, Wang C, Fortina P, Addya S, Pestell RG (2010) Loss of stromal caveolin-1 leads to oxidative stress, mimics hypoxia and drives inflammation in the tumor microenvironment, conferring the “reverse Warburg effect”: a transcriptional informatics analysis with validation. Cell Cycle 9:2201–2219

Pecqueur C, Oliver L, Oizel K, Lalier L, Vallette FM (2013) Targeting metabolism to induce cell death in cancer cells and cancer stem cells. Int J Cell Biol 2013: 805975

Pham PV, Vu NB (2019) Mesenchymal stem cells as vectors for cancer therapy. In: Pham PV, El-Hashash A (eds) Stem cells for cancer and genetic disease treatment. Springer, Cham

Phi L, Sari IN, Yang YG, Lee SH, Jun N, Kim KS, Lee YK, Kwon HY (2018) Cancer stem cells (CSCs) in drug resistance and their therapeutic implications in cancer treatment. Stem Cells Int 2018:5416923. https://doi.org/10.1155/2018/5416923

Piccirillo SG, Reynolds BA, Zanetti N, Lamorte G, Binda E, Broggi G et al (2006) Bone morphogenetic proteins inhibit the tumorigenic potential of human brain tumour-initiating cells. Nature 444:761–765

Prasetyanti PR, Medema JP (2017) Intra-tumor heterogeneity from a cancer stem cell perspective. Mol Cancer 16:41

Prince M, Sivanandan R, Kaczorowski A, Wolf G, Kaplan M, Dalerba P, Weissman I, Clarke M, Ailles L (2007) Identification of a subpopulation of cells with cancer stem cell properties in head and neck squamous cell carcinoma. Proc Natl Acad Sci U S A 104:973–978

Qin W, Huang G, Chen Z, Zhang Y (2017) Nanomaterials in targeting cancer stem cells for cancer therapy. Front Pharmacol 8:1

Raaphorst FM, Meijer CJ, Fieret E et al (2003) Poorly differentiated breast carcinoma is associated with increased expression of the human Polycomb group EZH2 gene. Neo- plasia 5:481–488

Saijo H, Hirohashi Y, Torigoe T, Horibe R, Takaya A, Murai A et al (2016) Plasticity of lung cancer stem-like cells is regulated by the transcription factor HOXA5 that is induced by oxidative stress. Oncotarget 7: 50043–50056

Sell S (2010) On the stem cell origin of cancer. Am J Pathol 176:2584–2594

Semenza GL (2014) Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. Ann Rev Pathol 9:47–71

Sharma S, Kelly TK, Jones PA (2009) Epigenetics in cancer. Carcinogenesis 31:27. https://doi.org/10.1093/carcin/bgp220

Sharma A, Goyal AK, Rath G (2018) Recent advances in metal nanoparticles in cancer therapy. J Drug Target 26:617–632

Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB (2003) Identification of a cancer stem cell in human brain tumors. Cancer Res 63: 5821–5828

Singh P, Pandit S, Mokkapati VRSS, Garg A, Ravikumar V, Mijakovic I (2018) Gold nanoparticles in diagnostics and therapeutics for human cancer. Int J Mol Sci 19. https://doi.org/10.3390/ijms19071979

Siziopikou K, Miao H, Rizzo P et al (2003) Notch signaling is a therapeutic target in breast cancer. In: Proceedings of the 94th annual meeting of the AACR, pp 1277–1278

Sparmann A, van Lohuizen M (2006) Polycomb silencers control cell fate, development and cancer. Nat Rev Cancer 6:846–856

Sun N, Zhao C, Cheng R, Liu Z, Li X, Lu A, Tian Z, Yang Z (2018) Cargo-free nanomedicine with pH sensitivity for codelivery of DOX conjugated prodrug with SN38 to synergistically eradicate breast cancer stem cells. Mol Pharm 15:3343–3355

Suzuki S, Okada M, Sanomachi T, Togashi K, Seino S, Sato A, Yamamoto M, Kitanaka C (2020) Therapeutic targeting of pancreatic cancer stem cells by dexamethasone modulation of the MKP-1-JNK axis. J Biol Chem 295(52):18328–18342. https://doi.org/10.1074/jbc.RA120.015223

Tsuchiya H, Shiota G (2021) Immune evasion by cancer stem cells. Regenerat Ther 17:20–33. https://doi.org/10.1016/j.reth.2021.02.006

Verma AH, Ganesh S, Venkatakrishnan K, Tan B (2022) Self-functional gold nanoprobes for intra-nuclear epigenomic monitoring of cancer stem-like cells. Biosensors & Bioelectronics 195:113644. https://doi.org/10.1016/j.bios.2021.113644

Vermeulen L, Felipe De Sousa EM, Van Der Heijden M, Cameron K, De Jong JH, Borovski T, Tuynman JB, Todaro M, Merz C, Rodermond H (2010) Wnt activity defines colon cancer stem cells and is regulated by the microenvironment. Nat Cell Biol 12:468–476

Villela-Martinez LM, Velez-Ayala AK, Lopez-Sanchez RD, Martinez-Cardona JA, Hernandez-Hernandez JA (2017) Advantages of drug selective distribution in cancer treatment: Brentuximab Vedotin. Int J Pharmacol 13:785–807

Wainwright EN, Scaffidi P (2017) Epigenetics and cancer stem cells: unleashing, hijacking, and restricting cellular plasticity. Trends Cancer 3:372–386

Walko CM, West H (2019) Antibody drug conjugates for cancer treatment. JAMA Oncol 5:1648

Wang J et al (2010) Notch promotes radioresistance of glioma stem cells. Stem Cells 28:17–28

Wang WJ et al (2013) MYC regulation of CHK1 and CHK2 promotes radioresistance in a stem cell-like population of nasopharyngeal carcinoma cells. Cancer Res 73:1219–1231

Wu X et al (2017) AKR1B1 promotes basal-like breast cancer progression by a positive feedback loop that activates the EMT program. J Exp Med 214: 1065–1010

Xiao M, Yan M, Zhang J, Xu Q, Qi S, Wang X, Chen W (2017) Cancer stem-like cell related protein CD166 degrades through E3 ubiquitin ligase CHIP in head and neck cancer. Exp Cell Res 353(1):46–53. https://doi.org/10.1016/j.yexcr.2017.03.005

Yang L, Shi P, Zhao G, Xu J, Peng W, Zhang J, Zhang G, Wang X, Dong Z, Chen F, Cui H (2020) Targeting cancer stem cell pathways for cancer therapy. Signal Transduct Target Ther 5(1):8. https://doi.org/10.1038/s41392-020-0110-5

Yokoyama A, Kakiuchi N, Yoshizato T, Nannya Y, Suzuki H, Takeuchi Y et al (2019) Age-related remodelling of oesophageal epithelia by mutated cancer drivers. Nature 565:312–317

Yu Z, Pestell TG, Lisanti MP, Pestell RG (2012) Cancer stem cells. Int J Biochem Cell Biol 44(12):2144–2151. https://doi.org/10.1016/j.biocel.2012.08.022

Zamur C, Topal U, Özdemir H, Altınay S (2020) Stem cell concept in thyroid cancer. Integr J Med Sci 7:5

Zhao Y, Zhao W, Lim YC, Liu T (2019) Salinomycin-loaded gold nanoparticles for treating cancer stem cells by ferroptosis-induced cell death. Mol Pharm 16: 2532–2539

Zhou HM, Zhang JG, Zhang X, Li Q (2021) Targeting cancer stem cells for reversing therapy resistance: mechanism, signaling, and prospective agents. Signal Transduct Target Ther 6(1):62. https://doi.org/10.1038/s41392-020-00430-1

Zhu P, Fan Z (2018) Cancer stem cells and tumorigenesis. Biophys Rep 4(4):178–188. https://doi.org/10.1007/s41048-018-0062-2

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Rafting on the Plasma Membrane: Lipid Rafts in Signaling and Disease

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Abstract

The plasma membrane is not a uniform phospholipid bilayer; it has specialized membrane nano- or microdomains called lipid rafts. Lipid rafts are small cholesterol and sphingolipid-rich plasma membrane islands. Although their existence was long debated, their presence in the plasma membrane of living cells is now well accepted with the advent of super-resolution imaging techniques. It is interesting to note that lipid rafts function to compartmentalize receptors and their regulators and substantially modulate cellular signaling. In this review, we will examine the role of lipid rafts and caveolae-lipid raft-like microdomains with a distinct 3D morphology—in cellular signaling. Moreover, we will investigate how raft compartmentalized signaling regulates diverse physiological processes such as proliferation, apoptosis, immune signaling, and development. Also, the deregulation of lipid raft-mediated signaling during tumorigenesis and metastasis will be explored.

Keywords

Cancer · Caveolae · Lipid rafts · Plasma membrane compartmentalization · Signal transduction

Abbreviations

CAF	Cancer-associating fibroblasts
Cav	Caveolin
CBM	Caveolin binding motif
CSD	Caveolin scaffolding domain
DISC	Death-inducing signaling complex
DRM	Detergent-resistant membranes
DSM	Detergent-soluble membranes
ECM	Extracellular matrix
EGF Signaling	Epidermal growth factor signaling
EMT	Epithelial-mesenchymal transition
FADD	Fas-associated death domain
GPI-anchor	Glycosylphosphatidylinositol anchor
IGF Signaling	Insulin-like growth factor signaling
IRS-1	Insulin receptor substrate protein 1
LAT	Linker for activation of T cells
Ld	Liquid disordered
Lo	Liquid ordered

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MAPK Signaling	Mitogen-activated protein kinase signaling
MβCD	Methyl-β-cyclodextrin
NSOM	Near-field scanning optical
PALM	Photoactivated localization microscopy
PI3K	Phosphatidylinositol-3 kinase
PM	Plasma membrane
PTEN	Phosphatase and tensin homolog
STED Microscopy	Stimulated emission depletion microscopy
STORM	Stochastic optical reconstruction microscopy
TEM	Tetraspanin-enriched membrane rafts

1 The Compartments of the Plasma Membrane as Organizers of Cellular Signaling

Plasma membrane (PM) has long been viewed as a homogenous phospholipid bilayer dispersed randomly with membrane-bound proteins (Singer and Nicolson 1972). This view was challenged by identification of many subcompartments of the plasma membrane with various compaction properties and protein composition (Yu et al. 1973). These subcompartments of PM—so-called lipid rafts—are laterally mobile, enriched in cholesterol and saturated sphingolipids. They are now recognized for their crucial roles as platforms recruiting regulatory signaling molecules and facilitating effective signal transduction.

2 Characterization of Lipid Rafts

Biochemical experiments initially revealed that extracting PM at cold temperatures by nonionic detergents, such as Triton X-100, yields two populations of PM lipids (Yu et al. 1973). These are named detergent-soluble membranes (DSMs) and detergent-resistant membranes (DRMs) (Bagatolli and Mouritsen 2013). This observation put forth the idea that the lipidic composition of PM is heterogenous rather than being a homogenous phospholipid bilayer as previously assumed (Ahmed et al. 1997; Brown and Rose 1992; Friedrichson and Kurzchalia 1998; Pralle et al. 2000; van Meer et al. 1987; Varma and Mayor 1998). Nevertheless, these findings were also challenged, as the protein composition of DRMs exhibits alterations depending on the detergent type and the detergent concentration used in lipid extraction (Mayor and Maxfield 1995; Schuck et al. 2003). The heterogeneous nature of PM lipids was explored and shown complementarily in model membranes (Feigenson and Buboltz 2001; McConnell et al. 1984; Tamm and McConnell 1985; Veatch and Keller 2003). Model membranes exhibit a phenomenon called liquid–liquid phase separation in which lipidic molecules spontaneously separate into Liquid ordered (Lo) (Hjort Ipsen et al. 1987) and Liquid disordered (Ld) (Kaiser et al. 2009; Veatch and Keller 2003) compartments. Interestingly, both Lo membranes and DRMs exhibit enrichment in terms of cholesterol and glycosylated lipids, lending credence to the lipid raft hypothesis. However, studying lipid rafts in model membranes has its handicaps. For example, sharp phase separation between compartments was observed in model membranes, yet in natural membranes, it is believed that phase separation exists as a more dynamic gradient (Sezgin et al. 2012). Also, the percentage of protein molecules incorporated into model membranes falls short compared to the protein percentage in natural membranes, which is around 25% (Dupuy and Engelman 2008). Therefore, most complex regulatory processes occurring during raft formation and cellular signal regulation through rafts are hard to examine in model membranes. More recently, a plethora of fluorescent microscopy, electron microscopy, and label-free spectroscopy techniques have been developed to study lipid rafts. And all were presented with their advantages and disadvantages (Sezgin and Schwille 2011).

However, with the advancement in super-resolution microscopy, the resolution limit of traditional fluorescence microscopy, which is

200 nm, has been overcome (Hell and Wichmann 1994; Klar et al. 2001). As a result, we are now able to resolve objects smaller than 200 nm and conduct in-depth research on lipid rafts. Stimulated emission depletion (STED) (Hell and Wichmann 1994; Klar et al. 2001), photoactivated localization microscopy (PALM) (Sengupta et al. 2011; Hess et al. 2006; Rust et al. 2006), stochastic optical reconstruction microscopy (STORM) (Rust et al. 2006), and near-field scanning optical (NSOM) (Sezgin 2017) are some of the super-resolution microscopy methodologies that are utilized in order to study PM biology and composition (you may refer to Sezgin 2017, for a more detailed review on the nuances between super-resolution microscopy techniques) (Sezgin 2017). For example, STED is used to characterize the tetraspanin-enriched membrane rafts (TEMs), a high-order organization of membranous tetraspanins (Zuidscherwoude et al. 2015). Before, all proteins associated with TEMs were believed to localize to the same raft compartment. Nonetheless, this study established the existence of several different types of TEMs (Zuidscherwoude et al. 2015). Similarly, using the PALM technique, it was shown that raft-resident T-cell receptor and LAT protein locate in different raft compartments in T lymphocytes (Lillemeier et al. 2010; Rossy et al. 2013; Sherman et al. 2011). Furthermore, the STORM and PALM techniques revealed the segmented arrangement of B lymphocyte and mast cell receptors in the PM (Dustin et al. 2017; Shelby et al. 2013). Additionally, NSOM reveals raft compositions in artificial membranes (Burgos et al. 2003; Coban et al. 2007; Hollars and Dunn 1997; Hwang et al. 1998). All in all, the development of these highly sensitive microscopy tools sheds light on the previously unknown life of lipid rafts and promises more exciting times for cellular biologists.

So, what exactly are "lipid rafts"? According to the Keystone Symposia on lipid rafts in 2006, lipid rafts are PM microdomains enriched in cholesterol, sphingolipids, and glycosylated lipids and are no larger than 200 nm. Furthermore, lipid rafts can recruit smaller rafts to construct larger platform-like PM domains, which constitute complex signaling hubs within PM (Pralle et al. 2000). Lipid rafts are separated into planar lipid rafts and flask-shaped non-planar lipid rafts (caveolae) (Ludwig et al. 2016; Simons and Ikonen 1997; Yamada 1955). Planar lipid rafts exist in all cell types and are marked by the presence of Flotillin protein (Babuke and Tikkanen 2007; Staubach and Hanisch 2011; Stuermer 2010, 2011). Caveolae is present only in specific tissues like endothelium and marked by Caveolin and Cavin proteins (Hill et al. 2008; Kurzchalia et al. 1992; Rothberg et al. 1992; Way and Parton 1995). Although they differ in morphology and chemical content, both lipid rafts have been shown to significantly modulate cellular signaling. In this review, the term "lipid raft" will be used for planar lipid rafts. In the following section, the role of lipid rafts in cellular signaling will be explained in different physiological contexts. In the last section of this review, caveolae and the role of those tiny pits in cellular signaling will be reviewed.

3 Lipid Rafts in Normal Physiology

The compartmentalization of the members of the cellular signaling machinery is crucial for receiving a specific signal and generating a specific response—the goal of signal transduction. Lipid rafts are one of the tools that are utilized by cells to spatially regulate the signal. Membrane proteins are targeted to PM through their membrane-spanning and hydrophobic transmembrane domains, glycosylphosphatidylinositol (GPI)-anchors, and the covalent attachment of lipid moieties (Levental et al. 2010a; Resh 2013). Among them, GPI anchors and a subset of lipid modifications are associated with targeting proteins further in lipid rafts (Levental et al. 2010a). For example, S-palmitoylation of proteins is a well-characterized lipid modification to target proteins into rafts. The long-saturated-fatty acyl chain of palmitate (with 16 carbon atoms) is hydrophobic enough and, therefore, can integrate into lipid rafts. Also, palmitoylation is a reversible PTM, which gives targeted proteins flexibility to move in or out of the lipid rafts

(Levental et al. 2010b). Besides palmitoylation, dual acylation of proteins (consecutively adding two lipid acyl moieties) is simultaneously associated with raft-targeting, such as myristate and isoprenoid groups. However, none of those modifications alone is sufficient for lipid raft compartmentalization (Levental et al. 2010a; Resh 2013). The myristate acyl lacks the required hydrophobicity, and isoprenoid groups have unsaturated acyl chains. Another strategy used to target proteins in the rafts is the addition of cholesterol (Porter et al. 1996). Upon secretion into the extracellular space, Hedgehog proteins are directed to PM of adjacent cells in this manner (Gallet et al. 2006; Porter et al. 1996).

Once inside the lipid rafts, receptors interact with their activators or inhibitors (Simons and Toomre 2000). Hence, the molecular processes taking place in the raft or non-raft portions of the plasma membrane are of paramount significance. One other way lipid rafts might affect signaling is that the folding and therefore the functions of PM resident proteins can be altered by specific lipid-protein interactions (Laganowsky et al. 2014; Lingwood et al. 2011). Although these are two general modes of regulation in lipid rafts, they have been specifically implicated in many signaling cascades involving proliferation, apoptosis, immune responses, and development (Fig. 1).

4 Proliferation

Cellular proliferation is primarily sustained by growth factor signaling through the receptor tyrosine kinases. The activities of many receptor tyrosine kinases (RTKs) are regulated through lipid rafts (Pike 2005; Simons and Toomre 2000). Epidermal growth factor (EGF) and insulin-like growth factor (IGF) are two examples of receptor tyrosine kinases associated with lipid rafts (Simons and Toomre 2000). These receptors activate effectors such as Ras-MAPK and PI3K/Akt to sustain the proliferative state (Pollak 2008; Wee and Wang 2017). EGFR is the most studied receptor tyrosine kinase in terms of its mode of activation. For the activation of downstream signaling components, EGFR molecules must be oligomerized and autophosphorylated. Both of these activities are demonstrated to be controlled by lipid rafts (Coskun et al. 2011; Kovacs et al. 2022). Many studies have shown that disruption of lipid rafts by a cholesterol-depleting reagent—MβCD (Methyl-β-cyclodextrin)—causes aberrant activation of EGFR in the presence or absence of EGF (Jans et al. 2004; Pike et al. 2005; Roepstorff et al. 2002; Waugh et al. 1999). Therefore, to be activated appropriately, EGFR first needs to be present in raft regions; then, it needs to move to the non-raft plasma membrane after being fully activated (Kovacs et al. 2022; Lambert et al. 2006; Roepstorff et al. 2002; Turk et al. 2012; Waugh et al. 1999). Several raft-dependent regulatory mechanisms are described for IGF signaling as well.

IGF signaling is initiated by insulin or IGF binding to the insulin receptor or IGFR, which results in receptor phosphorylation and activation. In 3T3-L1 adipocytes, IGFR1 is demonstrated to localize in lipid rafts (Hong et al. 2004; Huo et al. 2003). However, unlike EGFR, cholesterol depletion causes reduced IGF signal, and IGFR seems to be active only in raft regions (Huo et al. 2003). After IGFR activation, the primary effector phosphatidylinositol-3 kinase (PI3K) gets recruited to phosphorylated IGFR (therefore to PM). This recruitment occurs through the SH2 domain of the negative regulatory subunit of the PI3K-p85. On the PM, PI3K is activated after binding to IGFR (Alessi et al. 1997). Activated PI3K phosphorylates the membrane lipid PIP_2 and generates a lipidic secondary messenger PIP_3. Transiently produced PIP_3 is recognized by the PH domain, which is present within many proteins/kinases involved in cellular proliferation (Ebner et al. 2017). AKT is one of these proteins recruited to PM by its PH domain (Brazil and Hemmings 2001; Ebner et al. 2017). Upon recruitment to the PM, AKT is activated by two sequential phosphorylation events, initiates many pathways of ultimate proliferation, and inhibits apoptosis (Manning and Cantley 2007; Manning and Toker 2017). It is interesting to note that the rafts contribute to the AKT activation. Through microscopy-based methods, active AKT was demonstrated in lipid

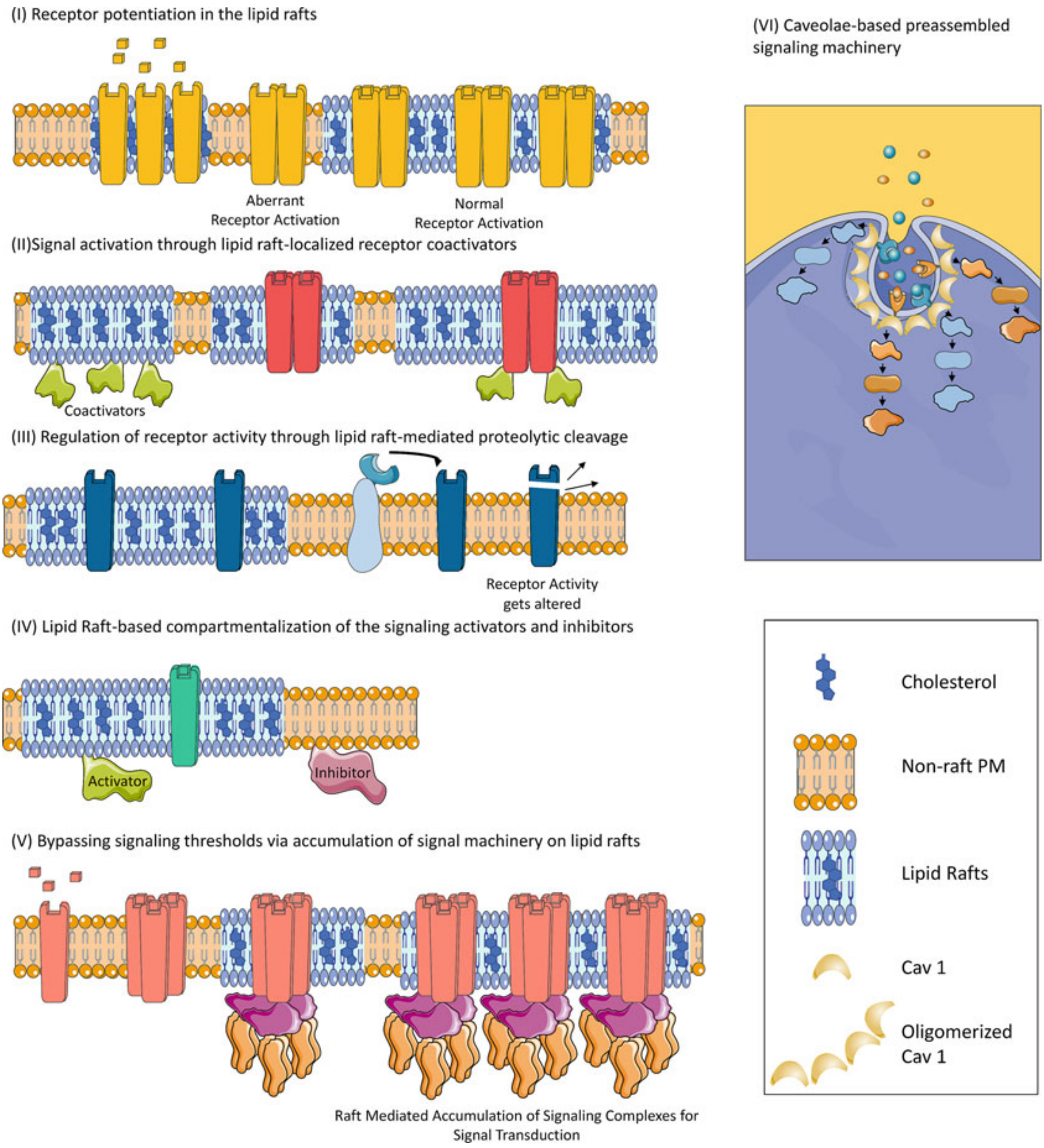

Fig. 1 Modes of signaling regulation related to lipid rafts. Receptors are potentiated within the lipid rafts. The immature association of receptors with non-raft PM causes aberrant overactivation, as in the case of EGFR (I). Both receptors and coactivators are localized to different species of lipid rafts and get associated within the same lipid raft as in the case of TCR and FcεRI signaling (II). Receptors move to non-raft PM and are proteolytically cleaved, leading EMT as in the case of CD44 signaling (III). Lipid raft-mediated differential localization of activators and inhibitors compartmentalizes signal transduction as in the case of PI3K signaling (IV). Accumulation of signaling complexes to initiate an intracellular response, as in the case of CD95/FasL signaling(V). Caveolar signal regulation occurs through caveolar coat protein Cav 1, which binds to many signaling molecules and establishes molecular machinery (VI)

rafts, and complementary studies revealed that cholesterol depletion ablates AKT activity (Gao et al. 2011; Lasserre et al. 2008). PI3K activity is reversed by phosphatase and tensin homolog (PTEN), which dephosphorylates PIP_3 lipids into PIP_2 (Song et al. 2012; Stambolic et al.

1998). Interestingly, PTEN, the negative regulator of AKT activation, has been shown to localize in non-raft membranes, whereas PDK1, the activator of AKT, has been shown to localize in raft regions. Of note, targeting PTEN to lipid rafts through molecular methods leads to PDK1 inhibition and abrogates AKT activation (Gao et al. 2011). PI3Ks have four isoforms, two of which are ubiquitously expressed (Fruman et al. 2017). However, these two isoforms—namely PI3Kα and PI3Kβ—have differential activities and regulations in cell signaling (Jia et al. 2008a, 2008b; Vanhaesebroeck et al. 2010; Zhao et al. 2006). For example, PI3Kα is the main effector and gets activated by RTK signaling (Jia et al. 2008a, 2008b; Zhao et al. 2006). On the other hand, PI3Kβ was found to signal through GPCRs (Jia et al. 2008a, 2008b). This was found to be mediated via lipid rafts. PI3Kα localizes in non-raft areas, whereas PI3Kβ interacts with Rac1 to localize to lipid rafts. This raft localization of PI3Kβ is crucial for GPCR coupling and appears to be important in a PTEN-null genetic background (Cizmecioglu et al. 2016) (Fig. 2).

The uptake of nutrients is also associated with the PI3K axis and lipid rafts. For example, in 3T3 adipocytes, when the IGF pathway is inactive, extracellular glucose transporter GluT4 is constitutively internalized. However, once activated, IGF signaling inhibits the internalization of GluT4. Uninternalized GluT4 gets colocalized with raft-resident protein flotillin or caveolae-resident protein caveolin3 and mediates glucose uptake (Cav-3) (Fecchi et al. 2006; Ribon et al. 2001). Also, long-chain fatty acid uptake can be regulated through lipid rafts. Fatty acid translocase (FAT)/CD36 was demonstrated to be extracted from the DRM fraction of PM but not from the DSM fraction (Pohl et al. 2004, 2005).

Other than receptor tyrosine kinases, some G-protein coupled receptors (GPCRs) were involved in raft-associated signaling (Ostrom and Insel 2004). Beta-adrenergic, oxytocin, serotonin, and dopamine receptors are among the GPCRs that regulate normal physiological states in the muscular and nervous systems (Björk et al. 2010; Chini and Parenti 2004; Cole and Sood 2012; Ostrom and Insel 2004). Also, several ligand-gated ion channels such as ionotropic receptors are associated with lipid rafts of neurons and muscle cells. Several glutamate receptors and GABA receptors are among them, which were demonstrated to localize to lipid rafts to relay proper excitatory signals in their target tissue

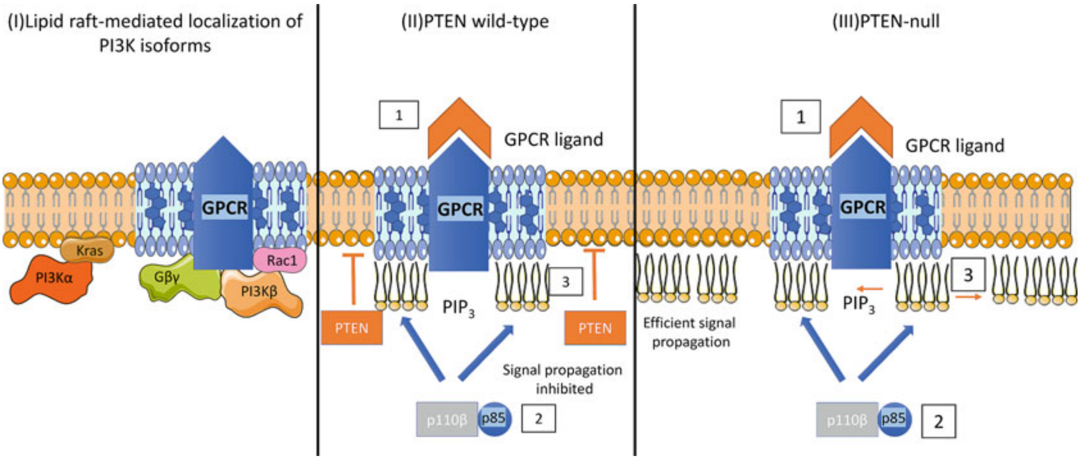

Fig. 2 Regulation of isoform-specific PI3K activity through lipid raft-based compartmentalization. Under resting conditions, PI3Kα localizes to non-raft PM, while PI3Kβ localizes to raft PM together with Gβγ and Rac1, promoting GPCR signaling (I). In PTEN wt cellular conditions, non-raft PM localized PTEN blocks propagation of PIP_3 production and signal (II). The blockage of PIP_3 production in non-raft PM is compromised in PTEN-null cells, and raft-associated GPCR/PI3Kβ signaling gets amplified (III)

(Chini and Parenti 2004; Dalskov et al. 2005; Xu et al. 2014). Aside from GPCRs, membrane compartmentalization has been shown to modulate their related heterotrimeric G proteins (Moffett et al. 2000). Gβγ subunit of heterotrimeric G associates mostly with non-raft PM, while Gα associates with lipid rafts or caveolae. Interestingly, Gα subtypes—$G\alpha_q$, $G\alpha_i$, $G\alpha_s$—also localize differentially to PM compartments. $G\alpha_i$ and $G\alpha_s$ localize to lipid rafts, while $G\alpha_q$ localizes to caveolae through its interactions with Caveolin 1 (a caveolae-resident protein) (Oh and Schnitzer 2001). Gq, on the other hand, has been shown to localize to planar lipid rafts via its interaction with the raft marker flotillin, which stimulates proliferation via the p38/MAPK axis (Sugawara et al. 2007).

5 Apoptosis

Apoptosis, or "programmed cell death," is a physiological process by which cells die during development to give rise to normal morphology or during the cell cycle when cells cannot pass the cell cycle checkpoint due to an acquired mutation. During type I apoptosis, the raft-resident CD95 (Fas) receptor needs to be bound by FasL. As a TNF family protein, CD95 possesses a cytosolic Death Domain (DD), yet the DD of CD95 functions to accumulate CD95 through protein interactions rather than induce apoptosis by itself (Ferrao and Wu 2012; Mollinedo and Gajate 2006). Liganded CD95 needs to accumulate in PM. In time, if apoptotic signals increase, accumulation of liganded CD95 reaches a threshold and recruits Fas-Associated Death Domain (FADD) (Kischkel et al. 1995; Tibbetts et al. 2003) and consecutively Caspase −8/−10 through FADD. With the recruitment of caspases, Fas/CD95, FADD, and Caspase −8/−10 form the death-inducing signaling complex (DISC) (Kischkel et al. 1995). Accumulated procaspase-8 self-cleavage occurs and initiates apoptotic signaling (Muzio et al. 1998). Following ligand interaction, CD95 travels to lipid rafts where its accumulation takes place, leading to the successful production of an apoptotic signal (Hueber et al. 2002). When cells are treated with cholesterol-depleting MβCD, it was demonstrated that apoptosis is inhibited because of the prevention of lipid raft formation, accumulation of CD95, and formation of DISC (Gajate et al. 2009; Gajate and Mollinedo 2015; Hueber et al. 2002; Wajant 2006).

6 Immune Signaling

Lipid rafts are involved in many immunogenic processes. Firstly, one of the members of the innate immune system—Toll-like receptor (TLR4), is shown to be activated by raft-resident protein-CD14 (Wright et al. 1990). CD14 is known for its role in the pathogen recognition process. For this purpose, it forms a ternary complex with bacterial lipopolysaccharide (LPS) and the host LPS binding protein to initiate the immune response (Płóciennikowska et al. 2015; Triantafilou et al. 2011; Wright et al. 1990). It is also worth mentioning that several TLRs have cholesterol-binding motifs, and cholesterol is found to regulate the responses of those TLRs (Wong et al. 2009). Aside from innate immunity, IgE signaling in mast cells is the first known lipid raft-dependent signaling. IgE ligand binds to its receptor FcεRI (Field et al. 1995). Once bound and activated, they go to DRMs to initiate the appropriate signaling (Field et al. 1995). Lymphocyte maturation appears to be through lipid rafts as well. In resting state, BCR and TCR are localized into DSM but once activated; they transfer into DRMs (Beck-García et al. 2015; Dustin et al. 2017; Sproul et al. 2000). Upon their transfer to DRMs, these receptors can colocalize with raft-resident downstream signaling elements of immune signaling such as LAT, LCK, and FYN kinases and start the immune response (Filipp et al. 2004; Levental et al. 2010b).

7 Lipid Rafts in Development and Stem Cell Biology

Embryonic development is one of the fascinating biological processes in which signaling events are regulated along the spatiotemporal dimension.

Although much is known about the signaling components involved in developmental processes, the control of these signals via lipid rafts is relatively unexplored. However, in a few studies, the presence of raft-enriched ganglioside-GM1 was demonstrated in both preimplantation mouse embryos and mouse oocytes (Comiskey and Warner 2007). Depleting cholesterol in these cells hampered raft formation (Comiskey and Warner 2007; Buschiazzo et al. 2013). Also suppressed is raft-resident c-Src, which is involved in the formation of the second polar body (Buschiazzo et al. 2013). Lipid rafts are also implicated in maintaining the identity and self-renewal of some stem cells. The existence of lipid rafts in mesenchymal stem cells and even embryonic stem cells is extensively recognized, although their regulatory roles remain largely unknown (Lee et al. 2010; Sohn et al. 2018). However, the regulation of hematopoietic stem cells (HSCs) by rafts is better characterized. It was shown that raft-resident integrin, CXCR, and CD117 receptors plays essential roles in the HSCs recognition of their stem cell niche (Alomari et al. 2019; Ratajczak and Adamiak 2015; Wysoczynski et al. 2005).

8 Lipid Rafts in Cancer and Metastasis

Cancer is uncontrollable, aberrant cellular proliferation. During carcinogenesis, mutated unhealthy cells are unable to be eliminated by apoptosis. Given the importance of lipid rafts in mitogenic signaling via RTKs and GPCRs, it is not surprising that lipid raft composition is greatly deregulated in tumor cells (Michel and Bakovic 2007; Staubach and Hanisch 2011). Of note, lipid rafts are highly abundant in tumor cells, and lipid rafts existent in tumor cells are highly enriched in cholesterol compared to their untransformed counterparts (Levin-Gromiko et al. 2014; Li et al. 2006; Owen et al. 2012). Furthermore, it has been established that raft integrity is crucial for proliferative signaling in cancer cells. For example, EGFR recruitment to the rafts was shown to be crucial for proliferation in prostate cancer (Zhuang et al. 2002; Zidovetzki and Levitan 2007), and complementarily, the disruption of lipid rafts by statins abolishes the EGF signal, leading to apoptosis (Hea et al. 2007; Hryniewicz-Jankowska et al. 2019; Zhuang et al. 2005). IGF signaling and its downstream PI3K/AKT pathway were affected by lipid raft-mediated regulation. As previously mentioned, AKT activity is controlled through lipid raft-mediated compartmentalization of its activator (PDK1) and negative regulator (PTEN) (Gao et al. 2011). However, this balance is lost in some cancers. For example, in prostate cancer LNCAP cells, raft-resident AKT and non-raft-resident AKT demonstrate different affinities to their substrates (Adam et al. 2007). These AKT pools highlight the presence of two distinct platforms for AKT signaling in the same cell population (Adam et al. 2007). Another example where alterations in raft-mediated regulation of AKT drive tumorigenesis is mantle cell lymphoma (MCL) (Reis-Sobreiro et al. 2013). In MCL cells, AKT is found to be constitutively active (Reis-Sobreiro et al. 2013; Rudelius et al. 2006), and raft-resident PDK1 and mTOR signaling components favor constant AKT activation (Reis-Sobreiro et al. 2013). Conversely, lipid raft disruption by alkyl phospholipid edelfosine displaces all AKT, PDK1, and mTOR from lipid rafts, leading to AKT inactivation and apoptosis (Reis-Sobreiro et al. 2013). Similarly, cholesterol extraction through MβCD prevents AKT activation in many transformed cell lines and promotes apoptosis (Calay et al. 2010; Motoyama et al. 2009).

Besides being an essential constituent of PM and lipid rafts, cholesterol is the precursor of steroid hormones such as estrogen and androgen (Chimento et al. 2019). Both estrogen and androgen-mediated signaling is associated with the development of gender-specific cancers like breast cancer due to aberrant estrogen signaling and prostate cancer due to aberrant androgen signaling in males (Pelton et al. 2012; Yager et al. 2006). Amplification of estrogen receptor α (ER α) occurs in 70% of all breast cancers (Lumachi et al. 2013). Furthermore, it was demonstrated that cholesterol is a direct agonist of estrogen receptor (Casaburi et al. 2018; Wei et al. 2016). Also, a similar relationship was observed between prostate cancer and cholesterol in prostate cancer mouse models. When fed high cholesterol diet, these mice develop more aggressive tumors compared to mice fed a regular diet (Llaverias et al. 2010). Canonically, steroid receptors belong to the nuclear receptor family and reside either in the nucleus or cytosol (Pietras and Márquez-Garbán 2007). However, in HeLa cells, a pool of Erα receptors was demonstrated to localize PM. Targeting these receptors to PM occurs through the palmitoylation modification carried out by palmityl-transferase (Acconcia et al. 2005; la Rosa et al. 2012). This pool of ERα receptors was shown to reside in lipid rafts and activate MAPK/ERK and PI3K/AKT axes (Márquez et al. 2006; Maselli et al. 2015). It is interesting to note that androgen receptors have also been shown to cluster close to lipid rafts where AKT signaling activity has been detected (Freeman et al. 2007; Pelton et al. 2012).

In addition, lipid rafts are shown to be essential for the metastatic behavior of tumor cells. CD44 is a transmembrane receptor that is involved in cell adhesion. In the clinic, it is used as a metastatic marker for some cancer types (Senbanjo and Chellaiah 2017). Through a mechanism that has been incompletely elucidated, it is believed to regulate the extracellular matrix and induce epithelial-to-mesenchymal transformation (Murai 2015). Proteolytic cleavage of CD44 is necessary for its activity in ECM (Sugahara et al. 2003). CD44 is a raft-resident protein typically, while its protease- ADAM10 resides in non-raft regions (Murai et al. 2011). However, in metastatic tissues, CD44 has been reported to migrate into non-raft areas where it is shed by ADAM10, causing migratory behavior and metastasis (Babina et al. 2014; Murai 2015).

The association of lipid rafts/cholesterol with various cancers is an active area of research. The use of drugs that inhibit cholesterol biosynthesis, like statins (Longo et al. 2020), or the use of raft-dispersing alkyl phospholipids-like edelfosine (Gajate and Mollinedo 2007; Saraiva et al. 2021; Selivanov et al. 2010; Udayakumar et al. 2016) in cancer treatment is currently being explored and discussed among cancer researchers and clinicians.

9 Caveolae

Caveolae is another compartmentalized plasma membrane microdomain described as cholesterol/sphingolipid-rich lipid rafts with cave-like invaginated morphology (Yamada 1955). The presence of caveolin proteins first molecularly characterized caveolae (Kurzchalia et al. 1992; Rothberg et al. 1992; Way and Parton 1995; Zurzolo et al. 1994). More recently, cavin proteins were discovered to be another significant constituent (Hill et al. 2008). For signal transduction, these specialized cellular morphologies present extensive importance. Several potent receptors were shown to be restricted and concentrated in caveolae, making caveolae a vital signaling hub for the cell (García-Cardeñ et al. 1996; Li et al. 1996a; Liu et al. 1996, 1997; Oh and Schnitzer 2001; Rizzo et al. 1998).

The morphological description of caveolae was first made in the gall bladder of mouse epithelium by Yamada and dates to 1955 (Yamada 1955). Later, caveolae were shown to be lipid-rich plasma membrane microdomains similar to lipid rafts, but consequent molecular characterization experiments showed that both have distinct molecular components (Liu et al. 1997; Oh and Schnitzer 2001; Zurzolo et al. 1994). The formation of functional caveolae requires the enrichment and oligomerization of a cholesterol-binding protein named Caveolin 1 (Cav1) (Fra et al. 1995; Kurzchalia et al. 1992; Monier et al. 1995; Murata et al. 1995; Rothberg et al. 1992). Cav1 was the first caveolin protein described, followed by the discovery of other homologs, Caveolin 2 (Cav2) and muscle-specific Caveolin 3 (Cav3) (Scherer et al. 1996; Way and Parton 1995). Much later than the discovery of Cav1, another essential caveolar protein-Cavin 1, was discovered (Hill et al. 2008; Liu et al. 2008). Further, Cavin 1 homologs-Cavin 2, Cavin 3, and Cavin 4 were discovered and found to have roles in caveolar function in mammals (Bastiani et al. 2009; Hansen et al. 2009; Kovtun et al. 2014; McMahon et al. 2009). Among all caveolin and cavin proteins, Cav1 and Cavin 1 were shown to be essential for the formation of a functional caveolae (Hill et al. 2008; Liu et al. 2008). Caveolae formation starts with binding caveolin proteins to cholesterol in a 1:1 ratio and budding of caveolin-rich vesicles from Golgi network membrane domains (Hayer et al. 2010; Murata et al. 1995). After caveolin accumulation around the cholesterol-rich lipid species, caveolin proteins self-assemble themselves into homo- and hetero-oligomers (Monier et al. 1995). Oligomerized caveolins recruit important lipid species such as phosphatidylserine (PS), phosphatidylinositol phosphate PIP_2, sphingomyelin, and gangliosides (Sohn et al. 2018). Caveolin-rich lipid rafts are further tightened and fixated by the recruitment and the binding of Cavin proteins. By following those steps, caveolae reach their well-known striated disc-like appearance under the electron microscope (Monier et al. 1995; Parton et al. 2021; Rothberg et al. 1992).

10 Compartmentalized Signaling Through Caveolae

Caveolae is a critical regulatory plasma membrane compartment for signal transduction. The regulation of cellular signaling gets affected by caveolae at two levels. First, preassembled signaling complexes are compartmentalized and enriched in the caveolar membrane. Some of the most studied signaling cascades get initiated from this membrane microdomain, and their receptors are restricted at the caveolar membrane (Martinez-Outschoorn et al. 2015; Okamoto et al. 1998). Insulin, EGF, PDGF, GPCR, and eNOS are some of the well-studied cellular signals that are relayed into the cells through caveolae (Couet et al. 1997b; Liu et al. 1996, 1997; Nystrom et al. 2013; Oh and Schnitzer 2001). Second, the activity of these signaling complexes is regulated by caveolae or, more specifically, caveolar protein Cav1. Over the years of research, it was shown that the receptors of the enlisted signaling pathways above, as well as H-Ras, K-Ras, Src-tyrosine kinase, and heme oxygenase, precipitate together with Cav1 in co-immunoprecipitation experiments (Li et al. 1996b; Song et al. 1996; Taira et al. 2011). Further molecular characterization of the interaction of Cav1 with the members of these signaling pathways revealed the Caveolin scaffolding domain (CSD), which is mapped to 82–101 residues of Cav1 and regulates all the Cav1-associated signaling. CSD was shown to have a negative regulatory role on Cav1-associated signaling. For this inhibitory regulation to occur, CSD binds to the caveolin binding motif (CBM), which is present in many Cav1-associated signaling components (Bernatchez et al. 2005; Kirkham et al. 2008; Nystrom et al. 2013; Song et al. 1996; Taira et al. 2011). Although Cav1 interaction was shown to inhibit the basal activation signaling elements such as Src and K-ras (Couet et al. 1997a; Li et al. 1996b), the inhibitory activity of CSD is best characterized in eNOS signaling. First, eNOS hydrophobic pocket, therefore catalytic activity, was shown to be blocked and inhibited by the side-chain extension of phenylalanine 92 (F92) of CSD (Bernatchez et al. 2005; Trane et al. 2014). Moreover, the presence of a peptide identical to CSD was shown to inhibit the eNOS signal. The mutated version of the CSD peptide, which lacks all the aromatic residues (including F92), was found to activate the eNOS signal due to a probable competition between the mutated peptide and Cav1 (Bernatchez et al. 2011). Although CSD is largely acknowledged as the caveolae's signal regulatory component, there is still some dispute in the caveolae field. The main cause of this contention is CSD itself, which may be buried inside the plasma membrane (Kirkham et al. 2008). Recent studies indicated that Cav1 has a more dynamic structure than initially contemplated and that CSD can exist in multiple states, which might support signaling switches in different contexts (Liu et al. 2016; Sinha et al. 2011).

Caveolar proteins other than Cav1 were also found to affect several cellular signals. For example, Cav2 fatty acylation and phosphorylation have a role in regulating insulin signaling pathway. These two post-translational modifications inhibit the insulin receptor-SOCS3 phosphatase interaction, leading to IRS-1 activation and activated Stat3 translocation to the nucleus (Kwon et al. 2009, 2015; Kwon and Pak 2010). Other studies indicated that Cav3 might function in carrying the membranal signal to the nucleus. For example, C-terminal 154–156 residues of Cav3 were shown to regulate insulin-induced phospho-ERK translocation to the nucleus (Kwon et al. 2011). Interestingly, Cav3 was also found to be crucial for activating the estrogen receptor by the hormone 17β-estradiol (Totta et al. 2016). Cavins as well were found to affect cellular signaling. Firstly, the essential Cavin component of caveolae-Cavin 1 determines the fully functional caveolae pool and, as a result, dictates the location of activated receptors signaling through caveolae (Li et al. 2014; Moon et al. 2014a). Also, Cavin-3 was found to be necessary for ERK activation over AKT activation by keeping caveolae anchored to the cortical cytoskeletal elements through myosin-1c (Hernandez et al. 2013).

Caveolae can also affect cellular signaling by regulating the actin cytoskeleton. As a result, it plays a role in lipid sorting and delivery (Echarri and del Pozo 2015). Consequently, caveolae can function in the organization of nanoscale membrane domains, which ultimately control the activation of signaling receptors (Blouin et al. 2016). Mechanical forces can as well affect caveolae dynamics, contributing to the organization of the plasma membrane by the caveolae (Nassoy and Lamaze 2012; Sinha et al. 2011). As an example, c-Src gets activated when caveolar disassembly occurs due to the distribution of Cav1 and membrane lipids like sphingolipids due to membranal stretch (Gervásio et al. 2011). More interestingly, the absence of Cav1 leads to the disorganization of Ras protein, lipid, and phosphatidylserine distribution (Ariotti et al. 2014). Finally, caveolar disassembly was shown to affect changes in Gaq-Cav1 interaction and result in reduced Ca^{+2} signaling, concomitant with perturbations in the localization of calcium pumps (Fujimoto 1993; Gervásio et al. 2011; Guo et al. 2015).

11 Caveolae in Cancer

Caveolar composition and signaling have long been related to cancer. First, it was observed that Cav1 levels decreased and caveolae got lost during the cellular transformation of NIH 3T3 cells after the expression of oncogenes (Koleske et al. 1995). Cav1 expression levels are inversely correlated with the colony size of the transformed cells (Koleske et al. 1995). Also, Cav1 knock-out mice exhibit hyperproliferation in the lung and vascular tissues (Drab et al. 2001; Razani et al. 2001). Next, a role for Cav1 in breast cancer is described. Cav1 deletion in MMTV-PyMT model of breast cancer in mice leads to a delayed initiation of tumor growth, an increase in tumor burden, ERK1 hyperactivation, and cyclinD1 overexpression (Williams et al. 2004). However, cancer progression and survival studies revealed that the main predictor of survival is the Cav1 level in stromal cells rather than epithelia (Sloan et al. 2009; Witkiewicz et al. 2009). The lower the expression of Cav1 in stromal cells, the faster the cancer progresses (Sloan et al. 2009; Witkiewicz et al. 2009). Lisanti and colleagues isolated breast stromal cells (cancer-associating fibroblasts (CAFs)) from patients and compared their metabolism to normal fibroblasts of the matched patient (Martinez-Outschoorn et al. 2015; Pavlides et al. 2009). CAFs represent significant metabolic/expressional differences compared to normal fibroblasts. Then, they devise a physiological mechanism named the “Reverse Warburg Effect” in which transformed cells induce a differentiation program in stromal cells (Martinez-Outschoorn et al. 2015; Pavlides et al. 2009) Therefore, the idea is that cancer cells induce tumor stromal cells to undergo a myofibroblast differentiation. Differentiated tumor stroma activates TGFβ signaling and goes through a metabolic transformation yielding a Warburg metabolism in stromal cells, although present in normoxic conditions. Metabolically altered stroma then sustains the tumor growth and progression by representing metabolites like lactate or pyruvate (Pavlides et al. 2009). Besides Cav1, caveolar protein cavin1 is considered a prognostic marker for prostate cancer (Moon et al. 2014b), while caveolar CD36 protein level is associated with breast cancer (DeFilippis et al. 2012).

12 Concluding Remarks

After its initial postulation in 1997 (Simons and Ikonen 1997), lipid rafts became a generally accepted concept in recent years. Advances in microscopy techniques revealed that PM is not only "not homogenous" but much more heterogenous than we thought before. There are probably different subtypes of lipid rafts. For example, a two-color PALM study has shown that TCR, Zap70, and LAT proteins are destined to different membrane microdomains upon activating TCR signaling (Sherman et al. 2011). The concept of distinct lipid raft types brings some provoking questions as "How can we differentially mark these raft species molecularly?", "How proteomics of distinct raft types differ?", "How can specific proteins are targeted to different raft species?", "Are these different subcompartments preassembled or co-assembled?" Another outstanding question is, "What would we encounter if we increase lipid raft resolution into atomic levels?". In other words, "Can we crystalize these solid-like microdomains and determine their structure by X-ray crystallography?". The crystallization of lipid rafts has been speculated earlier (de Almeida and Joly 2014). Moreover, there were few attempts to crystalize lipid rafts in model membranes, but the data acquired in these studies were restricted (de Almeida and Joly 2014; Park et al. 2020; Ziblat et al. 2012). However, recently developed co-Mesh X-ray crystallography could be a promising method to unravel the structures of lipid rafts. With this technique, serial X-ray images of multiple crystals are acquired and assembled so that they can present information on the dynamic nature of the molecules within the crystals (Sierra et al. 2015). As a result, this approach might be useful for determining the structure of lipid rafts.

Now, it is clear that planar lipid rafts and caveolae are both cholesterol-sphingolipid-rich membrane domains, one favoring the presence of flotillin and the other caveolin. However, we are unaware of how they are specifically localized to the PM, meaning if planar/non-planar rafts favor particular dimensional positioning within the cell. In those terms, we need more precise knowledge of the interactions between rafts and cytoskeleton. Another interesting and not thoroughly answered question is, "Within the same organism, why do some cells acquire caveolae and why do others not?" It is now known that the presence of both Cav 1 and Cavin 1 is a prerequisite for caveolae formation (Parton et al. 2021). However, we are still far from understanding how both proteins integrate at the membrane and enable the typical membrane curvature of caveolae.

In some disease conditions, it is now well characterized that lipid raft-mediated signaling gets perturbed. Mainly in many cancer types, it was observed that raft-mediated signaling is deregulated. It can be reflected by the increased cholesterol levels within the whole cells and cholesterol within the rafts of cancers compared to their untransformed counterparts. It is yet unclear what kind of dysregulations take place in the rafts of those cancer cells. For example, we know that when cells are depleted from cholesterol by MβCD, EGFR moves into non-raft regions, and tumorigenic signaling is activated. Nevertheless, quite the opposite effect is observed for other RTKs. For example, raft blockage results in the inhibition of proliferative signaling of IGFR. So, "What specific raft-mediated mechanisms distinguish these akin signaling molecules?"

Another topic open to discussion is the disruption of lipid rafts as a therapy against cancer. First, the use of raft inhibiting—alkyl phospholipid-like edelfosine—is being investigated as an anticancer drug (Mollinedo 2014). The anticancer effects of edelfosine are demonstrated in many studies in the cell culture or xenografted animals of several types of tumors (Mollinedo 2014). Also, another edelfosine analog, perifosine, is under investigation for its anticancer effects on hematological cancers and solid tumors (Richardson et al. 2012). A more dramatic approach is using cholesterol synthesis inhibitors-statins for cancer treatment. Like perifosine, statins are also investigated in numerous clinical trials (di Bello et al. 2020). Given the critical functions of lipid rafts and cholesterol, the value of inhibiting them will become evident in the coming decade.

References

Acconcia F, Ascenzi P, Bocedi A, Spisni E, Tomasi V, Trentalance A, Visca P, Marino M (2005) Palmitoylation-dependent Estrogen Receptor Membrane Localization: Regulation by 17-Estradiol. Mol Biol Cell 16:231–237. https://doi.org/10.1091/mbc.E04

Adam RM, Mukhopadhyay NK, Kim J, di Vizio D, Cinar B, Boucher K, Solomon KR, Freeman MR (2007) Cholesterol sensitivity of endogenous and myristoylated Akt. Cancer Res 67(13):6238–6246. https://doi.org/10.1158/0008-5472.CAN-07-0288

Ahmed SN, Brown DA, London E (1997) On the origin of sphingolipid/cholesterol-rich detergent-insoluble cell membranes: physiological concentrations of cholesterol and sphingolipid induce formation of a detergent-insoluble, liquid-ordered lipid phase in model membranes. Biochemistry 36:10944–10953

Alessi DR, James SR, Downes CP, Holmes AB, Gaffney PRJ, Reese CB, Cohen P (1997) Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase B. Curr Biol

Alomari M, Almohazey D, Almofty SA, Khan FA, al Hamad, M., & Ababneh, D (2019) Role of lipid rafts in hematopoietic stem cells homing, mobilization, hibernation, and differentiation. Cell 8(6). https://doi.org/10.3390/cells8060630

Ariotti N, Fernández-Rojo MA, Zhou Y, Hill MM, Rodkey TL, Inder KL, Tanner LB, Wenk MR, Hancock JF, Parton RG (2014) Caveolae regulate the nanoscale organization of the plasma membrane to remotely control Ras signaling. J Cell Biol 204(5): 777–792. https://doi.org/10.1083/jcb.201307055

Babina IS, McSherry EA, Donatello S, Hill AD, Hopkins AM (2014) A novel mechanism of regulating breast cancer cell migration via palmitoylation-dependent alterations in the lipid raft affiliation of CD44. Breast Cancer Res. http://breast-cancer-research.com/content/16/1/R19

Babuke T, Tikkanen R (2007) Dissecting the molecular function of reggie/flotillin proteins. Eur J Cell Biol 86(9):525–532). Elsevier GmbH. https://doi.org/10.1016/j.ejcb.2007.03.003

Bagatolli LA, Mouritsen OG (2013) Is the fluid mosaic (and the accompanying raft hypothesis) a suitable model to describe fundamental features of biological membranes? What may be missing? Front Plant Sci 4 (NOV) Frontiers Research Foundation. https://doi.org/10.3389/fpls.2013.00457

Bastiani M, Liu L, Hill MM, Jedrychowski MP, Nixon SJ, Lo HP, Abankwa D, Luetterforst R, Fernandez-Rojo M, Breen MR, Gygi SP, Vinten J, Walser PJ, North KN, Hancock JF, Pilch PF, Parton RG (2009) MURC/Cavin-4 and cavin family members form tissue-specific caveolar complexes. J Cell Biol 185(7):1259–1273. https://doi.org/10.1083/jcb.200903053

Beck-García K, Beck-García E, Bohler S, Zorzin C, Sezgin E, Levental I, Alarcón B, Schamel WWA (2015) Nanoclusters of the resting T cell antigen receptor (TCR) localize to non-raft domains. Biochim Biophys Acta, Mol Cell Res 1853(4):802–809. https://doi.org/10.1016/j.bbamcr.2014.12.017

Bernatchez PN, Bauer PM, Yu J, Prendergast JS, He P, Sessa WC (2005) Dissecting the molecular control of endothelial NO synthase by caveolin-1 using cell-permeable peptides. PNAS 102(3):761–766. www.pnas.orgcgi. https://doi.org/10.1073/pnas.0407224102

Bernatchez P, Sharma A, Bauer PM, Marin E, Sessa WC (2011) A noninhibitory mutant of the caveolin-1 scaffolding domain enhances eNOS-derived NO synthesis and vasodilation in mice. J Clin Investig 121(9): 3747–3755. https://doi.org/10.1172/JCI44778

Björk K, Sjögren B, Svenningsson P (2010) Regulation of serotonin receptor function in the nervous system by lipid rafts and adaptor proteins. Exp Cell Res 316(8): 1351–1356). Academic Press Inc. https://doi.org/10.1016/j.yexcr.2010.02.034

Blouin CM, Hamon Y, Gonnord P, Boularan C, Kagan J, Viaris de Lesegno C, Ruez R, Mailfert S, Bertaux N, Loew D, Wunder C, Johannes L, Vogt G, Contreras FX, Marguet D, Casanova JL, Galès C, He HT, Lamaze C (2016) Glycosylation-dependent IFN-γR partitioning in lipid and actin Nanodomains is critical for JAK activation. Cell 166(4):920–934. https://doi.org/10.1016/j.cell.2016.07.003

Brazil DP, Hemmings BA (2001) Ten years of protein kinase B signalling: a hard Akt to follow. Trends Biochem Sci 26(11) http://tibs.trends.com0968

Brown DA, Rose JK (1992) Sorting of GPI-anchored proteins to glycolipid-enriched membrane subdomains during transport to the apical cell surface. Cell 66:533–544

Burgos P, Yuan C, Viriot ML, Johnston LJ (2003) Two-color near-field fluorescence microscopy studies of microdomains ("rafts") in model membranes. Langmuir 19(19):8002–8009. https://doi.org/10.1021/la034551p

Buschiazzo J, Ialy-Radio C, Auer J, Wolf JP, Serres C, Lefèvre B, Ziyyat A (2013) Cholesterol depletion disorganizes oocyte membrane rafts altering mouse fertilization. PLoS One 8(4). https://doi.org/10.1371/journal.pone.0062919

Calay D, Vind-Kezunovic D, Frankart A, Lambert S, Poumay Y, Gniadecki R (2010) Inhibition of akt signaling by exclusion from lipid rafts in normal and transformed epidermal keratinocytes. J Investig Dermatol 130(4):1136–1145. https://doi.org/10.1038/jid.2009.415

Casaburi I, Chimento A, de Luca A, Nocito M, Sculco S, Avena P, Trotta F, Rago V, Sirianni R, Pezzi V (2018) Cholesterol as an endogenous ERRα agonist: a new perspective to cancer treatment. Front Endocrinol 9 (SEP) Frontiers Media S. https://doi.org/10.3389/fendo.2018.00525

Chimento A, Casaburi I, Avena P, Trotta F, de Luca A, Rago V, Pezzi V, Sirianni R (2019) Cholesterol and its metabolites in tumor growth: therapeutic potential of statins in cancer treatment. Front Endocrinol 10(JAN) Frontiers Media S. https://doi.org/10.3389/fendo.2018.00807

Chini B, Parenti M (2004) G-protein coupled receptors in lipid rafts and caveolae: how, when and why do they go there? J Mol Endocrinol 32:325–338. http://www.endocrinology.org

Cizmecioglu O, Ni J, Xie S, Zhao JJ, Roberts TM (2016) Rac1-mediated membrane raft localization of PI3K/p110β is required for its activation by GPCRs or PTEN loss. elife 5:1–21. https://doi.org/10.7554/elife.17635

Coban O, Burger M, Laliberte M, Ianoul A, Johnston LJ (2007) Ganglioside partitioning and aggregation in phase-separated monolayers characterized by bodipy GM1 monomer/dimer emission. Langmuir 23(12): 6704–6711. https://doi.org/10.1021/la0635348

Cole SW, Sood AK (2012) Molecular pathways: Beta-adrenergic signaling in cancer. Clin Cancer Res 18(5):1201–1206. https://doi.org/10.1158/1078-0432.CCR-11-0641

Comiskey M, Warner CM (2007) Spatio-temporal localization of membrane lipid rafts in mouse oocytes and cleaving preimplantation embryos. Dev Biol 303(2): 727–739. https://doi.org/10.1016/j.ydbio.2006.12.009

Coskun Ü, Grzybek M, Drechsel D, Simons K (2011) Regulation of human EGF receptor by lipids:108. https://doi.org/10.1073/pnas.1105666108/-/DCSupplemental

Couet J, Li S, Okamoto T, Ikezu T, Lisanti MP (1997a) Identification of peptide and protein ligands for the Caveolin-scaffolding domain (implications for the interaction of Caveolin with caveolae-associated proteins). J Biol Chem. http://www-jbc.stanford.edu/jbc/

Couet J, Sargiacomo M, Lisanti MP (1997b) Interaction of a receptor tyrosine kinase, EGF-R, with Caveolins (Caveolin binding negatively regulates tyrosine and serine/threonine kinase activities). J Biol Chem. http://www.jbc.org

Dalskov SM, Immerdal L, Niels-Christiansen LL, Hansen GH, Schousboe A, Danielsen EM (2005) Lipid raft localization of GABAA receptor and Na+, K+-ATPase in discrete microdomain clusters in rat cerebellar granule cells. Neurochem Int 46(6):489–499. https://doi.org/10.1016/j.neuint.2004.11.010

de Almeida RFM, Joly E (2014) Crystallization around solid-like nanosized docks can explain the specificity, diversity, and stability of membrane microdomains. Front Plant Sci 5(MAR). https://doi.org/10.3389/fpls.2014.00072

DeFilippis RA, Chang H, Dumont N, Rabban JT, Chen YY, Fontenay G v, Berman HK, Gauthier ML, Zhao J, Hu D, Marx JJ, Tjoe JA, Ziv E, Febbraio M, Kerlikowske K, Parvin B, Tlsty TD (2012) CD36 repression activates a multicellular stromal program shared by high mammographic density and tumor tissues. Cancer Discov 2(9):826–839. https://doi.org/10.1158/2159-8290.CD-12-0107

di Bello E, Zwergel C, Mai A, Valente S (2020) The innovative potential of statins in cancer: new targets for new therapies. Front Chem 8). Frontiers Media S. https://doi.org/10.3389/fchem.2020.00516

Drab M, Verkade P, Elger M, Kasper M, Lohn M, Lauterbach B, Menne J, Lindschau C, Mende F, Luft FC, Schedl A, Hailer H, Kurzchalia T v (2001) Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. Science 293(5539): 2449–2452. https://doi.org/10.1126/science.1062688

Dupuy AD, Engelman DM (2008) Protein area occupancy at the center of the red blood cell membrane. PNAS. www.pnas.orgcgi. https://doi.org/10.1073/pnas.0712379105

Dustin ML, Stone MB, Shelby SA, Nunez MF, Wisser K, Veatch SL (2017) Protein sorting by lipid phase-like domains supports emergent signaling function in B lymphocyte plasma membranes. https://doi.org/10.7554/eLife.19891.001

Ebner M, Lučić I, Leonard TA, Yudushkin I (2017) PI(3,4,5)P3 engagement restricts Akt activity to cellular membranes. Mol Cell 65(3):416–431.e6. https://doi.org/10.1016/j.molcel.2016.12.028

Echarri A, del Pozo MA (2015) Caveolae - mechanosensitive membrane invaginations linked to actin filaments. J Cell Sci 128(15):2747–2758). Company of Biologists Ltd. https://doi.org/10.1242/jcs.153940

Fecchi K, Volonte D, Hezel MP, Schmeck K, Galbiati F (2006) Spatial and temporal regulation of GLUT4 translocation by flotillin-1 and caveolin-3 in skeletal muscle cells. FASEB J 20(6):705–707. https://doi.org/10.1096/fj.05-4661fje

Feigenson GW, Buboltz JT (2001) Ternary phase diagram of dipalmitoyl-PC/dilauroyl-PC/cholesterol: Nanoscopic domain formation driven by cholesterol. Biophys J 80(6):2775–2788. https://doi.org/10.1016/S0006-3495(01)76245-5

Ferrao R, Wu H (2012) Helical assembly in the death domain (DD) superfamily. Curr Opin Struct Biol 22(2):241–247. https://doi.org/10.1016/j.sbi.2012.02.006

Field KA, Holowka D, Baird B (1995) FceRI-mediated recruitment of p53/56lyn to detergent-resistant membrane domains accompanies cellular signaling (mast cells/high-affinity IgE receptor/caveolae/tyrosine kinases). Immunology 92:9201–9205

Filipp D, Leung BL, Zhang J, Veillette A, Julius M (2004) Enrichment of Lck in lipid rafts regulates Colocalized Fyn activation and the initiation of proximal signals through TCRαβ. J Immunol 172(7):4266–4274. https://doi.org/10.4049/jimmunol.172.7.4266

Fra AM, Williamson E, Simons K, Parton RG (1995) De novo formation of caveolae in lymphocytes by expression of VIP21-caveolin. Cell Biol 92:8655–8659

Freeman MR, Cinar B, Kim J, Mukhopadhyay NK, di Vizio D, Adam RM, Solomon KR (2007) Transit of hormonal and EGF receptor-dependent signals through cholesterol-rich membranes. Steroids 72(2):210–217. https://doi.org/10.1016/j.steroids.2006.11.012

Friedrichson T, Kurzchalia TV (1998) Microdomains of GPI-anchored proteins in living cells revealed by crosslinking. Nature

Fruman DA, Chiu H, Hopkins BD, Bagrodia S, Cantley LC, Abraham RT (2017) The PI3K pathway in human disease. Cell 170(4):605–635. https://doi.org/10.1016/j.cell.2017.07.029

Fujimoto T (1993) Calcium pump of the plasma membrane is localized in caveolae. J Cell Biol

Gajate C, Mollinedo F (2007) Edelfosine and perifosine induce selective apoptosis in multiple myeloma by recruitment of death receptors and downstream signaling molecules into lipid rafts. Blood. https://doi.org/10.1182/blood-2006-04

Gajate C, Mollinedo F (2015) Lipid raft-mediated Fas/CD95 apoptotic signaling in leukemic cells and normal leukocytes and therapeutic implications. J Leukoc Biol 98(5):739–759. https://doi.org/10.1189/jlb.2mr0215-055r

Gajate C, Gonzalez-Camacho F, Mollinedo F (2009) Involvement of raft aggregates enriched in Fas/CD95 death-inducing signaling complex in the antileukemic action of edelfosine in Jurkat cells. PLoS One 4(4). https://doi.org/10.1371/journal.pone.0005044

Gallet A, Ruel L, Staccini-Lavenant L, Thérond PP (2006) Cholesterol modification is necessary for controlled planar long-range activity of hedgehog in drosophila epithelia. Development 133(3):407–418. https://doi.org/10.1242/dev.02212

Gao X, Lowry PR, Zhou X, Depry C, Wei Z, Wong GW, Zhang J (2011) PI3K/Akt signaling requires spatial compartmentalization in plasma membrane microdomains. Proc Natl Acad Sci U S A 108(35):14509–14514. https://doi.org/10.1073/pnas.1019386108

García-Cardeñ G, Fan R, Stern DF, Liu J, Sessa WC (1996) Endothelial nitric oxide synthase is regulated by tyrosine phosphorylation and interacts with Caveolin-1. J Biol Chem

Gervásio OL, Phillips WD, Cole L, Allen DG (2011) Caveolae respond to cell stretch and contribute to stretch-induced signaling. J Cell Sci 124(21):3581–3590. https://doi.org/10.1242/jcs.084376

Guo Y, Yang L, Haught K, Scarlata S (2015) Osmotic stress reduces Ca2+ signals through deformation of caveolae. J Biol Chem 290(27):16698–16707. https://doi.org/10.1074/jbc.M115.655126

Hansen CG, Bright NA, Howard G, Nichols BJ (2009) SDPR induces membrane curvature and functions in the formation of caveolae. Nat Cell Biol 11(7):807–814. https://doi.org/10.1038/ncb1887

Hayer A, Stoeber M, Bissig C, Helenius A (2010) Biogenesis of caveolae: stepwise assembly of large caveolin and cavin complexes. Traffic 11(3):361–382. https://doi.org/10.1111/j.1600-0854.2009.01023.x

Hea YO, Eun JL, Yoon S, Byung HC, Kang SC, Sung JH (2007) Cholesterol level of lipid raft microdomains regulates apoptotic cell death in prostate cancer cells through EGFR-mediated Akt and ERK signal transduction. Prostate 67(10):1061–1069. https://doi.org/10.1002/pros.20593

Hell SW, Wichmann J (1994) Breaking the diffraction resolution limit by stimulated emission: stimulated-emission-depletion fluorescence microscopy. Opt Lett 19(11)

Hernandez VJ, Weng J, Ly P, Pompey S, Dong H, Mishra L, Schwarz M, Anderson RGW, Michaely P (2013) Cavin-3 dictates the balance between ERK and Akt signaling. elife 2013(2). https://doi.org/10.7554/eLife.00905

Hess ST, Girirajan TPK, Mason MD (2006) Ultra-high resolution imaging by fluorescence photoactivation localization microscopy. Biophys J 91(11):4258–4272. https://doi.org/10.1529/biophysj.106.091116

Hill MM, Bastiani M, Luetterforst R, Kirkham M, Kirkham A, Nixon SJ, Walser P, Abankwa D, Oorschot VMJ, Martin S, Hancock JF, Parton RG (2008) PTRF-cavin, a conserved cytoplasmic protein required for caveola formation and function. Cell

Hjort Ipsen J, Karlström G, Mouritsen OG, Wennerström H, Zuckermann MJ (1987) Phase equilibria in the phosphatidylcholine-cholesterol system. Biochim Biophys Acta 905:162–172

Hollars CW, Dunn RC (1997) Submicron fluorescence, topology, and compliance measurements of phase-separated lipid monolayers using tapping-mode near-field scanning optical microscopy. J Phys Chem B. https://pubs.acs.org/sharingguidelines

Hong S, Huo H, Xu J, Liao K (2004) Insulin-like growth factor-1 receptor signaling in 3T3-L1 adipocyte differentiation requires lipid rafts but not caveolae. Cell Death Differ 11(7):714–723. https://doi.org/10.1038/sj.cdd.4401405

Hryniewicz-Jankowska A, Augoff K, Sikorski AF (2019) Highlight article: the role of cholesterol and cholesterol-driven membrane raft domains in prostate cancer. Exp Biol Med 244(13):1053–1061). SAGE Publications Inc. https://doi.org/10.1177/1535370219870771

Hueber A-O, Bernard A-M, Hérincs Z, Couzinet A, He H-T (2002) An essential role for membrane rafts in the initiation of Fas/CD95-triggered cell death in mouse thymocytes. EMBO Rep 3(2):190–196

Huo H, Guo X, Hong S, Jiang M, Liu X, Liao K (2003) Lipid rafts/caveolae are essential for insulin-like growth factor-1 receptor signaling during 3T3-L1 preadipocyte differentiation induction. J Biol Chem

278(13):11561–11569. https://doi.org/10.1074/jbc.M211785200

Hwang J, Gheber LA, Margolis L, Edidin M (1998) Domains in cell plasma membranes investigated by near-field scanning optical microscopy. Biophys J 74(5):2184–2190. https://doi.org/10.1016/S0006-3495(98)77927-5

Jans R, Atanasova G, Jadot M, Poumay Y (2004) Cholesterol depletion upregulates involucrin expression in epidermal keratinocytes through activation of p38. J Investig Dermatol

Jia S, Liu Z, Zhang S, Liu P, Zhang L, Hyun S, Zhang J, Signoretti S, Loda M, Roberts TM, Zhao JJ (2008a) Kinase-dependent and -independent functions of the p110β phosphoinositide-3-kinase in cell growth, metabolic regulation and oncogenic transformation. Nature 454(7205):776–779. https://doi.org/10.1038/nature07091.Kinase-dependent

Jia S, Liu Z, Zhang S, Liu P, Zhang L, Lee SH, Zhang J, Signoretti S, Loda M, Roberts TM, Zhao JJ (2008b) Essential roles of PI(3)K-p110β in cell growth, metabolism and tumorigenesis. Nature 454(7205):776–779. https://doi.org/10.1038/nature07091

Kaiser H-J, Lingwood D, Levental I, Sampaio JL, Kalvodova L, Rajendran L, Simons K (2009) Order of lipid phases in model and plasma membranes. PNAS 106(39)

Kirkham M, Nixon SJ, Howes MT, Abi-Rached L, Wakeham DE, Hanzal-Bayer M, Ferguson C, Hill MM, Fernandez-Rojo M, Brown DA, Hancock JF, Brodsky FM, Parton RG (2008) Evolutionary analysis and molecular dissection of caveola biogenesis. J Cell Sci 121(12):2075–2086. https://doi.org/10.1242/jcs.024588

Kischkel FC, Hellbardt S, Behrmann I, Germer M, Pawlita M, Krammer PH, Peter ME (1995) Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. EMBO J 14(22):5579–5588

Klar TA, Engel E, Hell SW (2001) Breaking Abbe's diffraction resolution limit in fluorescence microscopy with stimulated emission depletion beams of various shapes. Phys Rev E Statist Phys Plasmas Fluids Related Interdisciplin Topics 64(6):9. https://doi.org/10.1103/PhysRevE.64.066613

Koleske AJ, Baltimore D, Lisanti MP (1995) Reduction of caveolin and caveolae in oncogenically transformed cells (cancer/growth regulation/oncogene/potocytosis). Cell Biol 92:1381–1385. https://www.pnas.org

Kovacs T, Zakany F, Nagy P (2022) It takes more than two to tango: complex, hierarchal, and membrane-modulated interactions in the regulation of receptor tyrosine kinases. Cancers 14(4) MDPI. https://doi.org/10.3390/cancers14040944

Kovtun O, Tillu VA, Jung WR, Leneva N, Ariotti N, Chaudhary N, Mandyam RA, Ferguson C, Morgan GP, Johnston WA, Harrop SJ, Alexandrov K, Parton RG, Collins BM (2014) Structural insights into the organization of the cavin membrane coat complex. Dev Cell 31(4):405–419. https://doi.org/10.1016/j.devcel.2014.10.002

Kurzchalia TV, Dupree P, Parton RG, Kellner R, Virta H, Lehnert M, Simons K (1992) VIP21, a 21-kD membrane protein is an integral component of trans-golgi-network-derived transport vesicles. J Cell Biol

Kwon H, Pak Y (2010) Prolonged tyrosine kinase activation of insulin receptor by pY27-caveolin-2. Biochem Biophys Res Commun 391(1):49–55. https://doi.org/10.1016/j.bbrc.2009.10.159

Kwon H, Jeong K, Hwang EM, Park JY, Hong SG, Choi WS, Pak Y (2009) Caveolin-2 regulation of STAT3 transcriptional activation in response to insulin. Biochim Biophys Acta, Mol Cell Res 1793(7):1325–1333. https://doi.org/10.1016/j.bbamcr.2009.04.015

Kwon H, Jeong K, Hwang EM, Park JY, Pak Y (2011) A novel domain of caveolin-2 that controls nuclear targeting: regulation of insulin-specific ERK activation and nuclear translocation by caveolin-2. J Cell Mol Med 15(4):888–908. https://doi.org/10.1111/j.1582-4934.2010.01079.x

Kwon H, Lee J, Jeong K, Jang D, Pak Y (2015) Fatty acylated caveolin-2 is a substrate of insulin receptor tyrosine kinase for insulin receptor substrate-1-directed signaling activation. Biochim Biophys Acta, Mol Cell Res 1853(5):1022–1034. https://doi.org/10.1016/j.bbamcr.2015.02.002

la Rosa P, Pesiri V, Leclercq G, Marino M, Acconcia F (2012) Palmitoylation regulates 17β-estradiol-induced estrogen receptor-α degradation and transcriptional activity. Mol Endocrinol 26(5):762–774. https://doi.org/10.1210/me.2011-1208

Laganowsky A, Reading E, Allison TM, Ulmschneider MB, Degiacomi MT, Baldwin AJ, Robinson C v (2014) Membrane proteins bind lipids selectively to modulate their structure and function. Nature 510(7503):172–175. https://doi.org/10.1038/nature13419

Lambert S, Vind-Kezunovic D, Karvinen S, Gniadecki R (2006) Ligand-independent activation of the EGFR by lipid raft disruption. J Investig Dermatol 126(5):954–962. https://doi.org/10.1038/sj.jid.5700168

Lasserre R, Guo XJ, Conchonaud F, Hamon Y, Hawchar O, Bernard AM, Soudja SMH, Lenne PF, Rigneault H, Olive D, Bismuth G, Nunès JA, Payrastre B, Marguet D, He HT (2008) Raft nanodomains contribute to Akt/PKB plasma membrane recruitment and activation. Nat Chem Biol 4(9):538–547. https://doi.org/10.1038/nchembio.103

Lee MY, Ryu JM, Lee SH, Park JH, Han HJ (2010) Lipid rafts play an important role for maintenance of embryonic stem cell self-renewal. J Lipid Res 51(8):2082–2089. https://doi.org/10.1194/jlr.M001545

Levental I, Grzybek M, Simons K (2010a) Greasing their way: lipid modifications determine protein association with membrane rafts. Biochemistry 49(30):6305–6316. https://doi.org/10.1021/bi100882y

Levental I, Lingwood D, Grzybek M, Coskun Ü, Simons K (2010b) Palmitoylation regulates raft affinity for the majority of integral raft proteins. PNAS 107(51): 22050–22054. https://doi.org/10.1073/pnas

Levin-Gromiko U, Koshelev V, Kushnir P, Fedida-Metula S, Voronov E, Fishman D (2014) Amplified lipid rafts of malignant cells constitute a target for inhibition of aberrantly active NFAT and melanoma tumor growth by the aminobisphosphonate zoledronic acid. Carcinogenesis 35(11):2555–2566. https://doi.org/10.1093/carcin/bgu178

Li S, Couet J, Lisanti MP (1996a) Src tyrosine kinases, G (α) subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin: Caveolin binding negatively regulates the auto-activation of Src tyrosine kinases. J Biol Chem 271(46):29182–29190. https://doi.org/10.1074/jbc.271.46.29182

Li S, Couet J, Lisanti MP (1996b) Src tyrosine kinases, G (α) subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin: Caveolin binding negatively regulates the auto-activation of Src tyrosine kinases. J Biol Chem 271(46):29182–29190. https://doi.org/10.1074/jbc.271.46.29182

Li YC, Park MJ, Ye SK, Kim CW, Kim YN (2006) Elevated levels of cholesterol-rich lipid rafts in cancer cells are correlated with apoptosis sensitivity induced by cholesterol-depleting agents. Am J Pathol 168(4): 1107–1118. https://doi.org/10.2353/ajpath.2006.050959

Li Q, Bai L, Liu N, Wang M, Liu JP, Liu P, Cong YS (2014) Increased polymerase I and transcript release factor (Cavin-1) expression attenuates platelet-derived growth factor receptor signalling in senescent human fibroblasts. Clin Exp Pharmacol Physiol 41(3): 169–173. https://doi.org/10.1111/1440-1681.12202

Lillemeier BF, Mörtelmaier MA, Forstner MB, Huppa JB, Groves JT, Davis MM (2010) TCR and Lat are expressed on separate protein islands on T cell membranes and concatenate during activation. Nat Immunol 11(1):90–96. https://doi.org/10.1038/ni.1832

Lingwood D, Binnington B, Róg T, Vattulainen I, Grzybek M, Coskun Ü, Lingwood CA, Simons K (2011) Cholesterol modulates glycolipid conformation and receptor activity. Nat Chem Biol 7(5):260–262. https://doi.org/10.1038/nchembio.551

Liu P, Ying Y, Ko Y-G, Anderson RGW (1996) Localization of platelet-derived growth factor-stimulated phosphorylation cascade to caveolae. J Biol Chem

Liu J, Oh P, Horner T, Rogers RA, Schnitzer JE (1997) Organized endothelial cell surface signal transduction in caveolae distinct from glycosylphosphatidylinositol-anchored protein microdomains. J Biol Chem. http://www-jbc.stanford.edu/jbc/

Liu L, Brown D, McKee M, LeBrasseur NK, Yang D, Albrecht KH, Ravid K, Pilch PF (2008) Deletion of Cavin/PTRF causes global loss of Caveolae, Dyslipidemia, and glucose intolerance. Cell Metab 8(4): 310–317. https://doi.org/10.1016/j.cmet.2008.07.008

Liu H, Yang L, Zhang Q, Mao L, Jiang H, Yang H (2016) Probing the structure and dynamics of caveolin-1 in a caveolae-mimicking asymmetric lipid bilayer model. Eur Biophys J 45(6):511–521. https://doi.org/10.1007/s00249-016-1118-1

Llaverias G, Danilo C, Wang Y, Witkiewicz AK, Daumer K, Lisanti MP, Frank PG (2010) A western-type diet accelerates tumor progression in an autochthonous mouse model of prostate cancer. Am J Pathol 177(6):3180–3191. https://doi.org/10.2353/ajpath.2010.100568

Longo J, van Leeuwen JE, Elbaz M, Branchard E, Penn LZ (2020) Statins as anticancer agents in the era of precision medicine. Clin Cancer Res 26(22): 5791–5800). American Association for Cancer Research Inc. https://doi.org/10.1158/1078-0432.CCR-20-1967

Ludwig A, Nichols BJ, Sandin S (2016) Architecture of the caveolar coat complex. J Cell Sci 129(16): 3077–3083. https://doi.org/10.1242/jcs.191262

Lumachi F, Brunello A, Maruzzo M, Basso U, Basso SMM (2013) Treatment of estrogen receptor-positive breast cancer. Curr Med Chem 20:596–604

Manning BD, Cantley LC (2007) AKT/PKB Signaling: navigating downstream. Cell 129(7):1261–1274. https://doi.org/10.1016/j.cell.2007.06.009

Manning BD, Toker A (2017) Review AKT / PKB Signaling : navigating the network. Cell 169(3):381–405. https://doi.org/10.1016/j.cell.2017.04.001

Márquez DC, Chen HW, Curran EM, Welshons W v, Pietras RJ (2006) Estrogen receptors in membrane lipid rafts and signal transduction in breast cancer. Mol Cell Endocrinol 246(1–2):91–100. https://doi.org/10.1016/j.mce.2005.11.020

Martinez-Outschoorn UE, Sotgia F, Lisanti MP (2015) Caveolae and signalling in cancer. Nat Rev Cancer 15(4):225–237). Nature Publishing Group. https://doi.org/10.1038/nrc3915

Maselli A, Pierdominici M, Vitale C, Ortona E (2015) Membrane lipid rafts and estrogenic signalling: a functional role in the modulation of cell homeostasis. Apoptosis 20(5):671–678. https://doi.org/10.1007/s10495-015-1093-5

Mayor S, Maxfield FR (1995) Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment. Mol Biol Cell 6:929–944

McConnell HM, Tamm LK, Weis RM (1984) Periodic structures in lipid monolayer phase transitions (two-dimensional systems/lateral diffusion/fluorescence microscopy/membranes). PNAS 81:3249–3253

McMahon KA, Zajicek H, Li WP, Peyton MJ, Minna JD, Hernandez VJ, Luby-Phelps K, Anderson RGW (2009) SRBC/cavin-3 is a caveolin adapter protein that regulates caveolae function. EMBO J 28(8): 1001–1015. https://doi.org/10.1038/emboj.2009.46

Michel V, Bakovic M (2007) Lipid rafts in health and disease. Biol Cell 99(3):129–140. https://doi.org/10.1042/bc20060051

Moffett S, Brown DA, Linder ME (2000) Lipid-dependent targeting of G proteins into rafts. J Biol Chem. http://www.jbc.org

Mollinedo F (2014) Antitumor alkylphospholipid analogs: a promising and growing family of synthetic cell membrane-targeting molecules for cancer treatment. Anti Cancer Agents Med Chem 14:495–498

Mollinedo F, Gajate C (2006) Fas/CD95 death receptor and lipid rafts: new targets for apoptosis-directed cancer therapy. Drug Resist Updat 9(1–2):51–73. https://doi.org/10.1016/j.drup.2006.04.002

Monier S, Parton RG, Vogel F, Behlke J, Henske A, Kurzchalia T v (1995) VIP21-Caveolin, a membrane protein constituent of the caveolar coat, oligomerizes in vivo and in vitro. Mol Biol Cell 6:911–927

Moon H, Lee CS, Inder KL, Sharma S, Choi E, Black DM, Lê Cao KA, Winterford C, Coward JI, Ling MT, Craik DJ, Parton RG, Russell PJ, Hill MM (2014a) PTRF/cavin-1 neutralizes non-caveolar caveolin-1 microdomains in prostate cancer. Oncogene 33(27): 3561–3570. https://doi.org/10.1038/onc.2013.315

Moon H, Lee CS, Inder KL, Sharma S, Choi E, Black DM, Lê Cao KA, Winterford C, Coward JI, Ling MT, Craik DJ, Parton RG, Russell PJ, Hill MM (2014b) PTRF/cavin-1 neutralizes non-caveolar caveolin-1 microdomains in prostate cancer. Oncogene 33(27): 3561–3570. https://doi.org/10.1038/onc.2013.315

Motoyama K, Kameyama K, Onodera R, Araki N, Hirayama F, Uekama K, Arima H (2009) Involvement of PI3K-Akt-bad pathway in apoptosis induced by 2,6-di-O-methyl-β-cyclodextrin, not 2,6-di-O-methyl-α-cyclodextrin, through cholesterol depletion from lipid rafts on plasma membranes in cells. Eur J Pharm Sci 38(3):249–261. https://doi.org/10.1016/j.ejps.2009.07.010

Murai T (2015) Cholesterol lowering: role in cancer prevention and treatment. Biol Chem 396(1):1–11). Walter de Gruyter GmbH. https://doi.org/10.1515/hsz-2014-0194

Murai T, Maruyama Y, Mio K, Nishiyama H, Suga M, Sato C (2011) Low cholesterol triggers membrane microdomain-dependent CD44 shedding and suppresses tumor cell migration. J Biol Chem 286(3): 1999–2007. https://doi.org/10.1074/jbc.M110.184010

Murata M, Peranen J, Schreinert R, Wielandt F, Kurzchalia, T. v, & Simons, K (1995) VIP21/caveolin is a cholesterol-binding protein (membrane microdomains/intracellular transport/membrane reconstitution). PNAS 92:10339–10343

Muzio M, Stockwell BR, Stennicke HR, Salvesen GS, Dixit VM (1998) An induced proximity model for Caspase-8 activation. J Biol Chem. http://www.jbc.org

Nassoy P, Lamaze C (2012) Stressing caveolae new role in cell mechanics. Trends Cell Biol 22(7):381–389. https://doi.org/10.1016/j.tcb.2012.04.007

Nystrom FH, Chen H, Cong L-N, Li Y, Quon MJ (2013) Caveolin-1 interacts with the insulin receptor and can differentially modulate insulin signaling in transfected Cos-7 cells and rat adipose cells. Mol Endocrinol 13. https://academic.oup.com/mend/article/13/12/2013/2747792

Oh P, Schnitzer JE (2001) Segregation of heterotrimeric G proteins in cell surface microdomains Gq binds caveolin to concentrate in caveolae, whereas Gi and Gs target lipid rafts by default. Mol Biol Cell 12:685–698

Okamoto T, Schlegel A, Scherer PE, Lisanti MP (1998) Caveolins, a family of scaffolding proteins for organizing “preassembled signaling complexes” at the plasma membrane. J Biol Chem. http://www.jbc.org

Ostrom RS, Insel PA (2004) The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology. British J Pharmacol 143(2):235–245. https://doi.org/10.1038/sj.bjp.0705930

Owen DM, Rentero C, Magenau A, Abu-Siniyeh A, Gaus K (2012) Quantitative imaging of membrane lipid order in cells and organisms. Nat Protoc 7(1):24–35. https://doi.org/10.1038/nprot.2011.419

Park S, Sut TN, Ma GJ, Parikh AN, Cho NJ (2020) Crystallization of cholesterol in phospholipid membranes follows Ostwald’s rule of stages. J Am Chem Soc 142(52):21872–21882. https://doi.org/10.1021/jacs.0c10674

Parton RG, Tillu V, McMahon KA, Collins BM (2021) Key phases in the formation of caveolae. Curr Opin Cell Biol 71:7–14). Elsevier Ltd. https://doi.org/10.1016/j.ceb.2021.01.009

Pavlides S, Whitaker-Menezes D, Castello-Cros R, Flomenberg N, Witkiewicz AK, Frank PG, Casimiro MC, Wang C, Fortina P, Addya S, Pestell RG, Martinez-Outschoorn UE, Sotgia F, Lisanti MP (2009) The reverse Warburg effect: aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. Cell Cycle 8(23):3984–4001. https://doi.org/10.4161/cc.8.23.10238

Pelton K, Freeman MR, Solomon KR (2012) Cholesterol and prostate cancer. Curr Opin Pharmacol 12(6): 751–759. https://doi.org/10.1016/j.coph.2012.07.006

Pietras RJ, Márquez-Garbán DC (2007) Membrane-associated estrogen receptor signaling pathways in human cancers. Clin Cancer Res 13(16):4672–4676. https://doi.org/10.1158/1078-0432.CCR-07-1373

Pike LJ (2005) Growth factor receptors, lipid rafts and caveolae: an evolving story. Biochim Biophys Acta Mol Cell Res 1746(3):260–273. https://doi.org/10.1016/j.bbamcr.2005.05.005

Pike LJ, Han X, Gross RW (2005) Epidermal growth factor receptors are localized to lipid rafts that contain a balance of inner and outer leaflet lipids: a shotgun lipidomics study. J Biol Chem 280(29):26796–26804. https://doi.org/10.1074/jbc.M503805200

Płóciennikowska A, Hromada-Judycka A, Borzęcka K, Kwiatkowska K (2015) Co-operation of TLR4 and raft proteins in LPS-induced pro-inflammatory signaling. Cell Mol Life Sci 72(3):557–581. https://doi.org/10.1007/s00018-014-1762-5

Pohl J, Ring A, Ehehalt R, Schulze-Bergkamen H, Schad A, Verkade P, Stremmel W (2004) Long-chain fatty acid uptake into adipocytes depends on lipid raft function. Biochemistry 43(14):4179–4187. https://doi.org/10.1021/bi035743m

Pohl J, Ring A, Korkmaz U, Ehehalt R, Stremmel W (2005) FAT/CD36-mediated long-chain fatty acid uptake in adipocytes requires plasma membrane rafts. Mol Biol Cell 16:24–31. https://doi.org/10.1091/mbc.E04

Pollak M (2008) Insulin and insulin-like growth factor signalling in neoplasia. Nat Rev Cancer 8(12): 915–928. https://doi.org/10.1038/nrc2536

Porter JA, Young KE, Beachy PA (1996) Cholesterol modification of hedgehog signaling proteins in animal development. Science. https://www.science.org

Pralle A, Keller P, Florin E-L, Simons K, Hörber JKH, Hoirber JKH (2000) Sphingolipid-cholesterol rafts diffuse as small entities in the plasma membrane of mammalian cells. J Cell Biol 148(5):997–1007

Ratajczak MZ, Adamiak M (2015) Membrane lipid rafts, master regulators of hematopoietic stem cell retention in bone marrow and their trafficking. Leukemia 29(7): 1452–1457). Nature Publishing Group. https://doi.org/10.1038/leu.2015.66

Razani B, Engelman JA, Wang XB, Schubert W, Zhang XL, Marks CB, Macalusol F, Russell RG, Li M, Pestell RG, di Vizio D, Hou H, Kneitz B, Lagaud G, Christ GJ, Edelmann W, Lisanti MP (2001) Caveolin-1 null mice are viable but show evidence of Hyperproliferative and vascular abnormalities. J Biol Chem 276(41):38121–38138. https://doi.org/10.1074/jbc.m105408200

Reis-Sobreiro M, Roue G, Moros A, Gajate C, de La Iglesia-Vicente J, Colomer D, Mollinedo F (2013) Lipid raft-mediated Akt signaling as a therapeutic target in mantle cell lymphoma. Blood Cancer J 3(5). https://doi.org/10.1038/bcj.2013.15

Resh MD (2013) Covalent lipid modifications of proteins. Curr Biol 23(10) Cell Press. https://doi.org/10.1016/j.cub.2013.04.024

Ribon V, Kanzaki M, Thurmond DC, Mora S, Shigematsu S, Bickel PE, Pessin JE, Saltiel AR, Chiang SH, Baumann CA, Watson RT, Neudauer CL, Macara IG (2001) Lipid rafts are required for GLUT4 internalization in adipose cells. PNAS 407: 202–207. www.pnas.org

Richardson PG, Eng C, Kolesar J, Hideshima T, Anderson KC (2012) Perifosine, an oral, anti-cancer agent and inhibitor of the Akt pathway: mechanistic actions, pharmacodynamics, pharmacokinetics, and clinical activity. Exp Opin Drug Metabol Toxicol 8(5):623–633. https://doi.org/10.1517/17425255.2012.681376

Rizzo V, Sung A, Oh P, Schnitzer JE (1998) Rapid mechanotransduction in situ at the luminal cell surface of vascular endothelium and its caveolae. J Biol Chem. http://www.jbc.org

Roepstorff K, Thomsen P, Sandvig K, van Deurs B (2002) Sequestration of epidermal growth factor receptors in non-caveolar lipid rafts inhibits ligand binding. J Biol Chem 277(21):18954–18960. https://doi.org/10.1074/jbc.M201422200

Rossy J, Owen DM, Williamson DJ, Yang Z, Gaus K (2013) Conformational states of the kinase Lck regulate clustering in early T cell signaling. Nat Immunol 14(1):82–89. https://doi.org/10.1038/ni.2488

Rothberg KG, Heuser JE, Donzell WC, Ying Y-S, Glenney JR, Anderson RGW (1992) Caveolin, a protein component of caveolae membrane coats. Cell 68: 673–682

Rudelius M, Pittaluga S, Nishizuka S, Pham THT, Fend F, Jaffe ES, Quintanilla-Martinez L, Raffeld M (2006) Constitutive activation of Akt contributes to the pathogenesis and survival of mantle cell lymphoma. Blood 108(5):1668–1676. https://doi.org/10.1182/blood-2006-04-015586

Rust MJ, Bates M, Zhuang X (2006) Sub-diffraction-limit imaging by stochastic optical reconstruction microscopy (STORM). Nat Methods 3(10):793–795. https://doi.org/10.1038/nmeth929

Saraiva SM, Gutiérrez-Lovera C, Martínez-Val J, Lores S, Bouzo BL, Díez-Villares S, Alijas S, Pensado-López A, Vázquez-Ríos AJ, Sánchez L, de la Fuente M (2021) Edelfosine nanoemulsions inhibit tumor growth of triple negative breast cancer in zebrafish xenograft model. Sci Rep 11(1). https://doi.org/10.1038/s41598-021-87968-4

Scherer PE, Okamotot T, Chun M, Nishimotot I, Lodish HF, Lisanti MP (1996) Identification, sequence, and expression of caveolin-2 defines a caveolin gene family. Cell Biol 93:131–135

Schuck S, Honsho M, Ekroos K, Shevchenko A, Simons K (2003) Resistance of cell membranes to different detergents. PNAS. www.pnas.orgcgi. https://doi.org/10.1073/pnas.0631579100

Selivanov VA, Vizán P, Mollinedo F, Fan TWM, Lee PWN, Cascante M (2010) Edelfosine-induced metabolic changes in cancer cells that precede the overproduction of reactive oxygen species and apoptosis. BMC Syst Biol:4. https://doi.org/10.1186/1752-0509-4-135

Senbanjo LT, Chellaiah MA (2017) CD44: a multifunctional cell surface adhesion receptor is a regulator of progression and metastasis of cancer cells. Front Cell Dev Biol 5(MAR) Frontiers Media S.A. https://doi.org/10.3389/fcell.2017.00018

Sengupta P, Jovanovic-Talisman T, Skoko D, Renz M, Veatch SL, Lippincott-Schwartz J (2011) Probing protein heterogeneity in the plasma membrane using PALM and pair correlation analysis. Nat Methods 8(11):969–975. https://doi.org/10.1038/nmeth.1704

Sezgin E (2017) Super-resolution optical microscopy for studying membrane structure and dynamics. J Phys Condense Matter 29(27) Institute of Physics Publishing. https://doi.org/10.1088/1361-648X/aa7185

Sezgin E, Schwille P (2011) Fluorescence techniques to study lipid dynamics. Cold Spring Harb Perspect Biol 3(11). https://doi.org/10.1101/cshperspect.a009803

Sezgin E, Levental I, Grzybek M, Schwarzmann G, Mueller V, Honigmann A, Belov VN, Eggeling C, Coskun Ü, Simons K, Schwille P (2012) Partitioning, diffusion, and ligand binding of raft lipid analogs in model and cellular plasma membranes. Biochim Biophys Acta Biomembr 1818(7):1777–1784. https://doi.org/10.1016/j.bbamem.2012.03.007

Shelby SA, Holowka D, Baird B, Veatch SL (2013) Distinct stages of stimulated FceRI receptor clustering and immobilization are identified through superresolution imaging. Biophys J 105(10):2343–2354. https://doi.org/10.1016/j.bpj.2013.09.049

Sherman E, Barr V, Manley S, Patterson G, Balagopalan L, Akpan I, Regan CK, Merrill RK, Sommers CL, Lippincott-Schwartz J, Samelson LE (2011) Functional nanoscale organization of signaling molecules downstream of the T cell antigen receptor. Immunity 35(5): 705–720. https://doi.org/10.1016/j.immuni.2011.10.004

Sierra RG, Gati C, Laksmono H, Dao EH, Gul S, Fuller F, Kern J, Chatterjee R, Ibrahim M, Brewster AS, Young ID, Michels-Clark T, Aquila A, Liang M, Hunter MS, Koglin JE, Boutet S, Junco EA, Hayes B et al (2015) Concentric-flow electrokinetic injector enables serial crystallography of ribosome and photosystem II. Nat Methods 13(1): 59–62. https://doi.org/10.1038/nmeth.3667

Simons K, Ikonen E (1997) Functional rafts in cell membranes. Nature 387

Simons K, Toomre D (2000) Lipid rafts and signal transduction. Nat Rev Mol Cell Biol 1:1. www.nature.com/reviews/molcellbio

Singer SJ, Nicolson GL (1972) The fluid mosaic model of the structure of cell membranes. Science 175(4023)

Sinha B, Köster D, Ruez R, Gonnord P, Bastiani M, Abankwa D, Stan R v, Butler-Browne G, Vedie B, Johannes L, Morone N, Parton RG, Raposo G, Sens P, Lamaze C, Nassoy P (2011) Cells respond to mechanical stress by rapid disassembly of caveolae. Cell 144(3):402–413. https://doi.org/10.1016/j.cell.2010.12.031

Sloan EK, Ciocca DR, Pouliot N, Natoli A, Restall C, Henderson MA, Fanelli MA, Cuello-Carrión FD, Gago FE, Anderson RL (2009) Stromal cell expression of caveolin-1 predicts outcome in breast cancer. Am J Pathol 174(6):2035–2043. https://doi.org/10.2353/ajpath.2009.080924

Sohn J, Lin H, Fritch MR, Tuan RS (2018) Influence of cholesterol/caveolin-1/caveolae homeostasis on membrane properties and substrate adhesion characteristics of adult human mesenchymal stem cells. Stem Cell Res Ther 9(1). https://doi.org/10.1186/s13287-018-0830-4

Song KS, Li S, Okamoto T, Quilliam LA, Sargiacomo M, Lisanti MP (1996) Co-purification and direct interaction of Ras with Caveolin, an integral membrane protein of caveolae microdomains (detergent-free purification of caveolae membranes). J Biol Chem

Song MS, Salmena L, Pandolfi PP (2012) The functions and regulation of the PTEN tumour suppressor. Nat Rev Mol Cell Biol 13(5):283–296. https://doi.org/10.1038/nrm3330

Sproul TW, Malapati S, Kim J, Pierce SK (2000) Cutting edge: B cell antigen receptor Signaling occurs outside lipid rafts in immature B cells. J Immunol 165(11):6020–6023. https://doi.org/10.4049/jimmunol.165.11.6020

Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP, Mak TW (1998) Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. Cell 95(1):29–39. https://doi.org/10.1016/S0092-8674(00)81780-8

Staubach S, Hanisch FG (2011) Lipid rafts: Signaling and sorting platforms of cells and their roles in cancer. Expert Rev Proteom 8(2):263–277. https://doi.org/10.1586/epr.11.2

Stuermer CAO (2010) The reggie/flotillin connection to growth. Trends Cell Biol 20(1):6–13. https://doi.org/10.1016/j.tcb.2009.10.003

Stuermer CAO (2011) Reggie/flotillin and the targeted delivery of cargo. J Neurochem 116(5):708–713. https://doi.org/10.1111/j.1471-4159.2010.07007.x

Sugahara KN, Murai T, Nishinakamura H, Kawashima H, Saya H, Miyasaka M (2003) Hyaluronan oligosaccharides induce CD44 cleavage and promote cell migration in CD44-expressing tumor cells. J Biol Chem 278(34):32259–32265. https://doi.org/10.1074/jbc.M300347200

Sugawara Y, Nishii H, Takahashi T, Yamauchi J, Mizuno N, Tago K, Itoh H (2007) The lipid raft proteins flotillins/reggies interact with Gαq and are involved in Gq-mediated p38 mitogen-activated protein kinase activation through tyrosine kinase. Cell Signal 19(6):1301–1308. https://doi.org/10.1016/j.cellsig.2007.01.012

Taira J, Sugishima M, Kida Y, Oda E, Noguchi M, Higashimoto Y (2011) Caveolin-1 is a competitive inhibitor of heme oxygenase-1 (HO-1) with heme: identification of a minimum sequence in caveolin-1 for binding to HO-1. Biochemistry 50(32): 6824–6831. https://doi.org/10.1021/bi200601t

Tamm LK, McConnell HM (1985) Supported phospholipid bilayers. Biophys J 47(1):105–113. https://doi.org/10.1016/S0006-3495(85)83882-0

Tibbetts MD, Zheng L, Lenardo MJ (2003) The death effector domain protein family: regulators of cellular homeostasis. Nat Immunol. http://www.nature.com/natureimmunology

Totta P, Gionfra F, Busonero C, Acconcia F (2016) Modulation of 17β-Estradiol Signaling on cellular proliferation by Caveolin-2. J Cell Physiol 231(6):1219–1225. https://doi.org/10.1002/jcp.25218

Trane AE, Pavlov D, Sharma A, Saqib U, Lau K, van Petegem F, Minshall RD, Roman LJ, Bernatchez PN (2014) Deciphering the binding of Caveolin-1 to client protein endothelial nitric-oxide synthase (eNOS): scaffolding subdomain identification, interaction modeling, and biological significance. J Biol Chem 289(19):13273–13283. https://doi.org/10.1074/jbc.M113.528695

Triantafilou K, Triantafilou M, Lepper PM, Olden R, Dias IDSR (2011) Location, location, location: is membrane partitioning everything when it comes to innate immune activation? Media Inflam 2011. https://doi.org/10.1155/2011/186093

Turk HF, Barhoumi R, Chapkin RS (2012) Alteration of EGFR spatiotemporal dynamics suppresses signal transduction. PLoS One 7(6). https://doi.org/10.1371/journal.pone.0039682

Udayakumar TS, Stoyanova R, Shareef MM, Mu Z, Philip S, Burnstein KL, Pollack A (2016) Edelfosine promotes apoptosis in androgen-deprived prostate tumors by increasing atf3 and inhibiting androgen receptor activity. Mol Cancer Ther 15(6):1353–1363. https://doi.org/10.1158/1535-7163.MCT-15-0332

van Meer G, Stelzer EHK, Wijnaendts-Van-Resandt RW, Simons K (1987) Sorting of sphingolipids in epithelial (Madin-Darby canine kidney) cells. J Cell Biol 105(4): 1623–1635

Vanhaesebroeck B, Guillermet-Guibert J, Graupera M, Bilanges B (2010) The emerging mechanisms of isoform-specific PI3K signalling. Nat Rev Mol Cell Biol 11(5):329–341. https://doi.org/10.1038/nrm2882

Varma R, Mayor S (1998) GPI-anchored proteins are organized in submicron domains at the cell surface. Nature

Veatch SL, Keller SL (2003) Separation of liquid phases in Giant vesicles of ternary mixtures of phospholipids and cholesterol. Biophys J 85(5):3074–3083. https://doi.org/10.1016/S0006-3495(03)74726-2

Wajant H (2006) Fas signaling. Landes Bioscience/Eurekah.com

Waugh MG, Lawson D, Hsuan JJ (1999) Epidermal growth factor receptor activation is localized within low-buoyant density, non-caveolar membrane domains. Biochem J 337:591–597

Way M, Parton RG (1995) M-caveolin, a muscle-specific caveolin-related protein. FEBS Lett 376:108–112

Wee P, Wang Z (2017) Epidermal growth factor receptor cell proliferation. Cancers:1–45. https://doi.org/10.3390/cancers9050052

Wei W, Schwaid AG, Wang X, Wang X, Chen S, Chu Q, Saghatelian A, Wan Y (2016) Ligand activation of ERRα by cholesterol mediates statin and bisphosphonate effects. Cell Metab 23(3):479–491. https://doi.org/10.1016/j.cmet.2015.12.010

Williams TM, Medina F, Badano I, Hazan RB, Hutchinson J, Muller WJ, Chopra NG, Scherer PE, Pestell RG, Lisanti MP (2004) Caveolin-1 gene disruption promotes mammary tumorigenesis and dramatically enhances lung metastasis in vivo: role of Cav-1 in cell invasiveness and matrix metalloproteinase (MMP-2/9) secretion. J Biol Chem 279(49):51630–51646. https://doi.org/10.1074/jbc.M409214200

Witkiewicz AK, Dasgupta A, Sotgia F, Mercier I, Pestell RG, Sabel M, Kleer CG, Brody JR, Lisanti MP (2009) An absence of stromal caveolin-1 expression predicts early tumor recurrence and poor clinical outcome in human breast cancers. Am J Pathol 174(6):2023–2034. https://doi.org/10.2353/ajpath.2009.080873

Wong SW, Kwon MJ, Choi AMK, Kim HP, Nakahira K, Hwang DH (2009) Fatty acids modulate toll-like receptor 4 activation through regulation of receptor dimerization and recruitment into lipid rafts in a reactive oxygen species-dependent manner. J Biol Chem 284(40):27384–27392. https://doi.org/10.1074/jbc.M109.044065

Wright SD, Ramos RA, Tobias PS, Ulevitch RJ, Mathison JC (1990) CD14, a receptor for complexes of lipopolysaccharide (LPS) and LPS binding protein. Science 249(4975):1431–1433

Wysoczynski M, Reca R, Ratajczak J, Kucia M, Shirvaikar N, Honczarenko M, Mills M, Wanzeck J, Janowska-Wieczorek A, Ratajczak MZ (2005) Incorporation of CXCR4 into membrane lipid rafts primes homing-related responses of hematopoietic stem/progenitor cells to an SDF-1 gradient. Blood 105(1):40–48. https://doi.org/10.1182/blood-2004-04-1430

Xu C, Zhang W, Rondard P, Pin JP, Liu J (2014) Complex GABAB receptor complexes: how to generate multiple functionally distinct units from a single receptor. Front Pharmacol, Frontiers Media SA 5(FEB). https://doi.org/10.3389/fphar.2014.00012

Yager JD, Davidson NE, Hopkins Bloomberg J (2006) Estrogen carcinogenesis in breast cancer. N Engl J Med 354:270–282. www.nejm.org

Yamada E (1955) The fine structure of the gall bladder epithelium of the mouse. J Biophys Biochem Cytol 1(3):117–122

Yu J, Fischman DA, Steck TL (1973) Selective solubilization of proteins and phospholipids from red blood cell membranes by nonionic detergents. J Supramol Struct

Zhao JJ, Cheng H, Jia S, Wang L, Gjoerup O v, Mikaini A, Roberts TM (2006) The p110α isoform of PI3K is essential for proper growth factor signaling and oncogenic transformation. Proc Natl Acad Sci U S A 103(44):16296–16300. https://doi.org/10.1073/pnas.0607899103

Zhuang L, Lin J, Lu ML, Solomon KR, Freeman MR (2002) Cholesterol-rich lipid rafts mediate AKT-regulated survival in prostate cancer cells. Cancer Res 62:2227–2231. http://aacrjournals.org/cancerres/article-pdf/62/8/2227/2502141/ch0802002227.pdf

Zhuang L, Kim J, Adam RM, Solomon KR, Freeman MR (2005) Cholesterol targeting alters lipid raft composition and cell survival in prostate cancer cells and xenografts. J Clin Investig 115(4):959–968. https://doi.org/10.1172/JCI200519935

Ziblat R, Fargion I, Leiserowitz L, Addadi L (2012) Spontaneous formation of two-dimensional and three-dimensional cholesterol crystals in single hydrated lipid bilayers. Biophys J 103(2):255–264. https://doi.org/10.1016/j.bpj.2012.05.025

Zidovetzki R, Levitan I (2007) Use of cyclodextrins to manipulate plasma membrane cholesterol content:

evidence, misconceptions and control strategies. Biochim Biophys Acta Biomembr 1768(6):1311–1324. https://doi.org/10.1016/j.bbamem.2007.03.026

Zuidscherwoude M, Göttfert F, Dunlock VME, Figdor CG, van den Bogaart G, van Spriel AB (2015) The tetraspanin web revisited by super-resolution microscopy *Scientific Reports*:5. https://doi.org/10.1038/srep12201

Zurzolo C, Van't Hof W, van Meer G, Rodriguez-Boulan E (1994) VIP21 /caveolin, glycosphingolipid clusters and the sorting of glycosylphosphatidylinositol-anchored proteins in epithelial cells. EMBO J 13(1):42–53

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Sounding a New Era in Biomechanics with Acoustic Force Spectroscopy

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Abstract

The acoustic force spectroscopy (AFS) tool was recently introduced as a novel tool for probing mechanical properties of biomolecules, expanding the application of sound waves to high-throughput quantification of the mechanical properties of single cells. By using controlled acoustic forces in the piconewton to nanonewton range, tens to hundreds of cells functionalized by attached microspheres can simultaneously be stretched and tracked in real-time with sub millisecond time response. Since its first application, several studies have demonstrated the potential and versatility of the AFS for high-throughput measurements of force-induced molecular mechanisms, revealing insight into cellular biomechanics and mechanobiology at the molecular level. In this chapter, we describe the operation of the AFS starting with the underlying physical principles, followed by a run-down of experimental considerations, and finally leading to applications in molecular and cellular biology.

Keywords

Acoustic force spectroscopy (AFS) · Acoustic radiation force · Acoustic standing waves · Adhesion kinetics · Adhesion strength · Biomechanics · Lab-on-a-chip · Mechanobiology · Microfluidic · Single biomolecule · Viscoelasticity

Abbreviations

AFS	Acoustic force spectroscopy
ECM	Extracellular matrix
HEK	Human embryonic kidney
IPSCs	Induced pluripotent stem cells
LUT	Lookup table
NA	Numerical aperture
PDMS	Polydimethylsiloxane
RGD	Tripeptide Arg-Gly-Asp

1 Introduction

Acoustic wave generating instruments are non-invasive, versatile, and cost-effective tools offering excellent potential applications in biological sciences, such as medical diagnostic

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imaging (Sarvazyan et al. 2013), drug delivery (Mitragotri 2005), and cell manipulation (Mulvana et al. 2013). Recently, it has been shown that acoustic waves can also be used to exert forces on single molecules, expanding their application in biomechanics and mechanobiology studies.

Biological processes are known to be regulated by the behaviour of cells in reaction to the external mechanical environment. Cells are the structural and functional units of all living organisms, which have characteristic mechanical properties (e.g. viscosity and elasticity) that determine the ability of cells to withstand and respond to mechanical forces from the surrounding environment. How cells respond to these external cues will influence their behaviour, generating a complex cascade of cellular signalling events characterized by conversion of mechanical stimuli into intracellular biochemical signals (Eyckmans et al. 2011; Huang et al. 2004). This process of mechanosensory transduction involves a hierarchy of molecular complexes, including the extracellular matrix, membrane lipid bilayer containing force-sensitive molecules, and cytoskeletal network. Understanding the role mechanical stimuli, such as shear force or stretch, play in cellular homeostasis has required the development of new generation of biophysical tools that allow for probing cellular and subcellular mechanical properties at high spatial and temporal resolution on a biologically relevant force scale. However, conventional approaches for evaluating molecular mechanics use to ‘average’ the response of a group of molecules, which results in loosing key details due to the inhomogeneity and stochasticity of the measured ensemble properties. The development of single-molecule manipulation techniques, such as optical tweezers, magnetic tweezers, and atomic force spectroscopy (Neuman and Nagy 2008), has significantly increased the ability to study mechanical properties at the single molecule level, allowing for detailed investigations of force-induced molecular mechanisms in many biological processes. By exerting external mechanical forces, the displacements and the local forces associated with biological molecules are measured with nanometre resolution, providing unprecedented amount of information about the molecular events underlying mechanotransduction processes (Sen and Kumar 2010). Recently, a novel single-molecule force spectroscopy tool, the acoustic force spectroscopy (AFS), has been shown to have multiple advantages over traditional measuring techniques (Kamsma et al. 2016). Importantly, acoustic standing waves generated in microfluidic channels are used to trap a large number of functionalized particles at the acoustic nodes which in turn simultaneously stretch individual tethered biomolecules. The non-invasive nature of acoustic waves and the high-throughput functionality are the two core strengths of the AFS system. Another key aspect of the AFS system is the range of the applied acoustic forces between sub-piconewtons and nanonewtons, which enables researchers to induce conformational changes in biomolecules and monitor related events. It has been shown that the AFS can be used to investigate the strength of DNA-protein interactions (Sitters et al. 2015) as well as the real-time assembly of a virus particle around a DNA (Van Rosmalen et al. 2020). More recently, the AFS was shown to have great potential in measuring forces at cellular level by probing viscoelastic properties of erythrocytes and human embryonic kidney (HEK) cells in response to application of different drugs (Sorkin et al. 2018; Romanov et al. 2021) or the stiffness modulation of endothelial cells subjected to physiological fluid shear stress (Silvani et al. 2021). In the following section, we describe principles of the AFS operation and provide details of critical experimental parameters for applications in molecular and cellular mechanics.

2 Principles of the AFS Operation

Integrated in a lab-on-a-chip device, the AFS system consists of a piezoelectric element excited by an oscillatory voltage which generates a standing acoustic wave within a microfluidic channel made of glass (Fig. 1).

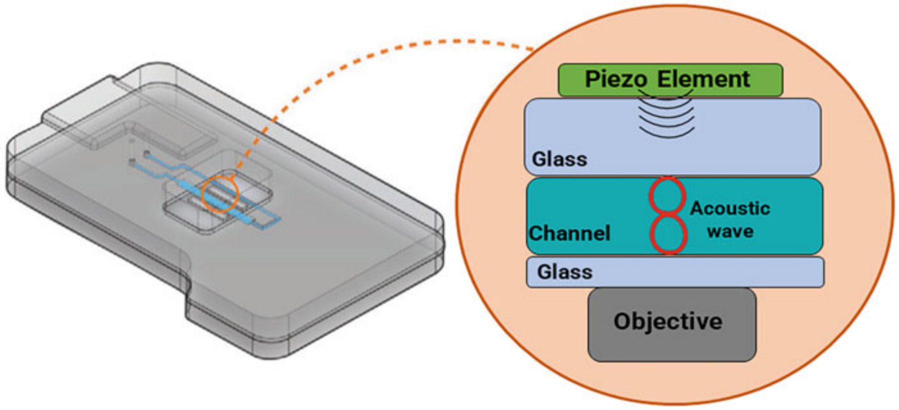

Fig. 1 General operational principle of the AFS system. A piezoelectric element is used to generate standing acoustic waves within a microfluidic channel of the experimental chip

Any particles (with volume V_p) floating into the channel having different density than the surrounding medium will experience an acoustic pulling force along the vertical (z) direction, which forces them to align at the acoustic node of the standing wave (Fig. 2a). The acoustic radiation force, F_{rad}, applied on each particle is described by the following equation:

$$F_{\text{rad}}(z) = -\nabla U_{\text{rad}}(z) \tag{1}$$

where U_{rad} is the energy of the acoustic wave defined as:

$$U_{\text{rad}} = V_p\left(\frac{1-k^*}{4}\kappa_f|P(z)|^2 - \frac{3}{4}\left(\frac{\rho^*-1}{2\rho^*+1}\right)\rho_f|v(z)|^2\right) \tag{2}$$

in which $P(z)$ is the acoustic pressure field, $v(z)$ is the velocity field, and ρ^* $(=\frac{\rho_p}{\rho_f})$ and k^* $(=\frac{k_p}{k_f})$ are the density ratio and compressibility ratio between the particle and the fluid, respectively (Settnes and Bruus 2012; Gor'kov 1962). The magnitude of the force applied to the microsphere can vary from the sub-piconewton to nanonewton and is determined by several factors, including the material and size of the microsphere, the medium inside the flow cell, the intensity of the acoustic wave, and the vibration of the piezoelectric element (Kamsma et al. 2018). Another way to look at this dependence is to take a force balance around a bead experiencing acoustic radiation within a fluid:

$$F_{\text{rad.}} + F_{\text{buoyancy}} - F_{\text{gravity}} - F_{\text{drag}} = 0 \tag{3}$$

$$F_{\text{rad}} = \left(v_p\gamma_{\text{brenner}}\right) - \frac{4}{3}\pi\, g\, r^3\left(\rho_p - \rho_f\right) \tag{4}$$

with

$$\gamma_{\text{brenner}} = \frac{6\pi\mu r}{1 - \frac{9r}{8h} + \frac{r^3}{2h^3} - \frac{57r^4}{100r^4} + \frac{r^5}{5h^5} + \frac{7r^{11}}{200h^{11}}} \tag{5}$$

where ρ_p and ρ_f are the density of particle and the fluid, respectively, μ is the viscosity of the media where particle is floating with a velocity v_p, r is the radius of the particle, and h is its height. The forces acting on a suspended particle in a fluid are the gravity force, the buoyancy force, the Stokes drag, and the acoustic radiation force (Fig. 2b). For a particle moving perpendicularly from the surface of the substrate at constant velocity, these forces cancel out, and the particle will experience an acoustic radiation force directly proportional to the drag force. Indeed, gravity and buoyancy forces are constant, while the drag force is directly related to the particle's velocity which in turn depends on the amplitude of the standing wave. Large amplitudes result in larger acoustic forces, thus leading to faster bead movement (↑v_p).

Holding the amplitude constant, material properties are the next major determinant of how

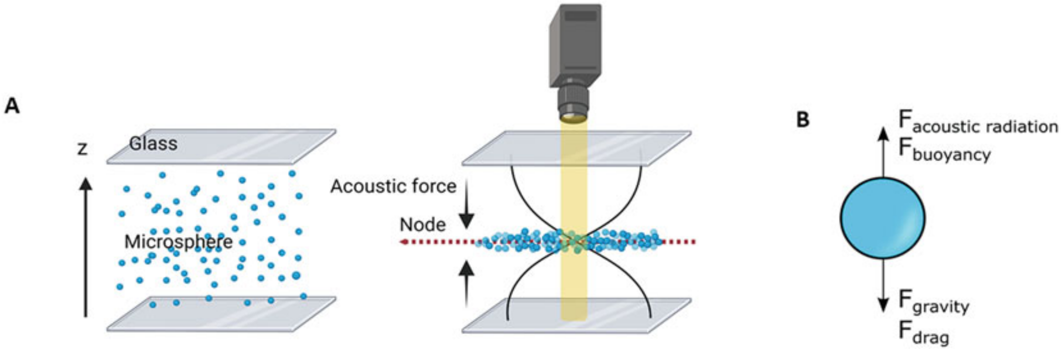

Fig. 2 Particle floating in a fluid upon the application of the acoustic radiation force. (**a**) Unbound particles will aggregate at nodal points throughout the chip. (**b**) A particle initially at rest will experience gravitational and buoyancy forces. Application of an acoustic force will exert radiation force on the particle, causing movement, leading to drag

much force an object will experience. A silica particle, moving through water (ρ_{water} = 1000 kg/m^3), with a density of 2000 kg/m^3 and a radius of 5 μm experiences a constant gravitational force of ~10 pN and a buoyancy force of ~5 pN. Hence, at least 5 pN of force is required to move the bead from the bottom of the channel. Referring to Eq. (4), the greater the difference between the densities of the object to the surrounding fluid, the greater the acoustic radiation force. Using the example above, $\rho_p - \rho_f = 2000 - 1000 = 1000$. Conversely, a red blood cell with a density of $\rho_{rbc} = 1110$ kg/m^3, surrounded by water, will have a difference of $\rho_p - \rho_f = 110$. Hence, to move a red blood cell with the same velocity as a silica bead, larger acoustic radiation force will need to be applied.

The microfluidic channel can be functionalized with several biologically interesting structures such as proteins or cells. Properties of either the protein or the cell can be probed via the attachment and movement of a functionalized bead (Fig. 3a), the latter being used as force transducers when excited by an acoustic wave at the proper frequency. The resonance frequency – the optimal excitation frequency of the system – is in MHz for a microsphere with diameter in the micrometre range. By tracking the microsphere's displacement using a digital camera and comparing the displacement of the microspheres with the magnitude of the applied force, hundreds of force/deformation curves are simultaneously collected using the AFS system and correlated with molecular structures, molecular bond strength, or the mechanical properties of a cell membrane. The slow deformation of a viscoelastic structure (Fig. 3b), such as a biological cell, is described by the creep function, which captures cell deformation as a function of constant force over a predefined time period.

First described in 2015 (Sitters et al. 2015), the AFS has been greatly improved, resulting in better optical and acoustic performance (Kamsma et al. 2016). First, a newly developed transparency piezo element improved the microsphere tracking accuracy as well as the measurable field of view, due to the compatibility of the AFS with trans illumination imaging. It has been shown that AFS can also be integrated with epifluorescence microscopes, in combination with high numerical aperture (NA) water and oil-immersion objectives, expanding its measurements applicability. However, this was only possible with a steep reduction in force magnitude (Kamsma et al. 2016). Second, the acoustic properties of AFS were improved by optimizing the thickness of the channel depth in order to generate a more efficient force at the bottom of the microfluidic channel, where cells and molecules are seeded. Finally, an external pump allows for complete

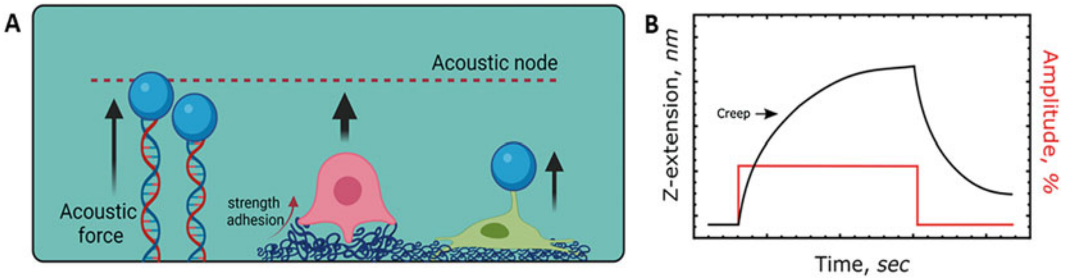

Fig. 3 Application of acoustic force spectroscopy in single-molecule and single-cell force measurements

control over fluid flow rate and pulsating conditions, better mimicking in vivo conditions (Silvani et al. 2021). Among the advantages, the AFS has several limitations. The optical setup determines the field-of-view, limiting the number of microspheres that can be tracked in real-time. Further, variations in bead size will affect the acoustic radiation force magnitude across the field-of-view. Also, prolonged measurements, at high operating voltages, can raise temperature, which may lead to sample heating. Nevertheless, at a maximum voltage of 42 V at 13 MHz applied for less than a few seconds, the resultant sample heating is less than 2 °C, a rise in temperature acceptable for most measurements of mechanical properties of cells, for example (Kamsma et al. 2018).

To better understand the functionality and applicability of AFS as a tool, the following section focuses on the experimental setup, providing details about critical AFS parameters, microsphere functionalization and tracking, and the model used to correlate deformational information to viscoelastic cell properties.

3 Experimental Setup and Analysis

3.1 Resonance Frequency

The resonance frequency is the optimal excitation frequency of the system. The resonance frequency depends on several factors, including channel geometry, temperature, and fluid properties. In the case of the AFS system, a small change in the thickness of the fluid layer will have minimal impact on the resonance frequency but can influence the resultant force output (Kamsma et al. 2016). Because the thickness of the microfluidic channel is constant, the main variable to consider is the temperature of the system. The AFS chip (Fig. 1) can be heated from room temperature up to about 40 °C. Each degree change in temperature changes the resonance frequency of the system.

The resonance frequency of an AFS chip can be determined by measuring the point of maximum displacement of a bead, measured from the surface of the channel. Each AFS chip comes pre-calibrated from the factory (water at 21 °C), using this frequency as the starting point, the actual resonance frequency is adjusted in steps of 0.01 MHz, either up or down (Fig. 4a). The change in resonance frequency as a function of increasing temperature is shown in Fig. 4b.

3.2 Temperature

Biological cells exhibit temperature-dependent properties, that is, temperature may drive changes in the overall elasticity and/or fluidity of the cell. These two properties are a measure of the viscoelasticity of the cell, the properties of which are also dependent on the cell area where the viscoelasticity is being measured. For example, viscoelasticity at the cell periphery may be predominantly driven by actomyosin cytoskeletal arrangement and may differ from the properties measured closer to the nucleus, where stresses are born not only by the nucleus but also by the cytoskeleton

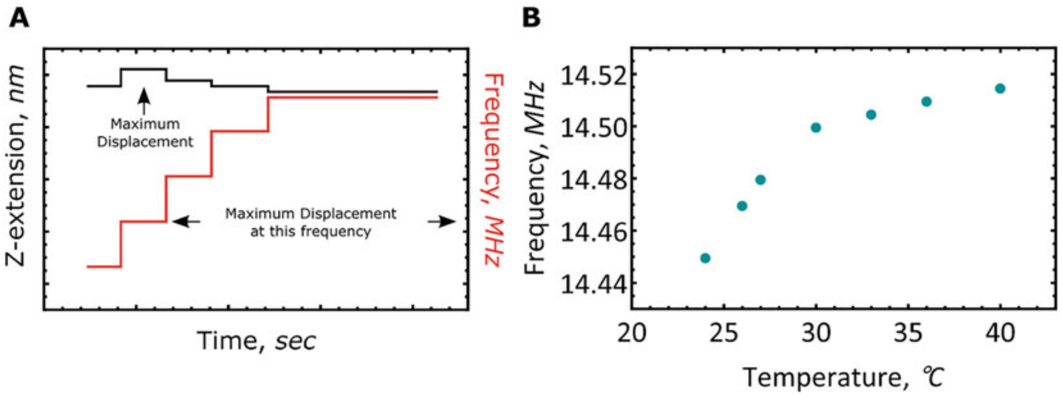

Fig. 4 Determining resonance frequency. (**a**) Maximum bead displacement as a function of frequency. The resonance frequency is the point at which the z-extension is maximum. Temperature is held constant. (**b**) Frequency vs temperature plot

(Sunyer et al. 2009). The absolute values of elasticity and fluidity are difficult to characterize as they are cell type dependent, influenced by passage number, cell cycle, media composition, etc. Several studies have been carried out to probe the effects of temperature on the viscoelasticity of several different cell lines with some studies showing an increase in stiffness and a decrease in fluidity, while other studies showing the opposite trend, a decrease in stiffness and an increase in fluidity (Romanov et al. 2021; Sunyer et al. 2009; Sunnerberg et al. 2019; Chan et al. 2014).

One potential issue with an acoustic driver is localized heating arising from the conversion of the mechanical energy into heat. Particularly relevant to the AFS measurements is the presence of a heatsink between the piezoelectric element-glue-glass and liquid layer within the microfluidic channel (Wiklund 2012). If thermal effects do arise, they tend to be present close to the surface of the glass channel, typically occupied by the object of study (DNA, cells, etc.). The greater the amplitude, the greater the chance of localized heating effects that may alter cell viscoelasticity (Nguyen et al. 2021), introducing a complex source of error. No systematic study utilizing the AFS system has been carried out to investigate the influence of increased amplitude, and hence temperature, on cell viscoelasticity.

3.3 Microsphere Coating

Depending on the experimental requirements, beads can be coated with a variety of molecules to promote adhesion to cells. Typically, fibronectin at a final concentration of 100 μg/mL is used for coating. Fibronectin can be used to coat both the glass microfluidic chip and the silica beads. The type and concentration of the coating is also dependent on the cell type. Different molecules can be used to study different aspects of the system. The physical properties (material and volume) of the beads play an important role in how the cell interacts with the bead. Smaller beads (<1 μm) have been shown to more rapidly undergo phagocytosis compared to larger beads (>6 μm) (Grinnell and Geiger 1986). While larger beads may not be phagocytosed to the same degree, partial ingestion or localized accumulation of actin patches have been demonstrated (Grinnell and Geiger 1986; Deng et al. 2005). The bead coating plays an important role in the cell structure that is ultimately probed by AFS or any method used for studies of molecular and cellular mechanics. For example, magnetic beads coated with RGD (peptide with sequence Arg-Gly-Asp) had the greatest effect on cytoskeletal rearrangement while also measuring stiffness values tenfold greater than the same beads coated with acetylated low-density lipoprotein (Puig-de-Morales et al. 2004).

Further consideration needs to be given to the coating of the microfluidic channel. We have found that fibronectin is sufficient for use with HEKT293T and endothelial cells (Romanov et al. 2021; Silvani et al. 2021). However, other extracellular matrix (ECM) components, such as collagen, laminin, and gelatin may sometimes be required to achieve the optimal attachment. Among those mentioned here, fibronectin and laminin are two of the most commonly employed ECM molecules that have been shown to greatly enhance attachment and proliferation of human-induced pluripotent stem cells (IPSCs) on polydimethylsiloxane (PDMS) surfaces, as compared to either collagen or gelatin (Yoshimitsu et al. 2014). Typically, these molecules are delivered to the surface of the substrate via diffusion and by chemical modification of the surface to promote binding. There are several limitations to these approaches including lack of control over patterning (non-uniformity), protein denaturing and mis-orientation, as well as protein density variations across the substrate (Cooke et al. 2008).

3.4 Microsphere Tracking

The AFS software utilizes a lookup table (LUT) for tracking the vertical displacement of particles within the microfluidic channel. The LUT is used to generate a z-stack of images, capturing the radial profile of the bead at pre-defined intervals. For typical cell work, 100 nm steps are used with a maximum height of 10 μm. The tracking accuracy will vary depending on experimental conditions (beads, cell type, buffer, etc.). For example, the z-positional resolution for microspheres in a simple buffer has been shown to be around 4 nm (Kamsma et al. 2016). However, in the presence of other objects, such as red blood cells, the resolution has been shown to reduce to about 20 nm (Sorkin et al. 2018). One of the main challenges with using the LUT is tracking of the bead over large distances. The tracking algorithm may clearly identify the bead in the first half of the LUT formation and then skip to a nearby object. As such, care should be taken to select the appropriate focus point on the bead, which typically is not the centre of the bead (Sarvazyan et al. 2013).

3.5 Determining Viscoelasticity

A variety of different models can be utilized to measure the creep response of cells. The linear viscoelasticity model utilizes springs to capture elastic responses and dashpots to capture viscous components. While simple and intuitive to use, the number of free-fit parameters makes it hard to assign a specific or a unique biological response to each parameter. On the other hand, the power law model contains two free-fit parameters, one describing the elasticity and the other describing the fluidity. The nature of the fluidity component is yet to be satisfactorily described, but a number of models have been proposed (Kollmannsberger et al. 2011a). In AFS experiments, we use the power law model to capture the creep response of cells under constant force loading conditions. In this experiment, the AFS is used to drive a bead to the acoustic node. If the bead is attached to the cell, the movement of the bead pulls a plasma membrane tether from the cell. The movement of the bead is tracked in real-time, yielding the strain.

The power law model describes the change in material compliance with time (Kollmannsberger et al. 2011a):

$$J(t) = J_0\left(\frac{t}{\tau}\right)^{\beta} \tag{6}$$

where J_0 is the material compliance, which is inverse of Young's modulus at time τ describing the normalizing time (t), usually set to 1 s, and β is the power-law exponent. Note that when β approaches zero, Eq. (6) will describe the deformation of a purely elastic material, which corresponds to the stiffness of the material, whereas when β approaches unity, then the equation describes the deformation of a purely viscous material having properties of a Newtonian fluid (Kollmannsberger et al. 2011b).

The creep compliance is fit to the above expression using (Kollmannsberger et al. 2011b):

$$J(t) = \frac{z(t)}{F} * \pi r \tag{7}$$

where $z(t)$ is the extension curve (z-height), F is the force, and r is radius of the particle. This expression is plotted as a function of time, and the power law model is then fitted to the resultant distribution allowing for the values of J_0 and β to be extracted for each cell.

In addition to the above model, fractional viscoelastic models for power law materials are also gaining popularity. Fractional models can accurately capture the response of viscoelastic materials across a number of time scales, using less parameters than traditional linear models. While the mathematical formalism is outside the scope of this article, we simply present the fractional model of creep compliance here:

$$J(t) = \frac{1}{c_\beta \Gamma(1+\beta)} t^\beta \tag{8}$$

where c_β represents the hardness of a material, Γ is the gamma function, t is time, and β is the power law exponent, where if $\beta = 0$, the material reduces to a simple elastic spring while for $\beta = 1$ the material reduces to a dashpot. For more information, we point the reader to this excellent review (Bonfanti et al. 2020).

4 Applications: Single-Cell and Single-Molecule Force Spectroscopy

Acoustic force spectroscopy is the latest addition to the myriad of other tools available for measuring the viscoelastic properties of single cells. Here, we aim to familiarize the reader with the various specific applications of the AFS system.

4.1 Measuring DNA Binding Activity

The AFS instrument was first used to demonstrate the stochasticity and binding activity of RecA filament protein to DNA (Sitters et al. 2015). As RecA activity is driven by DNA tension, binding and unbinding events under different loading conditions drive changes in tension, thus resulting in altered DNA extension profiles (Sitters et al. 2015). More recently, Van Rosmalen et al. (2020) utilized the AFS to follow, in real-time, assembly of virus-like particles (VLP) on DNA templates. Assembly of virus-like particles was monitored by comparing the force distribution on a bead tethered to DNA and its extension over time. Incubation of DNA + WT fragments of VLPs resulted in both reduction in the force and extensional distance of DNA.

4.2 Measuring Non-adherent Cells

Red blood cells circulate in the circulatory system throughout the body. The passage of a red blood cell through tight constrictions is driven by its ability to deform and to regain shape while retaining function. Diseases such as malaria have been shown to alter the deformability of red blood cells, making them stiffer. In one of the first demonstrations, the AFS was used to measure the mechanical properties of these circulating cells (Sorkin et al. 2018). The instantaneous elastic elongation (F/k_1 where k_1 is a spring constant in Burger's model) of red blood cells was used as a measure of elasticity and a way to differentiate between different pharmacological treatments. The AFS was used to demonstrate quantifiable differences in the elastic properties of red blood cells exposed to 0.04% formaldehyde.

4.3 Measuring Cell Adhesion Strength

The AFS is typically used to drive a bead attached to the object of interest. However, the same principles can be applied to moving the object of interest (if not immobilized on the surface). Kamsma et al. (2018) demonstrated an approach for measuring the binding kinetics and adhesion forces of CD4$^+$T lymphocytes to fibronectin. A linear force ramp was employed to measure the binding strength starting from ~10 pN up to ~100 pN. Importantly, it was demonstrated that around 300 cells could be tracked in real-time, simultaneously.

4.4 Measuring Adherent Cells

Recently, several articles have been published looking at the viscoelastic properties of adherent cells. Romanov et al. (2021) explored the creep response of adherent cells by measuring their elasticity and fluidity. The AFS instrument was used to measure the viscoelasticity of HEK293T cells under a variety of conditions and in the process demonstrating the effects of temperature, force, and pharmacological treatments on cell elasticity and fluidity. Significantly, changes in elasticity of cells overexpressing a mechanosensitive ion channel protein, Piezo1, could also be captured by the AFS system.

While the above study was performed under static conditions, dynamic perfusion is required for the formation of functional, mature endothelial cell monolayers. Silvani et al. (2021) developed further protocols and methods for characterizing the viscoelastic properties of two different types of endothelial cell lines, human aortic and human umbilical vein endothelial cells. Cells were cultured within the AFS chips for a period of 48 h under a constant flow rate of 6 dyn/cm^2. The authors reported immunofluorescent staining protocols and an approach for quantifying force-dependent stiffness of the membrane cortex.

Nguyen et al. (2021) expanded the utility of the AFS system by developing a new technique for frequency-dependent measurements of the viscoelasticity properties of human umbilical vein endothelial cells. The complex shear modulus was measured over a period of several hours after exposing cells between 70 and 100 h of fluid flow at 1.66 μL/min. Several shortcomings in the fundamental operation of the AFS instrument were also identified relating to non-homogenous force distribution within a single field of view, potential heating effects, and the overall impact of media properties, frequency, and temperature on the final force distribution.

5 Conclusions

The AFS instrument is just one of several techniques that employ sound waves to interact with physical objects. The power behind this approach lies in its ability to generate small (>1 pN) and large (> 1 nN) forces that interact with objects in a high-throughput manner, where hundreds or even thousands of objects can be moved and tracked in real time. As such, the AFS system is an excellent case study for the use of ultrasound waves in biology. Although a recent innovation, this technique has been successfully used to study DNA binding events and to characterize the viscoelasticity of a variety of different cell types exposed to many different physical and chemical conditions. We envision that future studies using AFS instrument and future advancements of the system will allow for deeper insights into the mechanobiology behind biological processes.

References

Bonfanti A, Kaplan JL, Charras G, Kabla A (2020) Fractional viscoelastic models for power-law materials. Soft Matter 16(26):6002–6020

Chan CJ, Whyte G, Boyde L, Salbreux G, Guck J (2014) Impact of heating on passive and active biomechanics of suspended cells. Interface Focus 4(2): 20130069

Cooke MJ, Phillips SR, Shah DS, Athey D, Lakey JH, Przyborski SA (2008) Enhanced cell attachment using a novel cell culture surface presenting functional domains from extracellular matrix proteins. Cytotechnology 56(2):71–79

Deng L, Fairbank NJ, Cole DJ, Fredberg JJ, Maksym GN (2005) Airway smooth muscle tone modulates mechanically induced cytoskeletal stiffening and remodeling. J Appl Physiol 99(2):634–641

Eyckmans J, Boudou T, Yu X, Chen CS (2011) A hitchhiker's guide to mechanobiology. Dev Cell 21(1):35–47

Gor'kov LP (1962) On the forces acting on a small particle in an acoustical field in an ideal fluid. Sov Phys Dokl 6: 773–775

Grinnell F, Geiger B (1986) Interaction of fibronectin-coated beads with attached and spread fibroblasts: binding, phagocytosis, and cytoskeletal reorganization. Exp Cell Res 162(2):449–461

Huang H, Kamm RD, Lee RT (2004) Cell mechanics and mechanotransduction: pathways, probes, and physiology. Am J Phys Cell Phys 287(1):C1–C1

Kamsma D, Creyghton R, Sitters G, Wuite GJ, Peterman EJ (2016) Tuning the music: acoustic force spectroscopy (AFS) 2.0. Methods 1(105):26–33

Kamsma D, Bochet P, Oswald F, Alblas N, Goyard S, Wuite GJ, Peterman EJ, Rose T (2018) Single-cell acoustic force spectroscopy: resolving kinetics and strength of T cell adhesion to fibronectin. Cell Rep 24(11):3008–3016

Kollmannsberger P, Mierke CT, Fabry B (2011a) Nonlinear viscoelasticity of adherent cells is controlled by cytoskeletal tension. Soft Matter 7(7):3127–3132

Kollmannsberger P, Mierke CT, Fabry B (2011b) Nonlinear viscoelasticity of adherent cells is controlled by cytoskeletal tension. Soft Matter 7(7):3127–3132

Mitragotri S (2005) Healing sound: the use of ultrasound in drug delivery and other therapeutic applications. Nat Rev Drug Discov 4(3):255–260

Mulvana H, Cochran S, Hill M (2013) Ultrasound assisted particle and cell manipulation on-chip. Adv Drug Deliv Rev 65(11–12):1600–1610

Neuman KC, Nagy A (2008) Single-molecule force spectroscopy: optical tweezers, magnetic tweezers and atomic force microscopy. Nat Methods 5(6):491–505

Nguyen A, Brandt M, Muenker TM, Betz T (2021) Multi-oscillation microrheology via acoustic force spectroscopy enables frequency-dependent measurements on endothelial cells at high-throughput. Lab Chip 21(10):1929–1947

Puig-de-Morales M, Millet E, Fabry B, Navajas D, Wang N, Butler JP, Fredberg JJ (2004) Cytoskeletal mechanics in adherent human airway smooth muscle cells: probe specificity and scaling of protein-protein dynamics. Am J Phys Cell Phys 287(3):C643–C654

Romanov V, Silvani G, Zhu H, Cox CD, Martinac B (2021) An acoustic platform for single-cell, high-throughput measurements of the viscoelastic properties of cells. Small 17(3):2005759

Sarvazyan AP, Urban MW, Greenleaf JF (2013) Acoustic waves in medical imaging and diagnostics. Ultrasound Med Biol 39(7):1133–1146

Sen S, Kumar S (2010) Combining mechanical and optical approaches to dissect cellular mechanobiology. J Biomech 43(1):45–54

Settnes M, Bruus H (2012) Forces acting on a small particle in an acoustical field in a viscous fluid. Phys Rev E 85(1):016327

Silvani G, Romanov V, Cox CD, Martinac B (2021) Biomechanical characterization of endothelial cells exposed to shear stress using acoustic force spectroscopy. Front Bioeng Biotechnol 4(9):21

Sitters G, Kamsma D, Thalhammer G, Ritsch-Marte M, Peterman EJ, Wuite GJ (2015) Acoustic force spectroscopy. Nat Methods 12(1):47–50

Sorkin R, Bergamaschi G, Kamsma D, Brand G, Dekel E, Ofir-Birin Y, Rudik A, Gironella M, Ritort F, Regev-Rudzki N, Roos WH (2018) Probing cellular mechanics with acoustic force spectroscopy. Mol Biol Cell 29(16):2005–2011

Sunnerberg JP, Moore P, Spedden E, Kaplan DL, Staii C (2019) Variations of elastic modulus and cell volume with temperature for cortical neurons. Langmuir 35(33):10965–10976

Sunyer R, Trepat X, Fredberg JJ, Farre R, Navajas D (2009) The temperature dependence of cell mechanics measured by atomic force microscopy. Phys Biol 6(2): 025009

Van Rosmalen MG, Kamsma D, Biebricher AS, Li C, Zlotnick A, Roos WH, Wuite GJ (2020) Revealing in real-time a multistep assembly mechanism for SV40 virus-like particles. Sci Adv 6(16):eaaz1639

Wiklund M (2012) Acoustofluidics 12: biocompatibility and cell viability in microfluidic acoustic resonators. Lab Chip 12(11):2018–2028

Yoshimitsu R, Hattori K, Sugiura S, Kondo Y, Yamada R, Tachikawa S, Satoh T, Kurisaki A, Ohnuma K, Asashima M, Kanamori T (2014) Microfluidic perfusion culture of human induced pluripotent stem cells under fully defined culture conditions. Biotechnol Bioeng 111(5):937–947

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Drug Therapeutics Delivery to the Salivary Glands: Intraglandular and Intraductal Injections

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Abstract

Salivary gland hypofunction and xerostomia following pathological conditions like Sjogren's syndrome or head and neck radiotherapy usually lead to tremendous impairment of oral health, speech, and swallowing. The use of systemic drugs to alleviate the symptoms of these conditions has been associated with various adverse effects. Techniques of local drug delivery to the salivary gland have grown enormously to address this problem properly. The techniques include intraglandular and intraductal injections. In this chapter, we will provide a review of the literature for both techniques while incorporating our lab experience in using them.

Keywords

Drug therapeutics · Head and neck radiotherapy · Intraductal · Intraglandular · Salivary gland · Salivary hypofunction · Sjogren's syndrome · Xerostomia

Abbreviations

AdMSCs	Adipose tissue derived mesenchymal stem cells
ALS	Amyotrophic lateral sclerosis
AMI	Amifostine
AMY	Alpha-amylase
BMSC	Bone marrow stem cells
BoNT, BTXA	Botulinum toxin
FDA	Food and Drug Administration
H&E	Hematoxylin and eosin
ID	Intraductal
IG	Intraglandular
IV	Intravenous
MSCs	Mesenchymal stem cells
NSSVAC	Immortalized human salivary gland acinar cells
PBS	Phosphate-buffered saline
PEG	Poly(ethylene glycol)
PLGA	Poly(lactic-co-glycolic acid)
RI	Rho kinase inhibitor
SDS	Sodium dodecyl sulfate
SGs	Salivary glands
SMG	Submandibular salivary gland

1 Introduction

Various disorders can affect the salivary glands (SGs) and they range from infections, obstructions, and tumors to autoimmune diseases (Ogle 2020).

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Despite the advances in systemic drug administration, it is still not perfectly reliable in terms of efficiency and associated adverse side effects (Muthumariappan et al. 2019; Diggelmann and Hoffman 2015; Teymoortash et al. 2007; Lynggaard et al. 2022). Recently, several local delivery approaches have been conducted for the treatment of salivary gland diseases. This review addresses these efforts using intraglandular (IG) and intraductal (ID) injections. For each approach, the methodology, targeted salivary cells or glands, the used drug, and the efficiency are fully discussed.

2 Intraglandular (IG) Injection for Drug Delivery

Intraglandular (IG) injections involve the process of injecting medication and various other cell therapies into the SGs. The advantage of this injection technique is that it allows for direct access to the SGs as well as reduction of the systemic side effects that are associated with many therapies. It is also less time-consuming and requires less of a dosage to be injected. The objectives of this section are:

1. To discover the different methods of IG injections that have been employed so far and the SGs they have targeted.
2. To review the literature on the different drugs and gene therapeutics that are currently being used or under development and their safety and efficiency.

In addition to the above objectives, we would like to introduce a novel transcutaneous non-surgical IG injection technique that can be used to deliver drugs to the submandibular SGs and how we intend to use this technique with radioprotective drug, amifostine (AMI).

2.1 Methods

IG injection is usually given surgically or non-surgically. The surgical route is primarily used for the submandibular SGs and involves a conservative horizontal incision in the neck area to expose and localize the SGs of interest (Tran et al. 2013). The non-surgical route, however, is the more common route that is employed, usually for the treatment of sialorrhea with botulinum toxin (Mueller et al. 2022). Injections are currently most performed using an ultrasound-guided approach to view the site of injection in the SGs (Mueller et al. 2022). Non-ultrasound-guided IG injections have been used earlier but have fallen out of contention due to less accuracy. A study by So et al. compared the accuracy between ultrasound-guided and non-ultrasound-guided IG injections by visual confirmation of dye accumulation in the submandibular and parotid SGs (So et al. 2017). They found that there was a marked reduction in the accuracy of the non-ultrasound-guided injections in comparison to the ultrasound-guided approach (So et al. 2017).

2.1.1 IG Non-surgical Transcutaneous Approach

Recently, our laboratory has been working on a novel IG technique for the submandibular glands (SMGs) to deliver therapeutic agents to mitigate radiation injury to the SGs in mice. The rationale behind developing this technique was to investigate a less invasive and localized method of delivering therapeutics directly to the submandibular SGs to mitigate side effects induced by systemic administration. Moreover, ultrasound-guided injections are usually operator dependent and require an experienced radiologist, which is not possible in many cases. Our laboratory, therefore, looks to develop an anatomical landmark-guided IG technique. To localize the right and left submandibular SGs, the mice were anaesthetized with a ketamine cocktail, and the hair was shaved in the neck region from the chest wall to the lower border of the mandible. A midline was drawn from the lower lip border passing through the inferior border of the mandible up to the chest wall. An incision was made to expose the right and left SMG, and the distance between the midline and the center of the gland was measured to determine the injection site. The mice were sacrificed, the glands were then harvested, and the thickness was measured using an endodontic file with a rubber stopper to mark on the syringe to be used for the injection (Fig. 1).

According to our preliminary results, the mean total distance of the midline from the lower lip to the chest wall was 1.9 cm, and on exposure of the SGs, the center of the gland was located 0.9 cm from the chest wall and 0.2 cm lateral to the midline. This meant that the center of the SGs was located at half the distance of the midline. As the gland thickness was 0.2 cm, and the thickness of the mouse skin was 0.1 cm, we concluded that the injection depth is 0.3 cm. Using the measurements above, the IG injection was verified by injecting Trypan Blue dye at a 90° angle to ensure that the drug reached the maximum areas of the gland. It was found that the Trypan Blue solution entered the gland at a success rate of 80%. While the success rate is encouraging, we do anticipate that there will be limitations. Due to the health or size of the mice, there could be variations in the SG location measurements, which could affect the area of administration. It also remains to be seen if there will be any difference if substances are injected in the center versus other areas of the gland, thus necessitating further studies.

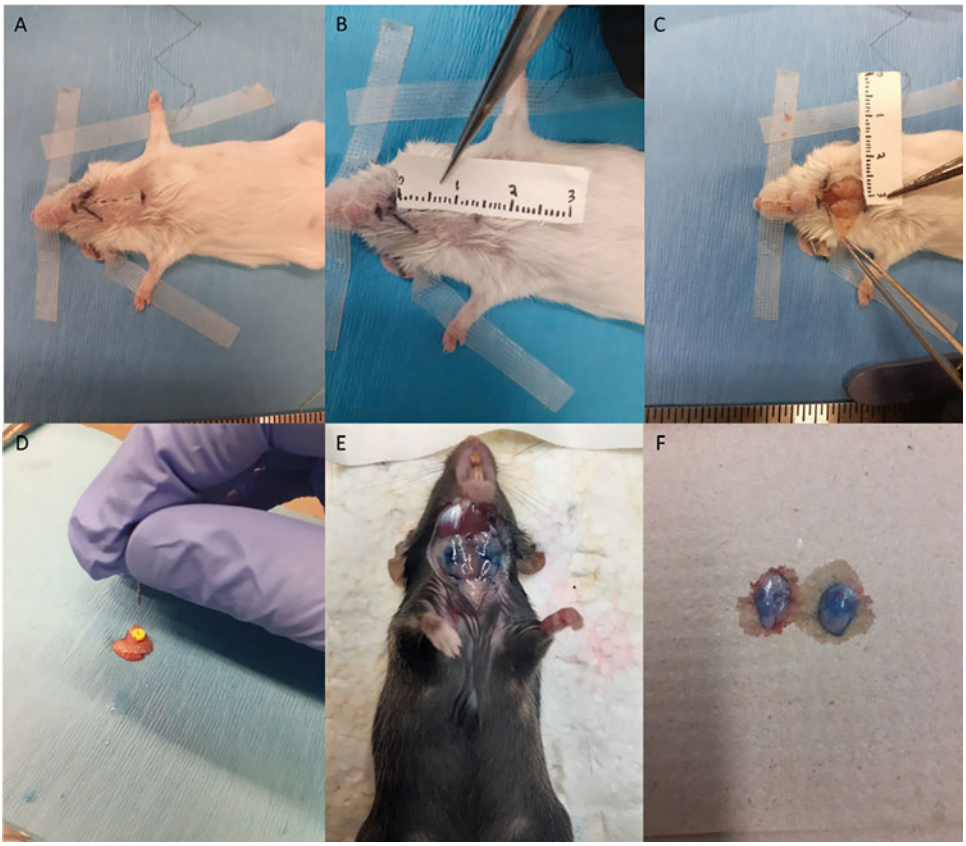

Fig. 1 Demonstration of IG injection using a mouse animal model. (**a**) The mouse's neck was shaved and outlined. (**b**) The distance from the base of the mandible to the sternum was measured. (**c**) The submandibular salivary glands were exposed and the distance from the neck midline to the center of the salivary gland was measured. (**d**) The thickness of the salivary gland was measured. (**e**) A mouse with both SMGs that were exposed after transcutaneous injection. (**f**) The glands were harvested to examine their saturation with the injected blue dye

2.2 Targeted Glands

IG injections have traditionally been employed in the parotid and SMGs; however, there are no reports of the involvement of the sublingual SG or minor SGs. Most studies that involve the injection into the parotid use non-ultrasound IG injections based on superficial landmarks, whereas ultrasound-guided injection is used for the SMGs (So et al. 2017). The injection site for the parotid is usually marked behind the ascending ramus or before the mastoid process (So et al. 2017; Jost et al. 2019). In the SMG, a reference line is made from the gnathion to the mandibular angle, following which, the injection site is marked at a distance that is 20–35% from the angle, 1.5 cm below the inferior mandible (So et al. 2017). In the ultrasound-guided approach for the SMG, the IG injection is given from a lateral approach, where the head of the patient is facing away from the injection site and the needle is inserted perpendicular to the ultrasound transducer (So et al. 2017).

2.3 Drugs and Agents Used

1. *Botulinum Toxin*

Botulinum toxin A (BoNT) is an FDA-approved drug that is indicated for aesthetic as well as clinical applications (Sherif et al. 2018). It is a neurotoxin that is produced by *Clostridium botulinum* and was first used for the treatment of strabismus and then subsequently, for disorders involving spasticity and cosmetic purposes (Nigam and Nigam 2021; Scott 1981). Bushara et al. in 1997 were the first group of researchers to use it in the treatment of sialorrhea or excessive salivation through surgical IG administration (Bushara 1997). It has, since then, been employed in neurological disorders such as Parkinson's disease, amyotrophic lateral sclerosis (ALS), and cerebral palsy, all of which have clinical features of sialorrhea (Fuster Torres et al. 2007). Traditionally, BoNT has been injected in either the parotid gland only, the submandibular gland only, or both glands, and it is still unclear as to which of these three scenarios is most effective (Ellies and Laskawi 2010).

In addition to Bushara's study, BoNT first displayed its effects of alleviating sialorrhea in patients with Parkinson's disease in a study by Pal et al., with minimal side effects and through intra-parotid injections (Pal et al. 2000). The potential of BoNT was further studied in a meta-analysis where it was found to significantly reduce drooling in both adults and patients with sialorrhea (Vashishta et al. 2013). Further studies confirmed the effectiveness of BoNT in treating sialorrhea by examining the histological changes in the SGs. Teymoortash et al. examined the structural and functional changes of IG administration of BoNT in acinar cells in rats. They found that there was atrophy of the acini, which they concluded was because of glandular denervation, explaining the reduced salivary secretion (Teymoortash et al. 2007). This was confirmed in a more recent study by Sherif et al., who observed that the acini lost their spherical morphology in the group of mice that received BoNT; however, they also noted that the morphology was recovered over time (Sherif et al. 2018). Recent studies have interestingly shown that while BoNT has uses in reducing salivary secretion, it has potential applications in radioprotection. Ultrasound-guided IG administration was able to offer radioprotection for both submandibular and parotid glands with minimal side effects. A clinical trial showed that injection of BoNT preserved gland function in patients undergoing radiotherapy for head and neck cancer, and further studies should explore the safety and efficacy of IG BoNT (Teymoortash et al. 2016). BoNT is also associated with some disadvantages, such as high costs and the development of antibodies against the botulinum toxin (Lakraj et al. 2013). Studies have shown that although uncommon, BoNT injections could lead to an increase in salivary thickness, dysphagia, pneumonia, and xerostomia (Vashishta et al. 2013; Lakraj et al. 2013).

2. *Stem Cell Transplantation*

A novel area of research includes the IG administration of stem cells to mitigate SG hypofunction, mainly due to radiotherapy. IG administration with stem cells has so far been employed in the submandibular SG through the surgical approach. Lombaert et al. were one of the pioneers in initiating the importance of stem cell transplantation into the salivary glands, by identifying markers that determined stem cell differentiation into acinar and ductal cells (Lombaert et al. 2008). Building on this concept, they were able to further enrich the cells using a progenitor cell marker, *c-kit*, to promote long-term restoration of an irradiated submandibular mouse SG in vivo (Lombaert et al. 2008). These findings contributed to future studies using different types of stem cells to be transplanted in the SGs through the IG route (Nanduri et al. 2013; Isabelle et al. 2013).

Bone marrow stem cells (BMSCs) and their extracts are an emerging research area for stem cell transplantation. Differentiation of BMSCs into salivary gland acinar cells have been observed, and the school of thought is that this is due to the secretion of paracrine factors like cytokines and growth factors, which are required for tissue regeneration and repair (Fang et al. 2015). While studies have displayed the success of BMSCs when given intravenously (IV), IG administration is still poorly understood (Fang et al. 2015; Tsutsui 2020). One of the first studies to test BMSCs on SGs was by Tran et al., where they showed that IG administration of BMSC from mice was able to restore SG hypofunction when administered post-irradiation (Tran et al. 2013). The IG method was compared to the IV approach and found that the number of injections and dosage to provide the same effects was significantly reduced (Tran et al. 2013). This laid the foundation for future studies on BMSC and more recent studies using mouse BMSC extracts agreed with previous findings, showing that they were able to ameliorate SG hypofunction post-IR at different time points when given IG (Lim et al. 2013; Mohamed et al. 2022). A study by Schwarz et al. also compared the IG administration of BMSC to intravenous (IV) administration in a surgical model of submandibular SG damage in mice and found that the presence of MSCs, leucocytes, and macrophages was more enhanced in the damaged SG when given IG, thus demonstrating the potential of this injection technique (Schwarz et al. 2014). In addition, a study by Rabea et al. demonstrated the regenerative capacity of BMSCs when given IG to the parotid glands of rats, which shows that the IG technique using stem cells could be useful for parotid glands as well (Rabea et al. 2022). A very recent study found that BMSCs derived from humans could also prevent SG hypofunction in mice post-IR when given IV, which has created avenues for testing BMSCs derived from humans for IG administration (Su et al. 2018).

The IG approach to preventing SG hypofunction has also been tested with stem cells from different sources. Adipose tissue MSCs (AdMSCs) have also been explored for tissue regeneration studies and are known to maintain characteristics of multipotent progenitor cells (Kim et al. 2019). Kim et al. examined whether AdMSCs could restore radiation-induced SG hypofunction by IG injection in mice and found that there were elevated expressions of epithelial markers, suggesting that localized delivery could regenerate SG damage (Kim et al. 2019). This was confirmed by Wang et al., who showed that AdMSCs along with plasma-derived fibrin extracts could restore SG hypofunction when given IG post-IR (Wang et al. 2017). Dental-pulpal stem cells have also been tested for their capacity to regenerate SG defects and are known to be less invasive to isolate than BMSC. A study on rats with induced-diabetic SG defects showed that when dental-pulpal stem cells were given by IG transplantation, they could restore the defects through reduced vacuolization of acinar cells and an increase in serum markers (Narmada et al. 2019). A unique study isolated and cultured stem cells from minor salivary glands (labial glands) in mice and injected them IV and found that they could mitigate injury to the SGs, which opens avenues for testing this method by IG administration (Su et al. 2020).

The success of stem cell transplantation has culminated with the start of in-human studies using the IG technique. Sumita et al. is currently performing the first-in-human transplantation of mononuclear cells using ultrasound-guided IG injection to submandibular SGs to mitigate injury due to irradiation (Sumita et al. 2020). It is expected to be the first instance of using a less invasive cell-based therapy for SG regeneration. Comella et al. performed the first-in-human clinical trial wherein they delivered AdMSCs with plasma-derived extracts by ultrasound-guided IG injection to both the parotid and submandibular glands and found that it improved the quality of life of patients undergoing radiotherapy as well as an increase in gland size (Comella and Bell 2017). A Danish-based clinical trial (MESRIX) is currently underway to test the effects of allogeneic AdMSCs to treat radiation-induced xerostomia through the IG injection (Lynggaard et al. 2022; Grønhøj et al. 2017). They have so far shown that it can be a feasible method of injection into the parotid and submandibular SGs with few adverse effects months after treatment and there was a significant increase in stimulated and unstimulated salivary flow rates (Lynggaard et al. 2022; Grønhøj et al. 2017). Positive results have shown that stem cell transplantation using the IG technique can be further investigated in larger clinical trials safely and effectively.

3. *IG Administration of Amifostine (AMI) to Prevent Radiation-Induced SG Injury*

Amifostine (AMI) is the only FDA-approved radioprotective drug to prevent radiation-induced xerostomia. Although its protective mechanism remains unclear, theories suggest that AMI scavenges oxygen-derived free radicals. It is usually administered 30 min before IR IV at a dose of 200 mg/m^2; however, it induces side effects like vomiting, nausea, and hypotension within 1 h of administration (King et al. 2020). Our laboratory is currently investigating whether the IG administration of AMI can provide comparable radioprotection to the SGs with reduced side effects. We plan to use the non-surgical transcutaneous IG injection technique to the submandibular SGs in mice to test AMI. The IG method of administering AMI will allow for more direct and less invasive access to the SGs, which could potentially limit side effects associated with the systemic administration. Also, AMI has shown to act synonymously with bone marrow stromal cells and suppress the effects of radiation, which could be explored in SGs as well using stem cell transplantation (Huang et al. 2019).

2.4 Safety and Efficacy of IG Injection

Initially, direct IG injection, using a surgical incision exposing the gland, was applied for various treatment modalities. The first study for BoNT injection into the parotids by Bushara et al. was performed using the surgical method but they noted that it could be associated with potential complications such as injury to the carotid artery or facial nerve branches (Bushara 1997). There was also the question of whether these injections could be well-tolerated in the pediatric population as well as the cost factor (Lakraj et al. 2013). Studies involving IG stem cell transplantations in mice also involved surgical incision into the mouse submandibular glands, and while they mention that it is relatively well-tolerated, they acknowledge that improved injection techniques may be useful (Lombaert et al. 2008).

The introduction of ultrasound-guided IG injections reduced the invasiveness of the surgical technique. BoNT injections for the treatment of sialorrhea have been approved as a mainstay of treatment with ultrasound guidance, and studies have shown that it was well-tolerated in general (Teymoortash et al. 2007). Especially in the case of providing radioprotection, it was found that IG BoNT through ultrasound guidance did not interfere with the radio-chemotherapy treatment in patients (Teymoortash et al. 2007; Mueller et al. 2022). There were also minimal incidences of facial weakness or dysphagia, both of which could be seen with the surgical IG technique (Mueller et al. 2022). However, further larger studies assessing the efficacy of the ultrasound technique in gland preservation are required (Teymoortash et al. 2007). Ultrasound-guided IG injection to deliver stem cells is also gaining importance, and recent clinical trials are starting to show strong safety profiles, thus warranting larger studies to assess efficacy and feasibility (Lynggaard et al. 2022; Comella and Bell 2017; Grønhøj et al. 2017).

It is still unclear as to whether the non-surgical IG technique is better performed with or without ultrasound guidance. So et al. demonstrated comparisons between the ultrasound- and non-ultrasound-guided IG injections to determine which would be a safer and more accurate method (So et al. 2017). There was a significant difference in accuracy while injecting the parotid glands, with the ultrasound guidance being the more accurate method; however, there was no significant difference in accuracy while injecting the submandibular gland (So et al. 2017). Therefore, further studies confirming whether ultrasound guidance is required for IG injections are required, especially since ultrasound can be largely operator-dependent and expensive. Our laboratory will look to address this with the development of a novel non-surgical transcutaneous IG technique for the submandibular glands.

3 Intraductal (ID) Drug Delivery

Intraductal (ID) infusion of the salivary glands is regularly performed with sialography (Song and Lee 2014). During this imaging technique, contrast agents are taken up in the gland ducts and acini producing a parenchymal clouding (Schwalje and Hoffman 2019). A water-based contrast has been shown to reach the intercellular spaces and the basal surface of the acini cells and connective tissue (Qwarnström 1986). Su et al. evaluated the effective drug delivery route in the management of SG diseases using a porcine cadaver and methylene blue and found it was evenly distributed throughout the salivary gland (Su et al. 2017). The authors suggested that "intraductal injections might serve as a potential therapeutic procedure in the management of salivary gland disease." (Schwalje and Hoffman 2019) Using this approach limits the use of systematic drugs with their inevitable side effect. It will also maximize the potential therapeutic effect by delivering a maximum dose to the gland only (Haroun and Brem 2000). The potential therapeutic application includes sialorrhea, sialadenitis, and Sjogren syndrome (Su et al. 2017).

3.1 Method

The ID infusion has been well performed for both parotid and submandibular salivary glands. Patients with active infection or inflammation are contraindicated from receiving this treatment. They are usually asked to attend well-hydrated and given a prophylactic antibiotic as indicated (Schwalje and Hoffman 2019). The patients are positioned semi-recumbent in preparation for the procedure (Schwalje and Hoffman 2019). For the parotid salivary gland, Stensen's duct papillae are identified in humans opposite to the second upper molar using a salivary duct probe. For the submandibular gland, Wharton's submandibular duct is identified in the floor of the mouth midway between the tongue ventral surface and lower incisors (Carlson 2000). Lidocaine-soaked gauze is applied to the duct papilla for topical anesthesia. The duct orifice is then dilated gradually with duct dilators (Su et al. 2017). A sialendoscopy might be used for proper viewing and drug infusion (Strychowsky et al. 2012). It is recommended to irrigate the gland with 1 ml of sterile saline or water before the drug infusion (Diggelmann and Hoffman 2015; Strychowsky et al. 2012). A 3 ml of the drug is then infused into the gland. A catheter might be introduced to keep the duct patent and left in place with a syringe attached for 2 min to prevent the immediate escape of the drug (Diggelmann and Hoffman 2015).

Clinically, dilation of the duct orifice might be difficult in patients with chronic submandibular sialadenitis and chronic parotitis (Zenk et al. 2009). Additionally, repeated traumatic attempts of cannulization may raise the risk of orifice stricture (Antoniades et al. 2004). However, this difficulty might be solved with the use of sialoendoscopy. Initially, any mucus and stones should be removed along with dilation of the ductal stenosis. Secondly, the drug is then infused into the gland duct using the sialendoscope working channel. Lastly, a duct stent can be introduced and secured for weeks to enable repeated drug delivery and prevent re-stenosis (Su et al. 2016; Beilvert et al. 2014).

3.2 Type of Drugs and Therapeutic Applications

The first reported study was about the intraductal delivery of penicillin and saline for the treatment of chronic sialadenitis. Forty-four patients received penicillin and 11 patients were given saline. Among them, 22 patients were followed up and found to be symptom-free for an average of 11 years after the intraductal treatment (Su et al. 2017).

Pilocarpine is one of the most used drugs for the treatment of xerostomia. It is given orally and might be associated with adverse side effects causing less patient compliance and tolerability to the drug (Wiseman and Faulds 1995; Berk 2008). The drug is also contraindicated in some medical conditions like chronic obstructive pulmonary disease and cardiovascular disease. The drug has a short duration of action (~4 h) and is required to be given two to three times daily. Therefore, locally administering pilocarpine into the gland with avoidance of systemic absorption will be much more beneficial. Muthumariappan et al. (2019) proposed a formulation consisting of pilocarpine-loaded poly(lactic-co-glycolic acid) (PLGA)/poly(ethylene glycol) (PEG) nanofiber mats. The outcomes of this in vitro study showed an initial release of pilocarpine at 26% (4.5 h), followed by a gradual increase (~46%) over 15 days. The pilocarpine-loaded nanofiber scaffold was compatible with the SG growth with normal cellular proliferation and homeostasis. Salivary secretion was significantly increased at 4.5 h after intradermal SG treatment with drug-loaded nanofibers in vivo. After 24 h, no difference was noticed between these two treatment formulations. Furthermore, the whole gland weight was comparable between the two formulations indicating no gross changes in SG composition and cellular content. Also, no histological differences were found between the treatment groups (Muthumariappan et al. 2019).

IG injections of botulinum toxin (BTXA) are reliable for the treatment of sialorrhea (excessive saliva secretion) (Fuster Torres et al. 2007). On the other hand, a case report has shown the treatment of two patients with an ID infusion of BTXA (Schwalje and Hoffman 2019). Twenty-five units in 1 cc of reconstituted BTXA were infused into one of the major salivary ducts, followed by titration of up to 6 cc saline to the subject’s reported level of discomfort using a disposable pressure transducer (Schwalje and Hoffman 2019).

Another recent study has also shown the use of intraductal corticosteroid infusion of triamcinolone for two patients with painful salivary swelling associated with Sjogren syndrome (Diggelmann and Hoffman 2015). In one patient, she was symptom-free for 2 months at the time of follow-up, and for the other patient, symptoms recurred 3 months later. The treatment was repeated three times and the patient remained symptom-free for 8 months since the last injection (Diggelmann and Hoffman 2015).

ID infusion of the salivary gland might not only be limited to medication. Cell-based therapies have been tried for the treatment of atrophic salivary glands (Almansoori et al. 2019). Recently, Kasamatsu et al. have evaluated the intraductal delivery of salivary cells for irradiated glands. They cultured rat salivary gland cells in a medium with Rho kinase inhibitor (RI) and then transplanted them via the duct into the submandibular glands of irradiated atrophic SG rats. Twelve weeks after the transplantation, immunohistochemical analysis was performed, and salivary flow rates were measured (Kasamatsu et al. 2022). The cells were found to be located in the ductal region following transplantation and alpha-amylase (AMY) expression, and the salivary flow was higher in the salivary cell transplantation group. The results showed that intraductal cell-based therapy using RI-treated SG cells was able to restore salivary secretion of the irradiated salivary gland. They suggested this therapy can be applied clinically by culturing the labial minor salivary gland cells of patients before the radiotherapy and transplanting them through the salivary gland duct later. The same therapy can be used for hyposalivation associated with aging and Sjogren syndrome (Kasamatsu et al. 2022).

In our lab, we have been using the intraductal route for decellularization and recellularization of the rat submandibular salivary gland. The purpose of this approach is to produce a bioengineered salivary gland to be used for transplantation purposes in absence of immune rejection. For decellularization, the submandibular salivary gland was harvested along with its vascular pedicles (linguofacial vein and carotid artery) and duct. The gland was flushed for 15 min using saline that contains 50 U/mL heparin. Two 26-gauge catheters were inserted into the gland artery, and duct, and fixed with 8/0 silk sutures. The gland was decellularized by infusion with 10% sodium dodecyl sulfate (SDS) for 2 h at room temperature. The gland was then infused through the duct with 1%Triton-X100 for 1 h at room temperature. The gland was then treated with benzonase (90 U/mL) for 60 min to remove remnant nuclei. Subsequently, the gland was rinsed with phosphate-buffered saline containing penicillin (100 U/mL), streptomycin (100 U/mL), and amphotericin B (2.5 mg/L) for 30 min and stored at 4 °C.

Recellularization was performed as mentioned in previous studies (Doi et al. 2017; Uygun et al. 2010; Gao et al. 2014). Briefly, the decellularized gland was soaked in phosphate-buffered saline (PBS) containing 1% penicillin-streptomycin-amphotericin at 4 °C for 1 h. The gland was then equilibrated in the cell culture medium for 3 h. A density of 13×10^6 immortalized human salivary gland acinar cells (NSSVAC) were seeded via the salivary gland duct. The cells were injected at a rate of 1 ml/min at four steps, 2 ml/step, with 10 min intervals between each step. The overnight static culture was conducted to allow the cell's attachment. The next day, the medium was perfused through the gland artery at a 0.5 ml/min rate while the gland is soaked within a culture medium in the glass jar. The whole system was placed in the incubator at 37 °C with 5% CO_2 and 95% O_2. The perfusion rate was changed to 4 ml/min from the second day for 14 days culture period. The medium was changed every 48 h.

Our primary results showed complete decellularization using the intraductal route shown in the hematoxylin and eosin (H&E) staining and DNA quantification assessment with preservation of the acinar and duct structures and removal of the cellular components. The decellularized salivary gland also preserved the collagen fibers well as shown by the trichrome stain and total collagen assay. The recellularized gland showed a grossly homogenous distribution of the seeded cells, and the H&E staining showed the cells well-attached and grown inside the gland (Fig. 2).

3.3 Efficiency and Limitations

The abovementioned studies have supported the use of intraductal injections as potential therapeutic approaches for the treatment of salivary gland diseases. They demonstrated the utility of intraductal infusion of penicillin, botulinum toxin, corticosteroid, and salivary cells for the treatment of sialadenitis, hypersalivation, Sjogren syndrome, and irradiated salivary glands. The intraductal approach is free of systemic side effects and ensures a maximum dose delivery to the salivary gland. It also decreases the risks of percutaneous needle injection. These studies have also provided evidence for the safety of intraductal salivary gland infusion.

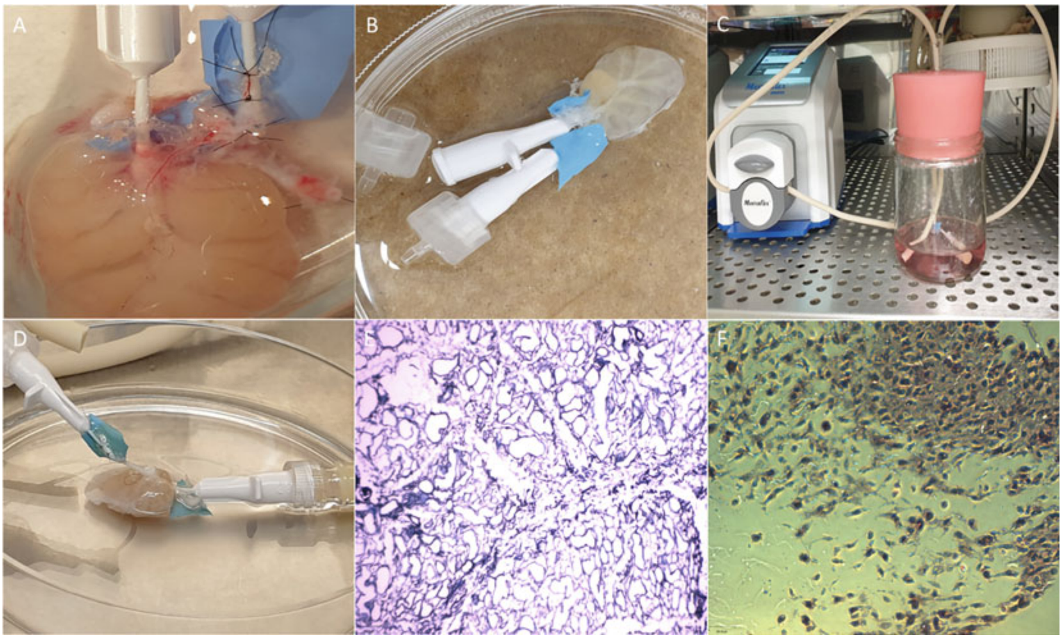

Fig. 2 Stages of decellularization and recellularization of rat SMG through intraductal route. (**a**) Two 26-gauge catheters were placed into the gland duct and artery. (**b**) White translucent SMG scaffold after completing the decellularization. (**c**) SMG scaffold during recellularization. (**d**) Brown-look SMG after recellularization. (**e**) Trichrome staining showing the preservation of collagen fibers and the absence of cellular content. (**f**) H&E staining showing the attachment of seeded salivary gland acinar cells

References

Almansoori AA, Khentii N, Kim B, Kim S-M, Lee J-H (2019) Mesenchymal stem cell therapy in submandibular salivary gland allotransplantation: experimental study. Transplantation 103(6):1111–1120

Antoniades D, Harrison JD, Epivatianos A, Papanayotou P (2004) Treatment of chronic sialadenitis by intraductal penicillin or saline. J Oral Maxillofac Surg 62(4): 431–434

Beilvert A, Faure F, Meddahi-Pellé A, Chaunier L, Guilois S, Chaubet F et al (2014) A resorbable shape-memory starch-based stent for the treatment of salivary ducts under sialendoscopic surgery. Laryngoscope 124(4):875–881

Berk L (2008) Systemic pilocarpine for treatment of xerostomia. Expert Opin Drug Metab Toxicol 4(10): 1333–1340

Bushara KO (1997) Sialorrhea in amyotrophic lateral sclerosis: a hypothesis of a new treatment – botulinum toxin A injections of the parotid glands. Med Hypotheses 48(4):337–339

Carlson GW (2000) The salivary glands: embryology, anatomy, and surgical applications. Surg Clin 80(1): 261–273

Comella K, Bell W (2017) First-in-man intraglandular implantation of stromal vascular fraction and adipose-derived stem cells plus platelet-rich plasma in irradiation-induced gland damage: a case study. Int Med Case Rep J 10:295–299

Diggelmann HR, Hoffman HT (2015) Intraductal infusion of steroids in patients with Sjögren syndrome to treat painful salivary swelling: report of 2 cases. Ear Nose Throat J 94(6):238–239

Doi R, Tsuchiya T, Mitsutake N, Nishimura S, Matsuu-Matsuyama M, Nakazawa Y et al (2017) Transplantation of bioengineered rat lungs recellularized with endothelial and adipose-derived stromal cells. Sci Rep 7(1):8447

Ellies M, Laskawi R (2010) Diseases of the salivary glands in infants and adolescents. Head Face Med 6(1):1

Fang D, Hu S, Liu Y, Quan V-H, Seuntjens J, Tran SD (2015) Identification of the active components in bone marrow soup: a mitigator against irradiation-injury to salivary glands. Sci Rep 5(1):16017

Fuster Torres MA, Berini Aytés L, Gay Escoda C (2007) Salivary gland application of botulinum toxin for the treatment of sialorrhea. Med Oral Patol Oral Cir Bucal 12(7):E511–E517

Gao Z, Wu T, Xu J, Liu G, Xie Y, Zhang C et al (2014) Generation of bioartificial salivary gland using whole-organ decellularized bioscaffold. Cells Tissues Organs 200(3–4):171–180

Grønhøj C, Jensen DH, Glovinski PV, Jensen SB, Bardow A, Oliveri RS et al (2017) First-in-man mesenchymal stem cells for radiation-induced xerostomia (MESRIX): study protocol for a randomized controlled trial. Trials 18(1):e108

Haroun RI, Brem H (2000) Local drug delivery. Curr Opin Oncol 12(3):187–193

Huang B, He T, Yao Q, Zhang L, Yao Y, Tang H et al (2019) Amifostine suppresses the side effects of radiation on BMSCs by promoting cell proliferation and reducing ROS production. Stem Cells Int 2019: 8749090

Isabelle S, Li L, Veraragavan A, Robert et al (2013) Combined KIT and FGFR2b signaling regulates epithelial progenitor expansion during organogenesis. Stem Cell Rep 1(6):604–619

Jost WH, Friedman A, Michel O, Oehlwein C, Slawek J, Bogucki A et al (2019) SIAXI. Neurology 92(17): e1982–e1991

Kasamatsu A, Fukushima R, Nakamura K, Kawasaki K, Yoshimura S, Koyama T et al (2022) A novel cell-based transplantation method using a Rho kinase inhibitor and a specific catheter device for the treatment of salivary gland damage after head and neck radiotherapy. Biochem Biophys Rep 32:101385

Kim JW, Kim JM, Choi ME, Kim S-K, Kim Y-M, Choi J-S (2019) Adipose-derived mesenchymal stem cells regenerate radioiodine-induced salivary gland damage in a murine model. Sci Rep 9(1):15752

King M, Joseph S, Albert A, Thomas TV, Nittala MR, Woods WC et al (2020) Use of amifostine for cytoprotection during radiation therapy: a review. Oncology 98(2):61–80

Lakraj A, Moghimi N, Jabbari B (2013) Sialorrhea: anatomy, pathophysiology and treatment with emphasis on the role of botulinum toxins. Toxins 5(5):1010–1031

Lim JY, Yi T, Choi JS, Jang YH, Lee S, Kim HJ et al (2013) Intraglandular transplantation of bone marrow-derived clonal mesenchymal stem cells for amelioration of post-irradiation salivary gland damage. Oral Oncol 49(2):136–143

Lombaert IMA, Brunsting JF, Wierenga PK, Faber H, Stokman MA, Kok T et al (2008) Rescue of salivary gland function after stem cell transplantation in irradiated glands. PLoS One 3(4):e2063

Lynggaard CD, Grønhøj C, Christensen R, Fischer-Nielsen A, Melchiors J, Specht L et al (2022) Intraglandular off-the-shelf allogeneic mesenchymal stem cell treatment in patients with radiation-induced xerostomia: a safety study (MESRIX-II). Stem Cells Transl Med 11(5):478–489

Mohamed NH, Shawkat S, Moussa MS, Ahmed N (2022) Regeneration potential of bone marrow derived mesenchymal stem cells and platelet rich plasma (PRP) on irradiation-induced damage of submandibular salivary gland in albino rats. Tissue Cell 76:101780

Mueller J, Langbein T, Mishra A, Baum RP (2022) Safety of high-dose botulinum toxin injections for parotid and submandibular gland radioprotection. Toxins 14(1):64

Muthumariappan S, Ng WC, Adine C, Ng KK, Davoodi P, Wang C-H et al (2019) Localized delivery of pilocarpine to hypofunctional salivary glands through electrospun nanofiber mats: an ex vivo and in vivo study. Int J Mol Sci 20(3):541

Nanduri LSY, Lombaert IMA, Van Der Zwaag M, Faber H, Brunsting JF, Van Os RP et al (2013) Salisphere derived c-Kit+ cell transplantation restores tissue homeostasis in irradiated salivary gland. Radiother Oncol 108(3):458–463

Narmada IB, Laksono V, Nugraha AP, Ernawati DS, Winias S, Prahasanti C et al (2019) Regeneration of salivary gland defects of diabetic wistar rats post human dental pulp stem cells intraglandular transplantation on acinar cell vacuolization and interleukin-10 serum level. Pesquisa Brasileira em Odontopediatria e Clínica Integrada 19(1):1–10

Nigam PK, Nigam A (2021) Botulinum toxin. Indian J Dermatol 55:8–14

Ogle OE (2020) Salivary gland diseases. Dental Clin 64(1):87–104

Pal PK, Calne DB, Calne S, Tsui JKC (2000) Botulinum toxin A as treatment for drooling saliva in PD. Neurology 54(1):244–244

Qwarnström E (1986) Experimental sialography: the effects of retrograde infusion of radiographic contrast media on salivary gland morphology and function: a review article. Oral Surg Oral Med Oral Pathol 62(6): 668–682

Rabea AA, Rashed L, Hassan R (2022) Regenerative capacity of bone marrow stem cells on aged albino rat's parotid excretory duct. Arch Oral Biol 141: 105470

Schwalje AT, Hoffman HT (2019) Intraductal salivary gland infusion with botulinum toxin. Laryngoscope Investig Otolaryngol 4(5):520–525

Schwarz S, Huss R, Schulz-Siegmund M, Vogel B, Brandau S, Lang S et al (2014) Bone marrow-derived mesenchymal stem cells migrate to healthy and damaged salivary glands following stem cell infusion. Int J Oral Sci 6(3):154–161

Scott AB (1981) Botulinum toxin injection of eye muscles to correct strabismus. Trans Am Ophthalmol Soc 79: 734–770

Sherif H, Esmail A, Abdelfattah M, Liu Z-J, Abouzid W (2018) Evaluation of the effects of Botulinum Neuro Toxin A (BoNTA) on submandibular salivary gland in rats by histological examination and semi-quantitative scoring method. Al-Azhar J Dental Sci 21(5):511–518

So JI, Song DH, Park JH, Choi E, Yoon JY, Yoo Y et al (2017) Accuracy of ultrasound-guided and non-ultrasound-guided botulinum toxin injection into cadaver salivary glands. Ann Rehabil Med 41(1):51

Song GG, Lee YH (2014) Diagnostic accuracies of sialography and salivary ultrasonography in Sjögren's syndrome patients: a meta-analysis. Clin Exp Rheumatol 32(4):516–522

Strychowsky JE, Sommer DD, Gupta MK, Cohen N, Nahlieli O (2012) Sialendoscopy for the management of obstructive salivary gland disease: a systematic review and meta-analysis. Arch Otolaryngol Head Neck Surg 138(6):541–547

Su C-H, Lee K-S, Tseng T-M, Hung S-H (2016) Post-sialendoscopy ductoplasty by salivary duct stent placements. Eur Arch Otorhinolaryngol 273(1):189–195

Su C-H, Lee K-S, Tseng T-M, Tseng H, Ding Y-F, Koch M et al (2017) Intraductal injection as an effective drug delivery route in the management of salivary gland diseases. Eur Arch Otorhinolaryngol 274(1):399–404

Su X, Fang D, Liu Y, Ruan G, Seuntjens J, Kinsella JM et al (2018) Lyophilized bone marrow cell extract functionally restores irradiation-injured salivary glands. Oral Dis 24(1–2):202–206

Su X, Liu Y, Bakkar M, Elkashty O, El-Hakim M, Seuntjens J et al (2020) Labial stem cell extract mitigates injury to irradiated salivary glands. J Dent Res 99(3):293–301

Sumita Y, Iwamoto N, Seki M, Yoshida T, Honma R, Iwatake M et al (2020) Phase 1 clinical study of cell therapy with effective-mononuclear cells (E-MNC) for radiogenic xerostomia (first-in-human study) (FIH study on E-MNC therapy for radiogenic xerostomia). Medicine (Baltimore) 99(26):e20788

Teymoortash A, Sommer F, Mandic R, Schulz S, Bette M, Aumüller G et al (2007) Intraglandular application of botulinum toxin leads to structural and functional changes in rat acinar cells. Br J Pharmacol 152(1): 161–167

Teymoortash A, Pfestroff A, Wittig A, Franke N, Hoch S, Harnisch S et al (2016) Safety and efficacy of botulinum toxin to preserve gland function after radiotherapy in patients with head and neck cancer: a prospective, randomized, placebo-controlled, double-blinded phase I clinical trial. PLoS One 11(3):e0151316

Tran SD, Liu Y, Xia D, Maria OM, Khalili S, Wang RW-J et al (2013) Paracrine effects of bone marrow soup restore organ function, regeneration, and repair in salivary glands damaged by irradiation. PLoS One 8(4): e61632

Tsutsui TW (2020) Dental pulp stem cells: advances to applications. Stem Cells Clon Adv Appl 13:33

Uygun BE, Soto-Gutierrez A, Yagi H, Izamis ML, Guzzardi MA, Shulman C et al (2010) Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. Nat Med 16(7):814–820

Vashishta R, Nguyen SA, White DR, Gillespie MB (2013) Botulinum toxin for the treatment of sialorrhea: a meta-analysis. Otolaryngol Head Neck Surg 148(2): 191–196

Wang Z, Xing H, Hu H, Dai T, Wang Y, Li Z et al (2017) Intraglandular transplantation of adipose-derived stem cells combined with platelet-rich fibrin extract for the treatment of irradiation-induced salivary gland damage. Exp Ther Med 15:795

Wiseman LR, Faulds D (1995) Oral pilocarpine: a review of its pharmacological properties and clinical potential in xerostomia. Drugs 49(1):143–155

Zenk J, Iro H, Klintworth N, Lell M (2009) Diagnostic imaging in sialadenitis. Oral Maxillofacial Surg Clin 21(3):275–292

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Negative-Pressure Wound Therapy: What We Know and What We Need to Know

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Abstract

Negative-pressure wound therapy (NPWT) promotes wound healing by applying negative pressure to the wound surface. A quarter of a century after its introduction, NPWT has been used in various clinical conditions, although molecular biological evidence is insufficient due to delay in basic research. Here, we have summarized the history of NPWT, its mechanism of action, what is currently known about it, and what is expected to be known in the future. Particularly, attention has shifted from the four main mechanisms of NPWT to the accompanying secondary effects, such as effects on various cells, bacteria, and surgical wounds. This chapter will help the reader to understand the current status and shortcomings of NPWT-related research, which could aid in the development of basic research and, eventually, clinical use with stronger scientific evidence.

Keywords

Angiogenesis · Biofilm · Epithelial-mesenchymal transition · Mechanotransduction · Microdeformational wound therapy · Surgical site infection · Topical negative pressure · Vacuum-assisted closure · Vacuum sealing drainage · Wound healing

Abbreviations

ALP	alkaline phosphatase
Ang-1	angiogenin-1
Ang-2	angiogenin-2
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
EGF	epidermal growth factor
IL	interleukin
iNPWT	incisional negative-pressure wound therapy
MAPK	mitogen-activated protein kinase
MDSC	muscle-derived stem cell
MMP	matrix metalloproteinase
NPWT	negative-pressure wound therapy

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NPWTi-d	negative-pressure wound therapy with instillation and dwell time
P-MSC	periosteum-derived mesenchymal stem cell
sNPWT	single-use negative-pressure wound therapy
SSI	surgical site infection
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor-alpha
VEGF	vascular endothelial growth factor
α-SMA	smooth muscle actin α

1 Introduction

Since the initial introduction of negative-pressure wound therapy (NPWT) in 1997 (Morykwas et al. 1997), this innovative treatment has been widely adopted in difficult-to-treat wounds, primarily those of the skin and soft tissue (Poteet et al. 2021). This approach, in which the wound surface is sealed with gauze, polyurethane foam, or other materials and a film dressing, followed by internal suction, is also called topical negative pressure (TNP), vacuum sealing drainage (VSD), or microdeformational wound therapy (MDWT) and is known to promote wound healing through various mechanisms. It is recognized as a clinically useful treatment in many areas of practice, including emergency, abdominal and thoracic surgery, orthopedic surgery, and plastic surgery. Specifically, clinical studies have shown its effectiveness in treating difficult-to-treat wounds, including diabetic foot lesions (Meloni et al. 2015; Chen et al. 2021); postoperative wound dehiscence (Seidel et al. 2020) and sternal osteomyelitis (Steingrimsson et al. 2012); trauma wounds, including burns (Kantak et al. 2016); skin and soft tissue defects resulting from resection of benign and malignant tumors (Fourman et al. 2022); and infected wounds (Faust et al. 2021) as well as preventing complications following primary closure surgery (Norman et al. 2022).

Although a quarter of a century has passed since its introduction, the molecular biological mechanism involved in NPWT remains unclear, and the level of evidence for its effectiveness remains insufficient, despite clinicians' reassurances of its effectiveness. This is partly due to the lack of basic research, including in vitro and in vivo studies, compared to the many clinical reports. Additionally, the complexity of the healing mechanism of NPWT and the difficulty of reproducing it in basic research may be one of the reasons for the small number of studies and low level of evidence. An increase in the level of evidence would greatly contribute to expanding the range of indications for NPWT by providing new therapeutic targets and reducing complications.

In this chapter, we first describe the history of NPWT and its status in clinical practice, followed by the molecular biological mechanisms currently known and areas expected to be elucidated in the future.

2 History and Variations of NPWT

Commercial negative-pressure therapy devices were available in 1995, and in 1997. Morykwas et al. (1997) first reported a novel treatment, later called NPWT. They reported that −125-mmHg negative pressure caused increased wound granulation and periwound blood flow in a porcine model. Argenta and Morykwas (1997) reported its clinical usefulness in a case study of 300 patients. Their treatment involved applying a porous sponge to the wound surface, sealing it with a film dressing, and applying constant negative pressure with a specialized treatment device. This new treatment method was rapidly accepted throughout the 2000s, and in the 2010s, various treatment variations appeared with equipment development. Unlike conventional NPWT, which involves a foam and film contact surface, single-use NPWT (sNPWT) (Fong and Marston 2012; Sharp 2013; van den Bulck et al. 2013) comprises a special dressing that integrates a nonadherent contact surface and a sponge portion that absorbs the exudate. Many of these devices are battery-operated and disposable; thus, they are portable and can be used in outpatients. Furthermore, sNPWT was found to be more effective than regular NPWT in patients with diabetic foot lesions (Kirsner et al. 2021). Recent reports indicate that sNPWT effectively prevents wound infection in primary closed wounds after abdominal surgery (Norman et al. 2022). Intermittent NPWT or variable NPWT (Borgquist et al. 2010a; Lee et al. 2015), in which the intensity of negative pressure is varied periodically, is said to be superior to continuous negative pressure in increasing periwound blood flow (Borgquist et al. 2010a; Sogorski et al. 2018). This was already pointed out by Morykwas et al. (1997) in their first report; however, only continuous negative pressure was widely known owing to the capability of the treatment device, and intermittent NPWT was brought back into focus only around 2010 (Ahearn 2009). Despite its superior therapeutic effect, it has been noted that patient discomfort is greater with intermittent NPWT than with continuous negative pressure due to repetitive pain at the start of each cycle (Borgquist et al. 2010a; Malmsjo et al. 2012).

A treatment combining NPWT with wound irrigation has been considered since the early 2000s (Wolvos 2004). A typical example is NPWT with instillation and dwell time (NPWTi-d), which has become easier to use since 2011, when a device with an improved irrigation system (from gravity to pump) was launched into the market. This treatment is based on the idea that the wound can be cleaned by repeating the cycle of injecting the cleaning solution into the wound, immersing it for a certain period, and then suctioning it under negative pressure (Wolvos 2013), making it possible to apply NPWT to wounds with infection (Kanapathy et al. 2020). NPWT is also used for dressing open abdominal wounds that cannot be closed primarily due to intestinal edema or other conditions that increase the risk of abdominal compartment syndrome (Bjarnason et al. 2011; Bertelsen et al. 2014). However, its indication, safety, and comparison to other treatments are still under debate (Cheng et al. 2022). All these treatment variations have gained wide acceptance in clinical practice. Thus, NPWT has evolved along with the development of commercial negative-pressure treatment devices. Although its usefulness is widely recognized in many clinical studies, only few basic research reports have examined its treatment mechanism in detail.

3 Principal Mechanisms of NPWT

The effects of NPWT in promoting wound healing are thought to involve four primary mechanisms and their accompanying secondary effects (Orgill et al. 2009; Huang et al. 2014; Normandin et al. 2021) (Fig. 1). The primary mechanisms are (1) coarse wound contraction (macrodeformation), (2) microscopic deformation of the wound surface (microdeformation), (3) fluid removal, and (4) stabilization of the wound environment (Fig. 2). The interaction of these mechanisms results in various secondary effects.

3.1 Macrodeformation

When a wound is filled with foam, such as gauze or porous sponge, covered with a film dressing, and negative pressure is applied, the wound shrinks as the foam shrinks. In experiments with polyurethane foam by Scherer et al. (2008), the negative pressure of -125 mmHg reduced the volume of the foam by approximately 80%. This gross contraction effect depends on the nature and mobility of the tissue surrounding the treatment site, suction pressure, and material and volume of the foam (Orgill et al. 2009; Borgquist et al. 2011; Anesater et al. 2011). The contraction effect is stronger in areas with thick subcutaneous fat, loose skin, and high flexibility, such as the abdominal wall, and less effective in areas with low flexibility, such as the scalp and scar-covered wounds (Orgill et al. 2009). This is supported by reports that subcutaneous fat exhibits a stronger contractile response than skin at the same site (Torbrand et al. 2010; Katzengold et al. 2018). With the wound shrinkage, the wound area is physically reduced due to edema reduction, granulation, and scarring (Torbrand et al. 2010; Katzengold et al. 2018; Borgquist et al. 2010b).

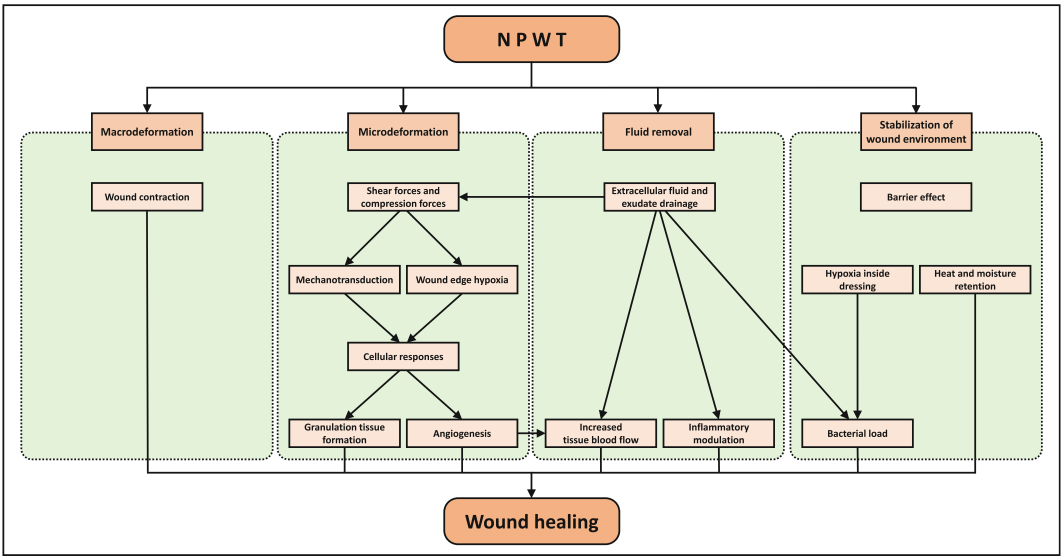

Fig. 1 Mechanisms of action of NPWT. Negative-pressure wound therapy (NPWT) promotes wound healing through four primary mechanisms and associated secondary effects

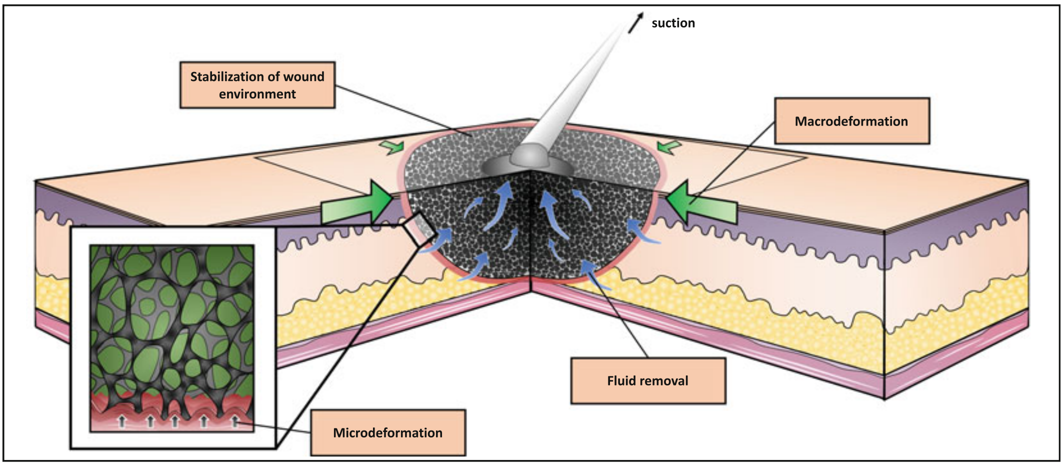

Fig. 2 Schematic illustration of NPWT. Negative-pressure wound therapy (NPWT) is associated with four primary mechanisms of action: (1) macrodeformation, (2) microdeformation, (3) fluid removal, and (4) stabilization of the wound environment

3.2 Microdeformation

Mechanical loading of the tissue causes deformation (McLeod et al. 1987). In NPWT, the pressure on the wound surface increases in contrast to the low-pressure environment inside the dressing (Kairinos et al. 2009a, b; Biermann et al. 2020; Sogorski et al. 2022). The minute tissue deformation at the foam-wound boundary resulting from this is called microdeformation (Borgquist et al. 2010b; Saxena et al. 2004). This mechanical force propagates into the tissue through the extracellular fluid and produces shear and deformation forces on individual cells (Orgill et al. 2009;

Wilkes et al. 2009a; Lu et al. 2011). Finite element studies have shown that under typical treatment conditions using porous sponges, approximately 20% strain is exerted on the wound surface (Saxena et al. 2004; Wilkes et al. 2009a, b). In response to this mechanical stimulation, effects, such as increased cell proliferation and migration, angiogenesis, and granulation, are observed (Saxena et al. 2004; Hsu et al. 2010; Toume et al. 2017). The process by which cells sense mechanical stimuli and convert them into physiological responses and gene expression is called mechanotransduction. It is believed to occur primarily in the cytoskeleton via mechanoreceptors, such as integrin β1 (Wang et al. 1993; Huang et al. 1998). Mechanotransduction is often mentioned in studies of fibroproliferative diseases characterized by excessive connective tissue accumulation and persistent tissue contraction, and signaling pathways, such as transforming growth factor-β (TGF-β)/Smad, mitogen-activated protein kinase (MAPK), Rho/ROCK, Wnt/β-catenin, and tumor necrosis factor-α (TNF-α)/nuclear factor-kappa B (NF-κB), are known to be involved (Huang and Ogawa 2012). For instance, in skin fibroblasts, integrins act as mechanical sensors for bidirectional signaling between cells and the extracellular matrix (ECM) (Katsumi et al. 2004). The ECM transduces mechanical stimuli to the cytoskeleton via the integrin-focal adhesion kinase (FAK) pathway (Santos and Lagares 2018), and ECM stiffness affects mechanical signaling through the TGF-β1 pathway. Integrins activated by the stiff ECM stimulate TGF-β1 release and bind to the TGF-β1 receptor on myofibroblasts. This induces a positive feedback that increases TGF-β1 and smooth muscle actin α (α-SMA) levels and stiffens the ECM. Conversely, a soft ECM suppresses TGF-β1 and α-SMA expression and further softens the ECM (Santos and Lagares 2018; Fu et al. 2021). In keratinocytes, mechanical stress-induced proliferative changes occur via mechanisms such as the ECM-integrin pathway, MAPK pathway, and epithelial-mesenchymal interactions (Reichelt 2007), while in endothelial cells, mechanical stimulation is known to cause angiogenesis and vascular remodeling (Fu et al. 2021). NPWT-induced cellular responses, such as increased cell proliferation and migration as well as changes in transcription factors associated with wound healing, are thought to be mainly the result of microdeformation and mechanotransduction induced by mechanical stimuli. For this reason, microdeformation has been considered the most important of the four main mechanisms (Orgill et al. 2009; Huang et al. 2014; Normandin et al. 2021; Wiegand and White 2013).

3.3 Fluid Removal

NPWT drains the exudate from the wound surface, and the pressure gradient allows the extracellular fluid in the tissue to discharge. This reduces tissue edema (Orgill et al. 2009; Labanaris et al. 2009), relieves microvascular compression caused by excess interstitial fluid, and may contribute to increased blood flow to the wound (Huang et al. 2014). The amount of exudate depends on the condition of the wound; thus, appropriate pressure settings are necessary (Borgquist et al. 2011). Wound exudate contains inflammatory cytokines and proteolytic enzymes that inhibit wound healing, such as TNF-α and matrix metalloproteinase (MMP). It is thought that NPWT promotes wound healing not only by promoting cell proliferation but also by eliminating and controlling these factors along with the exudate (Stechmiller et al. 2006; Moues et al. 2008; Glass et al. 2014).

3.4 Stabilization of the Wound Environment

In NPWT, dressings are made with a semipermeable polyurethane film to ensure airtightness in addition to the foam material. This partially restricts gas exchange and water vapor permeation, which provides heat and moisture retention and is beneficial for wound healing (Orgill et al. 2009; Kloth et al. 2002; Winter and Scales 1963; Hinman and Maibach 1963). The dressing is also impermeable to proteins and bacteria and acts as a physical barrier to the outside world (Orgill et al. 2009; Huang et al. 2014). Materials used for the foam in direct contact with the wound include polyurethane and polyvinyl alcohol. However, noncommercial alternatives, such as those using gauze and wall suction, are also used owing to cost concerns (Bui et al. 2006; Gibson 2022). Veerasubramanian et al. (2021) investigated the function of the foam material itself on the inflammatory response of macrophages in vitro and reported that it had an inhibitory effect on the inflammatory molecules TNF-α and interleukin-1β (IL-1β). It has also been noted that a decrease in partial pressure of oxygen in the dressing during negative-pressure treatment may affect bacterial activity (Biermann et al. 2019), suggesting that the therapeutic effect of NPWT is not solely due to mechanical stimulation.

4 Secondary Effects of NPWT

The four primary mechanisms described above interact to produce cellular responses and secondary effects that lead to wound healing (Fig. 1). In this section, we discuss the cellular response, immune responses, angiogenesis, and granulation, which have received particular attention, as well as the effects on epithelialization, osteogenesis, tumors, and bioburden and the latest findings on the effects on surgical procedures, such as primary closed wounds, skin grafts, and flap operation.

4.1 Cellular Responses

As mentioned previously, mechanical stimulation with NPWT alters cell function through microdeformation and mechanotransduction. Cytoskeletal changes affect cell function and, consequently, tissue function (Folkman and Moscona 1978; Alford et al. 2011); more highly distorted cells are more sensitive to soluble mitogens and promote cell proliferation (Chen et al. 1997; Ingber 2005). Saxena et al. (2004) reported, via finite element method simulations, that the 5–20% strain produced by NPWT was equivalent to the strain level that promotes cell proliferation in vitro. Toume et al. (2017) and Katzengold et al. (2021) confirmed that wound closure was accelerated in experiments where elongation forces equivalent to those during NPWT treatment were applied directly to cultured fibroblast wound models. Their studies mainly focused on strain, among the various complex therapeutic mechanisms of NPWT. Multiple experiments in mice showing that NPWT increased the expression of Ki-67, a marker of cell proliferative potential (Scherer et al. 2009; Dastouri et al. 2011; Shao et al. 2021), corroborate the biological effects. Growth factors, binding to ECM proteins, and isometric tension are required for cells to proliferate and differentiate effectively (Ingber 2005; Huang and Ingber 1999). In chronic wounds, the cell scaffold is unstable, resulting in inadequate isometric tension among these three factors, which prevents cell proliferation and differentiation. Mechanical stimulation with NPWT may compensate for this lack of isometric tension and further promote wound healing by affecting the mechanical environment within the tissue (Huang et al. 2014).

4.2 Immune Responses

As mentioned in Sect. 3.3, NPWT contributes to the regulation of wound inflammation by removing exudate containing inflammatory substances. In patients with traumatic soft tissue injuries treated with NPWT or a bilayer wound dressing made of Teflon and polyurethane, the amount of IL-8 in the exudate was found to be significantly higher in the NPWT group (Labler et al. 2009). In addition, biopsy experiments examining gene expression in human split-thickness skin wounds showed increased expression of leukocyte chemoattractants, such as IL-8 and CXCL5 (Nuutila et al. 2013). IL-8, a potent chemokine and angiogenesis-promoting factor, plays an important role in regulating the migration of neutrophils and macrophages (Koch et al. 1992; Mukaida et al. 1998). These findings indicate that NPWT may affect the inflammatory phase of wound healing by removing soluble chemokines and infiltrating leukocytes along with exudate (Glass et al. 2014).

4.3 Angiogenesis and Tissue Blood Flow

NPWT creates a hypoxic environment outside the wound (Biermann et al. 2019), and microdeformation also causes temporary localized hypoperfusion and hypoxia at the wound margins (Erba et al. 2011). Hypoxia increases vascular endothelial growth factor (VEGF) expression via the upregulation of hypoxia-inducible factor-1α (HIF-1α). This hypoxia and VEGF expression contribute to directional, more near-physiologic angiogenesis because it exhibits the strongest concentration gradient at the wound edge (Erba et al. 2011) (Fig. 3). Thus, NPWT increases microvascular density at the treatment site and increases wound blood flow (Greene et al. 2006; Malsiner et al. 2015). Ma et al. (2016) focused on changes in growth factors over time in an in vivo study involving patients. They reported that NPWT increased the expression of angiogenin-2 (Ang-2) and decreased the expression of angiogenin-1 (Ang-1) and the Ang-1/Ang-2 ratio in the early phase of treatment; meanwhile, it increased the expression of Ang-1, Ang-1/Ang-2 ratio, and phosphorylation level of the tyrosine kinase receptor Tie-2 in the late phase. This implies that NPWT increases angiogenesis by stimulating microvascular destabilization and regression in the early stages of treatment and promotes microvascular maturation by prioritizing microvascular stabilization in the later stages (Ma et al. 2016). They also demonstrated that pericytes and collagen IV are involved

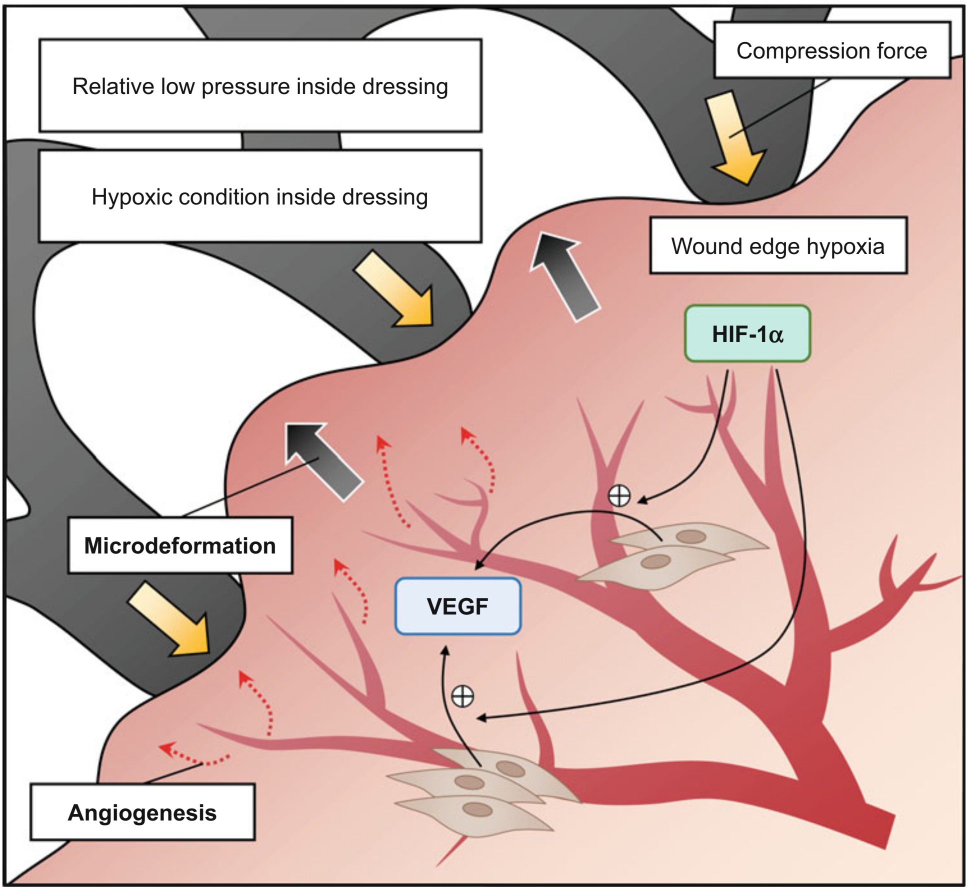

Fig. 3 Angiogenesis induced by NPWT. The hypoxic environment inside and outside the wound caused by negative-pressure wound therapy (NPWT) activates the HIF-1α-VEGF pathway, leading to angiogenesis. *HIF-1α* hypoxia-inducible factor-1α, *VEGF* vascular endothelial growth factor

in the maturation of microvessels in the late stages of NPWT using a rat diabetic wound model (Ma et al. 2017).

The effect of NPWT on tissue blood flow has long been a subject of debate. Morykwas et al. (1997) first reported that NPWT increases wound edge blood flow via evaluation with laser Doppler. Sogorski et al. performed experiments on healthy volunteers. They showed that the blood flow of their normal thigh skin under NPWT increased by up to +151% and tissue oxygen saturation increased by up to +28.2%, noting that this effect may be greater with variable negative pressure than with continuous negative pressure (Sogorski et al. 2018, 2022). Conversely, Wackenfors et al. (2004) reported that changes in blood flow were affected by the distance from the wound margin, with blood flow reduced at positions very close to the wound. Their results in porcine models are supported by in vitro simulation experiments (Biermann et al. 2020) and experiments that evaluated radioisotope perfusion and transcutaneous oxygen saturation in healthy volunteers (Kairinos et al. 2009b). Therefore, it is believed that, in NPWT, attention should be paid to the possibility of adverse effects on tissue blood flow when applied to ischemic lesions or when used circumferentially on the limb (Kairinos et al. 2009b; Livingstone et al. 2021; Singh et al. 2021). When considering these contradictory results regarding the effect of NPWT on tissue blood flow, it is necessary to focus on the timing of the blood flow measurements. When discussing NPWT today, it is generally considered that NPWT promotes angiogenesis and increases blood flow to the wound in the long term (Panayi et al. 2017).

4.4 Granulation Tissue Formation

Granulation tissue in wounds is specialized for wound healing and is associated with cellular findings, such as the proliferation of vascular endothelial cells, pericytes, fibroblasts, and myofibroblasts, and mobilization of macrophages (Diaz-Flores Jr. et al. 2009). Morykwas and Argenta first reported on the promotive effect of NPWT on granulation growth (Morykwas et al. 1997; Argenta and Morykwas 1997). This promotive effect on granulation growth has been investigated mainly through cell experiments using bioreactors that reproduce the NPWT environment in vitro. Wilkes et al. (2007) successfully reproduced a treatment environment similar to that of commercial devices by applying a negative-pressure load on 3D-cultured fibroblasts via foam dressing using 6-well plates and cell inserts. They observed that a 48-h treatment caused fibroblasts to thicken and become elongated and bipolar. Similarly, experiments with 3D-cultured fibroblasts have confirmed that NPWT improves the energetic state of fibroblasts by increasing adenosine triphosphate (ATP) and cytochrome c oxidase levels, significantly increasing TGF-β and platelet-derived growth factor (PDGF) α,β levels (McNulty et al. 2007; McNulty et al. 2009), and upregulating the expression of the messenger RNA of type 1 collagen α1 (*COL1A1*), basic fibroblast growth factor (*bFGF*), *TGFβ1*, and *α-SMA* (Lu et al. 2011). All these findings indicate the upregulation of collagen production, which is the basis for promoting granulation growth. Furthermore, experiments using mast cell-deficient mice have shown that NPWT induces wound tissue granulation, cell proliferation, and angiogenesis during the proliferative phase of wound healing and that mouse mast cell proteases 4, 5, and 6 (mMCP 4, 5, 6) in the secretory granules of mast cells may play an important role in this process (Younan et al. 2011; Succar et al. 2014).

4.5 Reepithelialization and Epithelial-Mesenchymal Transition

Transcriptome analysis in patients has shown that NPWT may promote reepithelialization during the wound inflammation phase by increasing epithelial cell migration and proliferation but may impair epidermal maturation in the long term by reducing differentiation (Nuutila et al. 2013). Gene expression profiling of 7-day

NPWT-adapted split-thickness skin graft donor site wounds showed that the most induced genes encoded ILs (*IL8*, *IL24*), prostaglandins (*COX2*), chemokines (*CXCL5*), osteostatins (parathyroid hormone-like hormone (*PTHLH*)), and MMPs (*MMP1*, *MMP3*, and *MMP10*), which revealed that factors associated with cell proliferation and inflammation were induced (Nuutila et al. 2013). Furthermore, genes particularly suppressed were those related to the formation of the cornified envelope (CE), which consists of stratified squamous cells and acts as a protective barrier to the epidermis (Sun and Green 1976) (*LOR*, *FLG*, annexin A9 (*ANX9*), and *LCE*) and cytokeratins (*KRT1*, *KRT2*, and *KRT10*), suggesting that the development and maturation of the neoepidermis may be disturbed (Nuutila et al. 2013).

Hsu et al. reported the results of a series of notable in vitro studies in which the behavior of keratinocytes in monolayer culture was analyzed using electrical cell-substrate impedance sensing (ECIS) methods and morphological observations (Hsu et al. 2010, 2013; Chow et al. 2016; Huang et al. 2016). A negative-pressure environment of −125 mmHg stimulated the appearance of cell division control protein 42 (Cdc42) at the leading edge of epithelial cells, which may facilitate epithelial cell migration by promoting actin polymerization and the formation of lamellipodia and filopodia (Hsu et al. 2010, 2013). They also found that negative-pressure treatment decreased the expression level of β-catenin in human keratinocytes at the plasma membrane, while it increased in the nucleus with elevated activity. They reported that Src-dependent phosphorylation of p120-catenin (p120ctn) at the plasma membrane caused a downregulation of the adhesion molecule E-cadherin followed by degradation of the adherens junction (Chow et al. 2016; Huang et al. 2016). Liu et al. (2022) also reported that negative pressure in vitro downregulates miR-203 expression and promotes cell proliferation and migration of human keratinocytes via upregulation of the p63 protein. We monitored human keratinocytes during negative-pressure treatment in real time and found that migration was enhanced under continuous and intermittent negative pressure (Yamashiro et al. 2022). In summary, these reports suggest that NPWT contributes to wound healing by weakening the intercellular junctions of epithelial cells and promoting cell movement.

This biomarker change in keratinocytes by NPWT recalls an association with epithelial-mesenchymal transition (EMT) (Zeisberg and Neilson 2009) (Fig. 4). EMT is a phenomenon in which epithelial cells acquire mesenchymal cell-like characteristics and is thought to be involved in embryonic development, fibrosis, wound healing, and cancer progression (Nieto et al. 2016; Dongre and Weinberg 2019). As a result of EMT, epithelial cells lose their cell polarity and cell adhesive function with surrounding cells and gain migration and invasive capacity, transforming into mesenchymal-like cells (Nieto et al. 2016; Dongre and Weinberg 2019) (Fig. 4). EMT-promoting factors include TGF-β, bFGF, epidermal growth factor (EGF), and hepatocyte growth factor (HGF) (Sistigu et al. 2017; Koike et al. 2020). EMT in wound healing may play an important role in reepithelialization from the wound margin (Haensel and Dai 2018). Despite these clues, the association between NPWT and EMT has not been examined. An in vitro study showed that EMT was promoted by intermittent negative pressure on triple-negative breast cancer cells (Liu et al. 2018a). EMT is also induced in human keratinocytes cultured in a microgravity environment through a decrease in levels of the epithelial marker E-cadherin; increase in the levels of mesenchymal markers, such as vimentin and α-SMA, as well as typical EMT transcription factors, such as *Snail1*, *Snail2*, and *ZEB2*; and increase in the levels of MMPs (Ranieri et al. 2017). These findings may lead to a discussion of the potential of EMT as a mechanism of action for NPWT.

4.6 Osteogenesis

Ilizarov reported that osteogenesis can occur following the application of a sustained elongation force to the bone (Ilizarov 1989a, b); bone lengthening is one of the most common examples of mechanotransduction being incorporated into

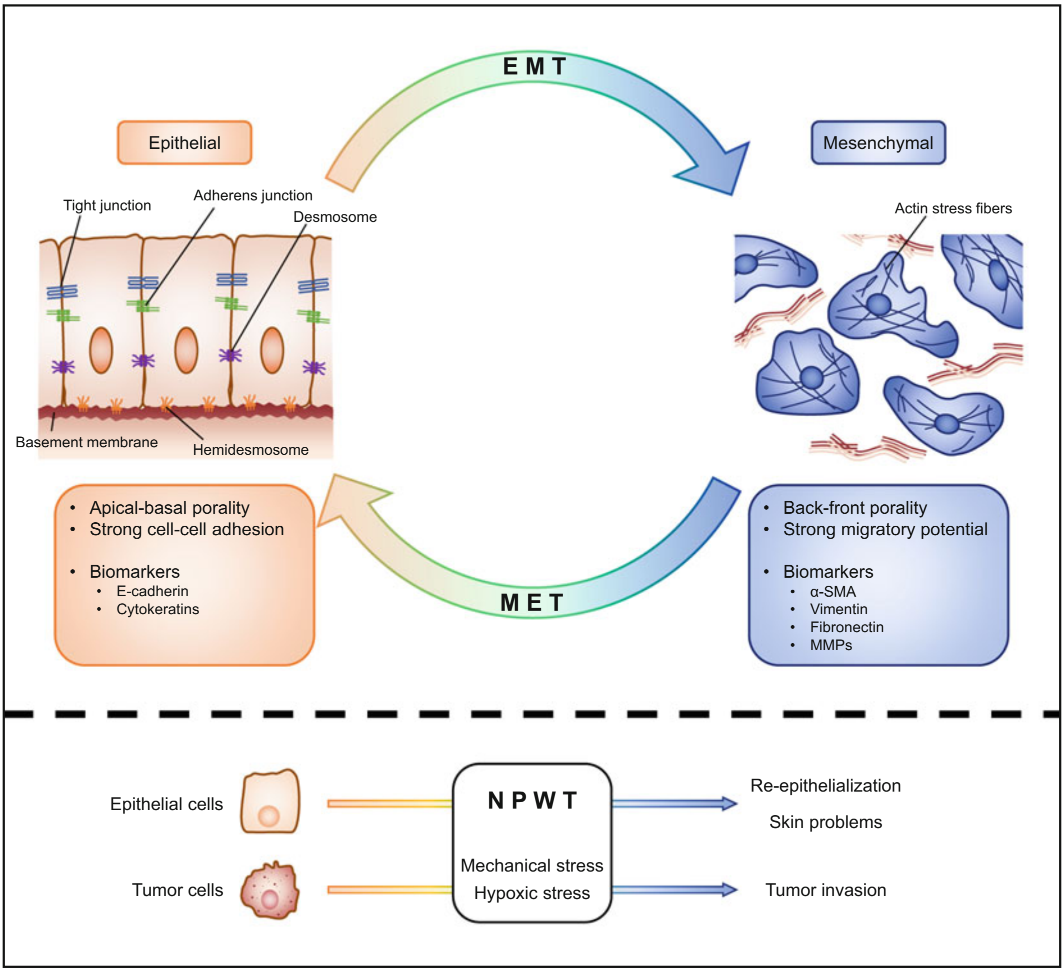

Fig. 4 Possible association of NPWT with EMT. Epithelial-mesenchymal transition (EMT) refers to the process by which epithelial cells acquire mesenchymal cell-like properties. EMT is a dynamic and transitional state between epithelial and mesenchymal phenotypes, and the opposing process is referred to as mesenchymal-epithelial transition (MET). The epithelial cell changes induced by negative-pressure wound therapy (NPWT) are similar to those observed in the process of EMT, and the same may apply to its effects on tumor cells. *EMT* epithelial-mesenchymal transition, *MET* mesenchymal-epithelial transition, *NPWT* negative-pressure wound therapy, *α-SMA* smooth muscle actin α, *MMP* matrix metalloproteinase

clinical practice (McNulty et al. 2007). Although the role of NPWT in bone healing remains unclear, several basic studies have confirmed that it promotes the differentiation of mesenchymal cells into an osteogenic phenotype and induction of osteoblast differentiation in vitro and promotes bone regeneration in vivo (Zhu et al. 2014; Liu et al. 2018b; Zhang et al. 2022; Yang et al. 2014; Wang et al. 2020). The group of Zhu et al. (2014) and Liu et al. (2018b) investigated the association between NPWT and osteogenesis using rat mesenchymal cells. Rat periosteum-derived mesenchymal stem cells (P-MSCs) and muscle-derived stem cells (MDSCs) were used to envisage closed fractures and open fractures with periosteal avulsion, respectively. Both cells were cultured under continuous negative pressure of −125 mmHg for 3 days, and cell proliferation was found to be significantly enhanced compared to that in culture under atmospheric pressure (Zhu et al. 2014; Liu et al. 2018b). Alkaline phosphatase (ALP) activity and mineralization were enhanced in P-MSCs, and increased expression of collagen type 1 and integrin β5, a mechanotransduction-related factor, was observed (Zhu et al. 2014). Experiments using MDSCs and inhibitors suggested that NPWT may promote osteogenic differentiation via the MAPK pathway (Liu et al. 2018b). It was also reported that NPWT induced MSC osteoblast differentiation in vitro and promoted bone regeneration in a rat model of cranial defect in vivo (Zhang et al. 2022). Yang et al. (2014) showed that intermittent negative-pressure exposure predominantly increased ALP activity and type I collagen and VEGF expression in MSCs as well as promoted and induced differentiation into osteoblasts. Wang et al. (2020) showed that 12 h of −125-mmHg negative-pressure exposure significantly increased osteoblast proliferation and ALP activity and promoted maturation.

4.7 Nerve Regeneration

The knowledge of the effects of NPWT on peripheral nerve regeneration is limited. A previous study indicated that NPWT for full-thickness skin wounds in diabetic mice significantly increased nerve fiber density and expression of substance P, calcitonin gene-related peptide (CGRP), and nerve growth factor (NGF) in the epidermis and dermis (Younan et al. 2010). The authors stated that NPWT may help in the healing of denervated wounds, such as pressure ulcers and diabetic foot lesions, by modulating the production of nerve fibers and neuropeptides.

The effects of NPWT on thicker nerve repair were studied using a rabbit sciatic nerve model. Hu et al. (2015) transected the sciatic nerve of a rabbit by 1 cm, sutured it in its original position, and treated it with NPWT and conventional methods. At 4 and 8 weeks post-operation, the NPWT group had significantly higher electrical nerve conduction velocity, higher intensity of immunohistochemical staining for brain-derived neurotrophic factor (BDNF), and higher number of myelinated nerve fibers than did the conventional group. Wu et al. (2018) also treated a rabbit sciatic nerve injury model for 12 weeks in three groups (0 kPa, −20 kPa, and −40 kPa) and reported that electrical nerve conduction velocity, myelinated nerve fiber count, and S100 and BDNF expression were significantly increased in the −20 kPa group compared with those in the 0 kPa group, similar to the findings of Hu et al. Conversely, the −40 kPa group had significantly lower electrical nerve conduction velocity and number of myelinated nerve fibers than did the 0 kPa group, and S100 and BDNF expression levels were not significantly different between −40 kPa and 0 kPa groups. Based on these results, they pointed out that NPWT may be beneficial for nerve repair when used at the appropriate strength but may not contribute to nerve repair when used at too high an intensity (Wu et al. 2018).

4.8 Effects on Bacterial Load

High bacterial loads inhibit wound healing; the effect of normal NPWT on bacterial load is debatable (Patmo et al. 2014). Morykwas et al. (1997) showed in their porcine experiments that negative-pressure treatment at −125 mmHg for 4 days significantly reduced bacterial counts. Ngo et al. (2012) reported a significant reduction of *Pseudomonas aeruginosa* biofilm in in vitro experiments using agar. Wang et al. (2016) further found that negative-pressure treatment significantly suppressed the secretion of virulence factors, such as exotoxin A, rhamnolipid, and elastase by *P. aeruginosa* as well as the expression levels of these regulatory genes. Conversely, Boone et al. (2010), in their experiments with pigs, reported that bacterial counts continued to increase after 7 days of NPWT despite gross and microscopic wound improvement. Fujiwara et al. (2013), in their in vitro study, reported that NPWT increased the growth potential of *Escherichia coli*, with this trend being stronger with intermittent negative pressure than with continuous negative pressure and with short cycles than with long cycles. In a prospective randomized trial using tissue bacterial culture specimens from patients with full-thickness wounds that could not be temporarily closed due to wound healing inhibitors, Mouës et al. (2004) reported that the −125-mmHg negative-pressure treatment group showed a significant decrease in gram-negative bacillus counts and a significant increase in *Staphylococcus aureus* counts compared to those in the conventional treatment group. Assadian et al. (2010) reported no difference in *S. aureus* abundance with or without negative pressure in an in vitro study with pork, noting that results may differ for live tissues. They mentioned that NPWT-induced changes in bacterial load may not be due to pure negative pressure alone. Biermann et al. (2019) described that the different responses among bacterial species to hypoxia in the dressing may be related to bioburden changes. Based on these considerations, the application of NPWT on wounds with strong infection is generally considered a relative contraindication (Normandin et al. 2021).

Efforts have been made to apply NPWT to difficult-to-heal wounds caused by infection, including using antimicrobial materials (Payne and Ambrosio 2009) and liquid infusion in combination with wound cleansing (Wolvos 2004). Silver-impregnated foam is often used as an antimicrobial material. It is assumed that silver ions released slowly into the exudate exert an antimicrobial effect. Most of the ions are recovered without deposition on the wound surface or distribution throughout the body (Abarca-Buis et al. 2014). In vitro studies have shown the antimicrobial efficacy of silver-impregnated foam against *S. aureus* colonies (Valente et al. 2016; Matiasek et al. 2017) and biofilms, such as those of methicillin-resistant *S. aureus* (MRSA) (Valente et al. 2016; Ellenrieder et al. 2015) and *P. aeruginosa* (Valente et al. 2016*;* Ngo et al. 2012). In clinical practice, its usefulness in acute lower extremity trauma is also described (Hahn et al. 2019). NPWT with irrigation includes (1) irrigation and application of negative pressure continuously and simultaneously (Kiyokawa et al. 2007) and (2) periodically and sequentially instilling washing solution, immersion, and negative-pressure treatment (Wolvos 2013; Jerome 2007; Kim et al. 2014); the latter approach is more popular. The infusion solution includes saline solution and disinfectants, such as sodium hypochlorite solution (Yang et al. 2017), povidone-iodine solution (Tahir et al. 2018), and polyhexanide (Timmers et al. 2009). These disinfectants are all considered more effective in wound healing than NPWT without irrigation (Tahir et al. 2018), but some reports suggest that patient outcomes are no different with antiseptic and saline (Kim et al. 2015; Lavery et al. 2020). It has been suggested that the effect cannot be explained by a reduction in bacterial count alone (Burusapat and Sringkarawat 2021), and further study is needed on the optimal composition of the infusion solution and treatment protocol (Saeg et al. 2021).

4.9 Effects on Tumorigenesis

As mentioned previously, since NPWT promotes cell proliferation and angiogenesis, applying NPWT to sites where malignant tumors are present is contraindicated as it may increase tumor cell growth (Normandin et al. 2021). However, owing to ethical concerns, there is little evidence of whether NPWT leads to proliferation of tumor cells in clinical practice; this aspect has also not been explored in vitro. Continuous or intermittent mechanical stimulation in breast cancer cells enhances invasiveness by promoting EMT (Liu et al. 2018a; Tse et al. 2012) (Fig. 4). In clinical practice, attempts have been made to use NPWT as coverage for wounds after resecting epithelial or mesenchymal malignancies until two-stage reconstruction, assuming complete resection of tumor cells (Fourman et al. 2022; Dadras et al. 2022). Several recent reports have documented that NPWT after resection of malignant tumors may predominantly reduce postoperative complications, such as infection, with no clear difference in the risk of local recurrence of tumors compared to that observed with conventional dressing (Hays et al. 2022; Wang et al. 2022).

4.10 Effects on Operative Wounds

As described previously, NPWT has traditionally been used for acute and chronic open wounds. In recent years, the effect of negative pressure on areas where no raw surface exists has been investigated. NPWT, particularly for primary closed wounds, called incisional NPWT (iNPWT), has attracted increasing attention. iNPWT is performed, instead of regular dressings, on primary closed incisional wounds and is thought to reduce wound complications by using negative-pressure loading, such as with sNPWT devices (Scalise et al. 2016). In the 2022 Cochrane review, it was noted that while iNPWT reduces the incidence of surgical site infection (SSI), wound dehiscence is similar to that observed with standard dressings and may increase blistering problems (Norman et al. 2022). There is no consensus regarding the effect of iNPWT on scar formation (Zwanenburg et al. 2021; Timmermans et al. 2022). These effects of iNPWT may result from tissue or bacterial responses to the primary mechanisms and secondary effects described so far, but further studies are needed to elucidate the detailed mechanisms (Scalise et al. 2016).

The efficacy of NPWT in skin graft surgery was first reported in 1998 by Blackburn et al. (1998). Many subsequent clinical studies have shown that fixing grafted skin with NPWT improves the rate of graft skin takes, compared to conventional skin fixation methods (Petkar et al. 2011; Yin et al. 2018). From a macroscopic perspective, several mechanisms have been considered, including suppression of seroma and hematoma development, which can cause poor graft implantation, and good fixation to the graft bed by compression (Yin et al. 2018). Although the molecular biological mechanism is currently unknown, ex vivo studies using patients' excess skin showed that NPWT promoted the expression of fibroblast growth factor 1 (*FGFR1*), endothelin 2 (*EDN2*), and 22 keratin-related proteins and genes in the graft (Rapp et al. 2020).

Animal studies have demonstrated that NPWT enhances the survival area of random pattern flaps (Morykwas et al. 1997). Some researchers believe that NPWT in flap surgery has benefits beyond the reduction in SSI obtained with iNPWT. In the literature comparing postoperative coverage of free myocutaneous flaps with NPWT and conventional treatment using biopsy specimens of the flap tissue, tissue infiltration of CD68-positive macrophages and expression of the inflammatory cytokines IL-1β and TNF-α were reduced in the NPWT group, and the number of apoptotic cells was also reduced due to improved microcirculation (Eisenhardt et al. 2012). Some clinical reports suggest that the drainage effect of NPWT is useful in reducing flap congestion, one of the major causes of flap failure (Yu et al. 2017; Boissiere et al. 2021). Overall, postoperative NPWT coverage after skin flap surgery may have beneficial effects, such as improving skin flap viability and reducing congestion through anti-inflammatory effects and improved microcirculation, but additional research is needed.

There have been other attempts at flap preconditioning (Rhodius et al. 2018), in which negative pressure is applied in advance to the area where the flap is to be harvested to obtain improved viability of the flap through the angiogenesis effect, and there is potential for further development (Rhodius et al. 2018; Aydin et al. 2019; Hong et al. 2019; Mohan et al. 2020; Brown and Ghareeb 2021).

5 Drawbacks of NPWT

As described thus far, NPWT works favorably for a variety of wounds through four main mechanisms and accompanying secondary effects; however, there are several drawbacks, including complications and conditions that require caution. We briefly introduce them in this section.

5.1 Complications

Several complications of NPWT have been reported, ranging from minor to serious ones, but most are due to human factors, such as poor patient selection or procedural problems, and almost all complications can be prevented or avoided (Normandin et al. 2021).

Heart rupture, one of the fatal NPWT-related complications, may be caused by contact between the right ventricle and the edge of incised sternum due to the change in wound geometry (Sartipy et al. 2006; Vos et al. 2013). Preventive measures include covering the heart and immobilizing the sternum (Vos et al. 2013); the use of protective devices inserted between the heart and sternum has also been reported to be effective (Lindstedt et al. 2011; Ingemansson et al. 2014).

Direct application of NPWT to exposed internal organs or blood vessels can cause organ damage, such as intestinal fistulas or major bleedings (White et al. 2005; Rao et al. 2007; Fischer 2008). Attention should be paid to vital structures by protecting organs with contact layers or covering them with grafts or flaps (Huang et al. 2014; Normandin et al. 2021).

As noted above, infection is one of the most well-known complications of NPWT, although there is ongoing debate. Clinically, the foam itself is a foreign material, and blood clots from bleeding can cause bacterial growth; therefore, treatment should be interrupted if bleeding is suspected (Li and Yu 2014).

Pain is another complication that should not be ignored. Pain in NPWT occurs at the start of treatment and during dressing changes. Pain during treatment has been reported to be stronger with intermittent negative pressure than with continuous negative pressure (Borgquist et al. 2010a; Malmsjo et al. 2012); starting treatment at low pressure and gradually increasing the pressure are considered effective (Normandin et al. 2021). There have been reports of the use of anesthetics during dressing changes (Tank et al. 2021); however, large studies are needed to draw any conclusions.

5.2 Contraindications

There are several conditions under which NPWT application requires caution; incorrect patient selection can not only prevent the expected therapeutic effect but also cause the aforementioned complications.

NPWT for wounds with malignant tumors or exposed vital organs is contraindicated owing to concerns regarding tumor growth and fatal complications, as mentioned above, and is considered only after thorough resection of tumor cells and organ protection (Normandin et al. 2021).

It is important to consider the possibility of rather harmful effects on tissue blood flow, such as when applying NPWT to ischemic lesions or when using NPWT in the extremity circumferentially (Kairinos et al. 2009a, b; Livingstone et al. 2021; Singh et al. 2021). If NPWT is to be performed on ischemic tissue, it should only be done after appropriate revascularization (De Caridi et al. 2016).

When NPWT is performed on wounds with infected or necrotic tissue, such as diabetic foot lesions, proper debridement and control of infection are necessary beforehand, as their persistence may lead to serious systemic complications (Meloni et al. 2015; Chen et al. 2021). With the advent of NPWTi-d, minor colonization can now be treated relatively safely (Kim et al. 2014).

6 Final Remarks

A quarter of a century has passed since the advent of NPWT, and it is time for basic research to support its effectiveness. NPWT has many variable parameters, such as the type of treatment device, dressing material, intensity and duration of negative pressure, duration of treatment, continuous or variable pressure, presence or absence of irrigation, composition of irrigation solution, and even patient-side factors, which must be fully considered when designing the study model. Moreover, when conducting in vivo experiments in mice, it is difficult to confirm the effects of the experimental model on the physiological functions of the whole body of the mouse and the possible adverse effects on the experimental results (Dastouri et al. 2011). Furthermore, different devices may have different mechanisms and effects (Nuutila et al. 2021), and it is important to consider whether the findings of each report apply to NPWT, as a whole. We believe that elucidation of the mechanism of NPWT through expansion of basic research will greatly contribute to the expansion of therapeutic indications, such as safer use with reduced complications, in addition to the improvement of devices or combination with other therapeutic methods with newly defined therapeutic targets.

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References

Abarca-Buis RF, Munguia NM, Gonzalez JM, Solis-Arrieta L, Osorio LS, Krotzsch E (2014) Silver from polyurethane dressing is delivered by gradient to exudate, tissue, and serum of patients undergoing negative-pressure wound treatment. Adv Skin Wound Care 27:156–162. https://doi.org/10.1097/01.ASW.0000444849.71374.bc

Ahearn C (2009) Intermittent NPWT and lower negative pressures – exploring the disparity between science and current practice: a review. Ostomy Wound Manage 55:22–28

Alford PW, Nesmith AP, Seywerd JN, Grosberg A, Parker KK (2011) Vascular smooth muscle contractility depends on cell shape. Integr Biol (Camb) 3:1063–1070. https://doi.org/10.1039/c1ib00061f

Anesater E, Borgquist O, Hedstrom E, Waga J, Ingemansson R, Malmsjo M (2011) The influence of different sizes and types of wound fillers on wound contraction and tissue pressure during negative pressure wound therapy. Int Wound J 8:336–342. https://doi.org/10.1111/j.1742-481X.2011.00790.x

Argenta LC, Morykwas MJ (1997) Vacuum-assisted closure: a new method for wound control and treatment: clinical experience. Ann Plast Surg 38:563–576; discussion 577

Assadian O, Assadian A, Stadler M, Diab-Elschahawi M, Kramer A (2010) Bacterial growth kinetic without the influence of the immune system using vacuum-assisted closure dressing with and without negative pressure in an in vitro wound model. Int Wound J 7:283–289. https://doi.org/10.1111/j.1742-481X.2010.00686.x

Aydin OE, Algan S, Tan O, Demirci E, Keles ON, Kantarci A (2019) A novel method for flap delay vacuum assisted flap delay: an experimental study in rabbits. J Plast Surg Hand Surg 53:208–215. https://doi.org/10.1080/2000656X.2019.1582425

Bertelsen CA, Fabricius R, Kleif J, Kristensen B, Gogenur I (2014) Outcome of negative-pressure wound therapy for open abdomen treatment after nontraumatic lower gastrointestinal surgery: analysis of factors affecting delayed fascial closure in 101 patients. World J Surg 38:774–781. https://doi.org/10.1007/s00268-013-2360-7

Biermann N, Geissler EK, Brix E, Schiltz D, Prantl L, Kehrer A et al (2019) Oxygen levels during negative pressure wound therapy. J Tissue Viability 28:223–226. https://doi.org/10.1016/j.jtv.2019.09.001

Biermann N, Geissler EK, Brix E, Schiltz D, Muehle C, Prantl L et al (2020) Pressure distribution and flow characteristics during negative pressure wound therapy. J Tissue Viability 29:32–36. https://doi.org/10.1016/j.jtv.2019.12.004

Bjarnason T, Montgomery A, Hlebowicz J, Lindstedt S, Petersson U (2011) Pressure at the bowel surface during topical negative pressure therapy of the open abdomen: an experimental study in a porcine model. World J Surg 35:917–923. https://doi.org/10.1007/s00268-010-0937-y

Blackburn 2nd JH, Boemi L, Hall WW, Jeffords K, Hauck RM, Banducci DR et al (1998) Negative-pressure dressings as a bolster for skin grafts. Ann Plast Surg 40:453–457. https://doi.org/10.1097/00000637-199805000-00001

Boissiere F, Gandolfi S, Riot S, Kerfant N, Jenzeri A, Hendriks S et al (2021) Flap venous congestion and salvage techniques: a systematic literature review. Plast Reconstr Surg Glob Open 9:e3327. https://doi.org/10.1097/GOX.0000000000003327

Boone D, Braitman E, Gentics C, Afthinos J, Latif J, Sordillo E et al (2010) Bacterial burden and wound outcomes as influenced by negative pressure wound therapy. Wounds 22:32–37

Borgquist O, Ingemansson R, Malmsjo M (2010a) The effect of intermittent and variable negative pressure wound therapy on wound edge microvascular blood flow. Ostomy Wound Manage 56:60–67

Borgquist O, Gustafsson L, Ingemansson R, Malmsjo M (2010b) Micro- and macromechanical effects on the wound bed of negative pressure wound therapy using gauze and foam. Ann Plast Surg 64:789–793. https://doi.org/10.1097/SAP.0b013e3181ba578a

Borgquist O, Ingemansson R, Malmsjo M (2011) The influence of low and high pressure levels during negative-pressure wound therapy on wound contraction and fluid evacuation. Plast Reconstr Surg 127:551–559. https://doi.org/10.1097/PRS.0b013e3181fed52a

Brown CA, Ghareeb PA (2021) Early experience with external negative pressure delay in free anterolateral thigh perforator flap reconstruction. Plast Reconstr

Surg Glob Open 9:e3606. https://doi.org/10.1097/GOX.0000000000003606

Bui TD, Huerta S, Gordon IL (2006) Negative pressure wound therapy with off-the-shelf components. Am J Surg 192:235–237. https://doi.org/10.1016/j.amjsurg.2006.01.013

Burusapat C, Sringkarawat S (2021) Efficacy of negative-pressure wound therapy with tetrachlorodecaoxygen-anion complex instillation compared with standard negative-pressure wound therapy for accelerated wound healing: a prospective, randomized, controlled trial. Plast Reconstr Surg 148:339–352. https://doi.org/10.1097/PRS.0000000000008233

Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE (1997) Geometric control of cell life and death. Science 276:1425–1428. https://doi.org/10.1126/science.276.5317.1425

Chen L, Zhang S, Da J, Wu W, Ma F, Tang C et al (2021) A systematic review and meta-analysis of efficacy and safety of negative pressure wound therapy in the treatment of diabetic foot ulcer. Ann Palliat Med 10:10830. https://doi.org/10.21037/apm-21-2476

Cheng Y, Wang K, Gong J, Liu Z, Gong J, Zeng Z, Wang X (2022) Negative pressure wound therapy for managing the open abdomen in non-trauma patients. Cochrane Database Syst Rev 5:CD013710. https://doi.org/10.1002/14651858.CD013710.pub2

Chow SE, Chen CP, Hsu CC, Tsai WC, Wang JS, Hsu NC (2016) Quantifying cell behaviors in negative-pressure induced monolayer cell movement. Biom J 39:50–59. https://doi.org/10.1016/j.bj.2015.08.005

Dadras M, Ufton D, Sogorski A, Wallner C, Wagner JM, Lehnhardt M et al (2022) Closed-incision negative-pressure wound therapy after resection of soft-tissue tumors reduces wound complications: results of a randomized trial. Plast Reconstr Surg 149:972e–980e. https://doi.org/10.1097/PRS.0000000000009023

Dastouri P, Helm DL, Scherer SS, Pietramaggiori G, Younan G, Orgill DP (2011) Waveform modulation of negative-pressure wound therapy in the murine model. Plast Reconstr Surg 127:1460–1466. https://doi.org/10.1097/PRS.0b013e31820a63cb

De Caridi G, Massara M, Greco M, Pipitò N, Spinelli F, Grande R et al (2016) VAC therapy to promote wound healing after surgical revascularisation for critical lower limb ischaemia. Int Wound J 13:336–342. https://doi.org/10.1111/iwj.12301

Diaz-Flores L Jr, Gutierrez R, Madrid JF, Varela H, Valladares F, Diaz-Flores L (2009) Adult stem cells and repair through granulation tissue. Front Biosci (Landmark Ed) 14:1433–1470. https://doi.org/10.2741/3317

Dongre A, Weinberg RA (2019) New insights into the mechanisms of epithelial-mesenchymal transition and implications for cancer. Nat Rev Mol Cell Biol 20:69–84. https://doi.org/10.1038/s41580-018-0080-4

Eisenhardt SU, Schmidt Y, Thiele JR, Iblher N, Penna V, Torio-Padron N et al (2012) Negative pressure wound therapy reduces the ischaemia/reperfusion-associated inflammatory response in free muscle flaps. J Plast Reconstr Aesthet Surg 65:640–649. https://doi.org/10.1016/j.bjps.2011.11.037

Ellenrieder M, Redanz S, Bader R, Mittelmeier W, Podbielski A (2015) Influence of antimicrobial coatings of vacuum-assisted closure dressings on methicillin-resistant Staphylococcus aureus growth kinetics: an in vitro study. Surg Infect 16:139–145. https://doi.org/10.1089/sur.2013.268

Erba P, Ogawa R, Ackermann M, Adini A, Miele LF, Dastouri P et al (2011) Angiogenesis in wounds treated by microdeformational wound therapy. Ann Surg 253:402–409. https://doi.org/10.1097/SLA.0b013e31820563a8

Faust E, Opoku-Agyeman JL, Behnam AB (2021) Use of negative-pressure wound therapy with instillation and dwell time: an overview. Plast Reconstr Surg 147:16S–26S. https://doi.org/10.1097/PRS.0000000000007607

Fischer JE (2008) A cautionary note: the use of vacuum-assisted closure systems in the treatment of gastrointestinal cutaneous fistula may be associated with higher mortality from subsequent fistula development. Am J Surg 196:1–2. https://doi.org/10.1016/j.amjsurg.2008.01.001

Folkman J, Moscona A (1978) Role of cell shape in growth control. Nature 273:345–349. https://doi.org/10.1038/273345a0

Fong KD, Marston WA (2012) SNaP((R)) wound care system: ultraportable mechanically powered negative pressure wound therapy. Adv Wound Care (New Rochelle) 1:41–43. https://doi.org/10.1089/wound.2011.0281

Fourman MS, Ramsey DC, Newman ET, Schwab JH, Chen YL, Hung YP et al (2022) Assessing the safety and utility of wound VAC temporization of the sarcoma or benign aggressive tumor bed until final margins are achieved. Ann Surg Oncol 29:2290–2298. https://doi.org/10.1245/s10434-021-11023-9

Fu S, Panayi A, Fan J, Mayer HF, Daya M, Khouri RK et al (2021) Mechanotransduction in wound healing: from the cellular and molecular level to the clinic. Adv Skin Wound Care 34:67–74. https://doi.org/10.1097/01.ASW.0000725220.92976.a7

Fujiwara T, Nishimoto S, Ishise H, Sotsuka Y, Kawai K, Fukuda K et al (2013) Influence of continuous or intermittent negative pressure on bacterial proliferation potency in vitro. J Plast Surg Hand Surg 47:180–184. https://doi.org/10.3109/2000656X.2012.748667

Gibson DJ (2022) A comparison of the biomechanical performance of 3 negative pressure wound therapy foams. J Wound Ostomy Continence Nurs 49:51–58. https://doi.org/10.1097/WON.0000000000000833

Glass GE, Murphy GF, Esmaeili A, Lai LM, Nanchahal J (2014) Systematic review of molecular mechanism of action of negative-pressure wound therapy. Br J Surg 101:1627–1636. https://doi.org/10.1002/bjs.9636

Greene AK, Puder M, Roy R, Arsenault D, Kwei S, Moses MA et al (2006) Microdeformational wound therapy: effects on angiogenesis and matrix metalloproteinases in chronic wounds of 3 debilitated patients. Ann Plast Surg 56:418–422. https://doi.org/10.1097/01.sap.0000202831.43294.02

Haensel D, Dai X (2018) Epithelial-to-mesenchymal transition in cutaneous wound healing: where we are and where we are heading. Dev Dyn 247:473–480. https://doi.org/10.1002/dvdy.24561

Hahn HM, Lee IJ, Woo KJ, Park BY (2019) Silver-impregnated negative-pressure wound therapy for the treatment of lower-extremity open wounds: a prospective randomized clinical study. Adv Skin Wound Care 32:370–377. https://doi.org/10.1097/01.ASW.0000569116.59534.a6

Hays TR, Singh G, Saragossi J, Park J, Shekar S, Marquez JE et al (2022) Negative-pressure wound therapy versus standard surgical dressings after malignant tumor resection: a systematic review and meta-analysis. Plast Reconstr Surg 150:655e–670e. https://doi.org/10.1097/PRS.0000000000009448

Hinman CD, Maibach H (1963) Effect of air exposure and occlusion on experimental human skin wounds. Nature 200:377–378. https://doi.org/10.1038/200377a0

Hong YG, Kim SC, Koh KS (2019) Flap preconditioning with the cyclic mode (triangular waveform) of pressure-controlled cupping in a rat model: an alternative mode to the continuous system. Plast Reconstr Surg 143:88e–98e. https://doi.org/10.1097/PRS.0000000000005104

Hsu CC, Tsai WC, Chen CP, Lu YM, Wang JS (2010) Effects of negative pressures on epithelial tight junctions and migration in wound healing. Am J Physiol Cell Physiol 299:C528–C534. https://doi.org/10.1152/ajpcell.00504.2009

Hsu CC, Chow SE, Chen CP, Tsai WC, Wang JS, Yu SY et al (2013) Negative pressure accelerated monolayer keratinocyte healing involves Cdc42 mediated cell podia formation. J Dermatol Sci 70:196–203. https://doi.org/10.1016/j.jdermsci.2013.03.007

Hu C, Zhang T, Deng Z, Ren B, Cai L, Zhang Y et al (2015) Study on the effect of vacuum sealing drainage on the repair process of rabbit sciatic nerve injury. Int J Neurosci 125:855–860. https://doi.org/10.3109/00207454.2014.979981

Huang S, Ingber DE (1999) The structural and mechanical complexity of cell-growth control. Nat Cell Biol 1:E131–E138. https://doi.org/10.1038/13043

Huang C, Ogawa R (2012) Fibroproliferative disorders and their mechanobiology. Connect Tissue Res 53:187–196. https://doi.org/10.3109/03008207.2011.642035

Huang S, Chen CS, Ingber DE (1998) Control of cyclin D1, p27(Kip1), and cell cycle progression in human capillary endothelial cells by cell shape and cytoskeletal tension. Mol Biol Cell 9:3179–3193. https://doi.org/10.1091/mbc.9.11.3179

Huang C, Leavitt T, Bayer LR, Orgill DP (2014) Effect of negative pressure wound therapy on wound healing. Curr Probl Surg 51:301–331. https://doi.org/10.1067/j.cpsurg.2014.04.001

Huang CH, Hsu CC, Chen CP, Chow SE, Wang JS, Shyu YC et al (2016) Negative pressure induces p120-catenin-dependent adherens junction disassembly in keratinocytes during wound healing. Biochim Biophys Acta 1863:2212–2220. https://doi.org/10.1016/j.bbamcr.2016.05.017

Ilizarov GA (1989a) The tension-stress effect on the genesis and growth of tissues. Part I. the influence of stability of fixation and soft-tissue preservation. Clin Orthop Relat Res 238:249–281

Ilizarov GA (1989b) The tension-stress effect on the genesis and growth of tissues: part II. The influence of the rate and frequency of distraction. Clin Orthop Relat Res 239:263–285

Ingber DE (2005) Mechanical control of tissue growth: function follows form. Proc Natl Acad Sci U S A 102:11571–11572. https://doi.org/10.1073/pnas.0505939102

Ingemansson R, Malmsjö M, Lindstedt S (2014) The HeartShield device reduces the risk for right ventricular damage in patients with deep sternal wound infection. Innovations (Phila) 9:137–141; discussion 141. https://doi.org/10.1097/imi.0000000000000054

Jerome D (2007) Advances in negative pressure wound therapy: the VAC instill. J Wound Ostomy Continence Nurs 34:191–194. https://doi.org/10.1097/01.WON.0000264834.18732.3b

Kairinos N, Solomons M, Hudson DA (2009a) Negative-pressure wound therapy I: the paradox of negative-pressure wound therapy. Plast Reconstr Surg 123:589–598. https://doi.org/10.1097/PRS.0b013e3181956551

Kairinos N, Voogd AM, Botha PH, Kotze T, Kahn D, Hudson DA, Solomons M (2009b) Negative-pressure wound therapy II: negative-pressure wound therapy and increased perfusion. Just an illusion? Plast Reconstr Surg 123:601–612. https://doi.org/10.1097/PRS.0b013e318196b97b

Kanapathy M, Mantelakis A, Khan N, Younis I, Mosahebi A (2020) Clinical application and efficacy of negative pressure wound therapy with instillation and dwell time (NPWTi-d): a systematic review and meta-analysis. Int Wound J 17:1948–1959. https://doi.org/10.1111/iwj.13487

Kantak NA, Mistry R, Halvorson EG (2016) A review of negative-pressure wound therapy in the management of burn wounds. Burns 42:1623–1633. https://doi.org/10.1016/j.burns.2016.06.011

Katsumi A, Orr AW, Tzima E, Schwartz MA (2004) Integrins in mechanotransduction. J Biol Chem 279:12001–12004. https://doi.org/10.1074/jbc.R300038200

Katzengold R, Topaz M, Gefen A (2018) Dynamic computational simulations for evaluating tissue loads applied by regulated negative pressure-assisted wound therapy (RNPT) system for treating large wounds. J Tissue Viability 27:101–113. https://doi.org/10.1016/j.jtv.2017.10.004

Katzengold R, Orlov A, Gefen A (2021) A novel system for dynamic stretching of cell cultures reveals the mechanobiology for delivering better negative pressure wound therapy. Biomech Model Mechanobiol 20:193–204. https://doi.org/10.1007/s10237-020-01377-6

Kim PJ, Attinger CE, Steinberg JS, Evans KK, Powers KA, Hung RW et al (2014) The impact of negative-pressure wound therapy with instillation compared with standard negative-pressure wound therapy: a retrospective, historical, cohort, controlled study. Plast Reconstr Surg 133:709–716. https://doi.org/10.1097/01.prs.0000438060.46290.7a

Kim PJ, Attinger CE, Oliver N, Garwood C, Evans KK, Steinberg JS et al (2015) Comparison of outcomes for normal saline and an antiseptic solution for negative-pressure wound therapy with instillation. Plast Reconstr Surg 136:657e–664e. https://doi.org/10.1097/PRS.0000000000001709

Kirsner RS, Zimnitsky D, Robinson M (2021) A prospective, randomized, controlled clinical study on the effectiveness of a single-use negative pressure wound therapy system, compared to traditional negative pressure wound therapy in the treatment of diabetic ulcers of the lower extremities. Wound Repair Regen 29:908–911. https://doi.org/10.1111/wrr.12966

Kiyokawa K, Takahashi N, Rikimaru H, Yamauchi T, Inoue Y (2007) New continuous negative-pressure and irrigation treatment for infected wounds and intractable ulcers. Plast Reconstr Surg 120:1257–1265. https://doi.org/10.1097/01.prs.0000279332.27374.69

Kloth LC, Berman JE, Nett M, Papanek PE, Dumit-Minkel S (2002) A randomized controlled clinical trial to evaluate the effects of noncontact normothermic wound therapy on chronic full-thickness pressure ulcers. Adv Skin Wound Care 15:270–276. https://doi.org/10.1097/00129334-200211000-00008

Koch AE, Polverini PJ, Kunkel SL, Harlow LA, DiPietro LA, Elner VM et al (1992) Interleukin-8 as a macrophage-derived mediator of angiogenesis. Science 258:1798–1801. https://doi.org/10.1126/science.1281554

Koike Y, Yozaki M, Utani A, Murota H (2020) Fibroblast growth factor 2 accelerates the epithelial-mesenchymal transition in keratinocytes during wound healing process. Sci Rep 10:18545. https://doi.org/10.1038/s41598-020-75584-7

Labanaris AP, Polykandriotis E, Horch RE (2009) The effect of vacuum-assisted closure on lymph vessels in chronic wounds. J Plast Reconstr Aesthet Surg 62:1068–1075. https://doi.org/10.1016/j.bjps.2008.01.006

Labler L, Rancan M, Mica L, Härter L, Mihic-Probst D, Keel M (2009) Vacuum-assisted closure therapy increases local interleukin-8 and vascular endothelial growth factor levels in traumatic wounds. J Traum Acute Care Surg 66:749–757. https://doi.org/10.1097/TA.0b013e318171971a

Lavery LA, Davis KE, La Fontaine J, Farrar JD, Bhavan K, Oz OK et al (2020) Does negative pressure wound therapy with irrigation improve clinical outcomes? A randomized clinical trial in patients with diabetic foot infections. Am J Surg 220:1076–1082. https://doi.org/10.1016/j.amjsurg.2020.02.044

Lee KN, Ben-Nakhi M, Park EJ, Hong JP (2015) Cyclic negative pressure wound therapy: an alternative mode to intermittent system. Int Wound J 12:686–692. https://doi.org/10.1111/iwj.12201

Li Z, Yu A (2014) Complications of negative pressure wound therapy: a mini review. Wound Repair Regen 22:457–461. https://doi.org/10.1111/wrr.12190

Lindstedt S, Ingemansson R, Malmsjö M (2011) Sternum wound contraction and distension during negative pressure wound therapy when using a rigid disc to prevent heart and lung rupture. J Cardiothorac Surg 6:42. https://doi.org/10.1186/1749-8090-6-42

Liu W, Fu X, Yang Z, Li S, Cao Y, Li Q, Luan J (2018a) Moderate intermittent negative pressure increases invasiveness of MDA-MB-231 triple negative breast cancer cells. Breast 38:14–21. https://doi.org/10.1016/j.breast.2017.11.011

Liu H, Zheng X, Chen L, Jian C, Hu X, Zhao Y et al (2018b) Negative pressure wound therapy promotes muscle-derived stem cell osteogenic differentiation through MAPK pathway. J Cell Mol Med 22:511–520. https://doi.org/10.1111/jcmm.13339

Liu L, Chen R, Jia Z, Li X, Tang Y, Zhao X, Zhang S et al (2022) Downregulation of hsa-miR-203 in peripheral blood and wound margin tissue by negative pressure wound therapy contributes to wound healing of diabetic foot ulcers. Microvasc Res 139:104275. https://doi.org/10.1016/j.mvr.2021.104275

Livingstone JP, Singh D, Murray PC (2021) An in vitro study measuring the effects of circumferential and near-circumferential closed incisional negative pressure wound therapy dressings. Cureus 13:e14389. https://doi.org/10.7759/cureus.14389

Lu F, Ogawa R, Nguyen DT, Chen B, Guo D, Helm DL et al (2011) Microdeformation of three-dimensional cultured fibroblasts induces gene expression and morphological changes. Ann Plast Surg 66:296–300. https://doi.org/10.1097/SAP.0b013e3181ea1e9b

Ma Z, Shou K, Li Z, Jian C, Qi B, Yu A (2016) Negative pressure wound therapy promotes vessel destabilization and maturation at various stages of wound healing and thus influences wound prognosis. Exp Ther Med 11:1307–1317. https://doi.org/10.3892/etm.2016.3083

Ma Z, Li Z, Shou K, Jian C, Li P, Niu Y et al (2017) Negative pressure wound therapy: regulating blood flow perfusion and microvessel maturation through microvascular pericytes. Int J Mol Med 40:1415–1425. https://doi.org/10.3892/ijmm.2017.3131

Malmsjo M, Gustafsson L, Lindstedt S, Gesslein B, Ingemansson R (2012) The effects of variable, intermittent, and continuous negative pressure wound therapy, using foam or gauze, on wound contraction, granulation tissue formation, and ingrowth into the wound filler. Eplasty 12:e5

Malsiner CC, Schmitz M, Horch RE, Keller AK, Leffler M (2015) Vessel transformation in chronic wounds under topical negative pressure therapy: an immunohistochemical analysis. Int Wound J 12:501–509. https://doi.org/10.1111/iwj.12143

Matiasek J, Domig KJ, Djedovic G, Babeluk R, Assadian O (2017) The effect of negative pressure wound therapy with antibacterial dressings or antiseptics on an in vitro wound model. J Wound Care 26:236–242. https://doi.org/10.12968/jowc.2017.26.5.236

McLeod KJ, Lee RC, Ehrlich HP (1987) Frequency dependence of electric field modulation of fibroblast protein synthesis. Science 236:1465–1469. https://doi.org/10.1126/science.3589667

McNulty AK, Schmidt M, Feeley T, Kieswetter K (2007) Effects of negative pressure wound therapy on fibroblast viability, chemotactic signaling, and proliferation in a provisional wound (fibrin) matrix. Wound Repair Regen 15:838–846. https://doi.org/10.1111/j.1524-475X.2007.00287.x

McNulty AK, Schmidt M, Feeley T, Villanueva P, Kieswetter K (2009) Effects of negative pressure wound therapy on cellular energetics in fibroblasts grown in a provisional wound (fibrin) matrix. Wound Repair Regen 17:192–199. https://doi.org/10.1111/j.1524-475X.2009.00460.x

Meloni M, Izzo V, Vainieri E, Giurato L, Ruotolo V, Uccioli L (2015) Management of negative pressure wound therapy in the treatment of diabetic foot ulcers. World J Orthop 6:387–393. https://doi.org/10.5312/wjo.v6.i4.387

Mohan AT, Zhu L, Michalak GJ, Laungani AT, Vercnocke AJ, Moran SL et al (2020) Preconditioning with foam-mediated external suction on flap microvasculature and perfusion in a rodent model. Plast Reconstr Surg Glob Open 8:e2739. https://doi.org/10.1097/GOX.0000000000002739

Morykwas MJ, Argenta LC, Shelton-Brown EI, McGuirt W (1997) Vacuum-assisted closure: a new method for wound control and treatment: animal studies and basic foundation. Ann Plast Surg 38:553–562. https://doi.org/10.1097/00000637-199706000-00001

Moues CM, Vos MC, van den Bemd GJ, Stijnen T, Hovius SE (2004) Bacterial load in relation to vacuum-assisted closure wound therapy: a prospective randomized trial. Wound Repair Regen 12:11–17. https://doi.org/10.1111/j.1067-1927.2004.12105.x

Moues CM, van Toorenenbergen AW, Heule F, Hop WC, Hovius SE (2008) The role of topical negative pressure in wound repair: expression of biochemical markers in wound fluid during wound healing. Wound Repair Regen 16:488–494. https://doi.org/10.1111/j.1524-475X.2008.00395.x

Mukaida N, Harada A, Matsushima K (1998) Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. Cytokine Growth Factor Rev 9:9–23. https://doi.org/10.1016/S1359-6101(97)00022-1

Ngo QD, Vickery K, Deva AK (2012) The effect of topical negative pressure on wound biofilms using an in vitro wound model. Wound Repair Regen 20:83–90. https://doi.org/10.1111/j.1524-475X.2011.00747.x

Nieto MA, Huang RY, Jackson RA, Thiery JP (2016) Emt: 2016. Cell 166:21–45. https://doi.org/10.1016/j.cell.2016.06.028

Norman G, Shi C, Goh EL, Murphy EM, Reid A, Chiverton L et al (2022) Negative pressure wound therapy for surgical wounds healing by primary closure. Cochrane Database Syst Rev 4:CD009261. https://doi.org/10.1002/14651858.CD009261.pub7

Normandin S, Safran T, Winocour S, Chu CK, Vorstenbosch J, Murphy AM, Davison PG (2021) Negative pressure wound therapy: mechanism of action and clinical applications. Semin Plast Surg 35:164–170. https://doi.org/10.1055/s-0041-1731792

Nuutila K, Siltanen A, Peura M, Harjula A, Nieminen T, Vuola J et al (2013) Gene expression profiling of negative-pressure-treated skin graft donor site wounds. Burns 39:687–693. https://doi.org/10.1016/j.burns.2012.09.014

Nuutila K, Broomhead M, Proppe K, Eriksson E (2021) Study comparing platform wound dressing, a negative-pressure device without a filler, with three conventional negative-pressure wound therapy systems in the treatment of excisional and incisional wounds. Plast Reconstr Surg 147:76–86. https://doi.org/10.1097/PRS.0000000000007450

Orgill DP, Manders EK, Sumpio BE, Lee RC, Attinger CE, Gurtner GC, Ehrlich HP (2009) The mechanisms of action of vacuum assisted closure: more to learn. Surgery 146:40–51. https://doi.org/10.1016/j.surg.2009.02.002

Panayi AC, Leavitt T, Orgill DP (2017) Evidence based review of negative pressure wound therapy. World J Dermatol 6:1–16. https://doi.org/10.5314/wjd.v6.i1.1

Patmo AS, Krijnen P, Tuinebreijer WE, Breederveld RS (2014) The effect of vacuum-assisted closure on the bacterial load and type of bacteria: a systematic review. Adv Wound Care (New Rochelle) 3:383–389. https://doi.org/10.1089/wound.2013.0510

Payne JL, Ambrosio AM (2009) Evaluation of an antimicrobial silver foam dressing for use with V.A.C. therapy: morphological, mechanical, and antimicrobial properties. J Biomed Mater Res B Appl Biomater 89:217–222. https://doi.org/10.1002/jbm.b.31209

Petkar KS, Dhanraj P, Kingsly PM, Sreekar H, Lakshmanarao A, Lamba S et al (2011) A prospective randomized controlled trial comparing negative pressure dressing and conventional dressing methods on split-thickness skin grafts in burned patients. Burns 37:925–929. https://doi.org/10.1016/j.burns.2011.05.013

Poteet SJ, Schulz SA, Povoski SP, Chao AH (2021) Negative pressure wound therapy: device design, indications, and the evidence supporting its use. Expert Rev Med Devices 18:151–160. https://doi.org/10.1080/17434440.2021.1882301

Ranieri D, Proietti S, Dinicola S, Masiello MG, Rosato B, Ricci G et al (2017) Simulated microgravity triggers epithelial mesenchymal transition in human keratinocytes. Sci Rep 7:538. https://doi.org/10.1038/s41598-017-00602-0

Rao M, Burke D, Finan PJ, Sagar PM (2007) The use of vacuum-assisted closure of abdominal wounds: a word of caution. Color Dis 9:266–268. https://doi.org/10.1111/j.1463-1318.2006.01154.x

Rapp SJ, Dershem V, Zhang X, Schutte SC, Chariker ME (2020) Varying negative pressure wound therapy acute effects on human split-thickness autografts. J Burn Care Res 41:104–112. https://doi.org/10.1093/jbcr/irz122

Reichelt J (2007) Mechanotransduction of keratinocytes in culture and in the epidermis. Eur J Cell Biol 86:807–816. https://doi.org/10.1016/j.ejcb.2007.06.004

Rhodius P, Haddad A, Matsumine H, Sakthivel D, Ackermann M, Sinha I et al (2018) Noninvasive flap preconditioning by foam-mediated external suction improves the survival of fasciocutaneous axial-pattern flaps in a type 2 diabetic murine model. Plast Reconstr Surg 142:872e–883e. https://doi.org/10.1097/PRS.0000000000005038

Saeg F, Schoenbrunner AR, Janis JE (2021) Evidence-based wound irrigation: separating fact from fiction. Plast Reconstr Surg 148:601e–614e. https://doi.org/10.1097/PRS.0000000000008331

Santos A, Lagares D (2018) Matrix stiffness: the conductor of organ fibrosis. Curr Rheumatol Rep 20:2. https://doi.org/10.1007/s11926-018-0710-z

Sartipy U, Lockowandt U, Gäbel J, Jidéus L, Dellgren G (2006) Cardiac rupture during vacuum-assisted closure therapy. Annal Thorac Surg 82:1110–1111. https://doi.org/10.1016/j.athoracsur.2006.01.060

Saxena V, Hwang CW, Huang S, Eichbaum Q, Ingber D, Orgill DP (2004) Vacuum-assisted closure: microdeformations of wounds and cell proliferation. Plast Reconstr Surg 114:1086–1096.; discussion 1097-1088. https://doi.org/10.1097/01.prs.0000135330.51408.97

Scalise A, Calamita R, Tartaglione C, Pierangeli M, Bolletta E, Gioacchini M et al (2016) Improving wound healing and preventing surgical site complications of closed surgical incisions: a possible role of incisional negative pressure wound therapy. A systematic review of the literature. Int Wound J 13:1260–1281. https://doi.org/10.1111/iwj.12492

Scherer SS, Pietramaggiori G, Mathews JC, Prsa MJ, Huang S, Orgill DP (2008) The mechanism of action of the vacuum-assisted closure device. Plast Reconstr Surg 122:786–797. https://doi.org/10.1097/PRS.0b013e31818237ac

Scherer SS, Pietramaggiori G, Mathews JC, Orgill DP (2009) Short periodic applications of the vacuum-assisted closure device cause an extended tissue response in the diabetic mouse model. Plast Reconstr Surg 124:1458–1465. https://doi.org/10.1097/PRS.0b013e3181bbc829

Seidel D, Diedrich S, Herrle F, Thielemann H, Marusch F, Schirren R et al (2020) Negative pressure wound therapy vs conventional wound treatment in subcutaneous abdominal wound healing impairment: the SAWHI randomized clinical trial. JAMA Surg 155:469–478. https://doi.org/10.1001/jamasurg.2020.0414

Shao PL, Liao JD, Wu SC, Chen YH, Wong TW (2021) Microplasma treatment versus negative pressure therapy for promoting wound healing in diabetic mice. Int J Mol Sci 22:22. https://doi.org/10.3390/ijms221910266

Sharp E (2013) Single-use NPWT for the treatment of complex orthopaedic surgical and trauma wounds. J Wound Care 22:S5–S9. https://doi.org/10.12968/jowc.2013.22.Sup10.S5

Singh D, Livingstone JP, Lautze J, Murray PC (2021) Measuring in-vivo foot perfusion distal to a near-circumferential negative pressure wound therapy dressing via thermal imaging. Cureus 13:e17720. https://doi.org/10.7759/cureus.17720

Sistigu A, Di Modugno F, Manic G, Nistico P (2017) Deciphering the loop of epithelial-mesenchymal transition, inflammatory cytokines and cancer immunoediting. Cytokine Growth Factor Rev 36:67–77. https://doi.org/10.1016/j.cytogfr.2017.05.008

Sogorski A, Lehnhardt M, Goertz O, Harati K, Kapalschinski N, Hirsch T et al (2018) Improvement of local microcirculation through intermittent negative pressure wound therapy (NPWT). J Tissue Viability 27:267–273. https://doi.org/10.1016/j.jtv.2018.08.004

Sogorski A, Becker A, Dadras M, Wallner C, Wagner JM, Lehnhardt M et al (2022) Superior enhancement of cutaneous microcirculation due to “cyclic” application of a negative pressure wound therapy device in humans – local and remote effects. Front Surg 9:822122. https://doi.org/10.3389/fsurg.2022.822122

Stechmiller JK, Kilpadi DV, Childress B, Schultz GS (2006) Effect of vacuum-assisted closure therapy on the expression of cytokines and proteases in wound fluid of adults with pressure ulcers. Wound Repair Regen 14:371–374. https://doi.org/10.1111/j.1743-6109.2006.00134.x

Steingrimsson S, Gottfredsson M, Gudmundsdottir I, Sjogren J, Gudbjartsson T (2012) Negative-pressure wound therapy for deep sternal wound infections reduces the rate of surgical interventions for early re-infections. Interact Cardiovasc Thorac Surg 15:406–410. https://doi.org/10.1093/icvts/ivs254

Succar J, Douaiher J, Lancerotto L, Li Q, Yamaguchi R, Younan G et al (2014) The role of mouse mast cell proteases in the proliferative phase of wound healing in microdeformational wound therapy. Plast Reconstr Surg 134:459–467. https://doi.org/10.1097/PRS.0000000000000432

Sun TT, Green H (1976) Differentiation of the epidermal keratinocyte in cell culture: formation of the cornified envelope. Cell 9:511–521. https://doi.org/10.1016/0092-8674(76)90033-7

Tahir S, Malone M, Hu H, Deva A, Vickery K (2018) The effect of negative pressure wound therapy with and without instillation on mature biofilms in vitro. Materials (Basel) 11(5). https://doi.org/10.3390/ma11050811

Tank JC, Georgiadis GM, Bair JM, Rice A, O’Mara Gardner K, Chen JT et al (2021) Does the use of ethyl chloride improve patient-reported pain scores with negative pressure wound therapy dressing changes? A prospective, randomized controlled trial. J Trauma Acute Care Surg 90:1061–1066. https://doi.org/10.1097/TA.0000000000003157

Timmermans FW, Mokken SE, Smit JM, Bouman MB, van de Grift TC, Mullender MG et al (2022) The impact of incisional negative pressure wound therapy on scar quality and patient-reported outcomes: a within-patient-controlled, randomised trial. Wound Repair Regen 30:210–221. https://doi.org/10.1111/wrr.13001

Timmers MS, Graafland N, Bernards AT, Nelissen RG, van Dissel JT, Jukema GN (2009) Negative pressure wound treatment with polyvinyl alcohol foam and polyhexanide antiseptic solution instillation in post-traumatic osteomyelitis. Wound Repair Regen 17: 278–286. https://doi.org/10.1111/j.1524-475X.2009.00458.x

Torbrand C, Ugander M, Engblom H, Arheden H, Ingemansson R, Malmsjo M (2010) Wound contraction and macro-deformation during negative pressure therapy of sternotomy wounds. J Cardiothorac Surg 5: 75. https://doi.org/10.1186/1749-8090-5-75

Toume S, Gefen A, Weihs D (2017) Low-level stretching accelerates cell migration into a gap. Int Wound J 14: 698–703. https://doi.org/10.1111/iwj.12679

Tse JM, Cheng G, Tyrrell JA, Wilcox-Adelman SA, Boucher Y, Jain RK et al (2012) Mechanical compression drives cancer cells toward invasive phenotype. Proc Natl Acad Sci U S A 109:911–916. https://doi.org/10.1073/pnas.1118910109

Valente PM, Deva A, Ngo Q, Vickery K (2016) The increased killing of biofilms in vitro by combining topical silver dressings with topical negative pressure in chronic wounds. Int Wound J 13:130–136. https://doi.org/10.1111/iwj.12248

van den Bulck R, Siebers Y, Zimmer R, Acton C, Janzing H, Lang W (2013) Initial clinical experiences with a new, portable, single-use negative pressure wound therapy device. Int Wound J 10:145–151. https://doi.org/10.1111/j.1742-481X.2012.00954.x

Veerasubramanian PK, Joe VC, Liu WF, Downing TL (2021) Characterization of macrophage and cytokine interactions with biomaterials used in negative-pressure wound therapy. Bioengineering (Basel) 9:9. https://doi.org/10.3390/bioengineering9010002

Vos RJ, Yilmaz A, Sonker U, Kloppenburg GTL (2013) Acute mediastinal bleeding during vacuum-assisted closure. Int Wound J 10:348–350. https://doi.org/10.1111/j.1742-481X.2012.00989.x

Wackenfors A, Sjogren J, Gustafsson R, Algotsson L, Ingemansson R, Malmsjo M (2004) Effects of vacuum-assisted closure therapy on inguinal wound edge microvascular blood flow. Wound Repair Regen 12:600–606. https://doi.org/10.1111/j.1067-1927.2004.12602.x

Wang N, Butler JP, Ingber DE (1993) Mechanotransduction across the cell surface and through the cytoskeleton. Science 260:1124–1127. https://doi.org/10.1126/science.7684161

Wang GQ, Li TT, Li ZR, Zhang LC, Zhang LH, Han L et al (2016) Effect of negative pressure on proliferation, virulence factor secretion, biofilm formation, and virulence-regulated gene expression of Pseudomonas aeruginosa in vitro. Biomed Res Int 2016:7986234. https://doi.org/10.1155/2016/7986234

Wang R, Thayer P, Goldstein A, Wagner WD (2020) Interaction of material stiffness and negative pressure to enhance differentiation of bone marrow-derived stem cells and osteoblast proliferation. J Tissue Eng Regen Med 14:295–305. https://doi.org/10.1002/term.2993

Wang YJ, Yao XF, Lin YS, Wang JY, Chang CC (2022) Oncologic feasibility for negative pressure wound therapy application in surgical wounds: a meta-analysis. Int Wound J 19:573–582. https://doi.org/10.1111/iwj.13654

White RA, Miki RA, Kazmier P, Anglen JO (2005) Vacuum-assisted closure complicated by erosion and hemorrhage of the anterior tibial artery. J Orthop Trauma 19:56–59. https://doi.org/10.1097/00005131-200501000-00011

Wiegand C, White R (2013) Microdeformation in wound healing. Wound Repair Regen 21:793–799. https://doi.org/10.1111/wrr.12111

Wilkes RP, McNulty AK, Feeley TD, Schmidt MA, Kieswetter K (2007) Bioreactor for application of subatmospheric pressure to three-dimensional cell culture. Tissue Eng 13:3003–3010. https://doi.org/10.1089/ten.2007.0036

Wilkes R, Zhao Y, Cunningham K, Kieswetter K, Haridas B (2009a) 3D strain measurement in soft tissue: demonstration of a novel inverse finite element model algorithm on MicroCT images of a tissue phantom exposed to negative pressure wound therapy. J Mech Behav Biomed Mater 2:272–287. https://doi.org/10.1016/j.jmbbm.2008.10.006

Wilkes R, Zhao Y, Kieswetter K, Haridas B (2009b) Effects of dressing type on 3D tissue microdeformations during negative pressure wound therapy: a computational study. J Biomech Eng 131: 031012. https://doi.org/10.1115/1.2947358

Winter GD, Scales JT (1963) Effect of air drying and dressings on the surface of a wound. Nature 197:91–92. https://doi.org/10.1038/197091b0

Wolvos T (2004) Wound instillation – the next step in negative pressure wound therapy. Lessons learned from initial experiences. Ostomy Wound Manage 50: 56–66

Wolvos T (2013) The use of negative pressure wound therapy with an automated, volumetric fluid administration: an advancement in wound care. Wounds 25: 75–83

Wu JP, Jiang ZH, Feng XJ, Jiang JN, Cheng MH (2018) Negative pressure therapy in the regeneration of the sciatic nerve using vacuum – assisted closure in a rabbit model. Med Sci Monit 24:1027–1033. https://doi.org/10.12659/msm.906696

Yamashiro T, Kushibiki T, Mayumi Y, Tsuchiya M, Ishihara M, Azuma R (2022) Novel cell culture system for monitoring cells during continuous and variable negative-pressure wound therapy. Skin Res Technol 29:e13262. https://doi.org/10.1111/srt.13262

Yang Z, Yao JF, Xu P, Zhang JB, Zhang YM, Zhu YJ et al (2014) Functions and mechanisms of intermittent negative pressure for osteogenesis in human bone marrow mesenchymal stem cells. Mol Med Rep 9:1331–1336. https://doi.org/10.3892/mmr.2014.1968

Yang C, Goss SG, Alcantara S, Schultz G, Lantis Ii JC (2017) Effect of negative pressure wound therapy with instillation on bioburden in chronically infected wounds. Wounds 29:240–246

Yin Y, Zhang R, Li S, Guo J, Hou Z, Zhang Y (2018) Negative-pressure therapy versus conventional therapy on split-thickness skin graft: a systematic review and meta-analysis. Int J Surg 50:43–48. https://doi.org/10.1016/j.ijsu.2017.12.020

Younan G, Ogawa R, Ramirez M, Helm D, Dastouri P, Orgill DP (2010) Analysis of nerve and neuropeptide patterns in vacuum-assisted closure-treated diabetic murine wounds. Plast Reconstr Surg 126:87–96. https://doi.org/10.1097/PRS.0b013e3181da86d0

Younan GJ, Heit YI, Dastouri P, Kekhia H, Xing W, Gurish MF et al (2011) Mast cells are required in the proliferation and remodeling phases of microdeformational wound therapy. Plast Reconstr Surg 128:649e–658e. https://doi.org/10.1097/PRS.0b013e318230c55d

Yu P, Yu N, Yang X, Jin X, Lu H, Qi Z (2017) Clinical efficacy and safety of negative-pressure wound therapy on flaps: a systematic review. J Reconstr Microsurg 33:358–366. https://doi.org/10.1055/s-0037-1599076

Zeisberg M, Neilson EG (2009) Biomarkers for epithelial-mesenchymal transitions. J Clin Invest 119:1429–1437. https://doi.org/10.1172/JCI36183

Zhang S, Xie Y, Yan F, Zhang Y, Yang Z, Chen Z et al (2022) Negative pressure wound therapy improves bone regeneration by promoting osteogenic differentiation via the AMPK-ULK1-autophagy axis. Autophagy 18:2229–2245. https://doi.org/10.1080/15548627.2021.2016231

Zhu J, Yu A, Qi B, Li Z, Hu X (2014) Effects of negative pressure wound therapy on mesenchymal stem cells proliferation and osteogenic differentiation in a fibrin matrix. PLoS One 9:e107339. https://doi.org/10.1371/journal.pone.0107339

Zwanenburg PR, Timmermans FW, Timmer AS, Middelkoop E, Tol BT, Lapid O et al (2021) A systematic review evaluating the influence of incisional negative pressure wound therapy on scarring. Wound Repair Regen 29:8–19. https://doi.org/10.1111/wrr.12858

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Vitamin A Deficiency, COVID-19, and Rhino-Orbital Mucormycosis (Black Fungus): An Analytical Perspective

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Abstract

Mucormycosis is a rare but serious opportunistic fungal disease characterized by rhino-orbito-cerebral and pulmonary involvement. It is mainly seen in people with secondary immunosuppression, isolated vitamin A deficiency, measles, and AIDS patients. It showed a rise during the second wave of the COVID-19 epidemic in the spring of 2021 in India, especially in diabetic COVID-19 patients. Vitamin A deficiency is known to cause nutritional immunodeficiency and hence leading the way to increased opportunistic fungal, bacterial, and viral infections. In the eye, it causes keratitis, night blindness, xerophthalmia, conjunctivitis, Bitot spots, keratomalacia, and retinopathy. It also causes decreased tear secretion and deterioration of the anatomical/physiological defense barrier of the eye. The negative impact of vitamin A deficiency has been previously demonstrated in measles, AIDS, and COVID-19. We think that mucormycosis in COVID-19 might be rendered by vitamin A deficiency and that vitamin A supplementation may have preventive and therapeutic values against mucormycosis and other ocular symptoms associated with COVID-19. However, any vitamin A treatment regimen needs to be based on laboratory and clinical data and supervised by medical professionals.

Keywords

Black fungus · Conjunctivitis · COVID-19 · Keratitis · Mucormycosis · Night blindness · Vitamin A deficiency · Xerophthalmia

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1 Introduction

The number of life-threatening fungal infections has increased dramatically over the past two decades. Recent estimates have identified a global disease burden of approximately two million patients with systemic and invasive fungal infections (Bongomin et al. 2017; Brown et al. 2012; Kim 2016). However, despite aggressive treatments, opportunistic fungal infections in high-risk patients have an unacceptably high mortality rate (Bongomin et al. 2017; Brown et al. 2012). The main reason for this is the rapid increase in the number of chronically immunocompromised patients (Brown et al. 2012; Mei-Sheng Riley 2021). Aggressive chemotherapy applications to treat leukemia and other hematological malignancies, the increase in bone marrow transplantations, and the widespread use of immunosuppressive drugs to prevent rejection of transplanted organs, as well as the persistence of AIDS as a global epidemic, have significantly increased the number of immunosuppressed patients who are susceptible to the opportunistic infections including mucormycosis (Brown et al. 2012; Mei-Sheng Riley 2021; Reid et al. 2020).

Mucormycosis, also known as zygomycosis or inappropriately black fungus, is a rare but serious opportunistic fungal disease characterized by rhino-orbito-cerebral, pulmonary, systemic, gastrointestinal, and cutaneous involvement (Binder et al. 2014; Petrikkos et al. 2012; Reid et al. 2020; WHO 2021). Mucorales spores are common in various environments and nasal flora of many people and normally do not cause invasive clinical pictures in immunocompetent individuals but can cause severe and fatal mucormycosis in the immunosuppressed individuals by invading the nasal cavities, paranasal sinuses, and from there to the orbit and the brain (Gandra et al. 2021; Jeong et al. 2019). As an opportunistic infection, mucormycosis is mainly seen in immunosuppressed individuals, such as those with AIDS, diabetes, and chemotherapy treatment for malignancies (Binder et al. 2014; Jeong et al. 2019; Reid et al. 2020). It is also seen in isolated vitamin A deficiency, non-diabetic COVID-19 patients, and measles (Dwivedi et al. 2023; Lagorce Pagès et al. 2000; Mei-Sheng Riley 2021).

The second wave of the COVID-19 pandemic had shaken India, reaching more than 400,000 daily COVID cases in the spring of 2021 leading to its healthcare system being on the verge of collapse. In this second wave, mucormycosis was observed at an alarming level between April and June of 2021, which had further shaken the country’s healthcare system (Bhattacharyya et al. 2021a, b; Dilek et al. 2021; Lima et al. 2021b; Nagalli and Kikkeri 2021; Sen et al. 2021). These cases of COVID-19-associated mucormycosis (CAM) increased simultaneously in multiple states, causing a second outbreak within the COVID-19 pandemic and constituting about 71% of global CAM cases (Gambhir et al. 2021). This sudden increase in mucormycosis in India was devastating as it led to orbital exenteration in some patients. With the spread of ocular infarction, some patients’ eyes need to be surgically removed, to prevent the dissemination of the infection into the brain (Gandra et al. 2021).

Although the cause of this sharp rise of mucormycosis in the COVID-19 epidemic is still unknown, its frequent occurrence in diabetic COVID-19 patients may indicate its possible association with diabetes (Al-Tawfiq et al. 2021; Bhattacharyya et al. 2021b; Dilek et al. 2021; Dwivedi et al. 2023). Diabetes, overuse of steroidal therapeutics, and tocilizumab have been attributed to the mucormycosis spike (Dwivedi et al. 2023; Gandra et al. 2021; Khichar et al. 2021). Diabetes is known to weaken the immune system and makes the individual susceptible to other diseases/infections (Rocha et al. 2021). It is also noted that the widespread use of anti-hyperglycemic and immunosuppressive drugs might weaken the immune system and lead to opportunistic fungal infections (Elhamamsy et al. 2021; Rocha et al. 2021).

We hypothesize that hypovitaminosis A-associated immune deficiency is most likely a major underlying cause of the increased mucormycosis cases during the second wave of the COVID-19 pandemic in India. A possible depletion of vitamin A in culminating COVID-19 infection may compromise a proper immune response leading to secondary infections as seen in measles and HIV infections (Bello et al. 2016; Mselle 1999; Rodrigues and Dohlman 2004).

In this study, we aim to bring attention to the possible role of vitamin A in COVID-19 and associated infections, including mucormycosis. We discuss the relationship between vitamin A deficiency and COVID-19, measles, AIDS, and other chronic diseases. In particular, the proven efficacy of vitamin A in reducing measles mortality and related eye complications is reviewed. In this context, we believe that vitamin A or retinoic acid supplementation may prove therapeutically beneficial in treating and preventing mucormycosis and other ocular infections associated with COVID-19 especially early during infection. We think this idea deserves a well-controlled comprehensive study. However, it should be noted that the therapeutic use of vitamin A has to be managed by medical professionals.

2 Mucormycosis, COVID-19, and Vitamin A Deficiency

COVID-19 infection displays a plethora of multiorgan involvement including pulmonary, cardiac, circulation, immune, and ocular involvement (Gavriatopoulou et al. 2020; Lanska 2010; Munjal et al. 2020; Romero-Sánchez et al. 2020). The common ophthalmic manifestations include central retinal artery occlusion, central retinal vein occlusion, cranial nerve palsy, follicular conjunctivitis, optic neuritis, conjunctivitis, and mucormycosis (Sen et al. 2021; Wan et al. 2022). Ocular mucormycosis is characterized by swelling and pain in the eyes, dark spots around the nose, and an excessively runny nose. Blurred vision, loss of vision, and eyelid loss have also been reported in some patients (Gandra et al. 2021). Ocular findings have been observed as late symptoms of the disease in COVID-19 and mucormycosis tends to occur approximately 2 weeks after recovery, leading to rehospitalization (Lima et al. 2021b; Sen et al. 2021; Wan et al. 2022).

Vitamin A has an important role in development, tissue repair, vision, and immunity (Ross and Stephensen 1996; Sommer 2008; Sommer and Vyas 2012; Tanumihardjo 2011). In the eye, it is essential in the biochemistry of the vision (Faustino et al. 2016). Its deficiency abrogates the biochemistry of the vision and leads to the development of microbial keratitis, night blindness, xerophthalmia, conjunctivitis, Bitot spots, keratomalacia, and retinopathy (Sherwin et al. 2012; Sommer 1983, 1993). Vitamin A deficiency is classically caused by food deprivation and is common in underdeveloped countries; rural areas and peripheries of large cities in South Asia, Africa, and Latin America; and poor communities of large cities in developed countries (Mason et al. 2015; Melo et al. 2004; Spannaus-Martin et al. 1997). It is a global health problem and has been the target of international preventive vitamin A supplements and periodic evaluation programs (Akhtar et al. 2013; Mason et al. 2015). The most vulnerable people are children, pregnant women, and the elderly. The prevalence of hypovitaminosis A in children under 6 years of age reaches 50% in some regions (Akhtar et al. 2013). In addition to ocular problems, hypovitaminosis A causes growth retardation, infertility, congenital malformations, susceptibility to infections, and premature death (Akhtar et al. 2013; Mason et al. 2015).

The risk of developing ocular symptoms in COVID-19 has been linked to high viral load (Wan et al. 2022). About one-fifth (21%) of the cohort of patients diagnosed with COVID-19 reported one or more symptoms related to dry eye disease, of which the most frequent ones were turbidity (9.2%), itching (6.1%), and pain or burning sensation (4.8%) (Wan et al. 2022). The clinical "pink eye" picture commonly seen in severe COVID-19 patients is similar to conjunctivitis observed in vitamin A deficiency (Al-Namaeh 2021; Sen et al. 2021). We believe that the cause of the eye findings in COVID-19 might be due to vitamin A deficiency (Sarohan 2020). We previously hypothesized that COVID-19 infection might cause depletion of vitamin A in the body leading to immunosuppression, biphasic immune dysregulation, defect in type-I interferon synthesis, cytokine storm, excessive inflammatory response, systemic organ damage, and aggravated clinical picture (Sarohan 2021b; Sarohan et al. 2021). In a recent study, we showed that vitamin A levels were low in severe COVID-19 patients (Sarohan et al. 2022). It will be important to elucidate if COVID-19 patients develop retinitis and other vision problems due to atrophy and necrosis of cells in the cornea and or retina resulting from vitamin A deficiency and consecutively disturbed retinoic acid signaling.

2.1 Mucormycosis Superinfection in COVID-19

Most CAM cases in India in 2021 were observed in diabetic COVID-19 patients (Banerjee et al. 2021; Bhattacharyya et al. 2021a; Hoenigl et al. 2022; Nagalli and Kikkeri 2021). The widespread use of corticosteroids in COVID-19 treatment to suppress the excessive inflammatory response and cytokine storm might have further predisposed diabetic patients to the fungal disease (Bhogireddy et al. 2021; Hoenigl et al. 2022; Patel et al. 2021). However, it is important to note that CAM is also seen in COVID-19 patients who do not have diabetes or have recently used corticosteroids (Nair et al. 2021). In this study, 13 otherwise immunocompetent COVID-19 patients with no previous diabetes nor a history of corticosteroid use were reported to develop a new onset of uncontrolled diabetes and rhino-orbital mucormycosis following COVID-19 infection, and some of these patients underwent orbital exenteration due to advanced orbital involvement despite maximal medical therapy (Nair et al. 2021). Some cases of rhino-orbital mucormycosis requiring aggressive surgery and medical intervention have also presented with a new onset of diabetes following COVID-19 infection (Nair et al. 2021). Age, rhino-orbital-cerebral involvement, and intensive care unit admission have been associated with increased mortality (Nair et al. 2021). In another study, among 287 mucormycosis patients, 187 (65.2%) had CAM (Patel et al. 2021). Uncontrolled diabetes was the most common underlying condition among CAM and non-CAM patients. In 32.6% of CAM patients, the only underlying disease was COVID-19. Hypoxemia associated with COVID-19 and inappropriate use of glucocorticoids have been reported to be independently associated with CAM (Patel et al. 2021). The case death rate due to mucormycosis was 45.7%, which was similar for CAM and non-CAM patients.

In addition to India, CAM cases have been reported in other countries such as the USA, the Netherlands, Brazil, England, Germany, Italy, and others (Hoenigl et al. 2022). Four cases of CAM were also reported in the Netherlands between December 2020 and May 2021 (Buil et al. 2021). *Mucorales* species have been identified as the causative agent of mucormycosis in these patients. Clinical presentations included pulmonary, rhino-orbital, cerebral, and disseminated infection. Three of them have died. All were men over 50 years of age (Buil et al. 2021). In another study, three non-diabetic cases of invasive mucormycosis with different outcomes were presented (Elhamamsy et al. 2021). In this study, the increase in CAM was attributed to both the immune system dysregulation in COVID-19 and the use of high-dose corticosteroids (Elhamamsy et al. 2021). In addition, the burden on the health system with the epidemic, the intense use of oxygen in intensive care units, and the use of non-sterilized water to humidify oxygen have been shown to be a factor in the transmission of mucormycosis (Elhamamsy et al. 2021).

2.2 Depletion of Vitamin A in COVID-19 Infection

Vitamin A depletion is a common pathogenetic mechanism observed during some infections, such as measles and HIV (WHO 2017). Depletion of vitamin A during infections may lead to systemic organ involvement associated with persistent infections, reinfections, secondary infections, and increased inflammation (Sarohan et al. 2021).

A few studies from Brazil and China show a high rate of vitamin A deficiency, especially in rural and urban areas (Chen et al. 2017; Custodio et al. 2009; Lima et al. 2021a; Nascimento et al. 2007). A 2009 screening study found that 17.4% of children and 12.3% of women were deficient in vitamin A throughout Brazil with high prevalence in rural and urban areas (Custodio et al. 2009). In another similar study, vitamin A deficiency was found in 26% of the elderly population aged over 60 years living in big cities (Nascimento et al. 2007). These data show a strong correlation between the epidemiology of vitamin A and the geographical and demographic course of COVID-19 and may suggest that vitamin A deficiency may be an underlying reason for the concentrated COVID-19 infection in urban areas and the elderly population. It is also well known that COVID-19 has a severe clinical course and high mortality in urban areas and especially in the elderly population (Nascimento et al. 2007). However, unfortunately, no epidemiological study has been conducted to prove this relationship.

In yet another study conducted in Brazil, the high risk of death from COVID-19 in the southeastern part of the country, where the major cities are located, was attributed to the high proportion of the elderly (Lima et al. 2021a). The same study also noted the high rates of infection and mortality for COVID-19 among young adults, people of low socioeconomic status, and people without access to healthcare in the less developed parts of the north of the country (Lima et al. 2021a). The relationship between COVID-19 and advanced age was especially emphasized in the study (Lima et al. 2021a). The result of this study indirectly points to a possible relationship between vitamin A deficiency and COVID-19. However, the study did not examine the relationship of COVID-19 with micronutrient deficiencies such as vitamins A, D, or others. The prevalence of vitamin A deficiency in the elderly population over 60 years old living in big cities was found to be as high as 22% in China and 26% in Brazil (Chen et al. 2017; Lata et al. 2021).

2.3 Role of Vitamin A in Immunity and Inflammation

Vitamin A has paramount importance in development, tissue repair, vision, and immunity. In immunity, vitamin A is recognized as an important factor for the development of a proper immune response, suppression of acute inflammation, and tissue repair after disease or injury (Green and Mellanby 1928; Stephensen and Lietz 2021; Timoneda et al. 2018). Vitamin A and its derivative retinoic acids have anti-infective, anti-inflammatory, and strong adjuvant activities (Hao et al. 2021; Li et al. 2020; Midha et al. 2020). Retinoic acid signaling is important in the synthesis of type-I interferon, which is a critical antiviral mediator in strengthening the immune system and clearing viruses (Kell and Gale Jr. 2015; Liu et al. 2016). In vitamin A deficiency, retinoic acid is depleted, type-I interferon synthesis is disrupted, and host defense is weakened (Sarohan et al. 2021). The decrease and/or depletion of vitamin A in the body disrupts type-I interferon synthesis and weakened immune response leading to an increased susceptibility to infections (Sarohan 2020; Sarohan et al. 2021). Therefore, vitamin A and retinoic acids are vital for an effective immune defense during viral infections. Vitamin A deficiency displays an acquired immunodeficiency-like picture (Oliveira et al. 2018; Stephensen 2001; Timoneda et al. 2018), which leads to increased opportunistic fungal, bacterial, and viral infections (Ross and Stephensen 1996; Stephensen 2001; Tanumihardjo 2011). While the earliest signs of vitamin A deficiency manifest themselves in immune cells (Hester et al. 2020; Timoneda et al. 2018), the eye-related signs are manifested later (Gilbert 2013; Sommer 2008). Vitamin A deficiency-related immune signs are decreased interferon response to viral infections and excessive chronic inflammation and cytokine storm leading to multiorgan damage, such as liver, lung, and kidney (Sarohan 2020; Sarohan et al. 2021).

Vitamin D has gained more attention than vitamin A in the treatment of COVID-19 infection (Abdulateef et al. 2021; Vyas et al. 2021). Both vitamin A and vitamin D are lipid in structure and relay their signaling through retinoid receptors. In a systematic review, the authors listed a number of candidate molecules, including vitamin A, vitamin D, and zinc, as essential components for the development of an adequate immune response against COVID-19 infection (Zhang and Liu 2020). Zinc is also shown to modulate vitamin A metabolism and immune system regulation in infections including fungal pathogenicity (Staats et al. 2013). The protective effect of vitamins A and D against COVID-19 supports our initial hypothesis in retinol depletion and retinoid signaling disorder that we initially proposed to explain the pathogenesis of COVID-19 (Sarohan et al. 2021).

Vitamin A deficiency is associated with a variety of infectious diseases, including diarrhea, respiratory diseases, measles, and HIV-1 infection (Bello et al. 2016; Stephensen 2001; Stephensen and Lietz 2021). Some infections, such as measles and HIV, may lower the systemic vitamin A levels and consequently disturbed retinoid signaling (Arrieta et al. 1992; D'Souza and D'Souza 2002b) that leads to systemic inflammation and severe clinical pictures (Stephensen 2001; Timoneda et al. 2018). This reciprocal relationship between vitamin A and infections in the host-pathogen interaction causes a vicious cycle during infections, increasing infection-induced morbidity and mortality (Timoneda et al. 2018; Wiseman et al. 2017). Similar studies point to the importance of vitamin A in the COVID-19 pathogenesis as well. More than 71% of COVID-19 patients had a low level of vitamin A, and decreased level of vitamin A was associated with the severity of COVID-19 infection (Tepasse et al. 2021; Tomasa-Irriguible et al. 2021). Furthermore, an in silico analysis suggested that vitamin A might be a candidate for the treatment of COVID-19 infection (Chakraborty et al. 2022).

Many trials have been conducted with vitamin A to treat and prevent various infections (Green et al. 1931; Sommer 2008; Sommer et al. 1983). In one of the most important studies, administering daily vitamin A reduced mortality rates by 50% in measles cases admitted to Grove Fever Hospital outside London (Ellison 1932). Sulfa-based antimicrobials introduced before the Second World War and some other antibiotics discovered afterward were found to be much more effective than vitamin A in the treatment of acute bacterial infections. With the increase in the welfare level of societies and the improvement in their nutritional status, vitamin A deficiency and its clinical findings (especially xerophthalmia) have almost completely disappeared. Afterward, clinical studies and reports on vitamin A have not attracted much attention (Sommer 2008). After these developments, research on the administration (and advocacy) of vitamin A to treat and prevent infections has also come to almost a standstill leading to a loss of interest in studies on vitamin A deficiency (Sommer 2008; Sommer and Vyas 2012). However, the epigenetic role and mechanisms of vitamin A in regulating gene functions remain a subject of exploration and interest (Sommer 2008). The association of chronic inflammatory diseases such as diabetes, autoimmune diseases, and chronic degenerative neurological diseases such as epidemics with malnutrition and vitamin A deficiency will revive the interest in vitamin A. In addition, the increased antibiotic resistance shifts the attention to alternative approaches in treating bacterial infections and boosting a proper immune response with vitamins being such a prominent approach (Mora et al. 2008).

2.4 Role of Vitamin A in Eye Health

Vitamin A is extremely important in the biochemistry of vision and the health of the eye (Faustino et al. 2016; Lanska 2010; Sommer 1983). Its deficiency leads to the development of night blindness, xerophthalmia, conjunctivitis, Bitot spots, microbial keratitis, corneal ulceration, keratomalacia, and retinopathy (Chung et al. 2022; Connell et al. 2006; Morjaria et al. 2011; Sherwin et al. 2012; Sommer 1993). Vitamin A deficiency also causes decreased tear secretion and deterioration of the anatomical/physiological defense barrier of the eye leading the way to opportunistic ophthalmic infections (Sherwin et al. 2012; Sirisinha 2015).

A central figure in human nutritional research, McLaren designated vitamin A as a representative vitamin for eye and vision (McLaren 2000). Mild xerophthalmic Indonesian children with night blindness and Bitot spots were reported to die at much higher rates than their non-xerophthalmic peers (Akhtar et al. 2013; Sommer et al. 1983). This association between mortality rates and the severity of xerophthalmia suggested that even "subclinical" vitamin A deficiency not accompanied by ocular changes could be associated with increased mortality, which reveals that prevention of vitamin A deficiency can lead to massive reductions in overall mortality rates (Stevens et al. 2015).

Night blindness and xerophthalmia are among the first ocular signs of vitamin A deficiency (Faustino et al. 2016; Sommer 1983). While night blindness is developed earlier, xerophthalmia is the most important expression of vitamin A deficiency and is pathognomonic for vitamin A deficiency (Sommer 1990, 2008). More severe forms of vitamin A deficiency, such as corneal xerosis, corneal ulceration, and keratomalacia, tend to occur with prolonged malnutrition. In its more severe forms, vitamin A deficiency causes dryness of the cornea, resulting in damage to the cornea and retina, with serious consequences that can lead to blindness (Awasthi et al. 2013; Lanska 2010; Sommer 1990). An estimated 250,000–500,000 children become blind each year due to vitamin A deficiency, and half of these die within 12 months of losing sight (WHO 2009, 2014). Vitamin A deficiency is associated with significant morbidity and mortality from common childhood infections and is the leading preventable cause of childhood blindness in the world (Imdad et al. 2017; WHO 2014). WHO still advocates for prophylactic vitamin A use for the prevention of night blindness and xerophthalmia in less developed countries (Awasthi et al. 2013; WHO 2009).

Vitamin A deficiency especially weakens its ability to fight infections. Vitamin A is essential for the differentiation of the mucus-secreting epithelium of the eye and the maintenance of tear secretion. Secondary and opportunistic infections, such as mucormycosis, develop in the eye as the anatomical and immunological defense barriers of the eye becomes impaired (Faustino et al. 2016; Imdad et al. 2017; WHO 2014). Night blindness more often occurs during pregnancy, possibly due to the deepening of pre-existing marginal maternal vitamin A deficiency because of the nutritional demands of pregnancy and intervening infections (Awasthi et al. 2013; Hussaini et al. 1978; Tielsch et al. 2008; Wolf 1978). It has been found that 80% of pregnant women develop vitamin A deficiency after week 34 of gestation (Awasthi et al. 2013; Villar et al. 2003). Vitamin A deficiency is associated with serious maternal and fetal complications of pregnancy such as preeclampsia and preterm birth and is also responsible for increased maternal and fetal death and other poor outcomes of pregnancy and lactation (Awasthi et al. 2013; Villar et al. 2003). One study reported that a 40% reduction in maternal mortality could be achieved with routine supplementation of vitamin A during pregnancy (West et al. 1999).

Although eye problems due to vitamin A deficiency are rare in developed countries, they usually present as superposed microbial keratitis. In addition, a wide spectrum of ocular findings can be encountered, ranging from simple night blindness, Bitot spots, and conjunctival and corneal xerosis to serious corneal ulcers, scar formation, and corneal perforation (Chung et al. 2022; WHO 2014). Sometimes, vitamin A deficiency manifests itself with subclinical findings. Interestingly, the patient may have severe vitamin A deficiency, despite the absence of classical ocular findings of hypovitaminosis A (Mason et al. 2015; WHO 2014). Late-occurring ocular findings can develop insidiously and rarely occur before the age of two (Diab and Krebs 2018). Therefore, vitamin A levels begin to decline before the classic ocular manifestations of vitamin A deficiency appear.

2.5 Vitamin A Deficiency in Measles and Eye Involvement

COVID-19 infection may mimic measles infection, especially for depleting vitamin A and dysregulating immune response. COVID-19 infection displays many similarities to that of measles. For example, the following findings are similarly observed in both infections: depletion of vitamin A, predisposition to secondary infections, aggravation of the clinical picture, pneumonia, and ARDS (Midha et al. 2020; Sarohan et al. 2022; Tepasse et al. 2021). It has been shown that the severity of measles, a disease characterized by the suppression of the immune system and infectious complications, is directly proportional to the degree of vitamin A deficiency (D'Souza and D'Souza 2002a; Imdad et al. 2017; Sherwin et al. 2012). As in measles, vitamin A deficiency causes the development of secondary infections such as mucormycosis in COVID-19 (Imdad et al. 2017; Sarohan 2020, 2021a; Sommer 1993; Stephensen and Lietz 2021). In an observational clinical study conducted at the beginning of the pandemic, it was found that serum vitamin A levels were low in severe COVID-19 patients (Sarohan et al. 2022). In another study, retinol levels were found to decrease in COVID-19 patients in correlation with the worsening of the clinical picture (Tepasse et al. 2021).

Measles is considered a global health threat (WHO 2017). It is a major ongoing problem in developing countries, affecting approximately 30 million children, and causing up to one million deaths annually (Angelo et al. 2019; Crecelius and Burnett 2020). Its prevalence in developed western countries has also risen in recent years (Angelo et al. 2019; Crecelius and Burnett 2020). Especially in the USA and European countries, its prevalence has recently increased (Angelo et al. 2019; Chovatiya and Silverberg 2020). In Ireland, it increased by 244% and became an important public health problem (O'Mahony et al. 2019). Concurrent with the COVID-19 pandemic in New Zealand, cases of measles outbreak-related keratitis occurred in 2020 (Ong et al. 2020). In measles, conjunctivitis and keratitis are the most common ocular manifestations, while encephalitis and pneumonia may cause mortality (Ong et al. 2020).

Measles blindness is the single leading preventable cause of blindness in children in low-income countries and is responsible for an estimated 15,000–60,000 cases of blindness per year (Semba and Bloem 2004). There is a close synergism between measles and vitamin A deficiency, which can result in xerophthalmia, corneal ulceration, keratomalacia, and subsequent corneal scarring and blindness (Crecelius and Burnett 2020; D'Souza and D'Souza 2001; Hussey and Klein 1993; Imdad et al. 2017). High-dose vitamin A supplements are given to all children with measles in developing countries (Bello et al. 2016; Benn 2012). In addition to preventing measles transmission and expanding the scope of measles immunization, interventions to improve children's vitamin A nutrition and vitamin A prophylaxis programs have gained important momentum among the main strategies to prevent measles blindness (Bello et al. 2016; Sherwin et al. 2012; Sommer 1990). Control of blindness in children has been recognized as a high-priority intervention under the World Health Organization's VISION 2020, Right to Sight Program (Semba and Bloem 2004; WHO 2007).

Measles is destructive to vitamin A metabolism leading to severe vitamin A deficiency in the host during measles infection (Bello et al. 2016; Imdad et al. 2017; Sherwin et al. 2012). During the 2015 measles outbreaks in California, about 50% of the infected children were found to be deficient in vitamin A during the screenings (Arrieta et al. 1992). The World Health Organization added prophylactic vitamin A supplements to their treatment protocols to prevent vision loss caused by vitamin A deficiency in children (Bello et al. 2016; Imdad et al. 2017; Sherwin et al. 2012). Prophylactic vitamin A applications aim to prevent measles-related blindness in countries where measles is common (Bello et al. 2016; D'Souza and D'Souza 2002a). For this purpose, 200,000 IU/day of vitamin A supplementation given to children with measles for

2 days resulted in a significant reduction in measles-related blindness as well as overall mortality (Bello et al. 2016; Semba and Bloem 2004).

2.6 Vitamin A Deficiency in Chronic Diseases and COVID-19

Chronic diseases such as obesity, diabetes, and cardiovascular diseases create comorbidity for COVID-19 and increase COVID-19 mortality by aggravating its clinical picture (Iadecola et al. 2020; Lim et al. 2020; Mertens and Peñalvo 2020; WHO n.d.). In recent years, chronic inflammatory and autoimmune diseases such as obesity, diabetes, cardiovascular diseases, and autism, which are not specifically caused by microorganisms, are associated with an unhealthy lifestyle, nutrition, and vitamin A deficiency (Snelson et al. 2021; WHO n.d.). The uncontrolled increase in chronic inflammatory diseases, which predispose to severe disease in COVID-19 and cause increased mortality and morbidity, especially in western societies, is attributed to unhealthy nutrition, such as high-calorie packaged foods (WHO n.d.). In this "healthy recovery from COVID-19" manifesto, WHO declared that the largest risk factor for mortality and morbidity for COVID-19 is chronic diseases such as obesity, diabetes, and cardiovascular diseases, which are caused by high-calorie unhealthy diet (WHO n.d.). Countries' vulnerability to the deadly COVID-19 is indicated to be higher with increasing vitamin A deficiency (Mertens and Peñalvo 2020). It has been reported in many recent studies that chronic diseases, which have the potential to harm human health and socioeconomic structure more than COVID-19, are associated with high-calorie malnutrition and vitamin A deficiency (Marley et al. 2021; Nugent 2008; Snelson et al. 2021; WHO 2020). The uncontrolled increase in chronic inflammatory diseases such as obesity and diabetes, which predispose to severe disease in COVID-19 and cause increased mortality and morbidity, especially in western societies, is attributed to unhealthy and high-calorie, packaged foods, whose deficiencies in vital nutrients such as vitamin A and vitamin D have been overlooked.

3 Discussion and Conclusion

Vitamin A was discovered 106 years ago (Semba 2012) and the prevention of vitamin A deficiency at population scales has been recognized as a public health priority for over six decades by the World Health Organization (Daré et al. 2019; Underwood 1994; WHO 2009). It is known that vitamin A supplements strengthen the immune system and protects patients from secondary infections, especially measles and AIDS. For this reason, we believe that vitamin A and retinoic acids can be useful in the prophylaxis and treatment of opportunistic infections and eye problems such as immunosuppression and mucormycosis occurring in association with COVID-19. A controlled randomized clinical trial may produce useful results not only for COVID-19 but also for similar possible future epidemics and pandemics.

Despite great combined efforts, the molecular pathogenesis of COVID-19 has not been well identified. In addition, the immune protection induced by the available COVID-19 vaccines wanes very fast within months, and the drugs adopted for the treatment are not very effective (Feikin et al. 2022; Prompetchara et al. 2020; Toor and Chana 2022). The large increase in the population of immunocompromised patients, the limited efficacy of current antifungals, and the increasing resistance to these drugs have prompted a search for alternative solutions for the prevention of invasive mucormycosis (Meir and Osherov 2018). The success of reduced blindness and mortality associated with the prophylactic use of vitamin A in measles outbreaks may serve as a model for the control of COVID-19 and COVID-19-related mucormycosis.

COVID-19 vaccines, which had been developed at an unprecedented speed, have not provided long-lasting effective immunity. Due to the long and costly drug development process, unfortunately, no fully effective drug has yet been found against

COVID-19. Despite the extraordinary measures, so far more than 650 million people have been infected and more than 6.6 million lost their lives (as of December 2022) (WHO n.d.). In addition, the pandemic has caused very serious socioeconomic losses. The most feared mutations in the pandemic had been our saviors. With this last mutation seen with the Omicron variant, the SARS-CoV-2 virus gained the ability to coexist with the host, as required by the evolutionary process, leading the pandemic toward its end (Fan et al. 2022; Singhal 2022; Tian et al. 2022). However, the virus is evolving, and the rise of dangerous mutants is not totally out of question yet. In addition, it should be kept in mind that the Omicron variant may cause severe infections in elderly and immunocompromised patients and individuals with comorbid diseases. Likewise, even though the COVID-19 pandemic is ending, chronic, autoimmune, and some other degenerative neurological diseases, which are seen after COVID-19 and defined as post-COVID syndromes, and some sequelae caused by vaccines will continue to be a problem for a long time. Therefore, elucidation of the pathogenesis of COVID-19 and associated diseases as well as its modulation of the immune system will be seminal for developing rational strategies for the prevention of COVID-19 infection, treatment of COVID-19-associated diseases such as mucormycosis and post-COVID symptoms, and coping with the negative side effects of vaccines.

Despite its important historical role, vitamin A has not attracted attention for the prophylaxis and treatment of COVID-19 during the pandemic. We think both vitamins A and D are important in regulating immunity as they act through retinoid signaling that orchestrates a proper immune response (Sarohan et al. 2021). Although some studies on vitamin D were carried out, these studies were not sufficient for the use of vitamins A and/or D against COVID-19 on a global scale. In addition, large-scale epidemiological studies have not been conducted to explain the geographical distribution of COVID-19, its relationship with comorbid diseases, demographic characteristics, and its relationship with nutritional status, and vitamin A and D deficiencies during the pandemic.

Conflicts of Interest The authors have no conflicts of interest to declare that are relevant to the content of this article.

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References

Abdulateef DS, Rahman HS, Salih JM, Osman SM, Mahmood TA, Omer SHS, Ahmed RA (2021) COVID-19 severity in relation to sociodemographics and vitamin D use. Open Med (Warsaw, Poland) 16: 591–609

Akhtar S, Ahmed A, Randhawa MA, Atukorala S, Arlappa N, Ismail T, Ali Z (2013) Prevalence of vitamin A deficiency in South Asia: causes, outcomes, and possible remedies. J Health Popul Nutr 31:413–423

Al-Namaeh M (2021) COVID-19 and conjunctivitis: a meta-analysis. Ther Adv Ophthalmol 13: 25158414211003368

Al-Tawfiq JA, Alhumaid S, Alshukairi AN, Temsah MH, Barry M, Al Mutair A, Rabaan AA, Al-Omari A, Tirupathi R, AlQahtani M et al (2021) COVID-19 and mucormycosis superinfection: the perfect storm. Infection 49:833–853

Angelo KM, Gastañaduy PA, Walker AT, Patel M, Reef S, Lee CV, Nemhauser J (2019) Spread of measles in Europe and implications for US travelers. Pediatrics 144:e20190414

Arrieta AC, Zaleska M, Stutman HR, Marks MI (1992) Vitamin A levels in children with measles in Long Beach, California. J Pediatr 121:75–78

Awasthi S, Peto R, Read S, Clark S, Pande V, Bundy D (2013) Vitamin A supplementation every 6 months with retinol in 1 million pre-school children in north India: DEVTA, a cluster-randomised trial. Lancet (London, England) 381:1469–1477

Banerjee I, Robinson J, Asim M, Sathian B, Banerjee I (2021) Mucormycosis and COVID-19 an epidemic in a pandemic? Nepal J Epidemiol 11:1034–1039

Bello S, Meremikwu MM, Ejemot-Nwadiaro RI, Oduwole O (2016) Routine vitamin A supplementation for the prevention of blindness due to measles infection in children. Cochrane Database Syst Rev 2016: CD007719

Benn CS (2012) Combining vitamin A and vaccines: convenience or conflict? Dan Med J 59:B4378

Bhattacharyya A, Sarma P, Kaur H, Kumar S, Bhattacharyya J, Prajapat M, Prakash A, Sharma S, Reddy DH, Thota P et al (2021a) COVID-19-associated rhino-orbital-cerebral mucormycosis: a systematic review, meta-analysis, and meta-regression analysis. Indian J Pharmacol 53:499–510

Bhattacharyya A, Sarma P, Sharma DJ, Das KK, Kaur H, Prajapat M, Kumar S, Bansal S, Prakash A, Avti P et al (2021b) Rhino-orbital-cerebral-mucormycosis in COVID-19: a systematic review. Indian J Pharmacol 53:317–327

Bhogireddy R, Krishnamurthy V, Jabaris SS, Pullaiah CP, Manohar S (2021) Is Mucormycosis an inevitable complication of Covid-19 in India? Braz J Infect Dis 25: 101597

Binder U, Maurer E, Lass-Flörl C (2014) Mucormycosis--from the pathogens to the disease. Clin Microbiol Infect 20(Suppl 6):60–66

Bongomin F, Gago S, Oladele RO, Denning DW (2017) Global and multi-national prevalence of fungal diseases-estimate precision. J Fungi (Basel, Switzerland) 3:57

Brown GD, Denning DW, Gow NA, Levitz SM, Netea MG, White TC (2012) Hidden killers: human fungal infections. Sci Transl Med 4:165rv113

Buil JB, van Zanten ARH, Bentvelsen RG, Rijpstra TA, Goorhuis B, van der Voort S, Wammes LJ, Janson JA, Melchers M, Heusinkveld M, et al. (2021) Case series of four secondary mucormycosis infections in COVID-19 patients, The Netherlands, December 2020 to may 2021. Euro Surveillance: bulletin Europeen Sur les maladies transmissibles = European communicable disease bulletin 26

Chakraborty R, Bhattacharje G, Baral J, Manna B, Mullick J, Mathapati BS, Abraham P, Madhumathi J, Hasija Y, Ghosh A et al (2022) In-silico screening and in-vitro assay show the antiviral effect of indomethacin against SARS-CoV-2. Comput Biol Med 147:105788

Chen J, Hu YC, Yang C, Yun CF, Wang R, Mao DQ, Li WD, Yang YH, Yang XG, Yang LC (2017) [Study on vitamin A nutritional status of Chinese urban elderly residents in 2010-2012]. Zhonghua yu fang yi xue za zhi [Chinese journal of preventive medicine] 51:121–124

Chovatiya R, Silverberg JI (2020) Inpatient morbidity and mortality of measles in the United States. PLoS One 15:e0231329

Chung IY, Tavassoli S, Wong N, Cleary G (2022) Vitamin A deficiency presenting with fungal keratitis and bilateral corneal perforations. BMJ Case Rep 15:e247853

Connell BJ, Tullo AB, Parry NR, Brown L, Osman A, Edwards M (2006) Vitamin A deficiency presenting with microbial keratitis in two patients in the UK. Eye (London, England) 20:623–625

Crecelius EM, Burnett MW (2020) Measles (Rubeola): an update. J Spec Operat Med 20:136–138

Custodio VI, Daneluzzi JC, Custodio RJ, Del Ciampo LA, Ferraz IS, Martinelli CE Jr, Ricco RG, Cupo P, Hering SE, Meirelles MS et al (2009) Vitamin A deficiency among Brazilian school-aged children in a healthy child service. Eur J Clin Nutr 63:485–490

D'Souza RM, D'Souza R (2001) Vitamin A for treating measles in children. Cochrane Database Syst Rev 2005:CD001479

D'Souza RM, D'Souza R (2002a) Vitamin A for the treatment of children with measles – a systematic review. J Trop Pediatr 48:323–327

D'Souza RM, D'Souza R (2002b) Vitamin A for treating measles in children. Cochrane Database Syst Rev 2005(4):CD001479

Daré LO, Bruand PE, Gérard D, Marin B, Lameyre V, Boumédiène F, Preux PM (2019) Co-morbidities of mental disorders and chronic physical diseases in developing and emerging countries: a meta-analysis. BMC Public Health 19:304

Diab L, Krebs NF (2018) Vitamin excess and deficiency. Pediatr Rev 39:161–179

Dilek A, Ozaras R, Ozkaya S, Sunbul M, Sen EI, Leblebicioglu H (2021) COVID-19-associated mucormycosis: case report and systematic review. Travel Med Infect Dis 44:102148

Dwivedi S, Choudhary P, Gupta A, Singh S (2023) The cross-talk between mucormycosis, steroids, and diabetes mellitus amidst the global contagion of COVID-19. Crit Rev Microbiol 49(3):318–333

Elhamamsy S, Bayer T, Al-Kaffas M, Hatahet S, Grover M, Samdhani S, Nanda A, Gravenstein S (2021) Rhino-orbital cerebral mucormycosis in non-diabetic patients with COVID-19. Rhode Island Med J (2013) 104:19–21

Ellison JB (1932) Intensive vitamin therapy in measles. Br Med J 2:708–711

Fan Y, Li X, Zhang L, Wan S, Zhang L, Zhou F (2022) SARS-CoV-2 Omicron variant: recent progress and future perspectives. Signal Transduct Target Ther 7: 141

Faustino JF, Ribeiro-Silva A, Dalto RF, Souza MM, Furtado JM, Rocha Gde M, Alves M, Rocha EM (2016) Vitamin A and the eye: an old tale for modern times. Arq Bras Oftalmol 79:56–61

Feikin DR, Higdon MM, Abu-Raddad LJ, Andrews N, Araos R, Goldberg Y, Groome MJ, Huppert A, O'Brien KL, Smith PG et al (2022) Duration of effectiveness of vaccines against SARS-CoV-2 infection and COVID-19 disease: results of a systematic review and meta-regression. Lancet (London, England) 399: 924–944

Gambhir RS, Aggarwal A, Bhardwaj A, Kaur A, Sohi RK, Mehta S (2021) Covid-19 and mucormycosis (black fungus): an epidemic within the pandemic. Rocz Panstw Zakl Hig 72:239–244

Gandra S, Ram S, Levitz SM (2021) The "black fungus" in India: the emerging syndemic of COVID-19-associated mucormycosis. Ann Intern Med 174:1301–1302

Gavriatopoulou M, Korompoki E, Fotiou D, Ntanasis-Stathopoulos I, Psaltopoulou T, Kastritis E, Terpos E, Dimopoulos MA (2020) Organ-specific manifestations of COVID-19 infection. Clin Exp Med 20:493–506

Gilbert C (2013) The eye signs of vitamin A deficiency. Community Eye Health J 26:66–67

Green HN, Mellanby E (1928) Vitamin A as an anti-infection agent. Br Med J 2:691–696

Green HN, Pindar D, Davis G, Mellanby E (1931) Diet as A prophylactic agent against puerperal sepsis. Br Med J 2:595–598

Hao X, Zhong X, Sun X (2021) The effects of all-trans retinoic acid on immune cells and its formulation design for vaccines. AAPS J 23:32

Hester GZ, Nickel AJ, Stinchfield PA, Spaulding AB (2020) Low use of vitamin A in children hospitalized for measles in the United States. Pediatr Infect Dis J 39: e45–e46

Hoenigl M, Seidel D, Carvalho A, Rudramurthy SM, Arastehfar A, Gangneux JP, Nasir N, Bonifaz A, Araiza J, Klimko N et al (2022) The emergence of COVID-19 associated mucormycosis: a review of cases from 18 countries. Lancet Microbe 3:e543–e552

Hussaini G, Tarwotjo I, Sommer A (1978) Cure for night blindness. Am J Clin Nutr 31:1489

Hussey GD, Klein M (1993) Routine high-dose vitamin A therapy for children hospitalized with measles. J Trop Pediatr 39:342–345

Iadecola C, Anrather J, Kamel H (2020) Effects of COVID-19 on the nervous system. Cell 183:16–27.e11

Imdad A, Mayo-Wilson E, Herzer K, Bhutta ZA (2017) Vitamin A supplementation for preventing morbidity and mortality in children from six months to five years of age. Cochrane Database Syst Rev 3:CD008524

Jeong W, Keighley C, Wolfe R, Lee WL, Slavin MA, Kong DCM, Chen SC (2019) The epidemiology and clinical manifestations of mucormycosis: a systematic review and meta-analysis of case reports. Clin Microbiol Infect 25:26–34

Kell AM, Gale M Jr (2015) RIG-I in RNA virus recognition. Virology 479–480:110–121

Khichar S, Samantaray S, Kumar D, Mobarsa V, Jain V, Sharma V, Soni K, Choudhury B, Goyal A, Shankar Meena D et al (2021) New-onset hyperglycaemia and prolonged systemic corticosteroids therapy in mild COVID-19 patients as major risk factors for invasive mucormycosis: a preliminary study. Curr Med Mycol 7:22–28

Kim JY (2016) Human fungal pathogens: why should we learn? J Microbiol (Seoul, Korea) 54:145–148

Lagorce Pagès C, Fabre A, Bruneel F, Zimmermann U, Hénin D (2000) Disseminated mucormycosis in AIDS. Ann Pathol 20:343–345

Lanska DJ (2010) Chapter 29: historical aspects of the major neurological vitamin deficiency disorders: overview and fat-soluble vitamin A. Handb Clin Neurol 95: 435–444

Lata S, Bafna RK, Asif MI, Sachan A (2021) Bilateral liquefactive corneal necrosis: a rare and devastating complication of vitamin A deficiency in the adult. BMJ Case Rep 14:e237343

Li R, Wu K, Li Y, Liang X, Tse WKF, Yang L, Lai KP (2020) Revealing the targets and mechanisms of vitamin A in the treatment of COVID-19. Aging 12: 15784–15796

Lim MA, Pranata R, Huang I, Yonas E, Soeroto AY, Supriyadi R (2020) Multiorgan failure with emphasis on acute kidney injury and severity of COVID-19: systematic review and meta-analysis. Can J Kidney Health Dis 7:2054358120938573

Lima EEC, Gayawan E, Baptista EA, Queiroz BL (2021a) Spatial pattern of COVID-19 deaths and infections in small areas of Brazil. PLoS One 16:e0246808

Lima LCF, Moraes Junior HV, Moraes HMV (2021b) COVID-19 ocular manifestations in the early phase of disease. Ocul Immunol Inflamm 29:666–668

Liu Y, Olagnier D, Lin R (2016) Host and viral modulation of RIG-I-mediated antiviral immunity. Front Immunol 7:662

Marley A, Smith SC, Ahmed R, Nightingale P, Cooper SC (2021) Vitamin A deficiency: experience from a tertiary referral UK hospital; not just a low- and middle-income country issue. Public Health Nutr 24:6466–6471

Mason J, Greiner T, Shrimpton R, Sanders D, Yukich J (2015) Vitamin A policies need rethinking. Int J Epidemiol 44:283–292

McLaren DS (2000) The antiinfective vitamin arises once more. Nutrition (Burbank, Los Angeles County, CA) 16:1110–1111

Meir Z, Osherov N (2018) Vitamin biosynthesis as an antifungal target. J Fungi (Basel, Switzerland) 4:72

Mei-Sheng Riley M (2021) Invasive fungal infections among immunocompromised patients in critical care settings: infection prevention risk mitigation. Crit Care Nurs Clin North Am 33:395–405

Melo AM, de Carvalho RA, Figueiredo JF, Vannucchi H, Jordão Junior A, Rodrigues ML (2004) Serum vitamin A levels in patients with ocular lesions attributable to non-complicated malaria in the Brazilian Amazon region. Trans R Soc Trop Med Hyg 98:485–488

Mertens E, Peñalvo JL (2020) The burden of malnutrition and fatal COVID-19: a global burden of disease analysis. Front Nutr 7:619850

Midha IK, Kumar N, Kumar A, Madan T (2020) Mega doses of retinol: a possible immunomodulation in Covid-19 illness in resource-limited settings. Rev Med Virol 31:e2204

Mora JR, Iwata M, von Andrian UH (2008) Vitamin effects on the immune system: vitamins A and D take centre stage. Nat Rev Immunol 8:685–698

Morjaria R, Mitra A, McDonnell P (2011) Bilateral microbial keratitis in a case of severe xerophthalmia following small bowel resection. J R Soc Med 104:124–126

Mselle J (1999) Fungal keratitis as an indicator of HIV infection in Africa. Trop Dr 29:133–135

Munjal M, Das S, Chatterjee N, Setra AE, Govil D (2020) Systemic involvement of novel coronavirus (COVID-19): a review of literature. Indian J Crit Care Med 24: 565–569

Nagalli S, Kikkeri NS (2021) Mucormycosis in COVID-19: a systematic review of literature. Infez Med 29: 504–512

Nair AG, Adulkar NG, D'Cunha L, Rao PR, Bradoo RA, Bapaye MM, Kothari A, Dave TV, Shinde CA (2021) Rhino-orbital mucormycosis following COVID-19 in previously non-diabetic, immunocompetent patients. Orbit (Amsterdam, Netherlands) 40:499–504

Nascimento AL, Diniz Ada S, Arruda I (2007) [Vitamin A deficiency in elderly attending the health family Programme in Camaragibe, PE, Brazil]. Archivos latinoamericanos de nutricion 57:213–218

Nugent R (2008) Chronic diseases in developing countries: health and economic burdens. Ann N Y Acad Sci 1136:70–79

O'Mahony AM, McSwiney TJ, Cosgrave E (2019) Measles and eye complications. Ir Med J 112:1007

Oliveira LM, Teixeira FME, Sato MN (2018) Impact of retinoic acid on immune cells and inflammatory diseases. Mediat Inflamm 2018:3067126

Ong APC, Watson A, Subbiah S (2020) Rubeola keratitis emergence during a recent measles outbreak in New Zealand. J Prim Health Care 12:289–292

Patel A, Agarwal R, Rudramurthy SM, Shevkani M, Xess I, Sharma R, Savio J, Sethuraman N, Madan S, Shastri P et al (2021) Multicenter epidemiologic study of coronavirus disease-associated mucormycosis, India. Emerg Infect Dis 27:2349–2359

Petrikkos G, Skiada A, Lortholary O, Roilides E, Walsh TJ, Kontoyiannis DP (2012) Epidemiology and clinical manifestations of mucormycosis. Clin Infect Dis 54 (Suppl 1):S23–S34

Prompetchara E, Ketloy C, Palaga T (2020) Immune responses in COVID-19 and potential vaccines: lessons learned from SARS and MERS epidemic. Asian Pac J Allergy Immunol 38:1–9

Reid G, Lynch JP 3rd, Fishbein MC, Clark NM (2020) Mucormycosis. Semin Respir Crit Care Med 41:99–114

Rocha ICN, Hasan MM, Goyal S, Patel T, Jain S, Ghosh A, Cedeño TDD (2021) COVID-19 and mucormycosis syndemic: double health threat to a collapsing healthcare system in India. Trop Med Int Health 26:1016–1018

Rodrigues MI, Dohlman CH (2004) Blindness in an American boy caused by unrecognized vitamin A deficiency. Archives of ophthalmology (Chicago, IL: 1960) 122: 1228–1229

Romero-Sánchez CM, Díaz-Maroto I, Fernández-Díaz E, Sánchez-Larsen Á, Layos-Romero A, García-García J, González E, Redondo-Peñas I, Perona-Moratalla AB, Del Valle-Pérez JA et al (2020) Neurologic manifestations in hospitalized patients with COVID-19: the ALBACOVID registry. Neurology 95:e1060–e1070

Ross AC, Stephensen CB (1996) Vitamin A and retinoids in antiviral responses. FASEB J 10:979–985

Sarohan AR (2020) COVID-19: endogenous retinoic acid theory and retinoic acid depletion syndrome. Med Hypotheses 144:110250

Sarohan AR (2021a) New strategies for COVID-19 vaccination and adjuvant prophylaxis. J Vaccines Vaccin 12:1–8

Sarohan AR (2021b) Systemic organ involvement and retinoid signaling disorder in COVID-19. Immunogenetics: Open Access 6:1–6

Sarohan AR, Kızıl M, İnkaya A, Mahmud S, Akram M, Cen O (2021) A novel hypothesis for COVID-19 pathogenesis: retinol depletion and retinoid signaling disorder. Cell Signal 87:110121

Sarohan AR, Akelma H, Araç E, Aslan Ö, Cen O (2022) Retinol depletion in COVID-19. Clin Nutr Open Sci 43:85–94

Semba RD (2012) On the 'discovery' of vitamin A. Ann Nutr Metab 61:192–198

Semba RD, Bloem MW (2004) Measles blindness. Surv Ophthalmol 49:243–255

Sen M, Honavar SG, Sharma N, Sachdev MS (2021) COVID-19 and eye: a review of ophthalmic manifestations of COVID-19. Indian J Ophthalmol 69:488–509

Sherwin JC, Reacher MH, Dean WH, Ngondi J (2012) Epidemiology of vitamin A deficiency and xerophthalmia in at-risk populations. Trans R Soc Trop Med Hyg 106:205–214

Singhal T (2022) The emergence of omicron: challenging times are here again! Indian J Pediatr 89:490–496

Sirisinha S (2015) The pleiotropic role of vitamin A in regulating mucosal immunity. Asian Pac J Allergy Immunol 33:71–89

Snelson M, Tan SM, Clarke RE, de Pasquale C, Thallas-Bonke V, Nguyen TV, Penfold SA, Harcourt BE, Sourris KC, Lindblom RS et al (2021) Processed foods drive intestinal barrier permeability and microvascular diseases. Sci Adv 7:eabe4841

Sommer A (1983) Mortality associated with mild, untreated xerophthalmia. Trans Am Ophthalmol Soc 81:825–853

Sommer A (1990) Xerophthalmia, keratomalacia and nutritional blindness. Int Ophthalmol 14:195–199

Sommer A (1993) Vitamin A, infectious disease, and childhood mortality: a 2 solution? J Infect Dis 167: 1003–1007

Sommer A (2008) Vitamin a deficiency and clinical disease: an historical overview. J Nutr 138:1835–1839

Sommer A, Vyas KS (2012) A global clinical view on vitamin A and carotenoids. Am J Clin Nutr 96:1204s–1206s

Sommer A, Tarwotjo I, Hussaini G, Susanto D (1983) Increased mortality in children with mild vitamin A deficiency. Lancet (London, England) 2:585–588

Spannaus-Martin DJ, Cook LR, Tanumihardjo SA, Duitsman PK, Olson JA (1997) Vitamin A and vitamin E statuses of preschool children of socioeconomically disadvantaged families living in the midwestern United States. Eur J Clin Nutr 51:864–869

Staats CC, Kmetzsch L, Schrank A, Vainstein MH (2013) Fungal zinc metabolism and its connections to virulence. Front Cell Infect Microbiol 3:65

Stephensen CB (2001) Vitamin A, infection, and immune function*. Annu Rev Nutr 21:167–192

Stephensen CB, Lietz G (2021) Vitamin A in resistance to and recovery from infection: relevance to SARS-CoV2. Br J Nutr 126(11):1663–1672

Stevens GA, Bennett JE, Hennocq Q, Lu Y, De-Regil LM, Rogers L, Danaei G, Li G, White RA, Flaxman SR et al (2015) Trends and mortality effects of vitamin A deficiency in children in 138 low-income and middle-income countries between 1991 and 2013: a pooled analysis of population-based surveys. Lancet Glob Health 3:e528–e536

Tanumihardjo SA (2011) Vitamin A: biomarkers of nutrition for development. Am J Clin Nutr 94:658s–665s

Tepasse P-R, Vollenberg R, Fobker M, Kabar I, Schmidt H, Meier JA, Nowacki T, Hüsing-Kabar A (2021) Vitamin A plasma levels in COVID-19 patients: a prospective multicenter study and hypothesis. Nutrients 13:2173

Tian D, Sun Y, Xu H, Ye Q (2022) The emergence and epidemic characteristics of the highly mutated SARS-CoV-2 Omicron variant. J Med Virol 94: 2376–2383

Tielsch JM, Rahmathullah L, Katz J, Thulasiraj RD, Coles C, Sheeladevi S, Prakash K (2008) Maternal night blindness during pregnancy is associated with low birthweight, morbidity, and poor growth in South India. J Nutr 138:787–792

Timoneda J, Rodríguez-Fernández L, Zaragozá R, Marín MP, Cabezuelo MT, Torres L, Viña JR, Barber T (2018) Vitamin A deficiency and the lung. Nutrients 10:1132

Tomasa-Irriguible TM, Bielsa-Berrocal L, Bordejé-Laguna L, Tural-Llàcher C, Barallat J, Manresa-Domínguez JM, Torán-Monserrat P (2021) Low levels of few micronutrients may impact COVID-19 disease progression: an observational study on the first wave. Metabolites 11:565

Toor R, Chana I (2022) Exploring diet associations with Covid-19 and other diseases: a network analysis-based approach. Med Biol Eng Comput 60:991–1013

Underwood BA (1994) Hypovitaminosis A: international programmatic issues. J Nutr 124:1467s–1472s

Villar J, Merialdi M, Gülmezoglu AM, Abalos E, Carroli G, Kulier R, de Onis M (2003) Nutritional interventions during pregnancy for the prevention or treatment of maternal morbidity and preterm delivery: an overview of randomized controlled trials. J Nutr 133:1606s–1625s

Vyas N, Kurian SJ, Bagchi D, Manu MK, Saravu K, Unnikrishnan MK, Mukhopadhyay C, Rao M, Miraj SS (2021) Vitamin D in prevention and treatment of COVID-19: current perspective and future prospects. J Am Coll Nutr 40(7):632–645

Wan KH, Lui GCY, Poon KCF, Ng SSS, Young AL, Hui DSC, Tham CCY, Chan PKS, Pang CP, Chong KKL (2022) Ocular surface disturbance in patients after acute COVID-19. Clin Exp Ophthalmol 50:398–406

West KP Jr, Katz J, Khatry SK, LeClerq SC, Pradhan EK, Shrestha SR, Connor PB, Dali SM, Christian P, Pokhrel RP et al (1999) Double blind, cluster randomised trial of low dose supplementation with vitamin A or beta carotene on mortality related to pregnancy in Nepal. The NNIPS-2 Study Group. BMJ (Clin Res Ed) 318:570–575

WHO (n.d.) WHO Coronovirus (COVID-19) dashboard. World Health Organization, Geneva

WHO (2007) Vision 2020: the right to sight. World Health Organization, Geneva

WHO (2009) Global prevalence of vitamin A deficiency in populations at risk 1995–2005: WHO global database on vitamin A deficiency. World Health Organization, Geneva

WHO (2014) Xerophthalmia and night blindness for the assessment of clinical vitamin A deficiency in individuals and populations. World Health Organization, Geneva

WHO (2017) Measles vaccines: WHO position paper – April 2017. Releve epidemiologique hebdomadaire 92: 205–227

WHO (2020) WHO manifesto for a healthy recovery from COVID-19. Prescriptions for a healthy and green recovery from COVID-19. World Health Organization, Geneva

WHO (2021) Mucormycosis_1. World Health Organization, Geneva

Wiseman EM, Bar-El Dadon S, Reifen R (2017) The vicious cycle of vitamin a deficiency: a review. Crit Rev Food Sci Nutr 57:3703–3714

Wolf G (1978) A historical note on the mode of administration of vitamin A for the cure of night blindness. Am J Clin Nutr 31:290–292

Zhang L, Liu Y (2020) Potential interventions for novel coronavirus in China: a systematic review. J Med Virol 92:479–490

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Treatment Strategies for Multiple Myeloma Treatment and the Role of High-Throughput Screening for Precision Cancer Therapy

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Abstract

In the past few years, development of approved drug candidates has improved the disease management of multiple myeloma (MM). However, due to drug resistance, some of the patients do not respond positively, while some of the patients acquire drug resistance, thereby these patients eventually relapse. Hence, there are no other therapeutic options for multiple myeloma patients. Therefore, this necessitates a precision-based approach to multiple myeloma therapy. The use of patient's samples to test drug sensitivity to increase efficacy and reduce treatment-related toxicities is the goal of functional precision medicine. Platforms such as high-throughput-based drug repurposing technology can be used to select effective single drug and drug combinations based on the efficacy and toxicity studies within a time frame of couple of weeks. In this article, we describe the clinical and cytogenetic features of MM. We highlight the various treatment strategies and elaborate on the role of high-throughput screening platforms in a precision-based approach towards clinical treatment.

Keywords

Drug screening · Drug sensitivity · Heterogeneity · Multiple myeloma · Precision medicine · Treatment strategies

Abbreviations

BM	Bone marrow
BMSC	BM-derived stem/stromal cells

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CAM-DR	Cell adhesion-mediated drug resistance
CDKN2A	Cyclin-dependent kinase inhibitors (CDKN2A, CDKN2C)
CRAB	Calcium, Renal, Anaemia, and Bone
DAPK	E-cadherin death-associated protein kinase
EC	Endothelial cells
ECM	Extracellular matrix
FISH	Fluorescence in situ hybridisation
HATs	Histone acetylases
HDACs	Histone deacetylases
HTS	High-throughput screening
IGF-1	Insulin-like growth factor 1
JAK/STAT	Janus kinase, signal transducer and activator of transcription proteins
LDH	Lactate dehydrogenase
MAPK	Mitogen-activated protein kinase
MGMT	O^6-Methylguanine-DNA methyltransferase
MGUS	Monoclonal gammopathy of undetermined significance
MM	Multiple myeloma
mTOR	Mammalian target of rapamycin
NF-κB	Nuclear factor kappa beta
OB	Osteoblasts
OC	Osteoclasts
OS	Overall survival
PDGF	Platelet-derived growth factor
Ras	Rat sarcoma virus
SOCS-1	Suppressor of cytokine signalling
ß2M	Beta 2-microglobulin
TERT	Telomerase reverse transcriptase
TIMP3	Tissue inhibitor of metalloproteinase 3
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen 4

1 Introduction

Multiple myeloma (MM) is a plasma cell-based malignant disorder in the bone marrow (BM), characterised by the accumulation of monoclonal protein in serum and urine (Gulla and Anderson 2020; Landgren et al. 2009). The Global Cancer Observatory (GLOBOCAN) reported 117,077 (66%) deaths from 176,404 MM cases worldwide in 2020 (The Global Cancer Observatory 2020). Data from the United States show an estimated death of 12,800 MM patients and account for 2.1% of all types of cancer deaths in 2020 (Padala et al. 2021). The median age of MM patients in the United States is 69, with more than 60% of MM are from patients older than 65 years (Padala et al. 2021). In 2010, the survival age range for older patients was 2 to 3 years, and for younger patients it was 5 to 6 years. Additionally, these survival ranges can depend on the stage of disease at diagnosis (Bladé et al. 2010; Padala et al. 2021). For a long time, MM patients have been treated with a first-line treatment chemotherapy combination of melphalan and prednisone, hoping to achieve remission (Gregory et al. 1992). Treatment options have since improved with inclusion of proteasome inhibitors, immunomodulatory drugs, and autologous stem cells transplantation; however, even with these options, most patients do not respond favourably to the treatment with most relapsing. Additionally, there are instances where the side effects of the drugs outweigh the benefits (Roussel et al. 2014). For example, most patients who had a high-dose therapy of lenalidomide, bortezomib, and dexamethasone combination supported by stem cell transplantation experienced a better response after induction, even though some common adverse events such as neuropathy, neutropenia, and thrombocytopenia can still be observed (Roussel et al. 2014). Drug regimen allocation to MM patients depends on drug availability, disease severity, age of the patient, and cytogenetics (Laudin et al. 2020; Mateo et al. 2005). If the treatment outcome is not favourable, patients are enrolled into clinical trials where treatment is in a randomisation format (Laudin et al. 2020; Mateo et al. 2005). As a result, there is an insufficient number of effective treatment options for MM patients capable of achieving a complete response and there is a critical need for a library of MM treatment data to reduce morbidity and mortality.

2 Clinical and Pathological Characteristics of Multiple Myeloma

The first diversity of MM patients is observed on signs and symptoms, for example, most MM patients in the 1999 Moosa Patel study had bone pain (94.1%), followed by anaemia (81.2%), infections (59.8%), while 40.6% had plasmacytomas and 88.4% showed lytic bone lesions (Patel 1999). Multiple myeloma patients can also be asymptomatic with less bone pain and just headaches (Rankapole et al. 2011). Additional features include bone fractures, which require the use of an orthotic device to effectively mobilise, and the incidence of an extramedullary disease (Laudin et al. 2020). These findings show that MM patients are a diverse population that would require extensive strategies of management for all the ailments.

Multiple myeloma is mainly characterised by an increase of clonal plasma cells in the BM (Matsue et al. 2017). Other diagnostic features include the detection of monoclonal protein in serum or urine (Reddy et al. 2021), and the evidence of end-organ damage, which includes hypercalcaemia, renal insufficiency, anaemia, and/or osteolytic bone lesions satisfying the CRAB criteria (Dinner et al. 2013). The CRAB criteria occurs because the neoplastic plasma cells of MM patients in the BM proliferate frequently and invade the adjacent bone, causing osteolytic lesions. Neoplastic plasma cell proliferation also interferes with the production of red blood cells causing anaemia (Kyle et al. 2003). The abundant production of the abnormal monoclonal proteins by MM cells instead of the normal proteins results in renal insufficiency and the occurrence of recurrent infections (Kyle et al. 2003).

Multiple myeloma is preceded by two premalignant phases, the monoclonal gammopathy of undetermined significance (MGUS) and smouldering multiple myeloma (SMM) (Prideaux et al. 2014). During these stages, plasma cells experience sequential genetic and epigenetic events, and the BM also changes to create a conducive environment for MM cells to survive and proliferate (Giannakoulas et al. 2021). Accordingly, the patients in the first phase (MGUS) are asymptomatic, and their serum monoclonal protein levels and plasma cell clones in the BM are lower than 30 g/L and 10%, respectively. In contrast, SMM patients' serum monoclonal protein levels and plasma cell clones are more than 30 g/L and 10%, respectively. To satisfy MM diagnosis, the patients must have a myeloma-related organ or tissue impairment (ROTI) and also have serum monoclonal protein levels and plasma cell clones at least >30 g/L and ≥10% in the BM, respectively (Chai et al. 2019; Matsue et al. 2017; Prideaux et al. 2014). Moreover, the clonal plasma cell percentage is also used during intensive care of MM patients, where a complete response is defined as <5% clonal plasma cells in the bone marrow (Joshi et al. 2008; Matsue et al. 2017). According to the WHO criteria, a move from the 10%-30% range to >30% BM plasma cell infiltration equates to a shift from minor to major disease, a criterion which is used as a predictor of relapse in cases of treated MM (Štifter et al. 2010). Therefore, it is crucial to assess the patient's clonal plasma cell percentages during treatment at specified time points to determine the effectiveness of the drug regimen.

In addition to different levels of plasma cell infiltration in the BM, MM is characteristically heterogeneous and exhibits diverse genetic, molecular, and clinical features (Giannakoulas et al. 2021). The different attributes are used in risk assessments and have been adopted in the revised International Staging System (R-ISS) to classify MM disease into three stages using beta 2-microglobulin (ß2M) and albumin, lactate dehydrogenase (LDH) serum levels, patients cytogenetics, gene expression profiles, plasma cell proliferation, and the presence of extramedullary disease (Giannakoulas et al. 2021; Moser-Katz et al. 2021). Patients at stages I, II, and III had a 5-year overall survival (OS) of 82%, 62%, and 40%, respectively, showing that the level of risk determines the prognosis (Giri et al. 2020).

3 Cytogenetics of Multiple Myeloma

Multiple myeloma cytogenetic aberrations are complex and crucial for determining disease severity and sensitivity to treatment. Newly diagnosed MM patients can be stratified into prognostic groups of genetic aberrations, which are attained using the standard cytogenetics and fluorescence in situ hybridisation (FISH) technique (Catamero 2018; Sawyer 2011). Multiple myeloma patients can be split into two main groups, the hyperdiploid and nonhyperdiploid based on their karyotype (Bianchi and Munshi 2015; de Smedt et al. 2018). The nonhyperdiploid karyotype group comprises the hypodiploid/pseudodiploid (which also includes the near-tetraploid karyotypes) (de Reave and Vanderkerken 2005).

The hyperdiploid karyotype group is characterised by trisomies in odd chromosomes (3, 5, 7, 9, 11, 15, 19, and 21) (Bianchi and Munshi 2015) and is displayed in 50% to 60% of MM patients at diagnosis (de Smedt et al. 2018). The nonhyperdiploid karyotype chromosomal abnormalities include monosomy 13, 14, and 22; deletions of 1p, 12p, 16q, and 17p; and gains of 1p defects (van Wier et al. 2013). Nonhyperdiploid aberrations are observed in 40% to 50% of MM patients (Corre et al. 2015). Hyperdiploid karyotype has a better prognosis compared to the nonhyperdiploid karyotype group (Sawyer 2011). Multiple myeloma patients with t(4;14), t(14;16), and t(14;20) translocations, del(17p), are classified as high-risk disease and have a poor prognosis, in contrast, patients with t(11;14), t(6;14), and/or hyperdiploidy are classified as standard-risk disease and have a better prognosis (Catamero 2018; Diamantidis et al. 2022).

4 Multiple Myeloma Clonal Expansion

Multiple myeloma evolves from the premalignant phases (Prideaux et al. 2014), at a rate of 1% per year from MGUS, and this goes with the idea that genetic changes such as t(4;14), t(14;16), and 17p deletion start at the MGUS phase (López-Corral et al. 2011). López-Corral et al. (2011) assessed the incidence of genetic abnormalities in 565 patients, 90 MGUS, 102 SMM, and 373 MM, and they also observed the frequency of 13q, 17p deletions, 1q gains, t(11;14), t(4;14), and 13q deletions were all significantly less in MGUS compared to SMM and MM indicating that cytogenetic alteration accumulation is a necessary step during disease progression (López-Corral et al. 2011). They also investigated if the accumulation of cytogenetic was related to the complications in MM. The authors demonstrated that the occurrence of t(14;16) translocation was associated with renal failure, and that, t(11;14) and t(6;14) were more common in patients with bone disease indicating that cytogenetics abnormalities are a good indicator of MM prognosis (Greenberg et al. 2014).

Furthermore, there is also a difference in genetic aberrations observed at MM diagnosis and relapse. At relapse, the clones can either gain or lose chromosomal/genetic aberrations constituting intra-tumoural heterogeneity depending on the treatment the patient has been subjected to (Corre et al. 2015). In patients who received 4 cycles of either immunomodulatory or proteasome inhibitors-based treatment, 13q deletion and 1q21 gain tended to appear earlier, followed by a later attainment of 16q and 17p deletion. The appearance of 13q deletion was associated with worse survival than 1q21 gain (Yan et al. 2022). However, the prognostic value of 1q21 gain was variable at relapse due to changes in copy number and clone size. Patients who had an increase in copy number of 1q21 or who developed de novo 1q21 gain at relapse experienced the poorest outcome when compared to patients who had a reduced 1q21 copy number or those without or those who experienced no change to 1q21 gain (Yan et al. 2022).

Genetic heterogeneity was further shown in MM patients who became double-refractory, triple-refractory, or quadruple-refractory to immunomodulatory and protease inhibitors at relapse (Giesen et al. 2022). When compared to newly diagnosed MM patients, the relapsed MM patients showed more aberrations, including gain (1q) and deletions of 1p, 13q, and 17p, and a high frequency of functional small nucleotide variants per patient (Giesen et al. 2022). The mechanism in which genetic aberrations develop in MM cells was perfectly demonstrated by Shen et al. (2021) using a "bone chip" xenograft mouse model named "MM PrEDiCT" for MM progression through evolution and dissemination of clonal tumour cells (Shen et al. 2021). The "MM PrEDiCT" model allows the implantation, dissemination, and easy tracking of fluorescent-tagged or DNA-barcoded cells on SCID mice. After cell transplantation, SCID mice BM was colonised at 6 weeks and on the 8th week and host mice presented with limb paralysis, multiple skeletal lytic lesions, and a patchy distribution of fluorescent-tagged cells with some colours surpassing other colours. Upon disease progression, there was less colour diversity, showing that the most competent cell clones could outcompete other clones (Shen et al. 2021). Dominant clones were the same, in both left and right femur bones, and only differed when the host is changed (Shen et al. 2021). This shows that when provided with conditions mimicking the BM microenvironment, dominant MM cell clones will prevail and that each host micro-microenvironment is different and therefore contributes to heterogeneity.

5 Genes Expression and Signalling

Some chromosome translocations may result in the overexpression of genes such as CCND1, CCND3, MAF, MAFB, WHSC1/MMSET, and FGFR3 via their juxtaposition to the immunoglobulin heavy chain (IgH) locus (Chapman et al. 2011). Other notable mutations occur in RAS family members (NRAS, KRAS, and BRAF), NFKB1, NFKB2, TRAF3, CHUK, and TP53, which can lead to the activation of signalling pathways (Giesen et al. 2022). In addition, during MM malignant progression, there is also MYC activation and loss-of-function mutations in the histone demethylase UTX/KDM6A (Chapman et al. 2011).

In broad terms, pathways involved in MM progression include the p53 signalling, nuclear factor kappa beta (NF-κB) pathway, the cell-cycle pathway, the p38 mitogen-activated protein kinase (MAPK) pathway, histone methyltransferase, the telomerase reverse transcriptase (TERT) pathway, Janus kinase, signal transducer and activator of transcription proteins (JAK/STAT), platelet-derived growth factor (PDGF) signalling pathways (Lam et al. 2018), rat sarcoma virus (Ras), mammalian target of rapamycin (mTOR), Notch-signalling pathway, vascular endothelial growth factor (VEGF) and insulin-like growth factor 1 (IGF-1) (Hou et al. 2019), and the cyclin-dependent kinases (CDKs), CDK1, and CDK2 (Lam et al. 2018). The functions of these pathways include tumour progression, survival, and escaping of tumour neutralising immune surveillance in the tumour microenvironment (Tai et al. 2018).

6 Heterogeneity in Multiple Myeloma: The Role of Bone Marrow Microenvironment

The BM microenvironment provides the extracellular matrix (ECM) a layered structure that supports the myeloma cells (Moser-Katz et al. 2021). The ECM is made up of proteins such as fibronectin, type1 collagen, osteopontin (Hiasa et al. 2021), hyaluronan, and laminin (Moser-Katz et al. 2021). Besides MM cells, the BM microenvironment also contains BM-derived stem/stromal cells (BMSCs), osteoclasts (OCs) (Hou et al. 2019), osteoblasts (OBs), adipocytes, vascular endothelial cells (ECs) (de Reave and Vanderkerken 2005), and immune cells (macrophages, neutrophils, natural killer (NK) cells, regulatory T cells (Tregs), etc.) (Giannakoulas et al. 2021) whose interactions are mediated by chemokines, cytokines, growth factors (Hou et al. 2019), receptors, and adhesion molecules (de Reave and Vanderkerken 2005).

In the pathophysiology of MM, interactions between MM cells and the BM microenvironment are crucial. These components help with the survival of MM cells through various strategies including homing of MM cells to the BM, spreading via microvasculature to other sites of the BM, and secretion of growth factors resulting in differentiation; proliferation; drug resistance; osteoclastogenesis; inhibition of osteogenesis, angiogenesis, humoral and cellular immunodeficiency; and anaemia (de Reave and Vanderkerken 2005).

7 Extracellular Matrix

The attraction of MM cells to the BM is mediated through the interaction of myeloma receptor CXCR4 with the chemokine stromal cell-derived factor 1 alpha (SDF1α) which results in migration towards stromal compartment of bone marrow (Moser-Katz et al. 2021), where they adhere to ECM proteins fibronectin and type I collagen (de Reave and Vanderkerken 2005), using the very late antigen 4 (VLA-4), an adhesion molecule expressed on MM cell surface (Ho et al. 2020). The VLA-4-fibronectin adherence results in the activation of NF-κB resulting in cell adhesion-mediated drug resistance (CAM-DR) and pro-survival signalling (Moser-Katz et al. 2021). Multiple myeloma cells also adhere to type I collagen via syndecan-1 or CD138, a heparan sulphate proteoglycan expressed on their surface (Moser-Katz et al. 2021) to induce the expression of matrix metalloproteinase 1 (MMP1), thereby promoting bone resorption, tumour invasion, angiogenesis, and ultimately MM cell survival (de Reave and Vanderkerken 2005). Additionally, MM cells adhere to the ECM via CD38 a hyaluronan receptor which is highly expressed in plasma cells but less in other lymphoid and myeloid cells (Moser-Katz et al. 2021).

8 Bone Marrow-Derived Stromal Cells

Multiple myeloma cells utilise VLA-4 to adhere to vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule 1 (ICAM-1) on BMSCs (Ho et al. 2020). The adhesion of MM cells to BMSCs results in the secretion of various growth and antiapoptotic factors, such as VEGF, IGF-1, basic fibroblast growth factor, angiopoietin 1, transforming-growth factor (TGF)-b, hepatocyte growth factor, stromal cell-derived factor 1 (SDF-1), B cell-activating factor (BAFF), IL-21, IL-6, and IL-1 (Ho et al. 2020). The adhesion of MM cells to BMSCs and the secretion of growth and antiapoptotic factors lead to the activation of several pathways including the NF-κB, PI3K/Akt that mediate MM cell growth and survival (Hiasa et al. 2021).

9 Cytokines

In MM, there are a number of cytokines which are crucial in proliferation, migration, and drug resistance, including IL-6, vascular endothelial growth factor (VEGF), tumour necrosis factor-α (TNF-α), BAFF, a proliferation inducing ligand (APRIL), colony stimulating factor-1 (CSF1), fibroblast growth factor-2 (FGF-2), angiopoietin-1, and others (Giannakoulas et al. 2021). Key cytokines like VEGF, IL-6, TNF-α, BAFF, and RANKL medium serum levels were shown to be higher in relapsed MM patients compared to *newly diagnosed* patients, indicating that they are crucial for MM progression (Jasrotia et al. 2020).

Interleukin-6 is the most important cytokine in MM, since it has several functions including growth, survival, migration, invasion, angiogenesis, apoptosis inflammation, and drug resistance (Hou et al. 2019; Moser-Katz et al. 2021). It is produced by osteoblasts, monocytes, macrophages, and BMSCs (Andrews et al. 2013). For example, the secretion of IL-1ß, TGF-ß, TNF-α, bFGF, and VEGF by MM cells results in the induction of the secretion of IL-6 by the BMSCs (de Reave and Vanderkerken 2005). The binding of the secreted IL-6 to its receptor IL-6Ro on MM cells triggers the activation of the PI3K/Akt/mTOR and Ras/Raf/MEK/Erk signalling pathways and its growth factor role is through the phosphorylation of STAT3 via JAK1 (Hou et al. 2019). The phosphorylation of STAT3 results in the activation of Bcl-xL and myeloid cell factor-1 (mcl-1) which are crucial for MM survival (de Reave and Vanderkerken

2005). The activation of the phosphoinositol 3 kinase (PI3K)-protein kinase B (PkB/Akt) pathway by IL-6 provides an antiapoptotic activity and activation of Ras result in its translocation to the plasma membrane where it activates Raf, mitogen-activated protein kinase (MEKK), and MAPK, leading to increased proliferation of MM cells (Andrews et al. 2013). Furthermore, IL-6 promotes osteolysis (bone resorption) through the induction and production of RANKL, found on the surface of BMSCs and osteoblasts (Andrews et al. 2013). In addition, IL-6 may also contribute to immune dysfunction in MM (Prabhala et al. 2010).

Tumour necrosis factor alpha is a self-regulating inflammatory cytokine that has a variety of biological functions, including angiogenesis, proliferation, immortalisation, and the induction of expression of adhesion molecules (Ho et al. 2020; Lemancewicz et al. 2013; Moser-Katz et al. 2021). The mechanism of TNF-α includes canonical NF-kB pathway activation and subsequent potent stimulation of autocrine IL-6 production (Ho et al. 2020).

BAFF and APRIL, which are members of the TNF-α family (Bolkun et al. 2014), are highly expressed in MM cells as compared to normal plasma cells of the BM (Moser-Katz et al. 2021), and their expressions are associated with increased BM microvascular density (Bolkun et al. 2014). Furthermore, BAFF and APRIL were shown to contribute to a shorter progression-free survival and poor disease outcome (Lemancewicz et al. 2013). The APRIL protein, which is characteristically secreted without cell surface expression, binds to transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI) and B cell maturation antigen (BCMA) (Moreaux et al. 2004; Moser-Katz et al. 2021). In contrast, BAFF is produced as both a membrane-bound and a proteolytically cleaved soluble protein and binds to BCMA, TACI, and a third receptor called BAFF-R (Moreaux et al. 2004). Even though there are minor differences between the two cytokines, they both activate the NF-κB, phosphatidylinositol-3 (PI-3) kinase/AKT, and MAPK kinase pathways in MM cells which result in promotion of cell growth, up-regulation of the Mcl-1 and Bcl-2 antiapoptotic proteins, and immunosuppression via programmed death-1, TGFß, and IL10 (Moreaux et al. 2004; Moser-Katz et al. 2021).

The role of insulin growth factor in MM includes regulating the activities of proteasome and telomerase by binding to tyrosine kinase receptor IGF-1R and the priming of MM cells for response of action towards other cytokines and to produce pro-angiogenic cytokines (Moser-Katz et al. 2021). The mechanism of IGF-1 is through the activation of MAPK and PI3K/AKT signalling leads to Bcl-xL and Bcl-2-like protein 11 (BCL2L11) or Bcl-2 interacting mediator of cell death (BIM) (Ho et al. 2020).

Vascular endothelial growth factor, on the other hand, is one of the pro-angiogenic cytokines produced in MM by multiple cell types and is responsible for paracrine and autocrine growth of MM cells (Bolkun et al. 2014) and is key for angiogenesis and supports both microvascular endothelial cells and BMSCs through RAS, focal adhesion kinase, PI3K/AKT, MAPK, and STAT signalling (Ho et al. 2020). The mechanism of VEGF is thought to be a feedback loop in which MM cells stimulated by IL-6 secrete VEGF, which causes microvascular endothelial cells and BMSCs to secrete IL-6 (Paesler et al. 2012).

10 Immunomodulatory Cells

Multiple myeloma progression is characterised by immune evasion, which occurs when dysfunctional effector lymphocytes, tumour-educated immunosuppressive cells, and soluble mediators work together to form a barrier to antimyeloma immunity (Nakamura et al. 2020). The macrophages whose population is the most abundant in the BM microenvironment have been shown to support MM cells (Kim et al. 2012). Macrophages induced an increase in the proliferation of MM cells, when co-cultured with BMSCs and showed a reduced proliferation when treated with IL-6 blocking antibody indicating that synergistic effect in the BM microenvironment is mediated by IL-6 (Kim et al.

2012). The role of macrophages on MM cell growth was also examined on chemotherapy-treated MM cells. Multiple myeloma cells co-cultured with macrophages resisted dexamethasone- and melphalan-induced apoptosis, to a greater extent with those co-cultured with tumour-derived macrophages than normal macrophages. Moreover, the co-culture of myeloma cells with tumour-derived macrophages also inhibited the activation and cleavage of caspase-3 and poly (ADP-ribose) polymerase PARP and maintained the levels of Bcl-xL and IL-6 (Zheng et al. 2009). Neutrophils isolated from MM patients express high levels of immunosuppressive molecule arginase-1 (Arg-1) as compared as those isolated from MGUS and healthy controls. Furthermore, they were also shown to have a reduced phagocytic activity and an immunosuppressive function on T lymphocytes (Parrinello et al. 2013). Eosinophils were shown to enhance the proliferation of MM cell lines and primary CD138$^+$ MM cells and more when co-cultured in direct contact compared to on transwells (Wong et al. 2013).

11 Epigenetics in Multiple Myeloma

Epigenetic alterations in tumours are much more frequent than the existing identified genetic aberrations, and these epigenetic variations are involved in signalling pathways for cell growth, proliferation, apoptosis, immune escape, energy metabolism disorders, and promotion of tumour inflammation (Liu et al. 2022). Similar to other cancers, the epigenetic machinery plays a crucial role in MM genomic instability and function (de Smedt et al. 2018). Epigenetic modifications include histone deacetylation/acetylation or DNA methylation (Liu et al. 2022). In addition, dynamic spatiotemporal positioning of nucleosomes, control of chromatin three-dimensional conformation and nuclear topology, control of non-coding RNA, microRNA, and enhancer RNA are all aspects of epigenetic regulation (Liu et al. 2022).

Histone deacetylation/acetylation processes are controlled by the activity of histone acetylases (HATs) and histone deacetylases (HDACs); therefore, the balance between these enzymes can alter the gene expression profile and some signalling pathways, including ERK (extracellular signal-regulated kinase) and Wnt (wingless/Int-1), and can affect proteasomal degradation, influence protein kinase C activity, and change the DNA methylation status (Eckschlager et al. 2017). Increased histone acetylation causes decondensation of the chromatin, a process that can be reversed by HDACs (Eckschlager et al. 2017). Histone deacetylases reverse the decondensation by removing acetyl groups from protein tails causing the lysine residues to regain a positive charge and the resumption of the electrostatic contact with the DNA molecules resulting in the suppression of transcription (Garmpi et al. 2018). The acetylation process can also happen to non-histone proteins, altering numerous cellular processes (Eckschlager et al. 2017). Histone deacetylases regulate the functionality of various cytoplasmic proteins and transcription factors including tumour protein 53 (p53), RUNX3, signal transduction and activation of transcription 3 (STAT3), β-catenin, oestrogen receptor, avian myelocytomatosis viral oncogene homologue (Myc), erythroid Kruppel-like factor (EKLF), GATA family (GATA-binding factors), HIF-1alpha (hypoxia-inducible factor 1alpha), myogenic regulatory factor (MyoD), NF-κB, forkhead box P3 protein (Foxp3), E2F, GATA1, Bcl-6, HMG, HSP90, tubulin, ibortine, nuclear hormone receptors, and β-vaccine (Eckschlager et al. 2017; Garmpi et al. 2018).

DNA methylation of the CpG dinucleotides in a gene's promoter region prevents the binding of transcription factors resulting in reduced expression of genes situated near and far from the methylation site of a chromosome (de Reave and Vanderkerken 2005). Genomic instability in MM is caused by both hypomethylation and hypermethylation of intergenic regions, associated CpG islands of tumour suppressor genes, and microRNAs (Wang et al. 2017). Several cancer related genes such as cell-cycle regulators (p15, p16, and p18, p53, p73), tissue inhibitor of metalloproteinase 3 (TIMP3), E-cadherin death-associated protein kinase (DAPK), suppressor of cytokine signalling (SOCS)-1, the oestrogen receptor and O^6-methylguanine-DNA methyltransferase (MGMT), and cyclin-dependent kinase inhibitors (CDKN2A, CDKN2C) are all affected by the methylation process (de Reave and Vanderkerken 2005; de Smedt et al. 2018). Patients with p16 methylation were shown to have inferior clinical outcomes compared to those whose p16 is unmethylated (de Reave and Vanderkerken 2005).

12 Multiple Myeloma Treatment Strategies

Since the 1960s, there has been an improvement in the strategy of managing MM (Kristinsson et al. 2007). Current strategies include radiation therapy, stem cell transplantation, cancer vaccines, and several compounds with distinctive mechanisms of action (Tai et al. 2018). In the subsequent sections, we discuss these methods in more detail.

13 Alkylating Agents

Alkylating agents are compounds that react with the nucleophilic moieties of DNA molecules, resulting in the covalent transfer of an alkyl group; this causes the cross-linking of DNA strands, abnormal base pairing, or DNA strand breakage, and therefore the inhibition of cell division (Chiorcea-Paquim and Oliveira-Brett 2023; Cucchiara et al. 2022). Before the introduction of the alkylating agent, melphalan, in the 1960s, MM patients could not survive more than a year (Kumar et al. 2008). Melphalan increased the median OS to a year or two (Kristinsson et al. 2007). Treatment with melphalan induces cell death in highly proliferative cells by adding an alkyl group to DNA, causing strand linking and inhibiting DNA and RNA synthesis (Schjesvold and Oriol 2021). Melphalan has since been used in combination with prednisone or for mobilisation of autologous stem cells before collection and transplantation (Schjesvold and Oriol 2021). However, melphalan treatment is associated with prolonged recovery time, morbidity, and increased mutational load at relapse and was followed by the introduction of cyclophosphamide, which has less myelotoxic effects (Medical Research Council 1971; Schjesvold and Oriol 2021). Treatment with cyclophosphamide is associated with high stem cell count irrespective of dosage; therefore, it is suitable for frail patients or those with impaired renal function (Zannetti et al. 2021).

14 Glucocorticoids

Glucocorticoids are steroid hormones known to bind to cytosolic glucocorticoid receptors (GRs), causing it to translocate to the nucleus to modulate gene expression of genes such annexin I, MAPK phosphatase 1, nuclear factor κB, and activator protein-1 (AP-1), leading to the promotion of MM anti-inflammatory and immunosuppressive activities (Burwick and Sharma 2019). Prednisone was first used in combination with melphalan, providing proof that appropriate drug cocktails make are effective in for MM treatment (Costa et al. 1973). Currently, dexamethasone is used in combination with the immunomodulatory agent lenalidomide (Holstein and McCarthy 2017). Dexamethasone is used in combination with other drugs because it has been shown to have haemato-protective activity, prevents chemotherapy-induced toxicity, and has also been shown to have antiangiogenic effects, which helps to inhibit tumour growth (Gong et al. 2020). Glucocorticoids have been shown to improve the severity of immune effector cell-associated neurotoxicity syndrome (ICANS) and cytokine release syndrome (CRS) caused by CAR T cell immunotherapy (Wang et al. 2022).

15 Antitumour Antibiotics (Anthracyclines)

Anthracyclines are antitumour antibiotics isolated from *Streptomyces peucetius* var. *caesius* (Sritharan and Sivalingam 2021). Doxorubicin (dox, Adriamycin) is a water-soluble anthracycline approved for MM treatment and its mechanism of action is through the inhibition of DNA, RNA protein synthesis (Plosker et al. 2008). The damage to proteins, lipids, and DNA is induced by the oxygen-derived free radicals produced by dox by using iron as a cofactor and the mitochondrial respiratory chain and they also act by DNA intercalation and through topoisomerase II poisoning (Martins-Teixeira and Carvalho 2020; Smith et al. 2010). In addition, dox also induces the increase in intracellular C6 ceramide levels that in turn aids the activation of AMP-activated protein kinase (AMPK), inhibition of mTORC1, chemo-sensitisation, and induction of cancer cell death (Sritharan and Sivalingam 2021). However, dox is mostly associated with adverse events, the prominent one being cardiotoxicity (Duggan and Keating 2011).

16 Protease Inhibitors

The ubiquitin proteasome pathway (UPP) is key in maintaining the normal cellular homeostasis in eukaryotic cells through the degradation of proteins that control cell cycle, apoptosis, and DNA repair by eliminating dysfunctional or misfolded proteins via proteasome (Moreau et al. 2012). There is a high level of proteasome activity in MM cells as compared to normal cells, thereby making proteasome inhibition a crucial therapeutic strategy (Moreau et al. 2012). The introduction of bortezomib in the early 2000s improved the outcomes of MM patients (Kumar et al. 2008). Bortezomib inhibits the 26S proteasome directly by inhibiting the degradation of IκBα, an inhibitory protein that is constitutively bound to cytosolic NF-κB, thereby inhibiting the nuclear translocation and activation of NF-κB (Yang and Lin 2015). Bortezomib's effects include the disruption of MM cells and BMSCs adhesion via IL-6 activated inhibition (Yang and Lin 2015). However, bortezomib causes peripheral neuropathy, and only a small proportion of MM patients benefit from its use (An et al. 2015). Carfilzomib was FDA approved in July 2012 to be a second-generation protease inhibitor for the treatment of MM patients who have relapsed or are refractory, particularly those who received prior bortezomib and thalidomide/lenalidomide treatment (Yang and Lin 2015). In addition, carfilzomib effects are irreversible, more selective, and have no off-target activity (Bai and Su 2021). Most importantly, carfilzomib causes limited neurotoxicity (Moreau et al. 2012). Ixazomib was introduced as the first oral protease inhibitor with reversible effects but more efficient for proteasome inhibition in MM than bortezomib (Bai and Su 2021). In addition, ixazomib has a shorter dissociation half-life, and it was shown to penetrate the cancerous tissue more efficiently (Moreau et al. 2012).

17 Immunomodulatory Agents

Immunomodulatory drugs have a pleiotropy of properties against MM, including the ability to modulate host immune responses; influence cytokine secretion, angiogenesis, and induce inflammation (Sedlarikova et al. 2012). In 1999, thalidomide was introduced as the first immunomodulatory agent for MM treatment, and it exhibited improved response rate and progression-free survival of patients when used in combination with melphalan-prednisone therapy (Kumar et al. 2008). Thalidomide is a synthetic derivative of glutamic acid comprised of a chiral centre and functional (S)- and (R)- optical isomers with teratogenic and sedative effects, respectively (Sedlarikova et al. 2012). Thalidomide targets MM cells in the BM microenvironment by inhibiting TNF-α production and angiogenesis by blocking the angiogenic growth factors bFGF and VEGF (Yang and Lin 2015). Lenalidomide was FDA approved in 2006 as a secondary generation immunomodulatory agent for the treatment of relapsed/refractory MM, and it was further approved for use in combination with dexamethasone for the treatment of newly diagnosed MM patients (Holstein and McCarthy 2017). Pomalidomide was added in 2013 for the treatment of relapsed/refractory myeloma in for patients who had received at least two prior regimens (Holstein and McCarthy 2017).

18 Monoclonal Antibodies

Monoclonal antibodies exert their action by binding to molecules expressed on MM cells and subsequently blocking the BM-MM cell interaction, producing a durable and less toxic response (Wudhikarn et al. 2020). The cluster of differentiation 38 (CD38) and signalling lymphocyte activation molecule family member 7 (SLAMF7) molecules are highly expressed on the surface of MM plasma cells, making them suitable for the development of cell surface targeted therapy (Romano et al. 2021). Daratumumab monoclonal antibody for MM treatment was approved in the United States by the FDA and by the European Medicines Agency (EMA) for Europe in 2015 and 2016, respectively (Federico et al. 2021). Daratumumab targets CD38 and induces an antitumour effect via several mechanisms, which include complement-dependent cytotoxicity, antibody-dependent cell-mediated cytotoxicity, and antibody-dependent cellular phagocytosis, through the activation of complement proteins, NK cells, and macrophages, respectively (Federico et al. 2021). Isatuximab is another CD38 monoclonal antibody targeting CD38 that is used together with pomalidomide and dexamethasone as drug combinations, for the MM patient treatments after at least two line of treatments (Romano et al. 2021). The advantages of isatuximab are that it is more epitope specific and can also induce direct apoptosis without cross-linking (Wudhikarn et al. 2020). Elotuzumab, on the other hand, targets SLAMF7 and exerts the antimyeloma activity primarily via NK-mediated antibody-dependent cellular cytotoxicity through both direct activation and engagement of NK cells (Wudhikarn et al. 2020).

19 Histone Deacetylase Inhibitors

Epigenetic regulation of gene expression is crucial for normal cell function. In cancer, there are aberrations in histone deacetylases (HDACs) (Garmpi et al. 2018), making them important therapeutic targets (Hideshima et al. 2011). Histone deacetylase inhibitors (HDACIs) reverse the effects of HDACS by inducing cell-cycle arrest, differentiation, and death, as well as reducing angiogenesis and modulating immune responses (Garmpi et al. 2018). For MM treatment, HDACIs such as panobinostat and vorinostat were shown to inhibit cell growth and induce apoptosis when used alone and in combination with bortezomib by interfering with protein degradation and preventing MM cells from interacting with the tumour microenvironment (Hideshima et al. 2011). The disadvantage of using HDACIs is that HDACs could contribute to cancer through other mechanisms other than overexpression, thus may not be effective (Garmpi et al. 2018).

20 Stem Cell Transplantation

Stem cell transplantation after treatment with a high-dose-melphalan was first reported in 1996 (Kumar et al. 2008). The transplantation of stem cells helps in salvaging the patient after the increase of melphalan dose (Child et al. 2003). Patients receiving high-dose-melphalan plus stem cell transplantations have a good prognosis compared to those on standard therapy, though they are still at risk of infection due to the high doses of melphalan administered (Child et al. 2003). Double transplantation improved OS relapse-free survival and event-free survival values compared to single transplantation (Attal et al. 2003). However, not everyone can benefit from stem cell transplantation, since eligibility can be affected by the age and performance status of the patient (Attal et al. 2003). Another therapeutic strategy that affects autologous stem cell collection is radiotherapy (Damron et al. 2021). Radiotherapy in MM is used as a curative treatment for plasmacytomas and as palliation for local symptoms due to certain bone or extramedullary lesions (Momm et al. 2020).

21 Cancer Vaccines

Since BM-MM interactions create an immunosuppressive microenvironment, the cancer vaccine treatment approach aims to stimulate the endogenous antimyeloma T cell responses by introducing MM antigens (Garfall and Stadtmauer 2016). Because MM resides primarily in the BM, where it is difficult to traffic cellular therapies to their targets, dendritic cells (DCs) augmented by differentiation/activation ex vivo before infusion for antigen presentation to elicit a cytotoxic T lymphocyte response are utilised (Garfall and Stadtmauer 2016). Various MM antigens that can effectively induce an immune response have been explored, and the importance of selecting suitable antigens for this purpose was observed by Lu et al. (2017). The study investigated whether multi-epitome peptide comprised of the MM special protein (MMSA-1) and Dickkopf-1 (DKK1) could induce an antitumour immune response (Lu et al. 2017). The MMSA-1 protein is predominantly distributed on cell membranes and to a lesser extent in the cytoplasm, while DKK1 is a key regulator of myeloma bone via the Wnt signalling pathway. The vaccine significantly alleviated bone destruction in MM and increased the frequencies of $CD4^+$ and $CD8^+$ T cells in the blood of mice, indicating that this vaccine elicits MM antitumour immune response (Lu et al. 2017). However, this process of dendritic cell generation would be time-consuming and expensive. Moreover, good manufacturing practice protocols and quality assurance measures of ex vivo DCs are yet to be established (Verheye et al. 2022).

22 CAR T Cell Therapy

The utilisation of the patient's own immune system is one recent method for treating cancer cells. Examples of these techniques include utilising bispecific T cell-engaging antibodies, chimeric antigen receptor (CAR) T or CAR NK cells, or T cell or macrophage checkpoint inhibitors (Daver 2020). The CAR T cells are being investigated for the treatment of MM because they have demonstrated promise in treating haematological malignancies (Sidana and Shah 2019), and they have since been FDA approved in 2017 for cancer treatment (Panch et al. 2019).

Normally, the patient's peripheral blood mononuclear cells obtained by leukapheresis are used to generate CAR T cells, starting with T cells selection, followed by stimulation, genetic modification, and expansion for infusion into the patient (Panch et al. 2019). CAR T cell therapy targets markers expressed on cancer cells, and for MM treatment, investigations have been directed on targeting BCMA, CD138, CD38, CD19, signalling lymphocyte activation molecule (SLAM) or CS1, κ light chain, GPRC5D, and NKG2D (Sidana and Shah 2019).

The limitation to the use of autologous peripheral blood mononuclear cells is because the patient's age, prior treatment regimens, and the degree of the disease can all have an impact on the quality of patient-derived T cells (Sommer et al. 2019). It might be advisable to isolate the T cells earlier before relapse (Battram et al. 2022). Nevertheless, there are still logistics problems in manufacturing and storing the CAR T cells (Panch et al. 2019; Sommer et al. 2019). In addition, individuals receiving CAR T cell immunotherapy experience side effects such as CRS, ICANS, cytopenias, tumour lysis syndrome, hypogammaglobulinaemia, hepatotoxicity, anaemia, and infections (Javed et al. 2020; Kambhampati et al. 2022).

23 Precision Medicine Approach: The Role of High-Throughput Screening

Multiple myeloma patients exhibit diverse cytogenetic abnormalities and distinct clinical and pathological characteristics, considering that clonal heterogeneity and evolution hints towards a precision-based approach to treatment. Precision medicine is an approach where a patient's clinical and molecular characteristics can be used to determine treatment and management of a disease. Precision-based approaches can ensure that effective drugs and drug combinations can be identified for a patient (Nweke and Thimiri Govinda Raj 2021). Over recent years, there are growing interests in the evaluation of drugs that could be effective for patients using screening platforms.

High-throughput screening (HTS) is a procedure of assaying many drug candidates using miniaturised in vitro assays capable of identifying compounds that modulate biological targets of interest (Armstrong 1999; Pereira and Williams 2007). The HTS technique can be performed in 96-well, 384-well, or 1,536-well microplates, thereby, increasing the turnover and minimising the cost. Additionally, the HTS procedure uses the common assay types, including ELISA, CellTiter-Glo® luminescent cell viability, CellTox™ green cytotoxicity assays, or PrestoBlue® viability assay (Armstrong 1999). Examples of cancer drugs discovered from HTS include tyrosine kinase targets, such as Gefitinib (Iressa; AstraZeneca), Erlotinib (Tarceva, Roche), Sorafenib (Nexavar; Bayer/Onyx Pharmaceuticals), Dasatinib (Sprycel; Bristol-Meyers Squibb), and Lapatinib (Tykerb; GlaxoSmithKline) (MacArron et al. 2011).

In HTS, MM cells are treated with various drugs at different concentrations in microplates and cell viability readouts for drug sensitivity obtainable in 24–72 h by using a variety of cell-based assays, such as CellTiter-Glo® luminescent cell viability assay or CellTox™ green cytotoxicity assay or PrestoBlue® viability assay (Fig. 1) (Meurice et al. 2017; Thimiri Govinda Raj et al. 2018a, b). Drug sensitivity for each patient sample can therefore be calculated by using the drug sensitivity score (DSS) method of calculation, an improved "Area Under the Curve (AUC)". The drug sensitivity score integrates multiple dose-response parameters for each drug to obtain drug efficacy and response data across patients' samples, using the IC_{50} value (half maximal inhibitory concentration), slope, and AUC (Majumder et al. 2017). A selective DSS (sDSS) can then be created for each drug by comparing the DSS values of MM patients to healthy controls (Thimiri Govinda Raj et al. 2018a, b).

Thus, with HTS, more drugs can be tested against MM patient samples, consequently circumventing the need to carry out randomisation studies. A HTS platform can be used to optimise treatment options for multiple myeloma patients. Multiple myeloma patients can therefore be clustered based on their varied drug sensitivities.

To obtain optimal treatment options for individual patients, Majumder et al. (2017) conducted a drug sensitivity and resistance study using 50 MM patient samples expressing $CD138^+$ against 308 drugs at the clinically relevant range of 1-10,000 nM in 384-well drug plates. The selected drugs were the approved oncology

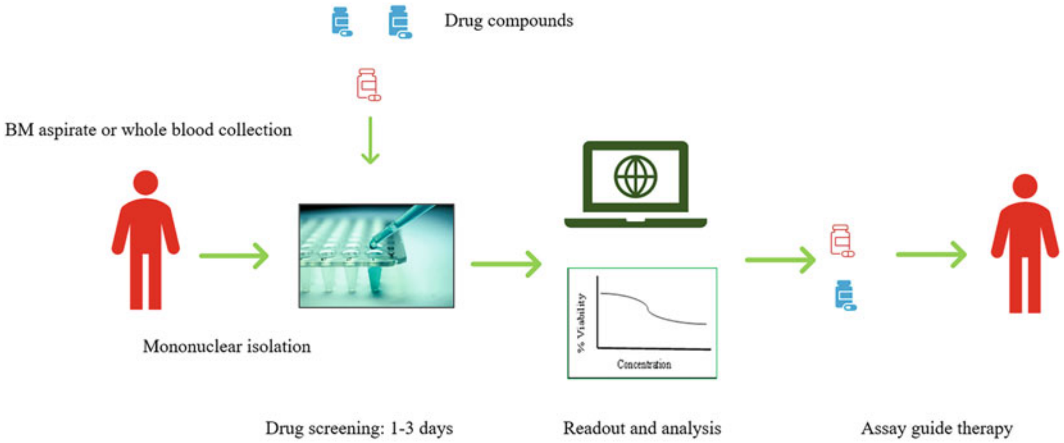

Fig. 1 Workflow for precision-based high-throughput screening platform. Patient samples are collected either from the bone marrow or whole blood. Mononuclear cells are then isolated by gradient separation and cultured for 1–3 days on precoated drug compounds microplates. After 1–3 days, cell viability readouts are obtained using CellTiter-Glo® or PrestoBlue® viability assay and results analysed to get drug sensitivities. Potent drugs are then recommended for treatment

drugs and novel agents meant to target multiple signalling pathways, including protease inhibitors, IMIDs, glucocorticoids, HDAC inhibitors, BCL2 inhibitors, PI3k-AKT-mTOR inhibitors, IGF1R inhibitors, MEK/ERK inhibitors, HSP90 inhibitors, CDK inhibitors, rapalogs, and others (Majumder et al. 2017). This allowed the authors to group each patient's samples into four (I-IV) groups based on drug sensitivity scores. Patient samples in group I ($n = 16$) had massive sensitivities, followed by group II ($n = 13$), which had moderate sensitivities, and those in group III ($n = 18$) were insensitive to several drugs, and the fourth group (IV) samples ($n = 3$) were insensitive to almost all the drugs (Majumder et al. 2017).

The authors also investigated the correlation between drug response profiles and karyotypes. Most patients with intermediate-risk t(4;14) cytogenic abnormality were in group II, and to a less extent in group III. Whereas those with the high-risk del (17p) cytogenic abnormality were predominant in groups III and IV (Majumder et al. 2017). The results show the heterogeneity of MM patient samples, and that prior drug sensitivity is required before subjecting the patients to a particular drug regimen.

One study sought to establish the feasibility of HTS as a precision medicine tool for informing treatment decisions in real time. A total of 177 compounds, including both FDA-approved and investigational drug compounds, were screened on 16 patient samples taken from MM patients, who had, on average, received six lines of prior medication. The median turnaround time to get drug screening results was 5 days. The assay-guided therapy was administered in 13 of the 16 patients and attained a 46% overall response rate (Coffey et al. 2021).

High-throughput screening platforms can also be beneficial in identifying effective synergistic drugs for combinatorial treatment. A recent study used a HTS platform to first identify a potent drug from single drug treatments and used it as a priming drug for a double- or triple-drug combination treatment screening. Carfilzomib, bortezomib, ixazomib, and panobinostat were found to be the most potent in single treatments, and in double combination treatments bortezomib-dexamethasone, panobinostat-melflufen, and carfilzomib-dexamethasone were more potent. Efficacy was widespread in triple-drug combination treatments bortezomib-dexamethasone-lenalidomide, bortezomib-dexamethasone-pomalidomide, dexamethasone-bortezomib-selinexor, bortezomib-prednisolone-melphalan, and carfilzomib-dexamethasone-panobinostat were more potent (Giliberto et al. 2022).

24 Future Perspectives and Conclusion

Over the years, the different treatment strategies discussed in this article have improved outcomes for MM patients. A study found that relative survival ratios have increased steadily from 1973 to 2003 (Kristinsson et al. 2007). There has also been an uptake in the utilisation of double- and triple-drug combinations, which have slightly improved patient outcomes. The use of proteasome inhibitors and immunomodulatory drugs supported by stem cell transplantation can enable improved leukemia treatment (Roussel et al. 2014). Taken together, these strategies still fail to significantly improve patient outcomes and quality of life. Some of the treated patients do not respond well, eventually develop resistance and the drugs themselves could be toxic. One major reason for the lack of favourable response to treatment is heterogeneity characteristic of MM. In this regard, a precision-based approach using platforms such as HTS could help in identifying optimal drug combinations.

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References

An G, Acharya C, Deng S, Yi S, Xu Y, Qin X, Sui W, Li Z, Shi L, Zang M, Feng X, Hao M, Zou D, Zhao Y, Qi J, Cheng T, Ru K, Wang J, Tai YT, Qiu L (2015) Cytogenetic and clinical marks for defining high-risk myeloma in the context of bortezomib treatment. Exp Hematol 43(3):168–176.e2. https://doi.org/10.1016/j.exphem.2014.11.004

Andrews SW, Kabrah S, May JE, Donaldson C, Morse HR (2013) Multiple myeloma: the bone marrow microenvironment and its relation to treatment. Br J Biomed Sci 70(3):110–120. Step Publishing Ltd. https://doi.org/10.1080/09674845.2013.11669945

Armstrong JW (1999) A review of high-throughput screening approaches for drug discovery. Am Biotechnol Lab 17:26–28

Attal M, Harousseau J-L, Facon T, Guilhot F, Doyen C, Fuzibet J-G, Monconduit M, Hulin C, Caillot D, Bouabdallah R, Voillat L, Sotto J-J, Grosbois B, Bataille R (2003) Single versus double autologous stem-cell transplantation for multiple myeloma. N Engl J Med 25(26):2495–2502. www.nejm.org

Bai Y, Su X (2021) Updates to the drug-resistant mechanism of proteasome inhibitors in multiple myeloma. Asia-Pacific J Clin Oncol 17(1):29–35. Blackwell Publishing Ltd. https://doi.org/10.1111/ajco.13459

Battram AM, Oliver-Caldés A, Suárez-Lledó M, Lozano M, Bosch i Crespo M, Martínez-Cibrián N, Cid J, Moreno DF, Rodríguez-Lobato LG, Urbano-Ispizua A, Fernández de Larrea C (2022) T cells isolated from G-CSF-treated multiple myeloma patients are suitable for the generation of BCMA-directed CAR-T cells. Mol Ther Methods Clin Dev. https://doi.org/10.1016/j.omtm.2022.06.010

Bianchi G, Munshi NC (2015) Pathogenesis beyond the cancer clone(s) in multiple myeloma. Blood 125(20):3049–3058. https://doi.org/10.1182/blood-2014-11

Bladé J, Teresa Cibeira M, Rosiñol L (2010) Novel drugs for the treatment of multiple myeloma. Haematologica 95(5):702–704. https://doi.org/10.3324/haematol.2009.021550

Bolkun L, Lemancewicz D, Jablonska E, Kulczynska A, Bolkun-Skornicka U, Kloczko J, Dzieciol J (2014) BAFF and APRIL as TNF superfamily molecules and angiogenesis parallel progression of human multiple myeloma. Ann Hematol 93(4):635–644. https://doi.org/10.1007/s00277-013-1924-9

Burwick N, Sharma S (2019) Glucocorticoids in multiple myeloma: past, present, and future. Ann Hematol 98(1):19–28. Springer Verlag. https://doi.org/10.1007/s00277-018-3465-8

Catamero D (2018) Multiple myeloma: detecting genetic changes through bone marrow biopsy and the influence on care. Clin J Oncol Nurs 22(3):263–265. https://doi.org/10.1188/18.CJON.263-265

Chai KY, Boyke Berahmana AA, Morison IM (2019) Haematologists usually over-estimate the percentage of CD138+ plasma cells in marrow biopsies. Pathology 51(6):647–648). Elsevier B.V. https://doi.org/10.1016/j.pathol.2019.05.010

Chapman MA, Lawrence MS, Keats JJ, Cibulskis K, Sougnez C, Schinzel AC, Harview CL, Brunet JP, Ahmann GJ, Adli M, Anderson KC, Ardlie KG, Auclair D, Baker A, Bergsagel PL, Bernstein BE, Drier Y, Fonseca R, Gabriel SB et al (2011) Initial genome sequencing and analysis of multiple myeloma. Nature 471(7339):467–472. https://doi.org/10.1038/nature09837

Child JA, Morgan GJ, Davies FE, Owen RG, Bell SE, Phil D, Hawkins K, Brown J, Drayson MT, Selby PJ (2003) High-dose chemotherapy with hematopoietic stem-cell Rescue for multiple myeloma. N Engl J Med 348(19):1875–1883. www.nejm.org

Chiorcea-Paquim AM, Oliveira-Brett AM (2023) Electrochemistry of chemotherapeutic alkylating agents and their interaction with DNA. J Pharm Biomed Anal 222:1–17). Elsevier B.V. https://doi.org/10.1016/j.jpba.2022.115036

Coffey DG, Cowan AJ, DeGraaff B, Martins TJ, Curley N, Green DJ, Libby EN, Silbermann R, Chien S, Dai J, Morales A, Gooley TA, Warren EH, Becker PS (2021) High-throughput drug screening and multi-Omic analysis to guide individualized treatment for multiple myeloma. JCO Precis Oncol 5:602–612. https://doi.org/10.1200/po.20.00442

Corre J, Munshi N, Hervé H, Avet-Loiseau H (2015) Genetics of multiple myeloma: another heterogeneity level? Blood 125(12):1870–1876. https://doi.org/10.1182/blood-2014-10

Costa G, Ralph Engle VL, Schilling A, Massachusetts Paul Carbone B, Maryland B, Va W, Harley JB, Krall JM, Ramanan SV, Hospital U, Carbone P, Serpick A, Shadduck R, Feldstein A (1973) Melphalan and prednisone: an effective combination for the treatment of multiple myeloma. Am J Med 54(5):589–599

Cucchiara F, Ferraro S, Luci G, Bocci G (2022) Relevant pharmacological interactions between alkylating agents and antiepileptic drugs: preclinical and clinical data. Pharmacol Res 175:105976

Damron EP, Qazilbash M, Fang P, Wu SY, Dabaja B, Rondon G, Hosing C, Champlin R, Bashir Q, Shpall EJ, Knafl M, Lee H, Manasanch EE, Patel K, Kaufman G, Thomas S, Orlowski R, Weber D, Pinnix CC, Gunther JR (2021) Radiation therapy can be safely incorporated into pre-transplant treatment regimens for patients with multiple myeloma. Int J Radiat Oncol Biol Phys 111(3):S81. https://doi.org/10.1016/j.ijrobp.2021.07.195

Daver N (2020) A bispecific approach to improving CAR T cells in AML. Blood 135(10):703–704). Elsevier B.V. https://doi.org/10.1182/blood.2020004791

de Reave HR, Vanderkerken K (2005) The role of the bone marrow microenviroment in multiple myeloma. Histol Histopathol 20:1227–1250

de Smedt E, Lui H, Maes K, de Veirman K, Menu E, Vanderkerken K, de Bruyne E (2018) The epigenome in multiple myeloma: impact on tumor cell plasticity and drug response. Front Oncol 8(DEC). Frontiers Media S.A. https://doi.org/10.3389/fonc.2018.00566

Diamantidis MD, Papadaki S, Hatjiharissi E (2022) Exploring the current molecular landscape and management of multiple myeloma patients with the t(11;14) translocation. Front Oncol 12. Frontiers Media S.A. https://doi.org/10.3389/fonc.2022.934008

Dinner S, Witteles W, Witteles R, Lam A, Arai S, Lafayette R, George TI, Schrier SL, Liedtke M (2013) The prognostic value of diagnosing concurrent multiple myeloma in immunoglobulin light chain amyloidosis. Br J Haematol 161(3):367–372. https://doi.org/10.1111/bjh.12269

Duggan ST, Keating GM (2011) Pegylated liposomal doxorubicin A review of its use in metastatic breast cancer, ovarian cancer, multiple myeloma and AIDS-related Kaposi's sarcoma. Drugs 71(18):2531–2558

Eckschlager T, Plch J, Stiborova M, Hrabeta J (2017) Histone deacetylase inhibitors as anticancer drugs. Int J Mol Sci 18(7). MDPI AG. https://doi.org/10.3390/ijms18071414

Federico V, Francesca F, Gianfranco L, Maria TP, Giovanni M, Claudio C (2021) Monoclonal antibodies in multiple myeloma. Panminerva Medica 63(1): 21–27. https://doi.org/10.23736/S0031-0808.20.04149-X

Garfall AL, Stadtmauer EA (2016) Cellular and vaccine immunotherapy for multiple myeloma. Hematology Am Soc Hematol Educ Program 1:521–527

Garmpi A, Garmpis N, Damaskos C, Valsami S, Spartalis E, Lavaris A, Patelis N, Margonis G-A, Apostolou KG, Spartalis M, Andreatos N, Diamantis E, Tsivelekas K, Moschos MM, Nonni A, Tsourouflis G, Markatos K, Antoniou EA, Kontzoglou K et al (2018) Histone deacetylase inhibitors as a new anticancer option: how far can we go with expectations? JBUON 23(4):846–861

Giannakoulas N, Ntanasis-Stathopoulos I, Terpos E (2021) The role of marrow microenvironment in the growth and development of malignant plasma cells in multiple myeloma. Int J Mol Sci 22(9). MDPI AG. https://doi.org/10.3390/ijms22094462

Giesen N, Paramasivam N, Toprak UH, Huebschmann D, Xu J, Uhrig S, Samur M, Bähr S, Fröhlich M, Mughal SS, Mai EK, Jauch A, Müller-Tidow C, Brors B, Munshi N, Goldschmidt H, Weinhold N, Schlesner M, Raab MS (2022) Comprehensive genomic analysis of refractory multiple myeloma reveals a complex mutational landscape associated with drug resistance and novel therapeutic vulnerabilities. Haematologica 107(8):1891–1901. https://doi.org/10.3324/haematol.2021.279360

Giliberto M, Thimiri Govinda Raj DB, Cremaschi A, Skånland SS, Gade A, Tjønnfjord GE, Schjesvold F, Munthe LA, Taskén K (2022) Ex vivo drug sensitivity screening in multiple myeloma identifies drug combinations that act synergistically. Mol Oncol 16(6):1241–1258. https://doi.org/10.1002/1878-0261.13191

Giri S, Huntington SF, Wang R, Zeidan AM, Podoltsev N, Gore SD, Ma X, Gross CP, Davidoff AJ, Neparidze N (2020) Chromosome 1 abnormalities and survival of patients with multiple myeloma in the era of novel agents. Blood Adv 4(10):2245–2253. https://doi.org/10.1182/bloodadvances.2019001425

Gong JH, Zheng YB, Zhang MR, Wang YX, Yang SQ, Wang RH, Miao QF, Liu XJ, Zhen YS (2020) Dexamethasone enhances the antitumor efficacy of gemcitabine by glucocorticoid receptor signaling. Cancer Biol Ther 21(4):332–343. https://doi.org/10.1080/15384047.2019.1702399

Greenberg AJ, Rajkumar S v, Therneau TM, Singh PP, Dispenzieri A, Kumar SK (2014) Relationship between initial clinical presentation and the molecular cytogenetic classification of myeloma. Leukemia 28(2):398–403. https://doi.org/10.1038/leu.2013.258

Gregory WM, Richards MA, Malpas JS (1992) Combination chemotherapy versus melphalan and prednisolone in the treatment of multiple myeloma: an overview of published trials. J Clin Oncol 10(2):334–342. https://doi.org/10.1200/JCO.1992.10.2.334

Gulla A, Anderson KC (2020) Multiple myeloma: the (r)evolution of current therapy and a glance into the future. Haematologica 105(10):2358–2367. https://doi.org/10.3324/haematol.2020.247015

Hiasa M, Harada T, Tanaka E, Abe M (2021) Pathogenesis and treatment of multiple myeloma bone disease. Jpn Dent Sci Rev 57:164–173). Elsevier Ltd. https://doi.org/10.1016/j.jdsr.2021.08.006

Hideshima T, Richardson PG, Anderson KC (2011) Mechanism of action of proteasome inhibitors and deacetylase inhibitors and the biological basis of synergy in multiple myeloma. Mol Cancer Therap 10(11):2034–2042. https://doi.org/10.1158/1535-7163.MCT-11-0433

Ho M, Goh CY, Patel A, Staunton S, O'Connor R, Godeau M, Bianchi G (2020) Role of the bone marrow milieu in multiple myeloma progression and therapeutic resistance. Clin Lymphoma Myeloma Leuk 20(10): e752–e768). Elsevier Inc. https://doi.org/10.1016/j.clml.2020.05.026

Holstein SA, McCarthy PL (2017) Immunomodulatory drugs in multiple myeloma: mechanisms of action and clinical experience. Drugs 77(5):505–520). Springer International Publishing. https://doi.org/10.1007/s40265-017-0689-1

Hou J, Wei R, Qian J, Wang R, Fan Z, Gu C, Yang Y (2019) The impact of the bone marrow microenvironment on multiple myeloma (Review). Oncol Rep 42(4):1272–1282). Spandidos Publications. https://doi.org/10.3892/or.2019.7261

Jasrotia S, Gupta R, Halder A, Kumar L (2020) Cytokine profile in multiple myeloma. Cytokine 136

Javed S, Syed TIS, Inam SHA, Abdullah SM, Najmuddin MM, Haider MZ, Fatima H, Neupane K, Sana MK, Tayyeb M, Dar AJ, Mirza MA, Anwer F (2020) Safety and adverse reactions of CAR T therapy for the treatment of multiple myeloma: a systematic review. Blood 136(Supplement 1):6–7. https://doi.org/10.1182/blood-2020-143045

Joshi R, Horncastle D, Elderfield K, Lampert I, Rahemtulla A, Naresh KN (2008) Bone marrow trephine combined with immunohistochemistry is superior to bone marrow aspirate in follow-up of myeloma patients. J Clin Pathol 61(2):213–216. https://doi.org/10.1136/jcp.2007.049130

Kambhampati S, Sheng Y, Huang CY, Bylsma S, Lo M, Kennedy V, Natsuhara K, Martin T, Wolf J, Shah N, Wong SW (2022) Infectious complications in patients with relapsed refractory multiple myeloma after BCMA CAR T-cell therapy. Blood Adv 6(7): 2045–2054. https://doi.org/10.1182/bloodadvances.2020004079

Kim J, Denu RA, Dollar BA, Escalante LE, Kuether JP, Callander NS, Asimakopoulos F, Hematti P (2012) Macrophages and mesenchymal stromal cells support survival and proliferation of multiple myeloma cells. Br J Haematol 158(3):336–346. https://doi.org/10.1111/j.1365-2141.2012.09154.x

Kristinsson SY, Landgren O, Dickman PW, Derolf ÅR, Björkholm M (2007) Patterns of survival in multiple myeloma: a population-based study of patients diagnosed in Sweden from 1973 to 2003. J Clin Oncol 25(15):1993–1999. https://doi.org/10.1200/JCO.2006.09.0100

Kumar SK, Rajkumar SV, Dispenzieri A, Lacy MQ, Hayman SR, Buadi FK, Zeldenrust SR, Dingli D, Russell SJ, Lust JA, Greipp PR, Kyle RA, Gertz MA (2008) Improved survival in multiple myeloma and the impact of novel therapies. Blood 111(5):2516–2520. https://doi.org/10.1182/blood-2007-10

Kyle RA, Child JA, Anderson K, Barlogie B, Bataille R, Bensinger W, Bladé J, Boccadoro M, Dalton W, Dimopoulos M, Djulbegovic B, Drayson M, Durie B, Facon T, Fonseca R, Gahrton G, Greipp P, Harousseau JL, Harrington D et al (2003) Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the International Myeloma Working Group. Br J Haematol 121(5):749–757. https://doi.org/10.1046/j.1365-2141.2003.04355.x

Lam C, Ferguson ID, Mariano MC, Lin YHT, Murnane M, Liu H, Smith GA, Wong SW, Taunton J, Liu JO, Mitsiades CS, Hann BC, Aftab BT, Wiita AP (2018) Repurposing tofacitinib as an anti-myeloma therapeutic to reverse growth-promoting effects of the bone marrow microenvironment. Haematologica 103(7): 1218–1228

Landgren O, Kyle RA, Pfeiffer RM, Katzmann JA, Caporaso NE, Hayes RB, Dispenzieri A, Kumar S, Clark RJ, Baris D, Hoover R, Rajkumar SV (2009) Monoclonal gammopathy of undetermined significance (MGUS) consistently precedes multiple myeloma: a prospective study. Blood 113(22):5412–5417. https://doi.org/10.1182/blood-2008-12-194241

Laudin GE, Levay PF, Coetzer B (2020) Globulin fraction and albumin: globulin ratio as a predictor of mortality in a South African multiple myeloma cohort. Int J Hematol Oncol 9(3):IJH27. https://doi.org/10.2217/ijh-2020-0003

Lemancewicz D, Bolkun L, Jablonska E, Kulczynska A, Bolkun-Skorncka U, Kloczko J, Dzieciol J (2013) The evaluation of TNF superfamily molecules in multiple myeloma patients: correlation with biological and clinical features. Leuk Res 37(9):1089–1093

Liu J, Li JN, Wu H, Liu P (2022) The status and prospects of epigenetics in the treatment of lymphoma. Front Oncol 12. Frontiers Media S.A. https://doi.org/10.3389/fonc.2022.874645

López-Corral L, Gutiérrez NC, Vidriales MB, Mateos MV, Rasillo A, García-Sanz R, Paiva B, San Miguel JF (2011) The progression from MGUS to smoldering myeloma and eventually to multiple myeloma involves a clonal expansion of genetically abnormal plasma cells. Clin Cancer Res 17(7):1692–1700. https://doi.org/10.1158/1078-0432.CCR-10-1066

Lu C, Meng S, Jin Y, Zhang W, Li Z, Wang F, Wang-Johanning F, Wei Y, Liu H, Tu H, Su D, He A, Cao X, Zhou F (2017) A novel multi-epitope vaccine from MMSA-1 and DKK1 for multiple myeloma immunotherapy. Br J Haematol 178(3):413–426. https://doi.org/10.1111/bjh.14686

MacArron R, Banks MN, Bojanic D, Burns DJ, Cirovic DA, Garyantes T, Green DVS, Hertzberg RP, Janzen WP, Paslay JW, Schopfer U, Sittampalam GS (2011) Impact of high-throughput screening in biomedical research. Nat Rev Drug Discov 10:188–195. https://doi.org/10.1038/nrd3368

Majumder MM, Silvennoinen R, Anttila P, Tamborero D, Eldfors S, Yadav B, Karjalainen R, Kuusanmäki H, Lievonen J, Parsons A, Suvela M, Jantunen E, Porkka K, Heckman CA (2017) Identification of precision treatment strategies for relapsed/refractory multiple myeloma by functional drug sensitivity testing. Oncotarget 8(34):56338–56350. https://doi.org/10.18632/oncotarget.17630. PMID: 28915594; PMCID: PMC5593565

Martins-Teixeira MB, Carvalho I (2020) Antitumour anthracyclines: progress and perspectives. ChemMedChem 15(11):933–948). John Wiley and Sons Ltd. https://doi.org/10.1002/cmdc.202000131

Mateo G, Castellanos M, Rasillo A, Gutiérrez NC, Montalbán MA, Martín ML, Hernández JM, López-Berges MC, Montejano L, Bladé J, Mateos MV, Sureda A, de La Rubia J, Díaz-Mediavilla J, Pandiella A, Lahuerta JJ, Orfao A, San Miguel JF (2005) Genetic abnormalities and patterns of antigenic expression in multiple myeloma. Clin Cancer Res 11(10):3661–3667. https://doi.org/10.1158/1078-0432.CCR-04-1489

Matsue K, Matsue Y, Kumata K, Usui Y, Suehara Y, Fukumoto K, Fujisawa M, Narita K, Takeuchi M (2017) Quantification of bone marrow plasma cell infiltration in multiple myeloma: usefulness of bone marrow aspirate clot with CD138 immunohistochemistry. Hematol Oncol 35(3):323–328. https://doi.org/10.1002/hon.2300

Medical Research Council (1971) Myelomatosis: comparison of melphalan and cyclophosphamide therapy. Br Med J 1(5750):640–641

Meurice N, Petit JL, de Campos CB, Polito AN, Lopez Armenta ID, Ahmann GJ, Bruins LA, Mikhael JR, Reeder CB, Mayo AA, Bergsagel PL, Fonseca R, Sepetov N, Romanov S, Stewart AK (2017) 'Direct to drug' screening as a route to individualized therapy in multiple myeloma. Blood 130:1–3. https://doi.org/10.1182/blood.V130.Suppl_1.3080.3080

Momm F, Greil C, Schäfer H (2020) Towards individualized radiation therapy in multiple myeloma. Haematologica 105(7):1763–1764). Ferrata Storti Foundation. https://doi.org/10.3324/haematol.2019.243451

Moreau P, Richardson PG, Cavo M, Orlowski RZ, San Miguel JF, Palumbo A, Harousseau JL (2012) Proteasome inhibitors in multiple myeloma: 10 years later. Blood 120(5):947–959). American Society of Hematology. https://doi.org/10.1182/blood-2012-04-403733

Moreaux J, Legouffe E, Jourdan E, Quittet P, Rème T, Lugagne C, Moine P, Rossi JF, Klein B, Tarte K (2004) BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone. Blood 103(8):3148–3157. https://doi.org/10.1182/blood-2003-06-1984

Moser-Katz T, Joseph NS, Dhodapkar MV, Lee KP, Boise LH (2021) Game of bones: how myeloma manipulates its microenvironment. Front Oncol 10. Frontiers Media S.A. https://doi.org/10.3389/fonc.2020.625199

Nakamura K, Smyth MJ, Martinet L (2020) Cancer immunoediting and immune dysregulation in multiple myeloma. Blood 136(24):2731–2740

Nweke EE, Thimiri Govinda Raj DB (2021) Drug sensitivity and drug repurposing platform for cancer precision medicine. Cell Biol Transl Med 12:47–53

Padala SA, Barsouk A, Barsouk A, Rawla P, Vakiti A, Kolhe R, Kota V, Ajebo GH (2021) Epidemiology, staging, and management of multiple myeloma. Med Sci 9(1):3. https://doi.org/10.3390/medsci9010003

Paesler J, Gehrke I, Poll-Wolbeck SJ, Kreuzer KA (2012) Targeting the vascular endothelial growth factor in hematologic malignancies. Eur J Haematol 89(5):373–384. https://doi.org/10.1111/ejh.12009

Panch SR, Srivastava SK, Elavia N, McManus A, Liu S, Jin P, Highfill SL, Li X, Dagur P, Kochenderfer JN, Fry TJ, Mackall CL, Lee D, Shah NN, Stroncek DF (2019) Effect of cryopreservation on autologous chimeric antigen receptor T cell characteristics. Mol Ther 27(7):1275–1285. https://doi.org/10.1016/j.ymthe.2019.05.015

Parrinello N, Romano A, Conticello C, Cavalli M, la Fauci A, Rizzo G, la Cava P, Chiarenza A, Tibullo D, Giallongo C, Palumbo GA, di Raimondo F (2013) Neutrophils of multiple myeloma are dysfunctional and immunosuppressive. Blood 122(21):3138–3138. https://doi.org/10.1182/blood.v122.21.3138.3138

Patel M (1999) An epidemiological study of multiple myeloma in Southern Africa [Doctor of Philosophy]. University of the Witwatersrand

Pereira DA, Williams JA (2007) Origin and evolution of high throughput screening. Br J Pharmacol 152(1):53–61. https://doi.org/10.1038/sj.bjp.0707373

Plosker GL, Palumbo A, Reece DE (2008) ADIS drug evaluation pegylated liposomal doxorubicin A review of its use in the treatment of relapsed or refractory multiple myeloma. Drugs 68(17):2535–2551

Prabhala RH, Pelluru D, Fulciniti M, Prabhala HK, Nanjappa P, Song W, Pai C, Amin S, Tai YT, Richardson PG, Ghobrial IM, Treon SP, Daley JF, Anderson KC, Kutok JL, Munshi NC (2010) Elevated IL-17 produced by TH17 cells promotes myeloma cell growth and inhibits immune function in multiple myeloma. Blood 115(26):5385–5392. https://doi.org/10.1182/blood-2009-10-246660

Prideaux SM, Conway O'Brien E, Chevassut TJ, Chapman MA, Lawrence MS, Keats JJ, Cibulskis K, Sougnez C, Schinzel AC, Harview CL, Brunet J, Ahmann GJ, Adli M, Anderson KC, Ardlie KG, Auclair D, Baker A, Bergsagel PL, Bernstein BE et al (2014) The genetic architecture of multiple myeloma. Advances in Hematology 2014(10):1–25. https://doi.org/10.1155/2014/864058

Rankapole RK, Torres Z, Sedumedi B (2011) The occurrence of multi[ple myeloam at Dr George Mukhari Hospital, Gauteng: a retrospective review (2004–2009). [Master of Medicine (M med) in Haematology]. University of Limpopo- Medunsa Campus

Reddy A, Rapiti N, Gounden V (2021) Comparison of 24-hour versus random urine samples for determination and quantification of Bence Jones Protein in a South African population. Afr J Lab Med 10(1):1–7. https://doi.org/10.4102/ajlm

Romano A, Storti P, Marchica V, Scandura G, Notarfranchi L, Craviotto L, di Raimondo F, Giuliani N (2021) Mechanisms of action of the new antibodies in use in multiple myeloma. Front Oncol 11. Frontiers Media S.A. https://doi.org/10.3389/fonc.2021.684561

Roussel M, Lauwers-Cances V, Robillard N, Hulin C, Leleu X, Benboubker L, Marit G, Moreau P, Pegourie B, Caillot D, Fruchart C, Stoppa AM, Gentil C, Wuilleme S, Huynh A, Hebraud B, Corre J, Chretien ML, Facon T et al (2014) Front-line transplantation program with lenalidomide, bortezomib, and dexamethasone combination as induction and consolidation followed by lenalidomide maintenance in patients with multiple myeloma: a phase II study by the Intergroupe Francophone du Myélome. J Clin Oncol 32(25):2712–2717. https://doi.org/10.1200/JCO.2013.54.8164

Sawyer JR (2011) The prognostic significance of cytogenetics and molecular profiling in multiple myeloma. Cancer Genet 204(1):3–12). Elsevier Inc. https://doi.org/10.1016/j.cancergencyto.2010.11.002

Schjesvold F, Oriol A (2021) Current and novel alkylators in multiple myeloma. Cancers 13(10). MDPI AG. https://doi.org/10.3390/cancers13102465

Sedlarikova L, Kubiczkova L, Sevcikova S, Hajek R (2012) Mechanism of immunomodulatory drugs in multiple myeloma. Leuk Res 36(10):1218–1224. https://doi.org/10.1016/j.leukres.2012.05.010

Shen YJ, Mishima Y, Shi J, Sklavenitis-Pistofidis R, Redd RA, Moschetta M, Manier S, Roccaro AM, Sacco A, Tai Y-T, Mercier F, Kawano Y, Su NK, Berrios B, Doench JG, Root DE, Michor F, Scadden DT, Ghobrial IM (2021) Progression signature underlies clonal evolution and dissemination of multiple myeloma. Blood 137(17):2361–2372. https://www.r-project.org/

Sidana S, Shah N (2019) CAR T-cell therapy: is it prime time in myeloma? Blood Adv 3(21):3473–3480). American Society of Hematology. https://doi.org/10.1182/bloodadvances.2019000370

Smith LA, Cornelius VR, Plummer CJ, Levitt G, Verrill M, Canney P, Jones A (2010) Cardiotoxicity of anthracycline agents for the treatment of cancer: systematic review and meta-analysis of randomised controlled trials. BMC Cancer 10. http://www.biomedcentral.com/1471-2407/10/337

Sommer C, Boldajipour B, Kuo TC, Bentley T, Sutton J, Chen A, Geng T, Dong H, Galetto R, Valton J, Pertel T, Juillerat A, Gariboldi A, Pascua E, Brown C, Chin SM, Sai T, Ni Y, Duchateau P et al (2019) Preclinical evaluation of allogeneic CAR T cells targeting BCMA for the treatment of multiple myeloma. Mol Ther 27(6):1126–1138. https://doi.org/10.1016/j.ymthe.2019.04.001

Sritharan S, Sivalingam N (2021) A comprehensive review on time-tested anticancer drug doxorubicin. Life Sci 278. Elsevier Inc. https://doi.org/10.1016/j.lfs.2021.119527

Štifter S, Babarović E, Valković T, Seili-Bekafigo I, Štemberger C, Načinović A, Lučin K, Jonjić N (2010) Combined evaluation of bone marrow aspirate and biopsy is superior in the prognosis of multiple myeloma. Diagn Pathol 5(1). https://doi.org/10.1186/1746-1596-5-30

Tai YT, Cho SF, Anderson KC (2018) Osteoclast immunosuppressive effects in multiple myeloma: role of programmed cell death ligand 1. Front Immunol 9 (Aug). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2018.01822

The Global Cancer Observatory (2020, December) Multiple myeloma. International Agency for Research on Cancer. https://gco.iarc.fr/today/data/factsheets/cancers/35-Multiple-myeloma-fact-sheet.pdf

Thimiri Govinda Raj DB, Giliberto M, Cremaschi A, Skanland SS, Gade A, Tjønnfjord GE, Schjesvold F, Munthe LA, Tasken K (2018a) Drug sensitivity screening on multiple myeloma for precision cancer therapy. Blood 132(Supplement 1)

Thimiri Govinda Raj DB, Giliberto M, Skånland SS (2018b) Drug sensitivity screening in multiple myeloma (MM) for precision cancer therapy. https://wkhealth.force.com/lwwonline/s/topic/0TO0V000001YgLZWA0/lww-online?tabset-

van Wier S, Braggio E, Baker A, Ahmann G, Levy J, Carpten JD, Fonseca R (2013) Hypodiploid multiple myeloma is characterized by more aggressive molecular markers than non-hyperdiploid multiple myeloma. Haematologica 98(10):1586–1592. https://doi.org/10.3324/haematol.2012.081083

Verheye E, Melgar JB, Deschoemaeker S, Raes G, Maes A, de Bruyne E, Menu E, Vanderkerken K, Laoui D, de Veirman K (2022) Dendritic cell-based immunotherapy in multiple myeloma: challenges, opportunities, and future directions. Int J Mol Sci 23(2). MDPI. https://doi.org/10.3390/ijms23020904

Wang S, Wu W, Claret FX (2017) Mutual regulation of microRNAs and DNA methylation in human cancers. Epigenetics 12(3):187–197). Taylor & Francis Inc. https://doi.org/10.1080/15592294.2016.1273308

Wang X, Qi Y, Li H, Liu F, Cao J, Chen W, Wang Y, Qi K, Yan Z, Zhu F, Li Z, Cheng H, Xu K (2022) Impact of glucocorticoids on short-term and long-term outcomes in patients with relapsed/refractory multiple myeloma treated with CAR-T therapy. Front Immunol:13. https://doi.org/10.3389/fimmu.2022.943004

Wong TW, Kita H, Hanson CA, Walters DK, Arendt BK, Jelinek DF (2013) Induction of malignant plasma cell proliferation by eosinophils. PLoS One 8(7). https://doi.org/10.1371/journal.pone.0070554

Wudhikarn K, Wills B, Lesokhin AM (2020) Monoclonal antibodies in multiple myeloma: current and emerging targets and mechanisms of action. Best Pract Res Clin Haematol 33(1). Bailliere Tindall Ltd. https://doi.org/10.1016/j.beha.2020.101143

Yan Y, Qin X, Liu J, Fan H, Yan W, Liu L, Du C, Yu Z, Xu Y, Hao M, Deng S, Sui W, Lyu R, Liu W, Wang Q, Zou D, Hu L, Wang J, Tai YT et al (2022) Clonal phylogeny and evolution of critical cytogenetic aberrations in multiple myeloma at single-cell level by QM-FISH. Blood Adv 6(2):441–451. https://doi.org/10.1182/bloodadvances.2021004992

Yang WC, Lin SF (2015) Mechanisms of drug resistance in relapse and refractory multiple myeloma. BioMed Res Int 2015). Hindawi Publishing Corporation. https://doi.org/10.1155/2015/341430

Zannetti BA, Saraceni F, Cellini C, Fabbri E, Monaco F, Guarini A, Laszlo D, Martino M, Olivieri A, Imola M, Tosi P, Chiarucci M, Zuffa E, Lanza F (2021) Low-dose cyclophosphamide versus intermediate-high-dose cyclophosphamide versus granulocyte colony-stimulating factor alone for stem cell mobilization in multiple myeloma in the era of novel agents: a Multicenter Retrospective Study. Transplantat Cell Therapy 27(3):244.e1–244.e8. https://doi.org/10.1016/j.jtct.2020.12.009

Zheng Y, Cai Z, Wang S, Zhang X, Qian J, Hong S, Li H, Wang M, Yang J, Yi Q (2009) Macrophages are an abundant component of myeloma microenvironment and protect myeloma cells from chemotherapy drug-induced apoptosis. Blood 114(17):3625–3628. https://doi.org/10.1182/blood-2009-05-220285

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