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Kursad Turksen *Editor*

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Cell Biology and Translational Medicine, Volume 16

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Preface

In this next volume in the Cell Biology and Translational Medicine series, we continue to explore fundamentals of stem cell biology as well as their potential utility in regenerative medicine applications. Amongst topics explored in this volume are recent advances in understanding of the epithelial stem cell niche, identifying small molecules for stem cell expansion in vitro, and recent developments in cell therapy, in particular islet cell transplant for treatment of diabetes. A major objective of the series continues to be to highlight timely, often emerging topics and novel approaches that can accelerate realizing the utility of stem cells in regenerative medicine. Amongst the latter is the use of zebrafish as a platform to screen drugs that regulate stem cell function.

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

I would also like to acknowledge and thank Mariska van der Stigchel, Assistant Editor, for her outstanding efforts in helping to bring this volume to the production stages.

A special thank you goes to Shanthi Ramamoorthy and Rathika Ramkumar for their outstanding efforts in the production of this volume.

The rapid and rigorous sharing of knowledge remains key to achieving the potential of stem cell-mediated therapeutic options. To this end, my 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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Epithelial Stem Cells: Making, Shaping and Breaking the Niche

Paula Ferraces-Riegas, Anona C. Galbraith, and David P. Doupé

Abstract

Epithelial stem cells maintain tissues throughout adult life and are tightly regulated by their microenvironmental niche to balance cell production and loss. These stem cells have been studied extensively as signal-receiving cells, responding to cues from other cell types and mechanical stimuli that comprise the niche. However, studies from a wide range of systems have identified epithelial stem cells as major contributors to their own microenvironment either through producing niche cells, acting directly as niche cells or regulating niche cells. The importance of stem cell contributions to the niche is particularly clear in cancer, where tumour cells extensively remodel their microenvironment to promote their survival and proliferation.

Keywords

Cancer · Epithelia · Extracellular matrix · Homeostasis · Niche · Signalling · Stem cells

Abbreviations

BMP	Bone morphogenetic protein
CAF	Cancer-associated fibroblast
CSC	Cancer stem cell
DMBA	7,12-Dimethylbenz(a)anthracene
EC	Enterocyte
ECM	Extracellular matric
EE	Enteroendocrine
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
FGF	Fibroblast growth factor
ISC	Intestinal stem cell
JAK/STAT	Janus kinase/signal transducer and activator of transcription
SOS	Son of Sevenless
TA	Transit amplifying
TIC	Tumour-initiating cell
TPA	12-O-tetradecanoylphorbol-13-acetate
VEGF	Vascular endothelial growth factor

1 Introduction

Adult tissue stem cells play critical roles throughout the body in maintaining normal tissue homeostasis and responding to challenges such as damage. The stem cells must generate sufficient cells to maintain the tissue without overproliferating, which is a hallmark of cancer (Hanahan and Weinberg 2017). Stem cells are therefore

Authors Paula Ferraces-Riegas and Anona C. Galbraith have equally contributed to this chapter.

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tightly regulated by their local microenvironment or niche. As misregulation of stem cells has major implications for both normal physiology and diseases such as cancer, a great deal of research has focused on understanding the stem cell niche. Classically, the niche has been thought of in terms of signalling from adjacent cells, but more recent work has led to an expanded definition that incorporates not only classical signalling pathways but also mechanical and metabolic inputs (Scadden 2014; Chacón-Martínez et al. 2018).

Even as understanding of what constitutes the niche has broadened, most studies have focused on the cells and tissues surrounding the stem cells and their role in creating the microenvironment. In this context, stem cells are viewed primarily as the recipients of chemical and mechanical cues. However, work in a range of systems has shown that stem cells themselves contribute substantially to the niche. In this review, we will take a stem cell-centric view of the niche and explore the roles of stem cells themselves in producing and regulating their niches. Taking examples of epithelial stem cells from a range of model organisms and tissues, we will discuss the roles of stem cell progeny, autocrine stem cell signals and stem cell regulation of the niche in tissue homeostasis and repair. We will also consider how these principles of stem cell niche regulation are subverted by cancer cells to enable overproliferation, invasion and metastasis with implications for therapeutics and prognostic biomarkers.

2 Making the Niche: Stem Cell Progeny as Niche Components

While niche signals often originate from surrounding tissues, such as the underlying mesenchyme for many epithelial stem cells, an increasing number of examples have emerged of epithelial stem cell progeny playing a major role as sources of niche signals (Fig. 1a). In the mammalian intestine, for example, both mesenchymal- and stem cell-derived epithelial cells supply signals to regulate the intestinal stem cells (ISCs) (Santos et al. 2018). The epithelium is maintained by multipotent Lgr5+ stem cells located in the spatially restricted crypts of Lieberkuhn at the base of the villi (Barker et al. 2007). ISCs generate both absorptive enterocyte (EC) cells and secretory cells, including enteroendocrine (EE) cells and Paneth cells. In the crypt base, stem cells are found intercalated between large Paneth cells with which they form extensive surface contacts (Sato et al. 2011). Expression profiling showed Paneth cells express many ligands for pathways involved in stem cell regulation in vivo, including EGF, Dll4 and Wnt3, and depletion of Paneth cells can result in reduced stem cell numbers (Sato et al. 2011). Further evidence for the niche role of Paneth cells comes from the growth of self-organizing intestinal organoid cultures in vitro. These organoids develop with the support of appropriate growth factors in the absence of mesenchymal cells, but the efficiency of their growth is enhanced by the addition of Paneth cells (Sato et al. 2009, 2011).

Other studies have shown that stem cell function can be retained in the absence of Paneth cells, suggesting a degree of redundancy in the sources of niche signals (Durand et al. 2012; Kim et al. 2012). Non-epithelial cell types, including telocytes and discrete subtypes of mesenchymal cells, are also sources of ligands such as Wnts (Aoki et al. 2016; Valenta et al. 2016; Degirmenci et al. 2018; Shoshkes-Carmel et al. 2018). While the relative importance of these sources of niche signals may be unclear, Paneth cells, produced by the ISCs, make a significant contribution to the stem cell niche. Indeed, increased mTORC1 activity in Paneth cells with age leads to elevated expression of the Wnt inhibitor Notum, reducing stem cell proliferation, which demonstrates that changes in stem cell progeny can impact the niche and hence stem cell fate (Pentinmikko et al. 2019).

The simpler intestinal model of the *Drosophila* midgut is also maintained by a population of ISCs that generate both absorptive EC and secretory EE cells (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006). While a direct equivalent of the Paneth cells is absent, both progeny types

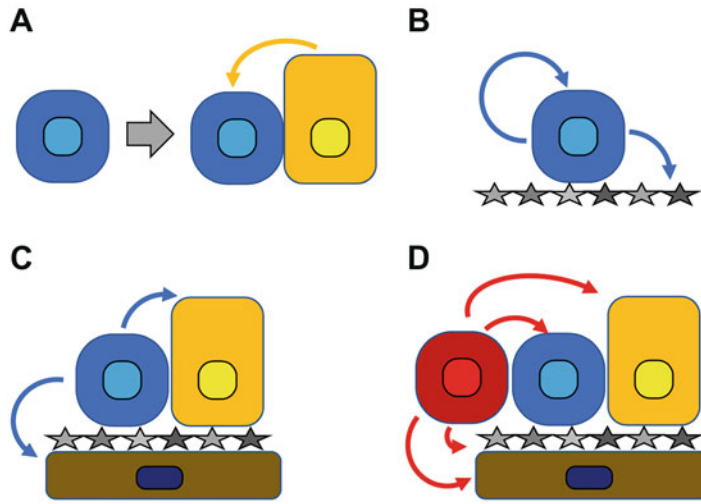

Fig. 1 Stem cell niche contributions from stem cells (**a**) Stem cells (blue) may generate progeny (yellow) that act as niche components regulating their own progenitor stem cells (**b**) Stem cells may act as their own niche cells producing autocrine signals or ECM components (grey stars) (**c**) Stem cells may regulate epithelial or non-epithelial (brown) niche cells or the ECM to influence their own environment (**d**) Cancer cells (red) may subvert the niche, exploiting any and all of the approaches **a**–**c** to create a microenvironment that favours their growth at the expense of normal cells

have been shown to express ligands critical for intestinal homeostasis. Slit-Robo signalling from EEs to ISCs acts as a negative feedback loop to regulate EE production (Biteau and Jasper 2014). EEs may also indirectly regulate ISCs via the secretion of tachykinin to regulate Dilp3 production by smooth muscle, which in turn promotes ISC maintenance (Amcheslavsky et al. 2014). Differentiated ECs are sources of BMP ligands that promote ISC maintenance and self-renewal (Tian and Jiang 2014), Hippo regulators (Karpowicz et al. 2010) and cytokine ligands that activate JAK/STAT signalling to promote tissue turnover (Jiang et al. 2009; Zhou et al. 2013). Differentiating progeny also contribute to the niche in the form of Wingless signals from enteroblast (EB) progenitors, which are required for ISC proliferation in regenerating midguts (Cordero et al. 2012).

As in mammalian intestinal epithelium, EGFR signalling is a critical regulator of *Drosophila* ISC proliferation. While the underlying visceral muscle is one source of the EGF ligand Vein, multiple ligands including epithelial Spitz and Keren expressed by differentiating EBs function redundantly to regulate the ISCs (Biteau and Jasper 2011; Jiang et al. 2011; Xu et al. 2011). Regulated secretion of EGF ligands from ECs is instrumental in driving turnover (Liang et al. 2017). These ligands are expressed in membrane-bound precursor forms that are unable to signal, but in response to EC apoptosis, the protease rhomboid is induced and cleaves the precursors to allow secretion of active EGF ligands. The resulting EGFR signalling in ISCs drives their proliferation (Liang et al. 2017), demonstrating the utility of feedback from progeny to the stem cells in coupling cell loss and replacement to maintain balance.

A similar coupling of differentiated progeny cell loss to stem cell proliferation has been demonstrated in the mouse epidermis. Live imaging studies were used to track thousands of nuclei in the basal layer of the epidermis over time (Mesa et al. 2018). It was found that driving cell differentiation caused a reduced local cell density in the basal layer, which in turn drove stem cell proliferation as contact inhibition was reduced. Whether this inhibition is through physical or

chemical signalling, it is the differentiating progeny of the stem cells that feed back to suppress their proliferation. A recent study has suggested that the mechanism may be based on intra-tissue tension (Ning et al. 2021). Signals from stem cell progeny are also important in the activation of quiescent mammalian hair follicle stem cells. Transit-amplifying (TA) cells secrete the Hedgehog ligand Shh to activate the hair follicle stem cells (Hsu et al. 2014).

Given the importance of coupling cell loss to new cell production, both in normal homeostasis and in response to challenges, it is perhaps not surprising that progeny should feed back to the stem cells (Fig. 1a). This form of coupling would allow the status of the epithelium to be communicated continuously to the stem cells, enabling them in turn to tune the production of new cells according to demand. The importance of stem cell progeny in the niche shifts our understanding from that of a fixed, clearly defined anatomical niche to that of a niche in continuous flux, able to act as rheostat, dynamically adapting to challenges and maintaining balance (Hsu and Fuchs 2012).

3 Being the Niche: Stem Cell-Derived Proteins as Niche Components

In addition to producing differentiated progeny that comprises part of the stem cell niche, there are an increasing number of examples of stem cells expressing their own niche components. In some cases, these act as autocrine signals; in others, they may contribute to the local environment, shaping the extracellular matrix (ECM) and modulating paracrine signals. In the mammalian interfollicular epidermis, stem cell self-renewal is driven by autocrine Wnt signalling (Lim et al. 2013). Wnt4 and Wnt10a are expressed by stem cells in both mouse epidermis in vivo and cultured human epidermal stem cells (Radoja et al. 2006; Lim et al. 2013). In the epidermal appendages, autocrine FGF18 and BMP6 act as niche factors to maintain quiescence of hair follicle bulge stem cells (Kimura-Ueki et al. 2012). One population of stem cells may in some contexts provide critical niche signals for another, for example, apoptotic hair follicle stem cells were recently shown to promote the proliferation of their non-apoptotic neighbours through Wnt signalling (Ankawa et al. 2021).

Similar mechanisms are found in the *Drosophila* intestine with Pvf2 acting as an autocrine stem cell maintenance factor. Overexpression of Pvf2 results in hyperproliferation, while depletion results in epithelial hypoplasia (Bond and Foley 2012). Increased expression of Pvf2 in aged intestines has been associated with stem cell misregulation, and knockdown of Pvf2 blocks increased proliferation observed with age, illustrating the importance of stem cell niche contributions in maintaining homeostatic balance (Choi et al. 2008). Autocrine signalling is not limited to Pvf2; the cytokine JAK/STAT ligand Upd1 is required autonomously for stem/progenitor cell maintenance (Osman et al. 2012). As in mammalian epidermis, there is some evidence that autocrine Wg signalling may contribute to stem cell regulation in the *Drosophila* midgut, particularly in anterior regions that may be further from a source of paracrine Wingless signals (Fang et al. 2016).

The role of stem cells in secreting their own niche proteins is not limited to signalling pathway ligands but also includes the contribution of stem cells to ECM composition. ECM components have been described as possible stem cell markers in the epidermal hair follicle bulge (Watt and Fujiwara 2011), with a range of collagens showing increased expression in hair follicle stem cells (Morris et al. 2004) and epidermal label-retaining cells (Tumbar et al. 2004). Given that integrin expression and adhesive properties are well-established markers of epidermal stem cells and that Ilk signalling in epidermal progenitor cells has been linked to the composition of the ECM, this may in effect represent an autocrine signalling mechanism with stem cells secreting their own ECM substrate to promote their maintenance (Jones and Watt 1993; Jones et al. 1995; Morgner et al. 2015). This may be a conserved principle of epithelial stem cell niches as *Drosophila* midgut ISCs are also maintained by

integrin signalling and express the ECM component laminin as an integrin ligand (Lin et al. 2013). In the mammalian intestine, both mesenchymal and epithelial cells contribute to the ECM components of the stem cell niche (Meran et al. 2017). Collagen VI is expressed in the basal epithelial layer of adult human intestine in the crypt region and in cultured human intestinal epithelial cells, suggesting it is produced either by the ISCs or their immediate progeny (Groulx et al. 2011). A similar pattern has been described for fibronectin in rat intestinal tissue and cultured cells (Quaroni et al. 1978), and some laminin subunits, such as α2, are expressed by epithelial cells and enriched around the crypt region (Lefebvre et al. 1999; Teller et al. 2007). Either by acting directly as autocrine signalling molecules or by creating a permissive physical environment, stem cell-derived ECM components contribute significantly to epithelial niches.

Autocrine signalling from stem cells (Fig. 1b) may be broadly associated with positive feedback mechanisms, serving to keep stem cells in a primed state where the removal of inhibitory factors could lead to rapid proliferation, for example, in the context of repairing tissue damage. However, such mechanisms pose risks for unregulated proliferation, and as discussed below, there are many examples of autocrine signalling within tumours.

4 Regulating the Niche: Stem Cell Signals to the Microenvironment

In addition to autocrine signals, stem cells also express a range of paracrine signals that play important roles in the regulation of their environment. Stem cell progeny in many cases constitute a critical niche component regulating their parental stem cells; in turn, it is perhaps not surprising that stem cells also regulate their progeny. *Drosophila* ISCs express the Notch ligand Delta, which signals to differentiating EBs to promote enterocyte fate (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006, 2007). Mammalian Delta-like 1 (DLL1) has been found to perform a similar function in mouse interfollicular epidermis, where it acts as a juxtacrine signal from stem cells in the basal layer to promote differentiation (Lowell et al. 2000). Recent work has identified an additional direct role for DLL1 in not only promoting differentiation of adjacent cells but actively inhibiting differentiation of the DLL1-expressing stem cells. Cis-inhibition by DLL1 in the stem cells inhibits the Notch signalling pathway to block differentiation (Negri et al. 2019). In effect, DLL1 is acting as both an autocrine inhibitory signal to maintain stemness and a paracrine Notch-activating signal to promote progeny differentiation. This complexity of local signalling is also observed for the Wnt pathway: as discussed in the previous section, mouse interfollicular epidermal stem cells express Wnt ligands that act as autocrine signals to promote stem cell fate; but they also secrete paracrine Wnt inhibitors that promote differentiation of their progeny (Lim et al. 2013). In addition to regulating cellular niche components, stem cells may also impact ECM composition through expression of regulators such as Timp2 in epidermal stem cells (Morris et al. 2004).

Expression profiling has identified a number of secreted proteins expressed by *Drosophila* ISCs that may act to regulate the local microenvironment (Doupé et al. 2018). The insulin-like peptide Ilp6 is expressed in the ISCs and was found to repress stem cell turnover, suggesting a non-autocrine role since ISC insulin signalling has previously been shown to be necessary for homeostatic turnover and growth (Choi et al. 2011; O'Brien et al. 2011). This study also identified the TNF ligand Eiger as a stem cell-expressed protein that promotes stem cell proliferation, possibly through indirect effects on stem cell enterocyte progeny (Doupé et al. 2018). Another group has further demonstrated that Eiger may be involved in both autocrine and paracrine positive feedback to promote stem cell divisions (Tamamouna et al. 2020).

Stem cell signalling to the microenvironment also plays a significant role in homeostatic turnover of the lung and airway epithelia. As in the examples above, Notch signalling from stem cells to their progeny occurs in the lung epithelium, but

it is required for the maintenance of secretory progeny rather than to promote differentiation (Pardo-Saganta et al. 2015). In co-culture assays, human airway stem/progenitor cells express VEGF-A and FGF ligands, which signal locally to endothelial cells to promote their function as sources of niche factors, such as MMP14 for the airway stem cell growth (Curradi et al. 2012; Ding et al. 2015).

Given the dynamic nature of epithelial turnover in homeostasis and the need to closely coordinate cell loss, differentiation and proliferation, it is perhaps not surprising that stem cells communicate their status to the niche, including their own progeny (Fig. 1c). In effect, this allows stem cells to answer back to the microenvironment, allowing a precise two-way feedback with the niche to offer a range of mechanisms that support balanced turnover.

5 Subverting the Niche: Carcinogenesis and Metastasis

The ability of stem cells to form, regulate or act as niche cells can be a double-edged sword. The potency that enables precise coordinated regulation in homeostasis and repair can be subverted in diseases such as cancers. Mutations not only trigger autonomous effects in the tumour itself but also impact the ability of the cancer stem cells (CSCs) or tumour-initiating cells (TICs) to communicate to and regulate their surroundings. A great deal of research has focused on understanding how tumour cells remodel their local microenvironment to promote their growth, invasion and metastasis at the expense of normal stem cells (Prager et al. 2019; López de Andrés et al. 2020; Winkler et al. 2020; Oshimori et al. 2021). CSCs have been shown to produce niche factors that either directly promote their self-renewal and survival or help create a favourable environment for cancer progression and invasion (Plaks et al. 2015). This complex network of cross-talk and signalling involves many of the key components of the tumour microenvironment, including mesenchymal stem cells, cancer-associated fibroblasts, immune cells and endothelial cells, along with the extracellular matrix and a wide range of cytokines.

The importance of cancer cell progeny as components of the niche that drive proliferation has been explored in the *Drosophila* midgut epithelium. In a *Drosophila* intestinal tumour model driven by loss of Notch function, which blocks differentiation, both autocrine and paracrine signals are responsible for tumour growth (Patel et al. 2015). Autocrine EGF signalling supports initial growth, but as tumour expansion displaces differentiated epithelial cells, these cancer cell progeny express cytokine ligands to further drive tumour growth. An alternative tumour model, in which loss of the transcription factor Sox21a causes accumulation of undifferentiated stem cell progeny, shows similar principles, with stem cell progeny expressing cytokine ligands, EGF ligands and Pvf2 to drive proliferation and matrix metalloproteases to drive invasion (Zhai et al. 2015; Chen et al. 2016). There is an extensive literature on tumour heterogeneity and the significance of communication between subclones within a tumour (Tabassum and Polyak 2015). In some cases, this is likely to include communication between cancer stem cells and their own progeny, for example, in a p53-null mouse model of breast cancer, mesenchymal-like progeny signal to their tumour-initiating cell parents to promote CSC self-renewal (Brooks and Wicha 2015; Zhang et al. 2015).

Autocrine signalling also plays key roles in the maintenance and proliferation of cancer stem cells. In SOS-driven or DMBA/TPA-induced mouse skin tumours, autocrine VEGF signalling drives tumour cell proliferation, and loss of VEGF or its receptor in the epithelium is sufficient to block tumour growth (Lichtenberger et al. 2010; Beck et al. 2011). Autocrine VEGF has roles in cell survival, proliferation or migration in a range of tumours, including the lung (Chatterjee et al. 2013; Barr et al. 2015), breast (Perrot-Applanat and Di Benedetto 2012) and oesophagus (Zhang et al. 2014). Oesophageal cancer stem cells upregulate both the cytokine CXCL2 and its receptor CXCR4 to promote invasion and metastasis (Wang et al. 2017). Advanced skin squamous cell carcinomas

express autocrine SDF-1 that signals through CXCR4 and CXCR7 to promote metastasis (Bernat-Peguera et al. 2019). Ovarian carcinomas express autocrine Interleukin-6 (IL-6), and inhibition of its receptor reduces metastasis (Mehner et al. 2020). Autocrine FGF signalling occurs in squamous cell carcinomas of the lung and head and neck (Gao et al. 2019; Li et al. 2020) and enables adaptive resistance to BRAF/MEK inhibition in BRAF tumours (Wang et al. 2019). Autocrine TGFβ signalling has been implicated in breast, colorectal and ovarian cancers (Matsumoto et al. 2018; Nakano et al. 2019; Woosley et al. 2019). IL-6 is also an important autocrine factor, in addition to its paracrine roles in regulating the stem cell niche (Yeh et al. 2006; Sansone et al. 2007; Sasser et al. 2007). The ECM protein periostin has been identified as another stem cell-secreted protein that promotes cancer stem cell fate in breast, colon and ovarian cancer through autocrine integrin signalling (Gillan et al. 2002; Bao et al. 2004; Lambert et al. 2016). Periostin is also expressed in mouse hair follicle stem cells, suggesting a role in normal stem cells that is subverted in cancer (Morris et al. 2004).

In addition to acting as a positive niche for their own maintenance through autocrine signalling, cancer stem cells can act as a restrictive niche to limit the proliferation and maintenance of normal stem cells. Three recent studies found that intestinal tumours alter the niche not only to promote their own growth but to drive the loss of adjacent wild-type stem cells. Mammalian ISCs mutant for Apc secrete Wnt antagonists such as Notum to inhibit Wnt signalling in wild-type ISCs, suppressing their proliferation and promoting their differentiation (Flanagan et al. 2021; van Neerven et al. 2021). The mutant ISCs are therefore acting as a repressive niche for normal ISC growth, but as the Apc mutant cells are no longer dependent on extrinsic Wnt signals, their own growth is not impeded. This remodelling was not specific to Apc cells or Wnt signalling, as clones of intestinal cells expressing KRAS/PI3K oncogenes have also been shown to suppress the growth of adjacent wild-type clones by secretion of BMP ligands (Yum et al. 2021). The Simons group also showed that in addition to directly acting as a repressive niche, cancer stem cells can regulate other niche components, indirectly repressing Wnt signalling in wild-type stem cells by remodelling of the stroma. Collectively, these three papers demonstrate that cancer stem cells remodel the local microenvironment to enhance their own competitive advantage over wild-type stem cells.

CSC- or TIC-derived signals play critical roles in regulating their own niche through recruitment or induction of cells in the microenvironment to express niche signals that promote tumour properties. CSCs in breast cancer and skin tumours secrete VEGF-A, which acts on endothelial cells to promote angiogenesis and the formation of a perivascular niche (Beck et al. 2011; Jiang et al. 2020). In addition to its role in promoting tumour endothelial niche function, mouse colon carcinoma-derived VEGF-A has also been shown to regulate T-cell immune checkpoints, creating an immune-protective environment (Voron et al. 2015). Squamous cell carcinoma TICs express the cytokine IL-33, which induces differentiation of macrophages that express TGFβ to promote cancer progression (Taniguchi et al. 2020). A similar loop is observed between breast CSCs and mesenchymal stem cells involving reciprocal IL-6 and CXCL7 signalling (Liu et al. 2011). Tumour-derived signals can also induce cancer-associated fibroblasts (CAFs), including inflammatory CAFs via IL-1α and myofibroblastic CAFs via TGFβ (Biffi et al. 2019). In the *Drosophila* midgut, Apc mutant cells subvert the normal EGF-driven feedback circuit that acts to couple cell loss to ISC proliferation by inducing rhomboid in adjacent cells to drive constitutive secretion of active EGF ligands (Ngo et al. 2020). Remodelling of the ECM elements of the niche is also critical in tumour development and progression, with contributions from both tumour cells themselves expressing ECM components or regulators such as MMPs and from tumour-associated cells, particularly CAFs (Winkler et al. 2020). In addition to its autocrine roles, the ECM component periostin is expressed by CAFs in the stroma of ovarian cancer in response to TGFβ1 signalling from the

tumour cells and promotes metastasis through integrin signalling (Yue et al. 2021).

In summary, cancer stem cells extensively remodel their niche in order to facilitate tumour progression and cancer cell survival through complex networks of interactions involving all of the contributions seen in the homeostatic niche (Fig. 1d). As a result, the cancer secretome is increasingly taking on therapeutic significance (López de Andrés et al. 2020). The multipotency and tight homeostatic regulation that characterizes stem cells and maintains tissue integrity is overturned in the CSC niche, promoting adverse effects of an otherwise beneficial characteristic of normal tissue stem cells.

6 Conclusions

The importance of stem cell-derived secreted proteins and stem cell progeny in the niche is increasingly recognized in the context of cancer cells. However, the extent to which this represents the misregulation of signalling circuits used in normal stem cell regulation is unclear. Studies from a range of systems have shown the significance of these mechanisms in normal homeostasis and tissue repair. Further work is needed to explore the extent of stem cell signalling as critical to the niche and the logic of two-way interactions between stem cells and the surrounding microenvironment. Coupling both at the level of two-way signalling between stem cells and the niche and between cell production and loss through niche roles of stem cell progeny provides mechanisms to maintain balance. Given the highly dynamic nature of these links, the development and application of novel approaches to study homeostasis in real time will be critical to explore the mechanisms of homeostasis and how it is perturbed. Many of the known mechanisms appear to be conserved from the relatively simple invertebrate model of the *Drosophila* midgut to mammalian systems, suggesting that complementary studies in both basic model organisms and more complex mammalian systems will be key to elucidating the full logic of these complex interactions.

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Parathyroid Cell Differentiation from Progenitor Cells and Stem Cells: Development, Molecular Mechanism, Function, and Tissue Engineering

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Abstract

Parathyroid glands are endocrine organs which are located posterior to thyroid glands and control secretion of parathyroid hormone (PTH) in order to regulate blood calcium level. PTH maintains calcium homeostasis by acting on the bone, kidney, and small intestine. PTH deficiency leads to chronic hypocalcemia, organ calcinosis, kidney and heart failure, painful muscle spasms, neuromuscular problems, and memory problems. Since parathyroid cells have inadequate proliferation potential in culture conditions, their utilization as a cellular therapy option is very limited. Although studies conducted so far include parathyroid cell differentiation from various cell types, problems related to successful cellular differentiation and transplantation still remain. Recently, parathyroid tissue engineering has attracted attention as a potential treatment for the parathyroid-related diseases caused by hypoparathyroidism. Although major progression is made in the construction of tissue engineering protocols using parathyroid cells and biomaterials, PTH secretion to mimic its spontaneous harmony in the body is a challenge. This chapter comprehensively defines the derivation of parathyroid cells from various cell sources including pluripotent stem cells, molecular mechanisms, and tissue engineering applications.

Keywords

Differentiation · Hypoparathyroidism · Parathyroid gland · Parathyroid hormone · Pluripotent stem cells

Abbreviations

BMP4	Bone morphogenetic protein 4
CaSR	Calcium-sensitive receptor
CCL21	C-C motif chemokine ligand 21
CRISPR	Clustered regularly interspaced short palindromic repeats
dTMSC	Differentiated TMSC
EBs	Embryoid bodies
ECF	Extracellular fluid
EYA1	EYA transcriptional coactivator and phosphatase 1
FGF23	Fibroblast growth factor 23
FOXN1	Forkhead box N1
GATA3	GATA binding protein 3
GCM2	Glial cells missing transcription factor 2
GFP	Green fluorescent protein

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GHH	Gelatin-hydroxyphenyl propionic acid (GH) hydrogels
hESC	Human embryonic stem cell
HOXA3	Homeobox A3
iPSCs	Induced pluripotent stem cells
IWP-2	Inhibitor of Wnt productions-2
MAFB	MAF BZIP transcription factor B
MEFs	Mouse embryonic fibroblasts
PAX1	Paired box 1
PAX9	Paired box 9
PKA	Protein kinase A
PTH	Parathyroid hormone
PTH1R	Type 1 parathyroid hormone receptor
PTH2R	Type 2 parathyroid hormone receptor
PTHrP	Parathyroid hormone-related protein
RA	Retinoic acid
SHH	Sonic hedgehog
TALEN	Transcription activator-like effector nucleases
TBX1	T-box transcription factor
TGFs	Transforming growth factors
TMSC	Tonsil-derived mesenchymal stem cells
Wnt3a	Wnt family member 3A

1 Introduction

Parathyroid glands are small endocrine organs which are located at the posterior part of the thyroid gland. Parathyroid glands secrete parathyroid hormone (PTH) in response to low calcium levels to regulate calcium metabolism (Cipriani 2014). Two different cell types such as chief cells and oxyphil cells are present in the parathyroid glands (Taterra et al. 2019). Although the function of oxyphil cells is unknown, several investigations indicate that oxyphil cells contribute to PTH secretion in secondary hyperparathyroidism (Tanaka et al. 1996). Chief cells produce PTH in response to low calcium which triggers the calcium-sensitive receptor (CaSR) and initiates the G-protein messenger pathway. In contrast, the G-protein messenger pathway is blocked and PTH secretion is diminished when the blood calcium level is high which is related to the secretion of calcitonin from the thyroid gland (Brown and Limaiem 2020). Calcium is one of the most widely distributed inorganic elements which regulates bone development and maintenance in mammals by targeting kidney and intestine for reabsorption of calcium (Khan and Sharma 2018). Calcium homeostasis is driven by four important factors such as PTH, calcitonin, metabolic D3, and fibroblast growth factor 23 (FGF23) (Shaker and Deftos 2013).

PTH is the hormone which raises the blood calcium concentration by three main mechanisms:

- PTH induces the generation of the active form of vitamin D, calcitriol, which plays role in the intestines to enhance calcium absorption.
- PTH simplifies the transportation of calcium from bone into the blood by stimulating osteoclasts when the blood calcium concentration is decreased.
- PTH increases the reabsorption of calcium directly in the kidney tubule. Therefore, the loss of calcium in the urine is decreased (Talmage and Mobley 2008).

Along with calcium homeostasis, PTH contributes to phosphate homeostasis. PTH inhibits phosphate reabsorption from the proximal tubule of the kidney, resulting in increased phosphate excretion through the urine. PTH, on the other hand, increases the absorption of phosphate from the stomach and bones into the blood. The final result of PTH release is a decrement in serum phosphate concentration (Jayakumar 2012). Parathyroid is a small endocrine organ that plays crucial roles in calcium and phosphate metabolism and regulates bone, kidney, and intestine tissue metabolism (Bro and Olgaard 1997). In the current chapter, we reviewed the differentiation of parathyroid cells from stem cells. We organized the current literature on development, molecular mechanism, differentiation, function, and tissue engineering of the parathyroid tissue.

2 PTH Mechanism and Biosynthesis

PTH is the main hormone which regulates calcium and phosphate homeostasis by acting on bone and kidney cells in the body (Renkema et al. 2008). PTH is a 115-amino acid precursor peptide that is extended at the amino (NH2)-terminus by a "pre" sequence that facilitates entry into the secretory system of cells. Propeptide PTH(1–84) is the main secreted and circulating form of the hormone, while the leader sequence is cleaved in the cell, giving rise to a straight peptide chain containing 84 amino acids, PTH(1–84). Ionized calcium, Ca+, in the extracellular fluid (ECF), appears to be the primary regulator of PTH generation and secretion.

Type 1 parathyroid hormone receptor (PTH1R) and type 2 parathyroid hormone receptor (PTH2R) which belong to the G-protein-coupled receptors family have been identified for PTH and parathyroid hormone-related protein (PTHrP) binding. PTHrP is secreted by a diverse range of cells throughout the life, including fetal and postnatal developmental stages. Surface epithelium of developing organs, mesenchyme, vascular smooth muscle, and the central nervous system are among the tissues that have been shown to release this hormone (Strewler 2009). PTH1R is activated by both PTH and PTHrP. Binding to this receptor triggers N-terminal PTH-like domain of the receptor which stimulates the cyclic AMP/Protein kinase A (PKA) pathway or calcium-dependent pathway (Mannstadt et al. 1999). PTH1R mRNA is mostly expressed in bone and renal tubules (Clemens et al. 2001). On the other hand, PTH2R has a very low affinity for PTHrP while it binds to PTH. Similar to PTH1R, it activates adenylyl cyclase and this causes an increase in intracellular cyclic AMP concentration (Mannstadt et al. 1999). PTH2R mRNA is expressed predominantly by adipocytes and keratinocytes in the skin (Sato et al. 2016).

By acting on the bone, kidney, and small intestine (Khan and Sharma 2018), PTH maintains calcium homeostasis. PTH exerts its effects on all bone cell types including osteocytes, osteoblasts, and osteoclasts. PTH first induces osteocyte osteolysis, resulting in the disintegration of the bone surface (Bellido et al. 2013). Ca is transported from the bone canalicular fluid into the osteocytes and then into the extracellular fluid as a result of this process. Then, PTH stimulates osteoclasts to promote bone resorption, releasing calcium and phosphate into the extracellular fluid. PTH exerts both anabolic and catabolic effects on bone tissue. PTH's catabolic effect on bone is an increase in bone resorption (Silva et al. 2011) in response to hypocalcemic stimuli. Numerous in vivo and in vitro studies have shown that PTH indirectly increases bone resorption via osteoclast activation (Chambers et al. 1985; McSheehy and Chambers 1986). On the other hand, preclinical and clinical investigations have demonstrated PTH's anabolic effect on bone tissue. In these studies, it was discovered that the use of PTH stimulates bone development (Neer et al. 2001; Greenspan et al. 2007; Iwaniec et al. 2007). The anabolic effect of PTH treatment is inversely proportional to the osteoblastic lineage in terms of bone mass (Yang et al. 2007). The hormone PTH, as previously stated, promotes and improves the growth of osteoblasts in primary calvarial cells as well as bone marrow-derived cells. Additionally, it has been demonstrated that intermittent PTH promotes the production of ossicles in immunodeficient animals following the implantation of bone marrow-derived cells on the dorsal surface of each mouse; mid-longitudinal skin incisions of approximately 1 cm in length were created (Pettway et al. 2005).

Physiological calcium reabsorption in the nephron occurs in the proximal convoluted tubule as well as the ascending Henle loop in the kidneys. By targeting the distal convoluted tubule and collecting duct, circulating PTH directly increases calcium reabsorption. In the proximal convoluted tubule, PTH inhibits phosphate reabsorption. Phosphate ions in serum form insoluble salts with calcium, lowering plasma calcium. As a result, phosphate ion reduction results in more ionized calcium in the blood (Khan and Sharma 2018). PTH increases the formation of 1-alpha-hydroxylase in the proximal convoluted tubule of

the kidneys. This enzyme, 1-alpha-hydroxylase, is essential for the catalysis of the conversion of 25-hydroxycholecalciferol to active vitamin D-1,25-dihydroxycholecalciferol. Vitamin D is active and participates in calcium reabsorption in the distal convoluted tubule. Vitamin D facilitates calcium absorption in the small intestine via an active transcellular pathway and a passive paracellular mechanism. The transcellular route demands energy, but the paracellular route allows calcium to pass past tight junctions (Khan and Sharma 2018).

3 Development of Parathyroid Glands

The 4 parathyroid glands are divided into pairs – upper parathyroid glands and lower parathyroid glands – which are located in close proximity to thyroid glands in the adult body (Rosen and Bordoni 2020). The parathyroid glands are originated from the endoderm layer of the third and fourth pharyngeal pouches (Casale and Giwa 2019). The third pharyngeal pouch and the fourth pharyngeal pouch give rise to the lower parathyroid glands and the upper parathyroids respectively. The third pharyngeal pouch is split into dorsal and ventral parts. The dorsal part leads to the lower parathyroid gland, while the ventral part leads to the formation of the thymus. In the seventh week of gastrulation, the lower parathyroid glands separate from the posterior pharyngeal wall and follow the thymus tract to reach their final destination in the posterior thyroid (Rosen and Bordoni 2020). The fourth pharyngeal pouch is divided into dorsal and ventral parts. The dorsal part differentiates into the superior parathyroid gland and the ventral part differentiates into the ultimobranchial body. The parathyroid glands are separated from the pharyngeal wall and attached to the posterior surface of the thyroid when the seventh week of development is completed (Rosen and Bordoni 2020).

In the pharyngeal endoderm, 4 main transcription factors are responsible for the development of parathyroid precursor. These are Homeobox A3 (HOXA3), Paired box 1 (PAX1), EYA Transcriptional Coactivator And Phosphatase 1 (EYA1), and Paired box 9 (PAX9), all of which are involved in thymus development at the pharyngeal endoderm stage (Bingham et al. 2009). HOXA3 is expressed in both the third pouch endoderm and the neural crest mesenchyme, and its function has been demonstrated to be upstream of PAX1 and PAX9 (Su et al. 2001). PAX1 and PAX9 are members of the paired-box gene family, which is involved in the development of numerous organs (Neubueser et al. 1995). PAX9 deficiency results in an early failure of thymus, parathyroid, and ultimobranchial body development (Peters et al. 1998), whereas PAX1 deficiency results in hypoplastic parathyroid and thymus, as well as abnormal thymocyte maturation (Su et al. 2001). At E9.5–10.5, Eya1 is highly expressed in the pharyngeal arches, pouch endoderm, and surface ectoderm, including pharyngeal clefts. Following that, expression of Eya1 was detected in structures arising from the pharyngeal area, including the thymus and parathyroid. Without Eya1, the thymus and parathyroid fail to develop concurrently with the early activation of organogenesis (Xu et al. 2002).

Parathyroid glands are developed in the third pharyngeal pouches in mice at E11.5 (Cordier and Haumont 1980) whereas in both third and fourth pouches in humans starting at 5–6 weeks (Gilmour 1937; Liu et al. 2010). The cell fate decision starts in E11.5 for parathyroid and thymus. Primordium cells located at the third pharyngeal pouches differentiate into the parathyroid and the thymus in the presence of glial cell missing 2 (GCM2) and forkhead box N1 (FOXN1), respectively (Gordon et al. 2001). Additionally, FGF (Gardiner et al. 2012) and bone morphogenetic protein 4 (BMP4) (Gordon et al. 2010) signaling pathways regulate the differentiation of the parathyroid-thymus complex from the pharynx. FOXN1 expression is seen in the ventral/posterial prospective third pharyngeal pouch, also known as the anterior thymus mass, at E10.5 through E12.5, with BMP4 expression found in the surrounding mesenchyme (Bleul and Boehm 2005). A recent study has shown that FGF feedback antagonists from the sprouty gene family are

important regulators of parathyroid organogenesis (Mason et al. 2006). During thymus/parathyroid organogenesis, sprouty proteins block FGF signaling at the cellular level. A decrease in the induction of GCM2 expression is caused by increased FGF signaling in the absence of sprouty proteins, which leads to the creation of a small GCM2+ domain by E11.5. GCM2 deficiency may be the cause of sprouty protein mutants' hypoplasia of the parathyroid gland (Gardiner et al. 2012).

GCM is a transcription factor which was first identified in Drosophila which regulates the determination of neuronal or glial fate (Hosoya et al. 1995). GCM1 and GCM2 were characterized in mammalians as homologs of GCM in Drosophila (Kim et al. 1998). GCM2 is slightly expressed in second and third dorsal pouches at E9.5 and upregulated at E10.5 followed by limited expression in dorsal-anterior of the third pouch endoderm (Gordon et al. 2001). GCM2 is important for the survival and differentiation of parathyroid cells. When GCM2 is upregulated, PTH and CaSR which are downstream of parathyroid development pathways are activated. In the absence or reduced expression of the parathyroid GCM2 gene, as seen in GCM2 knockout mice (Günther et al. 2000) or cultured human parathyroid cells treated with GCM2 siRNA (Mizobuchi et al. 2009), CaSR expression is reduced too. CaSR is a marker for early parathyroid differentiation (Liu et al. 2007). GCM2 activates the CaSR gene by binding to GCM response elements in the CASR promoters P1 and P2, which are located in the CASR promoter (Canaff et al. 2008). Thus, GCM2 and CaSR are mechanistically linked to the evolutionarily related parathyroid glands (Okabe and Graham 2004).

Besides, C-C motif chemokine ligand 21 (CCL21) activates the parathyroid domain of the receptor which is expressed at E11.5, and it is responsible for appealing lymphoid progenitors into the thymus domain (Liu et al. 2006). The conservation of CaSR and CCL21 expression in the parathyroid domain is a GCM2-dependent process, whereas the expression of CaSR and CCL21 is GCM2-independent. In discrepancy, PTH initiation and maintenance is a GCM2-dependent process at E12 (Liu et al. 2007). Because PTH is the primary target of GCM2, PTH expression was never observed in GCM2 null mice and parathyroid cells apoptosis was detected in GCM2 null mice (Liu et al. 2007). As a result of the absence of parathyroid glands in GCM2 null mice, it is believed that GCM2 gene is required for parathyroid gland development and survival at the earliest stage following organ specification during embryogenesis (Günther et al. 2000). Additionally, it is predicted that GCM2 plays a critical role in parathyroid function during mineral homeostasis. A series of analyses indicated that GCM2, in conjunction with MAF BZIP transcription factor B (MAFB) and GATA binding protein 3 (GATA3), regulates serum calcium concentration via CaSR binding (Mizobuchi et al. 2009) and followed by promoting PTH secretion (Yamada et al. 2019).

4 Derivation of Parathyroid Cells from Pluripotent Stem Cells

Elimination of parathyroid glands during thyroid surgery or radiotherapy of neck cancers results in parathyroid-related diseases such as hypoparathyroidism and hypocalcemia (Marx 2000). The lack of parathyroid glands and the resulting disorders lead to heart and kidney failure, painful muscle spasms, and neuromuscular problems (Khan and Sharma 2018). Development of parathyroid tissue engineering strategies, cellular therapy solutions, and successful treatment strategies is required to reduce or completely eliminate the hypoparathyroidism-related problems.

Therefore, derivation of parathyroid cells with parathyroid hormone production capacity in a laboratory might be a cell therapy-based solution. The recent studies on patient-specific therapies using human induced pluripotent stem cells are potential candidates for treatment (Fig. 1).

The molecular pathways that underlie parathyroid development were discovered using pluripotent stem cells by Bingham and colleagues in 2009. These molecular pathways can be used to mimic parathyroid development in vitro and

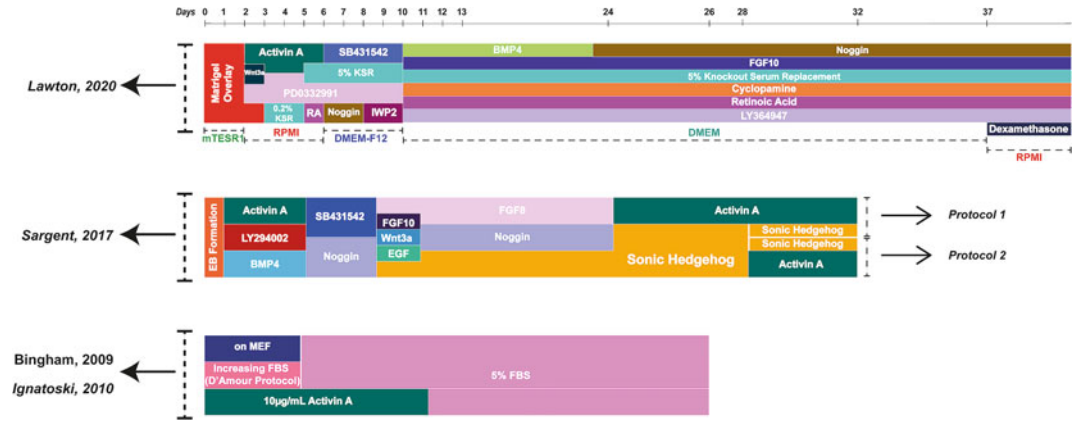

Fig. 1 The schematic representation of parathyroid cell differentiation from pluripotent stem cells

generation of cells for cellular replacement therapy. In vitro parathyroid regeneration was investigated using human embryonic stem cell (hESC) lines. BG01 cells were chosen as a model system because of their capacity to express several endoderm development markers during undirected differentiation into EBs, indicating that the cell line has not acquired abnormalities that would impair its ability to differentiate into endoderm. They optimized the expression of definitive markers of parathyroid development by modifying and extending existing techniques for the differentiation of mouse embryonic stem cells into definitive endoderm. The suitable technique was employing 100 ng/mL Activin A for 5 days with BG01 cells cultured on murine embryonic fibroblasts under circumstances of rising serum concentration. After 5 days in tissue culture, the cells were allowed to develop further without the addition of murine fibroblasts but with the presence of continuous Activin A. The differentiation protocol was known as Bingham protocol and cells were seeded onto mouse embryonic fibroblasts (MEFs) as feeder layer and treated with Activin A and SHH for a total of 26 days to obtain parathyroid-like cells (Fig. 1) (Bingham et al. 2009). Another study conducted by the same group found that another human embryonic stem cell line (H1) had comparable outcomes to their first findings with BG01 cells. According to the findings, differentiated cells exhibited parathyroid-related markers such as CasR, GCM2, and PTH and produced PTH hormone (Fig. 1) (Woods Ignatoski et al. 2010).

In a thesis study published in 2016, induced pluripotent stem cells (iPSCs) were reprogrammed from patients' somatic cells and then differentiated into parathyroid progenitors for personalized cellular therapy. The advantages of the utilization of iPSCs were to diminish immune rejection in this study. Clustered regularly interspaced short palindromic repeats (CRISPR) and transcription activator-like effector nucleases (TALEN) gene-editing technologies were used to insert green fluorescent protein (GFP) reporter into GCM2, PTH, and CasR regulatory sequences to detect differentiation of iPSCs into parathyroid gland cells and progenitors. They hypothesized that iPSCs were directly differentiated through the endodermal lineage with main parathyroid cell characteristics such as PTH secretion in low calcium level, CaSR, and vitamin D receptor expression. Additionally, GATA3 (+/−) mouse model was used to observe the effect of treatment for hypoparathyroidism. The functional efficacy of hypoparathyroidism in the GATA3 (+/−) mouse is critical to aid the production of parathyroid progenitors for transplantation. This model holds promise as the basis for developing a patient-specific cell-based therapy for hypoparathyroidism (Fig. 1) (Sargent 2017).

Lawton and coworkers developed parathyroid hormone expressing cells from hES and hiPS

cells in 2020. Definitive endoderm, anterior foregut endoderm, and pharyngeal endoderm differentiation were conducted at different time points of the protocol by using various differentiation medium cocktails. For definitive endoderm differentiation, Activin A, Wnt family member 3A (Wnt3a), PD0332991, and retinoic acid (RA) were added for 5 days. Between day 6 and 9, SB431542, Noggin, PD0332991, and inhibitor of Wnt productions-2 (IWP-2) were utilized for anterior foregut endoderm differentiation. Finally, in order to reach pharyngeal endoderm differentiation, LY364947, RA, FGF10, cyclopamine, BMP4, Noggin, and dexamethasone were used from day 10 to day 37. Each differentiation was confirmed with gene expression, immunofluorescence, flow cytometry, and PTH secretion analysis. This differentiation protocol allowed the proper treatment choices for patients through recognition of biological determinants of secretion and expression of PTH (Fig. 1) (Lawton et al. 2020).

5 Derivation of Parathyroid Cells from Various Cell Sources

In addition to pluripotent stem cells, various cell types including tonsil-derived mesenchymal stem cells (Park et al. 2015) and thymus epithelial cells (Ignatoski et al. 2011) have been used in the derivation of parathyroid-like cells, and these differentiation protocols were indicated in Table 1. Park et al. showed the differentiation of tonsil-derived mesenchymal stem cells into parathyroid-like cells. The differentiation was performed with Bingham protocol as described above. Briefly, Activin A and SHH combination was applied

Table 1 Parathyroid cell derivation from various sources

Differentiation conditions	Cell source	Duration	Ex vivo/ in vivo/in vitro	References
Activin A Sonic Hedgehog	H1 cells	26 days	In vitro	Woods Ignatoski et al. (2010)
Activin A Sonic Hedgehog	Thymus epithelial cells	21 days	In vitro	Ignatoski et al. (2011)
Activin A Sonic Hedgehog	hiPSC	–	In vitro	Shandiz et al. (2013)
Activin A Sonic Hedgehog	Tonsil-derived MSC	21 days	In vivo	Park et al. (2015)
Activin A LY294002 BMP4 SB431542 Noggin FGF8 Wnt3a EGF Shh	hiPSC	32 days	Ex vivo	Sargent (2017)
Activin A Wnt3a PD0332991 ATRA SB431542 Noggin IVP2 LY364947 FGF10 Cyclopamine BMP4 Dexamethasone	WA09 (H9) human ES cells, Y6-iPSCs, and CHOPWT10.2 iPSC	37 days	In vivo	Lawton et al. (2020)

for 21 days (Bingham et al. 2009). Differentiated cells secreted PTH and also when these cells were injected into rats that were fed a calcium-free diet, PTH level was renovated, and the survival rate was increased. This study has shown that these differentiated cells were new cell sources for parathyroid cell function restoration and osteoporosis therapy in the future for the first time in the literature (Park et al. 2015).

Ignatoski and colleagues indicated the new cell source as thymus epithelial cells for the differentiation of parathyroid-like cells by Bingham protocol (Bingham et al. 2009). This differentiation process was established based on the thymus development process in which thymus and parathyroid glands are arised from the same primordium. The important points of this protocol were summarized as follows. Firstly, this study had used human tissue and had controlled calcium-regulated PTH secretion. Finally, there is no genetic manipulation of cells. Taken together, this study had a potential candidate for the replacement of parathyroid function of patients by in vitro trans-differentiated cells (Table 1) (Ignatoski et al. 2011).

In 2016, Zhao and his coworker stated that adipose-derived stem cells can be used as a proper cell source for differentiation into parathyroid-like cells to treat hypoparathyroidism with autologous cell therapy. The reason for choosing the adipose tissue was their immunomodulatory effect and differentiation potential. The hypothesis of this study was the differentiated cells expressed PTH-related markers and composed blood calcium level (Table 1) (Zhao and Luo 2016).

6 Preclinical and Clinical Applications of Parathyroid Tissue Engineering

In 2020, 200.000 patients suffered from hypoparathyroidism in the United States, Europe, Japan, and South Korea (New Survey on World Hypoparathyroidism 2021). Only in the United States at least 80.000 patients had hypoparathyroidism in 2020 (New Survey on World Hypoparathyroidism 2021). Pre-clinical and clinical studies about parathyroid tissue engineering were summarized in Table 2.

Gökyürek and her co-workers managed to isolate parathyroid cells from human parathyroid tissue and created hydrogel scaffolds via 3D bioprinter in 2020. Because of the spherical shape of the parathyroid gland and the polygonal shape of the chief cells, cylindrical architecture with honeycomb design was picked for the scaffold rather than cornered designs such as square. To further reduce cell leakage and promote cell adherence to the scaffold, a close-pored first layer of the scaffold was printed on the first layer of the scaffold and broader pores were printed on the remaining two layers. The parathyroid cells were grown on alginate scaffolds that were 3D printed and had structural and mechanical qualities that

Table 2 Tissue engineering models for parathyroid-related diseases

Clinical application types of parathyroid glands	Cell source	Materials	References
Scaffold	Parathyroid cells	Hydrogel	Gökyürek (2020)
Allogenic transplantation	Allogeneic tissue particles (1–2 mm)	–	Agha et al. (2016)
Allogenic transplantation	Autologous and allogeneic parathyroid tissue fragments	Polytetrafluoroethylene membrane and a nonwoven polyester mesh	Khryshchanovich and Ghoussein (2016)
Macroencapsulation	Cells derived from adenoma	1.3% (w/v) sodium alginate	Picariello et al. (2001)
Embedded	Human tonsil-derived mesenchymal stem cells	Gelatin-hydroxyphenyl propionic acid hydrogels	Park et al. (2018)

were similar to those of the natural tissue. The capabilities of these 3D printed tissues substitute to support cell survival and PTH secretion. As a result, it has been demonstrated that functional parathyroid gland substitutes can be produced using autologous cells and 3D printing to replace the natural parathyroid gland (Table 2) (Gökyürek 2020).

Allotransplantation of parathyroid was successfully done from a 32-year-old woman to a living donor. There were no complications for both donor and recipient. After allotransplantation, the calcium level in serum and PTH secretion increased progressively. This study is one of the examples for transplantation of the parathyroid gland from healthy donors properly (Table 2) (Agha et al. 2016).

In another study, for severe hypoparathyroidism, allotransplantation of macroencapsulated parathyroid is a better option than substitution drug therapy. A 27-year-old male with renal failure-induced parathyroid hyperplasia donated his parathyroid tissue for study. Macroencapsulation was designed with 157 μM thick from polyvinylidene difluoride artificial membrane. This encapsulated graft was injected into the lumen of the deep femoral artery. After this allotransplantation, calcium level and PTH secretion turned to normal levels. Also, grafts still had functionality after 3 months (Table 2) (Khryshchanovich and Ghoussein 2016).

Picariello and co-workers conducted the microencapsulation of human parathyroid cells with alginate-polylysine-alginate membranes. 45 patients with primary and secondary hyperparathyroidism had surgery to collect pathological parathyroid tissues. Following the microencapsulation of cells, there was evidence of long-term survival, cell proliferation, and PTH secretion in vitro. This study is a potential candidate for patients suffering from hypoparathyroidism (Table 2) (Picariello et al. 2001).

In 2015, Park and colleagues showed that novel mesenchymal stem cells generated from human tonsillar tissues (tonsil-derived mesenchymal stem cells TMSC) were differentiated into PTH-secreting cells. As reported previously, differentiated TMSC (dTMSC) was only able to restore parathyroid function in vivo when placed on a Matrigel scaffold. In 2018, Park et al. improved gelatin-hydroxyphenyl propionic acid (GH) hydrogels (GHH) instead of Matrigel scaffolds in order to embed their PTH-producing TMSC for successful transplantation and therapeutic effects. Continuous PTH release from TMSC-embedded GHH sustained stable serum Ca2+ levels in transplanted mice. Taken together, their findings indicate that a GHH-based system is a more promising and superior stem cell scaffold than other hydrogels which is a requirement for successful clinical applications (Table 2) (Park et al. 2018).

In addition to cell therapy-based approaches, PTH itself is used in the treatment of many conditions. There is another approach for directly utilizing PTH in clinical trials for the treatment of bone and skeletal-related diseases (Arthur and Gronthos 2020). A previously submitted research showed the effect of PTH on bone fractures and mineralization capacity in postmenopausal women with osteoporosis. PTH was injected in 1637 postmenopausal women subcutaneously at two different doses. According to results, PTH injection caused decrement in risk of bone fractures and increment in the bone mineral density of total body (Neer et al. 2001).

Another study had developed the cell-free scaffold model to regenerate bone defects. For the first time in the literature, local pulsatile PTH delivery system was developed for supporting bone regeneration by mimicking bone remodeling. The daily PTH delivery was realized in a cell-free biomimetic nanofibrous scaffold for 21 days. This study is a novel model for local bone defects based on the significant results of enhancing bone regeneration (Dang et al. 2017).

Additionally, Ishizuya et al. demonstrated that the PTH had affected the osteoblast differentiation in a dose and time-dependent manner. Isolated osteoblastic cells from newborn rat calvaria were treated with PTH at a different time interval for 48 h. According to results, 6 h administration of PTH caused the osteoblast differentiation mostly compared to other groups. To conclude, PTH treatment affects the osteoblast differentiation based on the exposure time (Ishizuya et al. 1997).

Generation of parathyroid cells from pluripotent stem cells and other cell types might be a

source to produce PTH for clinical purposes. Production of the high amount of PTH in culture and further purification might be used in addition to cell therapy for therapeutic purposes.

7 Conclusion

Cell therapy is an emerging field for regenerative medicine in recent years. Stem cells, tissue-specific progenitors, stem cell-derived products, organoid systems, and tissue engineering applications are of great importance for clinical applications. Development of organ systems in controlled culture systems might be a solution for future organ replacement therapies. Parathyroid glands are small organs which could be a suitable target for cell and organ transfer therapies. Derivation of functional parathyroid cells and organ-like structures from appropriate cell sources are required to establish clinically relevant protocols for therapeutic applications. Pluripotent stem cells including embryonic stem cells and induced pluripotent stem cells, as remarkable cell sources with unlimited proliferation and differentiation potential might be used for parathyroid cell engineering. Derivation of induced pluripotent stem cells from adult cell sources enables the patient-specific cell therapy strategies. Even more crucially, because the donor cells and the tissues generated from iPSCs have identical genetic profiles, the immune system does not reject them (Taylor et al. 2011). ESCs, which are gold standard pluripotent stem cells, have limitless proliferation capacity and can transform into a variety of cell types (Zakrzewski et al. 2019). Understanding the molecular mechanisms of pluripotent stem cell fate decision might allow derivation of clinically relevant cell sources for regenerative medicine.

Investigation of culture and differentiation conditions might enable obtaining fully functional parathyroid cells, parathyroid organoids, and establishment of organ-like structures in scaffold systems. Because parathyroid is a small endocrine organ, transplantation of in vitro derived cells, organoids, and tissue engineering constructs might be a prospective solution.

In this chapter, we summarized the current literature for parathyroid cell differentiation from various sources which might help researchers and clinicians in the near future.

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Regenerative Medicine Application of Mesenchymal Stem Cells

Figen Abatay Sel and Fatma Savran Oguz

Abstract

Mesenchymal stem cell (MSC) has recently generated interest in regenerative medicine. For the definition of MSC, three criteria have been proposed – plastic adherent property, specific surface antigens, and multipotent differentiation capacity. MSC exists in almost all tissues such as synovium, fat, liver, dental pulp, cord blood, Wharton's jelly, and also differentiates into osteoblast, chondrocyte, adipocyte, epithelial, and neuron cells originating from three germ layers. The use of different MSCs for regenerative therapies has been studied over the years as a promising option for treatment of tissue damages and various diseases. Here, the most frequently applied and newly developed stem cell–based techniques are designated, and recent MSC applications knowledge for regenerative medicine in the field are explained.

Keywords

Cell therapy · Mesenchymal stem cell · Regenerative medicine · TERM

Abbreviations

AAV	adeno-associated virus
AD-MSC	adipose tissue–derived mesenchymal stem cell
AKI	acute kidney injury
BM-MSC	bone marrow–derived mesenchymal stem cell
CKD	chronic kidney disease
CRISPR/Cas9	clustered regularly interspaced palindromic repeats/CRISPR-associated protein 9
CXCR4	CXC chemokine receptor 4
G-CSF	granulocyte colony-stimulating factor
GIS	gastrointestinal system
HLA	human leukocyte antigen
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IFN-γ	interferon-γ
iPSC	induced pluripotent stem cell
MHC	major histocompatibility complex
MI	myocardial infarction
MMP-9	matrix metalloproteinase-9
MSC	mesenchymal stem cell
SDF-1	Stromal cell–derived factor-1
sgRNA	single-guide RNA
RM	regenerative medicine
TALEN	transcription activating like effector nuclease
TE	tissue engineering

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TERM	tissue engineering and regenerative medicine
TGF-β	transforming growth factor-β
tracrRNA	trans-activating CRISPR-RNA
TRAIL	tumor necrosis factor–related apoptosis-inducing ligand
UC-MSC	umbilical cord–derived mesenchymal stem cell
UCB-MSC	umbilical cord blood–derived mesenchymal stem cell
VCAM-1	vascular cell adhesion molecule-1
WJ-MSC	Wharton's jelly–derived mesenchymal stem cell
ZFN	zinc finger nucleases

1 Introduction

In recent years, besides pharmaceutical treatments, regenerative medicine (RM) has been an exciting research field by use of cell-based therapy and tissue engineering (TE). The emergence of tissue engineering and regenerative medicine (TERM) can be used for treatment of various diseases and tissue regeneration (Han et al. 2020; Pina et al. 2019).

The mesenchymal stem cell (MSC) was characterized for the first time in the 1970s (Friedenstein et al. 1974). Since then, it has been widely used in a large number of TERM studies. Not only has MSC been used for in vitro and in vivo experimental studies, but also for clinical trials.

The ability of MSC to differentiate has been important and valuable for tissue damages and several diseases. MSC has advantages, as it is easy to obtain compared to the other stem cells, such as induced pluripotent stem cell (iPSC) and embryonic stem cell (Ullah et al. 2015). It can be obtained without the need for an invasive procedure. In addition to these advantages, MSC has been obtained from different autologous and allogeneic sources, and isolated from various tissues, including synovium, fat, liver, dental pulp, adipose, bone marrow, cord blood, and Wharton's jelly (Kim and Park 2017). It can be distinguished with CD29+, CD44+, CD73+, CD90+, CD105+, and lack of CD14-, CD34-, CD45-, and HLA (human leukocyte antigen)-DR expression from other stem cells, and also the ability to adhere to plastic inherently (Dominici et al. 2006).

The extraction, culture, and transplantation of all MSC types have improved, with MSCs derived from different tissue sources. The ability to differentiate into osteocytes, chondrocytes, epithelial, and neuron from three germ layers–derived stem cells makes it a valuable tool for TERM (Futami et al. 2012; Kim et al. 2011) (Fig. 1). These features have gained MSC an effective tool for the different kinds of disease models (Li et al. 2019; Mathew et al. 2019; Zhao et al. 2019; Sun et al. 2018; Doeppner et al. 2015).

In addition to the capacity of differentiation, the variety of sources, and the easier-to-obtain factor, MSC has also immunomodulatory features. The MSC has the ability to secrete cytokines, anti-inflammatory molecules, and immune receptors that regulate the microenvironment of the host tissues and have low immunogenicity by lack of major histocompatibility complex (MHC) and co-stimulatory molecules of T-cell recruitment, which makes MSC safe for allogeneic transplantation. Furthermore, MSC has been reported to ensure immunomodulation in many diseases (Willis et al. 2018; de Witte et al. 2018; Ha et al. 2020; Du et al. 2018). In this present work, we assess the newest development of regenerative medicine application of mesenchymal stem cells in both clinical and experimental studies.

2 Human Mesenchymal Stem Cell Sources

Mesenchymal stem cells are isolated from many different adult tissues such as bone marrow, liver, dental pulp, adipose tissue, and from 'the younger tissues' including placenta, amniotic fluids, amniotic membrane, umbilical cord blood, and Wharton's jelly (Kim and Park 2017). Although obtained from different sources, MSCs share the common features: cell surface markers, a

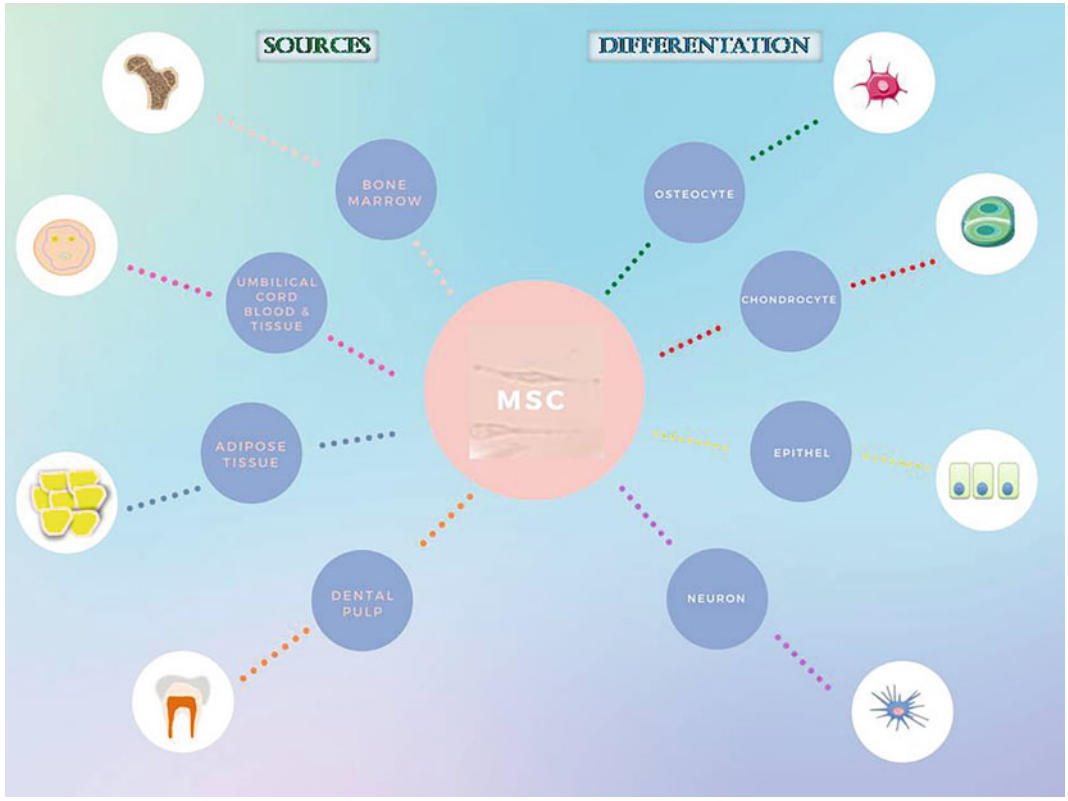

Fig. 1 The frequently used sources of MSC for TERM and their differentiation potentials

trilineage differentiation, plastic adherence, and spindle-shaped appearance (Naji et al. 2019).

Although MSC can be obtained from almost all tissues within the human tissues, the common sources of MSC have been accepted bone marrow and adipose tissue.

But there are some limitations such as invasiveness of obtaining procedure, hard to obtain, and adequate cell source. Bone marrow–derived MSC (BM-MSC) is harvested from the iliac crest of the healthy donors, so obtaining BM-MSC is painful and can cause nosocomial infections, whereas obtaining of MSC from younger birth–derived tissue does not cause any of them. BM-MSC is used mostly for clinical use (Barnhoorn et al. 2020; Wilson et al. 2015; Matthay et al. 2019; Liu et al. 2014).

Besides the invasiveness and possibility of infection of bone marrow–derived MSC, the use of anesthesia may also be necessary, and also the cell yield and potential for differentiation can vary from donor to donor and with donor age. BM-MSC represents 0.001–0.01% of BM mononuclear cells (Bhat et al. 2021). Because of its low abundance, in vitro culturing is required to obtain an adequate amount of MSC for clinical or research studies.

The other adult MSC source is adipose tissue–derived MSC (AT-MSC), usually obtained from the biological materials during liposuction or lipectomy procedures. This AT-MSC isolation approach is easier and safer than BM-MSC and also a considerably larger amount of MSC can be obtained than BM-MSC. Approximately 3.5×10^5 to 1×10^6 MSC can be obtained from 1 g of adipose tissue and the amount is much greater than that obtained from bone marrow (Tsuji et al. 2014). Currently, AT-MSC has become the most widely used approach than BM-MSC for clinical use. It is ideal because of its abundance and less-invasive surgical intervention. There are some reports that exosomes from

AT-MSC stimulate the cardioprotection factors after myocardial infarction (MI) (Xu et al. 2020; Lee et al. 2021).

The MSC can be also isolated from "the younger" neonatal tissues, including umbilical cord, umbilical cord blood, conjunctive tissues of umbilical cord, Wharton's jelly (Hass et al. 2011). These neonatal-derived MSCs are more easily accessible, noninvasive, and have larger amount than the adult-derived MSC. Umbilical cord–derived MSC (UC-MSC) and Wharton's jelly–derived MSC (WJ-MSC) are more superior than umbilical cord blood–derived MSC (UCB-MSC) in terms of its abundance (Naji et al. 2019; Hass et al. 2011; Laroye et al. 2019). WJ-MSC has been the focus for RM due to its abundance and easily accessible features. Although easily available, WJ-MSC can be isolated from Wharton's jelly after removal of blood vessels carefully which need to be performed by the experienced researchers.

3 Genetically Modified Mesenchymal Stem Cells Therapy

Mesenchymal stem cells have become important for TERM due to their self-renewal and multilineage differentiation. Recently, several genetic engineering techniques have been used for modifying the MSC gene expression profile. These techniques can be classified as the nonviral methods, the viral vectors, and the new-generation nonviral genetic methods, as seen in Fig. 2.

Nonviral methods can be performed by physical- or chemical-based methods. The most important advantages of both physical and chemical methods are that they have low immunogenicity and cause cytotoxicity. Nonviral methods are also easy to produce and have lower cost than viral methods that make them attractive for using MSC therapies. The physical methods used for MSC include electroporation, nucleofection, and sonoporation techniques. Electroporation is a specific technique which applies transient electric to some parts of cells in suspension, allowing to open the cell pores and the entry of nucleic acids from cell membrane to cytoplasm. Electroporation is widely used for MSC (Wang et al. 2014), but it has a hurdle to overcome the cytotoxicity. The latter technique is nucleofection, which enables to ease permeability of cell membrane. The short high-voltage pulses are used to increase the permeability, and thus target deoxyribonucleic acid/ribonucleic acid (DNA/RNA) transferred to the stem cell. The limitation is the technique is low cell viability (Aslan et al. 2006). The last and most interesting physical membrane disruption technique is sonoporation. Each tissue and organ of the human has different acoustics, all of which can generate specific echoes. This method aims to change the permeability of the cell membrane by acoustic microbubbles by using specific ultrasonic waves and allowing macromolecules, DNA, or RNA into the cell (Miller et al. 2002). Nonviral chemical methods are basically designated the same: A target molecule which is loaded by a carrier such as lipid, polymer, or nanoparticle, and these carriers with their cargo molecules enable cellular uptake (Ramamoorth and Narvekar 2015).

In genetically modified MSC therapy, viral vector methods are the most used tools due to their efficiency of transduction into the stem cells. This method preserves the transgene through several passages and provides the stable transgene expression during cell division and differentiation (Zare et al. 2016). So, compared to the nonviral methods, viral methods for genetic modification of MSC have greater transduction efficiency, as well as long-term and stable transgene expression. There are several types of vectors that include mainly retrovirus, lentivirus, adenovirus, and adeno-associated virus (AAV). Currently, AAV has been mostly used for gene therapy among these viral vectors.

Retroviruses are RNA viruses that have specific genetic organization including gag, pol, and env genes which encode the internal structural proteins, reverse transcriptase/integrase, and envelope glycoprotein, respectively. These genes are arranged in separate plasmids to avoid the recombination of the viral replication. Lentiviruses have similar genes to encode the related enzyme, protein, and envelope proteins,

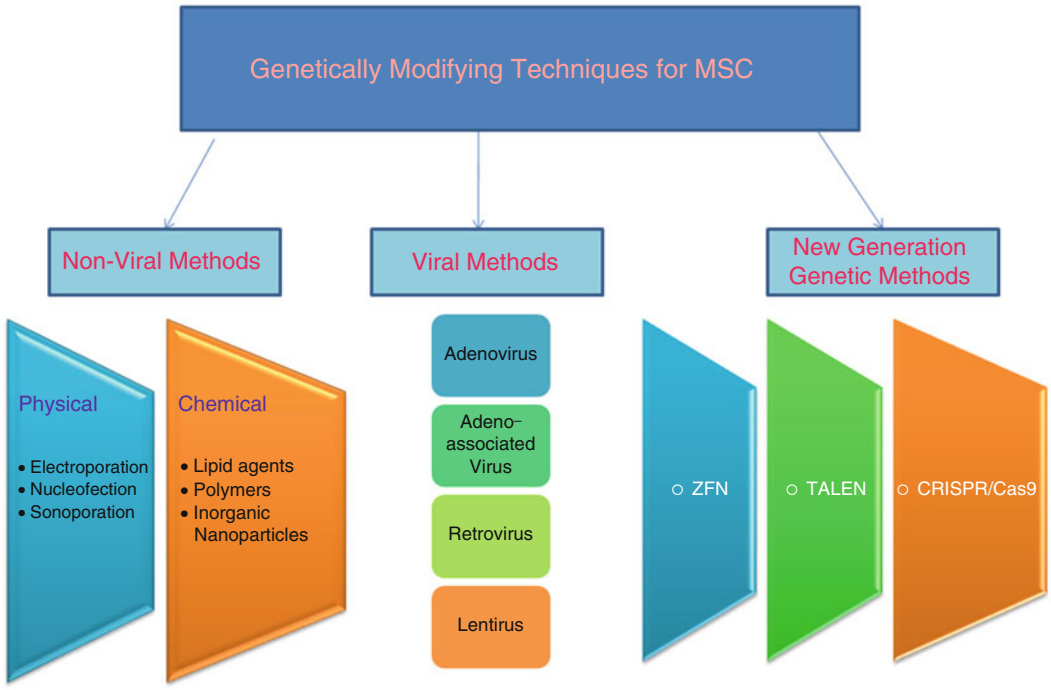

Fig. 2 Genetically modifying techniques for MSC sources

but retrovirus vector remains unable for quiescent cells, whereas lentivirus is capable of being transferred to dividing and quiescent cells (Balvay et al. 2007; Vargas et al. 2016). Unfortunately, studies with retroviruses have yielded unsatisfactory results. A clinical trial for nine patients with X-linked severe combined immunodeficiency, which used hematopoietic stem cells were genetically modified with retrovirus resulting in leukemia for four patients in follow-up period of 10 years (Hacein-Bay-Abina et al. 2010; Oggu et al. 2017, Hamann and Nguyen 2019). On the other hand, clinical stem cell trial with lentivirus vector for the patients with metachromatic leukodystrophy was applied and no adverse effects related to vector have been reported yet (Sessa et al. 2016).

Adenoviral vectors are not able to integrate into the host genome and can transfer into dividing and quiescent cells. This recombinant vector provides easy and high efficiency but high immunogenicity and transient expression of transgene are the limitations that must be overcome. In addition to these, adenovirus vector can activate both humoral and cellular immune responses which may increase the risk factors due to use of high-dose adenovirus vectors and finally autoimmunity and cytotoxicity responses may occur. AAV, which takes its place, versus these three frequently used vectors, has been accepted as the best solution for long-term and stable expression of transgene and inducing mild immune response (Colella et al. 2017). But there is a big limitation of AAV that neutralizing antibodies reduce their effectiveness in vivo. AAV vectors can also infect both dividing and quiescent cells and do not integrate into the genome of the host cell, like adenoviral vectors.

Recently, new-generation nonviral genetic modification methods arose for correction of mutation of a gene such as insertion, deletion, or translocation at a specific site of the genome. For several diseases, gene-modified MSC has been used as a tool for these new-generations methods (Torres-Ruiz et al. 2017; Hu et al. 2017; Lee et al. 2019b; Li et al. 2018; Cho et al. 2017; Benabdallah et al. 2010). The first generation of these methods was zinc-finger nucleases (ZFN).

ZFN are the most common DNA-binding domain among eukaryotes. ZFN also has a heterodimer structure that is composed of a zinc-finger domain and Fok1 endonuclease domain and endonuclease domains must dimerize when it can create the double-strand breaks (Carroll 2011). Each zinc-finger domain is capable of recognizing 3–6 nucleotide bases and they are composed of approximately 30 amino acids that interact with nucleotide triplets. After cleavage, MSC tries to repair the breaks by nonhomologous end joining or homology-directed repair mechanism to fix the double-strand break. Transcription activator like effector nuclease (TALEN) is a fusion protein that is similar to ZFN. TALEN utilizes DNA-binding motifs to cleave the genome at a specific site. Compared to ZFN, instead of recognizing the DNA triplets, each domain can recognize a single nucleotide. The interaction between DNA-binding domains and target nucleotides are less complex than ZFN, but the TALEN method is cheaper and can produce results faster than ZFN (Gaj et al. 2013). The latest new generation molecular-editing tool is clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9 (CRISPR/Cas9) which is derived from bacteria while both ZFN and TALEN are man-made. CRISPR/Cas9 system is based on an RNA-based bacterial defense mechanism designed to eliminate the foreign DNA from bacteriophage and plasmids. CRISPR/Cas9 system consists of two types of RNA (Trans-activating CRISPR-RNA(tracrRNA), also known as "tracer RNA," has the recognition sequence necessary for recognition by Cas9. The second RNA is the single-guide RNA (sgRNA) which is artificially programmed and can direct the target specific regions of DNA) and Cas9 endonuclease (Jiang and Doudna 2017). Briefly, the Cas9 enzyme can cleave the target sequences by sgRNAs. CRISPR/Cas9 system is cheaper and more efficient than other two methods and can be designed for many genomic targets and multiplexed by adding lots of numbers of sgRNAs. With the development of technology, it is estimated that the mechanisms of disease can be investigated by using genetically modified MSC, especially in disease model studies.

4 Applications of Mesenchymal Stem Cell for Cardiovascular and Cerebrovascular Diseases

Cardiovascular diseases are the leading cause of death around the world. According to the World Health Organization (WHO), approximately 18 million people died from cardiovascular diseases in 2019 (WHO https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)). Cardiovascular diseases are a group of disorders which includes of heart, blood, muscle, and vessels. The cardiovascular diseases can be divided six groups:

1. Coronary heart disease
2. Cerebrovascular disease
3. Congenital heart disease
4. Peripheral arterial disease
5. Rheumatic heart disease
6. Deep vein thrombosis and pulmonary embolism

Each of them affects different parts of the heart, tissues, and organs. Heart attacks and strokes are the most common events and caused by a blockage of vessels supplying heart and brain (Li et al. 2017). The most common reason for this blockage is lipid deposits on the inner walls of the vessels and also this dyslipidemia leads to a complicated disorder, called atherosclerosis, which causes chronic inflammation. Recently, there are promising MSC therapy studies on atherosclerosis (Frodermann et al. 2015). MSC has regenerative properties as well as immunomodulatory capacities (Mahdavi Gorabi et al. 2019). Especially experimental in vivo studies indicate that MSC therapy for atherosclerosis affects not only inflammatory responses but also reduces dyslipidemia, so MSC therapy has a multifactorial potential for inhibiting atherosclerosis (Frodermann et al. 2015).

On the other hand, there are other main goals for cardiac regeneration which are the production of the replacement myocardial mass, the formation of the functional vascular system, and the reformation of impaired vessels. Phenotypic differentiation of MSC plays an important role for

reformation of impaired vessels. Angiogenesis is a highly complex process and difficult to maintain the adequate tissue perfusion. A new promising pro-angiogenic MSC therapy provides the use of angiogenic cell sources to develop new vascularate from the sources of umbilical cord blood (Iqbal et al. 2017). According to the protocol, the aortic ring assay provides accessory cells and extracellular matrix to exclude inflammatory components while reformation of the vessels takes place. In this process, MSC acts as pericytes and perivascular cells are essential to vascularization for regeneration of the vascular networks. Myocardial infarction which is caused by the blockage of one or more coronary arteries cannot provide the heart with oxygenated blood and leads to heart tissue damages and finally can cause a heart attack. There are a number of studies to repair the tissue damage caused by MI (Luo et al. 2017; Adamiak et al. 2018; Xu et al. 2004; Toma et al. 2002). The paracrine effect of MSC plays an important role to repair wound and tissue damages when MI occurs (Dittmer and Leyh 2014).

Ischemic stroke is a serious and aggressively evolving cerebrovascular disease with high morbidity and disability. It is similar to heart attack but it occurs with blood clots in the vessels of the brain, and these clots block the blood flow to the brain's cells. The in vitro and in vivo MSC therapies have been a novel approach to cure ischemic stroke and the studies indicated that early administration of MSC within 7 days after ischemic stroke may be the best treatment approach (Guo et al. 2021). In the MSC-based treatment of ischemic stroke, there is more than one mechanism to establish and monitor. Immunoregulation, neuroprotection, angiogenesis, and neuronal circuit reconstructions have to be monitored for MSC therapy. Immunoregulation plays an important role in the stroke due to it releasing proinflammatory cytokines and chemokines from damaged brain tissue by disrupted blood–brain barrier. The continuous infiltration of proinflammatory cytokines and chemokines through the brain's cells can cause neuronal necrosis and even secondary brain injury (Shichita et al. 2014). Particularly, inflammatory cytokines affect the neuronal cells by causing invasion of chemokines and chemoattractants through the disrupted blood–brain barrier. MSC has also an important role for neuroprotection. Cheng et al. have found that MSC reduces the leakage of proinflammatory cytokines through the blood–brain barrier and also allows release of neutrophil matrix metalloproteinase-9, which plays a role in the recruitment of neutrophil to the inflammatory sites (Cheng et al. 2018). Furthermore, decreased neuronal apoptosis was indicated in the ischemic area after application of MSC (Zhu et al. 2014).

The other important issue after ischemic stroke is regeneration of the capillary vessels. The capillary vessels can be destroyed after stroke. The neovascularization helps to restore the blood and oxygen supply of the affected stroke area. After stroke, MSC administration can secrete angiogenic factors and differentiates into endothelial cells for inducing angiogenesis to promote tissue regeneration (Yong et al. 2018). The last and the most important issue is neuronal circuit reconstruction after ischemic stroke due to myelin destruction. It is believed that MSC can increase the oligodendrocyte progenitor cells in the ischemic area (Shen et al. 2006; Tobin et al. 2020).

For cardiovascular and cerebrovascular diseases, AD-MSC and BM-MSCs are the most commonly used sources. The abundance of MSC derived from adipose tissue and ease of acquiring adipose with the liposuction operation makes adipose tissue source feasible for experimental studies and clinical trials. There is a number of completed clinical trials as seen in Table 1.

5 Applications of Mesenchymal Stem Cell for Lung Diseases

Pulmonary disease is a general term for several disorders that include lung tissue diseases, lung circulation diseases, and also respiratory failure. Especially, pulmonary pathologies are commonly due to exposure of tobacco, smoke, and polluted air, which may lead to develop acute or chronic lung disorders. There is no cure for lung diseases but the inhalers and stopping smoking may relieve symptoms. Modified MSC can be used for lung diseases and there are a number of studies using MSC from different kinds of sources for

Table 1 Completed clinical trials related to several diseases

Application category	Trial identifier	Condition/disease	Sources of treatment	Enrollment
CV–CV	NCT01913886	Ischemic cardiomyopathy	Autologous BM-MSC	10 participants
CV–CV	NCT02501811	Ischemic cardiomyopathy	Autologous BM-MSC	125 participants
CV–CV	NCT02509156	AI cardiomyopathy	Allogeneic MSC	46 participants
CV–CV	NCT01739777	Dilated cardiomyopathy	UC-MSC	30 participants
CV–CV	NCT02635464	Chronic ischemic cardiomyopathy	Allogeneic UC-MSC	50 participants
CV–CV	NCT00768066	CILVD	Autologous BM-MSC	65 participants
CV–CV	NCT01302015	Buerger's disease	Autologous AT-MSC	15 participants
CV–CV	NCT00114452	Myocardial infarction	Adult MSC	60 participants
CV–CV	NCT01392625	Nonischemic dilated cardiomyopathy	Autologous and allogeneic BM-MSC	37 participants
CV–CV	NCT02467387	Nonischemic heart failure	Allogeneic BM-MSC	23 participants
CV–CV	NCT03418233	Heart failure	WJ-MSC	115 participants
CV–CV	NCT02387723	Heart failure	Allogeneic AT-MSC	10 participants
CV–CV	NCT01484574	Critical limb ischemia Buerger's disease	Ex vivo Cultured Adult BM-MSC	90 participants
CV–CV	NCT00629018	Dilated cardiomyopathy	Autologous BM-MSC	110 participants
CV–CV	NCT00644410	Congestive heart failure	BM-MSC	59 participants
CV–CV	NCT02013674	CILVD, MI	BM-MSC	30 participants
CV–CV	NCT01392105	Acute myocardial infarction	Autologous BM-MSC	80 participants
CV–CV	NCT03371329	Hemorrhagic stroke, intracerebral hemorrhage	Allogeneic BM-MSC	9 participants
CV–CV	NCT01076920	CILVD, MI	BM-MSC	10 participants
CV–CV	NCT04421274	MI	BM-MSC	43 participants
CV–CV	NCT00911365	Multiple system atrophy	Autologous MSC	27 participants
CV–CV	NCT00875654	Ischemic stroke	Autologous BM-MSC	31 participants
CV–CV	NCT01678534	Ischemic stroke	Allogeneic AT-MSC	19 participants
CV–CV	NCT01291329	MI	Human WJ-MSC	160 participants
CV–CV	NCT00260338	Myocardial ischemia, coronary heart disease	Autologous MSC	31 participants
CV–CV	NCT00883727	MI	Ex vivo cultured adult allogeneic MSC	20 participants
CV–CV	NCT00877903	Acute MI	Ex vivo cultured adult allogeneic MSC	220 participants
CV–CV	NCT02672267	MI	Allogeneic BM-MSC	50 participants
CV–CV	NCT01753440	CAD, ICM	Mesenchymal Precursor cells	11 participants
CV–CV	NCT01351610	PAD, critical limb ischemia	Autologous CD34-MSC	25 participants
CV–CV	NCT02378974	Cerebral infarction	Human UC-MSC	19 participants
CV–CV	NCT01449032	Chronic ischemic heart disease	AT-MSC	60 participants
CV–CV	NCT00629096	Dilated cardiomyopathy	Autologous BM-MSC	27 participants
CV–CV	NCT01468064	Stroke, infarction, middle cerebral artery	Autologous BM-MSC and Endothelial progenitor cells	20 participants
CV–CV	NCT01297413	Ischemic stroke	Allogeneic adult BM-MSC	38 participants
CV–CV	NCT02032004	Chronic heart failure	Allogeneic BM-MSC	566 participants
CV–CV	NCT00135850	Ischemic heart disease	HSC and MSC	48 participants
LUNG	NCT02594839	Interstitial lung disease	BM-MSC	20 participants

(continued)

Table 1 (continued)

Application category	Trial identifier	Condition/disease	Sources of treatment	Enrollment
LUNG	NCT04382547	COVID-19-associated pneumonia	Allogenic pooled olfactory mucosa MSC	32 participants
LUNG	NCT01919827	Idiopathic pulmonary fibrosis	Autologous BM-MSC	17 participants
LUNG	NCT01385644	Idiopathic pulmonary fibrosis	Placental MSC	8 participants
LUNG	NCT02625246	Bronchiectasis	Allogeneic BM-MSC	6 participants
LUNG	NCT02181712	Lung transplant rejection–bronchiolitis obliterans	Allogeneic BM-MSC	19 participants
LUNG	NCT02804945	ARDS	BM-MSC	20 participants
LUNG	NCT04898088	COVID-19 pneumonia	MSC	30 participants
LUNG	NCT04288102	Severe COVID-19	UC-MSC	100 participants
LUNG	NCT04349631	COVID-19	Autologous AT-MSC	56 participants
LUNG	NCT04522986	Severe pneumonia caused by SARS-CoV-2 infection	AT-MSC	6 participants
LUNG	NCT04355728	COVID-19	UC-MSC	24 participants
LUNG	NCT04573270	COVID-19	UC-MSC	40 participants
LUNG	NCT05019287	COVID-19	Menstrual blood MSC	29 participants
LUNG	NCT04400032	COVID-19, ARDS	UC-MSC	15 participants
KIDNEY	NCT02166489	Chronic renal failure due to polycystic kidney disease	Autologous BM-MSC	6 participants
KIDNEY	NCT02195323	Chronic kidney disease	Autologous BM-MSC	7 participants
GIS	NCT01157650	Fistulous Crohn's disease	Autologous AT-MSC	15 participants
GIS	NCT01342250	Liver cirrhosis	UC-MSC	20 participants
GIS	NCT04243681	Cirrhosis	Autologous HSC and MSC	5 participants
GIS	NCT02445547	Crohn's disease	UC-MSC	82 participants
GIS	NCT01220492	Liver cirrhosis	UC-MSC	266 participants
GIS	NCT01062750	Liver cirrhosis	Autologous AT-MSC	4 participants
GIS	NCT01591200	Liver cirrhosis	Allogeneic BM-MSC	40 participants
CANCER	NCT01089387	Prostate cancer	BM-MSC	18 participants
CANCER	NCT01207193	Bone cyst	BM-MSC	6 participants
CANCER	NCT00498316	Myelodysplastic syndrome	CB-MSC	98 participants
CANCER	NCT02530047	Ovarian cancer	Human Interferon-beta Induced MSC	5 participants
CANCER	NCT03106662	Hematological malignancies, GVHD, Haploidentical HSCT transplantation	BM-MSC	6 participants
CANCER	NCT01844661	Solid tumors with metastasis in pediatric patients	Autologous BM-MSC	20 participants
CANCER	NCT02509156	Cardiomyopathy due to anthracyclines	Allogeneic MSC	46 participants

CV–CV cardiovascular and cerebrovascular diseases, *GIS* gastrointestinal diseases, *AI cardiomyopathy* anthracycline-induced cardiomyopathy, *CILVD* chronic ischemic left ventricular dysfunction, *MI* myocardial infarction, *CAD* coronary artery disease, *ICM* ischemic cardiomyopathy, *PAD* peripheral artery disease,*COVID*-19 Corona virus disease 2019, *ARDS* acute respiratory distress syndrome, *SARS-CoV*-2 severe acute respiratory syndrome coronovirus-2, *GVHD* graft-versus-host disease, *BM-MSC* bone marrow–derived mesenchymal stem cell, *UC-MSC* umbilical cord–derived mesenchymal stem cell, *AT-MSC* adipose tissue–derived mesenchymal stem cell, *WJ-MSC* Wharton's jelly–derived mesenchymal stem cell, *HSC* hematopoietic stem cell, *CB-MSC* Cord blood–derived MSC

the treatment of lung diseases (Guo et al. 2013; Varshney et al. 2016).

Pulmonary arterial hypertension is one of the life-threatening conditions which leads to high blood pressure in the lungs. It has four stages, and the fourth stage is the worst among them. The blood vessels that carry blood from heart to lungs become narrow and harder and it affects any type of physical activities. Although there is no cure for pulmonary arterial hypertension, there are medications and procedures that can slow the progression. In an experimental treatment of pulmonary arterial hypertension, they found that in human HGF-expressing mouse bone marrow–derived MSCs reduced pulmonary arterial pressure (Guo et al. 2013). In the study, they have established five groups to determine the pulmonary arterial hypertension healing. To elucidate molecular mechanism of pulmonary arterial hypertension, they have evaluated the gene and protein levels of vascular cell adhesion molecule-1 (VCAM-1), endothelin-1, transforming growth factor-β (TGF-β), and matrix metalloproteinase-9 (MMP-9). They have observed that vessel muscularization of thickening, collagen deposition, TGF-β, and endothelin-1 concentrations in the pulmonary hypertensive rats were decreased. When they have compared the five groups, they have concluded that HGF + MSC and HGF + MSC + granulocyte colony–stimulating factor (G-CSF) groups exhibited significantly reduced right ventricular hypertrophy and pulmonary arterial pressure. For another and similar study based on monocrotaline-induced pulmonary hypertension rat, Zhang et al. have observed that pulmonary arterioles endothelial mesenchymal transition was suppressed and human umbilical cord–derived MSC exosomes upregulated the hypoxic pulmonary vascular cells (Zhang et al. 2020). An interesting antiaging gene, Klotho, slows the aging when overexpressed and when it is disrupted, it causes premature aging process (Xu and Sun 2015; Buchanan et al. 2020). In addition to this antiaging process, Klotho gene mutation causes systemic inflammation in the lungs. Varshney et al. have established five groups of monocrotaline-induced pulmonary arterial hypertension rats and also have used genetically modified MSC with lentiviral vectors expressing secreted Klotho protein for treatment of pulmonary (Varshney et al. 2016). In conclusion, they have suggested that genetically modified MSC treatment was an effective therapy option for pulmonary arterial hypertension, vascular endothelial dysfunction, and decreasing macrophage infiltration in the lungs.

Chronic obstructive pulmonary disease is a serious detrimental lung disease which leads to airflow blockage and breathing difficulty. People who suffer from chronic obstructive pulmonary disease are at increased risk of lung cancer, heart diseases, chronic bronchitis, and several other conditions. Experimental and clinical MSC therapy holds great promise for the treatment of chronic obstructive pulmonary disease at early stages (Weiss et al. 2013; Liu et al. 2016). A recent pilot clinical umbilical cord–derived MSC study has indicated that safe therapy options for the patients with moderate or severe chronic obstructive pulmonary disease (Le Thi et al. 2020). They have suggested that this option may improve the quality of patients' life as well. On the contrary, Khedoe et al. have established a lipopolysaccharide-induced inflammation experimental model in mice which leads to chronic obstructive pulmonary disease and obtained bone marrow–derived MSC from female mice 8–10 weeks of age. They have observed that MSC treatment results showed no effect on inflammation or emphysema.

In conclusion, MSC therapy shows promise for the treatment of lung diseases, especially when administered at early stages in clinical trials (Cruz and Rocco 2020). Moreover, clinical MSC administration has been reported as safe but associated with limited effects on clinical outcomes. Further studies are required to understand the best source option and optimal dose of MSC and standardization of MSC application for lung diseases.

6 Applications of Mesenchymal Stem Cell for Kidney Diseases

There are two main types of kidney diseases: acute kidney injury (AKI) and chronic kidney disease (CKD). Both of them have different mechanisms in kidney damage formation. AKI is temporary kidney function loss whereas patients with CKD have irreversible and lifelong damage in kidney. Many patients fully recover from AKI and continue to live fully healthy. However, if significant damages have occurred, there is a high risk of developing CKD after AKI. So, after AKI, it is highly recommended to monitor the health of the kidney.

Also known as acute renal failure, AKI is a public health problem. Despite supportive therapy administrations, the morbidity and mortality among patients still remain high. Although renal transplantation has been used to support life of patients with AKI, there are no specific therapies approved for kidney regeneration. Recently, lots of experimental stem cell–based studies have been reported for the treatment of kidney regeneration. An important research related to MSC therapy to porcine renal ischemia–reperfusion model has indicated that MSCs are capable of delivering to glomeruli and can survive for 8 h after intravenous administration (Sierra-Parraga et al. 2019). In this study, MSCs were obtained from male pig adipose tissue and fluorescently labeled. Then, they injected MSCs to female pigs to trace the MSCs by Y chromosomes. In conclusion, they have reported that a low percentage of MSCs were shown in the kidney 14 days after the injection, and the MSCs can allow them to ameliorate the injured kidney and cause a regenerative effect.

The researchers also monitor several chemotactic signals or protection factors as a sign of MSC to detect the treatment efficacy. The mobilization of MSC is associated with the CD44 and stromal cell–derived factor-1 (SDF-1)/CXC chemokine receptor 4 (CXCR4) interactions (Sun et al. 2019). MSC can migrate with CD44/SDF-1 axis. Liu et al. have reported that CXCR4 expression in BM-MSC increases migration in mice and compared to the control group, increased numbers of CXCR4 expression were determined in kidneys at 7 days after transplantation (Liu et al. 2013). Roudkenar et al. have generated a modified MSC that Lipocalin-2 was upregulated and they injected it to female rats. Finally, their study has indicated that Lipocalin-2-mediated MSC ameliorates kidney functions (Roudkenar et al. 2018).

The number of individuals with CKD has become a worldwide public health problem and affects approximately 10% of the world population (Bochon et al. 2019; Peired et al. 2016). CKD is characterized by reduced renal regeneration and is more progressive than AKI. The patients diagnosed with CKD generally develop end-stage renal disease, which leads to administration of renal transplantation in time. However, the increase in the organ transplantation waiting list due to the increasing number of CKD cases inversely proportional to the insufficient organ donation volunteers has pushed the researchers to apply MSC for experimental treatment of CKD. Recently, pioglitazone treatment of CKD-MSC has been reported. Yoon et al. have isolated MSC from CKD patients and they used pioglitazone to protect from endoplasmic reticulum (ER) stress and oxidative stress (Yoon et al. 2019). In this study, they have used immunofluorescence staining to measure the mitochondrial dynamics of CKD-MSC, and overall results have indicated that the pioglitazone can be used as a protector agent for the mitochondrial function of CKD-MSC against ER stress. Similar experimental treatments of tauroursodeoxycholic acid–treated MSC have been used for in vivo CKD-MSC in murine CKD model (Lee et al. 2019b). In this murine model study, they have isolated AD-MSC from CKD patients and treated it with tauroursodeoxycholic acid for 24 h. They have found that treated CKD-MSC prevented ER stress in the hippocampus of CKD mice and concluded that this treatment could be a powerful strategy to ameliorate CKD as well as neuronal dysfunction related to ER stress.

To summarize, although experimental and clinical data on the safety of the MSC

administration are encouraging, further studies are needed with prudence (Makhlough et al. 2018).

7 Applications of Mesenchymal Stem Cell for Gastrointestinal Diseases

The gastrointestinal system (GIS) consists of more than one organ, such as mouth, pharynx, stomach, small and large intestines, rectum, liver, and pancreas. The principal functions of the GIS are to digest the nutrients and to excrete the waste products of digested nutrients, and their mechanisms are extremely complex. The conditions and GIS-related diseases can affect GIS and the balance of its mechanisms.

Intestinal diseases can affect the several organs from the duodenum to the rectum. Constipation, diverticular diseases, irritable bowel syndrome (IBS), and inflammatory bowel disease (IBD) are very frequent among the population. IBS is described as abdominal discomfort including altered bowel habits (Ford et al. 2020). It can take years to diagnose IBS, because symptoms are very similar to other conditions, such as giardia and food allergies. Some of them are more likely constipation, others diarrhea, and a few of them both constipation and diarrhea. These altered stool conditions cause the damage in the bowel. The health of patients with IBS is affected as well as their social lives. The dietary changes or medication are recommended for patients with IBS and also the treatment depends on what type of IBS one has and how many years passed when they get diagnosed. On the other hand, IBD can be defined as the destruction of bowel wall–related inflammation which can lead to narrowing of the intestine and MRI and CT are used to determine how much of the bowel is affected for IBD diagnosis. IBD can cause destructive inflammation and permanent harm to the bowel wall which can lead to colon cancer. There are a number of experimental studies for bowel and mucosal tissue related to IBS and IBD to solve the problem related to healing damaged tissue. One of them has been conducted by Yang et al. in which they have developed human iPSC-derived MSC and injected intraperitoneally into IBD mice model. In conclusion, they have reported that iPSC-MSC provided epithelial cell proliferation and healed the mucosal colitis via tumor necrosis factor-α-stimulated gene 6 (Yang et al. 2019).

A malign type of IBD, Crohn's disease causes GIS to become inflamed and irritated. In many people, Crohn's disease is monitored as a chronic condition, while others may have remission status. There is no currently accepted cure for Crohn's disease, but the steroids are used for the relief of inflammation pain. Recently, the clinicians have conducted clinical trials as there are no approved drugs for Crohn's disease. A controlled randomized clinical trial based on UCB-MSC for Crohn's disease has showed great success (Zhang et al. 2018). They have used UCB-MSC on 24 male and 17 female patients and in the control group, 26 male and 15 female patients were added to the study. For the control group, they continued to give background treatment while in the trial group, the patients received UC-MSC infusion, once a week, four times in total, and they have been followed 3, 6, 9, and 12 months after the infusion doses were completed. Finally, they have reported that only four patients had fever after the infusion, which was relieved after symptomatic treatment and no adverse effects. Further, they have compared the two groups in the aspect of blood count and liver and renal function and reported that there were no significant differences. Furthermore, they have stated that six patients with concomitant anal fistula showed great improvement. In another double-blind, 49-center participated study, it was reported that allogeneic, expanded AD-MSC can be promising for the treatment of patients with Crohn's disease (Panés et al. 2016). They have used AD-MSC to the patients' perianal fistula area and reported that 53 of 107 patients achieved remission.

The late stage of scarring of the liver is known as cirrhosis and caused by long-term liver damage. Common causes of liver cirrhosis are excessive alcohol consumption, hepatitis, and nonalcoholic fatty liver diseases, and there is no approved therapy for liver cirrhosis (Basaranoglu

et al. 2015; Roehlen et al. 2020). However, liver transplantation is an option for the end-stage liver cirrhosis when the liver is failing. In clinical trials on cirrhosis, the therapeutic potential of MSC based on differentiation capacity to hepatocytes has been investigated, and so far the results are satisfactory for liver regeneration. MSCs increase liver regeneration, reduce liver apoptosis, thereby improving liver functions (Yang et al. 2021). For MSC-related liver regeneration, it is believed that MMPs play a great role in the damaged area of the liver (Fukushima et al. 2021). In this study, the liver fibrosis murine model was established by the administration of carbon tetrachloride, and then BM-MSC was examined as a therapeutic option. They have reported that MMP-14, which is secreted by BM-MSC into the targeted area of the liver, improved survival rates and reduced the fibrosis. It is believed that liver regeneration of MSC is related to its immunomodulatory features (Sharma et al. 2014). Besides its tendency to migrate to the damaged area, it also secretes some soluble factors such as nitric oxide, HLA-G, IL-6, and IL-10. It is suggested that these factors regulate the proliferation and functions of immune cells in liver regeneration. Further studies, even multicenter clinical trials, should be developed to better understand this issue and investigate the basis of liver regeneration.

8 Applications of Mesenchymal Stem Cell for Cancer

One of the causes of more than 10 million deaths per year worldwide is cancer (Sung et al. 2021). Metastasis can be defined as the uncontrolled growth of a cell and its infiltration to other tissues and organs. It is one of the major factors influencing the malignancy of cancer and was first suggested by the British surgeon Stephen Paget with the phenomenon of metastasis, the "seed and soil" theory (Paget 1989; Ribatti et al. 2006). Metastasis and tumor microenvironment in cancer formation are affected by mechanisms such as unlimited angiogenesis, inhibition of tumor suppressor genes, activation of cancer-initiating oncogenes, suppression of apoptosis known as programmed cell death, and thus unlimited growth (Quail and Joyce 2013).

Chemotherapy and radiotherapy frequently have been used for cancer patients so far, but these conventional therapies have shown poor efficacy especially for the cancer of end/advanced stages. In recent years, the researchers have been attracted stem cell applications in the field of cancer. Among these cell therapies, MSC therapy has been used as "Trojan horses" due to its potential feature related to chemical agent secretion to the tumor microenvironment (Hmadcha et al. 2020). The changes in the tumor microenvironment during tumor growth are important for MSC tropism against tumor. The MSCs express the CXCR4 receptors for antitumor effect in the tumor microenvironment (Zheng et al. 2019). Especially for leukemia, the CXCR4/CXCL12 axis is believed to be protective when chemotherapeutic drugs are used (Cancilla et al. 2020).

In many hematologic cancer types, T-cell activation is important for immune response and interferon-γ expression is the first cytokine secreting after T-cell activation. IFN-γ secreting is booster signal for T-cell activation to promote the TRAIL pathway. Yenilmez et al. have reported that MSC combined with IFN-γ induced apoptosis in breast cancer cell lines by tumor necrosis factor–related apoptosis-inducing ligand (TRAIL) pathway (Yenilmez et al. 2020). MSC combined with IFN-γ have been also suggested that they may ameliorate the graft-versus-host disease after allogeneic hematopoietic stem cell transplantation (Kim et al. 2018).

MSC application on solid tumor is controversial, and epithelial–mesenchymal transition feature of MSC is held responsible for tumor growth. On solid tumors, the MSC-based therapies are dependent on the migration feature of MSC into the tumor sites. MSCs are attracted toward tumor sites as a result of various molecules secreted by the tumor changing the tumor microenvironment. A subgroup of naïve MSCs arriving in the tumor microenvironment are able to transform into cancer-associated fibroblast (CAF). The CAFs are believed to contribute to the development of cancer, angiogenesis, metastasis, and the

suppression of apoptosis (Hass 2020). On the other hand, some studies have shown that MSC can change its cell fate. In an in vitro study by Hogan et al., it was indicated that human BM-MSC changed their cell fate differently for HT-29 and HCT-116 colorectal cell lines (Hogan et al. 2013). BM-MSCs have been observed to have increased tumor proliferation for HT-29, while BM-MSC have been indicated for decreased HCT-116 tumor growth. This double-edged sword of MSC feature has not been clarified yet. However, the researchers have noted that these different results might depend on many criteria, such as in vitro/in vivo studies, the type of MSC source used, the choice of MSC isolation, especially the medium in the culture stage (pH change, hypoxia conditions, etc.), and the selection and content of the medium used in the culture stage of MSC (Hass 2020).

On the other hand, there are also groups that report satisfactory results related to MSC as a strong tool for cancer treatment. Kostadinova et al. have reported that the long-term cultivation of BM-MSC and AD-MSC decreased the prostate carcinoma cell growth (Kostadinova et al. 2020). In the study, they have obtained MSCs from human adipose tissue and human bone marrow. Then, they have incubated MSC and PC-3 cell lines for 5 or 9 days and finally reported that increased apoptosis rate of prostatic cancer cells can be considered as an antitumor effect of MSCs. Similar to the antitumorigenic effect of MSC, there are also studies showing that UCB-MSC induces apoptosis in glioblastoma (Fan et al. 2020; Kim et al. 2010).

In conclusion, it has been suggested that the further studies are needed to understand mechanisms by which MSC is effective in cell fate, and also integrative technologies and MSC has to be generated for cancer treatment such as drug carrier MSC, genetically modified MSC, etc. (Ho et al. 2020; Srifa et al. 2020).

9 Conclusion

Regenerative medicine is a promising multidisciplinary field for tissue damage and several diseases. The continuous studies on MSC and the development of technology have increased the potential use of MSC in regenerative medicine. In years, many MSC studies have described the features and potential use of MSC. Then, many stem cell sources have been used in this field, experimental or clinical for the treatment options.

In the near future, it is estimated that MSCs combined with gene therapy, particularly, can be a great tool for several diseases. In addition to this, with the technology development, it is reported that MSC also can be used as a tool in disease models and shed light on solving the causes and mechanisms of the diseases. Therefore, as regenerative medicine continues to discover the different uses of MSC and the mechanisms that play on differentiation, plasticity, and double-edged sword features of MSC, as well as developments to evaluate and control these factors, the potential MSC use of regenerative medicine will grow.

Conflict of interest Authors declared no conflict of interest.

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Identification of Small Molecules That Enhance the Expansion of Mesenchymal Stem Cells Originating from Bone Marrow

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Abstract

Mesenchymal stem cells (MSCs) have been shown to be promising for regenerative medicines with their immunomodulatory characteristics. They may be obtained from a variety of tissue types, including umbilical cord, adipose tissue, dental tissue, and bone marrow (BM). BM-MSCs are challenging in terms of their ex vivo expansion capability. Thus, we aimed to improve the expansion of BM-MSCs with small molecule treatments. We tested about forty small molecules that are potent quiescence modulators, and determined their efficacy by analysis of cell viability, cell cycle, and apoptosis in BM-MSCs. We also examined gene expression for selected small molecules to explore essential molecular pathways. We observed that treatment with SB203580 increased BM-MSCs expansion up to two fold when used for 5 days. SB203580 decreased the proportion of cells in the G1 phase of the cell cycle and substantially increased the ratio of cells in the S-G2-M phase. Enhanced MSC expansion with SB203580 therapy was associated with the lower expression of CDKIs like p15, p18, p19, p21, p27, and p57. In conclusion, we have developed a new approach to facilitate the expansion of BM-MSCs. These results could enhance autologous and immunomodulation therapy involving BM-MSCs.

Keywords

MSCs · Small molecules · Stem cell expansion

Abbreviations

BM	Bone marrow
BM-MSCs	Bone Marrow Mesenchymal Stem Cells
CFU-F assay	Colony forming unit – fibroblast assay
DMSO	Dimethyl sulfoxide
MS	Muscle dystrophy
MSCs	Mesenchymal stem cells

1 Introduction

The interest around MSCs and their therapeutic potential increased significantly over the last decade. Ease of accessibility, ex vivo expansion, ease of isolation techniques, their immunomodulatory abilities and tissue repair potential made MSCs an important source for cell-based therapy

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applications. While our understanding of MSCs improved, new therapeutic applications were developed not only in the tissue engineering area, but also on MSCs immunomodulatory and reparative traits on wound healing and rectification of defective immune systems. Several studies have reported that there is an urgent need for optimization of MSC culture conditions before clinical studies (Laube et al. 2016). It is clear that regulations and innovations of new techniques are required to optimize MSC culture conditions in terms of passage number dependency, elimination of aging factors, improvement of cryopreservation and preconditioning, and the determination of optimal tissue origin suitable for each specific context.

The use of MSCs increased rapidly due to the clinical studies that demand a large population of MSCs. Although they are dividing cells, their expansion capability does not match always with the requirement of clinical studies. While a minimal dosage requirement for transplantation is 1-2 $\times$ 10^6 cells per kilogram, the bone marrow cell population consists of only 0.3% of MSCs. In the cord blood and peripheral blood samples, the ratio is even lower (Bonab et al. 2006). To reach the sufficient amount for transplantation, either long-term culture of MSC is required, which is unsafe due to senescence, loss of potency, tumor growth potential, or it is needed to develop new and safe expansion techniques.

Small molecules could be used to target biological pathways associated with growth, expansion, signal transduction, apoptosis, cell cycle, and differentiation (Huber and Superti-Furga 2016). In this study, previously identified and characterized small molecules which are potentially targeting cellular quiescence (Sidal et al. 2020) were tested in the expansion of bone marrow derived mesenchymal stem cells. We isolated and characterized BM-MSCs, which are known to be difficult to expand ex vivo. This is followed by testing small molecules in varying doses and assessing their effect in MSC growth, cell cycle, and apoptosis. In addition, we studied expression of genes relevant to improved MSC proliferation with selected small molecules. These studies led to the discovery of two small molecules SB203580 and SKF96395 that induce ex vivo BM-MSC expansion up to two fold by improving cell cycle progression. This was also associated with the upregulation of S-phase genes and downregulation of CDKIs such as p15, p21, and p57.

2 Materials and Methods

2.1 Animals and Ethical Approval

BALB/c mice have been used throughout this study. Animal studies were used under decision number 592 which was approved by the Institutional Clinical Studies Ethical and the Institutional Animal Care and Use Committee of Yeditepe University (YUDHEK).

2.2 MSC Isolation, Culture, and Characterization

BALB/c mice were dissected; femur and tibia bones were separated from flesh. Cold DPBS (Dulbecco's Phosphate-Buffered Saline, Gibco cat. no. 14190250) were flushed through the bone marrow cavity using a 26-G needle. The marrow cell suspension was filtered through a 70 μM cell strainer (Falcon cat. no. 0877102). The cell suspension was centrifuged at 1500 rpm for 5 min. Supernatant was removed. Pellet was resuspended in low-glucose DMEM media (Gibco, cat. no. 10567014), supplemented with 15% FBS (Gibco, cat. no. 10082147), 1% PSA (Gibco, cat. no. 15240062). Then, cells were counted on a hemocytometer. The cells were cultured in T75 flasks (20 million cells per flask) with the media volume of 8 mL, or T25 flasks (eight million cells per flask) with the media volume of 3 mL. Flasks were placed in an incubator set at 37 °C and 5% CO_2 overnight. Twenty-four hours later, media with non-adherent cells was discarded, fresh media was added to flasks. Media was replaced with fresh media every 3-4 days. After 2 weeks of initial culture, adherent cells (MSCs) were collected.

At P2, BM-MSCs were seeded at 20,000 per well density at a 96-well plate. Each was stained separately with anti-mouse CD90 (Thy-1)-PE, anti-mouse CD105 – PECy7 and anti-mouse CD11b-PE (Invitrogen cat. no. in order; 4,280,969, 12,105,742, 11,011,842) and diluted in DPBS at 1:2000 ratio. 50 μL of diluted antibodies was added to each well containing 200 μL of media. The cells were incubated in ice, in a dark place for 15 min. Later on, flow cytometry analysis was conducted.

2.3 MSC CFU-F Assay

BM-MSCs were seeded on 6-well plates at 25,000 cells per well density to assess their **CFU-F**. The cells were incubated at 37 °C and 5% CO_2 conditions for 12 days. Media was changed with a fresh media every 3–4 days. After 12 days, cells were washed gently with DPBS twice. Cells were incubated and fixed at room temperature for 20 min with 2 mL of 1% formaldehyde. Formaldehyde was discarded. Giemsa (Thermo Scientific, cat. no.10434969) was diluted with dH_2O at 1:20 ratio and added to the wells and incubated for 30 min. Giemsa was rinsed thoroughly with distilled H_2O. Later on, colonies were observed and counted on a confocal microscope. Colony condition was 100 cells or higher.

2.4 Small Molecule Treatments

Small molecules were dissolved in DMSO at 20 mM concentration (Table 1) as we have done previously (Sidal et al. 2020). Dilutions were done with DPBS. BM-MSCs were seeded at the density of 5,000 cells per well to 96-well plates. Twenty-four hours later, the media was replaced with fresh media and cells were treated with small molecules to the final concentrations of 0.1 μM, 1 μM, or 10 μM in triplicates for a dose. Thereafter, the cells were incubated at 37 °C and 5% CO_2 conditions for 5 days.

2.5 MSC Growth Analysis

WST-1 Reagent (Cell Proliferation Reagent WST-1, Roche, cat. No. 11644807001) was used and diluted in 1:10 ratio with cell culture media and added at 100 μL to each well. Samples were incubated in a humidified incubator at 37 °C and 5% CO_2 in the dark. After 4 h, the absorbance was measured using a spectrophotometer microplate reader (Thermo Scientific, Varioskan Lux) at 420-480 nm as we have done previously (Kalkan et al. 2020).

2.6 Cell Cycle Analysis

BM-MSCs were seeded at 20,000 per well density at a 24-well plate. Twenty-four hours later, cells were treated with small molecules. Five days later, cells were trypsinized, centrifuged at 1500 rpm for 5 min at room temperature. Supernatant was discarded. Pellet was resuspended with 200 μL of fresh media. Suspension was placed in 37 °C and 5% CO_2 incubator for 15 min. 1.5 μL of 200X Hoechst stain (Thermo Scientific, cat. no. 62249) was added per well. Plate was placed in the incubator for 30 min (in dark). After that step, 2 μL of 100X Pyronin Y (Sigma-Aldrich, cat. no. 213519) was added per well. The cells were incubated at 37 °C and 5% CO_2 for 15 min. Flow cytometry device was used for analysis (Beckman Coulter, Cytoflex S) as we have done previously (Turan et al. 2020).

2.7 Apoptosis Analysis

BM-MSCs were seeded at 20,000 per well density at a 96-well plate. Twenty-four hours later, cells were treated with small molecules. Five days later, cells were trypsinized, centrifuged at 1500 rpm for 5 min at room temperature. Supernatant was discarded. For this analysis, Invitrogen's Annexin V-FITC Apoptosis Detection Kit (cat. no. BMS500FI-100) was used. Pellet was resuspended with 50 μL of 1X Binding Buffer. 2.5 μL of Annexin V and 2.5 uL of PI was

Table 1 List of small molecules number and name of small molecules

1	Alexidine Dihydrochloride	**21**	EX 527&Nicotinamide
2	HAT inhibitor – 505,298/p300&CBP inhibitor VI	**22**	Tauroursodeoxycholic acid (TUDCA)
3	AS1949490	**23**	SB203580
4	RG 108	**24**	α-Tocopherol
5	HAT inhibitor-Garcinol	**25**	L-N&-(1-iminoethyl)-lysine hydrochloride (L-NIL)
6	CHIR-99021	**26**	BIO (6-bromoindirubin-30-oxime)
7	c-Myc inhibitor	**27**	SKF96395 hydrochloride
8	SC1 (Pluripotin)	**28**	Mdivi-1
9	Pifithrin-α	**29**	(5Z)-7-Oxozeaenol
10	Ro 26-4,550 trifluoroacetate	**30**	SB239063
11	GANT 61	**31**	Trans-2-phenylcyclopropyşamine hydrochloride
12	400,083 I HIF-1 inhibitor	**32**	Tetraethylammonium chloride
13	StemRegenin 1 (SR1)	**33**	K252c
14	CASIN	**34**	BML-260
15	CXCR4 antagonist 1, AMD3100	**35**	N-4-Tosyl-L-arginine methyl ester hydrochloride
16	Cannabidiol (CBD)	**CM1**	MEIS inhibitor-1 (MEISi-1, 25 mg)
17	Ro5-3,335	**CM2**	MEIS inhibitor-2 (MEISi-2, 25 mg)
18	SKP2-C25	**NAE**	MLN4924 (NAE inhibitor)
19	bpV (Hopic)		
20	2-aminoethoxydipenyl-borade (2APB)		

added to each well. The cells were incubated at room temperature, in the dark for 15 min. 200 µL of 1X Binding Buffer was added to each well. Flow cytometry device (Beckman Coulter Cytoflex S) was used for analysis as we have done previously (Boztas et al. 2013).

2.8 RNA Isolation, cDNA Preparation and RT-PCR

Total **RNA isolation** was done by NucleoZOL kit (Macerey-Nagel, cat. no. 740404200). Cell culture media was removed and 1 mL of NucleoZOL was added to the wells. Complete lysis was ensured by vigorous pipetting. 200 µL of RNase free water was added per 500 µL of NucleoZOL/lysed cell suspension. The suspension was incubated at room temperature for 5 min then centrifuged at 12,000 xg for 15 min. 500 µL of supernatant was transferred to a fresh tube, 500 µL isopropanole was added. The suspension was incubated at room temperature for 10 min and centrifuge at 12,000 xg for 10 min. Supernatant was discarded and 500 µL of 75% ethanol was added. Sample was centrifuged for 3 min at 8,000 xg. The ethanol-washing step was repeated two times. The RNA pellet was dissolved in RNase free water. Concentration and purity (A230/A280) results were obtained by Nanodrop. **cDNA synthesis** was done using ProtoScript II, First Strand cDNA Synthesis Kit (cat. no. E6560S). Up to 1 µg of template RNA, 2 µL d(T)23VN, 10 µ L of ProtoScript II Reaction Mix (2X), and 2 µL ProtoScript Enzyme Mix (10X) was mixed in a PCR tube. Nuclease free water was added as necessary to complete the mixture to a total volume of 20 µL. The mixture was incubated first at 42 °C for 1 h, then at 80 °C for 5 min. Promega, GoTaq **qPCR** Master Mix, cat. no. A6001 was used for RT-PCR. 4 µL of nuclease free water, 0.75 µL of Forward Primer (100 mM), 0.75 µL of Reverse Primer (100 mM) (Table 2: Primer list), 7.5 µL of Master Mix (Syber Green), and 2 µL of the sample's cDNA was mixed. 1 cycle of Hot Start Activation at 95 °C for 2 min, 50 cycles of Denaturation (95 °C for 15 s) and Annealing (60 °C for 1 min) protocol was set on Roche, LightCycler 96. GAPDH and β-actin were used as internal controls. Data was analyzed by using the 2-ΔΔCt Method as we have done previously (Uslu et al. 2020).

Table 2 List of primer sets used

Gene	Forward primer (5′to3′)	Reverse primer (5′to3′)
GAPDH	TTGATGGCAACAATCTCCAC	CGTCCCGTAGACAAAATGGT
β-ACTIN	ATGGAGGGGAATACAGCCC	TTCTTTGCAGCTCCTTCGTT
RAD51	GGTGGTCTGTGTTGAACCCT	ACACCGAGGGCACCTTTAG
PCNA	GGAGACAGTGGAGTGGCTTT	GGAGACAGTGGAGTGGCTTT
MCM2	TCAGCTCCTCCACATCTTCA	TCAGCTCCTCCACATCTTCA
p15	CAGTTGGGTTCTGCTCCGT	AGATCCCAACGCCCTGAAC
p18	CTCCGGATTTCCAAGTTTCA	GGGGGACCTAGAGCAACTTAC
p19	TCAGGAGCTCCAAAGCAACT	TTCTTCATCGGGAGCTGGT
p19 Arf	GTTTTCTTGGTGAAGTTCGTGC	TCATCACCTGGTCCAGGATTC
p21	ATCACCAGGATTGGACATGG	CGGTGTCAGAGTCTAGGGGA
p27	GGGGAACCGTCTGAAACATT	AGTGTCCAGGGATGAGGAAG
p57	TTCTCCTGCGCAGTTCTCTT	CTGAAGGACCAGCCTCTCTC

2.9 Statistics

Two tailed t-test was used to determine the significance of the data. The results were labeled as significant if the t-test value was less than 0.05, and high significant if the value is less than 0.01.

3 Results

3.1 Analysis and Growth of Primary Bone Marrow MSCs

We used BM-MSCs because they are among the hard-to-grow MSCs and needed more development in the expansion procedures. Primary MSC culture and characterization is regularly conducted by surface antigen analysis by flow cytometry, morphology and colony forming capacity in vitro.

BM-MSCs were isolated and cultured for 14 days on 37 °C and 5% CO_2 incubator then immediately after trypsinizing, they were stained with CD90-PE and CD105-PECy7 for positive MSC marker analysis. CD90-PE yielded >98% of positivity (Fig. 1a); while CD105-PECy7 showed >85% of positivity (Fig. 1b). Negative MSC marker staining of CD11b-PE showed that MSCs were < 95% negative (Fig. 1c).

After 7 days of initial culture following isolation, adherent MSCs and spindle shaped colony formation were seen. Cells were observed on brightfield microscopy to validate the morphology of the isolated cells (Fig. 1d). We also determined CFU-F values after 12 days of culture of passage number 3 on T25 flask and found over 25 colonies with >100 cells per colony as we counted under brightfield microscopy (Fig. 1e). These showed that we have successfully able to grow BM-MSCs, which could be further studied with small molecules to determine potent small molecules that could induce MSC expansion.

3.2 Screening of Small Molecules in BM-MSC Expansion

WST-1 cell viability assay was performed at the first stage of the experiments to evaluate and select the effective small molecules (Fig. 2). First, cells were treated for 5 days with a single dose (1 μM) of small molecules to see the overall effects of the molecules on BM-MSC (Fig. 2). These studies showed that several compounds (#23, #27, and #30) could increase MSC growth up to 1.4 fold at 1 μM dose. Second set of experiments was done with 0.1 μM, 1 μM, and 10 μM doses of small molecules numbered for selected small molecules (Fig. 3). Significant and up to two fold increased in MSC growth were seen for #23 and #27 treatments at 10 μM concentrations (Fig. 3a, b). Since optimum expansion effects in MSCs were seen after 5 days of treatments and indicated doses, the

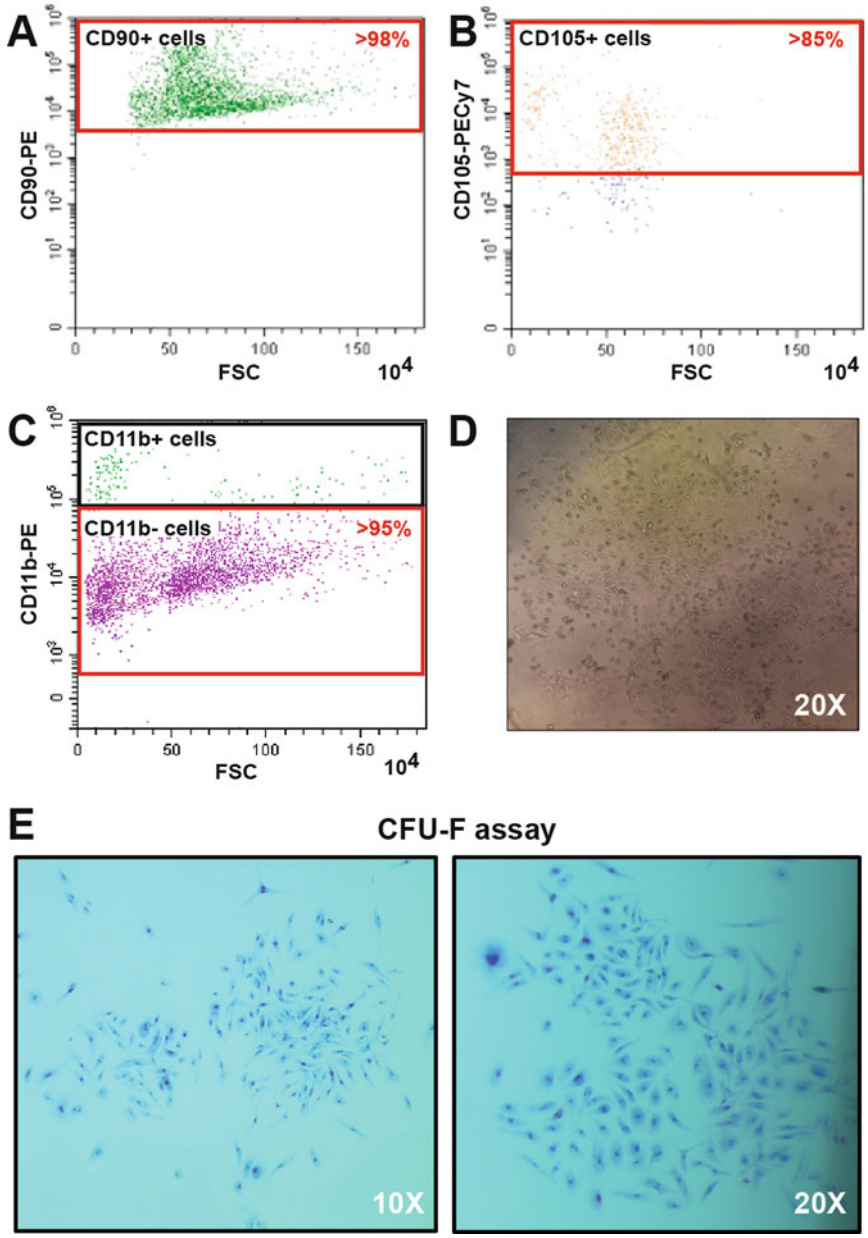

Fig. 1 Analysis of BM-MSCs by flow cytometry and colony forming assay. MSC surface antigen analysis by flow cytometry for (**a**) CD90 and (**b**) CD105 markers which are positive markers for MSCs. (**c**) BM-MSC characterization with anti-mouse antibody CD11b as a negative MSC marker. Analysis of (**d**) BM-MSCs at passage 1 at day 7 image of BM-MSC on bright field microscopy (20X magnification). (**f**) Analysis of colony forming unit. Representative CFU-F assay images taken on brightfield microscopy on the day 12 of culture (Left: 10X, and Right: 20X magnification)

rest of the studies were performed after 5 days of treatment throughout this study. These findings showed that #23 and #27 treatments could be an ideal approach for ex vivo BM-MSC expansion procedures. Next, we sought to determine potential mechanism of these compounds by studying

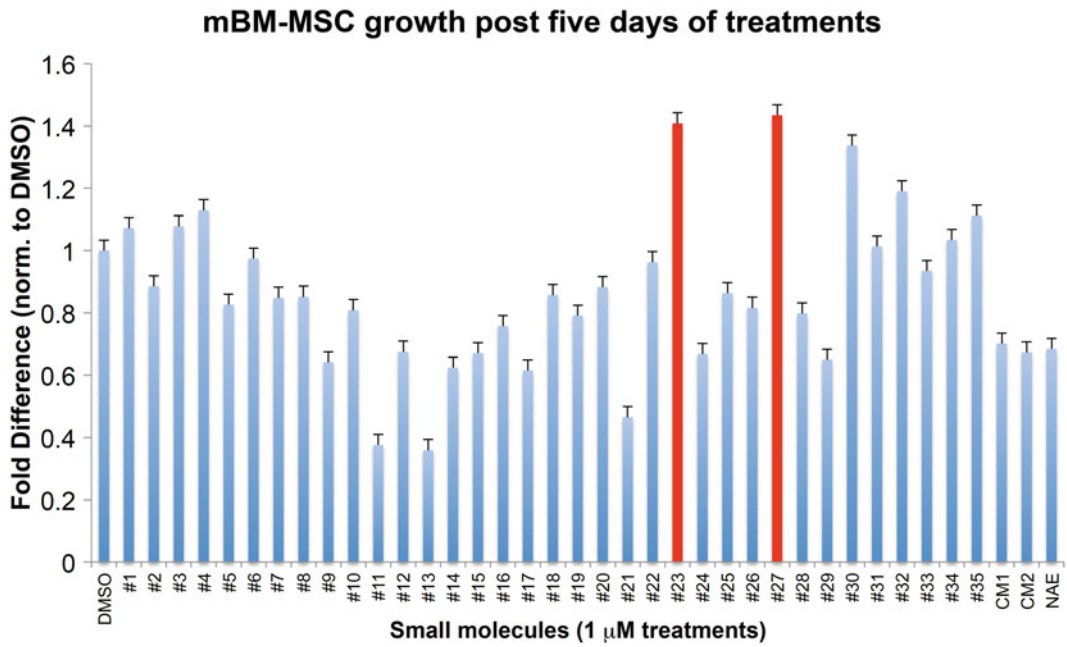

Fig. 2 Treatment of BM-MSCs with various small molecules and analysis for cell growth. WST-1 cell viability assay shows fold difference following 5 days of treatment with all of the small molecules at 1 μM final concentration. Note that molecules #23 and #27 showed an increased MSC growth compared to control as well as other small molecules. n = 3

cell cycle, apoptosis (viability), and gene expression.

3.3 Small Molecule Treatment Enhances Cell Cycle in BM-MSCs

After 5 days of treatments, BM-MSC cell cycle analysis was done with Hoechst and Pyronin-Y staining. The results were obtained by flow cytometry. Significant changes were seen on #23 and #27 treatments in comparison to DMSO control treatment (Fig. 4a, b, c). Treatment with #27 caused a reduced number of cells in G0 and an increased number of cells in G1 phase (Fig. 4d). On the other hand, #23 doubled the S/G2/M phase population ratio indicating that MSCs were indeed expanding rapidly (Fig. 4d).

Rate of apoptosis was also determined by Annexin V-FITC and PI staining by flow cytometry post treatment with DMSO (Fig. 5a), #23 (Fig. 5b) and #27 (Fig. 5c). Early apoptosis, late apoptosis, and necrotic cell rate was coherent with the control for #23; while #27 treated MSCs showed decreased dead/necrotic cell percentage (Fig. 5d). In conclusion, #23 and #27 molecules act by inducing cell cycle progression rather than altering ex vivo cell viability.

3.4 Small Molecule Treatment Alters CDKI and S-Phase Related Gene Expression in MSCs

RNA isolation was done following 5 days of selected small molecule treatment to BM-MSCs. Thereafter, cDNA was synthesized, Real Time-PCR was conducted to determine CDKI and S-Phase/HDR related gene expression alterations in comparison to DMSO control treated group (Fig. 6). #27 treatments increased expression of key S-Phase genes RAD51, PCNA, and MCM2 in BM-MSCs (Fig. 6a). On the other hand, analysis of CDKI gene expression in BM-MSCs

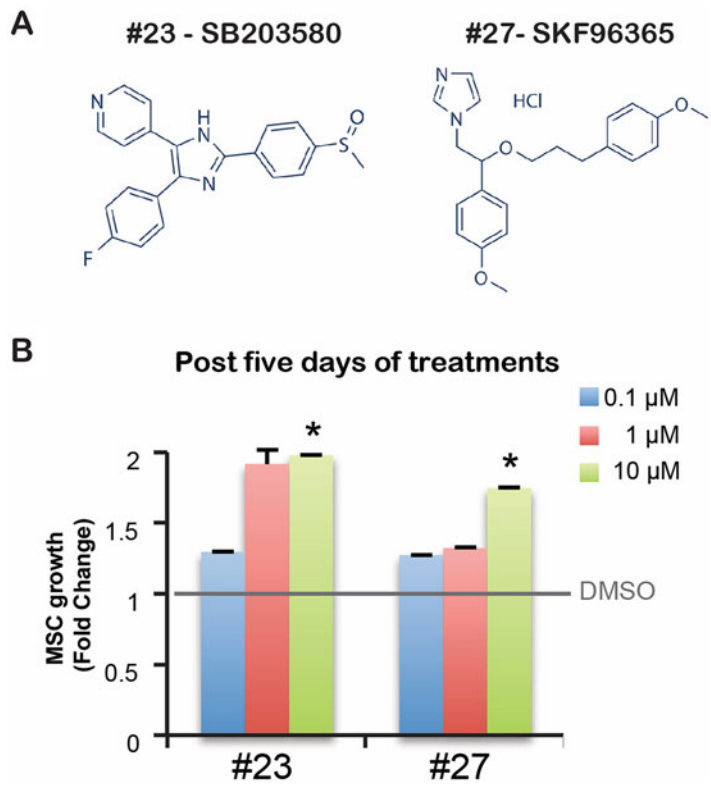

Fig. 3 Treatment with selected small molecules for the analysis of improved BM-MSC growth. (**a**) Structure of selected molecules. (**b**) MSC growth analysis by WST-1 following 5 days of treatments in increasing doses (0.1 μM, 1 μM, and 10 μM) of selected small molecules. Note that small molecules #23 and #27 shows both dose dependent increase and significant increase in BM-MSC number at 10 μM dose. n = 3. *p < 0.05

showed that #23 treatment reduced expression of several CDKI genes including p15, p18, p19, p21, p27 and p57 (Fig. 6b). This was consistent with increased cells S-G2-M phases and reduction in G1 phases of cell cycle post #23 treatments (Fig. 5). However, #27 treatments in BM-MSCs did not alter expression of CDKI genes.

4 Discussion

The progression of stem cell technologies have let stem cell therapy to grow over the past decade (Ringdén et al. 2006). The ease of access, low immunogenic effects, reliable ways of in vitro and in vivo transplantation abilities are set to be the desired requirements for application. With more research being done, it was concurred that mesenchymal stem cells were one of the most suitable stem cell sources. Immunomodulatory traits of MSCs present a great potential for treating various diseases including immune disorders, and it enables them to act as a co-transplanted cell population to reduce the immune response. Homing capability of MSCs is found to be advantageous, hence their interaction with the host-tissues (Yagi et al. 2010). On the other hand, there is a significant decline in the expansion ability of mesenchymal stem cell cultures through time. MSCs show potency loss during sub-culturing and at high passage numbers. MSCs' becoming senescent at long-termed cultures is seen after passage 5 (Ahmad et al. 2013). Although they are dividing cells, their expansion capability does not always match with the requirement of clinical studies.

The development of commercially available stem cell sources has led the researchers to modulate the immune system and provide valuable assets for regenerative medicine and cell-based tissue repairing systems. MSCs have been shown to be important for various diseases and conditions. Recently, the scientists have shown the importance of MSCs in the treatment of

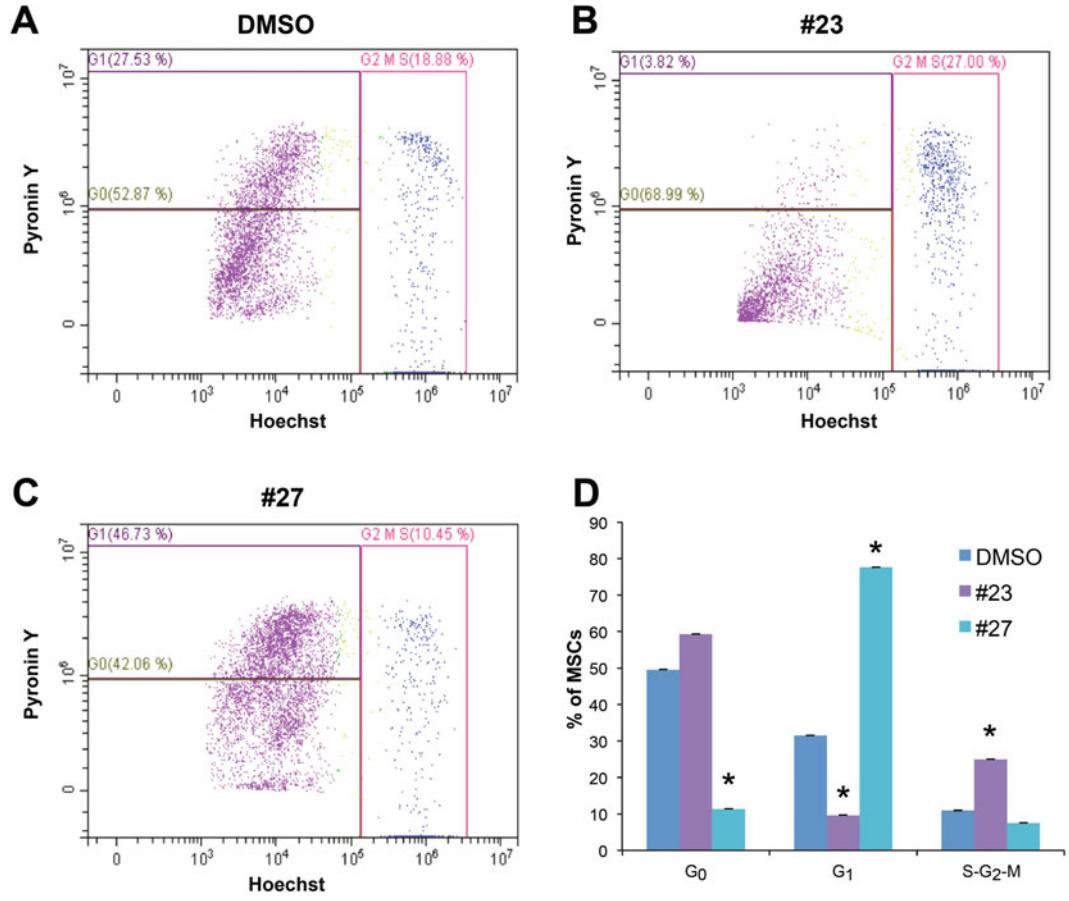

Fig. 4 Analysis of BM-MSCs cell cycle following treatment with selected small molecules. BM-MSCs were treated with (**a**) DMSO as control and (**b**) #23, (**c**) #27, and were quantified for (**d**) the distribution of cells in cell cycle phases by Hoechst and Pyronin Y staining. Note that #23 decreased the percentage of cells in G0. On the other hand, #27 increased the percentage of cells in G1 and S-G2-M phases of cell cycle. n = 3, *p < 0.05

muscle dystrophy (MS) (Shabbir et al. 2009). Treatments for muscle dystrophy mainly depend on activating the host satellite cells, forming new myofibers at the injury zone. These applications reveal the rapid loss in the grafted MSC population, suggesting the need for the improvements in MSC therapeutics (Shabbir et al. 2009).

Initial improvements in MSC expansion was established with a simple adjustment on the oxygen levels of cell culture conditions (Chow et al. 2001). The standard O_2 levels in bone marrow cavity is known to be around 5%. Yet, O_2 gradient has been seen throughout the bone marrow. Hematopoietic stem cell niche is extremely hypoxic, and it has been shown that culturing HSC at low O_2 percentage helps to protect the potency of the cells (Hawkins et al. 2013). Based on this information, MSCs have also been cultured on hypoxic conditions and the results indicated that hypoxia does not only promote MSC expansion, but it also allows the cells to preserve their potency (Grayson et al. 2006).

Platelet lysate was also proposed as an FBS subrogate for MSC expansion. The hypothesis was that platelet lysate growth factors can support MSC expansion (Abdelrazik et al. 2011). Studies suggested that the expansion of MSC is quicker and faster in this case compared to

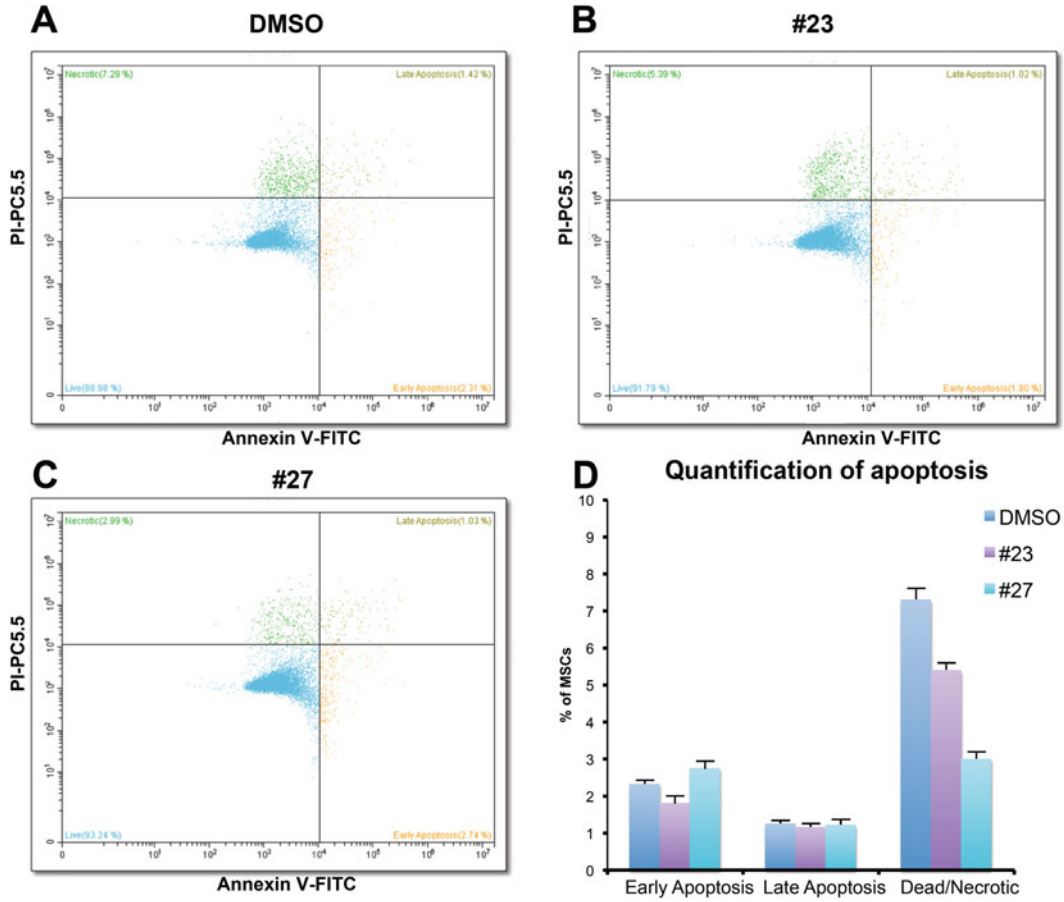

Fig. 5 Analysis of apoptosis in BM-MSCs treated with selected small molecules. (**a**) DMSO, (**b**) 10 μM dose of #23, and (**c**) 10 μM dose of #27 treated BM-MSCs were analyzed by flow cytometry after Annexin V-FITC and PI staining. (**d**) Quantification of apoptotic and necrotic BM-MSCs treated with DMSO and selected molecules. Note that there is no change in the apoptosis profile of #23 or #27 treated molecules compared to DMSO control treatment

FBS-supplemented media. However, platelet lysate also results in the alterations of gene expressions on BM-MSC leading to a decrease in their immunomodulatory effects (Abdelrazik et al. 2011). Suppressive immunoregulatory activities of MSCs may arise with the presence of soluble factors which are substantively expressed and secreted by MSCs or released after the presence of a stimulatory factor on an inflammatory environment (Aggarwal and Pittenger 2005). For instance, IDO and PGE2 expressions have been seen to increase in MSCs (Spaggiari et al. 2008). Immunomodulatory effects of PGE2 are associated with releases of cytokines, particularly IFN-γ and IL-2. PGE2 alters an inflammatory environment to an anti-inflammatory state (Meisel et al. 2004). Recent studies also indicate that when IFNγ is present, it stimulates HGF (hepatocyte growth factor) and TGFβ1 expressions and with that suppresses allo-responsiveness (Polchert et al. 2008).

In this study, a list of small molecules associated with stem cell quiescence was analyzed to identify novel BM-MSC expanding small molecules. For this purpose, bone marrow mesenchymal stem cells were isolated, cultured, and treated with small molecules in a dose-dependent manner. Following cell viability

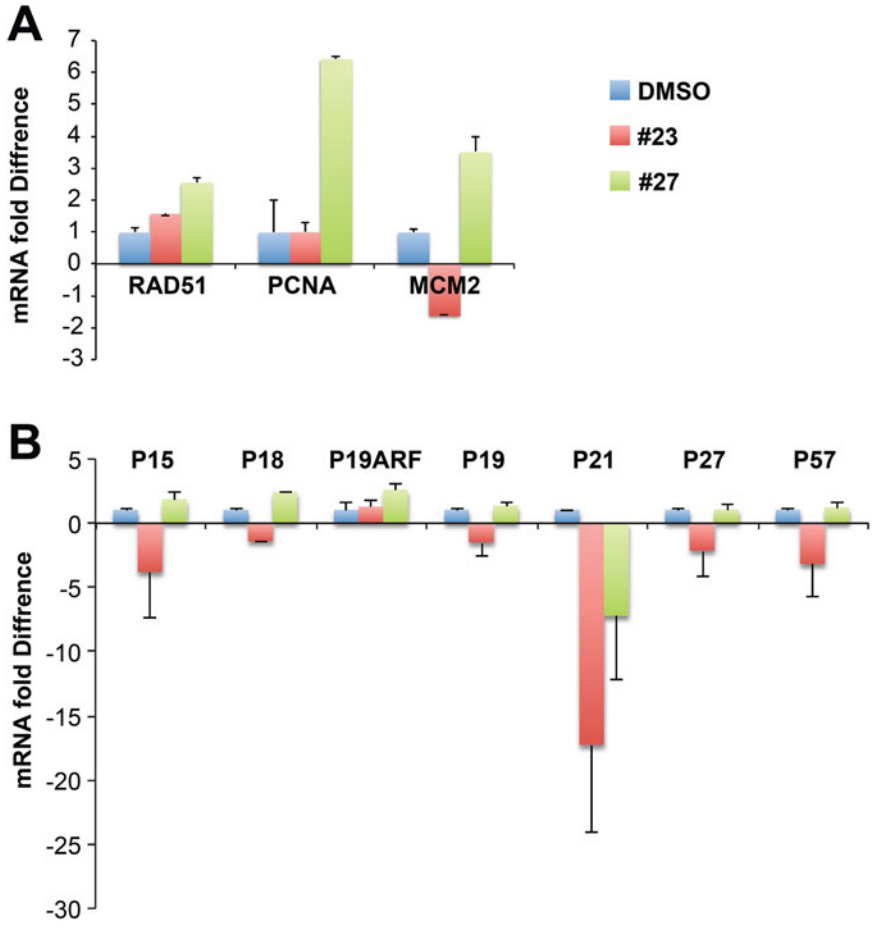

Fig. 6 Gene expression analysis in BM-MSCs treated with selected small molecules. (**a**) Analysis of S-Phase related gene expression, and (**b**) CDKI gene expression post treatment with DMSO, #23, and #27. Note that #27 induced expression of S-phase genes while #23 decreased expression of CDKIs, namely, p15, p18, p19, p21, p27, and p57

assays, we decided to continue to further investigate #23 known as SB203580 and #27 known as SKF96365. SKF96395 is a capacitative Ca^{2+} entry inhibitor and an appealing new anti-cancer drug candidate (Jing et al. 2016; Singh et al. 2010). SB203580 is a P38/MAPK inhibitor. MAPK is connected to differentiation, apoptosis, autophagy, and ageing. Its inhibition by SB203580 showed activity against fibrosis as well as muscle regeneration (Clerk and Sugden 1998). It has been shown that SB203580 evokes a therapeutic response in chronic airway disease (Underwood et al. 2000). In addition, SB203580 shows inhibition potential on cardiac stress activated protein kinases and C Jun N Terminal Kinases. These effects could be the results of elimination of the ageing-inducing persistently active p38/MAPK pathway (Clerk and Sugden 1998). Our findings also suggest that there might be an uncovered effect and associated benefits observed in these studies due to MSC expansion and growth that is triggered by SB203580 treatments.

In this study, the expansion capability of SB203580 was one of the highest among the tested small molecules. #23 on the optimum dose of 10 μM expanded BM-MSC safely. More specifically, 5 days was the most convenient

timespan for treatment to observe the significant difference. Cell cycle analysis results showed a significance increase on G2/M phases. Apoptosis analysis demonstrates that #23 treated BM-MSCs did not show any irregular patterns on apoptosis analysis. CDKI gene expressions were down regulated post #23 treatments when compared with control (DMSO-treated culture). We believe use of SB203580 in clinical studies that require larger number BM-MSCs could be beneficial. However, further studies are needed for long-term treatment with SB203580.

Overall, this study assessed the premise of finding a novel small molecule for BM-MSC expansion. Further studies should be conducted after the treatment of small molecules to see the effects on the immunomodulation and stemness aspects of BM-MSCs. In addition, in vivo effect of SB203580 or SKF96365 in MSCs or other cell types would be intriguing to study. Making a cocktail of the complementing molecules or approaches might result in higher expansion potential for hard to expand BM-MSCs. In addition to these, the effects of #27's on the induction of key HDR and S-Phase gene expressions could be exploited in MSC gene editing studies.

Declarations
Funding: FK was supported by Yeditepe University.

Conflicts of Interest/Competing interests: All authors declare no conflict of interest.

Availability of Data and Material: Supporting data is provided in the supporting materials. Further data is available upon request.

Code Availability: Not applicable.

Authors' Contributions: LYA planned the experiments, collected and analyzed data, and wrote the manuscript. FK designed the studies and wrote the manuscript.

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Human-Induced Pluripotent Stem Cell–Based Models for Studying Sex-Specific Differences in Neurodegenerative Diseases

Erkan Kiris

Abstract

The prevalence of neurodegenerative diseases is steadily increasing worldwide, and epidemiological studies strongly suggest that many of the diseases are sex-biased. It has long been suggested that biological sex differences are crucial for neurodegenerative diseases; however, how biological sex affects disease initiation, progression, and severity is not well-understood. Sex is a critical biological variable that should be taken into account in basic research, and this review aims to highlight the utility of human-induced pluripotent stem cells (iPSC)–derived models for studying sex-specific differences in neurodegenerative diseases, with advantages and limitations. *In vitro* systems utilizing species-specific, renewable, and physiologically relevant cell sources can provide powerful platforms for mechanistic studies, toxicity testings, and drug discovery. Matched healthy, patient-derived, and gene-corrected human iPSCs, from both sexes, can be utilized to generate neuronal and glial cell types affected by specific neurodegenerative diseases to study sex-specific differences in two-dimensional (2D) and three-dimensional (3D) human culture systems. Such relatively simple and well-controlled systems can significantly contribute to the elucidation of molecular mechanisms underlying sex-specific differences, which can yield effective, and potentially sex-based strategies, against neurodegenerative diseases.

Keywords

Sex-specific differences · Neurodegeneration · Induced pluripotent stem cells (iPSC) · iPSC-based neuronal and glial models · Neurodegenerative diseases · Central nervous system diseases

Abbreviations

AD	Alzheimer's disease
PD	Parkinson's disease
MS	multiple sclerosis
HD	Huntington's disease
ALS	amyotrophic lateral sclerosis
SMA	spinal muscular atrophy
PMA	progressive muscular atrophy
ES	embryonic stem
iPSC	induced pluripotent stem cells
APOE	apolipoprotein E
BDNF	brain-derived neurotrophic factor
ER	estrogen receptor
CSF	cerebrospinal fluid

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1 Introduction

Neurodegenerative diseases are characterized by progressive loss of neurons in the central nervous system (CNS), associated with irreversible defects in cognitive and motor functions (Dugger and Dickson 2017). Research shows an increasing burden of neurodegenerative diseases worldwide (Deuschl et al. 2020; Collaborators et al. 2021), highlighting the urgent need for effective prevention and treatment options. Toward that goal, there has been significant research interest to elucidate factors causing or contributing to disease initiation, progression, and severity. Aging is considered a primary risk factor in neurodegenerative diseases (Hou et al. 2019); however, many other potential risk factors have been suggested, including biological sex as a critical variable contributing to the diseases (Vegeto et al. 2020). Epidemiological studies demonstrate the striking prevalence and incidence differences between males and females in many neurodegenerative diseases (Hanamsagar and Bilbo 2016; Collaborators 2019b). Additionally, there are sex-specific differences concerning the age of disease onset, symptoms, progression, severity, and response to available treatment options (Pinares-Garcia et al. 2018). Understanding the effects of biological sex differences on disease initiation and progression is crucial to develop effective modalities against neurodegenerative diseases. Simple, well-controlled, species-specific, and physiologically relevant model systems can facilitate such studies.

The vast majority of clinical trials aiming to develop treatment options against neurodegenerative conditions have been unsuccessful despite large investments, with a failure rate exceeding 99% in clinical trials (Huang et al. 2020; Aldewachi et al. 2021). Failures in the drug development processes have been attributed in part to the animal models being extensively utilized in the relevant fields (Drummond and Wisniewski 2017). Although these models provided critical knowledge on neurodegenerative diseases, especially rodent models do not fully recapitulate the disease, indicating the need for species-specific model systems to complement, or if possible, replace, existing model systems (Veening-Griffioen et al. 2019). Additionally, sex has not been routinely taken into account in animal studies (Bhargava et al. 2021). Human neural cells and CNS samples represent valuable resources for neurobiology research, with advantages and disadvantages. Post-mortem human tissue samples are highly valuable; however, difficulties associated with obtaining high-quality post-mortem CNS tissues make such samples less attractive, especially for large-scale studies. Immortalized human neural cell lines can be utilized in large quantities in a cost-effective manner for neurodegenerative disease research and drug discovery; however, cancerous propensities of the lines make them less desirable. Advances in pluripotent stem cell biology with two-dimensional (2D) and, more recently, three-dimensional (3D) human culture systems offer unique approaches to study neurodegenerative diseases in a species-specific manner, with a potentially limitless supply of patients' own neurons and glial cells derived from human-induced pluripotent stem cells (iPSC) (Penney et al. 2019). It has been suggested that the 3D culture systems can represent a topological environment that allows cell-to-cell interactions and the formation of neuronal networks in a more physiologically relevant system than 2D culture (Gopalakrishnan 2019). However, 2D systems also provide certain advantages, including mechanism of action studies in relatively pure and better controlled neuronal or glial culture systems (Essayan-Perez et al. 2019). Therefore, it may be critical to test specific hypotheses in 2D and 3D systems and compare/contrast the results, which is not yet common in the literature.

This review focuses on sex-specific differences in neurodegenerative diseases and the importance of *in vitro* modeling, with an emphasis on the utility of human iPSC-based model systems to study sex-specific differences at the cellular level. Gender and sex are not interchangeable concepts, which have been discussed in elegant reviews (Bhargava et al. 2021; Mauvais-Jarvis et al. 2020), and this review uses

"sex" to emphasize biological differences. Although the main focus here is neurodegenerative diseases, we also touch upon other selected CNS disorders, including some neurodevelopmental and mental disorders. Here we summarize our current knowledge on the sex-specific differences in the prevalence and incidence of selected CNS diseases, molecular mechanisms implicated in sex-specific differences, and the importance of human model systems and sex of the cell. We also provide an overview of human cellular systems for studying sex-specific differences in CNS diseases, and the main focus is the advantages and disadvantages of human iPSC-based 2D and 3D model systems, which represent highly promising platforms for mechanistic studies, toxicity testings, and drug screening.

2 Sex as a Risk Factor in CNS Diseases

A better understanding of the biological basis of sex-specific differences observed in neurodegenerative conditions is crucial to develop efficient and potentially sex-dependent therapeutic options against such diseases. Many neurodevelopmental, psychiatric, and neurodegenerative diseases exhibit sex-based differences, and here we focus on some selected CNS diseases.

2.1 Alzheimer's Disease

Alzheimer's disease (AD), a chronic neurodegenerative condition without a cure, has a substantial burden for patients, families, and communities worldwide (Collaborators 2019b; Cummings et al. 2020). Approximately two-thirds of AD patients are females (Fig. 1), and importantly, the sex gap in the number of AD patients is expected to increase according to the estimations covering the range between 2020 and 2060 (Rajan et al. 2021). Interestingly, the treatment of patients with AD drugs also exhibits sex-specific differences (Scacchi et al. 2014). Although higher life expectancy may partially explain the greater prevalence in women, an increasing body of literature suggests that biological sex differences, beyond longevity and other gender-related effects, could significantly contribute to underlying reasons (Laws et al. 2016). Females are estimated to have a two-fold greater risk than males for developing AD at ages 45 and 65 (Sampathkumar et al. 2020; 2021 Alzheimer's disease facts and figures 2021; Chene et al. 2015). However, overall AD incidence differences between sexes are unclear as there have been conflicting studies, suggesting no difference, higher incidence for males or females (Mielke et al. 2018; Rocca 2017). Nonetheless, previous literature strongly suggests that AD is sex-biased and sex also affects the disease's progression and severity. For example, after diagnosis, AD progresses faster in females as compared to males (Ardekani et al. 2016), and cognitive decline is more significant in females than males at the same stage of AD (Levine et al. 2021). It has also been reported that brain atrophic rates are faster in female AD patients, and females exhibit higher AD tau pathology as compared to males (Barnes et al. 2005; Hohman et al. 2018; Oveisgharan et al. 2018). Taken together, females are more adversely affected than males by the disease, and biological sex-specific differences may play an important role in AD (Ferretti et al. 2020).

2.2 Parkinson's Disease

Parkinson's disease (PD), the second most common neurodegenerative disease following AD, develops upon progressive deterioration and death of dopaminergic neurons in the brain, leading to movement disability (Dickson et al. 2009). PD is considered a multifactorial disease in which multiple genetic and environmental factors play a role in disease initiation and progression (Poewe et al. 2017). In addition to motor symptoms, PD patients also suffer from cognitive and mood-related issues, including anxiety, depression, and sleep problems (Postuma et al. 2012). Opposite to AD, PD disproportionally affects men, as roughly 3/5 patients are males (Elbaz et al. 2016) (Fig. 1).

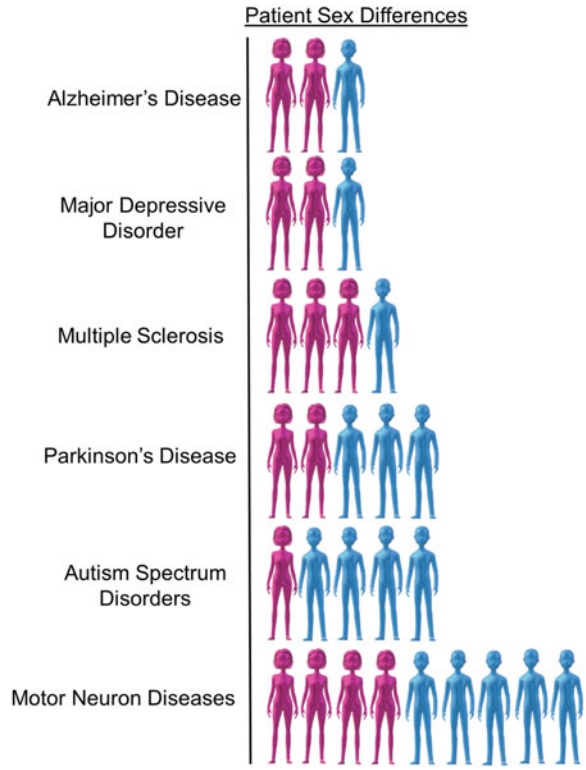

Fig. 1 Patient sex ratio differences in selected CNS diseases. Approximately 2/3 of Alzheimer's disease and major depressive disorder patients, and 3/4 of multiple sclerosis patients are females (see Sects. 2.1, 2.5, and 2.4., respectively). The age-standardized male-to-female incidence ratio in PD is approximately 1.5, as discussed in Sect. 2.2. Autism spectrum disorder affects males about four times more than females, although lower and higher male-to-female prevalence ratios have also been reported (Sect. 2.5). The global male-to-female prevalence ratio of motor neuron diseases is calculated to be 1.25; however, as discussed in Sect. 2.3, the male-to-female ratio in ALS is higher, especially at the age group of 20–49 years

The age-standardized PD male-to-female incidence ratio is about 1.5 (Picillo et al. 2017), and it has been demonstrated that the male-to-female ratio in terms of both prevalence and incidence increases with age (Moisan et al. 2016). Sex appears to be important for the disease progression as well. For example, females experience relatively milder motor deterioration, especially at the earlier stages of the disease (Haaxma et al. 2007). Non-motor symptoms of PD also exhibit sex-specific differences, affecting females more negatively (Meoni et al. 2020). The degree of cognitive impairments in PD is also sex-dependent, affecting males more severely (Reekes et al. 2020). It has been shown that sexual dimorphism exists in the gene expression profiles of basal ganglia in PD patients and healthy individuals (Cantuti-Castelvetri et al. 2007; Simunovic et al. 2010). Also, male PD patients exhibit a significantly higher neuronal loss in many different cortical areas compared to female PD patients (Tremblay et al. 2020). It has been suggested various factors play a role in the observed sex-specific differences in the prevalence and severity of the disease, including estrogen levels and signaling (Vaidya et al. 2021). The lack of treatment options causes a severe economic burden on patients and societies, especially with the increase in the elderly population. Numerous model systems have been utilized in PD research (Thomas et al. 2020), and among these, human iPSC-based modeling offers highly

useful systems for PD research and drug discovery.

2.3 Motor Neuron Diseases

Motor neuron disease is an umbrella term including various diseases affecting upper and lower motor neurons, including amyotrophic lateral sclerosis (ALS), spinal muscular atrophy (SMA), and progressive muscular atrophy (PMA) (Dion et al. 2009). Degeneration of motor neurons, which control muscle cell contraction, eventually leads to progressive weakness and respiratory failure (van Es et al. 2017). Motor neuron diseases disproportionally affect males compared to women, and the global male-to-female prevalence ratio is calculated to be 1.25 (Fig. 1). ALS is the most studied motor neuron disease, and both incidence and prevalence of ALS are higher among males (Deuschl et al. 2020). However, there are significant differences in male-to-female ratios depending on age (Manjaly et al. 2010). One study demonstrated while the male-to-female ratio in the age group 20–49 years is 2.26, the number drops to 1.41 in the age category of 50–84 years (Ahmadzai et al. 2018). The lifetime risk of developing motor neuron disease is also higher in males (Alonso et al. 2009; Johnston et al. 2006). Sex is an important variable for ALS disease phenotype (Trojsi et al. 2020), and future work elucidating biological sex-specific differences in ALS may be critical for better understanding the disease and developing effective modalities.

2.4 Multiple Sclerosis

Multiple sclerosis (MS) is another CNS disease that exhibits apparent sexual dimorphism (Gilli et al. 2020). MS is characterized by inflammation in the CNS and demyelination of axons, leading to axonal damage and neuronal loss that cause neurological symptoms (Filippi et al. 2018). Although many genetic and environmental factors have been hypothesized as the underlying cause of the disease, specific factors critical for disease initiation or progression are not well understood (Thompson et al. 2018). There is currently no cure for MS, and existing drugs generally aim to suppress the immune system to prevent acute attacks (Hauser and Cree 2020). The disability that develops over time in MS is mainly due to the loss of the myelin layer (demyelination) of the axons (Lubetzki et al. 2020). Approximately 3/4 of patients diagnosed with MS are females (Orton et al. 2006; Krysko et al. 2020) (Fig. 1). Although various female-to-male ratios have been reported from different geographic locations and age groups (Asmarian et al. 2021; Romanelli et al. 2020; Collaborators 2019a), it is well established that both incidence and prevalence of the disease are significantly higher in females than in males (Rotstein et al. 2018). Previous literature demonstrated that sexual dimorphism also affects the susceptibility and progression of the disease (Gold et al. 2019). For example, the age at the onset of the disease and the frequency of attacks differ in male and female patients, suggesting sex as an important variable for MS (Gilli et al. 2020).

2.5 Other CNS Diseases

Sex-specific differences have been reported in a range of neurological diseases in addition to the diseases mentioned above, some of which exhibit significantly higher prevalence in one of the sexes. For example, migraine is 2–3 times more prevalent in females than males, and disease severity also exhibits sex-specific differences (Vetvik and MacGregor 2017). On the other hand, stroke disproportionally affects males in terms of incidence of the disease, with variations by age (Vyas et al. 2021). CNS tumors also exhibit sex-dependent differences in disease incidence (Ostrom et al. 2020). For example, glioblastoma multiforme (GBM), the most aggressive CNS cancer, affects males more than women, with the male-to-female incidence ratio of 1.58 (Ostrom et al. 2020). There are also CNS diseases exhibiting roughly similar prevalence and incidence between females and males. For example, Huntington's disease (HD), a progressive

neurodegenerative condition caused by the polyglutamine repeat expansion in the huntingtin gene (Bates et al. 2015), affects both sexes roughly equally. However, recent analyses of a large cohort in the United States have suggested that HD prevalence is slightly higher in females than males (Bruzelius et al. 2019). HD symptoms also appear to be sex-specific (Hentosh et al. 2021), and there is growing interest in understanding sex-specific differences in the disease (Zielonka and Stawinska-Witoszynska 2020).

Neurodevelopmental disorders, including attention deficit hyperactivity disorder, intellectual disability, and autism spectrum disorder, have also been reported to exhibit prevalence differences between males and females, with significantly higher prevalence in males (May et al. 2019). For example, autism affects males about four times more than females (Fig. 1), although lower and higher male-to-female prevalence ratios have been reported in different geographic locations (Fombonne 2009; Elsabbagh et al. 2012; Baio et al. 2018). Similarly, there are sex differences in mental disorders regarding disease severity and prevalence. For example, schizophrenia symptoms, age at onset, and response to treatment differ between sexes, although prevalence appears to be similar in males and females, with slightly higher numbers in males (Hsu et al. 2019; Gonzalez-Rodriguez et al. 2020; Sommer et al. 2020; Wittchen et al. 2011). Major depressive disorder also exhibits symptom differences and significant prevalence differences between sexes, which is nearly doubled in females (Wittchen et al. 2011; McLean et al. 2011; Boyd et al. 2015; Kuehner 2017) (Fig. 1).

3 Mechanisms Implicated in Sex Differences in Neurodegenerative Diseases

As summarized above, many neurological disorders exhibit significant differences in males and females in terms of disease prevalence, symptoms, disease progression, and severity. It has been suggested that biological sex differences beyond gender-related effects might have a role in the outcome, and sex is an important biological variable that should be taken into account in CNS disease research (Rich-Edwards et al. 2018). Many pathways have been implicated in sex-dependent differences in neurodegenerative processes, including potentially female and male sex-specific risk genes and other differentially regulated pathways (Guo et al. 2021; Vaidya et al. 2021; Meoni et al. 2020). Here we briefly focus on some selected factors commonly associated with sex-specific differences.

3.1 Sex and Genetic Factors

Previous literature suggests a correlation between sex and known genetic risk factors for many CNS diseases, including AD (Guo et al. 2021) and PD (Vaidya et al. 2021). A great example comes from the AD field. Several groups have demonstrated that the best-known genetic risk factor for AD, apolipoprotein E (APOE)-ε4 genotype, may have sex-dependent effects, making females more vulnerable to both dementia progression (Altmann et al. 2014; Cho et al. 2021; Wang et al. 2019) and neurodegeneration (Hohman et al. 2018). A meta-analysis study suggested that females with APOE ε3/ε4 genotype have a particularly increased risk of developing AD between the ages of 65 and 75 compared to males with the same genotype (Neu et al. 2017). APOE-ε4 association with cerebrospinal fluid (CSF) biomarkers, especially tau, also appears to be sex-specific, with a stronger association in females (Hohman et al. 2018; Liu et al. 2021; Altmann et al. 2014). Taken together, a large body of literature suggests that effect(s) of APOE genotype on the risk of developing AD is more significant in females (Belloy et al. 2019); however, molecular mechanisms underlying the sex-specific effects of APOE in AD are not clear. One potential hypothesis involves the cross-talk between APOE genotype and the sex hormone estrogen signaling. Estrogen is reported to be a regulator of APOE gene expression (Stone et al. 1997). Importantly, estrogen receptor (ER)α activation leads to enhanced APOE mRNA and protein expression, while the activation of ERβ leads to opposite results (Wang

et al. 2006), suggesting a tight regulation of APOE levels by estrogen signaling. The potential cross-talk between APOE and estrogen might be indirect as well. A recent study identified a significant positive correlation with CSF APOE and sex hormone-binding globulin (SBHG), which inhibits the functions of biologically active testosterone and estrogen (Liu et al. 2021). Plasma levels of AD patients have been reported to have higher levels of SBHG, which may potentially lead to the inactivation of testosterone and estrogen and thereby bioavailability of the hormones (Xu et al. 2016). Overall, a better understanding of the correlation between biological sex and genetic risk factors may open new research avenues for CNS diseases.

3.2 Sex Hormones

Potential roles of sex hormones, especially estrogen, have long been recognized and studied in the context of various CNS diseases, including AD, PD, ALS, and anxiety and related disorders (Li and Graham 2017; Vegeto et al. 2020; Trojsi et al. 2020). Estrogen signaling attracts significant attention in AD research since a potential mechanism underlying the sex-specific differences in AD is the rate of sex hormone depletion with age (Uddin et al. 2020). Male hormone levels gradually decline with aging (Morley et al. 1997). However, the depletion is more severe for females as menopause is a critical contributor, in addition to aging, to the hormone levels (Russell et al. 2019). Estrogen, mainly 17-β-estradiol (E2) as the main circulating female sex hormone, is produced in the brain and gonadal tissues and is neuroprotective against neurodegeneration (Raghava et al. 2017; Akwa 2020). E2 exerts its effect primarily through ERα and ERβ, mediating membrane-initiated and transcription-dependent pathways (Yasar et al. 2017). E2 is involved in various vital physiological functions, including regulating CNS homeostasis (Russell et al. 2019). Therefore, it has been suggested that such sharp depletion of E2 could make females more vulnerable to AD than males of the same age. E2 is indeed lower in female AD patients compared to controls, while there is no difference between healthy and AD males (Yue et al. 2005; Rosario et al. 2011), and therefore, the level of E2 has been associated with sex-dependent differences observed in AD (Sahab-Negah et al. 2020). The decline of E2 correlates with the severity of AD pathogenesis (Pike 2017). Studies in *in vitro* and animal models have provided compelling evidence that E2 has neuroprotective effects against AD (Azcoitia et al. 2019). However, clinical studies with estrogen-based approaches have yielded controversial results as some researchers found the hormone therapy beneficial effects against AD (Tang et al. 1996; Song et al. 2020), while some other researchers reported either no benefit (Wang et al. 2000; Mulnard et al. 2000) or, worse, increased risk for AD or some other conditions (Resnick et al. 2009; Savolainen-Peltonen et al. 2019; Espeland et al. 2004). It has been suggested that the time window of the therapy might be crucial (Merlo et al. 2017). Regardless, a better understanding of the biological basis of sex-specific differences observed in AD is crucial to determine whether E2-mediated neuroprotection can indeed be an attractive approach for AD. In addition to the neuroprotective roles of estrogen in neuronal cells, the signaling pathway has been shown to also play important roles in glial cells. For example, estrogen signaling appears to be critical for the differentiation and myelination processes of oligodendrocyte precursor cells (OPC), relevant to MS disease (Khalaj et al. 2016; Kumar et al. 2013). Although the basis for sex-specific differences in MS are not well understood, it has been suggested that sex hormones may play an important role in the disease initiation and progression (Bove and Gilmore 2018; Miller et al. 2014; Triantafyllou et al. 2016).

3.3 Inflammation

Inflammation has been implicated in many CNS diseases, and sex has been reported to be an important factor for inflammatory dysregulation (Hanamsagar and Bilbo 2016). There are sex differences in immunological responses in

diseases (Klein and Flanagan 2016), and it has long been suggested that sexual dimorphism in the immune system may be critical for CNS diseases (Lopez-Lee et al. 2021). Inflammatory dysregulation is reported to be sex-biased. For example, female MS patients suffer from higher inflammation compared to males (Ramien et al. 2016). Microglia is the primary immune cell in the CNS, which reacts differently under homeostatic and pathological conditions (Kettenmann et al. 2011). Under pathological conditions, microglia is highly active with many functions, including the destruction of pathogens (Boche et al. 2013). Microglia dysfunction has been implicated in various neurodegenerative diseases (Ashford et al. 2021; Salter and Stevens 2017). Female and male microglia exhibit significant differences at the molecular level (Guneykaya et al. 2018; Villa et al. 2018; Hammond et al. 2019), and importantly sex differences in microglia may be contributing to well-established sex bias in neurodegenerative diseases (Kodama and Gan 2019).

3.4 Neurotrophin Signaling

Another crucial pathway implicated in sex-specific differences in neurodegenerative diseases is neurotrophin signaling (Chan and Ye 2017). The neurotrophin family of growth factors is composed of brain-derived neurotrophic factor (BDNF), nerve growth factor, neurotrophin-3, and neurotrophin-4 (Tessarollo 1998); and among these, BDNF is the most associated one with sex-specific differences in neurodegeneration (Wei et al. 2017). BDNF has a central role in brain development, physiology, and pathology (Miranda et al. 2019) and transduces its effect primarily through its high-affinity tyrosine kinase receptor, TrkB, mediated signaling (Chao 2003). BDNF has a neuroprotective role against neurodegeneration, and defects in BDNF and TrkB levels and functions have long been associated with many CNS diseases (Mitre et al. 2017). For example, BDNF and TrkB are downregulated in AD, and the correlation between AD and defects in BDNF signaling have been reported (Ginsberg et al. 2019; Caffino et al. 2020). Previous studies suggest BDNF-TrkB signaling may contribute to sex-specific differences in cells that make up the nervous system (Chan and Ye 2017). Involvement of BDNF in AD, in a sex-specific manner, may involve a cross-talk with estrogen signaling, potentially through many mechanisms. The first potential mechanism involves the regulation of BDNF expression by estrogen. Indeed, it has been shown that E2 can modulate the expression of both BDNF and its receptor TrkB (Milne et al. 2015; Bora et al. 2005; Sohrabji et al. 1995), and BDNF is regulated in a sex-specific manner in the brain (Liu et al. 2012). The second potential mechanism is the BDNF association with APOE in a sex-specific manner. Potential cross-talk of APOE and estrogen signaling is discussed above (Sect. 3.1.). It has been shown that APOE regulates BDNF processing and release, suggesting a direct regulation between two factors in AD (Sen et al. 2017). Lastly, there may also be a sex-specific convergence between the E2-ER and BDNF-TrkB signaling at the cytoplasmic level regardless of gene expression changes (Chan and Ye 2017; Wong et al. 2011). ER and TrkB receptors are co-localized in hippocampal and cortical neurons (Miranda et al. 1993), which are highly affected in AD. It has been shown that TrkB overexpression in cells can induce ERα phosphorylation, suggesting the cytoplasmic signaling cross-talk (Wong et al. 2011). Importantly, phosphorylation and the distribution of TrkB in the brain is sex-specific (Spencer-Segal et al. 2011; Hill and van den Buuse 2011), and TrkB and ERα signaling share common downstream effectors in neurons (Chan and Ye 2017). Additionally, small molecule-based TrkB activation exhibits sex-specific effects in hypoxia-ischemia-induced brain injury models, and observed TrkB-mediated protective effect requires ERα signaling (Uluc et al. 2013; Cikla et al. 2016). Lastly, a recent study utilizing two different neuroimaging

methods demonstrated that estrogen modulates the effects of BDNF in hippocampal function in women's brains (Wei et al. 2018), further suggesting the convergence of the two pathways in the human brain. Despite these efforts, the molecular mechanism(s) underlying the potential intersection between BDNF-TrkB and E2-ER signaling in neurons is still largely unexplored, and their potential integrated effects in CNS diseases are not well understood.

Sex-specific effects of BDNF have also been implicated in other molecular mechanisms. For example, BDNF has been shown to affect inflammatory responses in a sex-specific manner (Rossetti et al. 2019), and BDNF polymorphism Val66Met also leads to sexually dimorphic effects, affecting females more negatively (Chen et al. 2014; Fukumoto et al. 2010). Although the examples provided above have focused on AD, BDNF pathways have also been implicated in many other CNS diseases. For example, the BDNF-TrkB signaling pathway affects OPC differentiation and myelination in the context of MS (Van't Veer et al. 2009; Xiao et al. 2010); however, the sex-specific effects of the pathway are not well understood. Generally speaking, neurotrophin signaling is considered a therapeutic target for many neurodegenerative diseases (Mitre et al. 2017). Therefore, there are efforts to modulate the pathways *in vivo* through various mechanisms, including agonists and antagonists (Saragovi et al. 2019), and identification of critical residues of Trk receptors for signaling and their degradation (Kiris et al. 2014; Yu et al. 2014). A better understanding of truncated Trk receptors in neural cells may also open avenues to better control neurotrophin signaling in neurons (Palko et al. 1999; Yanpallewar et al. 2021; Yanpallewar et al. 2012; Esteban et al. 2006; Galan et al. 2017; Bai et al. 2010; Brahimi et al. 2016; Dorsey et al. 2006; Ferrer et al. 1999; Quarta et al. 2018; Tomassoni-Ardori et al. 2019). In addition to such efforts, elucidation of potential sex-specific roles of neurotrophin signaling can pave the road for neurotrophin-mediated and potentially sex-specific neuroprotection.

4 Model Systems to Study Sex-Specific Differences in Neurodegenerative Diseases

4.1 Importance of Human Model Systems

Many model systems, including numerous animal models, have been developed to study neurodegenerative diseases, which have been extensively discussed in excellent reviews (Scearce-Levie et al. 2020; Drummond and Wisniewski 2017; Cenci and Bjorklund 2020). However, there has been growing literature demonstrating significant differences between animal models and humans, which may hamper the scientific development to understand the disease mechanisms and the pace of drug development efforts (Drummond and Wisniewski 2017). A great example of rodent models vs. human differences comes from the MS field. Studies focusing on remyelination in MS have often used rodent models (Sanabria-Castro et al. 2020), which has been crucial for scientific development in the field; however, recent studies have pointed to significant differences between human and animal models, particularly in cell types (OPCs vs. surviving, existing oligodendrocytes) carrying out remyelination (Yeung et al. 2019). Additionally, species-specific differences may be critical for drug discovery. For example, significant potency differences have been reported for some compounds between mouse and human stem cell–derived neuronal systems (McNeish et al. 2010). Therefore, the utility of biologically relevant human neural cell types may enhance scientific development in the neurodegenerative disease fields. Such platforms may also be utilized to validate observations made using non-human mammalian systems.

4.2 Sex of the Cell Matters

There has been a sex bias in basic and clinical research, which extensively utilized male cells

and male animal model systems (Will et al. 2017; Klein et al. 2015). Male sex bias has been more prominent in neuroscience; however, the inclusion of both sexes in studies appears to be increasing over the years (Woitowich et al. 2020; Mamlouk et al. 2020). Despite the mandates from funding sources such as the U.S. National Institutes of Health (Clayton and Collins 2014) and the increased awareness of sex as a biological variable, the sex of the cells utilized in research is still largely unreported. For example, James *et al*. surveyed 303 papers utilizing *in vitro* cell culture experiments in the field of biomaterials and found that 96.3% of the articles did not report the sex of the utilized cells (James et al. 2021).

Previous literature suggests that the sex of the cell matters as male and female cells exhibits different characteristics under the same conditions in various contexts (Lee 2018). Indeed, cellular sex differences have been reported in many different cell types, including osteoblasts (Berger et al. 2018), cardiac cells (Walker et al. 2021), neurons (Fairbanks et al. 2012), and glial cells (Yasuda et al. 2020). There are differences in cellular pathways in male and female cells, which can exhibit sex-specific effects. For example, the sex of the cell appears to be important for impairments in mitochondrial metabolism (Yao et al. 2009; Demarest et al. 2020) and neuronal death pathways (Demarest and McCarthy 2015). Some of the sex-specific differences could be due to the differential expression of specific receptors. For example, ERα protein levels are reported to be significantly higher in female neurons (Bryant and Dorsa 2010), which may be critical for sex-specific neuroprotection. Female and male neurons in culture exhibit differences in various characteristics, including dendritic morphology (Keil et al. 2017), pool size of recycling synaptic vesicles (Sertel et al. 2021), GABA receptor activation (Mir et al. 2020), survival under normoxia and hypoxia conditions (Heyer et al. 2005), oxygen-glucose deprivation (Fairbanks et al. 2012), cell death pathways (Sharma et al. 2011), and dopamine-induced neurotoxicity (Lieb et al. 1995).

In addition to neurons, glial cells also exhibit sexually dimorphic characteristics that are hypothesized to be important for sex-specific differences in neurodegeneration (Chowen and Garcia-Segura 2021). Recent genomics studies at single-cell resolution utilizing female and male AD patient samples identified subpopulations of human brains cells, particularly in glial cells in AD (Mathys et al. 2019; Grubman et al. 2019; Nott et al. 2019), exhibiting disease-specific changes in gene expressions. Among the glial cells, microglia is increasingly implicated in sex-specific differences in various neurodegenerative diseases (Delage et al. 2021; Kodama and Gan 2019), with implications that female microglia has differences compared to male microglia (Guneykaya et al. 2018; Villa et al. 2018; Hammond et al. 2019). For example, there are differences in expressed miRNAs in male and female microglia, which is critical for sex-specific microglia responses to AD tau pathology (Kodama et al. 2020). Genetic factors and sex may combinatorially affect microglia function in pathological conditions. For example, APOE and sex are important factors regulating TREM-2-dependent microglia activation in AD (Stephen et al. 2019). Oligodendrocytes also exhibit sexual dimorphism in normal and pathological conditions. For example, it has been shown that female oligodendrocytes are short-lived compared to that of males in rodents (Cerghet et al. 2006), and there are differentially regulated signaling pathways between the two sexes (Swamydas et al. 2009). Sexual dimorphism also exists in OPCs, and differential effects can be observed in cell culture studies. For example, female OPCs are more resistant to hyperoxia-induced damage (Sunny et al. 2020) and oxygen-glucose deprivation (Yasuda et al. 2020) in culture. These differences might be important for remyelination in male and female MS patients. Sex-specific differences in astrocytes have also been reported in culture. For example, female primary astrocytes respond to lipopolysaccharide-induced inflammatory challenges differently than males (Santos-Galindo et al. 2011; Chistyakov et al. 2018). Cultured astrocytes respond to oxygen-glucose

deprivation (Liu et al. 2007), a toxic molecule, insecticide dimethoate, (Astiz et al. 2014), and chronic alcohol exposure (Wilhelm et al. 2016) in a sex-specific manner. Interestingly, the phagocytic activity of cultured primary astrocytes also differs in male and female cells (Crespo-Castrillo et al. 2020). Taken together, previous literature strongly suggests that the sex of the cell matters and sex-specific differences can be assayed in culture.

4.3 Utilization of Human Cellular Platforms for Sex-Specific Differences

Considering the rodent and human neuronal cell differences and the documented translational challenges between rodents to humans, *in vitro* model systems utilizing human neuronal and glial cells have long been suggested as a potentially optimal strategy to complement existing *in vivo* models. Human cells and samples from various sources are being utilized in research, all of which have advantages and limitations. Analyses on the human post-mortem tissue are highly useful; however, they are limited to relatively small-scale studies due to the availability of high-quality post-mortem tissue. Obtaining samples from brain surgeries is also not a viable option for well-controlled and large-scale studies. Immortal human cell lines are also being extensively utilized for neurodegenerative disease research as they offer simple, cost-effective, and easily manipulatable cell environments. However, their genetic background differences from native neuronal and glial cells and tumorigenic propensities make them less attractive. An alternative methodology to generate human neuronal and glial cell types is the directed differentiation of human pluripotent cells, i.e., human ES cells (Sect. 4.3.1.) or induced pluripotent stem cells (iPSC) (Sect. 4.3.3). Pluripotent stem cells have two unique characteristics. First, these cells can be differentiated into specific cell types, including neuronal types and glial cells. Secondly, the cells have potentially unlimited self-renewing capacity, suggesting that these cells can be propagated in large quantities to generate a large number of cells for large-scale studies. In addition to the directed differentiation of pluripotent stem cells, neuronal and glial cells can also be directly reprogrammed from somatic cells (Sect. 4.3.2.) (Mertens et al. 2018).

4.3.1 Human Embryonic Stem (ES) Cell–Derived Neural Systems

There have been extensive research efforts to obtain specific neuronal cell types from human ES cells, and such model systems have been successfully utilized to study neurodegenerative diseases. It is well established that human ES cells can be differentiated into functional neuronal subtypes, including cortical neurons (Shi et al. 2012), motor neurons (Li et al. 2008), basal forebrain cholinergic neurons (Bissonnette et al. 2011), midbrain dopaminergic neurons (Kriks et al. 2011), and striatal neurons (Delli Carri et al. 2013). In addition to neurons, human ES cells have also been successfully differentiated to microglia (Muffat et al. 2016), astrocytes (Shaltouki et al. 2013; Emdad et al. 2012), and oligodendrocytes (Gorris et al. 2015; Hu et al. 2009). Such cells provide excellent culture systems for mechanistic studies (Hardingham et al. 2010), toxicity testings (Deshmukh et al. 2012), and drug screening (Kiris et al. 2015a, b; Little et al. 2019) in a human neuronal system. It has been shown that the sex of the ES cell significantly affects the directed differentiation of the pluripotent cells. For example, one study demonstrated sex-biased gene expression and differentially regulated cellular pathways in trophoblastic progenitor cells differentiated from male and female human ES cells (Syrett et al. 2018). Sex-specific differences have also been reported in human ES cell differentiation toward endothelial progenitor cells (Randolph et al. 2019) and fetal germ cells, and human primordial germ cell-like cells (Mishra et al. 2021). These examples suggest that neuronal and glial cells derived from male and female human ES cells can provide useful platforms to study sex-specific differences; however, its applications might be somewhat limited compared to the sex-matched patient and control iPSC-derived culture platforms.

4.3.2 Direct conversion of Somatic Cells to Neurons and Glial Cells

In addition to pluripotent stem cell–based approaches, it is also possible to directly convert somatic cells to neural lineages. This approach has certain advantages, including the shorter culture and maintenance of cells, which is both time- and cost-effective compared to pluripotent stem cell–based differentiation. Additionally, it has been suggested that reprogramming somatic cells to iPSCs may affect the age-related epigenetic landscape, a limitation that can be potentially overcome with directly converted neural cells (Mertens et al. 2015). Directly converted neurons are functional (Pang et al. 2011), and many neuronal subtypes have been generated with this approach (Mertens et al. 2018). Similarly, direct conversion of fibroblasts to oligodendrocyte precursors (Yang et al. 2013; Najm et al. 2013) and astrocytes (Caiazzo et al. 2015; Tian et al. 2016) have been achieved. Induced microglia-like cells have also been generated, utilizing monocytes as the starting cells (Sellgren et al. 2019; Ryan et al. 2017; Ohgidani et al. 2014; Banerjee et al. 2021). While direct reprogramming is an exciting approach, a significant limitation is that human cells' conversion efficiency and yield are lower than the directed differentiation of human pluripotent stem cells. However, there are continuing efforts to improve the protocols for efficient conversion (Ng et al. 2021). Induced neuronal cell types, induced astrocytes, and induced oligodendrocytes can also be potentially utilized to study sex-specific differences in neurodegenerative diseases. For example, a recent study generated iNeurons from fibroblasts of 16 AD patients and 19 healthy controls, exhibiting defects in AD iNeurons compared to controls (Mertens et al. 2021). Such experimental setup utilizing induced neurons, oligodendrocytes, or astrocytes can also include analyses to examine sex-specific differences in AD and other CNS diseases. Importantly, it has been shown that induced cells can be utilized to study biological sex-specific differences. For example, a recent study utilizing mouse-induced hepatocyte-like cells reprogrammed from male or female primary mouse embryonic fibroblasts demonstrated sex-specific expression patterns (Ullah et al. 2021).

4.3.3 Induced Pluripotent Stem Cell (iPSC)–Based Systems Can Be Utilized to Assess Sex-Specific Differences

Obtaining neurons or glial cells affected by specific diseases starting from the patients' cells constitutes a crucial resource for modeling CNS diseases. Since the initial studies demonstrated reprogramming adult cells with mitotic propensities to a pluripotent state, there has been significant scientific advancement to optimize reprogramming and differentiation protocols. iPSCs have been generated from patients suffering from many CNS diseases, including AD (Penney et al. 2020), PD (Avazzadeh et al. 2021), motor neuron diseases (Karpe et al. 2021), and MS (Begentas et al. 2021; Douvaras et al. 2014; Miquel-Serra et al. 2017; Morales Pantoja et al. 2020; Mozafari et al. 2020; Mutukula et al. 2021; Lopez-Caraballo et al. 2020; Nicaise et al. 2017). Directed differentiation of human iPSCs to various human cell types, including neurons, has been achieved, with specific efforts to develop methodologies to obtain relatively pure cultures for CNS disease modeling (D'Souza et al. 2021). Previous studies have demonstrated successful derivation of neuronal subtypes from iPSCs, including cortical neurons (Shi et al. 2012), motor neurons (Dimos et al. 2008), basal forebrain cholinergic neurons (Duan et al. 2014), midbrain dopaminergic neurons (Devine et al. 2011), and striatal neurons (Delli Carri et al. 2013). Recent efforts have also established efficient protocols to generate glial cells from iPSCs. Functional microglia have been efficiently generated from human iPSCs (Muffat et al. 2016; Abud et al. 2017; Pandya et al. 2017; Haenseler et al. 2017; Douvaras et al. 2017; McQuade et al. 2018). Similarly, human iPSCs have been differentiated to astrocytes (Shaltouki et al. 2013; Tcw et al. 2017; Emdad et al. 2012), and oligodendrocytes (Gorris et al. 2015; Ehrlich et al. 2017).

Importantly, numerous studies have demonstrated that iPSC-derived neuronal and glial cultures can be utilized to observe neurodegenerative disease-specific phenotypes. For example, neurons differentiated from iPSCs derived from sporadic and familial AD patients exhibit disease characteristics, including increased tau protein phosphorylation and amyloid levels (Ochalek et al. 2017; Ortiz-Virumbrales et al. 2017; Wezyk et al. 2018; Israel et al. 2012). Similarly, iPSC-derived astrocytes have provided critical knowledge about the diseases, including AD (Jones et al. 2017; Oksanen et al. 2017; Konttinen et al. 2019b), Schizophrenia (Akkouh et al. 2020), and Down syndrome (Kawatani et al. 2021) in human culture systems. Microglia cells differentiated from iPSCs have also been shown to be highly useful in disease modeling (Reich et al. 2020; Konttinen et al. 2019a; Garcia-Reitboeck et al. 2018). Directed differentiation of human iPSCs toward oligodendrocytes generates functional oligodendrocytes that provide critical information for relevant diseases, such as MS and other myelin diseases (Mozafari et al. 2020; Lopez-Caraballo et al. 2020). iPSC-derived *in vitro* culture systems has also been successfully utilized in drug screening in the context of neurodegenerative diseases (Little et al. 2019).

Previous work demonstrated that iPSC-derived cells could exhibit sex-specific differences. For example, an important study revealed that male and female iPSCs could form smooth muscle progenitor cells without significant differences in the differentiation efficiency; however, there are sex-specific differences in the derived male and female cells, which differentially respond to E2 stimulation (Li et al. 2017). The ability to generate specific neuronal subtypes and glial cells exhibiting disease phenotype using iPSC-based models are crucial for CNS disease research (Vadodaria et al. 2020), and such systems can be utilized for assessing sex-specific differences in culture. A representative workflow of neuronal and glial cell derivation from iPSCs generated from the male and female patients and healthy individuals to study sex-specific differences is illustrated in Fig. 2. Peripheral blood mononuclear cells (PBMCs) serve as a common source cell type to generate iPSCs. PBMCs can be isolated from peripheral blood samples, cultured in particular media for erythroblast enrichment, and then reprogrammed to iPSCs (Begentas et al. 2021). To study sex-specific differences in CNS diseases, such iPSC lines can be differentiated to neuronal and glial cultures in 2D and 3D environments (Fig. 2). Despite the great potential of such systems, there are still a limited number of publications evaluating sex-specific differences in patient and control iPSC-derived neural culture; however, such literature is expected to increase as recent work sets excellent examples. A great example comes from the schizophrenia field. Recent work demonstrates sex-specific differences in the gene expression profiles of cortical neurons differentiated from healthy control and schizophrenia patient iPSCs (Tiihonen et al. 2019). Furthermore, in a follow-up paper which is a preprint at the time of the composition of this review, the authors utilized the same iPSC lines derived from schizophrenia twin pairs and healthy controls and demonstrated that human iPSC-derived astrocytes also exhibit sex-specific differences (Koskuvi et al. 2020). Although both patient and control iPSC can differentiate into astrocytes with similar efficiency, expression profiles of certain genes and some specific cellular pathways are differentially regulated in male and female schizophrenia astrocytes (Koskuvi et al. 2020).

Genetic Manipulation of iPSCs to Generate Isogenic Controls

Since the initial discovery and development of genome editing methodology clustered regularly interspaced short palindromic repeats (CRISPR)-Cas9 (Barrangou et al. 2007; Jinek et al. 2012), there have been significant efforts to make the approach more precise and well controlled. Apart from its potential in treating CNS diseases (Lubroth et al. 2021), CRISPR-Cas9-based gene editing approach has great value in generating neurodegenerative disease models, which can be at least in two ways. First, WT cells, such as iPSCs from healthy control, can be genome engineered to knock in specific disease-associated

mutations. For example, it has been shown that homozygous and heterozygous mutations could be introduced to iPSCs with the CRISPR-Cas9 approach that can model AD (Paquet et al. 2016). Second, isogenic controls of iPSCs can be generated using CRISPR-Cas9 (Fig. 2), which are highly crucial for *in vitro* modeling. This is important as the healthy donor iPSCs may not be the optimal control cells to compare to patient iPSCs in studies due to variations in the genetic landscape. Taken together, considering the genetic diversity between healthy and patient-specific iPSCs, and many unknown molecular determinants, which may include sex-specific differences, in disease characteristics, isogenic controls of patient iPSCs may provide valuable information, as documented in the context of many CNS diseases, including AD (Kwart et al. 2019; Ortiz-Virumbrales et al. 2017), and Down Syndrome (Kawatani et al. 2021).

Three-Dimensional (3D) Modeling

Recent studies have suggested that 3D human neuronal systems can provide a brain-like model for research on neurodegenerative diseases (Siney et al. 2018). Such organoids represent a topological environment that allows cell-to-cell interactions and the formation of neuronal networks, which may provide a more physiologically relevant system than 2D culture (Gopalakrishnan 2019). Indeed, cerebral organoids exert neurodegenerative disease-specific phenotypes (Chang et al. 2020). For example, recent studies demonstrated that such systems exhibit both Aβ and tau pathologies and can model AD (Ghatak et al. 2019; Raja et al. 2016; Yin and VanDongen 2021). Importantly, neuroinflammatory components have also been introduced in 3D AD models, which exhibited critical hallmarks of AD, i.e., Aβ42 accumulation, tau phosphorylation, and microglia-induced

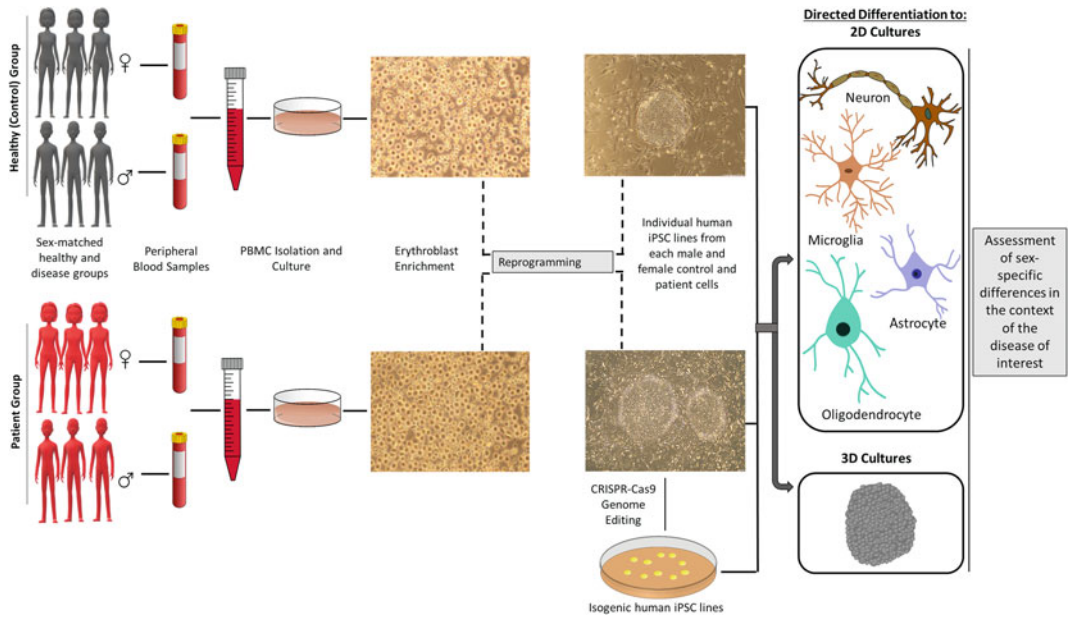

Fig. 2 Modeling sex-specific differences *in vitro* using human-induced pluripotent stem cell (iPSC)–derived cellular platforms. PBMCs can be isolated from the blood samples of male and female patients and healthy individuals, cultured for erythroblast enrichment, and then reprogrammed to iPSCs. The representative iPSC colonies in the figure are co-cultured with mitotically inactivated mouse embryonic fibroblast cells. Such iPSCs derived from the sex-matched healthy and patient cohorts, and gene-corrected isogenic controls, can be utilized to generate neuronal and glial cell types in 2D and 3D cell culture platforms to study sex-specific differences. These models can be utilized in drug screening assays, toxicity testings, and mechanism of action studies

neurite degeneration and cell death (Park et al. 2018). Similarly, 3D culture platforms have been successfully utilized for many other neurodegenerative disease modeling, including PD (Kim et al. 2019), ALS (Osaki et al. 2018), and SMA (Hor et al. 2018). There is also interest in glial cells in 3D cultures, and it has been shown that myelinating oligodendrocytes can form in such systems (Madhavan et al. 2018; Marton et al. 2019). Similarly, astrocytes (Lin et al. 2018; Quadrato et al. 2017; Sloan et al. 2017) and microglia (Ormel et al. 2018; Xu et al. 2021) can form in 3D-cultures. Isogenic 3D cultures have also been utilized in neurodegenerative disease modeling (Yin and VanDongen 2021; Kim et al. 2019). Taken together, recent advancements suggest that 3D culture models can provide powerful experimental systems to study sex-specific differences in the context of neurodegenerative diseases (Fig. 2). However, it is important to note that 3D models also have many limitations, including variability, cell diversity, and neuronal circuit connectivity (Kim et al. 2021), and such shortcomings need to be overcome to generate better models of neurodegenerative diseases.

Taken together, comparing and contrasting female and male neuronal subtypes and glial cells derived from patient and healthy iPSCs, and isogenic controls, in 2D and 3D cultures can potentially significantly enhance our scientific understanding of CNS diseases.

Limitations of Human İpsc-based Models to Study Biological Sex-Linked Differences in Culture

Despite the great potential, there are also several limitations of human iPSC-based cell culture platforms to study sex-specific differences. First, epigenetic imprinting already present in the donor material to begin with for iPSC generation may affect the phenotype in culture (Braverman-Gross and Benvenisty 2021). Second, the sex of the human iPSC can affect the differentiation capacity of the line (Randolph et al. 2019), which may hamper disease modeling under identical conditions, suggesting that there may be a need to optimize directed differentiation conditions for male and female human pluripotent cells. Third, variability between controls and disease iPSC lines, and even clones of the same line, may complicate data analyses (Volpato and Webber 2020). Fourth, aging is a critical factor for neurodegenerative diseases; however, aging in culture is challenging and may not recapitulate the disease conditions. Fifth, the genomic stability of iPSC lines, in the long run, is questionable. Therefore, regular karyotyping is a must to detect potential chromosomal abnormalities as there are many examples of Y chromosomes being lost in male human and mouse cells in culture (Shah et al. 2014). Although this phenomenon has been mostly reported in cancer cell lines, regular determination of the sexual identity of human iPSC in culture is still critical. Lastly, differentiation of human iPSCs toward neurons or glial cells typically gives rise to heterogeneous cultures. Although there have been efforts to generate more homogenous cultures and accelerate the differentiation protocols (Walsh et al. 2020), the vast majority of current directed differentiation protocols are long, expensive, and laborious.

Researchers must also be careful about factors affecting reproducibility in cell culture when assessing sex-specific differences. Culture medium contains steroids, growth hormones, and insulin that might influence the expression of various genes. For example, phenol red, commonly used in cell culture media, is reported to have an estrogen-like effect in culture (Welshons et al. 1988; Berthois et al. 1986). This is critical as estrogen differentially affects male and female cells in culture (Hong et al. 2009), and phenol red in culture media has been shown to affect neuronal cultures (Liu et al. 2013). Similarly, the serum is another source of factors that potentially confound reproducibility in culture. Given that serum contains many factors, including sex steroids, and there may be significant batch-to-batch differences, the utility of serum-free culture media may be useful for *in vitro* studies assessing sex-specific differences (De Souza et al. 2018). It has also been suggested that certain plastics may release estrogenic chemicals (Bittner et al. 2014; Yang et al. 2011), indicating the importance of plastic products used in media bottles. Regular tests for contaminations, such as mycoplasma, are

also crucial for maintaining high-quality iPSCs. As mentioned above, genomic instability is a critical issue for iPSC-based methodologies (Yoshihara et al. 2019; Rebuzzini et al. 2016), and therefore cultures should be regularly tested for genomic variations. Taken together, assessment of sex-specific effects in culture requires rigorous evaluation of external factors that may affect experimental outcomes, and culture conditions should be kept constant.

5 Concluding Remarks

Neurological diseases are among the top 5 most common causes of disability and death, and their prevalence and economic and social burden are predicted to increase (Deuschl et al. 2020; Collaborators et al. 2021). There are male or female-biased CNS diseases, including striking differences between the sexes regarding the age of onset, symptoms, disease severity, progression, and response to pharmacological treatments (Hanamsagar and Bilbo 2016; Collaborators 2019b; Pinares-Garcia et al. 2018). It has long been suggested that biological sex differences at the cellular level may be critical for disease initiation; however, sex-specific differences in neurodegeneration are still poorly studied (Rich-Edwards et al. 2018; Vegeto et al. 2020). Unfortunately, most CNS disease literature used sex-matched conditions in the analyses without comparisons between the sexes; however, sex is an important variable that should be taken into account for neurodegeneration research (Nebel et al. 2018). Understanding the role of biological sex differences in CNS diseases may be critical for earlier diagnosis and sex-specific treatments (Bartz et al. 2020). Indeed, sex-based neuroprotection has been demonstrated in the context of several neurological disorders. For example, a recent study demonstrated that Transferrin/Transferrin Receptor 2 (TfR2) deletion leads to neuroprotection against PD neurodegenerative process, and the observed effect is sex-dependent, providing higher protection in female mice (Milanese et al. 2021). A better understanding of molecular mechanisms underlying sex-specific differences in CNS diseases requires well-established and controllable model systems. There has been criticism in animal models that they do not recapitulate all pathophysiological and clinical aspects of neurodegenerative diseases. However, *in vivo* model systems are currently indispensable, and it may be best to complement the animal studies with human cellular systems as carefully as possible to study sex-specific differences. Integrating sex into analyses of molecular events at the cellular level may open new avenues for developing better and potentially sex-specific therapeutic options. Modeling sex differences in CNS diseases with human iPSC-based 2D and 3D model systems offers unique approaches to study neurodegenerative diseases in a species-specific manner, with a potentially limitless supply of patients' own neurons or glial cells.

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Current Status, Barriers, and Future Directions for Humanized Mouse Models to Evaluate Stem Cell–Based Islet Cell Transplant

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Abstract

Islet cell transplant (ITx) continues to improve, with recently published long-term outcomes suggesting nearly 80% graft survival, leading to improvements in glycemic control, reductions in insulin doses, and near-complete abrogation of severe hypoglycemia. Unfortunately, access to ITx remains limited by immunosuppression requirements and donor supply. Discovery of stem cell–derived functional islet-like clusters with the capacity to reverse diabetes offers a renewable, potentially immunosuppression-free solution for future widespread ITx. Evaluation and optimization of these therapies is ongoing, but may one day provide a realistic cure for type 1 diabetes. However, stem cell–based ITx has unique immunologic questions that remain unanswered. Here, we briefly synthesize current approaches for stem cell–derived ITx, review humanized mice models, and elaborate on the potential of humanized mice models for bridging the gap between current small rodent models and human clinical trials for allogeneic and autologous inducible pluripotent stem cell (iPSC)–based ITx while highlighting limitations and future directions.

Keywords

Diabetes · Humanized mouse model · Immunosuppression · Inducible pluripotent stem cells · Islet cell transplant · Transplant

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Abbreviations

APC	Antigen presenting cell
BLT	Bone liver thymus mouse model
BRG	BALB/c-Rag2 gamma
ESC	Embryonic stem cell
HSC	Hematopoietic stem cell
Hu-PBL-SCID	Human peripheral blood lymphocyte mouse model
Hu-SRC-SCID	Human SCID repopulating cell mouse model
iPSC	Inducible pluripotent stem cell
ITx	Islet cell transplantation
MHC	Major histocompatibility complex
NRG	NOD-Rag1 gamma (NRG)
NSG	NOD-scid gamma
PBL	Peripheral blood lymphocyte
T1D	Type 1 diabetes

1 Introduction

Twenty two years ago, islet cell transplant (ITx) provided proof-of-concept for a cell-based cure of type 1 diabetes (T1D), when 100% insulin independence was achieved 1 year post-ITx in a small number of subjects using glucocorticoid-free immunosuppression (Shapiro et al. 2000). Although long-term insulin independence only occurred in some patients, ITx has since proven to be a highly efficacious treatment for T1D patients with severe and recurrent hypoglycemia or severe glycemic lability (Marfil-Garza et al. 2020a). With ongoing technical, immune, and engraftment techniques, substantial advances continue to be recognized (Verhoeff et al. 2021a, b; Markmann et al. 2020; Marfil-Garza et al. 2021a); new evidence has demonstrated comparable 20-year patient survival between ITx and other T1D patient cohorts and 10-year graft survival rates of nearly 80% with sustained improvements in glycemic control, reductions in insulin doses, and near-complete abrogation of severe hypoglycemia (Verhoeff et al. 2021b; Vantyghem et al. 2019; Lemos et al. 2021; Collaborative Islet Transplant Registry 2017). The primary barriers to a more widespread use of ITx are lifelong immunosuppression requirements and limited donor supply. Embryonic stem cells (ESCs) and inducible pluripotent stem cells (iPSCs) present an exciting development and a path to simultaneously overcoming both barriers (Verhoeff et al. 2021a, b; Latres et al. 2019; Shapiro et al. 2021). With iPSC-based ITx, the Yamanaka transcription factors (Oct3/4, Sox2, Klf4, and c-Myc) are overexpressed in diverse mature cells (e.g., peripheral blood cells) to reverse them into a pluripotent state (Takahashi et al. 2007; Dadheech et al. 2022). Using ESCs and iPSCs functional islet cell clusters for transplant can be generated following a well-defined protocol (Verhoeff et al. 2021a; Rezania et al. 2014; Hogrebe et al. 2020). However, as stem cell–based ITx advance toward clinical trials, the question remains whether evaluation in rodents is sufficient or indeed confounding to demonstrate translational efficacy and safety.

While mice offer economical and ethical models to study ITx, their immune systems are dissimilar to those of humans, which limits their utility to evaluate immunogenic responses related to transplantation (Mestas and Hughes 2004; Cantarelli et al. 2013; Montanari et al. 2019). Historically, chimpanzees have helped bridge the gap between small rodent animal models and clinical trials in humans; however this has become less feasible due to ethical and funding limitations (Shultz et al. 2012; National Institutes of Health 2011). These limitations have led to an expanding interest in chimeric and humanized mouse models that could better replicate the human immune system.

In this review, humanized mice models refer to immunodeficient mice engrafted with portions of a human immune system. Ideally, these models would display an entire, innate, and acquired human immune system. Current efforts have focused on generating mouse strains capable of representing important aspects of human immune responses, more than the whole immune system. While it has been postulated that humanized mice may provide a way of evaluating stem cell–based ITx (Flahou et al. 2021), it remains unclear whether they can be utilized for evaluation of

autologous iPSC–based ITx. Here, we briefly synthesize current approaches for stem cell–based ITx, review humanized mice models, and elaborate on the potential of humanized mice models for bridging the gap between current small rodent models and human clinical trials for allogeneic and autologous stem cell–based ITx, while highlighting limitations and future directions.

2 Stem Cell–Based Islet Cell Transplantation

Currently, clinical ITx demonstrates proof-of-concept for a cell-based treatment for T1D (Marfil-Garza et al. 2020a, 2021a; Markmann et al. 2020; Vantyghem et al. 2019; Lemos et al. 2021; Shapiro et al. 2017; Zarinsefat and Stock 2020). As a way of addressing limitations in organ donor supply, ongoing work with ESCs and iPSCs have shown capacity to differentiate into functional islet-like clusters capable of reversing diabetes (Fig. 1) (Verhoeff et al. 2021a; Dadheech et al. 2022; Rezania et al. 2014; Hogrebe et al. 2020; Kroon et al. 2008; Sui et al. 2018). Their promise offers a path to personalized cell–based therapies that could ultimately offer a true cure for T1D (Verhoeff et al. 2021a; Latres et al. 2019; Takahashi et al. 2007). Herein, we discuss ESCs and iPSCs as they relate to humanized mouse models; for a recent review on stem cell–based cellular therapies in diabetes, see Verhoeff et al. (Verhoeff et al. 2021a, b). As it relates to humanized mouse models, two approaches for stem cell–based ITx have been suggested: allogeneic and autologous, each with unique immune-related considerations that require further evaluation.

Allogeneic stem cell–based ITx involves generating human leukocyte antigen (HLA)-matched or immune-silenced ESC or iPSC banks that can be used as a source of islet differentiation for all patients. It is highly likely that matching for major HLA antigens alone would be doomed to failure and mismatched minor antigens still generate potent, destructive immune response. Therefore generalized immunosuppression or alternatively some local or CRISPR-Cas9 genetic edit-based approaches would be needed to sustain allograft cell survival. Allogeneic approaches do however offer a technique that would simplify upscaling stem cell–based ITx to provide cells for >4 million T1D patients (Mobasseri et al. 2020). However, modified allogeneic islets represent a technically difficult approach, and currently only offer reduction and not complete elimination of immunosuppression. Several approaches to generating immune-tolerated allogeneic stem cell-based islets are being considered. Genetically modified stem cells may enable expression of immunotolerant molecules such as IL-10 or PD-L1 (Xu et al. 2015; Falcone and Fousteri

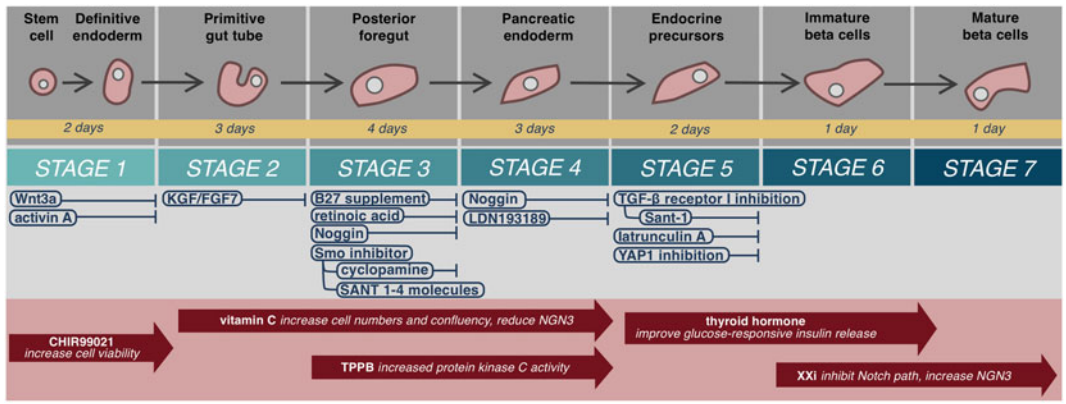

Fig. 1 Embryological differentiation and maturation of islet cells. (Previously published by Verhoeff et al. (2021a) with permission for reuse)

2020). Alternatively, generating ESCs or iPSCs without HLA class 1 molecule expression may further reduce graft rejection and immunosuppression requirements. Han et al. (2019) and others have recently generated iPSCs without HLA class I molecules and expressing the immunomodulatory factors PD-L1, HLA-G, and CD47, which resulted in with blunted T-cell reactivity, minimal NK cell–mediated death, and macrophage phagocytosis (Han et al. 2019; Deuse et al. 2019; Shi et al. 2020). Similarly, ViaCyte's PEC-QT multitiered approach also takes advantage of these concepts and combines a genetically modified clonal ESC line expressing PD-L1 and lacking HLA class I molecules (i.e., β microglobulin), with their PEC-Direct subcutaneous macroencapsulation device, and is expected to enter clinical trials soon (Verhoeff et al. 2021a, b; Shapiro et al. 2021; Sluch et al. 2019). However, islet cell maturation and their physiologic capacity to maintain normoglycemia after genetic modification remains unproven. Similarly, it remains uncertain whether allograft rejection will still occur despite genetic manipulation. Ongoing studies are certainly required prior to clinical implementation of allogeneic stem cell–based ITx.

Alternatively, individual iPSCs could be generated for each patient to create personalized islet-like cells for autologous ITx, which may eliminate the need for immunosuppression altogether. However, autologous islet cell clusters may still be subjected to recurrent autoimmune graft destruction (Verhoeff et al. 2021a, b). While autologous iPSC–derived islets would not face allogenecity, similar to what occurs following autologous ITx after total pancreatectomy, the effect of recurrent autoimmunity represents remains a key issue with uncertain effects. However, if recurrent autoimmunity does prove to be a barrier, combining "immune reset" approaches with autologous ITx may provide an effective solution to control recurrent autoimmunity (Marfil-Garza et al. 2021b, c; Moore et al. 2015; Voltarelli et al. 2007; Couri et al. 2009). Additionally, autologous iPSC–based ITx is more difficult for scale-up since it requires generation of unique iPSC lines for each patient, amplification, maturation, and subsequent safety screening prior to ITx to identify and prevent genetic mutations or off-target effects. Automation, artificial intelligence (i.e., machine learning), process automation, large-scale bioreactors, standardized protocols, and increased efficiency of processes will be required to enable cost-efficient autologous iPSC–based ITx (Verhoeff et al. 2021a). Despite substantial promise, there remains unanswered questions regarding recurrent autoimmunity and uncertainty regarding feasibility of upscaling with research to evaluate these questions ongoing.

3 Immune Responses

Differences between allogeneic and autologous responses for stem cell–based ITx are key when evaluating the utility of humanized mice models for preclinical evaluation. Alloimmune responses occur through direct recognition of donor major histocompatibility complex (MHC) molecules and indirect recognition of graft-derived peptides through recipient MHC molecules. In humans, the key MHC molecule is the human leukocyte antigen (HLA). Recognition of MHC molecules by recipient antigen-presenting cells (APCs) and co-stimulatory signals leads to activation and amplification of recipient effector T-cells with ensuing direct cytotoxic graft destruction (Li et al. 2018). Alloimmunity begins immediately after islet infusion and can lead to massive islet cell destruction that could prevent any substantial benefit following ITx. In contrast, autoimmune responses, such as in T1D, involves destruction of pancreatic β-cells by infiltrative mononuclear inflammatory cells, including macrophages, CD4+, and CD8+ T-cells (Itoh et al. 1993); high concentrations of interleukin 2 (IL-2) lead to activated CD4+ cells, that stimulate CD8+ direct-cell-mediated apoptosis, and inflammation-driven insulitis within the pancreas (Thivolet et al. 1991). While macrophages and dendritic cells act as antigen-presenting cells (APCs) that initiate the cytotoxic T-cell response (Knip and Siljander 2008; Echeverri and Tobón 2013; Gagnerault et al. 2002), it is T-cells that are

central in the pathogenesis of T1D. In support of the key role of T-cells, inhibiting T-cells with cyclosporine slows T1D onset, and agammaglobulinemic patients without B-cells but with T-cells can still develop T1D (Mandrup-Poulsen et al. 1990; Martin et al. 2001). Furthermore, transferring T-cells from a patient with T1D to a nondiabetic patient has shown to induce T1D in the recipient (Lampeter et al. 1993). Secondary to insulitis and β-cell destruction by T-cells, specific antigens are exposed and patients acquire antibodies to insulin, islet cells, the cation efflux pump ZnT8, isoforms of glutamic acid decarboxylase 65 or 67 (GAD65 or GAD67), or the IA-2 secretory protein (Echeverri and Tobón 2013). Autoimmunity occurs over months, with patients eventually becoming symptomatic once islet mass is reduced significantly. Recurrent autoimmunity in patients with T1D is largely understudied and remains difficult to evaluate, as patients receiving ITx are currently immunosuppressed. Concerning autologous iPSC–based ITx, theoretically, these patients continue to express islet specific antibodies, and may mount autoimmune responses to autologous iPSC–derived islets, but this remains an unanswered question.

4 Differences in Human and Murine Immune Systems

While human and murine immune systems are highly conserved and unique with regards to only approximately 300 genes (Chinwalla et al. 2002), acknowledging these differences provides context to current findings obtained from immunodeficient mouse. Additionally, understanding these differences highlights the role of humanized mice to enable ITx evaluation under the effects of a human immune system. Differences between mouse and human immune systems is only discussed briefly here, and specifically as they pertain to ITx. Reviews by Mestas and Hughes (2004), and Haley (2003) provide complete evaluation of differences between human and mouse immune systems (Mestas and Hughes 2004; Haley 2003).

The first major difference between humans and mice is the composition of circulating white blood cells (WBCs). Mice have a much higher proportion of lymphocytes (75–90% in mice vs. 30–50% in humans) and lower neutrophils (10–25% in mice vs. 50–75% in humans) (Table 1). For ITx, a lower proportion of neutrophils may explain successful subcutaneous ITx in mice due to less neutrophil-directed foreign body response (Yasunami et al. 2018; Jhunjhunwala et al. 2015; Yu et al. 2020; Pepper et al. 2017), while device and device-free techniques have shown less promising results in humans (Marfil-Garza et al. 2020b). The proportion of lymphocytes and neutrophils that exist in humanized mouse models varies, and should be considered when evaluating outcomes; higher or lower lymphocyte populations may inadequately represent allograft responses or recurrent autoimmunity in these models.

Important differences for innate immunity are also present when comparing humans to mice (Table 1). Neutrophils, one of the primary cells responsible for early graft death and the instant blood-mediated inflammatory reaction, express defensins as one of the key effector molecules in humans but not in mice (Risso 2000). Similarly, activation pathways for NK cells and macrophages are different between humans and mice (Mestas and Hughes 2004; Weinberg 1998; Lanier 1998). The clinical significance of this for ITx evaluation remains uncertain; however, both NK cells and macrophages play key roles in islet cell alloimmunity and autoimmunity (Knip and Siljander 2008; Poirot et al. 2004; Beilke et al. 2005; Azzi et al. 2010), with macrophages also contributing to islet angiogenesis and survival (Tessem et al. 2008). These cells also play an important role as antigen-presenting cells (APCs), with species specific antigen identification and presentation capabilities (Azzi et al. 2010; Monteiro and Van De Winkel 2003).

Acquired immunity also has notable differences between species (Table 1). Immunoglobulin (Ig) subtype activation pathways are different between mice and humans; as an example, interleukin 13 induces IgE class switching in humans but has no effect for mice (Mestas and

Table 1 Differences in human and murine immune systems

Immune system difference	Mouse	Human	Effect for islet cell transplantation
Proportion of neutrophils and lymphocytes in peripheral blood	10–25% neutrophils 75–90% lymphocytes	50–75% neutrophils 30–50% lymphocytes	Improved outcomes in subcutaneous and implantable devices for mice (less foreign body response)
Neutrophils with leukocyte defensins	Not present, defensins are expressed within small intestine	Present	Potentially reduced effect of IBMIR and early graft apoptosis
Macrophage and NK cell activation pathways	Different cytokines and messenger peptides lead to activation of macrophages and NK cells in mice and humans		Unclear clinical significance but both are crucial for alloimmunity and autoimmunity, including antigen presentation
Immunoglobulin class-switching pathways	Interleukins and inflammatory markers produce variable immunoglobulin classes		Unclear clinical significance but may produce variable alloimmune or autoimmune reaction.
T-cell differentiation and function	Interferon-alpha produces Th1 T-cells Th1 and Th2 cells make IL-10	Interferon-alpha has no effect Only Th2 cells make IL-10	Inflammatory markers and cell types found to cause insulitis or allograft rejection may be variable between groups

Hughes 2004). In evaluation of ITx, this may lead to different Ig being activated with allogeneic or autoimmune reaction, although this has never been evaluated. Potentially most significant is that the differentiation of T-cells occurs via stimulation by different cytokines in each species. For example, in humans interferon-alpha produces Th1 T-cells, while it does not have an effect in mice (Farrar et al. 2000). Additionally, differentiated T-cells release species-specific inflammatory molecules (Mestas and Hughes 2004). Again, Th1 and Th2 cells make IL-10 in humans, while only Th1 cells release IL-10 in mice (Del Prete et al. 1993). While the effect of these differences have not been studied specifically for T1D or ITx, we know their outcomes are crucial. The balance of Th1 and Th2 cells is directly related to development of T1D and modification of cytokine expression, including IL-10, and has clearly been shown to affect autoimmunity and allograft survival in islet cell transplant (Echeverri and Tobón 2013; Nitta et al. 1998; Zhang et al. 2003; Cote-Sierra et al. 2004).

These differences highlight the need for evaluating interventions and novel immune therapies within a true human-immune environment. The importance of these humanized mouse models has become patent in research related to human immunodeficiency virus vaccines (Gonzalez et al. 2013; Victor Garcia 2016), targeted oncologic immunotherapies (Wang et al. 2018), and human immunity (Pearson et al. 2008a), among other. However, the use of humanized models in the field of islet transplantation remains in its infancy and continues to face significant barriers. Understanding the history, current status, and major limitations is paramount to move this area of study forward.

5 Current Humanized Mouse Models

It has been nearly 20 years since the discovery that immunodeficient mice would accept, engraft, and display specific aspects of the human immune system (Ito et al. 2002; Shultz et al. 2005; Traggiai et al. 2004). Since then, substantial work has focus on optimizing both the recipient mice and the methods for immune system engraftment. In this section, we review historical and current immunocompromised mice that accept immune system engraftment with evaluation of efficacy between types. We also review

techniques used for human immune system engraftment into these recipient mice, with discussion of the benefits and drawbacks of these approaches specific to stem cell–based ITx evaluation. Finally, we discuss current evidence evaluating humanized models for ITx evaluation and consider the feasibility of these models for autologous and allogeneic stem cell–based ITx evaluation.

5.1 Immunodeficient Mice: Suitable Homes for a Human Immune System?

Early generation of humanized mouse models began with engraftment of NOD-scid mice. These models are homozygous for the severe combined immunodeficiency (*scid*) mutation and do not display any functional lymphoid tissue, which precluded development of both T- and B-cells. Given their highly immunocompromised state (Bosma and Carroll 1991), these mice can accept engraftment of diverse tissues and cell types, including those from a human immune system. However, these models have numerous limitations that preclude their use to evaluate the complexity of a fully functional human immune system. Firstly, NOD-*scid* mice "leak" native T- and B-cells, which means that these cells slowly reappear and destroy human tissues and cells (Flahou et al. 2021; Ito et al. 2012; Shultz et al. 1995). These mice also reintegrate Emv30 into their genome leading to thymic lymphoma and early death (Shultz et al. 1995; Serreze et al. 1995). Finally, NOD-*scid* mice continue to exhibit NK cell function, leading to high rates of hematopoietic stem cell (HSC) and stem cell death, which severely limits efficient immune system engraftment (Shultz et al. 1995, 2007; Christianson et al. 1996). Ongoing investigations have led to development of improved mouse strains to act as recipients for immune system engraftment.

To eliminate the initial barriers for humanization using of NOD-*scid* mice, the addition of *Rag1* and *Rag2* knockout mutations eliminated adaptive immune system "leak" (Ito et al. 2012; Katano et al. 2011; Bosma 1992). Subsequent manipulation of NOD-*scid-Rag* knockout mice with targeted disruption of the IL-2 gamma-chain markedly reduced occurrence of lymphoma (Katano et al. 2011; Kato et al. 2009). Combinations of the NOD-*scid*, *Rag* knockout, and IL-2-gamma disrupted mice have now generated the NOD-*scid* gamma strain (NSG), the NOD-*Rag1* gamma (NRG), and BALB/c-*Rag2* gamma (BRG) strains, which now represent the primary recipient strains for humanization (Traggiai et al. 2004; Katano et al. 2011; King et al. 2008). Evaluation of immune system engraftment into NSG, NRG, and BRG immunodeficient mouse strains has demonstrated that NSG mice, and a similar NOG model, accept engraftment better than others (Traggiai et al. 2004; Ito et al. 2012; Brehm et al. 2010a; Allen et al. 2019; Pearson et al. 2008b). As outlined by Ito et al. (2012), immune system engraftment success appears to occur with greatest success in NSG and NOG mice, followed by NRG>BRG>NOD/SCID (Ito et al. 2012).

5.2 Approaches to Engraftment

Optimization of immune system engraftment techniques have also occurred since the original description of HSC injection into immunocompromised mice (Fig. 2). Importantly, each technique to generate humanize mouse models creates a unique immune system with relevant benefits and drawbacks, which can be exploited to evaluate allogeneic and autologous stem cell–based ITx (Table 2).

Human peripheral blood lymphocyte SCID (Hu-PBL-SCID) mice, first described by Mosier et al. (1988), are generated through intravenous, intraperitoneal or intrasplenic injection of peripheral blood lymphocytes (PBLs) (Mosier et al. 1988). This technique offers nearly 100% engraftment success, is technically simple, and enables engraftment of already activated T-cells (King et al. 2008; Mosier et al. 1988). However, Hu-PBL-SCID mice have a shortened lifespan due to graft-versus-host disease (GVHD), and live approximately 1 month before meeting end points

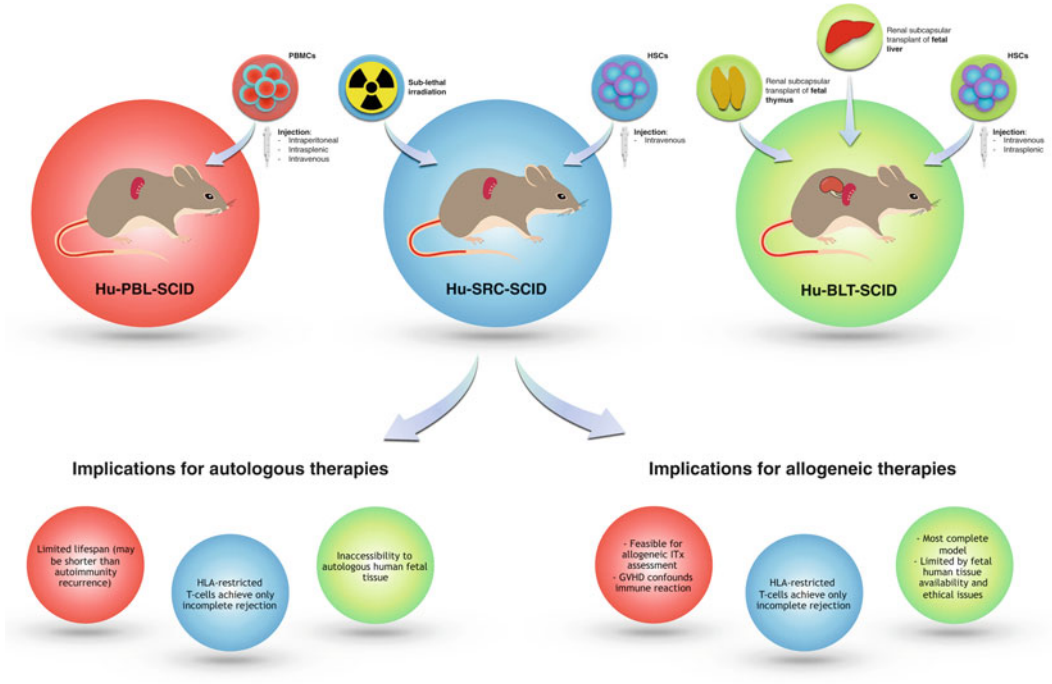

Fig. 2 Techniques used to engraft the human immune system into immunocompromised mice and key considerations for their use to evaluate allogeneic and autologous iPSC–based ITx

Table 2 Benefits and limitations to various human immune system engraftment techniques to generate humanized mouse models

Engraftment technique	Benefits	Limitations
Hu-PBL-SCID	100% engraftment success Engraftment of activated T-cells	Limited lifespan due to GVHD Lack of humoral immunity HLA-restricted immune responses and APC presentation
Hu-SRC-SCID	Enables engraftment of multiple immune cell lineages (T-cells, B-cells, NK-cells)	HLA-restricted immune responses and APC presentation Lack of class switching
BLT	Only model with mucosal human immune system Most complete immune system	Limited lifespan due to GVHD Requires fetal tissue Technically challenging

iPSC inducible pluripotent stem cells, *GVHD* graft-versus-host disease, *HLA* human leukocyte antigen, *APC* antigen presenting cell, *ITx* islet cell transplant

requiring euthanasia (Shultz et al. 2007; King et al. 2008, 2009; Allen et al. 2019; Sandhu et al. 1995). Naturally, GVHD confounds assessment of immune responses in these mice (King et al. 2009). Additionally, although Hu-PBL-SCID models enable engraftment of preactivated T-cells, antigen-specific T-cell activation, and humoral immunity within the engrafted mouse does occur (Shultz et al. 2012; Tary-Lehmann et al. 1994); this happens because the mice display antigens on H2 molecules whereas engrafted APCs are human-HLA restricted (Shultz et al. 2007; Allen et al. 2019; King et al. 2009; Banuelos et al. 2004).

The second approach is the human SCID repopulating cell scid mice (Hu-SRC-SCID), and involves engraftment of CD34+ HSCs injected into recipients via an intravenous or intrafemoral route into mice pretreated with sublethal irradiation. This approach benefits from technical feasibility, and development of a broader human immune system displaying all lineages of hematopoietic cells, including T-cells, B-cells, NK cells, myeloid cells, and precursors for red blood cells, megakaryocytes and granulocytes (Shultz et al. 2012; Allen et al. 2019). The most significant limitation with Hu-SRC-SCID mice is that, although they produce adequate T-cell populations, those T-cells fail to recognize, migrate, and reject allogeneic human antigens (Jacobson et al. 2010; Legrand et al. 2006). This is likely due to an absence of human thymic cells and lack of T-cell education to human MHC molecules (Traggiai et al. 2004; Allen et al. 2019; Chicha et al. 2005; Watanabe et al. 2009). Others have also postulated that lack of human-specific cytokines to direct these engrafted human T-cells limits their migration, or that lack of peripheral lymph tissues in the recipient mouse strains limits their maturation (Mestas and Hughes 2004; Allen et al. 2019; Shultz et al. 2019).

To resolve HLA-restriction and partially developed acquired immunity, SCID-Hu and BLT models were developed. These involve implantation of fetal liver and thymus fragments under the renal capsule in adult mice to enable HLA expression, immunoglobulin class switching, and T-cell activation (Shultz et al. 2012, 2019; Allen et al. 2019). The BLT model adds intravenous injection of HSCs from the same fetal liver. The BLT model displays the most complete human immune system and is also the only model to have a mucosal immune system. These models have been useful for evaluation of immune response to vaccination and HIV, but are severely limited due to their need for embryonic tissues, technical complexity, and reproducibility (Allen et al. 2019).

6 Humanized Mouse Models to Study Stem Cell–Based Islet Cell Transplantation

6.1 Current Evidence

Initial studies evaluating the utility of humanized mouse models utilized NOD-SCID and NRG mice with engraftment of human peripheral blood mononuclear cells (PBMC) (Banuelos et al. 2004). After xenotransplantation of transgenic mouse islets expressing human HLA-A2.1 allograft rejection occurred, but only in 70% of mice (Banuelos et al. 2004). In this study, high PBMC doses were required and engraftment was highly variable, likely due to the use of NOD-SCID and NRG mice strains. Since then, several studies evaluating the utility of humanized mouse models have been developed and tested, each with their own benefits and limitations (Table 3). King et al. (2008) improved upon this study substantially by first testing engraftment in NOD-SCID versus NSG mice, showing better success with NSG strains (King et al. 2008). They also evaluated intraperitoneal, intravenous, and intrasplenic PBMC engraftment and demonstrated optimal results with intravenous injection (King et al. 2008). Using those techniques, they achieved 100% human immune system engraftment and after injection of human islets, all mice with humanized immune systems demonstrated islet graft rejection in 21 days with microscopic evidence of allogeneic response (King et al. 2008). Unfortunately, mice in this study died due to GVHD after approximately 1 month after immune system engraftment. However, utilizing this model, Nadig et al. (2010) transplanted skin allografts onto humanized mice and demonstrated rejection; subsequent co-transplantation with regulatory T-cells (Tregs) prevented graft rejection (Nadig et al. 2010). This technique was evaluated with ITx, showing human islet allograft rejection after Hu-PBL-SCID generation and prolonged islet allograft survival (45 days) when ex vivo–

Table 3 Studies evaluating humanized mouse models in islet cell transplant, their benefits, and drawbacks

Study	Key findings	Limitations	Could this be used for IPSC evaluation?	
			Allogeneic	Autologous
Hu-PBL-SCID models				
Banuelos et al. (2004)	First study showing islet allograft rejection	Variable engraftment High PBMC doses required	No, inconsistent immune engraftment	No, inconsistent immune engraftment
King et al. (2008)	Demonstrated the NSG mice has improved immune system engraftment Proved IV PBMC injection was superior to intrasplenic or intraperitoneal 100% allograft rejection in 21 days	Early mouse death after approximately 1 month due to GVHD	Possibly, although examination beyond 1 month would be limited	Probably not, GVHD occurs soon after transplant and could confound assessment of autoimmunity
Wu et al. (2013)	Demonstrated human islet allograft rejection in Hu-PBL-SCID model Improved allograft survival with Treg co-transplantation	Study only evaluated mice for 45 days	Yes, effective model for early allograft rejection but not studied beyond 45 days, likely due to GVHD	No, GVHD is likely a limitation
Brehm et al. (2019)	Developed a Hu-PBL-SCID model with MHC class 1 and 2 knockout that rejects islet allografts	Chronic GVHD still occurs but is significantly decreased	Yes, however, chronic GVHD may confound later results	Possibly, however the lifespan is currently limited to 4 months and chronic GVHD may confound results
Hu-SRC-SCID models				
Jacobson et al. (2010)	Generated Hu-SRC-SCID model with 100% immune engraftment	Failed to reject islet allografts	No, allografts were not rejected, likely due to inadequate human-specific MHC activation and T-cell education	No, allografts were not rejected, likely due to inadequate human-specific MHC activation and T-cell education
Brehm et al. (2010a, b)	Developed mice that became diabetic spontaneously and accepted immune engraftment	Only achieved ~60% islet allograft rejection	No, allografts were not rejected, likely due to inadequate human-specific MHC activation and T-cell education	No, allografts were not rejected, likely due to inadequate human-specific MHC activation and T-cell education
BLT models				
Tonomura et al. (2008)	Developed a BLT model that rejected porcine islets (xenorejection)	Unclear pathophysiology of xenograft rejection and whether it would apply to human allografts	Unclear, no evidence of allograft rejection. The study only showed xenograft rejection	No, acquiring fetal tissues for autologous iPSC donor/recipients is not possible. GVHD is also a limitation
Tan et al. (2017)	Developed a BLT model with spontaneous development of T1D in DQ8+ transgenic mice	No evaluation of islet cell transplant to reverse spontaneous diabetes	Possible, but has yet to be tested and would be limited by ethical concerns and fetal tissue availability	No, acquiring fetal tissues for autologous iPSC donor/recipients is not possible. GVHD is also a limitation

iPSC inducible pluripotent stem cells, *GVHD* graft-versus-host disease, *MHC* major histocompatibility complex

expanded Tregs were co-transplanted with PBLs (Wu et al. 2013). These studies demonstrate successful use of humanized mouse models to evaluate islet allograft rejection, and similar techniques have been postulated to be helpful to evaluate allogenic stem cell–based ITx (Flahou et al. 2021). However, no study has demonstrated evaluation of recurrent autoimmunity in autologous iPSC–based ITx. This is partly due to restricted lifespan of current humanized models. While allogeneic responses occur soon after transplant, recurrent autoimmunity is delayed and occurs later, requiring models to evaluate it to have longer lifespans (Burke 3rd et al. 2011; Sundkvist et al. 1998).

Alternatively, using Hu-SRC-SCID models has been attempted but remains highly limited due to the immature immune system they possess and lack human-specific MHC activation and T-cell education (Legrand et al. 2006; Manz 2007). Because of this, most Hu-SRC-SCID models have failed to completely reject allografts (Jacobson et al. 2010) (Table 3). The most promising Hu-SRC-SCID model has been developed by Brehm et al. (Brehm et al. 2010b), where NRG mice crossed with mice heterozygous for *Ins2*^{Akita} showed spontaneous hyperglycemia similar to T1D in immunodeficient mice capable of being engrafted with human immune system cells; however, when they received human ITx, only 60% of islets were rejected, likely owing to poor T-cell function in Hu-SRC-SCID models (Brehm et al. 2010b). In these models, lack of T-cell education directed toward human MHC molecules within the thymus means that although human T-cells develop, they do not mount a robust peripheral immune response directed at human tissues (Shultz et al. 2007, 2019; Allen et al. 2019; Chicha et al. 2005). Additionally, others have postulated that poor peripheral lymphatic development in immunosuppressed recipient mouse strains (Cupedo and Mebius 2005), and the poor interaction of murine cytokines with both human immune cells and cytokine receptors may further limit allograft responses (Mestas and Hughes 2004; Allen et al. 2019; Shultz et al. 2019). To resolve these barriers, several genetically modified Hu-SRC-SCID models have been developed to express or interact with human cytokines (Allen et al. 2019; Willinger et al. 2011; Drake et al. 2012; Huntington et al. 2009; Nicolini et al. 2004; Ito et al. 2013; Rongvaux et al. 2014), or express important human MHC molecules (Shultz et al. 2019; Akkina et al. 2016; Patton et al. 2015; Jaiswal et al. 2009). Overall, similar to Hu-PBL-SCID models, Hu-SRC-SCID models may be of use to evaluate alloimmunity but are unlikely to display adequate immunogenicity to fully comprehend recurrent autoimmunity after autologous iPSC–based ITx.

Finally, evaluation of BLT models in the context of ITx remains highly limited. A single study has demonstrated islet xenograft rejection in a BLT model, but no studies evaluating human allografts have been conducted (Tonomura et al. 2008). A recent BLT model has demonstrated capacity to induce immune-mediated T1D in humanized mice without concerns for GVHD (Tan et al. 2017). In their model, transplantation of HLA-DQ8+ human fetal thymus and CD34+ cells into HLA-DQ8 transgenic mice developed hyperglycemia and diabetes (Tan et al. 2017). While testing allogeneic stem cell–based ITx in this model could be attempted, technical and ethical concerns regarding BLT models, as well as restrictions on the source of fetal liver or thymus fragments from patients with T1D profoundly limit their use for the study of immune responses following autologous iPSC–based ITx.

6.2 Future Directions

An ideal humanized mouse model for preclinical evaluation of autologous iPSC–based ITx currently does not exist. While generating individualized Hu-PBC-SCID mice for each iPSC donor is theoretically possible, the utility of those models remains highly limited by their shortened lifespan, while Hu-SRC-SCID models remain limited by their incomplete immune response (Fig. 3). When evaluating recurrent autoimmunity, humanized models with up to a year (or more) lifespan will be required. Current research is attempting to produce Hu-PBL-SCID

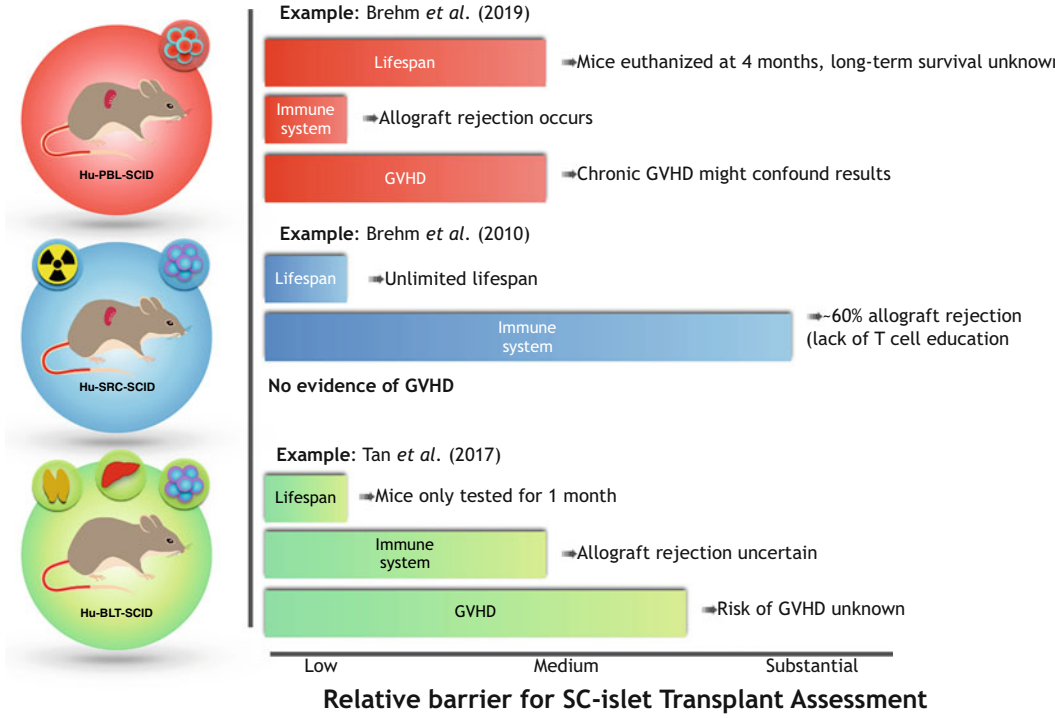

Fig. 3 Relative barrier for SC-islet transplant assessment

models with MHC knockout mice that do not express murine MHC class I or II molecules, which limits GVHD and prolongs their lifespans (Shultz et al. 2019; Yaguchi et al. 2018; Brehm et al. 2019; Goettel et al. 2015). Yaguchi et al. (2018) generated a NOG mouse strain deficient in MHC class I and II molecules and have demonstrated life spans of over 4 months (Yaguchi et al. 2018). Their model also showed human antigen-specific immune activation including primary T-cell responses and B-cell activation. Other similar models without MHC class I or II molecules have also demonstrated human islet allograft rejection (Brehm et al. 2019). However, it should be noted that while diminished, chronic GVHD still occurred with T-cell infiltration throughout the model's organs. It remains unclear how long these new models can live, and what effect GVHD plays, especially when evaluating alloimmune or autoimmune reactions. Alternatively, transgenic Hu-SRC-SCID models expressing human MHC molecules may enable improved T-cell immune response and enable primary immune responses within mouse models (Shultz et al. 2019; Akkina et al. 2016; Patton et al. 2015; Jaiswal et al. 2009). However, in transgenic mice expressing human MHC molecules, HSC engraftment is variable and may only occur for fetal-sourced cells (Patton et al. 2015). Future work is needed to demonstrate humanization in transgenic Hu-SRC-SCID models and subsequent evaluation of their utility for evaluation of immune responses following ITx.

Regardless of ongoing investigations to improve humanized mouse models, their use for evaluating stem cell–based ITx remains uncertain. The primary limitations of stem cell–based ITx remain off-target growth, allograft rejection in the case of allogeneic transplant, and recurrent autoimmunity for autologous iPSC–directed grafts. Fortunately, islets can be implanted locoregionally and not systemically, and initial evaluation in humans could be conducted subcutaneously. While subcutaneous ITx technologies have thus far not matched intraportal efficacy,

they have demonstrated acceptable islet growth and islet cell function (Marfil-Garza et al. 2020b). Even if these subcutaneous devices never achieve efficacy similar to intraportal transplantation, they already provide a safe, true human model with complete human immune system for clinical assessment prior to intraportal transplant. In cases where aberrant cells or graft complications occur, removing them and eliminating risk is feasible. We believe this allows for safe in-human trials to be conducted, potentially within subcutaneous devices initially with subsequent intraportal infusion once safety is demonstrated. This may limit the need for humanized mouse models in this area of medicine entirely.

7 Conclusion

Stem cell–derived islet cells are generating optimism for a potential true cure for T1D. The question remains whether efficacy and safety demonstrated in rodent models is sufficient to implement this technique in clinical trials. Humanized mice models appear on the surface to offer an opportunity to further test these techniques prior to implementation. However, human immune system models remain limited; current models display only certain elements of the immune system. While utilization of Hu-PBL-SCID models may allow evaluation of recurrent autoimmunity following transplant of autologous iPSC–based islets, they offer limited information regarding long-term efficacy or safety due to their brief lifespan. Unfortunately, other models do not appear useful for autologous iPSC–based ITx evaluation. The Hu-SRC-SCID models fail to achieve adequate T-cell responses, while Hu-BLT-SCID models are limited primarily by their need for embryonic tissues, and their technical and resource heavy requirements. Work is ongoing to generate Hu-PBL-SCID mice that do not present GVHD to enable longer lifespans, and to produce Hu-SRC-SCID models with normal T-cell activity; however, efficacy of these models for evaluation of ITx remains unrealized. Thorough demonstration of efficacy and safety in current mouse models, potentially followed by human ITx within subcutaneous devices to demonstrate safety currently offers a reliable pathway to obtain sufficient evidence to support stem cell–based ITx with intraportal infusion toward human clinical trials.

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Ethical Approval The authors declare that this article does not contain any studies with human participants or animals and was exempt from ethics board review.

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Mesenchymal Stem Cell Therapy for Osteoarthritis: Practice and Possible Promises

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Abstract

Osteoarthritis (OA) is a common progressively degenerative joint disease that affects more than 300 million people worldwide. OA is manifested by articular cartilage degradation, chronic pain, deformity, functional disability, and decreased quality of life. A real challenge in OA management is the lack of an effective cure because exiting therapeutics often provide symptom control rather than disease modification; therefore, they fail to prevent disease progression. The inadequate treatments for OA management have encouraged researchers to study mesenchymal stem cells (MSCs) as an investigational treatment for OA. MSCs are a promising tool for OA because of their availability; expand cultivation and multi-lineage differentiation capacity as well as well-documented paracrine function have made MSCs a promising tool in this field. Accordingly, MSCs application has been successfully utilized in a broad range of pre-clinical OA animal models as well as clinical studies with the aim of cartilage repair which had not previously been achieved using classical treatments. Here, the brief scientific review of MSC role in the control of OA as well as the proposed mechanisms are discussed. We provide an insight into the last 10 years' studies conducted on preclinical and clinical OA treatment as well as future opportunities in OA management strategies employing MSCs.

Keywords

Animal model · Cell therapy · Immunomodulatory effect · Mesenchymal stem cell · Osteoarthritis

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Abbreviations

3D	Three-dimensional
ACI	Autologous chondrocyte implantation
ACLT	Anterior cruciate ligament amputation
ADMSCs	Adipose-derived mesenchymal stem cells
BMSCs	Bone marrow mesenchymal stem cells
CDMP1	Cartilage-derived morphogenetic protein 1
CTGF	Connective tissue growth factor
CXCR4	C-X-C chemokine receptor 4
ECM	Extracellular matrix
EGF	Epidermal growth factor
ESWT	Extracorporeal shockwave therapy
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
GAG	Glycosaminoglycan
GO	Graphene oxide
HA	Hyaluronic acid
IA	Intra-articular
IGF-1	Insulin-like growth factor 1
IV	Intravenous
KOOS	Knee Injury and Osteoarthritis Outcome Score
MIA	Monosodium iodoacetate
MRI	Magnetic resonance imaging
MSCs	Mesenchymal stem cells
NPs	Nanoparticles
OA	Osteoarthritis
PBS	Phosphate-buffered saline
PGE2	Prostaglandin E2
PLMSCs	Placenta-derived mesenchymal stem cells
PRP	Platelet-rich plasma
TGFβ	Transforming growth factor beta
UCMSCs	Umbilical cord mesenchymal stem cells
VAS	Visual analog scale
VEGF1	Vascular endothelial growth factor1
WJMSCs	Wharton's jelly mesenchymal stem cells
WOMAC	Western Ontario and McMaster Universities Osteoarthritis Index
μCT	Micro-CT

1 Introduction

Osteoarthritis (OA) is one of the most prevalent degenerative joint diseases that affects approximately one-quarter of the world's adult population. OA is considered to be a leading cause of pain, disability, social isolation, and depression among people over the age of 40 (Chen et al. 2017; Glyn-Jones et al. 2015; Wieland et al. 2005). The annual cost of OA management in terms of healthcare and functional disability is estimated to be 1–2.5% of the gross domestic product (Woolf and Pfleger 2003). Limited therapeutic approaches along with their inability to successfully prevent OA progression make it necessary to develop new therapeutic strategies instead of traditional pharmaceutical and surgical approaches.

Thus far, the limited therapeutic options for OA treatment that include pharmaceutical treatments, non-pharmacological methods, and surgical interventions have failed to modify its effects and complete tissue regeneration (Wieland et al. 2005); therefore, the efficacy of available treatments is limited to pain management during the early disease stages and joint replacements at end-stage OA (Glyn-Jones et al. 2015). This limitation highlights the need to design more effective, alternative therapies. Considering this challenge, the emerging field of regenerative medicine has encouraged clinicians to focus on cell/stem cell therapy as a promising alternative treatment for OA because of its minimal risk for patients. Mesenchymal stem cell (MSC) therapy has been introduced as an easily accessible source with potential benefits that has led to promising long-term therapeutic success in both preclinical and clinical studies. The literature presented here is a review of evidence published during the previous 10 years in terms of the efficacy of

MSC-based therapeutics for the management of symptomatic and radiologic OA. We also intend to discuss various available approaches for MSC administration and possible mechanisms for their regenerative effects in OA treatment.

1.1 Osteoarthritis (OA): Biochemical and Biomechanical Modification of the Joint

OA progression is accompanied by deep degeneration of joint cartilage and underlying bone. Given the modified biochemistry and biomechanics of the osteoarthritic joints, OA has long been identified by a lack of intrinsic repair processes for damaged articular cartilage (Bijlsma et al. 2011). Although the exact pathophysiology of OA is poorly defined and under research, susceptibility to OA is determined by multiple risk factors including age, genetics, obesity, physical inactivity, poor diet, and joint trauma (Mobasheri et al. 2015; van der Kraan et al. 2017). Among these, age is the greatest risk factor for OA progression because of the reduced regenerative capacity of cartilage in elderly people (Lawrence et al. 2008).

Pathological changes in OA affect whole joint tissues such as synovium and subchondral bone. These changes are highly associated with metabolic alterations in chondrocytes, which are a small number of resident cells found in avascular, alymphatic, aneural, and hypocellular articular cartilage. In the metabolically challenging environment of an osteoarthritic joint, chondrocytes may activate their regulatory metabolic state and lead to progressive cartilage destruction, osteophyte formation, synovitis or synovial inflammation, joint capsule hypertrophy, and destruction of joint menisci and ligaments (Loeser et al. 2012; Mobasheri et al. 2017). The most important tissue damage from OA occurs in cartilage, which undergoes unregulated biochemical changes and loss of function. In this regard, chondrocytes and their environment play an important role in the development of this phenotype (McAlindon et al. 2000). Activated chondrocytes produce anti-anabolic and pro-catabolic agents such as inflammatory cytokines and matrix-degrading enzymes, including matrix metalloproteinases and aggrecanases, in the disturbed pathophysiological environment of an osteoarthritic joint (Biggee and McAlindon 2004). This altered chondrocyte phenotype is called chondrosenescence, and it can disrupt the ability of cartilage to tolerate mechanical stresses, disorganize cartilage homeostasis and metabolism, and increase its damage (Mobasheri et al. 2015). Synovial inflammation, which appears as hyperplasia of the synoviocytes and increased vascularity, may play a role in OA symptoms such as pain, joint swelling, and production of more inflammatory mediators through the accumulation of synovial macrophages (Bondeson et al. 2006; Scanzello and Goldring 2012). There is often a delay in diagnosis despite the progressive and extensive tissue destruction in OA, which is mainly due to the annularity of articular cartilage. Clinical signs appear when adjacent innervated tissues become involved (Bijlsma et al. 2011).

2 Cell/Stem Cell Therapy for Knee Osteoarthritis (OA): A Brief Scientific Overview

All commonly accepted strategies for OA treatment have failed to prevent disease progression and only provide symptom control. Tremendous challenges exist with finite treatment of OA because of the complications from arthroplasty surgery that include limited lifetime of the prostheses, high cost, and complexity of the procedure. Therefore, treatments are currently limited to lifestyle modifications and maintenance of an optimal weight (Glyn-Jones et al. 2015). Over the last two decades, cell or stem cell therapy have been used as potent therapeutic strategies in regenerative medicine. Various basic and translational studies have been conducted with therapeutic cells in order to achieve real tissue regeneration for musculoskeletal diseases in the clinic setting (Kitta et al. 2018).

The first report on cellular intervention was published in 1994 and it pertained to an autologous chondrocyte implantation (ACI) (Brittberg

et al. 1994). Despite promising results and relative advantages over conventional methods that included lack of stimulation of the host immune response, promotion of hyaline-like cartilage formation, and improved pain and joint function, ACI had limited use because of donor site morbidity, inability to restore tissue integrity and articular congruity, formation of fibrocartilage tissue, and repair of a focal cartilage lesion rather than regeneration of generalized cartilage loss (Niemeyer et al. 2016; Niethammer et al. 2014; Roberts et al. 2003). Given the limitations accompanied by ACI, in recent years MSC-based therapies have emerged as exciting possibilities for OA treatment along with evidence that favors disease modification and inhibition of disease progression (Freitag et al. 2016).

2.1 Mesenchymal Stem Cells (MSCs)

MSCs were first characterized and recognized as adult multipotent stem cells in bone marrow over 40 years ago. Similar cells were subsequently identified in the peripheral blood, skeletal muscle, adipose tissue, cord blood, and heart (Väänänen 2005). According to the International Society for Cell Therapy (ISCT), MSCs must have three necessary criteria: (1) plastic-adherent; (2) high expression levels of differentiation markers (CD105, CD90, and CD73) and inability to express CD45, CD11b, CD34, CD19, and HLA-DR surface molecules; and (3) high potential for in vitro differentiation into osteoblasts, adipocytes, and chondrocytes (Dominici et al. 2006). The contribution of MSCs to the tissue regeneration process mainly depends on their ability to be easily harvested from different types of adult tissues; high proliferation; in vitro expansion without loss of function; strong potential for differentiation into mesodermal lineages; immunomodulatory effects; and ability to migrate toward the injury site in response to homing signals from damaged tissue (Eom et al. 2015; Nasiri et al. 2019). These therapeutic benefits are mainly mediated by their trophic activity and secretion of factors for endogenous precursor cell recruitment, vascularization, apoptosis, and modulation of the immune response (Fu et al. 2017). Their trophic function is based on the secretion of an array of cytokines and growth factors such as epidermal growth factor (EGF), transforming growth factor beta (TGFβ), and vascular endothelial growth factor (VEGF), which participate in local tissue repair (Caplan and Correa 2011; Nakagami et al. 2006). The immunomodulatory actions of MSCs may differ among individuals and donor species, in addition to the tissue origin, culture situation, and activation modes (Zhao et al. 2016). Moreover, a lack of ethical issues with MSCs use makes them promising candidates for cell-based tissue engineering approaches in various diseases, including premature ovarian failure, nervous system injuries, amyotrophic lateral sclerosis, and OA (Mamidi et al. 2016).

2.2 Safety Issues That Pertain to Mesenchymal Stem Cell (MSC) Use in Regenerative Medicine

Apart from the effectiveness of MSCs for the tissue repair process and symptom relief, patients' wellbeing and lack of serious adverse effects following cell administration are important issues that must be addressed in preclinical studies. Despite the safety of different sources of MSCs for treatment of damaged tissues, complications such as tumorigenesis in immunocompromised mice have been reported (Pan et al. 2014). Administration of MSCs for OA has caused rare and uncommon adverse effects such as pain and slight bleeding at the injection site (Emadedin et al. 2018; Kuah et al. 2018) and knee edema (Jo et al. 2017). Except for these limited and rare cases, MSCs appear to be safe when used to treat OA. However, some of the studies that confirmed the relative safety of MSC applications had limitations such as a small sample size, lack of appropriate controls, or short-term follow-up. Transplantation of MSCs has been assessed in clinical trials of various neurological, cardiovascular, and musculoskeletal diseases. With the exception of rare cases of pulmonary embolism and mild infection, no acute adverse effects or malignancies were reported after transplantation of the MSCs (Peeters et al. 2013).

2.3 Effect of Mesenchymal Stem Cells (MSCs) on Symptomatic and Radiologic Osteoarthritis (OA)

MSCs have been safely and successfully administered for different inflammatory conditions, including OA. Various techniques have been used to guide and facilitate the differentiation of MSCs toward the formation of chondrocytes and hyaline cartilage for OA treatment. The cartilaginous differentiation of MSCs appears to be closely related to their trophic activity as well as cell-cell interactions between MSCs and residual chondrocytes in osteoarthritic cartilage (de Windt et al. 2015; Wu et al. 2011). Thus far, various administration modalities have been used in various animal and human studies to increase cell participation for management of OA and tissue repair. Proposed techniques to improve the effectiveness of MSC therapy include: accurate selection of MSCs density; MSCs source; selection of the best time for the MSC injection; selection of the most efficient cell administration route (intravascular, intra-articular [IA] or scaffold embedded); genetic modifications to increase the MSC therapeutic potential; pre-treatment of MSCs with inflammatory cytokines; MSCs combined with cartilage-derived morphogenetic protein and growth factors (GF), platelet concentrate/platelet-rich plasma (PRP) and hyaluronic acid (HA); and biomechanical stimulation and application of hydrogels and nanoparticles (NPs).

Briefly, the results of animal, preclinical, and clinical studies show that MSCs therapy for OA can alleviate pain, induce regrowth of hyaline-like cartilage, and eventually lead to real tissue regeneration and improved joint function (Grigolo et al. 2009, Nasiri et al. 2019.

3 Mesenchymal Stem Cell (MSC) Administration Routes

There are two major routes for MSCs transplantation in both animal and clinical applications – systemic injection or local administration. Systemic administration includes intravenous (IV) or IA injections of MSCs into the vasculature. This is a minimally invasive approach that can obligate MSC distribution throughout the body. However, there are several obstacles associated with systemic MSC injections and these include untargeted cell delivery, poor homing, and MSCs becoming trapped in the lungs through the "first pass" effect (Nasiri et al. 2019).

During local administration, MSCs are either directly injected into the damaged tissue or embedded in scaffolds prior to transplantation. For a direct injection, MSCs are suspended in phosphate-buffered saline (PBS), HA, and PRP before they are injected into the articular space. Compared to the systemic approach, local IA injections of MSCs provide an easy, simple route that has been reported to inhibit the degeneration of osteoarthritic cartilage and induce therapeutic outcomes in different animal studies and clinical trials (Davatchi et al. 2016; Emadedin et al. 2012).

OA induces diffuse chondral degeneration; therefore, MSC therapy can be performed alone or mixed with HA or PRP, which is the most common and beneficial treatment. HA can stimulate the production of glycosaminoglycans (GAG) and collagen (Erickson et al. 2012), and PRP is an active substance that can stimulate cell proliferation, inflammatory chemotaxis, and collagen synthesis (Wang et al. 2019a, 2019b), which may enhance the rate of the normally limited cartilage repair process (Kurtz et al. 2007). In addition, as therapeutic cells, MSCs can be seeded onto three-dimensional (3D) scaffolds and then used locally to repair osteoarthritic cartilage through their trophic activity or ability to differentiate (Demoor et al. 2014).

Articular cartilage is a tissue with biomechanical function. Therefore, it seems to be essential that tissue engineering and cell therapy techniques ultimately produce a new structure that has the capability to adequately simulate the necessary mechanical properties of native tissue. This challenge can be addressed by MSC seeding on 3D scaffolds that provide the required mechanical strength (Diekman and Guilak 2013). Moreover, the therapeutic effects of MSCs can further be increased by seeding on

scaffolds loaded with GF to assist tissue repair and elevate the integration of newly formed cartilage with the surrounding tissue (Barron et al. 2015; Qi et al. 2013). For example, scaffolds with chondrogenic growth factor TGFβ1 on their margins and the osteogenic factor BMP-2 on the bottom layer could increase tissue integration between regenerated cartilage and old surrounding tissue (Chen et al. 2011). Furthermore, scaffolds can guide both homing and differentiation of endogenous progenitor cells; this capacity seems to be the reason for the observed repair ability of cell-free scaffolds during OA treatment (Lee et al. 2010; Mendelson et al. 2011; Re'em et al. 2012). The different commercial scaffolds currently available for treatment of cartilage defects include: collagen, polylactic acid, polylactic-co-glycolic acid, and polyethylene glycol (Tribe et al. 2017). Despite the primary therapeutic outcomes of various cell-seeded or cell-free scaffolds in animal and preclinical studies, they are not used as current treatments in clinical practice; this is mainly due to concerns about safety and the possibility of a graft-versus-host immune response. Moreover, local administration of MSCs by using scaffolds is currently employed to treat small area cartilage defects rather than the large, diffuse cartilage losses observed in OA.

4 Mesenchymal Stem Cell (MSC)-Derived Extracellular Vesicles (EVs): A New Cell-Free Therapeutic for Osteoarthritis (OA)

In addition to anti-inflammatory and local immune-regulation activities, MSCs also exert their function by production and release of extracellular vesicles (EVs). EVs are intracellular communication vehicles enclosed in a phospholipid bilayer. They are rich in nucleic acids (mRNAs and microRNAs), lipids, proteins, and other extracellular matrix (ECM) components and have anti-apoptotic, antifibrotic, and pro-angiogenic activities to restore the anabolic and catabolic balance of the ECM and alleviate disease progression (Toh et al. 2017). EVs can be used as either a vehicle in drug delivery or therapeutic agents that contain a set of cellular products. EVs are representative of original cell components that can be used for disease diagnosis. In contrast to cell therapy, EVs do not have any risk for uncontrolled cell proliferation and tumorigenesis (Gomzikova and Rizvanov 2017). Therefore, by considering their tremendous healing potential, lack of toxicity, and minimal risk for immunogenicity, which is attributed to MSC exosomes, EVs are considered to be one of the simplest ways to treat OA and other inflammatory diseases. OA treatment with MSC-derived EVs appears to maintain the homeostasis of hyaline cartilage through SOX9 and aggrecan overexpression, as well as microRNA-140-5p (Liang et al. 2016; Tao et al. 2017). microRNA-140-5p seems to have a cartilage protective role that may effectively promote cartilage repair and inhibit OA progression (Tao et al. 2017).

MSCs can produce and secrete various factors under different environmental conditions that enter the exosomes and affect their therapeutic potentials. Thereby, the efficacy of exosome therapy can be enhanced by preconditioning of MSCs with hypoxia (Almeria et al. 2019) to stimulate the production of a large number of cytokines, as well as genetic modification of cells in the form of GATA-4-overexpressing MSCs (Li et al. 2010) and CXCR4-overexpressing MSCs (Kang et al. 2015) and by adding chemicals or cytokines including tumor necrosis factor-α (TNF-α), interferon ɣ (IFN-ɣ), IL-6, TGF-β, and IL-1β (Joo et al. 2020). Accordingly, these manipulated MSCs can produce exosomes enriched in pro-inflammatory cytokines, RNAs, and proteins to manage inflammatory micro-environment and anabolic events. Recent preclinical studies on OA animal models revealed that stem cell-derived exosomes by similar therapeutic function with original stem cells inhibited the OA progression and promote cartilage repair (Cosenza et al. 2017). Despite promising early results, the extraction of large-scale purified exosomes in a clinic is a challenging issue which should be more developed. Furthermore, the exact action mechanism of MSC-derived exosomes on OA is still unclear

and needs further in-depth research. It seems that MSCs exosome will soon become one of the promising therapeutic modalities for future OA management.

5 Therapeutic Effects of Apoptotic MSCs

According to the literature, most MSCs disappear a few days post administration in OA animal models as well as human studies (Toupet et al. 2015). Despite these cell clearance, significant therapeutic effects of MSCs have been observed frequently (Schelbergen et al. 2014). Accordingly, the attention has been attracted to the therapeutic effects of apoptotic MSCs which seems to be mediated through both direct effects including secretion of IL-10 and TGF-b, and thereby induction of immunosuppressive microenvironment (Korns et al. 2011), as well as indirect effect which is associated with inflammatory to anti-inflammatory switch by immune cells that phagocytose apoptotic MSCs (Fadok et al. 1998). Swallowed MSCs release IDO in the cytoplasm of the host cell and subsequently secrete it into extracellular environment, which leads to T-cell inhibition (Laing et al. 2018). Apoptotic MSCs may exert immunomodulatory effects and participate in alleviation of OA and inhibition of disease progression.

6 How Can Mesenchymal Stem Cells (MSCs) Be More Involved in the Process of Tissue Regeneration?

Despite their potential, the therapeutic outcome of the initial MSC therapies was not satisfactory; this limitation was the cause for subsequent development of novel strategies that could enhance the contribution of MSCs to the tissue regeneration process. Accordingly, accurate determination of their density; the adult tissue origin of MSCs; administration route; genetic correction; preconditioning of MSCs; combined administration of MSCs with different GFs, hydrogels, and NPs; and biomechanical stimulation have been employed to enhance the efficiency of MSC therapy (Nasiri et al. 2019). For example, researchers determined the proper cell dose of MSCs to prevent possible unwanted complications such as tumor formation or cartilage regeneration failure from insufficient numbers of cells, in different preclinical and clinical studies. Genetic manipulation of MSCs enhanced their cartilaginous ability both in terms of trophic function and differentiation potential (Wojdasiewicz et al. 2014). MSCs transduced with the CXCR4 were transfected to overexpress IL-10, CTLA4Ig, US6, and US11 to promote MSC migration toward fibrillated osteoarthritic cartilage (Li et al. 2017; Sullivan et al. 2013). Despite promising preliminary results from preclinical studies, safety concerns have restricted the use of genetically manipulated MSCs in the clinic. Alternatively, MSC priming with GFs and cytokines is used to increase their repair capacity. Various conditions and chemicals, including hypoxia, oxidative stress, heat shock, nutrient-deficient media, and inflammatory cytokines such as IL-1β, IFN-γ, and TNF-α, could boost the immunosuppressive effects of MSCs and promote both cell migration and differentiation capacities, and cell survival and homing after transplantation (Fan et al. 2012). The therapeutic effects of MSCs can also be enhanced by combined MSC-GFs treatment (Li et al. 2016). In this approach, fibroblast growth factors (FGFs) and their receptors (FGFRs), cartilage-derived morphogenetic protein 1 (CDMP1), VEGF1, TGFβ1, TGFβ3, insulin-like growth factor 1 (IGF-1), SOX 5, SOX 6, SOX 9, BMP-2, BMP-4, BMP-7, anti-apoptotic proteins, connective tissue growth factor (CTGF) and platelet-derived growth factor (PDGF) have been used in clinical trials (Hu et al. 2010; Wu et al. 2014) with promising regenerative results.

To date, numerous studies used the above strategies to increase the therapeutic effectiveness of MSC therapy; however, the efficacy of MSC participation in damaged tissue regeneration remains low and it appears that the cell therapy techniques should employ more efficient cell delivery approaches in order to access targeted

cell therapy and enhance cell migration, engraftment, and differentiation potential prior to the use of MSCs in the clinic setting.

7 Preclinical Data on Mesenchymal Stem Cell (MSC) Treatment of Osteoarthritis (OA) and Cartilage Defects

Recent preclinical studies on various animal models confirmed that administration of MSCs via direct IA injection of cells suspended in carrier media or seeded on scaffolds, and MSC-derived exosomes often improve OA symptoms and joint function; in rare cases, they can induce tissue regeneration. Different small (mouse, rat, rabbit) and large (goat, dog, sheep, horse) animal models have been generated by oophorectomy; anterior cruciate ligament amputation (ACLT); or injection of monosodium iodoacetate (MIA), collagenase, quinolone, or papain (Kim et al. 2018). Cheng et al. reported that treatment of rat knee model of early OA with Wharton's jelly mesenchymal stem cells (WJMSCs) combined with extracorporeal shockwave therapy (ESWT) significantly improved pathological and immunohistochemistry (IHC) findings, micro-CT (μCT), and reduced synovitis compared with ESWT or WJMSCs alone (Cheng et al. 2019). Xing et al. compared treatment with a single dose (10^6 cells) versus three doses of embryonic stem cell-derived MSCs (ES-MSCs) for a rat OA knee model. Six weeks after treatment, they observed improved modified Mankin scores and International Cartilage Repair Society (ICRS) macroscopic scores for both the single and multiple doses. At 10 weeks after treatment, the multiple-dose administration had a significantly greater ICRS score, lower Mankin score, and improved histological analysis (Xing et al. 2021). Table 1 shows a summary of preclinical studies from 2011 to 2021 that used MSCs to treat animal models of OA.

8 Clinical Trials of Cultured Mesenchymal Stem Cells (MSCs) for Osteoarthritis (OA) Treatment

Clinical trials are conducted after initial approval and promising therapeutic results are obtained from studies on small and large OA animal models. Successful clinical trial results can subsequently be implemented in the clinic setting. MSCs obtained from human bone marrow, synovial fluid, and adipose tissue have been directly injected into the IA space. MSCs are ideal cell therapy candidates for the treatment of OA in humans because of the ease of their extraction from different adult tissues, in addition to their high differentiation potential into chondrocytes and special capacity for immunomodulatory activity (Kobolak et al. 2016). Several clinical trials have been conducted that examined the therapeutic potential of MSCs in the clinical management of OA with short-term (weeks) and long-term (years) follow-up. Bastos et al. reported that IA injections of bone marrow-derived culture-expanded MSCs with and without PRP improved the Knee Injury and Osteoarthritis Outcome Score (KOOS) symptom domain and global score, which led to a decrease in OA symptoms and repaired joint function at the 12-month follow-up (Bastos et al. 2020). Recently, Hernigou et al. reported that subchondral bone marrow MSCs compared with total knee arthroplasty (TKA) had a significant effect on pain relief and enabled patients with bilateral OA to postpone or avoid TKA (Hernigou et al. 2021a, b). Briefly, administration of MSCs appears to reach the alleviated pain, repaired physical function of the joint, and formation of the hyaline-like cartilage in human. Table 2 summarizes findings from past

Table 1 Preclinical studies of mesenchymal stem cell (MSC) applications in osteoarthritis (OA) animal models (2011 to 2021)

OA animal model	MSC source	Intervention	Main results	References
Sheep	Autologous BMSCs	BMSCs with base medium	Better macroscopic and histological score in BMSCs	Al Faqeh et al. (2012)
Rabbit	Xenogenic UCMSCs	BMSCs with PBS	Better macroscopic and histochemical scores and less synovitis in cartilage fibrillation in the UCMSCs transplanted group.	Saulnier et al. (2015)
Mouse	Allogeneic ADMSCs	ADMSCs/mouse serum	Less synovitis and better macroscopic score in ADMSCs transplanted group.	ter Huurne et al. (2012)
Rat	Human MSCs or rat MSCs	Human MSCs versus rat MSCs in PBS	Meniscal regeneration in both human and human MSCs, improved histochemical score and inhibited OA progression in human MSCs compared with rat MSCs.	Horie et al. (2012)
Rat	Allogeneic BMSCs	BMSCs/self-assembled peptide hydrogels as a scaffold	Better histologic and macroscopic score in BMSCs transplanted group	Kim et al. (2014)
Sheep	Autologous ADMSC or BMSCs	AMSC versus BMSCs	Cartilage regeneration and ECM synthesis in both ADMSCs and BMSCs transplanted groups	Ude et al. (2014)
Guinea pigs	Human MSCs	MSCs/HA versus MSCs/PBS	Cartilage regeneration in both MSCs/HA and MSCs/PBS	Sato et al. (2012)
Rabbits	ADMSCs	ADMSCs/PRP versus PRP	Better histological evidence for ADMSCs/PRP compared with PRP.	Hermeto et al. (2016)
Dogs	ADMSCs	ADMSCs/PRP versus PRP	Improved function and focal compressive strength, reduced pain in ADMSCs/PRP compared with PRP.	Yun et al. (2016)
Rabbit	Allogeneic BMSCs	BMSCs/HA versus HA	Better histological score in BMSCs/HA compared with HA	Chiang et al. (2016)
Rat	Allogeneic ADMSCs	ADMSCs/PBS	Delayed joint degeneration and chondrocyte protection in ADMSCs transplanted group	Mei et al. (2017)
Rabbit	BMSCs	BMSCs/HA versus PRP versus PRP/HA	Better histologic and histochemical scores in BMSCs/HA, PRP/HA, and PRP.	Desando et al. (2018)
Sheep	Allogeneic ADMSCs	ADMSCs/HA versus HA	Improved results visualized by MRI in AD-MSCs/HA Compared with HA	Feng et al. (2018)
Sheep	ADMSCs	ADMSCs/HA versus HA	Better MRI, μCT, and histochemical score in ADMSCs/HA compared with HA.	Lv et al. (2018)
Dog	Allogeneic MSCs	MSCs/PBS	No observed histologic or behavior differences after MSCs administration.	Cabon et al. (2019)
Rat	WJMSCs	WJMSCs versus WJMSCs/ESWT or ESWT	Improved μCT and histological scores in ESWT, WJMSCs and the combined groups, better immunohistologic score in WJMSCs.	Cheng et al. (2019).
Rat	Human ADMSCs	ADMSCs/PBS	Reduction of joint pain in the human ADMSCs transplanted group.	Wang et al. (2020)
Rat	Human UCMSCs	UCMSCs/HA versus HA or PBS	Higher cartilage regeneration and histological score, lower OA score in UCMSCs/HA compared with HA or PBS	Xing et al. (2020)
Rat	Allogeneic BMSCs	BMSC-derived exosomes	Improved cartilage repair, ECM synthesis, and alleviated knee pain in exosome-treated group	He et al. (2020)
Rabbit	UCMSCs	UCMSC versus GO/UCMSC or GO	Higher ECM syntheses in UCMSCs and less inflammation in UCMSC and GO/UCMSC compared with GO.	Wang et al. (2021)

(continued)

Table 1 (continued)

OA animal model	MSC source	Intervention	Main results	References
Rat	hESC-derived ESMSCs	Single-dose ESMSCs versus three doses of ESMSCs	Better macroscopic, histological, and behavioral scores in the repeated dose group.	Xing et al. (2021).
Rabbit	Allogeneic ADMSCs	ADMSCs/PBS	Decreased cartilage destruction and OA score, and chondrocyte protective effects in ADMSCs	Zhang et al. (2021)

BMSCs Bone marrow mesenchymal stem cells, *ECM* Extracellular matrix, *UCMSCs* Umbilical cord mesenchymal stem cells, *PBS* Phosphate-buffered saline, *ADMSCs* Adipose-derived mesenchymal stem cells, *HA* Hyaluronic acid, *PRP* Platelet-rich plasma, *WJMSCs* Wharton's jelly mesenchymal stem cells, *μCT* Micro-CT, *ESWT* Extracorporeal shockwave therapy, *GO* Graphene oxide

5 years of clinical trial results for MSC treatment of human osteoarthritic joints.

9 Proposed Mechanisms of Action by Mesenchymal Stem Cells (MSCs) in the Treatment of Osteoarthritis (OA)

Although the exact mechanisms by which MSCs regenerate articular cartilage are unknown, evidence suggests that injected exogenous MSCs primarily regulate the local inflammatory microenvironment, inhibit tissue damage, decrease apoptosis, stimulate progenitor cell recruitment and proliferation, improve angiogenesis, and reduce oxidative stress. MSCs mainly regulate these activities via paracrine signaling, regulation of anabolic and catabolic factors, and direct MSC-chondrocyte interactions instead of direct differentiation into chondrocytes (Ozeki et al. 2016; Saulnier et al. 2015). Apparently, MSCs are not specifically designed to engraft into the empty spaces of the lost cartilage; rather they predominantly regulate the repair response via paracrine activity and immunomodulatory functions. Clearance of MSCs and inability to trace them shortly after administration support this hypothesis. Therefore, the trophic activity of MSCs rather than differentiation to tissue-specific cells appears to be fundamental mechanism for their therapeutic effects in OA. To conduct such therapeutic effects, MSCs secrete an abundant cytokine, chemokine, anti-inflammatory, and other soluble factors including prostaglandin E2 (PGE2), GAL3, GAL 9, HO-1, nitric oxide (NO), interferon (IFN)-γ, interleukins (ILs) (involved in anti-inflammatory function), IDO, TGF-β, TSG-6, IGF, HLA-G5, HGF, IL-6 (involved in immunomodulatory function), SDF-1α, MCP-1, and MCP-2 (involved in cell recruitment), VEGF (involved in angiogenesis) (Jo et al. 2014; Wang et al. 2019a, b), as well as other chondrogenic factors involved in chondrocyte proliferation and ECM synthesis (Im 2018). Figure 1 shows the paracrine activities of MSCs to induce tissue repair in an osteoarthritic knee joint.

10 Future of Mesenchymal Stem Cells-Directed Therapeutics for Osteoarthritis

Nowadays our current knowledge or application of exiting cell therapy techniques are failed to the treatment of large and diffuse cartilage defects which is observed in end-stage OA. Moreover, inadequate homing capacity and survival rate of MSCs have restricted the results and on the other hand, MSC-derived exosomes have not received permission to enter the clinic (Silva et al. 2018). However, given the existing knowledge and unresolved challenges, drawing the conclusion on the final effectiveness of MSC therapy in the treatment of OA is very early and precipitant and, in this regard, we need more extensive studies to guide the future of the path.

Despite the limited published evidence that supports the effectiveness of MSCs in OA

Table 2 Five years of clinical trial results with mesenchymal stem cells (MSCs) for treatment of osteoarthritic joints (2015–2021)

MSC source	Cell density	Number of patients	Mode of MSC delivery	Main results	References
Allogeneic BMSCs	40×10^6	30	IA injection	Higher cartilage quality and improved joint function in BMSCs group versus the control.	Vega et al. (2015) PMID: 25822648
Autologous BMSCs	5×10^6	30	IA injection	Improved VAS and WOMAC scores seen by MRI, improved cartilage quality, knee function, and decreased pain level in BMSCs group versus the control.	Emadedin et al. (2015) PMID: 26058927
Autologous BMSCs	50×10^6	15	IA injection	Reduced pain and remarkable improvement in clinical and quantitative MRI outcomes over 2 years in BMSCs group versus the control.	Soler et al. (2016) PMID: 26783191
Autologous ADMSCs	2×10^6 (low-dose), 10×10^6 (mid-dose) and 50×10^6 (high-dose)	18	IA injection	Only the low dose of ADMSCs exhibited statistically significant improvements in WOMAC index, VAS pain score, and KOOS score.	Pers et al. (2016)
Allogeneic UCMSCs	$20–30 \times 10^6$	36	IA injection	Improved joint function and overall quality of life in UCMSCs group versus the control.	Wang et al. (2016)
Autologous BMSCs	$8–9 \times 10^6$	3	IA injection	The treated knee had a higher VAS score, better functional results, and lower pain levels at 5 years after the BMSCs injection.	Davatchi et al. (2016)
Allogeneic BMSCs	25, 50, 75 or 150×10^6	60	IA injection	Improvement of VAS, ICOAP, and WOMAC scores was observed only in the 25 x 10^6 cell dose group at the 12-month follow-up	Gupta et al. (2016)
Autologous BMSCs	30×10^6	13	IA injection	The KOOS score and knee cartilage thickness were dramatically improved in the BMSCs group versus the control.	Al-Najar et al. (2017)
Autologous ADMSCs	10×10^6 (low-dose), 50×10^6 (mid-dose), 100×10^6 (high-dose)	18	IA injection	Improved function, pain, and WOMAC score in the high-dose group, which was confirmed by MRI and second look arthroscopy. Hyaline-like cartilage regeneration in the high-dose group.	Jo et al. (2017)
Autologous ADMSCs	10×10^6 (low-dose), 20×10^6 (mid-dose) and high-dose, 50×10^6 (high-dose)	18	IA injection	Improved pain, function, and cartilage volume at different doses. The most improvement was seen in the high-dose group.	Song et al. (2018)
Autologous BMSCs	Not mentioned	61	IA injection	Improved quality of life, VAS, WOMAC in X-rays, decreased pain at the six-month follow-up	Garay-Mendoza et al. (2018)
Autologous BMSCs	$10–100 \times 10^6$	30	IA injection	No adverse effects after BMSCs throughout the follow-up period. Improved VAS, WOMAC, and KOOS scores in BMSCs versus the control.	Lamo-Espinosa et al. (2016)

(continued)

Table 2 (continued)

MSC source	Cell density	Number of patients	Mode of MSC delivery	Main results	References
Allogeneic PLMSCs	500–600 × 10^6	20	IA injections	No adverse effects after PLMSCs after 24 weeks, clinical outcome, and better joint function in PLMSCs versus control	Khalifeh Soltani et al. (2019)
Autologous ADMSCs	100 × 10^6	30	IA injection	Significantly reduced pain and improved joint function at 12 months, better KOOS score in MRI. No adverse effects were observed during follow up	Freitag et al. (2019)
UCMSCs	20 × 10^6	40	IA injection	Frequent injections of UC-MSCs had lower ratings than other study groups during 12 months; progress in pain and knee surgery in OA patients were monitored at 12 months	Khalifeh Soltani et al. (2019)
BM-MSCs	1 × 10^6 (low-dose), 10 × 10^6 (mid-dose), and 50 × 10^6 (high-dose)	12	IA injection	Some cases of edema upon injection were reported. Patients who received higher doses of MSCs experienced reduced symptoms, greater improvements in KOOS, WOMAC score, and quality of life.	Chahal et al. (2019)
ADMSCs	100 × 10^6	24	IA injection	Decreased cartilage lesion size, better WOMAC and VAS scores in ADMSCs group vs. control. No significant adverse effects were reported.	Lee et al. (2019)
ADMSCs	80 × 10^6	34	IA injection	Improved pain and symptoms, higher VAS and KOOS score in ADMSCs group vs. control.	Higuchi et al. (2020)
BMSCs or BMSCs +PRP	40 × 10^6	47	IA injection	Improved KOOS and global symptom, decreased intra-articular levels of pro-inflammatory cytokine in BMSC and MSC + PRP groups after 12 months	Bastos et al. (2020)
BMSCs	5.8× 10^3/mL	60	IA injection	Both clinical and MRI improvement in BMSC group vs. control	Hernigou et al. (2021a, b)
BMSCs	7.8 × 10^3/mL	140	IA injection	Pain relief in BMSCs versus TKA	Hernigou et al. (2021b)

OA Osteoarthritis, *BMSCs* Bone marrow mesenchymal stem cells, *MRI* Magnetic resonance imaging, *IA injection* Intra-articular injection, *ADMSCs* Adipose-derived mesenchymal stem cells, *UCMSCs* Umbilical cord mesenchymal stem cells, *PLMSCs* Placenta-derived mesenchymal stem cells, *VAS* Visual analog scale, *KOOS* Knee Injury and Osteoarthritis Outcome Score, *WOMAC* Western Ontario and McMaster Universities Osteoarthritis Index, *PRP* Platelet-rich plasma, *TKA* Total knee arthroplasty

management, 80% of OA patient participants in a study of MSC therapy reported "symptomatic improvement," or "good results" after treatment (Piuzzi et al. 2018). The results of similar studies indicate relative patient satisfaction, and this reveals a disparity between the literature and marketing claims.

Future MSC-based therapeutics for OA should focus on increasing MSC survival and targeted cell delivery to the injured tissue. Immobilization of cells by encapsulation with semi-permeable membranes may help increase MSC survival and targeted MSC therapy may significantly improve therapeutic outcomes in the future.

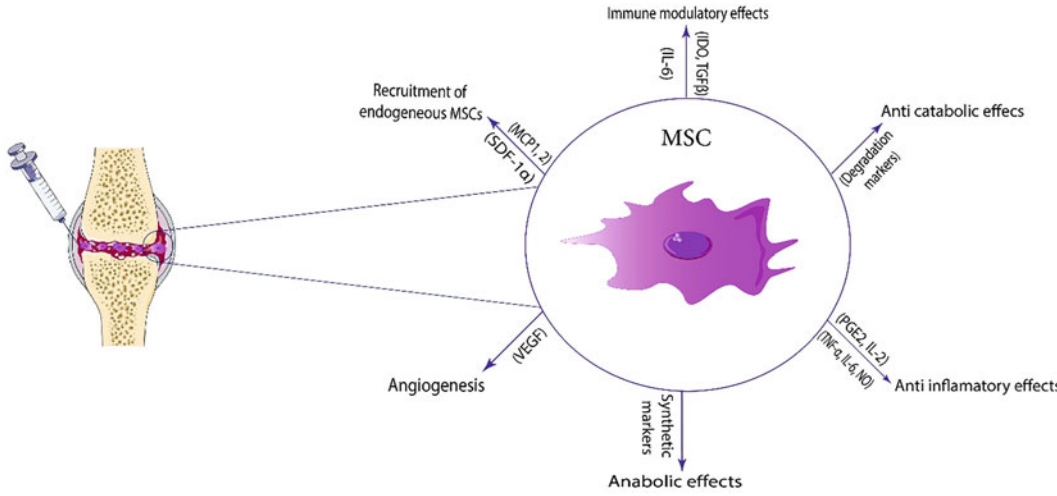

Fig. 1 Schematic representation of proposed mechanisms of action for tissue regeneration in an osteoarthritic joint by mesenchymal stem cells (MSCs). MSCs stimulate the angiogenesis and recruitment of endogenous precursor cells, in addition to their anti-inflammatory and anti-catabolic roles which enables them to participate in tissue repair and disease control of the osteoarthritic environment in a joint

Targeted MSCs delivery to osteoarthritic cartilage can be performed in two ways, by surface modification of MSCs and NPs (Nasiri et al. 2019). MSC surface modification can occur by expression induction of special ligands, antibodies, or peptides on the MSC surface with the intent to increase its binding affinity to desired ligands on the target tissue (Ansboro et al. 2012). NPs are connector interfaces that bind to both the MSCs and the tissue, and cause MSCs to bind indirectly to the surface of the target tissue and inhibit off-target attachment of MSCs to an undesired surface (Nasiri et al. 2019).

In conclusion, the future of MSC therapy for OA has uncertainties and challenges. The results of many studies have described the hypo-immunogenic nature of MSCs; however, others reported the production of antibodies in the body and a host immune response after allogenic MSC administration (Zhang et al. 2009), which seems to be a cause for rapid cell clearance. Despite compatibility of autologous MSC's with the patient's immune system, there are barriers to the use of autologous MSCs in the clinic, including an inadequate number of cells for administration, decreased therapeutic potential of autologous MSCs in elderly or unhealthy patients, and lack of time and cost for the treatment of acute disease with autologous MSCs because of the costly and time-consuming process for culturing MSCs. These obstacles restrict the future of MSC therapy in OA. However, it would be promising to develop a targeting system that would provide more efficient targeting of endogenous stem/progenitor cells and advance the tissue regeneration process, even without the need for administration of MSCs.

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The Historical Relationship Between Meis1 and Leukemia

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Abstract

Acute leukemia (AL) is a poor progressive resistant hematological disease, which has different subtypes and immunophenotypic properties according to leukemic blasts. AL is caused by genetic changes and associated with leukemia stem cells (LSCs), which determine its prognosis and endurance. LSCs are thought to be hematopoietic progenitor and stem cell (HPSCs)-like cells that underwent a malignant transformation. In addition to their low number, LSCs have the characteristics of self-renewal, resistance to chemotherapy, and relapse of leukemia. The myeloid ecotropic integration site-1 (MEIS1) protein is a member of the three-amino acid loop extension (TALE) family of homeodomain (HD) proteins that can bind to DNA sequence-specific manner. Studies have shown that overexpression of MEIS1 and associated cofactors involves tumorigenesis of numerous cancers. Historically, increased expression of Meis1 transcript as well as protein has been determined in acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) patients. Moreover, resistance to conventional chemotherapy was observed in leukemic blast samples with high Meis1 content. In this review article, the molecular mechanism of the oncological role of the MEIS1 protein in leukemia and LSC is discussed. In addition, it was suggested that MEIS1 protein could be utilized as a possible treatment target in leukemia with an emphasis on the inhibition of MEIS1, which is overexpressed in LSC.

Keywords

Leukemia · Leukemia stem cells · MEIS1

Abbreviations

AL	Acute leukemia
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
AML-IC	Aml-initiating cells
BCL-2	B-cell lymphoma-2
BM	Bone marrow
CSCs	Cancer stem cells
CXCR-4	C-x-c chemokine receptor type 4
DFS	Disease-free survival
DNMT3a	DNA methyltransferase 3 alpha
FAB	French-American-British
FLT3	Fms-like tyrosine kinase 3

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FLT3-ITD	Fms-like tyrosine kinase 3-internal tandem duplication
HD	Homeodomain
HIF	Hypoxia-inducible factor
Hif-1α	Hypoxia-inducible factor 1 alpha subunit
Hif-2α	Hypoxia-inducible factor 2 alpha subunit
HoxA9	Homeobox a9
HSC	Hematopoietic stem cell
HSPCs	Hematopoietic stem and progenitor cells
LICs	Leukemia initiating cells
LSCs	Leukemia stem cells
MDS	Myelodysplastic syndromes
MEIS1	Myeloid ecotropic integration site-1
MEIS1i	MEIS1 inhibitors
MLL	Mixed lineage leukemia
MRD	Minimal residual disease
NOD/SCID	Nonobese diabetic/severe combined immunodeficiency
NPM1	Nucleophosmin 1
OS	Overall survival
PB	Peripheral blood
Pbx1	Pre-b-cell leukemia transcription factor 1
ROS	Reactive oxygen species
TALE	Three-amino acid loop extension
Tdt	Terminal deoxynucleotidyl transferase

1 Introduction

1.1 Leukemia

Leukemia is a life-threatening hematological malignant disease (Juliusson and Hough 2016). Acute (fast progressive) and chronic (more slowly progressing) leukemia are divided into myeloid and lymphoid origin. Acute and chronic leukemias have different mechanisms and different treatment options. According to the 2021 data from the *American Cancer Society*, the number of newly diagnosed acute myeloid leukemia (AML) patients was 20,240, while the number of patients diagnosed with acute lymphocytic leukemia (ALL) was 5,690 in the USA. While the number of patients who died from AML was 11,400 in 2021, the number of patients who died from ALL was 1,580. The risk of most leukemias increases, as people get older. The typical age of a patient with AML, chronic lymphocytic leukemia (CLL), or chronic myeloid leukemia (CML) is 65 years or older. People under the age of 20 are more likely to develop ALL. The average age of an ALL patient at the time of diagnosis is 15. Mutagenic factors, genetic combinations, and environmental factors are among the known causes of leukemia (Kreile et al. 2016). Besides all these reasons, somatic mutations are accepted as the major factor in the development of leukemia. Some common somatic mutations include chromosomal translocations (fusion proteins, abnormal gene expressions) (Kumari et al. 2012; Nickoloff et al. 2008), numerical chromosomal aberrations (deletions, aneuploidy) (Simonetti et al. 2014; Zhang et al. 2011b), and mutations (Point-,ITD.) in known oncogenes or tumor suppressor genes (Osato 2004; Panuzzo et al. 2020).

1.1.1 Leukemia Stem Cells (LSCs) and Leukemia-Initiating Cells (LICs)

LSCs are usually characterized by their quiet nature, immature phenotype, distinct metabolic characteristics, and reliance on certain signaling pathways (Polak et al. 2020). In addition to these characters, LSCs have properties such as self-renewal, undifferentiation, drug resistance, as well as initiating leukemia when transported to NOD/SCID mice (Gupta et al. 2009; Hanekamp et al. 2017).

Self-renewal refers to the ability to transfer illness into secondary/tertiary recipients, whereas leukemia initiation refers to the ability to engraft and reconstitute the heterogeneous leukemia disease (Chung and Park 2014). LSCs and HSCs could share the same niche in the bone marrow (BM) (Vormoor et al. 1994). Both LSCs and HCSs demonstrate the similar CD34+CD38- immunophenotype. This strengthens the hypothesis that LSCs are likely to originate from HSCs and subsequently undergo a series of malignant transformations (Passegué et al. 2005; Weissman

2005). The LSCs that originate from the myeloid line have self-renewal and other biological features of HSCs (Ayton and Cleary 2003; Kumar et al. 2004; Passegué et al. 2005; Weissman 2005). The response of healthy and leukemic cells to hematopoietic growth factors, as well as their sensitivity to cytokines in stem cells, is the most significant difference (Jordan et al. 2000). Interleukin-3 receptor alpha chain (CD123) is the most prominent marker of LCSs and is not highly expressed in CD34+CD38- hematopoietic cells (Jordan et al. 2000; Okada et al. 2005; Wang et al. 2005). Furthermore, studies suggest that LSCs exist in the CD34+CD38- population (Hanekamp et al. 2017). For AML, LSCs are well understood, while their existence and significance for ALL are less clear (Bernt and Armstrong 2009). Human AML-IC was first found in 1994 by Lapidot and colleagues in immunodeficient SCID mice in vivo (Shultz et al. 2007) (Lapidot et al. 1994).

LSCs, defined as leukemia-initiating cells (LIC) from time to time, represent a subset of leukemia cells and are distinguished from other leukemia cells because they have some characteristics of the stem cell (Chung and Park 2014). Ng et al. (2016) studied four patients with AML, and CD34+/CD38- leukemic cells were injected into the mouse (Ng et al. 2016). Transfected mice were identified with LICs (Ng et al. 2016). LSCs obtained from transplanted xenograft specimens were found to be similar to the original patient LSCs (Buss and Ho 2011). Recent research have also confirmed the occurrence of CD34+CD38- LSCs and parallel correlation with minimal residual disease (MRD) levels after chemotherapy, disease responsiveness, and poor survival (Terwijn et al. 2014). In the presence of LSCs, the treatment of AML patients is different, and resistance to treatment increases (Buss and Ho 2011; Terwijn et al. 2014). In a broader gene study with 1,047 AML patients, the expression of 52 genes representing LSCs and non-LSCs was determined, and it was found to be related that patients with high-score LSCs had poor OS (overall survival) and DFS (disease-free survival) (Gentles et al. 2010).

Although xenograft transplantation experiments confirmed the presence of LCSs in CD34+CD38- cells, Taussig et al. (2010) found the existence of LSCs in the samples taken from NPM-mutated AMLs in low CD34- expression (Taussig et al. 2010; Jin et al. 2009; Lechman et al. 2016). In some instances, only the presence of LSCs in CD34- cells was observed, while in some samples, the presence of LSCs in both CD34+ and CD34- cells emphasized that the phenotyping of LSCs was heterogeneous. It has also been shown that the phenotype of LSCs has changed and the existence of LSCs differs in CD34 and CD38 expressions (Buss and Ho 2011). LSCs observed different ratios of AML subtypes, and the ratio of LSCs was found to be directly proportional to high-risk AML (Gentles et al. 2010; Taussig et al. 2010). In addition, LSCs-associated microRNAs obtained from samples known to be LSCs are associated with OS (Lechman et al. 2016). All these studies allow the patient to determine the treatment modality and identify risky groups according to the genetic and other characteristics of AML LSCs.

1.1.2 Biomarkers of Leukemia Stem Cells in AML and ALL

The identification of LSCs in leukemia is particularly important in disease diagnosis, prognosis, monitoring, and drug screening of leukemia. Multicolor flow cytometry, side population assays, and ALDH assays are all used to identify LSCs. Despite the variability of AML/ALL, multiple cell surface markers that are increased on CD34+CD38- LSCs when compared to normal CD34+CD38- HSPCs have been found. The most common surface markers in the LSCs are shown in Table 1.

Many of these markers related to relapses of the disease have not been studied in detail. However, the combination of CD123+CD34+CD38- is known to increase once the disease relapsed (Buss and Ho 2011; Kinstrie et al. 2015). In addition, CD123 positive cells and FLT3-internal tandem duplication (ITD) mutation come together in the CD34+CD38- LSC population (Al-Mawali et al. 2016) suggests that CD123 is a strong LSCs marker in FLT3-ITD mutated AML. In several studies to identify myeloid originated leukemic

Table 1 Common leukemia stem cell markers

Marker	Description	References
CD34	Marker antigen for HSPCs and LSCs	Taussig et al. (2010)
CD38	This is a maturation marker. Typical HSC is negative for CD38. This marker was found negative in AML-LSCs as well	Takagi et al. (2017)
CD123 (IL3RA)	LSCs are frequently identified by this marker	Han et al. (2017)
CD33	Positive for patients with AML-LSCs	Ehninger et al. (2014); Walter et al. (2012)
TIM3 (T-cell Ig mucin3)	Surface molecule expressed on LSCs in most forms of AML except acute promyelocytic leukemia but not on HSCs	Jan et al. (2011) and Kikushige et al. (2010)
Anti-BCL-2	The BCL-2 is highly expressed in CLL patients	Marschitz et al. (2000)
CD96	Cell surface marker present on many AML-LSCs	Du et al. (2015), Hosen et al. (2007) and Zeijlemaker et al. (2016)
CD47	Upregulated in murine and human myeloid leukemia	Jaiswal et al. (2009) and Majeti et al. (2009)
CLL-1	A stem cell and myeloid LSC marker	Moshaver et al. (2008), Taussig et al. (2010), Van Rhenen et al. (2007) and W. Zeijlemaker et al. (2016)
ALDH	ALDH activity is high in both myeloid LSCs and HSCs	Cheung et al. (2007) and Ran et al. (2009)
CD133	CD133 expression on LICs in children with ALL	Cox et al. (2009)
CD99	Allows functionally normal hematopoietic stem cells to be distinguished from leukemic stem cells in AML	Chung et al. (2017)
CD32	In some AML patients, the expression is linked to LSC activity	Saito et al. (2010a)
CD93	Novel biomarker of LSC in CML	Kinstrie et al. (2015)
CD22 CD19 Anti-Tdt CD10 CD20	These markers in combination are used for B-ALL classification	Mannelli (2016)
CD2 CD5 CD7 CD8 CD10 Anti-Tdt	These markers are used for T-ALL classification	Mannelli (2016)

cells, CD123 and CD33 were used as LCSs determinants (Han et al. 2017; Somervaille et al. 2009; Takagi et al. 2017; Testa et al. 2002). In sum, these studies indicate that CD123 is a crucial determinant in the targeting and identification of LSCs.

It has been reported that TIM3 is highly expressed on the surface of a large number of LCSs but not in normal BM-HSCs (Jan et al. 2011). TIM3 is defined as a new surface marker in the determination of AML-LCSs. Saito et al. (2010a, b) examined that CD32 and CD25 are highly expressed on the surface of human LCSs (Saito et al. 2010b). Although LSCs were defined in CD34+CD38- phenotype, Taussig et al. (2008) showed that CD34+ CD38+ leukemic cells were engrafted in all seven AML samples in their investigation (Taussig et al. 2008). Furthermore, technologies based on the flow cytometer analysis showed that LSCs were observed to be quite high in aldehyde dehydrogenase activity (ALDH) (Gerber et al. 2012).

1.1.3 AML and LSC

AML is defined as an aggressive neoplastic disease characterized by elevated proliferation, discontinuance of differentiation, and disruption of apoptosis mechanism (Weinstein et al. 2013). Although the treatment of AML has robustly increased in recent years, its cause has sufficiently not been determined (Döhner et al. 2017). Although transplantation is still an important treatment modality in relapsed and high-risk AML disease, side effects associated with transplantation and refractory diseases cause morbidity and mortality (Breems et al. 2005). AML patients are treated with various treatment methods and hematopoietic stem cell transplantation, but the relapse of leukemia is still an insoluble problem of AML treatment (Liu et al. 2017). The most important reason for the relapse of the disease after treatment is the presence of LSCs, which enable the regrowth of leukemia. For the discovery of new drugs to be developed for the treatment of AML subtypes (Table 2), CD34+ CD38-LSCs with leukemic blasts belonging to AML patients in xenograft models can be examined (Zhang et al. 2011a). With the disruption of hematopoiesis, the number of hematopoietic cells (red blood cells, platelets, and granulocytes) is reduced, which contributes to the development of the disease. Due to their resistance to chemotherapy drugs, LSCs were difficult to respond to treatment. However, OS rates have decreased in AML (Liu et al. 2017; Zhang et al. 2011a). CD34 is a trans-membrane sialomucine protein and as a cell-cell adhesion factor and is a protein that is expressed on the surface of HSCs, which allows stem cells to bind to the extracellular matrix (Aljurf et al. 2011). The CD34 monoclonal antibody is important in the identification of

Table 2 WHO classification of AML

Recurrent genetic anomalies in AML
AML with t(8;21)(q22;q22.1);RUNX1-RUNX1T1
AML with inv(16)(p13.1q22) or t(16;16)(p13.1;q22);CBFB-MYH11
APL with PML-RARA
AML with t(9;11)(p21.3;q23.3);KMT2A-MLLT3
AML with t(6;9)(p23;q34.1);DEK-NUP214
AML with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); GATA2, MECOM
AML (megakaryoblastic) with t(1;22)(p13.3;q13.3);RBM15-MKL1
Provisional entity: AML with BCR-ABL1
AML with mutated NPM1
AML with biallelic mutations of CEBPA
Provisional entity: AML with mutated RUNX1
Myelodysplasia-related alterations in AML
Myeloid neoplasms caused by therapy
AML, Not Otherwise Specified
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukemia
Acute monoblastic/monocytic leukemia
Pure erythroid leukemia
Acute megakaryoblastic leukemia
Acute basophilic leukemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Transient abnormal myelopoiesis (TAM)
Myeloid leukemia associated with down syndrome

immature and mature cells in LSCs and in identifying residual immature blasts for MRD disease (Basso et al. 2001).

The etiology of AML is influenced by chromosomal structural variations and genetic abnormalities (Rowley 2008). The five most common mutated genes in AML comprise NPM1, Idh1, Idh2, DNA methyltransferase 3 alpha (Dnmt 3a), and FLT3, according to The Cancer Genome Atlas project. In addition to all these, the classification of leukemia is an important issue. For this purpose, the *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues*, fourth edition, was originally released in 2016, with the whole description of the classification being published in 2017 (Aljurf et al. 2011; Basso et al. 2001; Rowley 2008) (Table 2).

CD34+ AML

The majority of AML-LCSs samples (about 75%) were positive for CD34 expression, and the blast rate was above > 10% (Thomas and Majeti 2017). In CD34+CD38- AML cells, a higher percentage of LSCs are observed than CD34+CD38+ cells, whereas these cells are immature cells with higher self-renewal properties (Arber et al. 2016; Thomas and Majeti 2017).

CD34- AML

CD34- leukemia cells are defined as cells that are resistant to treatment and have the potential to initiate leukemia (Hanekamp et al. 2017). 20% of patients with AML lacks neoplastic CD34+ cells (Eppert et al. 2011; Sarry et al. 2011). No LSCs were found in samples with CD34+ and a blast population below 1% (Sarry et al. 2011; Wendelien Zeijlemaker et al. 2015; Zhu et al. 2013). Patients with CD34- except for CD34+CD38- or CD34+CD38+ leukemic populations potentially have a population of LCSs. Apart from all these, it is thought that more small cell groups in the AML patient group are not yet defined, which are more leukemic and much more resistant to treatment (Hanekamp et al. 2017). Quek and colleagues studied 49 samples of CD34-AML cohort, and they compared the mutation profile with that of a distinct cohort of 84 sequential AML samples with more than 2% CD34+ blasts. There was enrichment of CD34- versus CD34+ AML with NPM1, TET2 mutations (Quek et al. 2016). Besides, the presence of NPM1 mutation was found to be inversely proportional to CD34 expression (Falini et al. 2005). In addition, cells identified by flow cytometry using Hoechst dye 33,342 were found to be resistant to AML treatment, and these cells were found to have leukemia initiation when NOD/SCID mice were administered (Boesch et al. 2012; Falini et al. 2005; Quek et al. 2016; Richard et al. 2013). These studies suggest that CD34+ and CD34- cells (Jaiswal et al. 2009; Wang et al. 2013) are to found to be neoplastic (Moshaver et al. 2008).

1.1.4 Acute Lymphocytic Leukemia and LSC

ALL is defined as a hematological disease that is seen in 20% of all adult AL and 80% of children AL (Feuring-Buske and Hogge 2001; Liew et al. 2012; Roshal et al. 2013). ALL diseases are classified as follows: T-lineage ALL and B-lineage ALL (Table 3). LSCs are found in a large proportion of AML but are less defined in ALL (Bernt and Armstrong 2009). Satake et al. (2014) have developed a new method of separating ALL LSCs and found that they had in vivo LICs cells obtained by this new method (Satake et al. 2014). They also demonstrated that the LSCs isolated from the same investigation had different transcriptome profiles and also showed that LSCs had subclones (Satake et al. 2014).

There are genetic variations that can be observed by cytogenetic, FISH, or molecular tests in around 80% of ALL patients. Five significant genetic mutations in B-cell leukemia/lymphoma including hypodiploidy and hyperdiploidy are established in the WHO 2008 classification as t(9;22) (q34;q11.2) BCR-ABL1, t(v;11q23) MLL rearranged, t(12;21) (p13;q22) TEL-AML1 (ETV6-RUNX1), t(5;14)(q31;q32) IL3-IGH, and t(1;19) (q23;p13.3) E2A-PBX1 (TCF3-PBX1) (Vardiman et al. 2009). Besides these, Philadelphia chromosome-like B-lineage ALL is one of the latest high-risk subtypes characterized by genetic changes that cause

Table 3 WHO classification of ALL

WHO types	Immunophenotyping
B- ALL	
Early precursor B-ALL	Pro-B-ALL, CD10−, CD19+, cCD79a+, cCD22+, TdT+
Common B-ALL	$CD10^+$
Precursor B-ALL (pre-B-ALL)	Pre-B-ALL, cytoplasmic μ^+, sIg^-, $CD10^{+/-}$)
T- ALL	
Immature T-cell	CD1a, CD2, CD3 (membrane and cytoplasm), CD4, CD5, CD7,CD8 (Chiaretti et al. 2014)

different signaling pathways, including those involving cytokine receptors, tyrosine kinases, and epigenetic modifiers (Yokota and Kanakura 2016).

CD34 is an important marker for B-ALL between ages 1 and 10 (Pui et al. 1993). In low-risk adult B-ALL patients, CD34 expression was correlated with higher WBC cell count and high peripheral blood (PB) LSCs (Thomas et al. 1995). In most of the ALL patients, CD34 is not expressed on the surface of LSCs (Aljurf et al. 2011).

1.2 MEIS1

Homeodomain transcription factors play an important function in embryogenesis and differentiation. The 60-amino acids-long homeodomain is characterizing the class of TALE that includes three extra amino acids between the first and the second a-helix. This class comprises two major subfamilies, PBC (Pbx1-4 in human) and MEINOX, further divided into sub-families PREP (PREP1-2) and MEIS (MEIS1-3) (Blasi et al. 2017).

Belonging to the MEIS subfamily, MIES1 protein has important roles in both hematopoiesis and leukemogenesis, including differentiation and regulation of apoptosis mechanism (Blasi et al. 2017; Schulte and Geerts 2019). The MEIS1 protein is a 43KDa protein of 137,360 base-pair gene length, consisting of 13 exons and containing 390 amino acids (Moskow et al. 1995; Rosales-Avĩa et al. 2011). The canonical DNA binding sequence of MEIS1 is TGACAG (Shen et al. 1997). The MEIS1 protein plays a crucial role in numerous types of cancers including neuroblastomas, nephroblastomas, ovarian carcinomas, prostate cancer, non-small cell lung adenocarcinoma, and leukemia (Geerts et al. 2003; Jones et al. 2000; Kroon et al. 1998; Shen et al. 1997; Sitwala et al. 2008; Spieker et al. 2001). Moreover, in recent research, it was found that MEIS1 controls both the development of papilloma and the malignant conversion from papilloma to carcinoma in papilloma. Despite all these studies, the role and mechanism of MEIS1 protein in tumor formation and development are not fully known (Okumura et al. 2014).

MEIS1 protein was first discovered in the BXH-2 mouse leukemia model as a common viral integration site (Moskow et al. 1995). The normal Meis1 mRNA or Meis1-proviral fusion mRNA level increases with viral integration. Recent studies have shown increased cytoplasmic glycolysis by overexpression of MEIS1 protein, which is thought to be involved in the spread and progression of cancer. The increase in resistance to chemotherapy treatment is also parallel to the increased expression of Meis1 than healthy counterparts. In addition, the MEIS1 protein modulates stem cell homeostasis by maintaining stem cell glycolytic metabolism (Aksoz et al. 2017; Crijns et al. 2007; Kocabas et al. 2012a, b, 2015; Okumura et al. 2014).

1.2.1 The Role of MEIS1 on Normal Hematopoiesis

Recent studies suggest that MEIS1 is a protein that performs a crucial role in the normal HSC self-renewal/proliferation. MEIS1 co-works with the Hox genes in the differentiation stages of hematopoiesis (Imamura et al. 2002; Kocabas et al. 2014; Mahmoud et al. 2013). This idea is supported by a high rate of bleeding due to the

lack of megakaryocytes in mouse 14.5 embryonic days, which has not supplemented the MEIS1 protein (Hisa et al. 2004). While the presence of myeloerythroids in dead embryos was observed, the total number of colony-forming cells was observed to be quite low.

1.2.2 The Oncogenic Role of MEIS1 in Leukemia

The onset of cancer is initiated by its overexpression in various tumors as a result of numerous genetic alterations in the genome. The excessive expression of MEIS1 causes cell proliferation and apoptosis resistance (Rosales-Avĩa et al. 2011).

Cancer cells, unlike healthy cells, use cytoplasmic glycolysis to digest large amounts of glucose, a phenomenon known as the Warburg effect in biology. With this effect, cancer cells can survive in the hypoxic tumor microenvironment. Pernilla Eliasson et al. (2010) revealed that when they performed hypoxic conditions to LSCs, they noticed that HSC proliferation had reduced by the accumulation of the cells to G0 phase (Eliasson et al. 2010; Aksoz et al. 2017; Crijns et al. 2007; Hisa et al. 2004). Supporting previous observations, increased MEIS1 expression in the BMs of ALL and AML patients was observed (Kocabas et al. 2014; Thorsteinsdottir et al. 2001). In the experiments with murine AML models, it was found that high expression of MEIS1 is involved in the onset of the disease (Rozovskaia et al. 2001; Wong et al. 2007).

MEIS1 and Hox proteins, especially Homeobox A9 (HoxA9) and Pre-B-Cell Leukemia Transcription Factor 1 (Pbx1), work together in leukemia and accelerate leukemogenesis by activating target genes (Imamura et al. 2002; Jones et al. 2000). HoxA9 transcription factor is important in HSCs replication, and its regulation mostly degenerates in AL (Collins and Hess 2016). The higher expressions of MEIS1 and HoxA9 were observed in aggressive murine and human ALL samples (Blasi et al. 2017; Collins and Hess 2016; Pineault et al. 2004). Intriguingly, decreased activity of fumarate hydratase (Fh1) (TriCarboxylic Acid (TCA) cycle and fumarate metabolism causes activation of MEIS1/HoxA9 oncogenic pathway in LSCs (Guitart et al. 2017). In this case, the change in metabolic activity is thought to trigger the activity of MEIS1 during the development of leukemia. In another study, even in the absence of fusion, the Dntm3a mutation in AML has been shown to reactivate the oncogenic MEIS1 pathway (Ferreira et al. 2016). PU.1, a tissue-specific transcription factor that is expressed in cells of the hematopoietic cells, has a role in leukemia by increasing the expression of MEIS1 in non-MLL U937 cells (Ferreira et al. 2016; Shen et al. 1999). Although there is a relationship between the high expression of MEIS1 protein in cancer cells and cytoplasmic glycolysis, the effects of MEIS1 and its cofactors on cancer stem cells and cancer metabolism still require further investigation (Aksoz et al. 2017).

1.3 Relapse-Refractory AML/ALL and MEIS1

One of the biggest problems encountered in the treatment of leukemia disease in the clinic is the resistance of LSCs in the blast population of patients to traditional chemotherapy. As a result of this resistance, the disease appears to be relapsed, known as *relapse or recurrence.* There are still uncertainties regarding the relapse of the disease, and it is known that the MEIS1 gene and its family are involved in development of resistance to traditional chemotherapy regimens (Liu et al. 2017). According to studies, patients with a high MEIS1 expression level were more resistant to chemotherapy than those with a low MEIS1 expression level. The connection between MEIS1 and relapse/refractory is strengthened as a result of this (Liu et al. 2017). According to the investigation of Adamaki et al. (2015), high HoxA9 and MEIS1 gene expressions are associated with relapse and OS in childhood AL (Adamaki et al. 2015).

1.4 Molecular Mechanism of Leukemia Associated with MEIS1

1.4.1 Relationship of MLL with MEIS1, HoxA9, and PBX

MLL contains chromosomal translocations that contain >50 different genes associated with leukemia (Adamaki et al. 2015; Guzman and Jordan 2004). Chromosomal translocations involving the MLL gene (11q23) are found in 8–19% of ALL patients and 3% of AML patients. Chromosomal translocations involving the MLL gene (11q23) fuse N-terminal sequences of MLL to one of >40 functionally diverse groups of C-terminal fusion partners (Daser and Rabbitts 2005). AFL, AF9, or ENL translocation of the MLL gene in AML patients contributes to the development of both lymphoid and myeloid disease. Gene analysis of all leukemias associated with MLL fusion indicated that Hox gene dysregulation was an integral factor for MLL fusion-related leukemias (Aoki et al. 2015; Daser and Rabbitts 2005; Ernst et al. 2002; Kawagoe et al. 1999). In other studies, both the wild-type MLL and MLL oncogenic fusions have been associated with the Hox genes and active transcription of these genes (Ferrando et al. 2003; Yeoh et al. 2002; Yu et al. 1995, 1998). Upregulation of HoxA9 and MEIS1 is seen in leukemias associated with MLL rearrangements (Aoki et al. 2015; Caslini et al. 2007; Milne et al. 2005; Weissman 2005). Recently, various genetic techniques are used to investigate the contribution of the TALE family of Pbx1, Pbx2, and Pbx3 and MEIS1 to the formation of MLL-induced AML. The presence of the relationship defines MLL fusion genes and their capacity to induce MEIS1 transcription was promising to find the contribution of MEIS1 to the formation of MLL oncogenesis (Wong et al. 2007). This hypothesis is not valid in all myeloid leukemias. In the recent study with the AML mouse model, it was found that the MLL-AF9 oncogene was effectively induced in FAB-M4 or M5 subtypes of human AML (Kohlmann et al. 2003; Tsutsumi et al. 2003) (Swansbury et al. 1998).

1.4.2 Relation with FMS-Like Tyrosine Kinase 3 (FLT3) with MEIS1, HoxA9, and PBX

FLT3 is a member of the class III tyrosine kinase receptor family. Although it is temporarily secreted during HSC maturation, it is much more expressed in AML disease (Somervaille and Cleary 2006; Swansbury et al. 1998). FLT3 is expressed in leukemic blasts even in AL groups with negative CD34 (Drexler 1996; Gary Gilliland and Griffin 2002; Ozeki et al. 2004). In human ALs, the MEIS1 protein and Hox gene are high, which is associated with the high expression of FLT3 mRNA (Roche et al. 2004). On the contrary, Morgado et al. (2007) found that FLT3 was independent of MEIS1 and HoxA9 oncologic cooperation, and leukemogenesis was independent of FLT3 (Morgado et al. 2007). Garcia-Cuellar et al. (2015) conducted important research on the regulation of Hox function with HoxA9-MEIS1-Pbx3 interaction (Garcia-Cuellar et al. 2015). According to this study, Pbx3 binds to MEIS1 and protects it from ubiquitination and then inhibits its proteasome-mediated degradation, which is greatly extending the half-life of MEIS1. When there is a mutation in MEIS1 (i.e., MEIS1 Δ), the association of Pbx3-MEIS1is disrupted, and the ubiquitination and degradation of MEIS1 are halted. Pbx3-MEIS1 binding is required for MEIS1-HoxA9 communication. As a result of Pbx3 stabilizing the MEIS1 protein level, MEIS1-HoxA9 communication and gene regulation activity are increased (Garcia-Cuellar et al. 2015). Similarly, Wang et al. (2005) suggested that Meis1-Pbx complexes control FLT3 transcription, expansion of normal short-term HSCs, and expansion of leukemia-initiating progenitors **in vivo** regulation of hypoxic phenotype in hematopoietic cells with MEIS1 (Wang et al. 2005a).

The MEIS1 protein is involved in the transcriptional control of the Hif-1 and Hif-2 genes. (Aksoz et al. 2017; Kocabas et al. 2012a, 2015; Zhang et al. 2012). MEIS1 and its cofactors (Pbx1, HoxA9) work together to activate Hif-1α by binding to the reserved sites on the Hif-1α gene in this mechanism (Kocabas et al. 2015).

One characteristic of the hematopoietic niche is the low-oxygen partial pressure. Hypoxia provides long-term protection of HSPCs. Hypoxia is a powerful signal and is provided by members of the hypoxia-inducible factor (HIF) family (Deynoux et al. 2016). High expression of Hif-1α was detected in leukemia and especially in AML, acute promyelocytic leukemia (APL), and ALL. Thus, response to chemotherapy was decreased, and poor prognosis was observed in these samples (Deeb et al. 2011; Deynoux et al. 2016; Gao et al. 2015; Li and Ye 2010; Thorne and Milne 2015). The severity and survival of AML and myelodysplastic syndromes (MDS) diseases are affected by the level of Hif-1α (Forristal et al. 2015; Wang et al. 2011; Zhe et al. 2015). While Hif-2α expression has not been observed in normal cells, it has been readily detected in AML and ALL samples (Li and Ye 2010; Song et al. 2014; Tong et al. 2012). Several studies have shown that when Hif-1α was inhibited by RNAi or small molecules, the primary cells did not form colonies in vitro, and tumor growth and leukemic development were reduced. In these studies, the disease was significantly regressed in AML xenograft samples, and leukemia was eliminated by inhibition of Hif-1α in second transplantation (Deynoux et al. 2016; Wang et al. 2005b; Zhe et al. 2015). This is backed up by research in ALL (Rouault-Pierre et al. 2013; Wellman et al. 2004) and CML (Giambra et al. 2015; Zou et al. 2013). Similarly, limited in vitro cell proliferation or weak or incomplete in vivo engraftment was observed by knockdown of Hif-2α with shRNA resulting in leukemic inhibition (Kawada et al. 2013; Li and Ye 2010; Song et al. 2014).

Hypoxia environment created by the HIF family allows the progression of disease and maintenance through a series of mechanisms effective in the deregulated and normal physiology of cancer, energy metabolism, cycle, quiescence, and immunological function are only a few examples (Coltella et al. 2014; Zhang et al. 2012). Hif-1α and Hif-2α activate the signaling pathways involved in the protection and spread of leukemia. Hif-1α influences the Notch1 pathway to activate the Wnt pathway that induces leukemia propagation and formation (Rouault-Pierre et al. 2013; Wellman et al. 2004). On the contrary, Hif-1α inhibits the expression of tumor suppressor genes such as p15, p16, p19, and p53 (Deynoux et al. 2016; Zou et al. 2013). Expressing HIFs allows cells to remain in the quiescence phase. The leukemia cells in the quiescence phase gain resistance to chemotherapy drugs. Here, hypoxia induces members of the HIF family, and Hif-1α keeps the cells in the G0/G1 phase and prevents AML cells from passing to the S phase (Aksoz et al. 2017; Kocabas et al. 2012a; Zhang et al. 2012).

Intriguingly, the expressions of Hif-1α and Hif-2α decrease with the tissue-specific deletion of the Meis1, thus increasing ROS content and hematopoietic cell apoptosis (Kocabas et al. 2012b). ROS activity in metabolism plays a main role in the functioning of stem cells. ROS activity is also effective in the onset and progression of leukemia. ROS levels are reduced in LSCs than HSCs, and agents that escalate the activity of ROS may contribute to the elimination of AML stem cells.

1.4.3 Effect of MEIS1 Protein in Cell Cycle and Apoptosis

Cell replication and apoptosis are necessary processes for the development of the organism. However, in cancer cells, cell proliferation goes out of control, and the pathways that provide the apoptosis mechanism are disrupted. Likewise, uncontrolled cell proliferation in cancer cells involves a change in the activity of cell cycle regulations. Meis1 is a protein that is significantly expressed in a variety of cancers, causing cell proliferation and apoptosis resistance (Rosales-Avĩa et al. 2011). In a research conducted by Kumar et al. (2009), cell cycle and apoptosis investigations were done to determine the processes by which Meis1 suppression leads to reduced cell proliferation. The findings obtained as a result of PI-stained nuclei analysis in flow cytometry show that inhibition of MEIS1 caused a cell cycle arrest in the G0/G1 phase. It was observed that G0/G1 phase was escalated and S and G2/M phase was reduced (Kumar et al. 2009). Other studies showed that the G0 phase was decreased

and the G1 phase was doubled after silencing of the Meis1 gene in the K562 cells. On the other hand, Wermuth and Buchberg (2005) found that HoxA9 and MEIS1 could eliminate the caspase-mediated apoptosis pathway (Wermuth and Buchberg 2005).

1.5 Leukemia Stem Cells (LSCs) Inhibition Methods

While developing strategies to selectively eliminate LSCs, it is believed that the maintenance of normal hematopoietic stem cells is also necessary for the long-term survival of leukemia patients. Thus, methods targeting LSCs should be more specific to LSCs by allowing survival of HSPCs.

1.5.1 Targeting Surface Markers Expressed by LSCs

It is a common approach to treat LCSs with monoclonal antibodies targeting markers on their surface. The effects of the newly developed humanized monoclonal anti-CD44 antibody, RG7356, on AML patients were investigated (Vey et al. 2016). At the end of this investigation, it was shown that RG7356 increased macrophage uptake and decreased AML blasts especially in CD34-CD38+ cells (Saygin et al. 2019). The anti-CD123 drugs developed for the CD123 antigen, the specific marker of LSC, were promising for the treatment of leukemia (Thomas and Majeti 2017). IL-3 is a cytokine that performs a function in myeloid differentiation, and blocking the IL-3 receptor CD123 has been shown to lessen the growth of LSCs in NOD/SCID mice (Jin et al. 2009). New drugs targeting LCSs are still in phase 1–2 stages in clinical studies (Thomas and Majeti 2017).

1.5.2 Targeting the LSC Microenvironment

CSCs are believed to reside in niches, as is the case for normal stem cells. Niches are specialized microenvironments that regulate the fate of adult stem cells by providing indications in the form of both contacts between cells and secreted variables (Plaks et al. 2015). C-X-C chemokine receptor type 4 (CXCR-4) has a significant impact on homing and quiescence of HSCs (Moriuchi et al. 1997; Saini et al. 2010). Blocking CXCR4 allows LCSs to move out of the protected stromal niche and allows the disease to be eliminated by chemotherapy. 52 AML patients were treated with CXCR4 antagonist *plerixafor* and salvage chemotherapy, and 46% of the patients showed improvement in one phase 1–2 study (Uy et al. 2012).

1.5.3 Targeting the MEIS1 Protein via Small Molecules Called MEIS1i

Inhibitors have been developed for MEIS1 protein, which has an important role in leukemia. Small molecules called MEIS1i-1 and MEIS1i-2 show an inhibition effect on MEIS1 protein activity over 95% of MEIS-luciferase activity at doses of 100 nM (Turan et al. 2020). MEIS1i-1 has been found to inhibit the activity of MEIS1, reducing the expression of hypoxia-inducible factors (Hif-1α and Hif-2α). The negative effects of newly developed drug-structured MEIS inhibitors on MEIS protein activity, LSCs cell survival, and the apoptotic pathway in LSCs should be tested with more comprehensive and in-depth studies. In addition, it should be shown that MEIS inhibitors reduced levels of expression of known genes related to cancer growth and survival in patient samples.

1.5.4 Other Methods for the Elimination of LSCs

Membrane markers, transcriptional factors, ROS activity, telomerase, and microRNAs have been targeted to eliminate LCSs in AML. In addition, transcription factors NF-κB, Hif-1α, B-catenin, and histone deacetylases (HDACs) are therapeutic targets for the elimination of LSCs (Ding et al. 2017). LSCs relied on telomerase to continue to self-renew and proliferate. Targeting telomerase as a therapeutic strategy for eradicating LSCs in AML is considered to be ideal (Kuo and Bhatia 2014). Furthermore, microRNAs such as miR-34a and miR-126 performed a crucial function in the regulation of LSCs and could be used to develop new anti-LSC therapies (Terwijn et al.

2014; Wang et al. 2016; Aksoz et al. 2017; Kocabas et al. 2012a; Zhang et al. 2012).

2 Conclusions

This review article summarizes the MEIS1 protein and its relationship with leukemia. As in many solid cancer types, MEIS1 protein is extremely expressed in leukemia and causes cell proliferation. In addition to that, MEIS1 gene expression performs a major function in the relapse of leukemia disease and its resistance to chemotherapy treatment. With the knowledge of the MEIS1 protein and related regulatory pathways and its gene family, it will increase the possibility of being a potential treatment target for leukemia treatment in the clinic.

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An Overview of Zebrafish Modeling Methods in Drug Discovery and Development

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Abstract

Animal studies are recognized as a significant step forward in the bridging between drug discovery and clinical applications. Animal models, due to their relative genetic, molecular, physiological, and even anatomical similarities to humans, can provide a suitable platform for unraveling the mechanisms underlying human diseases and discovering new therapeutic approaches as well. Recently, zebrafish has attracted attention as a valuable experimental and pharmacological model in drug discovery and development studies due to its prominent characteristics such as the high degree of genetic similarity with humans, genetic manipulability, and prominent clinical features. Since advancing a theory to a valid and reliable observation requires the manipulation of animals, it is, therefore, essential to use efficient modeling methods appropriate to the different aspects of experimental conditions. In this context, applying several various approaches such as using chemicals, pathogens, and genetic manipulation approaches allows zebrafish development into a preferable model that mimics some human disease pathophysiology. Thus, such modeling approaches not only can provide a framework for a comprehensive understanding of the human disease mechanisms that have a counterpart in zebrafish but also can pave the way for discovering new drugs that are accompanied by higher amelioration effects on different human diseases.

Keywords

Animal modeling · Danio rerio · Drug development · Drug discovery · In vivo studies · Zebrafish

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Abbreviations

5-ASA	5-aminosalicylic acid
6-OHDA	6-hydroxydopamine
AD	Alzheimer's disease
ADHD	Attention-deficit/hyperactivity disorder
AI	Artificial intelligence
AKI	Acute kidney injury
ALD	Alcoholic liver disease
ALS	Amyotrophic lateral sclerosis
ASD	Autism spectrum disorders
BA	Biliary atresia
BMAA	β-methylamine-alanine
cAMP	Cyclic adenosine monophosphate
CNS	Central nervous system
COPAS	Complex object parametric analysis and sorting
CRISPR / Cas9	Clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein 9 (Cas9)
DILI	Drug-induced liver injury
DM	Diabetes mellitus
DSS	Dextran sulfate sodium
E. coli	Escherichia coli
ENU	N-Ethyl-N-nitrosourea
GAS	Gastrodin
GFP	Green fluorescent protein
GIOP	Glucocorticoid-induced osteoporosis
HUVEC	Human umbilical vein endothelial cells
IACUCs	Institutional animal care and use committees
IBD	Inflammatory bowel disease
IND	Investigational new drug
LOC	Lab on a chip
LPS	Lipopolysaccharide
MPTP	1-methyl 4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	Messenger RNA
MRSA	Anti-methicillin-resistant Staphylococcus aureus
NAC	N-acetylcysteine
NAFLD	Non-alcoholic liver disease
NDA	New drug application
NPs	Nanoparticles
PAM	Protospacer adjacent motif
Pcna	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PD	Parkinson's disease
PDE	Pyridoxine-dependent epilepsy
PNS	Peripheral nervous system
PTZ	Pentylenetetrazole
RNAi	RNA interference
ROS	Reactive oxygen species
sgRNA	Single guide RNA
TALE	Transcription activator-like effector
TALENs	Transcription activator-like effector nucleases
TB	Tuberculosis
TILLING	Targeting induced local lesions in genomes
TNBS	2,4,6-trinitrobenzenesulfonic acid
TOR	Target of rapamycin
TRACP	Tartrateresistant acid phosphatase
UCS	Unpredictable chronic stress
VRI	VEGFR tyrosine kinase inhibitor II
ZFN	Zinc finger nuclease
ZFP	Zinc finger protein

1 Introduction

Drug discovery as a multi-process platform leads to identifying new candidate drugs for therapeutic purposes. Principally, the sequence of processes that constructs the basis of drug discovery pipeline is generally aimed at:

1. The selection and validation of the efficacy and druggability of a suitable biological target.
2. The identification and validation of a suitable target drug by screening a large library of chemical compounds.
3. The generation and optimization of the lead compounds that have the desired interaction with the drug target to enter the development

platform as a candidate drug (Sinha and Vohora 2018; Hughes et al. 2011).

The platform of drug development consists of different stages that principally include preclinical study, investigational new drug (IND), clinical phases, new drug application (NDA), and premarket approval, in which various therapeutic aspects are considered for the commercialization of pharmaceuticals (Tamimi and Ellis 2009). It is noteworthy that both drug discovery and development platforms are accompanied by in vivo experiments (Paramasivam 2020; Giacomotto and Ségalat 2010). In this regard, animal models create the possibility to advance the current knowledge of mechanisms underlying various diseases (Khorramizadeh and Saadat 2014; Arjmand et al. 2019; Larijani et al. 2021a, b; Roudsari et al. 2021; Mansouri et al. 2020) and assess the pharmacological and toxicological effects of drugs to ensure their safety and efficacy (Greek 2013). To promote in vivo researches, choosing an appropriate animal model must first be considered in line with adherence to the ethical guidelines in scientific researches to examine the different aspects of a therapeutic approach (Goodarzi et al. 2019; Larijani et al. 2019). One of the most commonly known ethical principles is named the principle of the 3Rs that are first and foremost. Generally, the 3R ethical principles include reduction, refinement, and replacement of animals. "Reduction" involves improving appropriate experimental methods or applying new techniques to use the minimum possible number of animals in experiments. Further, "Refinement" refers to the methods that provide suitable conditions to keep and care for the animal, reduce pain and stress, and increase animal welfare. Finally, "Replacement" refers to applying substitute methods such as using cell lines, in silico modeling, and young or animal embryonic forms instead of vertebrates or complex animal models in terms of physiology as much as possible (Sneddon et al. 2017; Graham and Prescott 2015). Also, it should be mentioned that the types of disease and target drugs are as indispensable as the physiological similarity between humans and animals to choose an appropriate in vivo model (Nematizadeh et al. 2020). To get more accurate results, animal unusual biological features and the results obtained from previous experiments on the selected model should also be considered. Additionally, it is necessary to pay attention to available experimental methods, modern biomedical techniques, and financial constraints in this field (Ericsson et al. 2013). Hence, in the light of the above-mentioned ethical considerations and many efforts in the field of translational medicine, recently, zebrafish (Danio rerio) has gained increasing interest as a candidate animal model in scientific researches such as drug discovery (Macrae and Peterson 2015; Hickman et al. 2017; Cassar et al. 2019) and development to make further advances and alleviate the challenges in an in vivo research (Cassar et al. 2019). In the context of in vivo experiments, zebrafish represents attractive characteristics that can pave the way in drug discovery and development platforms. In the past decade, as a result of continuous efforts to evaluate the zebrafish and human relationships at the genomic level, we were the witnesses of achieving a complete genome sequence of zebrafish and decoding the information behind it, which was unprecedented in the genome sequencing of a vertebrate. These efforts reflect a wealth of information, such as the high degree of genome similarity between zebrafish and humans (Howe et al. 2013). In addition, genetic manipulability is considered one of the advantages of working with zebrafish (Larijani et al. 2019), which can pave the way for the evaluation of human-related diseases (Bradford et al. 2017). Consequently, a variety of genetic methods, such as the use of mutagenic or transgenic procedures, have been developed, which are intended to create a suitable zebrafish model in preclinical studies (Tavakoli et al. 2017). In addition to genetic similarities, zebrafish have high homology to several human body systems in terms of physiological characteristics. Consequently, it can not only provide deeper insights into the pathophysiology of various diseases but can also contribute valuable information to the current biological and pharmaceutical knowledge about the various effects of drugs on the target diseases (Khan and

Alhewairini 2018). Moreover, the presence of remarkable unique characteristics such as transparent body, the short cycle of the reproductive period, and even low maintenance costs of this vertebrate, has made it possible to be considered as a suitable animal model in target-based phenotypic screening with high-throughput approaches (Williams and Hong 2016). Hereupon, to promote research in this field, this review will discuss the advantages and disadvantages of zebrafish and highlight the 3R ethical principles in zebrafish modeling. Then, the discussion particularly is focused on some modeling methods for zebrafish modeling, which can provide an opportunity to simulate human disease. Finally, this review provides an overview of some pharmaceutical findings that implicate zebrafish as a preclinical model in drug discovery and development.

2 A Prominent In Vivo Model

In recent years, zebrafish has been identified as a valuable vertebrate animal model based on its remarkable features in the field of in vivo experiments (Chakraborty et al. 2009; Arjmand et al. 2020). Some of the unique features which have made zebrafish a powerful vertebrate model in preclinical studies are as detailed below:

1. The small size of zebrafish and their natural aquatic environment have made it easier to house a large number of them in a small aquarium (Arjmand et al. 2020). Therefore, the maintenance costs and the materials used during experiments can be decreased (Avdesh et al. 2012).
2. The short cycle of the reproductive period and high fertility have made it possible to screen the different chemical compounds in several model groups at the same time (Littleton and Hove 2013; Kari et al. 2007).
3. Unlike many mammalian embryos, zebrafish embryos grow outside the mother's wombs. Therefore, this allows for a variety of studies, such as birth defects in the embryogenic stage.
4. Some cellular and molecular mechanisms involved in the biological function represent similarities in zebrafish and mammals (Kari et al. 2007).
5. Zebrafish embryonic development, which is accompanied by organ growth, can be completed within about 5 days after fertilization. Therefore, it is possible to perform the various experiments during the embryonic stage (Kari et al. 2007).
6. Transparent body of the zebrafish embryo compared to the other mammals has made it possible to evaluate drug toxicity and the physiological functions of various organs.
7. The body of a zebrafish embryo can penetrate small pharmaceutical and chemical compounds from the aquatic environment during the formation and development of organs (Littleton and Hove 2013, Kari et al. 2007).
8. Genetic comparisons between humans and zebrafish indicate high organ and genetic homology between them (Goldsmith and Jobin 2012). In addition, 82% of the genes involved in human disease show orthology with zebrafish (Howe et al. 2013; van Wijk et al. 2016).
9. Several body systems such as the cardiovascular, gastrointestinal, musculoskeletal, central nervous system (CNS), and peripheral nervous system (PNS) or some organs such as the pancreas, liver, and gall bladder have been preserved between zebrafish and human beings (Teame et al. 2019; Williams and Hong 2011).
10. Zebrafish are classified as living organisms that have the ability to regenerate some organs, such as the heart (Beffagna 2019) and CNS after injuries (Tayanloo-Beik et al. 2021).

Despite many advantages, zebrafish is still not recognized as a perfect model in animal studies due to some disadvantages and this can affect various aspects of preclinical and clinical studies as well. Therefore, examining the disadvantages along with the advantages of zebrafish should be taken into consideration in preclinical studies, which are described below:

1. Despite orthologous genes between zebrafish and humans, zebrafish is located at a more distant branch from that of humans in phylogenetic analyses.
2. Unlike humans, which are known as endothermic organisms, zebrafish is an ectothermic animal and its body temperature is more affected by the external environment.
3. The absence of some organs such as limbs, lungs, and synovial joints in zebrafish cannot allow the preclinical study of some human diseases related to these organs.
4. Some studies have highlighted that the zebrafish genome has been affected by genome duplication during vertebrate genome evolution (Ali et al. 2011). Consequently, concerning some genes, two copies of each gene reside on a single chromosome in each of two different species. Therefore, instead of a single copy, both copies of the targeted gene should be studied at genome-associated studies, which will be time-consuming (Postlethwait et al. 1998).

3 Ethical Principles

In vivo studies on zebrafish, like any other biological organism, must be consistent with ethical considerations. Therefore, in conforming to the 3R principles, this review has outlined some of the basic ethical and professional principles of working with zebrafish as a valuable vertebrate model in animal experiments:

- **Replacement:** Cultured fish cells can be an alternative approach to a living model. Since the cell lines have been developed from most fish tissues and organs, they effectively contribute to mimic some physiological features of zebrafish in an in vitro condition. Alternatively, the use of perfused organs of fish could also be a suitable platform for examining the physiology of the animal model. Indeed, a perfused organ can offer a higher level of tissue complexity than the cell culture in an in vitro experiment. In addition to the above, the embryo of zebrafish can be replaced with the adult type in some cases. Compared to the other species embryos, zebrafish embryos can reach the feeding stage in a shorter period (about 4 to 5 days post-fertilization). Also, studies indicate that the zebrafish in embryonic stages can lead to reliable results in toxicological studies, the same as the adults. On the other side of the coin, the complete replacement of zebrafish with leading-edge technologies can also be an advantageous solution in animal experiments. Recently, in light of the development of new biomedical technologies, computer-based modeling strategies have emerged that promise fundamental advances in in vivo studies with less dependence on animal models in the future. Although these technologies may not currently be a complete alternative to animal models, they have a key and cooperative contribution to in vivo modeling (Sloman et al. 2019).
- **Reduction:** In general, although a large number of animal models can increase the validity and accuracy of the results, this can lead to suffering and damaging of many models as a living organism. Conversely, the small number of animal models, in some cases, can also call into question the concept of results validity of scientific research (Larijani et al. 2020). Therefore, some statistical calculations such as power analyses can be a big step toward applying a reasonable and reliable number of animal models in the experiments so that neither a large number of living organisms are suffered nor the validity of the results obtained decreases. On the other hand, careful model and experiment designing by the researcher during experiments, based on data or experiences from previous researches, can also have a great contribution to minimizing the number of animal models per study (Sloman et al. 2019).
- **Refinement:** The discussion of zebrafish refinement should be assessed in several different aspects. First, in terms of in vivo research, the use of zebrafish bred in laboratory conditions has a higher priority than the

species of zebrafish bred in nature. Since any displacement of a living organism from its natural habitat to a new and unknown environment will lead to distress and consequently adverse effects on the final results in the experiments. Even if possible, it is recommended to use zebrafish bred in the study area. However, in case of impracticality, zebrafish eggs or larvae transferring is recommended. If there are still no options other than using off-site zebrafish, all the principles related to care and maintenance of the organism during displacement or even after displacement should be taken into consideration, according to the guidelines. Second, the zebrafish housing conditions should always be carefully examined in terms of the suitability of the storage tank, water-related parameters, amount of light, environmental disturbances, and humidity. In addition, other maintenance conditions such as the type of food and related diets, examining the health conditions, and providing suitable conditions for reproduction, must be closely monitored. Third, animal modeling requires well-organized modeling methods appropriate to the research purposes with minimal damage to the animal. Even in some cases, appropriate anesthetic and analgesic agents should be used to relieve the pain. Fourth, hiring skilled staff and researchers who adhere to ethical and professional principles of working with animal models can play a key role in maintaining the model in a healthy condition, timely reviewing of animal model phenotypes, taking steps toward instantly reducing pain and injury, recording all observations during the experiment (Reed and Jennings 2011), and applying an appropriate euthanasia procedure for inducing humane death under moral and environmental principles with the least suffering to the animal model in accordance with the principles approved by the Institutional Animal Care and Use Committees (IACUCs) (Moosapour et al. 2020).

4 Human Disease Modeling Strategies in Zebrafish

4.1 Experimental Environment

The relationship between environmental stimuli and neuropsychiatric disorders in humans is undeniable. Therefore, a comprehensive recognition of neuropsychiatric diseases such as anxiety can be a big step toward discovering effective therapeutic approaches for patients (Hollander et al. 2020). Accordingly, the use of animal models appropriate to the evaluation objectives of such diseases can be of great help in neuroscience studies. Over the past years, in the light of preclinical studies development, it has become evident that zebrafish can be an appropriate model for examining psychological and brain-related diseases by providing some mammalian-like neurotransmitters and hormones (Cachat et al. 2011). It has gradually become clear that anxiety-like behavior, same as rodents, can be induced in zebrafish, which is possible by applying the almost same stimuli (Stewart et al. 2012). For this purpose, due to the aquatic nature of zebrafish, the novel tank test has performed the suitable conditions almost similar to the open-field test apparatus to measure the anxiety-like behavior responses to novel environments. The novel tank test, in combination with video-aided analysis, can provide accurate facts about how zebrafish behave in the face of the new environment. For instance, examination of time and distance traveled by fish, behavioral reactions, and physical measurement of zebrafish movement velocity in different directions can be obtained from the videos recorded (Cachat et al. 2011). In addition to the novel tank test, which is intended to evaluate the animal's reactions against novel environmental conditions, exposure to alarm pheromones can also be an effective environmental factor in anxious behavior modeling. In a simple definition, alarm pheromones (e.g., hypoxanthine 3-N-oxide and Phoxinus phoxinus) are a group of chemical compounds that are released

into aquatic habitat as a result of injuries to the zebrafish body. Accordingly, the release of alarm pheromones into the surrounding water can trigger a risk response in the body of other zebrafish by stimulating their chemical receptors. Hence, anxiety-like behaviors such as prolonged immobility and irregular movements are displayed (Cachat et al. 2011; Speedie and Gerlai 2008). Another environmental factor that can have a major contribution to the study of emotional responses, such as fear on zebrafish, is the presence of a predator. Initially, it is worth mentioning that zebrafish can distinguish between a natural predator and a predator that does not belong to their habitat. Nevertheless, elevated levels of neuroendocrine responses, such as cortisol in the body of the zebrafish as a hormone involved in fight-or-flight responses, are among the available clues that confirm the emergence of fear towards the predator. Additionally, zebrafish display some behaviors (either instinctively or as a result of training), such as erratic movements or moving away from the predator due to fear responses. Therefore, by analyzing antipredator behaviors, researchers can take fundamental steps to examine various aspects of emotional reactions such as fear (Cachat et al. 2011).

4.2 Chemicals

The effects of chemical compounds evaluated in preclinical studies can lead to molecular, biochemical, and subsequently phenotypic changes in the body of an in vivo model such as zebrafish. In some cases, these changes can lead to the appearance of symptoms of various diseases. Accordingly, some chemical compounds can provide opportunities to establish a suitable model to investigate the effects of other candidate drugs on the target diseases (Table 1) (Mussulini et al. 2013b; Anichtchik et al. 2004; Fénero et al. 2016).

4.3 Approaches for Genetic Manipulation

In recent years, zebrafish have found a privileged position in human disease pathophysiology researches and subsequently in accelerating and facilitating the assessment of different drug compounds' effects on a living organism (Miyawaki 2020). The high percentage of gene homology with humans, easy genetic manipulability, body optical transparency, and high-fecundity are some of the main reasons to draw the attention of researchers to the production of genetically engineered zebrafish by employing extensive spectrum of genome engineering approaches (Rafferty and Quinn 2018). To acquire more knowledge in this field, some of the common approaches for zebrafish genome targeting will be outlined in the following.

4.3.1 N-Ethyl-N-Nitrosourea (ENU)

ENU, as a chemical compound with high mutagenic potential, aims at random mutagenesis in the target genome (DE Bruijn et al. 2009). One of the common ENU applications in zebrafish animal model is neoplasia induction and cancer researches (Beckwith et al. 2000). Although the ENU is considered as one of the effective factors in the forward genetic to identify the genetic causes of a particular abnormal phenotypic manifestation, ENU-induced mutations can be used as one of the genetic tools in the reverse genetic approaches to examine the particular phenotypes which have been caused by changes in the function of a specific gene (Varshney and Burgess 2014). To facilitate the identification process of induced-point mutations on the target gene, targeting induced local lesions in genomes (TILLING) techniques can be applied, which has a great contribution to the reverse genetics. In this method, applying restriction enzymes such as Cel1 and different polymerase chain reaction (PCR) methods can pave the way for detecting the

Table 1 Modeling human diseases by chemical exposure

Compounds	Disease-induced	Clinical signs and phenotypes in zebrafish	References
Soluble PTZ	Epilepsy	Fall to the bottom of the tank Loss of body posture Irregular movement Behaviors similar to the tonic seizure process High rate of swimming Rapid opercular movement Behaviors similar to the clonic seizure process High speed swimming Snake-like swimming Death	Mussulini et al. (2013a)
6-OHDA	PD	Alteration in the locomotor behaviors Reduction in dopamine and noradrenaline levels Complete or partial loss of nigral dopaminergic neurons Increase of *tnfα, il1β,* and *il10* transcripts	Anichtchik et al. (2004) and Soto-Otero et al. (2000)
MPTP	PD	Alteration in the locomotor behaviors Reduction in dopamine and noradrenaline levels	Anichtchik et al. (2004)
LPS	Intestinal inflammatory	High expression of *tnfa* High neutrophil recruitment	Marjoram and Bagnat (2015)
0.2% Oxazolone/ 50% ethanol	Intestinal inflammatory	Bowel-wall thickening Elimination of intestinal folds Goblet cells destruction Penetration of granulocytes, eosinophils, macrophages and lymphocytes into the intestinal tissue	Fénero et al. (2016)
TNBS in a 30% ethanol solution	Intestinal inflammatory	Ulcerations, swelling, thickening, and separation of villi in epithelial tissue in adult type Increase of *IL1β, IL8* and *IL10* transcripts in adult type Impaired epithelial integrity in adult type Decreased survival rate in relation to dose in both adult and larvae Expansion of intestinal lumen in larvae Villi destruction in larvae Increase of *IL1β, TNFα, IL8*, and *MMP9* in larvae Penetration of myeloid cells into larvae intestinal tissue High TNFα expression in the larvae intestinal lumen	Fénero et al. (2016)
DSS	Intestinal inflammatory	Representation of mucus production activity without any alteration in goblet cell rates Increase in intestine-infiltrating neutrophils count and the levels of *ccl20, il1 β, il23, il8, mmp9,* and *tnf α* transcripts in larvae Reduction in the *pcna* gene in larva	Fénero et al. (2016)
Glafenine	Intestinal inflammatory	Endoplasmic reticulum stress in epithelial cells Emergence of apoptotic phenotype in epithelium cells	Fénero et al. (2016)
Ethanol	ALD	Abnormal body curvature Hepatomegaly Transformation of behavior Turning on the activity of stellate cells in prolonged exposure process Liver toxicity in prolonged exposure process Increase in ROS level Increase in the expression of endoplasmic reticulum	Goessling and Sadler (2015)

(continued)

Table 1 (continued)

Compounds	Disease-induced	Clinical signs and phenotypes in zebrafish	References
		stress genes Increase in oxidative stress Reduction in one of the markers associated with the process of liver cells secretions Steatosis	
Acetaminophen	DILI	Increase in the serum levels of aminotransferase Histological alterations Necrosis Hemorrhage Death	Goessling and Sadler (2015)
Prednisolone-21phosphate	Osteoporosis	Small and irregular shape in regenerated scales of prednisolone exposed fish A three-dimensional figure whose plane sections are ellipses or circles in scales of some treated fish Scales with irregular perimeter in some treated fish More irregularly shaped scales and even fusion of two scales in fish just treated with prednisolone Presence large amount of calcium phosphate minerals in scales of prednisolone treated fish Reduction in transcriptions of glucocorticoid receptor-α Help in staining by increase in TRACP activity Increase in osteoclast activity Increase in matrix resorption Change in crystal maturation	de Vrieze et al. (2014)
Ferric chloride	Thrombosis	Injuries in the tail caudal vessels	Jagadeeswaran et al. (2016)
Phenylhydrazine	Thrombosis	Flippase activation Placing phosphatidylserine on the outer surface of red blood cells and platelets Adherence of thrombocytes to the endothelial surface Blockage of vessel in the caudal part	Jagadeeswaran et al. (2016)
Laser-induced venous thrombosis	Thrombosis	Crescent-shaped fibrin formation from the vascular endothelial surface toward the lumen	Jagadeeswaran et al. (2016)
Human amyloid light-chain proteins	Cardiomyopathy	Edema in pericardium Cardiac dysfunction Myocardial cells death	Asnani and Peterson (2014)
Doxorubicin	Cardiomyopathy	Decrease in myocardial and endocardial volumes Change in the structure or form of ventricle Ventricle regeneration Hypertrophy in myocardial cells Shutdown of cell proliferation activity	Dvornikov et al. (2018)
Streptozotocin	DM	Fasting glucose levels enhancement An excess of glucose in the bloodstream Reduction in insulin levels Increase the thickness of glomerular basement membrane	Heckler and Kroll (2017)
Gentamicin	AKI	Flattening of the epithelium of the distal tubules Increased tubular and glomerular diameter Lysosomal phospholipidosis Debris development in the nephron lumen White blood cells accumulation High renal dysfunction Pericardial effusion	Poureetezadi and Wingert (2016)

(continued)

Table 1 (continued)

Compounds	Disease-induced	Clinical signs and phenotypes in zebrafish	References
Cisplatin	AKI	Elimination and flattening of the tubular epithelium brush borders The formation of vacuoles or vacuole-like structures in the cells Nephron tubule distention High renal dysfunction Pericardial effusion	Poureetezadi and Wingert (2016)

Abbreviation: *6-OHDA* 6-hydroxydopamine, *AKI* Acute kidney injury, *ALD* Alcoholic liver disease, *DSS* Dextran Sulfate Sodium, *DILI* Drug-induced liver injury, *DM* Diabetes mellitus, *LPS* Lipopolysaccharide, *MPTP* 1-methyl 4-phenyl-1,2,3,6-tetrahydropyridine, *pcna* proliferating cell nuclear antigen, *PD* Parkinson's disease, *PTZ* Pentylenetetrazole, *ROS* Reactive oxygen species, *TNBS* 2,4,6-trinitrobenzenesulfonic acid, *TRACP* Tartrateresistant acid phosphatase

mutations created in zebrafish by a mutagen such as ENU (Moens et al. 2008). For instance, identifying the *SOD1* mutated gene that causes amyotrophic lateral sclerosis (ALS) is one of the applications of this reverse genetic approach in zebrafish (DA Costa et al. 2014).

4.3.2 Insertional Mutagenesis

Insertional mutagenesis is recognized as one of random mutations procedures, in which target DNA can be manipulated by accommodating additional base pairs. Although insertional mutagenesis is not as effective as mutagenesis by ENU, a comparison of these two methods reveals that the exogenous DNA injection procedure may also be superior to the ENU in the process of mutagenesis. For instance, the determination of the mutated genes induced by ENU faces many challenges and requires gene extensive sequencing methods such as whole-genome sequencing to trace the random gene disruptions (Varshney and Burgess 2014). Rather, the implementation of an exogenous DNA in the target, with its molecular tagging capability in which is empowered by some markers such as green fluorescent protein (GFP), allows tracing mutated genes with fewer constraints in comparison to the ENU method (Varshney and Burgess 2014) by novel high-throughput sequencing (Varshney and Burgess 2014). To promote mutagenesis based on the insertional method, the retroviruses (particularly pseudotyped retroviral agents) (Sivasubbu et al. 2007) and transposable elements are selected as the suitable platforms, which are marked with traceable labels. These insertion factors can represent a different range of effects on the targeted gene depending on the type of transposable elements and their insertion site into the gene. The effects of this mutagenesis method can be in the form of destruction of gene functionality (null allele) or partial loss of function (hypomorphic allele). In some cases, these agents are not destructive and do not alter the function of the gene (Varshney and Burgess 2014). Moreover, it is worth mentioning that pseudotyped retrovirus (Suster et al. 2009) and transposons, in addition to their important role in insertional mutagenesis, can also be categorized as transgenic agents in animal modeling, which will be discussed later in detail in the transgenic section (Varshney and Burgess 2014).

4.3.3 Transgenic Methods

The creation of a transgenic organism is essentially based on the insertion of one or more DNA sequences into the genome content of the host organism, which is typically applicable in the laboratory environment through certain methods. In recent years, the use of transgenic zebrafish has been able to occupy an important place to unravel the mechanisms underlying various diseases. In fact, the existence of outstanding features such as the short reproductive cycle with a large number of offspring in each generation can make zebrafish suitable as a genetically engineered organism (Chen et al. 2016). In addition, since the identification of genes transferred into the host organism is based on the expression of marker

labels (e.g., GFPs), an organism such as zebrafish, with its body transparency feature, can facilitate and accelerate the tracking of these labels in experimental researches (Higashijima 2008). Therefore, in recent decades and in accordance with these outstanding features of zebrafish, many efforts have been made to produce transgenic zebrafish lines to promote the accurate evaluation of toxological effects of candidate drugs in different organs (Lee et al. 2021; Poon et al. 2017). In order to advance a transgenic study, there are different exogenous DNA sequences that can be injected and transferred into the genome content of zebrafish to produce a genetically engineered fish. For instance, in vivo delivery of linear and purified plasmid is one of the transgenic approaches which is able to produce the target protein in the zebrafish body (Suster et al. 2009) with the cooperation of CMV promoter. Studies on transgenic zebrafish with such naked DNA have indicated that fluorescent labels and, as a result, products of the inserted gene show a mosaic expression pattern in the zebrafish. Therefore, it can be concluded that the method of using an expression plasmid is more preferable to study the various functional aspects of cells on a small scale within zebrafish body (Sassen and Köster 2015). In addition to plasmids, transposons, especially *Tol2* transposable element, have a major contribution in transgenic zebrafish development line. Insertion of *Tol2* transposable elements is mainly accomplished in collaboration with a variety of vectors that can facilitate and accelerate the integration of the desired gene into the host's genomic content (Suster et al. 2009). Additionally, *Tol1* and *Sleeping Beauty* are some of the other examples of transposons which are common in zebrafish genetic modification process. Detailed studies of zebrafish cells combined with transposons indicate that the use of transposons for tissue-limited studies may be appropriate (Sassen and Köster 2015).

4.3.4 Morpholino

Morpholinos are also known as novel mutagenic techniques that can speed up the emergence of phenotypic effects by injection into zebrafish emberyos. Morpholinos are antisense oligonucleotides that can make it possible to evaluate phenotypic changes in the early stages of zebrafish growth (Corey and Abrams 2001) by their translation-blocking and splice-blocking function during gene expression. The Morpholino splice-blocking function is intended to inhibit the pre-mRNA processing by inhibiting the spliceosome components. Also, in the translation-blocking process, Morpholino prevents the ribosomal components assembly. In in vivo studies, Morpholino can play an important role in discovering genes on a large scale, corroborating candidate gene function, and evaluating mutant phenotypes (Bill et al. 2009; Santoriello and Zon 2012). Nevertheless, the Morpholino-induced mutations are transient and cannot be passed on to subsequent generations. Eventually, this is one of the major drawbacks in using this genetic approach (Timme-Laragy et al. 2012). Inactivating the gene encoding dystroglycan by Morpholino is a good example of using this antisense knockdown tool to design zebrafish as an animal model for muscular dystrophy disease (Rubinstein 2003). Additionally, attention-deficit/hyperactivity disorder (ADHD) (Norton 2013), PD (Xi et al. 2011; Bretaud et al. 2004), and epilepsy are some of CNS disorders that can be inoculated by Morpholino in zebrafish (Zhang et al. 2015).

4.3.5 RNAi (RNA Interference) and Capped Messenger RNA (mRNA)

In line with other mutagenesis methods in zebrafish, procedures such as the injection of RNAi and capped mRNA can be considered as creative techniques, which can lead to gene silencing and over-increasing gene products, respectively. Therefore, they can ultimately lead to the emergence of phenotypes that result from alterations in the target gene function. Regarding capped mRNA, since it is injected into the body of the zebrafish in the early stages of embryonic formation, it has only a short-term effect on the zebrafish embryo along with general gene expression in all cells. Therefore, injection of capped mRNA is beneficial for studies associated with embryonic stages. It should also be noted that, in some cases, this method is employed in parallel

with the method of injecting transposons such as *Tol2* into the body of the zebrafish, which increases the possibility of transmitting the manipulated gene to the next generation (Sassen and Köster 2015).

4.3.6 Zinc Finger Nuclease (ZFN)

With the advancement of technology and the emergence of novel methods, a variety of genome engineering approaches have been considered in biological researches. ZFN is one of these approaches which is applied to create mutant zebrafish. In general, the structure of a ZFN comprises two main parts, which include a DNA-binding finger domain and a Fok1 restriction endonuclease. To initiate ZFN function and create double-strand breaks in the target DNA, two ZFNs need to be attached to the target DNA and nuclease domains cooperate as a dimer (Woods and Schier 2008). In this process, the Zinc finger protein (ZFP) has the task of identifying specific sequences of double-stranded DNA. Subsequently, the nuclease domains cleave the DNA (Meng et al. 2008). One of the advantages of ZFN is that in contrast to the function of restriction enzymes, there is no need for palindromic sequences to DNA cleavage (Leong et al. 2011). one of the examples in creating mutant zebrafish is the mutation in the *clrn1* gene, which ultimately leads to Usher syndrome type III (Zheng et al. 2018). Additionally, non-alcoholic liver disease (NAFLD) can also be inoculated by ZFN (Asaoka et al. 2013).

4.3.7 Transcription Activator-like Effector Nucleases (TALENs)

TALENs approach is also known as one of the gene-editing approaches that can lead to DNA break in both strands of targeted DNA by its protein-DNA binding interaction like ZFN. Structurally, TALENs consist of a transcription activator-like effector (TALE) and a DNA nuclease domain that the combination of these two parts can ultimately result in the target DNA cleavage at a specific region (Hwang et al. 2014). it should also be noted that the shear activity of DNA nuclease domains in TALENs is possible due to dimer cooperation same as in ZFN (Sertori et al. 2016). Studies indicate that the TALE protein consists of three domains, including a translocation domain at the amino-terminus, a DNA binding domain at the center of the sequence, and a transcriptional activation domain at the carboxyl-terminus (Auer and DEL Bene 2014). Additionally, the DNA binding domain of TALE protein mainly comprises multiple repeats of 33–35 amino acids with variable residues in 12 and 13 positions (Hwang et al. 2014). Accordingly, the type of these variable amino acids in the domains is among the main factors in the specificity of the TALENs function (Auer and DEL Bene 2014). One of the advantages of this bioengineering tool is that, unlike morpholino, TALENs-induced mutations can descend into to the next generation (Hwang et al. 2014). In addition, compared to the ZFN, not only the TALENs target binding sequence is longer (Sertori et al. 2016), but also the function of TALENs is ultimately associated with less cytotoxicity (Mussolino et al. 2011). One of the applications of the TALENs approach is in modeling human tumors, such as brain tumors by the inactivation of *rb1* tumor suppressor gene (Solin et al. 2015). Additionally, mutant models of NAFLD can be developed by TALENs in zebrafish (Asaoka et al. 2013).

4.3.8 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)/CRISPR-Associated Protein 9 (Cas9)

In recent years, another approach of genetic engineering called CRISPR has been widely applied to manipulate the targeted genes and create mutant zebrafish, likewise the TALENs and ZFN gene-editing approaches. The common CRISPR bioengineering tool consists of a single guide RNA (sgRNA) and a Cas9 protein that is responsible for identifying and cleaving the target sequence near the protospacer adjacent motif (PAM), respectively (Liu et al. 2017; Chusilp et al. 2020). Similar to the other gene-editing approaches, the induced mutations by CRISPR / Cas9 can be passed down to offspring (Liu et al. 2017). By contrast, CRISPR is more efficacious, low-cost, and user-friendly than the previous two

gene-editing approaches. Additionally, much labor requirement for designing and constructing the ZFN and TALENs is one of the significant drawbacks of these genome-engineering approaches in comparison to the CRISPR. Eventually, in contrast to the nuclease enzymes used in ZFN and TALENs approaches, Cas9 did not need to be reconstructed for each target sequence (Chusilp et al. 2020). One of the applications of the CRISPR /Cas9 mutagenesis approach is the inoculation of autism-like behaviors (Liu et al. 2018) and intestinal dysmotility related to autism spectrum disorders (ASD) by inducing mutation in the *shank3* gene (James et al. 2019). Furthermore, modeling skeletal diseases (Wu et al. 2019), pyridoxine-dependent epilepsy (PDE) (Zabinyakov et al. 2017), Diabetes mellitus (DM) (Heckler and Kroll 2017), and anxiety-like behavior are examples of applying CRISPR/Cas9 for human disease modeling in zebrafish (Fig. 1) (Wang et al. 2016).

4.4 Bacterial Pathogenesis

In the past few years, zebrafish embryos have been used as an appropriate model in many laboratory studies to investigate the effects of various pathogens on living organisms (Benard et al. 2012). In light of previous studies, it is conceivable that there are 82% disease-related orthologous genes between humans and zebrafish (Howe et al. 2013). Additionally, immunological studies indicate that, like mammals, zebrafish have an innate or nonspecific immune system whose function depends on the role of macrophage and neutrophil phagocytic cells (Torraca and Mostowy 2018). Today, zebrafish can provide wealth of information about how pathogens act in a physiological platform within a vertebrate, due to its prominent features such as a transparent body. In addition, the possibility of implementing genetic engineering and even tracing zebrafish genome by advanced screening and imaging techniques can be a big step toward finding therapeutic approaches to combat human infections (Gomes and Mostowy 2020; van der Vaart et al. 2012).

4.4.1 Mycobacterium Marinum

Tuberculosis (TB) is known as one of the top causes of death in the humans. The causative agent of TB is a bacterium called *Mycobacterium tuberculosis* that is categorized as an infectious agent and transmitted by airborne from person to person (Kaufmann et al. 2017). In recent years, animal models such as rodents, insects, cattle, sheep, pigs, goats, dogs, and non-human primates were common to use in pulmonary infections studies and the evaluation of therapeutic options. Despite the phylogenetic similarities, these animal models are not appropriate for complete coverage of all aspects of human lung diseases and infections. Therefore, zebrafish could have a superior position in the study of these infections based on the features described in the preceding sections (López Hernández et al. 2015). It must be mentioned that zebrafish do not possess lungs. Instead, their gills perform similar respiratory functions as that of the lungs and also possess the same tissues as compared to the pulmonary system in humans (Progatzky et al. 2016). *Mycobacterium marinum,* as a natural zebrafish pathogen, can cause infections similar to human TB in zebrafish (Gomes and Mostowy 2020). Zebrafish's gastrointestinal tract is the natural procedure to enter the causative agent of TB, but intraperitoneal or intramuscular injection in adults and caudal vein injection in embryos can also be used to cause infection. Furthermore, yolk injection in the early stages of development can lead to early infections (van Leeuwen et al. 2015). In order to fight the infections caused by *Mycobacterium marinum*, Rifampicin is known as an effective antibiotic drug in zebrafish, which is useful against some detrimental effects of *Mycobacterium marinum*, when it is loaded by nanoparticles (NPs). This antibiotic reduces bacterial load and also enhances the survival of the embryo (Fenaroli et al. 2014).

4.4.2 Shigella Flexneri

Shigella flexneri is a strain of *Escherichia coli (E. coli)* with the similar characteristics that can normally enter through the fecal-oral route and causes shigellosis in humans by making

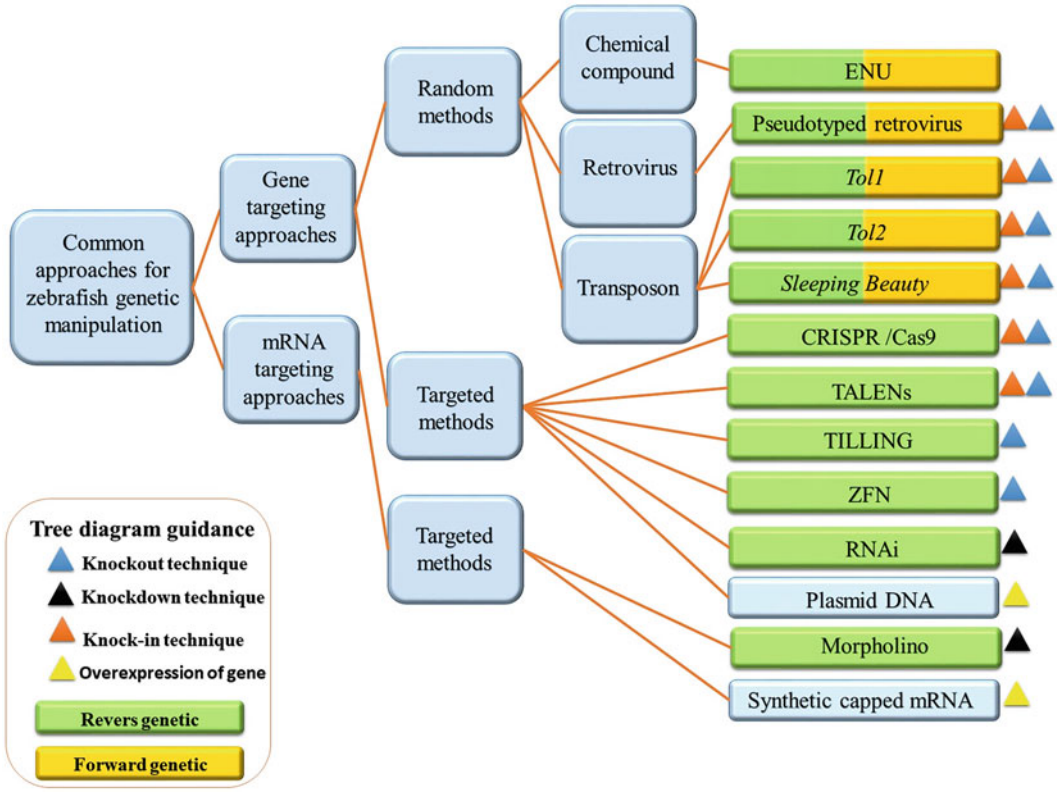

Fig. 1 Genetic manipulation approaches in zebrafish modeling. The common approaches for zebrafish genetic manipulation are divided into two approaches: Gene and mRNA targeting. Gene targeting approaches can be divided into two methods including random and targeted methods. Random methods include chemical compounds, retroviruses, and transposons. ENU is a chemical compound that is used in both forward and reverse genetic approaches. Pseudotyped retrovirus is one of the most common retroviruses in generating transgenic zebrafish, which is used as knockout and knock-in techniques in both forward and reverse genetic approaches. The *Tol1, Tol2*, and *Sleeping Beauty* are three transposons, which are used as knockout and knock-in techniques in both forward and reverse genetic approaches. Targeted methods in gene targeting approaches can be divided into six methods: CRISPR /Cas9, TALENs, ZFN, TILLING, plasmid DNA, and RNAi. CRISPR /Cas9 and TALENs are knockout and knock-in techniques in reverse genetic approach. TILLING and ZFN are also knockout techniques in reverse genetic approach. Plasmid DNA is used for overexpressing the gene of desired. RNAi is a knockdown technique in reverse genetic approach. The mRNA targeting approaches include targeted methods that are divided into two Morpholino and synthetic capped mRNA. Morpholino is a knockdown technique in reverse genetic approach. Synthetic capped is used for overexpressing the gene of desired. Forward genetic is related to the identification of the genetic causes of a particular abnormal phenotypic manifestation. Reverse genetic indicates the particular phenotypes which have been caused by changes in the function of a specific gene. Gene knockout refers to a genetic method in which a gene will be deactivated. Gene knock-in refers to a set of techniques in which the insertion of sequence occurs or in which homologous recombination occurs to replace the target gene. Gene knockdown technique leads to the reduction in the expression of the targeted gene (Haiyong 2018; Auer and DEL Bene 2014; Skromne and Prince 2008; Kawakami et al. 2017; Lawson and Wolfe 2011; Sassen and Köster 2015; Sivasubbu et al. 2007; Woods and Schier 2008; Sood et al. 2006; Stainier et al. 2017; Raby et al. 2020; Varshney and Burgess 2014; Andrews et al. 2014; Suster et al. 2011; Cornet et al. 2018; Argmann et al. 2006; Sawitzke et al. 2013; Roebroek et al. 2011). Abbreviation: *ENU* N-Ethyl-N-nitrosourea, *EMS* ethyl methanesulfonate, *TALENs* Transcription activator-like effector nucleases, *ZFN* Zinc-finger nuclease, *TILLING* Targeting induced local lesions in genomes, *CRISPR/Cas9* Clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9), *RNAi* RNA interference

inflammatory destruction of the intestinal epithelium. Recent studies have shown that zebrafish can create an opportunity for in vitro imaging of Shigella infections (Duggan and Mostowy 2018). In some studies, intravenous microinjection of this bacterium near cloaca or urogenital orifice has been used for investigating the pathogenicity of this bacterium in zebrafish larvae (Mostowy et al. 2013). *Shigella flexneri* induced inflammatory response, lowered by a biopharmaceutical drug called Anakinra. As a result, this drug can protect zebrafish from Shigella infection (shigellosis) (Mazon-Moya et al. 2017).

4.4.3 Staphylococcus Aureus

Staphylococcus aureus is a gram-positive bacterium and a human pathogen that can establish life-threatening infections. This pathogen is the main cause of bacteremia, osteoarticular, pneumonia, skin, and soft tissue infections (Tong et al. 2015). Additionally, various bacterial infections such as septic arthritis/sepsis, staphylococcal-related endocarditis, and toxic shock syndrome have been studied with Staphylococcus aureus origin in different animal models (Prajsnar et al. 2008). Recently, in addition to other animal models, zebrafish has also attracted attention as a suitable in vivo model for examining various infections caused by this type of bacterium. In order to advance studies in this field, some researches have mentioned methods like an intravenous injection to wild-type zebrafish larvae (Prajsnar et al. 2012) and automated microinjection system with complex object parametric analysis and sorting (COPAS) technology as the methods used for inducing and investigating *Staphylococcus aureus* infections in zebrafish (Veneman et al. 2013). Producing the extracted compound from *Streptomyces rubrolavendulae ICN3* and its assessment on zebrafish embryo can be a positive step in antibacterial therapy against anti-methicillin-resistant Staphylococcus aureus (MRSA). In this context, the effect of anti-MRSA compound has been examined by monitoring heart rate, enumerating blood cells, and calculating LD50 values of the embryo. All in all, the result of this compound assessment has shown the success of this novel compound with its MRSA inhibition performance (Kannan et al. 2014).

4.4.4 Salmonella Typhimurium

Salmonella typhimurium is known as a gram-negative and intracellular anaerobic bacterium that can induce inflammation in the digestive system by its primary mechanism of virulence (Patel and Mccormick 2014). Some methods have included inoculation of zebrafish in water (Howlader et al. 2016), injection of the bacterium Lipopolysaccharide (LPS) in the yolk zebrafish embryos (van der Sar et al. 2003), and inoculation by microinjection; and static immersion have been used to inoculate *Salmonella typhimurium* infectivity in zebrafish (Varas et al. 2017). Recent studies indicate that a heat-killed immunogen can be a protective agent against *Salmonella typhimurium*-induced infections. This immunogen is applied in different doses with different procedures, such as intraperitoneal injections and bath vaccination. Accordingly, bath vaccination can be more effective in protection; on the other side, intra-peritoneal techniques can virtually have a protective effect (Howlader et al. 2016).

4.4.5 Aeromonas Hydrophila

One of the natural pathogens of zebrafish is *Aeromonas hydrophila*. Aquatic environments such as freshwater are known as the natural habitat of these gram-negative bacteria (Li et al. 2011). Additionally, humans can be exposed to dangerous infections by consuming certain usable products or contacting infected animals (Rowe et al. 2014). In recent years, researchers use zebrafish as an appropriate animal model to study the infectious pathogenicity of the *Aeromonas hydrophila* and also to discover new therapeutic options for dangerous infections caused by this bacterium in humans. Microinjection and tail damage are some of the *Aeromonas hydrophila* inoculation methods which are used in some studies. Water immersion is another method for modeling zebrafish larvae. In this procedure, the bacteria in the water could easily

enter the body by mouth (Saraceni et al. 2016). Studies show that one of the functional treatment options that can affect the *Aeromonas hydrophila* in zebrafish is flumequine which can lessen the numbers of this bacteria, so in this respect, this drug can have a positive effect on the functioning of the immune system against the Aeromonas clinical infections, while drugs like tetracycline, trimethoprim, and sulphonamide are some ineffective therapeutants of *Aeromonas hydrophila* bacteria (Cantas et al. 2012). In line with the ideas of bacterial pathogenesis, it can be concluded that bacterial diseases modeling allows researchers to study the behavior and function of pathogens like bacteria in the body of the zebrafish as a preclinical animal model. This will be a step toward making vaccines or producing drugs to treat bacterial diseases in humans (Bailone et al. 2020).

5 Resultant Drugs from Disease Modeling on Zebrafish

Drug discovery is a promising platform with multi-scale approaches that have a considerable role in the field of human disease researches and therapies, especially when it is going to apply in preclinical phases through drug screening in zebrafish (Rubinstein 2003; Guo 2009). In conformity with zebrafish's unique superiorities, this animal has become an operational and even cost-effective model in the pharmaceutical and therapeutic fields of human diseases (Guo 2009; Rubinstein 2003; Das et al. 2013; Collier et al. 2014). Accordingly, by converting zebrafish into an animal model of human diseases, the opportunity for prescreening various drugs is created to realize whether they can potentially be used for human therapeutic purposes or not (Menke et al. 2011; Gut et al. 2017). In the following, there are some explanations and assessments which depict the efficacy of drug discovery in various human diseases (Table 2).

6 Conclusion and Future Perspective

Preclinical studies are one of the important processes of drug discovery and development platforms, intending to achieve new drugs with more effective therapeutic potential that rely mainly on the study of candidate drugs in the body of a living organism (Deore et al. 2019; Aghayan et al. 2021; Arjmand et al. 2021a; Arjmand et al. 2021b). Due to the necessity of efficient and appropriate animal models with different aspects of the preclinical stage, zebrafish has gained increasing interest over the last decades as a suitable vertebrate model with a high degree of genetic and clinical similarities with human beings (Zon and Peterson 2005). Despite some considerable differences between zebrafish and humans, such as the absence of breast tissue, prostate, and lung, zebrafish still represents many prominent features appropriate with drug discovery and development purposes. It is therefore tempting to apply zebrafish as a suitable preclinical model in many scientific types of research (Delvecchio et al. 2011). Although preclinical testing can be a key step in the development of new drugs and therapeutic strategies for human diseases such as determining the appropriate dosage and the preferred route of drug administration (Mohamadi-Jahani et al. 2020), the use of animals in preclinical studies, especially in the field of drug testing and toxicity evaluation, is still a major challenge. Particularly, in some cases, the results of a drug investigation in preclinical studies may not be the same as those in clinical trials. Therefore, this will lead researchers to step back and change their strategy to discover more compatible drugs with human diseases. Additionally, in recent years, by examining the public opinions regarding the use of animals as a laboratory platform for testing a variety of chemicals, it can be comprehended how animal experiments have become less acceptable. However, some people are in agreement with scientists

Table 2 Drugs and chemical compounds discovered through preclinical studies on zebrafish and their pharmaceutical functions

Drugs / Chemical compounds	Examples of disease	Pharmaceutical functions	References
NAC	Depression and anxiety	An antidote for paracetamol overdose Preventing stress Protection against the oxidative balance relevant to anxiety	Khan et al. (2017)
MEK inhibitors	Depression and anxiety	Lessen the cAMP -induced anxiety-like response	Khan et al. (2017)
Vasodilators	Epilepsy	Antiepileptic drug Reduction in PTZ-induced epilepsy seizures	Khan et al. (2017)
Antioxidants	Epilepsy	Antiepileptic drug Reduction in PTZ-induced epilepsy seizures	Khan et al. (2017)
Antipsychotic-related drugs	Psychosis	Treatment of impaired pre-pulse inhibition in schizophrenia	Khan et al. (2017)
Quercetin	AD	Pretreatment of scopolamine-induced-memory impairment	Khan et al. (2017)
Rutin	AD	Pretreatment of scopolamine-induced-memory impairment	Khan et al. (2017)
Physostigmine	AD	Amelioration of scopolamine-induced memory impairment	Khan et al. (2017)
Fatty acid amide hydrolase inhibitor	ALS	Inhibiting the BMAA-induced heart rate reduction	Khan et al. (2017) and Frøyset et al. (2016)
Bromazepam	UCS	Anxiolytic drug Preventing UCS-induced symptoms	Marcon et al. (2016)
Fluoxetine	UCS	Antidepressant drug Preventing UCS-induced symptoms	Marcon et al. (2016)
Nortriptyline	UCS	Antidepressant drug Preventing UCS-induced symptoms	Marcon et al. (2016)
Apomorphine	PD	Dopaminergic agonist Preventing MPP+ − induced symptoms	Pinho et al. (2016)
Antioxidants	Cardiotoxicity	Inhibiting destructive effects of chemotherapeutical agents on cardiovascular system	Zakaria et al. (2018)
Endothelial- or cardio myocyte-protective compound	Cardiotoxicity	Inhibiting destructive effects of chemotherapeutical agents on cardiovascular system	Zakaria et al. (2018)
Rapamycin	Cardiomyopathy	Inhibition in TOR signaling	Kushwaha and Xu (2012)
Human antithrombotic drugs (e.g., injections of aspirin, clopidogrel, diltiazem hydrochloride, xuanshuantong, salvianolate, astragalus)	Thrombosis	Preventive and therapeutic effect on disease Effective in the prevention of ADP-induced blood clots	Zhu et al. (2016) and Rubinstein (2003)

(continued)

Table 2 (continued)

Drugs / Chemical compounds	Examples of disease	Pharmaceutical functions	References
Calycosin	Myocardial ischemia	Post-treatment of VRI-induced vascular blood vessel loss Post-treatment of VRI-induced cytotoxicity and apoptosis in HUVEC	Zhou et al. (2014)
Antibiotics (e.g., ampicillin and kanamycin)	IBD	Diminishing TNBS-induced side effects Maintaining larvae from TNBS-related mortality	Oehlers et al. (2011)
5-ASA	IBD	Anti-inflammatory drug Diminishing TNBS-induced side effects	Oehlers et al. (2011)
Prednisone	IBD	Anti-inflammatory drug Diminishing TNBS-induced side effects	Oehlers et al. (2011)
Nitric oxide synthase (NOS) inhibitors	IBD	Anti-inflammatory effects Remission and prevention of IBD by rescuing in vivo disease phenotypes	Fleming et al. (2010)
Thalidomide andParthenolide	IBD	Failed to rescuing in vivo disease phenotypes Downregulation of TNF-α	Fleming et al. (2010)
Rhein	Ulcerative colitis	Reduction in the tail cutting-induced migration of immune cells Inhibition of some inflammasome factors Attenuate inflammations Decreased the levels of IL-6, IL-1β, and TNF-α	Ge et al. (2017)
Vancomycin	Oxazolone-induced enterocolitis	Decrease in enter colitis scores and intestinal damage Decrease in percentages of infiltrating neutrophils and eosinophils Intestinal damage	Brugman et al. (2009)
Colistin sulphate	Oxazolone-induced enterocolitis	No reduction in enter colitis scores Reduction in eosinophil and lymphocyte infiltration	Brugman et al. (2009)
Prednisolone + alendronate	GIOP	Increase in alkaline phosphatase and up-regulate the cAMP Prevention abnormal muscle structure in dystrophin-null fish Reduction in tartrate-resistant acid phosphatase	Pasqualetti et al. (2015)
GAS	NAFLD	Anti NAFLD Weight losing Lipid regulation effect Suppression of oxidant attack	Ahmad et al. (2019)
Metformin	DM	Type 2 DM treatment Improvement of hyperglycemia	Heckler and Kroll (2017)
Glimepiride	DM	Type 2 DM treatment Improvement of hyperglycemia	Heckler and Kroll (2017)

(continued)

Table 2 (continued)

Drugs / Chemical compounds	Examples of disease	Pharmaceutical functions	References
Aminoguanidine	DM	Lessen the methylglyoxal-induced vascular damage Prevention of methylglyoxal formation increment Decrease in the inducing improper blood vessel in larvae	Heckler and Kroll (2017)
Aminoglycosides (gentamicin and paromycin)	Human coloboma	Therapeutic effect on zebrafish *lamb* and *pax2* mutants Human coloboma therapy	Chhetri et al. (2014)
Sunitinib	Hypoxia-induced retinopathy	Anti-angiogenesis inhibitor Retina's neovascularization destroyer	(Chhetri et al. 2014)
Small drug-like molecule PROTO1	Hearing loss	Protection against neomycin induced damage	Owens et al. (2009)
Vemurafenib + DMSO	Melanoma	Reduction in tumor burden	Dang et al. (2016)
Prednisone	BA	Reduction in DNA methylation	Matthews et al. (2011)

Abbreviation: *5-ASA* 5-aminosalicylic acid, *AD* Alzheimer's disease, *BA* Biliary atresia, *BMAA* β-methylamine-alanine, *cAMP* Cyclic adenosine monophosphate, *DM* Diabetes mellitus, *GAS* Gastrodin, *GIOP* Glucocorticoid-induced osteoporosis, *HUVEC* Human umbilical vein endothelial cells, *IBD* Inflammatory bowel disease, *NAC* N-acetylcysteine, *NAFLD* Non-alcoholic liver disease, *PD* Parkinson's disease, *PTZ* Pentylenetetrazole, *TNBS* 2,4,6-trinitrobenzenesulfonic acid, *TOR* Target of rapamycin, *UCS* Unpredictable chronic stress, *VRI* VEGFR tyrosine kinase inhibitor II

and still consider the use of animals to be an integral part of toxicity experiments in some cases. Furthermore, the issue of cost and time spent on drug discovery studies confronts many pharmaceutical companies with major problems in the processes (van Norman 2019). Therefore, scientists believe that owing to the rapid technological advances, non-animal models might be replaced by animal models. For instance, due to the enormous impact of computational approaches, some advanced technologies such as artificial intelligence (AI) are maturing rapidly in various fields of biomedical sciences in recent years. AI can be considered a novel and cutting-edge technology which is concerned with simulating human intelligence by computer and smart machine processing. Today, to reduce costs and speed up drug production, AI, along with new technologies, is expected to have a positive impact on human studies. Based on the computational power of AI, scientists believe that AI will revolutionize the drug detection process and make it possible to conduct more accurate clinical trials in the future (Gilvary et al. 2019; Bonde 2019). Also, by focusing on technological aspects of the science world, Lab on a chip (LOC) is a novel in silico model platform with the power of earlier and cost-effective disease diagnosing, which results in advanced health care. In this context, the possibility of modeling diseases on a micro-physiological system by applying different tissue engineering technologies allows recognizing LOC technology as a promising tool in the revolution of the drug discovery and development realm (Azizipour et al. 2020).

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Cellular Stress Responses in Oocytes: Molecular Changes and Clinical Implications

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Abstract

The oocyte may be exposed to several sources of stress during its growth and maturation, which may lead to reduced fertility. Unfolded protein responses (UPRs) play a central role to maintain cell survival and repair. Transcription of heat shock proteins (HSPs) is a key element to facilitate reestablishment of cellular homeostasis. Unlike somatic cells, cellular mechanisms by which oocytes can sense and respond to stress are not well described. In here, we provide an overview about the impact of cellular stress, particularly due to lipotoxicity, oxidative stress, and heat stress on oocyte developmental competence. Next, we focus on the expression of HSPs in oocytes and their potential role in UPRs in oocytes and embryos. This is based on a comprehensive shotgun proteomic analysis of mature bovine oocytes performed in our laboratory, as well as a literature review. The topic is discussed in light of our understanding of similar mechanisms in other cell types and the limited transcriptional activity in oocytes. More fundamental research is needed both at the transcriptomic and proteomic levels to further understand cell stress response mechanisms in oocytes and early developing embryos, their critical interactions, and their long-term effects. Strategies to provide targeted external support to prevent or reduce cell stress levels during oocyte maturation or early embryo development under maternal metabolic stress conditions should be developed to maximize the odds of producing good quality embryos and guarantee optimal viability.

Keywords

Cell stress · Embryos · ER · Heat shock proteins · Mitochondria · Oocytes · Unfolded protein response

Abbreviations

ATF4	activating transcription factor 4
ATF6	activating transcription factor 6
COCs	cumulus oocyte complexes
eIF2α	eukaryotic translation initiation factor 2 alpha
ER	endoplasmic reticulum
ERAD	ER-associated degradation
FFAs	free fatty acids
FSH	follicle-stimulating hormone

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HS	heat stress
HSPs	heat shock proteins
IRE1	inositol-requiring enzyme 1
IVF	in vitro fertilization
MMP	mitochondria membrane potential
NEB	negative energy balance
OPU	ovum pick-up
OS	oxidative stress
PA	palmitic acid
PERK	protein kinase RNA-like endoplasmic reticulum kinase
ROS	reactive oxygen species
SCNT	somatic cell nuclear transfer
UPR^{er}	UPRs induced by the ER
UPR^{mt}	UPRs induced by the mitochondria
UPRs	unfolded protein responses
XBP1	X-box-binding protein 1

1 Introduction

1.1 The Vulnerable Oocyte and the Increasing Prevalence of Infertility

Oocyte quality is a key factor that determines female fertility. Acquisition of oocyte developmental competence is a cumulative process that takes place in the ovarian follicle during oocyte growth and maturation (Fulka et al. 1998; Watson 2007). This involves a sequence of complex cytoplasmic and molecular changes that are essential to make the oocyte fertilizable, ultimately leading to a viable offspring. Any perturbation in the microenvironment of the oocyte within the ovarian follicle has a potential impact on oocyte quality and developmental competence (Krisher 2013; Mermillod et al. 2008), which puts fertility at risk.

Female infertility is a global public health issue. According to recent WHO reports, over 10% of women in reproductive age are infertile or sub-fertile, and the prevalence of infertility could be as high as one in every four couples in some developing countries (Mascarenhas et al. 2012). Reduced fertility is a multifactorial problem; however, more and more research clearly indicate that reduced oocyte quality is a major factor (for a review, see Leroy et al. 2008; Wu et al. 2011). For example, in humans, maternal metabolic syndrome and the concomitant metabolic disorders associated with Western-type diets, obesity, and type 2 diabetes are main causes of subfertility and are strongly linked with reduced oocyte quality. Even when clinical-assisted reproductive services are provided, poor oocyte quality results in disappointing IVF success rates (Pandey et al. 2010).

Disappointing fertility results are not only relevant in human clinical settings. In livestock, fertility results determine the farmer's income, management efficiency, and environmental impact. Reproductive failure in pig and cow farming is now recognized as a main burden and has serious economic consequences. Metabolic stress due to, e.g., negative energy balance (NEB) status, has been strongly correlated with disappointing fertility outcome in modern dairy industry worldwide (Berry et al. 2016). Excessive fat mobilization in NEB cows elevates blood free fatty acid (FFA) concentrations, which results in a state of inflammation and lipotoxicity in several tissues and organs, including the ovary. This lipotoxicity has a direct negative impact on the oocyte. The dairy cow model showed to be an interesting and ethically acceptable model to learn more about the impact of lipotoxicity on oocyte and embryo quality (Leroy et al. 2015). Like in dairy cows, metabolic disturbances in women, associated with a significantly upregulated lipolysis due to reduced insulin sensitivity, obesity, and chronic stress, have been associated with disappointing fertility (Gambineri et al. 2019).

The resulting hyperlipidemia and elevated lipotoxic concentrations of FFAs in blood are reflected in the ovarian follicular fluid and thus can have a direct negative impact on oocyte quality (Leroy et al. 2005; Valckx et al. 2015). In women, high FFA concentrations in follicular fluid were associated with a reduction in nuclear maturation and subsequent embryo cleavage rates (Jungheim et al. 2011). In IVF cycles, oocytes are routinely collected by ovum pick-up (OPU) after hormonal stimulation (i.e., after maturation), and thus oocyte quality might be already

metabolically compromised if the mother suffers from a metabolic stress condition. The phenomenon of impaired developmental capacity of oocytes exposed to a lipotoxic microenvironment has been investigated in a series of studies in our laboratory and by other research groups. Exposing oocytes in vitro during long-term (13 days) murine follicle cultures (Valckx et al. 2014) or short-term (24 h) bovine oocyte maturation (Valckx et al. 2015; Van Hoeck et al. 2011, 2014) to pathophysiologically relevant concentrations of FFA or follicular fluid collected from obese women (Yang et al. 2012) resulted in a lower percentage of good-quality embryos after IVF. In these studies, embryo development to the blastocyst stage was significantly decreased, and higher embryo fragmentation rates were observed. In addition, surviving blastocysts had higher apoptotic cell indices, lower cell numbers, altered energy, and amino acid metabolism and exhibited different methylation and transcription patterns compared to blastocysts derived from healthy oocytes (Desmet et al. 2020; Van Hoeck et al. 2013, 2015). This can lead to a high incidence of early embryonic mortality and low pregnancy rates.

In addition, with the increasing importance of global warming, heat stress (HS) has also been documented to have adverse effects on oocyte quality and subsequent embryonic development (Yin et al. 2019). This is due to the direct impact of heat at the level of the oocyte or due to indirect stress-induced reduction in dry matter intake and the concomitant (exacerbation of) NEB status (Abdelatty et al. 2018). It has been shown that exposure of oocytes to HS either in vivo or in vitro impairs follicular development, reduces cumulus cell expansion and oocyte quality with lower maturation and polar body extrusion rates, and hampers early embryo development (Al-Katanani et al. 2002; Yin et al. 2019). Heat stress also resulted in a lower embryo yield and quality following hormonal stimulation in cows (Benyei et al. 2003).

Although the patterns of development of the pathogenesis of reduced fertility under metabolic and HS conditions may vary, it is commonly accepted that elevated levels of reactive oxygen species (ROS) and reduced mitochondrial functions are key underlying causes at the cellular level (Long et al. 2015). This appears to be also true for oocytes. Oocytes and their surrounding cumulus cells derived from obese mouse models or in vitro-exposed to pathophysiological concentrations of FFAs displayed altered mitochondrial inner membrane potential (MMP) (Fig. 1) and elevated ROS levels (Igosheva et al. 2010; Marei et al. 2012). Similarly, the impact of HS on oocyte quality and early embryo development has been shown to be mainly mediated through oxidative stress (OS), mitochondrial dysfunctions, and apoptosis in oocytes (Li et al. 2015; Tseng et al. 2006) and in cumulus cells (Rispoli et al. 2013; Shaeib et al. 2016).

Cells can sense and respond to increased intracellular stress (e.g., due to lipotoxicity, oxidative damage, or direct effect of heat). These responses are mainly initiated in the endoplasmic reticulum (ER) (Ozcan et al. 2004; Zhang and Kaufman 2008) and the mitochondria (Runkel et al. 2014) due to the accumulation of misfolded or unfolded proteins. Hence, they are called unfolded protein responses (UPRs). A mild degree of stress stimulates transcription of nuclear-encoded chaperones (mainly heat shock proteins (HSPs)) to facilitate repair mechanisms and to reestablish cellular homeostasis as described hereafter. Whereas high stress levels activate programmed cell death (apoptosis) to eliminate damaged cells.

Cellular stress responses have been mainly investigated and described in somatic cells. Our understanding of the mechanisms by which HSPs influence oocytes and embryo developmental capacity under different pathophysiological conditions is still emerging. Recent data from studies investigating the impact of metabolic or heat stress on oocyte and embryo quality and the underlying molecular changes provide a better insight and will be the focus of this chapter.

2 Heat Shock Proteins and Cell Stress Responses

Heat shock proteins (HSPs) are universally expressed across most cell types and within virtually all organisms. They are known to act as

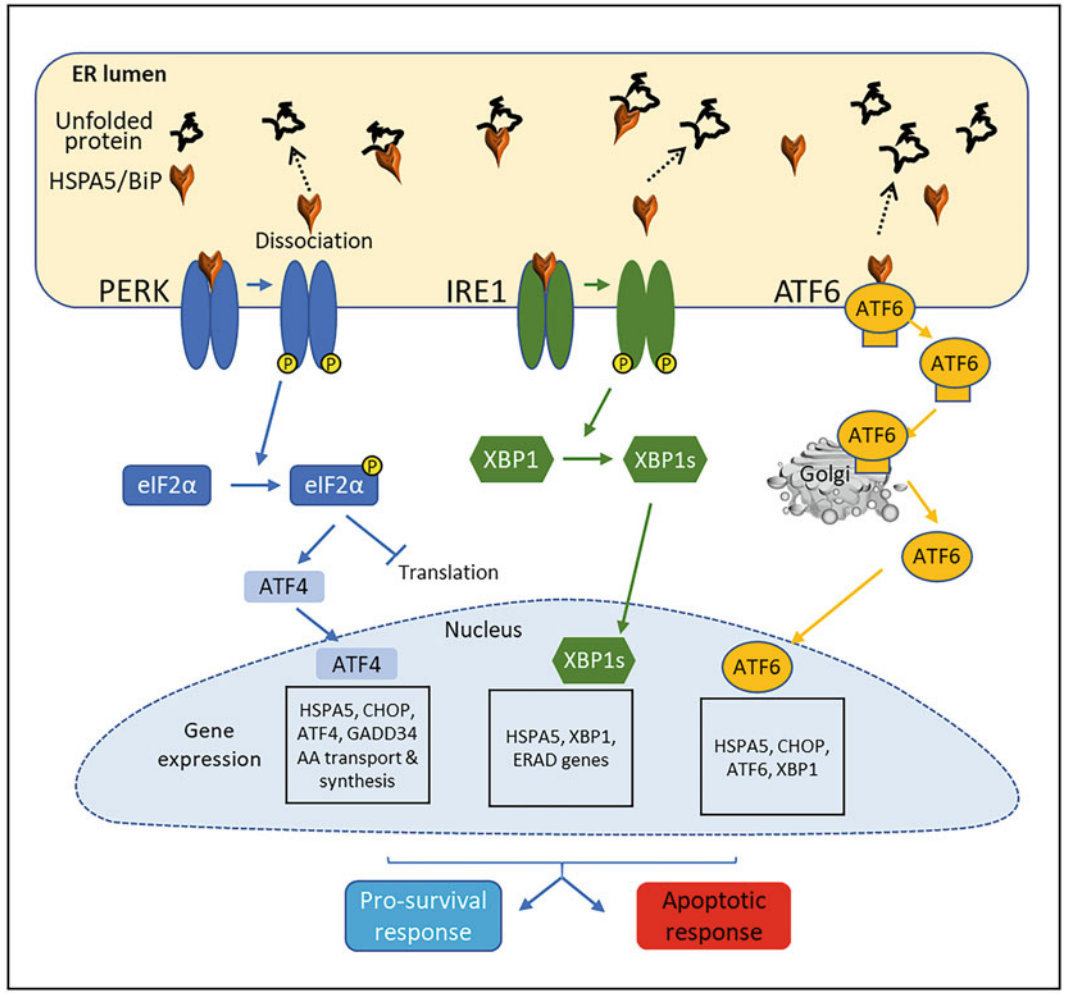

Fig. 1 Unfolded protein responses induced by the endoplasmic reticulum (ER) in response to the accumulation of misfolded proteins in its lumen. Dissociation of HSPA5 from the ER transmembrane sensors PERK, IRE1, and ATF6 results in activation of downstream signaling that stimulates the expression of nuclear-encoded chaperons. According to the level of stress, these mechanisms can lead to reestablishment of cellular homeostasis and cell survival or induction of programmed cell death (apoptosis)

molecular chaperons and are involved in several cellular functions related to protein homeostasis (proteostasis), such as facilitating protein and peptide transport, stabilization of nascent proteins, inhibition of protein aggregation, and mediation of protein complex formation (Becker and Craig 1994). Thus, HSPs are basically responsible for facilitating the biogenesis, stabilization, folding, trafficking, and degradation of proteins within the cell (Nixon et al. 2017).

Members and families of HSPs are classified mainly according to their molecular weight, which varies from 10 kDa (HSP10) to over 100 kDa (e.g., HSP110). Recently, a new nomenclature has been proposed (Kampinga et al. 2009) where HSPs are divided into the following families: HSPH (HSP110), HSPC (HSP90), HSPA (HSP70), DNAJ (HSP40), and HSPB (small HSP), as well as the chaperonin families HSPD (HSP60), HSPE (HSP10), and CCT

(TRiC). Members of the HSPA family are most abundant, with at least 13 gene products characterized in the human proteome (Radons 2016).

The core structural elements of most HSP classes display a considerable degree of conservation, and their expression and molecular functions are interconnected and complementary (Bukau et al. 2006). For example, DNAJ (HSP40) family are known to play a key regulatory role in modulating HSP70 activity, directing substrates to the protein, and focusing this family to discrete subcellular compartments (Kampinga and Craig 2010). The HSP40-HSP70 partnership can subsequently engage HSP90s to assist in further folding or protein degradation pathways (Nixon et al. 2017). This illustrates the importance of estimating, not only the function of individual HSPs but also their network with other HSPs and regulatory molecules that interact within the proteostasis pathways.

Importantly, in addition to the ability of HSPs to enhance the recovery of stressed cells, constitutively expressed HSPs and their chaperoning function appear to be also essential under physiological conditions for the housekeeping roles and for protection against stress (reviewed in Kampinga and Craig 2010; Radons 2016). These maintenance functions include transport of proteins between cellular compartments, folding and refolding of newly synthetized proteins, degradation of unstable and misfolded proteins, prevention and dissolution of protein aggregates, and control of regulatory proteins.

3 Expression and Abundance of HSP in Oocyte and Their Surrounding Somatic Cells

Cell stress responses in oocytes and early developing embryos are expected to be different from those described in somatic cells. This is because growing oocytes are arrested at the prophase of the first meiotic division and are known to be transcriptionally inactive particularly during the final stages of growth and maturation and until embryonic genome activation (Funaya and Aoki 2017). During this phase of transcriptional silence, the oocyte relies on stored mRNA transcribed earlier during oogenesis (Peaston et al. 2010). It has been recently shown that the surrounding cumulus cells may also deliver cargos containing mRNA molecules through the trans-zonal projections, which may contribute to the oocyte's mRNA reserve (Macaulay et al. 2016). As mentioned above, UPRs involve stimulation of de novo expression of nuclear-encoded factors, which may not be feasible at late stages of oocyte maturation and shortly after fertilization due to the transcriptional inactivity. For this reason, studying cell stress responses in oocytes and early embryos can be challenging and may be particularly misleading if investigated at the transcriptomic level. More importantly, investigating these mechanisms in whole cumulus-oocyte complexes (COCs) can be even more misleading due to the marked evident differences between the two cell types. Nevertheless, the transcriptional inactivity per se does not rule out the potential ability of oocytes to respond to cell stress by adaptive changes in translation and protein synthesis.

A comprehensive study performed in our laboratory was the first to look into the molecular changes that occur in metabolically compromised oocytes at the proteomic level as compared to their surrounding layers of somatic (cumulus) cells (Marei et al. 2019c). We used tandem mass tag (TMT) labeling and shotgun mass spectrometry-based analysis to screen the proteomic profile of cumulus cells, and the enclosed denuded oocytes isolated from bovine COCs matured in the presence or absence of pathophysiological, lipotoxic concentration of Palmitic acid (PA). We could identify 1703 and 1185 proteins in cumulus cells and oocytes, respectively, 679 of which were common. The database of this analysis offers a unique insight into the relative abundance of these proteins, their functional annotations, and how they are differentially regulated in response to PA-induced metabolic and oxidative stress (Marei et al. 2019c). Several members of HSP families could be detected in cumulus cells as well as in the oocytes. The relative abundance of

Table 1 The relative abundance (RA) of members of different HSP families that were identified in a shotgun proteomic analysis of bovine denuded oocytes and cumulus cells isolated after in vitro maturation of intact COCs

ID	Description	Localization*	RA in Oocytes**	RA in Cumulus cell**
HSPH1	Heat shock protein 105 kDa	C;N	2,84	0,59
HSP90A	Heat shock protein 90kDa alpha	C;N	2,65	0,97
HSP90AA1	heat shock protein 90 alpha family class A member 1	C;X;m;N	25,86	9,10
HSP90AB1	Heat Shock Protein 90 Alpha Family Class B Member 1	C;X;m;M;N	2,45	0,00
HSP90B1	Heat Shock Protein 90 Beta Family Member 1	C;ER;m;N;L	10,26	23,18
CDC37	Hsp90 co-chaperone Cdc37	C	0,43	0,12
AHSA1	AHA1, activator of heat shock 90kDa protein ATPase homolog 1	C	0,37	0,27
HSPA1A	Heat shock 70kDa protein 1A	C	0,34	0,48
HSPA1B	Heat shock 70 kDa protein 1B	C;S	5,27	1,24
HSPA1L	Heat shock 70 kDa protein 1-like	C	0,05	0,00
HSPA2	Heat shock 70kDa protein 1A	C	2,94	0,16
HSPA4	Uncharacterized protein	C	1,86	0,95
HSPA4L	Uncharacterized protein	C	1,29	0,00
HSPA5	78 kDa glucose-regulated protein	C;ER;L	15,80	27,29
HSPA6	Uncharacterized protein	C	0,01	0,00
HSPA8	Heat shock cognate 71 kDa protein	C;D;m;N	10,62	7,88
HSPA8	HSPA8 protein (Fragment)	C;D;m;N	0,02	0,00
ST13	Heat shock 70kD protein binding protein	C	0,36	0,97
HSPB1	Heat shock protein beta-1	C;m;N	1,58	14,78
HSPBP1	HSPA (Heat shock 70kDa) binding protein, cochaperone 1	C	0,07	0,00
HSPD1	60 kDa heat shock protein, mitochondrial	C;D;m;M;L	7,05	5,10
HSPE1	10 kDa heat shock protein, mitochondrial	C;m;M;L	1,82	1,74
HSPA9	Stress-70 protein, mitochondrial	M;N	3,99	3,71
TRAP1	Heat shock protein 75 kDa, mitochondrial	m;M;L	1,44	0,51
DNAJA3	DnaJ (Hsp40) homolog, subfamily A, member 3	C;M;N;L	0,24	0,00

*Cellular localization of the identified HSPs is shown: *C* cytoplasm, *D* endosomes, *ER* endoplasmic reticulum, *L* organelle lumen, *M* mitochondria, *m* membranes, *N* nucleus, *S* cytoskeleton, *X* extracellular

**Relative abundance (RA) is calculated as a percentage of the total relative abundance of the detected HSPs (i.e., not from the total proteins) in oocytes and cumulus cells, respectively

these proteins in oocytes and cumulus cells is shown in Table 1.

The unique insight from the data displayed in Table 1 suggests that oocytes exhibit a very similar expression pattern of most HSP family members at the protein level compared to cumulus cells. In addition, the illustrated relative abundance indicates that few specific HSPs are particularly highly expressed, suggesting that they may play an important role in cellular homeostasis in oocytes. These specific HSPs are namely related to the HSP70 (HSPA5 and HSPA8) and HSP90 families (HSP90AA1 and HSP90B1). These molecules are crucial for UPR signaling induced in the ER (UPR^{er}). In addition, few HSPs related to mitochondrial functions (e.g., HSPD1 and HSPE1) could also be detected in the oocytes. This chapter will further expand on ER and mitochondrial stress responses in oocytes and the involvement of HSPs.

4 Heat Shock Proteins, UPRs, and ER Stress in Oocytes

4.1 Mechanisms of UPR^{er}

The ER is a major intracellular organelle mainly responsible for protein synthesis and is also involved in lipid synthesis and metabolism, as well as in cellular Ca^{2+} homeostasis (Ozcan et al. 2004). Oocyte growth and maturation and early embryo development require active de novo protein synthesis. Synthesis of soluble and membrane proteins by translation of maternal mRNA, or embryonic mRNA after embryo gene activation, is essential for several cytoplasmic and molecular changes that occur during oocyte development and for embryonic cell replication and differentiation (Schultz and Wassarman 1977). Newly synthesized proteins must be

properly folded in the ER to acquire their functional 3D conformation (Lin et al. 2019).

ER functions and protein folding processes are sensitive to several pathophysiological conditions, such as lipotoxicity, OS, HS, as well as glucose deprivation and aberrant Ca^{2+} regulation (Adachi et al. 2009; Pagliassotti et al. 2016). Increased stress level results in failure of protein folding or protein misfolding, which are then retained in the ER to be either repaired or degraded by the ER-associated degradation (ERAD) machinery (Nakatsukasa et al. 2008). Accumulation of unfolded or misfolded proteins within the ER induces the ER stress responses, which constitute an important quality control adaptive mechanism that allows the cell to sense and respond to stress (Huang et al. 2017; Lin et al. 2019).

Unfolded protein responses induced by ER stress are mainly cytoprotective and aim at reestablishment of cellular homeostasis by attenuation of protein synthesis (translation) to reduce the folding load within the ER and by activation of transcription of chaperone proteins and folding catalysts to enhance the folding capacity (Trusina and Tang 2010). These cytoprotective UPRs are usually efficient under transient or mild degree of stress. Prolonged exposure or high levels of stress, in contrast, stimulate cyto-destructive UPRs, which induce apoptosis to eliminate the cells that cannot be repaired (Pagliassotti et al. 2016). Although these mechanisms are vital to maintain normal tissue and body functions, excessive ER stress-induced cell death has been shown to cause diseases such as diabetes mellitus (Brozzi and Eizirik 2016).

The mechanism by which misfolded proteins stimulate ER stress response signaling cascade is initially mediated by three ER transmembrane sensors, namely, PERK, IRE1α, and ATF6, and by their interaction with the heat shock 70 kDa protein 5 (HSPA5). The subsequent downstream signaling transduction is initiated by destabilization and dissociation of HSPA5 from these transmembrane sensors to bind the accumulated unfolded or misfolded proteins in the ER lumen (Lin et al. 2019) (Fig. 1).

Upon activation of UPR^{er}, the dissociation of HSPA5 (BiP) from the transmembrane sensor PERK to bind misfolded proteins triggers autophosphorylation of PERK, which then causes phosphorylation of the eukaryotic translation initiation factor 2 alpha (eIF2α). Activation of eIF2α subsequently activates ATF4 (activating transcription factor 4), which stimulates expression of nuclear-encoded chaperons. Dissociation of HSPA5 from IRE1 and ATF6 similarly activates XBP1 and ATF6, respectively, which in turn upregulate nuclear expression of HSPs and chaperons, including HSPA5 itself (Gardner et al. 2013).

4.2 Constitutive Role of HSPs and UPR^{er} in Oocytes

ER-induced UPRs have been shown to play important physiological roles in reproductive functions (for a review, see Huang et al. 2017). HSPs have been shown to be essential to protect the gametes and embryos from damage during gametogenesis and embryogenesis (Neuer et al. 2000). HSPs, especially those related to the 70 kDa family, were found to be among the first genes expressed at the zygotic genome activation (Christians et al. 1995), and their protective role during mammalian embryo development is well described (Christians et al. 2003). In this chapter, we specifically focus on the described role of ER stress and UPRs at the oocyte level. As shown in Table 1, we have identified several members of different HSP families in mature bovine oocytes at the proteomic level.

The heat shock 70 kDa protein 5 (HSPA5), which was found to be highly abundant in cumulus cells and in oocytes, is a key molecule in ER stress responses. HSPA5 is one of the most abundant members of the HSP70 (HSPA) family and is located in the lumen of the ER. HSPA5 is also known as binding immunoglobulin protein (BiP) or glucose-regulated protein, 78 kD (GRP-78). HSPA5/BiP binds newly synthesized proteins as they are translocated into the ER and maintains them in a state competent for subsequent folding

and oligomerization (Gething 1999; Ng et al. 1992).

Apparently, the activation of ER-induced UPR mechanisms plays important physiological role and during ovarian follicle growth and maturation. HSPA5 and other UPR^{er} markers, such as ATF4, ATF6, and XBP1, have been shown to be expressed in immature and mature oocytes and during all stages of preimplantation embryo development in mice, pigs, and cows (Yang et al. 2012; Yoon et al. 2014; Zhang et al. 2012a, b). It has been recently shown that HSPA5 expression is upregulated in granulosa and cumulus cells during mice antral follicle development together with the activation of other ER stress sensors (IRE1 and PERK) (Harada et al. 2015). This could be required to cope with the high rate of de novo protein synthesis, which is vital during follicular development for the acquisitions of oocyte developmental competence. Knockout of UPR-associated genes such as HSPA5 (*Grp78*$^{-/-}$*mice*) resulted in cell proliferation defects and a massive increase in apoptosis in the ICM and failure of peri-implantation embryo development (Luo et al. 2006). Moreover, the expression level of XBP1 was found to be doubled in human cumulus cells enclosing oocytes that were successfully fertilized compared to those that were unfertilized following ICSI (Harada et al. 2015). Relative high expression of HSPAs in oocytes was linked with better adaptation of cattle breeds to environmental stress and to the production of good-quality oocytes during ovum pick-up programs (Souza-Cacares et al. 2019). Altogether, this illustrates the important constitutive role of UPR^{er} for oocyte quality.

4.3 The Role of HSPs and UPR^{er} Under Stress Conditions

Systemic effects of maternal metabolic alterations due to, e.g., obesity, are strongly connected to increased levels of ER stress in many cell types. The ER membrane has low cholesterol content and is composed mainly of unsaturated phosphatidylcholine (Higgins 1981; Koh et al. 2018). This unique lipid composition is important to maintain a high degree of membrane fluidity, which is essential for the ER functions. High-fat diet feeding and obesity have been shown to increase FFA concentrations in blood and ovarian follicular fluid (Ferrannini et al. 2007; Valckx et al. 2012). These elevated FFA concentrations are predominantly saturated (e.g., composed of high PA concentrations). PA is known to be a pathophysiological inducer of ER stress in different types of cells and tissues (Haywood and Yammani 2016). In vitro exposure of murine (Yang et al. 2012) and bovine (Valckx et al. 2015) COCs to follicular fluid from obese individuals or to equivalent FFA concentrations has been shown to increase lipid droplet accumulation in the enclosed oocytes. This may lead to hardening of cell membranes and intracellular membranous structures including the ER by increasing phosphatidylcholine-phosphatidylethanolamine ratio and can directly impact ER functions for protein synthesis, lipid metabolism, and signal transduction in the affected oocytes.

High levels of ER stress have been associated with inhibition of granulosa cell response to FSH in vitro and in vivo (Babayev et al. 2016) and may thus have a negative impact on follicle development and steroidogenesis. In addition, ER stress and the associated UPRs were evident in metabolically compromised oocytes collected from obese mouse models (Robker et al. 2011; Wu et al. 2011; Yang et al. 2012). This effect was mimicked by direct exposure of bovine COCs to lipotoxic concentrations of FFAs in vitro during maturation (Marei et al. 2017, 2019c; Sutton-McDowall et al. 2016).

Interestingly, functional analysis of the proteomic profile of oocytes from PA-exposed bovine COCs (Marei et al. 2019c) showed that the enclosed oocytes undergo activation of the EIF2 signaling, which is a feature of PERK-ATF4-mediated ER stress known to inhibit protein synthesis (Fig. 1). Importantly, although the reduction in protein synthesis is an attempt to reduce protein folding load in the ER and reestablishment

of cellular homeostasis, this may have long-term consequences on oocyte developmental competence in surviving oocytes and embryos, due to the highly dynamic nature of development.

ATF4 was found to be particularly upregulated in granulosa cells and COCs collected from HFD-induced obese mice and was associated with lower fertilization rates (Wu et al. 2010). ATF4 is a key UPR-mediated gene expression; however, it is difficult to interpret the significance of its upregulation in metabolically stressed COCs. Firstly, upregulated gene expression of ATF4 in the affected COCs is most likely to be in cumulus cells rather than in oocytes. Secondly, ATF4 can activate both cell survival and cell death mechanisms depending on the degree and duration of stress exposure. Under mild stress, ATF4 induces cell survival by stimulating mRNA expression of genes involved in stress responses, protein secretion, and amino acid metabolism (Harding et al. 2003). Interestingly, we observed that in PA-exposed COCs, pathways related to the degradation of phenylalanine, isoleucine, tryptophan, and threonine and the biosynthesis of tyrosine were among the top canonical pathways reported in the enclosed oocytes based on the changes in their proteomic profile (Marei et al. 2019a). This indicates that the downstream effect of ATF4 is somehow active in the affected oocytes. Embryos produced by IVF of FFA-exposed bovine COCs also exhibited long-term effects on amino acid turnover detected at the blastocyst stage (Van Hoeck et al. 2011).

On the other hand, dissociation of ATF6, another ER transmembrane sensor, from HSPA5 results in cleavage, activation, and translocation of ATF6 to the nucleus where it is known to induce transcriptional activation of ER stress response genes, particularly HSPA5, XBP1, and CHOP (Fig. 1). The expression of *HSPA5* is thus expected to be significantly increased under stress conditions and is therefore extensively used as a master marker for detecting the induction of ER stress (Lee 2005). COCs from mice fed a high-fat diet had increased mRNA expression of *HSPA5* (Wu et al. 2010). We have also shown that HSPA5 protein was significantly high in bovine COCs (as a whole unit) exposed to elevated concentrations of FFAs and was linked with reduced developmental competence (Marei et al. 2017). However surprisingly, we found that the protein abundance of HSPA5 itself was significantly reduced (fold change = 0.65, adjusted p value <0.05) in oocytes in PA-exposed COCs compared to controls (Marei et al. 2019c). As mentioned above, this may reflect HSPA5 utilization for misfolded protein transport and degradation in the absence of active HSPA5 transcription leading to depletion of this important chaperon in the affected oocytes and in the subsequent stages of early embryo development.

The fact that the expression of some HSPs may actually decrease (and not increase) in oocytes under stress conditions has also been reported in other studies. Yin et al. (2019) examined gene expression of *HSP70, HSP90α*, *HSP90*β, *HSP40*, *HSP40*, *HSPA4*, *HSPA4L*, *HSPH1*, and *HSF1* in porcine COCs subjected to HS at 41.5 °C for 24 h. All of these mRNAs were found to be significantly downregulated in oocytes and upregulated in cumulus cells. The stage of oocyte growth was also suggested to play a role. For instance, after 1 h exposure to HS, *HSP70* was reported to increase in growing porcine oocytes but decrease in the fully grown ones (Lanska et al. 2006), which could be related to the different transcriptomic activities at these stages. In contrast, Pennarossa et al. (2012) found that *HSP70*, *HSP40*, *HSPH1*, *HSPA4*, *HSPA4L*, *HSF1,* and *HFS2* genes (but not HSP90A and HSP90B, which were also constitutively expressed) were upregulated in porcine oocytes upon exposure to HS (41.5 °C for 1 h) but surprisingly were not affected in the surrounding CCs (Pennarossa et al. 2012). In studies showing a reduced HSP mRNA expression or increased HSP protein expression, this might be due to increased translation of those HSPs and thus depletion of the corresponding mRNA reserve. In contrast, the increase in HSP mRNA expression in oocytes reported in some studies is more difficult to explain but may be due to the transfer of HSP mRNA molecules from cumulus cells to oocytes, while trans-zonal projections are still intact (Macaulay et al. 2016).

What is even more perplexing are the conflicting responses of cumulus cells themselves to different types of metabolic stress in different studies. While some studies show increased HSP mRNA expression in cumulus cells under stress conditions, e.g., as shown by Yin et al. (2019), we found that cumulus cells in PA-exposed COCs exhibited a significant reduction in protein expression of HSP90B1, HSPA9, HSPB1, and HSPE1, as well as several other pro-survival proteins involved in ER UPRs, such as CALR, P4HB, PDIA6, and CANX (Marei et al. 2019c). This was associated with increased apoptosis in cumulus cells and was considered to be a sign of ER stress-induced cell death mechanisms mediated by "regulated IRE1α-dependent decay." This mechanism degrades mRNA encoding for crucial pro-survival proteins such as ER chaperons and is induced under high levels of ER stress (Hollien and Weissman 2006; Moore and Hollien 2015).

It is also important to notice that the effect of metabolic stress or HS is not homogenous across all cumulus cells of the same COC. Using specific fluorescence markers, we have demonstrated that cumulus cells exhibiting different levels of ROS, mitochondrial MMP, and caspase-3 expression (apoptosis marker) co-exist in a heterogeneous pattern within the same metabolically compromised COCs (Leroy et al. 2005; Marei et al. 2019c). Therefore, the overall expression of HSPs either at the transcriptomic or proteomic level in cumulus cells will depend on the proportion of cells experiencing low level of stress (and upregulated chaperon expression) and those already at an apoptotic stage (exhibiting regulated IRE1-α-dependent decay).

Focusing back at HSP-induced responses in stressed oocytes, and in contrast to the expression of HSPA5, we found that PA-exposed oocytes exhibited significant upregulation in protein expression of HSPA8 (1.52X increase, adjusted p value <0.05). HSPA8 is a chaperon located in the ooplasm, nucleus, and endosomes and plays an important role in the regulation of protein import and stability, chaperon-mediated protein transport and refolding, chaperon-mediated autophagy, and chaperone cofactor-dependent protein refolding. Interestingly, STIP1 (stress-induced phosphoprotein 1), which acts as a co-chaperone for HSPA8 and for HSP90AA1, was also upregulated (1.26X) in PA-exposed oocytes (Marei et al. 2019c). As shown in Table 1, HSP90AA1 is constitutively highly abundant in the oocytes but was not upregulated in response to PA exposure (Marei et al. 2019c). Other upregulated factors involved in chaperon-mediated protein folding were detected: FKBP4 (FK506 binding protein 4, 1.28X), CCT8 (chaperonin containing TCP1 subunit 8, 1.36X), and NASP (nuclear autoantigenic sperm protein, 1.52X), as well as those involved in ER-associated ubiquitin-dependent protein catabolic processes, such as BCAP31 (B-cell receptor-associated protein 31, 1.64X). These changes clearly illustrate the capacity of the oocyte proper to sense and respond to the metabolic stress at the protein level. Of course this is dependent on the oocyte's mRNA reserves.

Interestingly, HSPA8, which was specifically upregulated, is also known to be involved in the positive regulation of mRNA splicing via spliceosomes. mRNA splicing is a process by which pre-mRNA transcripts are transformed into a mature mRNA and could be needed to activate the maternal pre-mRNA reserve for translation. We could also detect more evidence of the proteomic changes that support RNA activation, binding and stabilization, and RNA transport: including the increased poly(A)-binding protein PABPC4 (1.34X) and nucleoprotein 35 (1.51X), as well as the regulation of protein translation via the increased ribosomal proteins RPL27A (1.45X) (Marei et al. 2019c). This illustrates the ability of the oocytes to strategically use their RNA reserve for adaptive mechanisms under stress conditions.

As mentioned above, ER stress does not only occur in response to lipotoxicity, HS, and oxidative stress. Several other physicochemical alterations may result in ER stress, such as osmotic stress, abnormal pH, and chemical exposure (Brozzi and Eizirik 2016). There are several ER stress-inducing compounds that have been characterized and are widely used in research. Chemical stimulation of ER stress by, e.g., tunicamycin (which inhibits N-glycosylation

needed for protein folding), has been shown to negatively affect oocyte maturation and resulted in marked increase in embryonic arrest at the two-cell stage and reduced blastocyst rates due to increased apoptosis in mice, pigs, and cattle (for a review, see Nixon et al. 2017). It appears that the impact of such experimental chemical induction of ER stress may go beyond the expected stress levels induced by pathophysiological perturbations and induce apoptosis in the oocytes and higher rates of apoptosis during subsequent embryo development.

5 HSPs, UPRs, and Mitochondrial Stress in Oocytes

As mentioned above, several studies have shown that the metabolic stress associated with high-fat diet (HFD)-induced obesity causes altered mitochondrial functions in oocytes (Igosheva et al. 2010; Luzzo et al. 2012; Mitchell et al. 2009). Mitochondrial dysfunction clearly plays a central role in the pathogenesis of reduced oocyte quality and subfertility. Oocytes from obese mice exhibit altered mitochondrial membrane potential (MMP) and reduced ATP production, as well as increased mitochondrial ultrastructural abnormalities, altered mitochondrial biogenesis, and mtDNA copy numbers, compared to mice fed control diet (Igosheva et al. 2010; Wu et al. 2010, 2011, 2015). Similarly, in vitro studies have shown evidence of mitochondrial dysfunction (altered MMP and elevated ROS levels) when bovine oocytes are exposed during final oocyte maturation to elevated pathophysiological concentrations of FFAs (Marei et al. 2017, 2019b, c; Van Hoeck et al. 2011).

Mitochondria are the main source of energy (ATP) through oxidative phosphorylation, and they also regulate Ca^{2+} homeostasis and cellular metabolism. After fertilization, sperm mitochondrial mitophagy occurs, and embryo development entirely depends on the mitochondria inherited from the oocyte. Therefore, altered mitochondrial activity and ATP availability in stressed oocytes are directly responsible for a cascade of defects associated with infertility in maternal metabolic disorders including chromosomal aneuploidy (Zhang et al. 2006), failure of fertilization, increased embryo cell fragmentation, and developmental delay or arrest (McPherson et al. 2014).

Importantly, it is not all about energy. Similar to the ER, mitochondria are recognized as cellular sensors for stress, which is currently an area of intense research. These mechanisms are usually overlooked and not clearly defined in the pathogenesis of reduced oocyte quality and embryo developmental failure associated with metabolic stress. As mentioned above, oxidative damage and misfolding of proteins results in ER stress, which stimulates UPR^{er}. Mitochondria also incorporate de novo synthesized proteins in the complexes of the electron transport chain in the inner mitochondrial membrane. Similar UPR responses occur in the mitochondria (UPR^{mt}), which constitute the molecular mitochondrial quality control machinery (Runkel et al. 2014). Elevated ROS levels beyond a certain threshold will lead to mitochondrial uncoupling. This uncoupling coincides with dissipation of MMP, which is suggested to be an actively regulated process in response to stress as an attempt to reduce ROS production and maintain cell survival (Brand 2000). Dissipation of MMP not only results in low ATP production, but it also elicits stress signals. Depending on the degree of stress, a series of sequential responses are induced (Fig. 2).

The transcription factor ATFS-1 is postulated as a key regulator of the initial step in UPR^{mt} (Nargund et al. 2012). Low MMP reduces ATFS-1 transport to the mitochondria for its proteolytic inactivation. ATFS-1 is then translocated to the nucleus where it induces the expression of nuclear-encoded mitochondrial chaperones (mitochondrial heat shock protein 70 (mtHSP70), HSP60, and HSP10) and proteases. These molecular chaperons are translocated back to the mitochondrial matrix to alleviate stress-induced damage and inhibit induction of apoptosis. This has been correlated with cellular lifespan extension. Higher stress levels may induce ultrastructural damage in the mitochondria and stimulate organellar mitochondrial quality control mechanisms, which require activation of mitochondrial biogenesis. This involves fission and

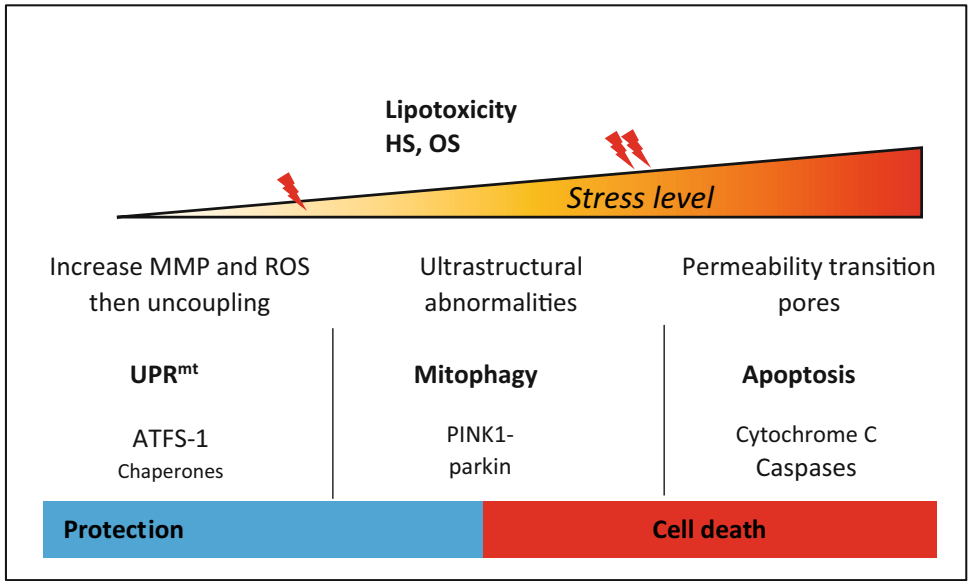

Fig. 2 Mitochondrial quality control mechanisms in response to stress. According to stress levels, mitochondria can stimulate protective chaperones via unfolded protein response (UPR^{mt}), undergo mitophagy, or induce apoptosis. *HS* heat stress, *OS* oxidative stress

fusion of mitochondria to allow repair or exclusion of damaged parts of mitochondria by PINK1/Parkin-mediated mitophagy. For stress levels that cannot be tolerated, mitochondria will initiate apoptotic pathways for removal of the damaged cell (Runkel et al. 2014). This occurs by the induction of mitochondrial membrane permeability transition pores and the release of cytochrome-C, which in turn activates caspases. This will ultimately end in the induction of apoptosis (Hirsch et al. 1997). We have shown evidence that mitochondrial uncoupling occurs in oocytes matured under metabolic stress conditions (Marei et al. 2017) or shortly after their fertilization (Marei et al. 2019b). However, importantly, embryos lack the machinery for mitochondrial biogenesis until blastocyst stage (St John et al. 2010) and are unable to eliminate damaged mitochondria due to the inability to activate mitophagy (Boudoures et al. 2017). This means that if mitochondrial molecular UPR fails, thresholds to induce apoptosis can be abruptly reached.

As stated above, mitochondrial heat shock proteins (HSPD1/HSP60, HSPE1/HSP10, as well as HSPA9 and TRAP1) were all detected at abundant levels in both oocytes and cumulus cells at the end of IVM of bovine COCs. HSPD1 (HSP60), which was more abundant, is known to be involved in re-folding of proteins and formation of oligomeric protein complexes in the mitochondria (Boilard et al. 2004). Both HSPD1 ($FC = 1.22$, $P = 0.065$) and HSPE1 ($FC = 1.62$, $P = 0.09$) tended to increase in oocytes after PA exposure compared to the control oocytes, suggesting that UPR^{mt} mechanisms may also be actively involved in stress responses in oocytes (Marei et al. 2019c). Again the level of protein expression of these markers could be limited by the transcriptional inactivity.

6 Consequences of UPR^{er} and UPR^{mt} in Oocytes on Further Embryo Development

The most logical question now is whether these limited transcription-free translation-based UPR^{er} and UPR^{mt} signaling in oocytes are sufficient (or efficient) for the reestablishment of protein

homeostasis and cell repair. It is evident that oocytes matured under metabolic stress or HS conditions have reduced developmental capacity and undergo embryonic arrest during cleavage, leading to reduced blastocyst rates (Krisher 2013; Valckx et al. 2014; Van Hoeck et al. 2011). In some cases, oocytes collected from obese women can be fertilized in vitro and undergo relatively normal early embryo development, sometimes with slightly lower cleavage rates or higher fragmentation rates but finally producing morphologically acceptable transferable embryos (Zander-Fox et al. 2012). However, the resultant embryos achieve significantly lower pregnancy rates after transfer and have a higher risk for preterm birth compared to embryos from metabolically healthy patients (Zander-Fox et al. 2012). The same observations have been recorded in bovine in vitro studies, where many COCs exposed to elevated levels of FFAs during IVM had acceptable cleavage rates (Marei et al. 2019b). Despite the arrest of some of the affected embryos during culture, the reduction in blastocyst rate is usually limited to a 10% difference compared with the control group, resulting in a relatively moderate yield of blastocyst production, which are morphologically normal and transferable (Marei et al. 2017, 2019b, c; Van Hoeck et al. 2011). Importantly, more in-depth investigation of the quality of these surviving blastocysts has shown that they exhibit high levels of apoptotic cell index, altered cellular metabolism, and altered transcriptomic and epigenetic patterns despite fertilization and culture in FFA-free standard conditions (Desmet et al. 2020; Marei et al. 2017, 2019b, c; Van Hoeck et al. 2011, 2015).

Interestingly, we have also noticed that blastocysts produced under FFA-free standard culture conditions but originating from PA-exposed COCs (24 h exposure) exhibited upregulated mRNA expression of both ATF6 and HSPA5 (but not ATF4), indicating persistent ER stress until day 8 of development. Nevertheless, these surviving blastocysts exhibited a 6X increase in the proportion of apoptotic cells compared with control embryos (Marei et al. 2019b). In another study, we investigated post-hatching development of PA-derived morphologically normal surviving blastocysts by transferring them at day 7.7 post-fertilization to uterine horns of healthy cows and flushing them back at day 14 (Desmet et al. 2020). We found that PA-derived embryos had retarded post-hatching growth and were less elongated, displayed altered cellular metabolism, and had altered transcriptome profile particularly in pathways related to redox-regulating mechanisms, apoptosis, cellular growth, interaction and differentiation, energy metabolism, and epigenetic mechanisms, compared to control embryos (Desmet et al. 2020). Collectively, it is evident that embryos produced from metabolically stressed oocytes might be partially able to maintain their survival early during development. However, the underlying sublethal stress levels appear to have persistent long-term effects that can be detected at the blastocyst stage or even later despite further development in stress-free conditions.

When COCs are matured in vivo or in vitro under metabolic stress conditions, cumulus cells are exposed first and are relatively more affected compared to the enclosed oocytes. Many of the exposed cumulus cells undergo apoptosis. In the same time, cumulus cells protect oocyte quality against oxidative stress and against lipotoxic effects in their microenvironment by internalizing and neutralizing FFAs (Aardema et al. 2013; Shaeib et al. 2016). Our proteomic data clearly show that despite the evident oxidative stress, altered mitochondrial activity, and signs of UPR^{er} and UPR^{mt}, PA-exposed oocytes exhibit several antiapoptotic changes, such as increased relative abundance of mitochondrial antioxidative proteins (particularly, peroxiredoxin 3 (PRDX3), NRF2-mediated oxidative stress response, activation of p70S6K-14-3-3 signaling (Marei et al. 2019c)), all of which are known to be pro-survival mechanisms (Amin et al. 2014; Chang et al. 2004; Lim et al. 2013). In addition, PA-exposed oocytes had upregulated SLC24A5, a negative regulator of apoptosis (Landes and Martinou 2011) as well as upregulated NLRP5 (NLR family pyrin domain containing 5), which plays an essential role for zygotes to progress

beyond the first embryonic cell division (Marei et al. 2019c). This illustrates the intrinsic ability of the oocytes to survive certain level and duration of stress. Oocytes may not reach thresholds of intracellular stress that are enough to induce apoptosis; however, as described above, intracellular stress appears to stay persistently high during fertilization and further embryo development even in FFA-free culture conditions. This results in persistent UPR signaling, which is apparently then not sufficient to combat the damage or prevent its further aggravation, leading to failure of embryo development at some stage.

In addition to the carryover of stress induced during oocyte maturation, embryos may be exposed to several additional sources of stress during fertilization and early development that may aggravate the damage and accelerate embryonic loss. For example, due to mitochondrial dysfunction and altered cellular metabolism in embryos derived from stressed oocytes, accumulation of increased concentrations of lactate (due to Warburg metabolism) or ammonia (from amino acid degradation) may progressively change pH and osmolality in the microenvironment (Cagnone and Sirard 2016), creating additional secondary factors that may have primary effects due to physicochemical stress. In fact, embryos appear to tolerate a range of suboptimal physicochemical conditions such as reduced osmolality during IVC without compromising their developmental capacity (Brinster and Troike 1979); however, embryos derived from metabolically compromised oocytes may not have the same level of tolerance.

It is also worth mentioning that embryos produced after somatic cell nuclear transfer (SCNT) were found to be more sensitive to tunicamycin-induced ER stress compared to in vitro-produced embryos (Babayev et al. 2016). This was suggested to be caused by the electrofusion step which is known to increase intracellular calcium ions and activate ER stress (Yin et al. 2019). SCNT-derived embryos exhibit increased *HSPA5*, *ATF6*, and *XBP1* gene expression, yet they have low developmental competence and quality (Lin et al. 2019). Importantly, ICSI has been also recently linked with increased levels of ER stress (Deng et al. 2020). Both mRNA and protein expression of GRP78 were found to be significantly higher in ICSI-produced embryos compared to IVF. ICSI embryos also showed higher apoptotic rates compared to IVF (Deng et al. 2020). Therefore, it is likely that a combination of these factors can amplify stress responses to a threshold beyond which embryo development is arrested. For example, it was found that human oocytes collected from follicles with high PA concentrations resulted in lower pregnancy rates following ICSI compared to those from low PA follicles (Mirabi et al. 2017). ICSI as such could be a contributing factor in this observation.

7 Therapeutic Applications Linked with Cellular Stress in Oocytes and Embryos

Fundamental understanding of the mechanisms by which oocytes and embryos handle cellular stress and how this may subsequently affect developmental competence is important to develop novel treatment strategies and identify molecular targets. This is particularly important in human settings when preventive measures are not possible or when preconception care strategies do not yield the expected results (Einarsson et al. 2017; Sim et al. 2014). Since it is clear that the intrinsic UPR mechanisms in oocytes have limited capacity to protect the oocyte and the growing embryo under cellular stress conditions, these treatment strategies should aim to prevent metabolic stress or at least to eliminate additional or persistent sources of stress during early embryo development and to optimally support oocyte and embryonic vital metabolic activities.

There are several periconceptional care strategies, such as weight loss, caloric restriction, or exercise, proposed for obese women to correct their metabolic profile and oocyte quality (Sim et al. 2014). In dairy cattle production, strategies to control excessive change in body condition

score and excessive fat mobilization during the transition period have been shown to limit metabolic stress and protect oocyte quality (Barletta et al. 2017).

On the other hand, several in vitro treatments have been proven effective in improving oocyte developmental competence under metabolic stress conditions. For example, the use of ER stress inhibitor (salubrinal) was shown to alleviate FFA-induced defects on mitochondrial activity, fertilization, and development to the blastocyst stage (Wu et al. 2012). Treatment of ICSI embryos with tauroursodeoxycholic acid (TUDCA, another ER stress inhibitor) could also alleviate ICSI effects on ER stress markers and enhance ICSI embryo development and quality to rates similar to IVF embryos (Deng et al. 2020). On the other hand, mitochondria have also been proposed as an important target for treatments aiming at enhancement of oocyte quality and fertility under metabolic stress conditions (Giannubilo et al. 2018; Legro 2019). Mitochondrial targeted therapies have been shown to protect oocyte developmental competence under elevated FFA concentrations in vitro (Marei et al. 2019a). In this study, such embryo treatment could eliminate persistent oxidative stress and rescue embryo development when supplemented during in vitro culture of zygotes derived from metabolically compromised oocytes (Marei et al. 2019b). Recent technologies may additionally offer tools that may prevent the buildup of physicochemical stress during culture by using microfluidic culture systems for continuous renewal of culture medium.

8 Conclusions

Collectively, when studying the pathogenesis of reduced oocyte and embryo developmental competence under maternal metabolic stress conditions and looking at the underlying cellular stress responses, it is very difficult to interpret what is a cause and what is a consequence. Nevertheless, there is enough evidence to show that oocytes and early developing embryos have an intrinsic pro-survival machinery that may temporarily extend the cellular lifespan in response to stress but is apparently not enough to support embryo development on longer terms. External support to prevent or reduce cell stress levels is thus inevitable to maximize the odds of producing good quality embryos, guaranteeing optimal viability. The insights, concepts, and speculations drawn in this chapter about the role of HSPs and UPRs in determining oocyte developmental competence are just the tip of the iceberg. More fundamental research is needed both at the transcriptomic and proteomic levels to further elucidate these responses and understand their critical interactions and long-term effects.

Conflict of Interest The authors have no conflict of interest to declare.

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