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Kursad Turksen *Editor*

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Stem Cells in Tissue Regeneration

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Stem Cells in Tissue Regeneration

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Preface

In this next volume in the Cell Biology and Translational Medicine series, we continue to explore the potential utility of stem cells in regenerative medicine. Chapters in this volume cover advances and challenges in applications of particular stem cell populations in a variety of diseases and conditions, and certain governance and policy issues and options.

I remain very grateful to Gonzalo Cordova, the Associate Editor of the series and acknowledge his continuous support.

I would also like to acknowledge and thank Sara Germans-Huisman, Assistant Editor, for her outstanding efforts in helping to get this volume to the production stages.

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

Finally, sincere thanks to the contributors not only for their support of the series, but also for their insight and effort to capture both the advances and 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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Apelin Receptor Signaling During Mesoderm Development

Derya Sağraç, Hatice Burcu Şişli, and Ayşegül Doğan

Abstract

The Apelin receptor (Aplnr) is a G-protein coupled receptor which has a wide body distribution and various physiological roles including homeostasis, angiogenesis, cardiovascular and neuroendocrine function. Apelin and Elabela are two peptide components of the Aplnr signaling and are cleaved to give different isoforms which are active in different tissues and organisms.

Aplnr signaling is related to several pathologies including obesity, heart disases and cancer in the adult body. However, the developmental role in mammalian embryogenesis is crucial for migration of early cardiac progenitors and cardiac function. Aplnr and peptide components have a role in proliferation, differentiation and movement of endodermal precursors. Although expression of Aplnr signaling is observed in endodermal lineages, the main function is the control of mesoderm cell movement and cardiac development. Mutant of the Aplnr signaling components results in the malformations, defects and lethality mainly due to the deformed heart function. This developmental role share similarity with the cardiovascular functions in the adult body.

Determination of Aplnr signaling and underlying mechanisms during mammalian development might enable understanding of regulatory molecular mechanisms which not only control embryonic development process but also control tissue function and disease pathology in the adult body.

Keywords

Apelin · Apelin receptor · Development · Elabela · Mesoderm

Abbreviations

ACTH	*Adrenocorticotropic Hormone*
Aplnr	Apelin Receptor
AVP	Arginine Vasopressin Hormone
bFGF	Basic Fibroblast Growth Factor
BMP-4	Bone Morphogenic Protein-4
CD	Cluster of Differentiation
CNS	Central Nervous System
DNA	Deoxyribonucleic Acid
Ela	Elabela
EMT	Epithelial to Mesenchymal Transition
eNOS	Endothelial Nitric Oxide Synthase
FGF	Fibroblast Growth Factor
FOXF-1	Forkhead Box F-1
GATA-4	GATA Binding Protein-4

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HAND-1	Heart- and Neural Crest Derivatives-Expressed Protein-1
hESCs	Human Embryonic Stem Cells
HIV	Human Immunodeficiency Virus
HUVEC	Human Umbilical Vein Endothelial Cells
IFN	Interferon
IL	Interleukin
IRX-3	Iroquois-class Homeodomain Protein-3
KO	Knock Out
MEOX-1	Mesenchyme Homeobox-1
mESCs	Mouse Embryonic Stem Cells
mRNA	Messenger Ribonucleic Acid
MYH-6	Myosin Heavy Chain-6
Nkx-2.5	NK-2 Homeobox-5
OXY	Oxytocin
PAX-3	Paired Box-3
PHA	Phytohemagglutinin
PVN	Paraventricular Nucleus
siRNA	Small Interfering Ribonucleic Acid
SON	*Supraoptic* Nucleus
TBX-5	T-box transcription factor-5
TCF-15	Transcription Factor-15
TDGF-1	Teratocarcinoma-Derived Growth Factor-1
TGF-β	Transforming Growth Factor Beta
VSMCs	Vascular Smooth Muscle Cells

1 Apelin Receptor Signaling Pathway

1.1 Components of Apelin Receptor Signaling

In 1993, Apelin receptor (Aplnr) was introduced as a member of class A rhodopsin-like guanine nucleotide binding (G) protein coupled receptors and the 377 amino acids long receptor was assumed orphan due to lack of its specified ligand (O'Dowd et al. 1993). Apelin, the first identified endogenous ligand of Aplnr, was obtained from the extract of bovine stomach in 1998 (Tatemoto et al. 1998). The 9698 bp Apelin gene, making up two introns and three exons, was revealed to encode a prepropeptide, consisting of 77 amino acids, including a signal peptide consisting of 22 amino acids at N terminal. Following the processes of proteolytic cleavage and maturation, three main isoforms of Apelin among various others were characterized as 36 amino acids long Apelin-36, 17 amino acids long Apelin-17 and 13 amino acids long Apelin-13, which is known as the most active form of the peptide (Tatemoto et al. 1998; Hosoya et al. 2000; Mesmin et al. 2011; Zhen et al. 2013; Shin et al. 2018). Another endogenous ligand of Aplnr, which was named as Elabela (Ela), also called as Apela or Toddler, was revealed in zebrafish in 2013. This peptide hormone was reported to consist of 54 amino acids, including N terminal signal peptide, and makes the active forms of Ela consisting of 32 amino acids (Ela-32), 21 amino acids (Ela-21) and 11 amino acids (Ela-11) (Chng et al. 2013; Pauli et al. 2014a).

After the activation of heterotrimeric Aplnr by its ligand, the signal is delivered to downstream pathways to regulate cellular events. $G\alpha_i$ subunit, coupled to Aplnr, activates PI3K/Akt pathway and PKC/Erk pathway and inhibits adenylyn cyclase, leading to inhibition of PKA by downregulating CAMP level (Hamada et al. 2008; Masri et al. 2002; O'Carroll et al. 2013). On the other hand, $G\alpha_q$ subunit, coupled to Aplnr, leads to activation of PKC pathway and upregulation of intracellular Ca^{2+} level by inducing PLCβ activation. Also, Aplnr, activated under mechanical stress, transduce signal in non-canonical pathway via β-arrestin to downstream pathways, which are yet to be elucidated (Chapman et al. 2014; Murza et al. 2016).

1.2 Tissue Distribution of Apelin Receptor Signaling

Since the discovery of the Aplnr, its distribution in various tissues have been reported including heart, endothelium, lung, brain, kidney, adipose tissue and plasma (Edinger et al. 1998; Novakov et al. 2015). Rat, mouse and human tissue distribution analysis of Aplnr showed that the highest similarity of Aplnr expression was observed in central nervous system (CNS). The distribution of

Aplnr mRNA in rat brain has shown that the highest level of mRNA expression has been shown in hypothalamus (De Mota et al. 2000). Similarly, mouse Aplnr mRNA expression has demonstrated a wide distribution in different CNS regions such as hypothalamus, pituitary, cerebral cortex, and spinal cord (Medhurst et al. 2003; Regard et al. 2008). The detailed investigation of Aplnr distribution in mice revealed that Aplnr was found at the lowest level in the posterior pituitary but relatively higher in the CNS and in the anterior pituitary (Pope et al. 2012). Furthermore, the corpus callosum and the spinal cord express highest Aplnr mRNA level in human CNS (Edinger et al. 1998; Medhurst et al. 2003; Matsumoto et al. 1996). Moreover, Choe and colleagues reported that the human Aplnr mRNA was highly expressed in oligodendrocytes and was relatively low in astrocytes (Choe et al. 2000). The high Apelin mRNA levels in both human and rodent CNS sections such as spinal cord (Pope et al. 2012), hippocampus (O'Donnell et al. 2007) and olfactory system (Cheng et al. 2012) may demonstrate that Aplnr pathway contributes to the development of nervous system in both human and rodent species. All these similarities suggest that Aplnr and its ligands are preserved among the high vertebrate throughout evolutionary processes.

The general distribution of Aplnr between human and rat tissues is similar, but this similarity was not seen in the peripheral organ distribution. In human tissues, Aplnr mRNA level is high in the spleen and relatively low in the small intestine, colon mucosa and ovaries. Moreover, Aplnr mRNA has not been reported in bone marrow, thymus, or epididymis tissues (The Human Protein Atlas n.d.). Also, Aplnr locus is methylated in undifferentiated human embryonic stem cells (hESCs) and therefore it is not present on the surface of hESCs. On the contrary, Ela is highly expressed and required for the self-renewal of hESCs (Ho et al. 2015). Subsequent studies have reported that placenta has high level of Aplnr expression, whereas low level of Aplnr mRNA expression were found in heart, lung, stomach, liver, pancreas, intestines and kidneys (Medhurst et al. 2003). It has been reported that in human placenta, Ela is mainly expressed in cytotrophoblasts until the first trimester (Wang et al. 2019; Georgiadou et al. 2019), while Aplnr and Apelin are expressed in syncytiotrophoblasts, cytotrophoblasts and endothelial cells of placenta as well as fetal vessels until birth (Eberlé et al. 2019). Similarly, Ela exists in serum of pregnant mice during E7.5 to E12.5 of gestation (Ho et al. 2017). In rats, Aplnr mRNA expression level was high in the lung and the heart and was not observed in colon, testes, stomach and liver (Hosoya et al. 2000; Medhurst et al. 2003). Besides, the rat Aplnr mRNA was expressed in the kidney and in corpora lutea of ovary (Różycka et al. 2018). Also, Medhurst and colleagues reported that except for thymus, testis and bladder, almost all tissues of mice expressed Aplnr mRNA and heart is the source of the highest level of Aplnr mRNA (Medhurst et al. 2003).

Immunofluorescence analysis of human heart has shown that proapelin peptide is expressed in the endocardial endothelium and cardiomyocytes. However, studies about Apelin peptide in vascular smooth muscle cells (VSMCs) are controversial. It has been reported that vascular smooth muscle cells expressed Apelin peptide (Li et al. 2008; Luo et al. 2018) whereas, Mughal and Pitkin determined separately that Apelin peptide is absent in VSMCs and only expressed in vessels of patients with atherosclerosis (Mughal and O'Rourke 2018; Pitkin et al. 2010). On the other hand, Aplnr is detected not only in human vascular endothelial cells and cardiomyocytes but also in smooth muscle cells (Folino et al. 2015; Kidoya and Takakura 2012; Kleinz et al. 2005). Thus, the elevated levels of Aplnr transcripts and proteins might be a reason why lungs and the heart have a high vascularization network as found in the spleen and placenta. During the mouse embryonic development (E9.5–10.5), Aplnr mRNA is mainly found in the endothelial layer of the vessels in the cardiovascular system (Kang et al. 2013) and in the retinal vessels after birth (Saint-Geniez et al. 2002). Inductive neovascular roles of the Aplnr signaling have been directed scientists to investigate the Aplnr-cancer relationship. Aplnr signaling is enriched in

tumor blood vessels (Uribesalgo et al. 2019) and expression level of Apelin peptide is elevated in various cancers such as colon, glioblastoma, non-small cell carcinomas, and hepatocellular carcinoma (Read et al. 2019). Human Aplnr mRNA is rarely detected in peripheral blood cells, although Aplnr is located on peripheral blood mononuclear cells when activated by Interleukin-2 (IL-2) and Phytohemagglutinin (PHA) (Choe et al. 1998; Masri et al. 2005). Moreover, Aplnr and Apelin signal was detected in human pancreatic islet cells (Ringström et al. 2010), human osteoblasts cells (Xie et al. 2007) and as well as both human and mouse adipose tissue (Boucher et al. 2005). Overall, the appearance of Aplnr in almost the whole body reveals the importance of Aplnr signaling for various processes in the body.

1.3 Physiological Role of Apelin Receptor Signaling

Based on the aforementioned structural properties and tissue distribution, Aplnr signaling has been reported to regulate fluid homeostasis (Roberts et al. 2010), body temperature (Kidoya and Takakura 2012), energy metabolism (Boucher et al. 2005), hormone (Wang et al. 2004) and neuropeptide (Masri et al. 2005) release, as well as cardiovascular function (Perjés et al. 2016) including vasodilation (Read et al. 2019), increased myocardial contractility (Maguire et al. 2009), angiogenesis (Saint-Geniez et al. 2002) and placental development (Wang et al. 2019; Eberlé et al. 2019; Ho et al. 2017).

The involvement of Apelin signaling in cardiac development was first described in intersomitic vein development of frog (Inui et al. 2006). Subsequently, it was demonstrated that mutation in the Aplnr leads to a decrease in the number of cardiomyocytes and may result in pericardial edema in Zebrafish (Scott et al. 2007). The effects of the Aplnr signaling in the cardiovascular system were also examined in mouse embryos, mouse embryonic stem cells (mESCs) and human embryonic stem cells (hESCs). In a study, cardiomyocytes were differentiated from hESC by adding Activin-A, bone morphogenic protein-4 (BMP-4) and basic fibroblast growth factor (bFGF). Ela expression was upregulated in cardiomyocytes along with cardiac transcription factors such as GATA Binding Protein-4 (GATA-4), myosin heavy chain-6 (MYH-6) and T-box transcription factor-5 (TBX5). After treatment with exogenous Ela, cardiomyocytes exhibited functional characteristics including contraction and releasing the cardiac troponin T protein into medium (Wang and Huang 2019).

Most of Aplnr-null mouse embryos have been reported to die in the prenatal state due to cardiovascular developmental disorders ranging from impaired development of the yolk sac and embryo vasculature to the progression of defective atrioventricular cushion and abnormally formed in right ventricles (Kang et al. 2013; Read et al. 2019; Charo et al. 2009; Wang et al. 2012). Interestingly, while Aplnr deficient mice have cardiovascular development defects, Apelin ligand deficient mice demonstrate normal development and become fertile (Folino et al. 2015). Therefore, it might be hypothesized that Aplnr plays a vital role in the developmental process and the signaling is regulated by other Apelin receptor ligands such as Ela. Besides the developmental process, recombinant Ela fusion protein has improved the function of infarcted heart in adult rats by injections everyday during 4 weeks (Xi et al. 2019). According to an *in vitro* study, Aplnr signaling has a role in human mesodermal projector cells during cardiac tissue differentiation and regulates the migration of progenitor cells that have been destined for cardiomyocyte differentiation (Vodyanik et al. 2010).

As mentioned before, Ela has vital roles in embryonic and placental development of mammals. Specifically, human Ela is responsible for the early cardiovascular system formation, cytotrophoblast proliferation, and invasion of trophoblasts into decidua (Eberlé et al. 2019). In the second trimester, placental Ela expression replaces with placental Apelin expression. Apelin induces the maturation of placental vessels to facilitate the transportation of nutrients. Besides, it has been revealed that a mutation in human Ela

causes preeclampsia which is a disorder characterized by abnormal cell movement and high blood pressure during the second trimester of pregnancy (Wang et al. 2019; Eberlé et al. 2019; Ho et al. 2017; Sharma et al. 2017). To determine the role of Ela in preeclampsia, Wang and colleagues collected patient tissues and blood samples and they revealed that placental Ela mRNA and protein levels decreased in patients with preeclampsia compared to healthy pregnants (Wang et al. 2019). In parallel, placental Apelin levels also decreased in early-onset pre-eclampsia. Although the plasma Apelin level was high in preeclamptic patients, there was not a clear difference between the patient and healthy women in terms of plasma Ela level (Georgiadou et al. 2019). Another study with preeclamptic mice revealed that Ela-KO pregnant mice with normal Apelin expression displayed impaired placental angiogenesis, which is a reason for high blood pressure (Ho et al. 2017). It is probably due to the diverse effects of Ela and Apelin in embryonic and placental development. Also, the role of Ela in early stage of embryonic development was demonstrated with the exogenous application of Ela which not only enhanced invasion and differentiation of throphoblasts but also supported cardiovascularization during pregnancy (Zhou et al. 2019). In a recent research, it was reported that overexpression of Ela induced invasion and migration of human trophoblast cells, HTR-8/SVneo, via the Akt-mTOR pathway, whereas knockdown of Ela interrupted the cellular movement of HTR-8/SVneo cells (Wang et al. 2019). The abovementioned results indicate the importance of Ela-Aplnr signaling in early and middle development of embryo.

The effects and roles of the Aplnr signaling pathway in the cardiovascular area were mostly studied on blood pressure regulation (Tatemoto et al. 2001) and cardiac muscle contraction (Ladeiras-Lopes et al. 2008). As mentioned earlier, human Aplnr is commonly found in endothelial cells and smooth muscle cells of the vascular wall (Wu et al. 2014) and human Aplnr mRNA was detected in endothelial cells in the endocardium of the large arteries, coronary vessels, and right atrium (Iturrioz and Llorens-Cortes 2013). Aplnr signaling regulates blood pressure through the endothelial Nitric Oxide Synthase (eNOS) dependent manner. Upon activation of Aplnr in endothelial cells of wild-type mice, eNOS phosphorylation is induced through several processes, including PI3K/Akt activation (Zhong et al. 2007). eNOS spreads to VMSCs where it exerts vasodilating effects and lowers blood pressure. Since Aplnr-Knockout (KO) mice do not have Apelin signaling in vascular endothelial cells, eNOS synthesis is not observed and this causes a hypertensive phenotype. Therefore, vascular smooth muscle cells which are located under the endothelial cells created the contraction response through Aplnr signaling (Hashimoto et al. 2006). Likewise, Aplnr is necessary for functional angiogenesis. It was shown that Apelin-13 ligand promotes thymidine incorporation into the DNA of human umbilical vein endothelial cells (HUVECs) where Aplnr is structurally expressed and provides vascular cell proliferation by inducing the p70S6 kinase through the activation of the PI3K and ERK pathways (Masri et al. 2004). Also, in Aplnr-KO mice, CD31 endothelial cells were present, but the number of vascular smooth muscle cells decreased and blood vessels did not form properly (Kasai et al. 2010). The elevated level of Aplnr and Apelin peptide expressions in the cardiac system suggested that the Aplnr signaling has regulative roles in cardiac functions. In addition, arterial-venous vascular alignment in the skin is processed correctly through the Aplnr signaling. Kidoya and colleagues reported that both Aplnr-KO and Apelin-KO mice did not tolerate the hot and cold stress compared to wild type mice. Therefore, Aplnr signaling has been reported to have an indirect effect on the regulation of body temperature (Kidoya et al. 2015).

Aplnr and its ligands have a crucial role for fluid homeostasis of mammals. In adult rats, it was reported that. Ela, which is highly expressed in kidney of rats, enhanced the water intake and diuresis via both ERK1/2 and G_i mediated Aplnr signaling pathways (Deng et al. 2015). Moreoever, studies with mice showed that when water was continuously supplied, the osmolality and urine volume of Aplnr-KO mice did not differ, but the water-drinking behavior was

lower than wild-type mice (Roberts et al. 2010; Roberts et al. 2009). Although these results indicated the important role of the Aplnr in fluid homeostasis, controversial studies about Apelin-induced water consumption in rats have been published. Reaux and colleagues showed that there was no difference in the level of water-balancing hormone, arginine vasopressin (AVP), among the rat groups which are free to access water (Reaux et al. 2001). On the other hand, Taheri and colleagues indicated that homeostatic behaviors such as releasing of adrenocorticotropin (ACTH) and AVP hormones into the bloodstream, the regulation in the volume and concentration of urine, and level of water intake improved dose-dependently with exogenous Apelin-13 (Taheri et al. 2002). Finally, Clarke and colleagues found that the water consumption of Apelin administered rats was lower compared to wild-type rats (Clarke et al. 2009). Therefore, the Aplnr receptor signal function needs to be investigated more thoroughly in fluid homeostasis to understand its osmoregulatory role.

Energy metabolism and food intake are also key fields in which Aplnr signaling pathway is involved. Expression of the Aplnr in the hypothalamus and the distribution of the Apelin expressed neurons to the regions related to food intake have led to the investigation of the effect of the Apelin signaling in food intake. Apelin is expressed in human adipocytes and mouse 3T3L-1 cells. The adipose tissue is one of the reservoirs of plasma Apelin (Boucher et al. 2005). Interestingly, insulin secretion as well as starvation and refeeding, stimulates the expression of Apelin in adipose tissue (Boucher et al. 2005; Wei et al. 2005), whereas Apelin (Apelin-36) has an inhibitory effect on insulin secretion (Winzell et al. 2005). It was also reported that Apelin-36 regulates the blood glucose by improving glucose homeostasis in obese mice (Yang et al. 2017). Studies on both human and murine pancreatic islet demonstrated that Apelin peptide expression is observed in α and β islet cells (Ringström et al. 2010), while the Aplnr expression is abundant in acinar cells, pancreatic duct, and islet cells (Kapica et al. 2012). However, the pancreas and adipocyte-based studies revealed that Aplnr deletion caused low islet density as well as insulin disorders and glucose intolerance in both normal and obese organisms (Han et al. 2015). Both the widespread distribution of Aplnr and its ligand in the pancreas and fatal results due to Aplnr deletion highlight the vital role of the Aplnr signaling in glucose/energy metabolism.

Aplnr signaling pathway has immunoregulatory roles (Choe et al. 1998; Masri et al. 2005; Edinger et al. 1998). Aplnr and downstream signals have protective functions as a major receptor with CD-4 for Human Immunodeficiency Virus (HIV) infection, which is inhibited by different Apelin isoforms (Zou et al. 2000). Induction of Aplnr expression by stimulating peripheral blood mononuclear cells suggests that Apelin signaling plays a role in the number of active T lymphocytes (Edinger et al. 1998). Moreover, it has been reported that isoforms of different Apelin ligands suppress cytokine production. In response to CD-3 cross-linking, which enables the activation of T cells, mouse spleen cells partially suppressed the production of cytokines, including IL-2, IL-4, and Interferon-γ (IFN-γ), by both Apelin-36 and Apelin-13 (Habata et al. 1999).

It is known that the Aplnr and its ligands regulate the hormone release of the pituitary and peripheral organs, as mentioned before. The Apelin ligand and its receptor are highly co-expressed in corticotrope cells in the pituitary (Le Goazigo et al. 2007) and Aplnr signaling involves release of ACTH (Iturrioz and Llorens-Cortes 2013). In the gastric enterochromaffin-like cells, Aplnr encoding gene is highly expressed. Thus, both histamine release and acid secretion (Lambrecht et al. 2006), as well as gastric cells proliferation and cholecystokinin secretion (Wang et al. 2004) were reduced. Besides, the Apelin ligand was co-expressed with neurophysin 1, which is a nerve cell hormone related to oxytocin metabolism, in the magnocellular oxytocin (OXY) neurons. During breastfeeding, Apelin ligand released from OXY neurons inhibited the effect of OXY neurons in autocrine or paracrine manner by binding to the Aplnr on OXY neuron surface (Iturrioz and Llorens-Cortes 2013). These results indicate that there is a strong relationship

between mechanisms of releasing various hormone and Aplnr signaling. Studies intended for all Aplnr functions show that Aplnr signaling provide the body homeostasis from embryonic to adulthood. A disruption in signaling process disrupts developmental or physiological functions.

2 Apelin Receptor Signaling in Endoderm

Gastrulation and germ layer specification are regulated by various signaling events. According to data accumulated in the last decade, Aplnr signaling also takes part during early embryogenesis. In early development, Ela/Aplnr signaling in zebrafish embryo was reported to affect migration, proliferation and differentiation of endodermal precursors for early cardiac development (Chng et al. 2013; Xu et al. 2018). Pauli and colleagues also demonstrated that activation of Ela/Aplnr signaling induces mesendodermal cell movement and internalization during gastrulation of zebrafish embryo (Pauli et al. 2014b). Furthermore, it was reported that Ela/Aplnr signaling, as a downstream of Nodal signaling, primarily controls mesodermal cell movement yet, endodermal cell movement is also affected indirectly through Cxcr4a signaling during zebrafish gastrulation (Norris et al. 2017). Freyer and colleagues investigated the role of Aplnr signaling in mammalian development. They reported that despite the expression of Ela in definitive endoderm of mouse embryo, absence of functional Ela affected Aplnr expressing mesodermal cells instead of defect in endoderm lineage (Hassan et al. 2010; Freyer et al. 2017). Moreover, hESCs were utilized to show the role of Ela in endodermal differentiation (Liu et al. 2020). Ela expression peaks at human blastocyte and undergoes a rapid decrease along with the differentiation (Miura et al. 2004). Interestingly, although Ela is expressed in undifferentiated hESCs, Aplnr is not expressed due to methylation. Ho et al. revealed that Ela interacts with and internalized by another receptor, which was not identified yet, and acts through PI3K pathway to mediate self-renewal effect of Ela in hESCs. They also demonstrated that Ela promoted endoderm lineage differentiation of hESCs through the activation of TGFβ pathway (Ho et al. 2015; Liu et al. 2020). These researches suggest that although Aplnr signaling is required mainly for mesodermal cell migration during gastrulation, it also mediates endodermal cell movement and differentiation.

3 Apelin Receptor Signaling in Mesoderm

Gastrulation is a vital process of embryogenesis that three germ layers are formed by the invagination of blastula cells (Ang S-LBRR 2002). In the gastrulation process, the mesodermal layer begins to form within the epiblast and hypoblast cells. In detail, a part of epiblast cells that will generate the mesoderm layer begin to migrate between the epiblast and hypoblast layer in lateral and cranial direction. The epiblast cells migrate towards the primitive streak and slide under the layer through the invagination process (Gilbert 2000). A part of migrating cells enters the inner cell area and form the endoderm layer. Other migrating cells spread between endoderm and epiblast in order to create mesoderm layer. The remaining cells at the outside form ectoderm layer. To form the notochordal plate and the notochord, which induces the formation of the neural tube and creates the anterior-posterior axis, prechordal cells move to the midline. After notochord was created, the mesoderm cells proliferate to form paraxial mesoderm. Mesoderm areas on both sides of the notochord are known as lateral plate mesoderm (Bellairs and Osmond 2014). Paraxial mesoderm forms somitomers that create dermatomes (skin) (Rehman and Muzio 2019) and sclerotomes (bone) (Maroto et al. 2012). The lateral plate mesoderm leads to the formation of both the circulatory system and mesodermal units (Stricker et al. 2017). In other words, the mesoderm layer is the main source of the middle line of the embryonic body plan; the skeleton, muscle, connective tissues (Carlson 2015), kidneys, circulatory and reproductive systems,

and the dermis precursors (Bakkum and Bachop 2013). Mesoderm formation occurs mainly by the migration of the cells existing in the epiblast and filling the intermediate section. This migration uses a mechanism called Epithelial-mesenchymal transition (EMT) (Nebigil and Désaubry 2019). EMT plays an important role in germ layer specification (ectoderm, mesoderm, endoderm) (Gheldof and Berx 2013). Epithelial cells in the primitive epiblast layer migrate to the midline and undergo EMT to create mesoderm and endoderm layers (Fig. 1) (Ohta et al. 2007). In this process, EMT forms a pool of primitive progenitor cells at the precise anatomical positions of the embryo, which constitutes the primordium of the developing organs. As mentioned before, the lateral plate mesoderm causes the formation of the heart and hematopoietic cells. As definitive germ layers emerge in the developing embryo, cardiac progenitors are the first cells among epiblast cells that undergo EMT and migrate from the primitive streak (Tam et al. 1997). This mesenchymal cardiac precursor population migrates bilaterally to the anterior pole of the embryo in the lateral plate to merge into an anterior cardiac in mammals (Kovacic et al. 2012). Besides EMT, several signaling pathways have been shown to contribute the germ layers specification in vertebrates, including Stat3, Prostaglandin E2, non-canonical Wnt and Fibroblast Growth Factor (FGF) signals. The specification of both endoderm and mesoderm layers mainly depends on a Transforming Growth Factor Beta (TGF-β) family member, Nodal signaling pathway (Wu and Hill 2009). The Nodal signaling uses ligands of TGF-β superfamily. These ligands phosphorylate transmembrane serine/threonine kinase receptors, which will subsequently phosphorylate Smad2 and Smad3. Then, Smad4 is induced and translocated to the nucleus, where the mesendoderm genes will begin to be expressed (Dunn et al. 2004). Nodal signaling is responsible for mesoderm formation and the expression of Aplnr and its ligands (Pauli et al. 2014b). Aplnr-Nodal signaling activates the genes necessary for cell movement and cardiac development of both zebrafish (Chng et al. 2013) and mice (Deshwar et al. 2016). Considering its known role in induction and migration of nodal signaling in mesoderm, Aplnr signaling has a role in parallel or upstream with the Nodal pathway. Therefore, the Nodal-mediated induction of Aplnr expression can make cells more active and responsive to Ela. It has been informed that an additional G-protein coupled receptor pathway regulated by Ela is required for suitable cell migration and

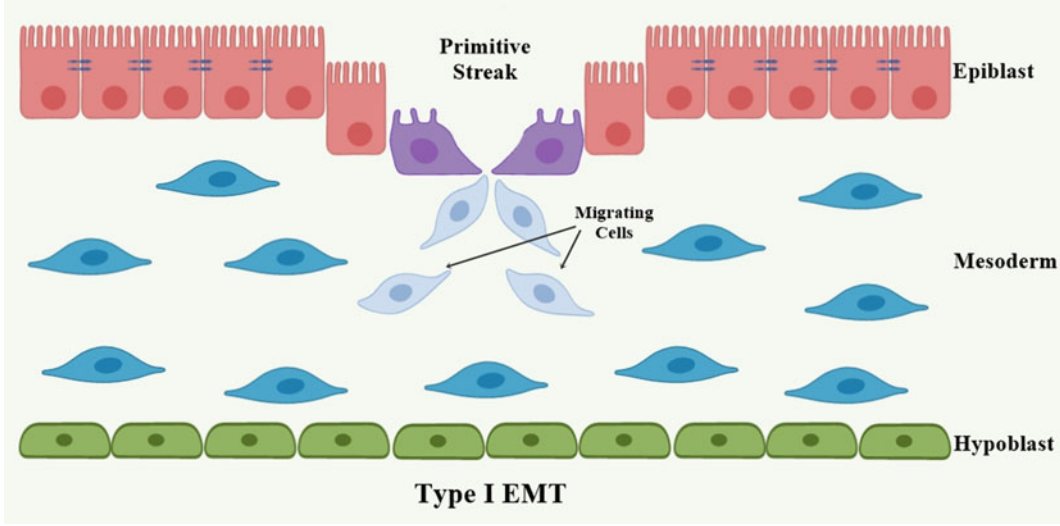

Fig. 1 The schematic illustration of cell migration during EMT in the gastrulation. Epiblast cells invaginate into the hypoblast layer to form the mesoderm and the migration of the cells creates the primitive streak

formation of mesendoderm. In this pathway, Ela, which is highly conserved during the evolution of vertebrates, generates downstream signals through the Aplnr (Chng et al. 2013; Pauli et al. 2014b). The initial specification of endoderm and mesoderm is formed normally in Ela-KO embryos (Pauli et al. 2014b). However, within the mid-gastrulation, it was observed that the mesodermal cell migration of Ela mutant embryos decreased, and the embryo had fewer endodermal cells than wild type. Ela mutants usually die 7 days after fertilization due to abnormal formation of cardiovascular units such as denuded vessels, deformed hearts, or formation of edema (Chng et al. 2013; Pauli et al. 2014b).

Aplnr signaling has important roles in mesoderm formation and angiogenesis during embryogenesis (Table 1). It was firstly reported in a study with *Xenopus laevis*. The expression of the Apelin receptor of *X. leavis,* homologus with the human Aplnr, was detected in the lateral plate mesoderm cells in which cardiac precursors develop (Devic et al. 1996) and then in the development of vascular structures of the somitic vessel (Inui et al. 2006). During gastrulation of zebrafish, Aplnr has been reported to be expressed not only in lateral plate mesoderm but also in the adaxial and intermediate mesoderm (Zeng et al. 2007). In another study with Zebrafish, it has been reported that Aplnr regulate the transcription factors of cardiac projectors, such as Nkx2.5 during gastrulation. When Aplnr was deleted in embryos, cell migration and mesoderm formation as well as cardiac development disrupted (Deshwar et al. 2016). Likewise, impair cell movement was observed in Aplnr-KO zebrafish embryos (Chng et al. 2013). Aplnr and heart relationship was also examined in zebrafish and Zeng and colleagues revealed that Apelin-KO zebrafish embryos exhibit late migration and abnormal distribution of anterior lateral plate mesoderm cells including cardiac precursors during mid-blastula. Besides, it has been reported that Apelin overexpression strongly inhibits some gastrulation movements of mesodermal cells and causes abnormal morphology and activity (Zeng et al. 2007). An example of Aplnr signalin related to cell migration as a result of EMT is the regulation of mesodermal cell migration in zebrafish using the Aplnr signal pathway with Ela. Aplnr gene is expressed in mesodermal progenitors, and the migration of mesodermal cells to the animal pole in the Ela-KO group was slow and exhibited in limited regions compared to wild type (Norris et al. 2017).

Table 1 Studies explaining the role of Aplnr signaling in mesodermal layer development

Species		References
Frog	Aplnr expression is limited to lateral plate mesoderm in the gastrulation process and has associated with the cardiovascular formation and endothelial lineage of *Xenopus leavis* embryos	Devic et al. (1996)
Zebrafish	Aplnr signaling regulates heart formation in the gastrulation process. Aplnr is expressed in LPM while apelin peptide is located in mid-line. Increase and decrease in Aplnr signaling cause disruption of heart formation	Zeng et al. (2007)
Zebrafish	Aplnr is located in the animal pole of the embryo after 4-h post-fertilization. At 10-h post-fertilization, Aplnr mRNA expression is detected in presomitic mesoderm and in lateral plate mesoderm cells at the somitogenesis stage. Also, the loss of Aplnr signalization results in abnormal cardiogenesis features due to a decline in the level of cardiac mesoderm markers	Scott et al. (2007)
Mouse	Aplnr-KO mice have exhibited reduced birth ratio due to developmental defects	Ishida et al. (2004); Kang et al. (2013)
Mouse	Aplnr transcripts have found in extraembryonic mesoderm, adjacent mesoderm, and primitive streak in E8 mouse embryos	D'Aniello et al. (2009)
Mouse	Loss of Ela causes embryonic lethality due to the circulation defects in mice. Apelin did not compensate for the lack of Ela in Aplnr signaling during development	Freyer et al. (2017)
Human	Aplnr transcripts found on day 2–3 of differentiation of hESCs whereas undifferentiated cells did not express Aplnr mRNA. Differentiation of Aplnr expressing cells has exhibited important markers for lateral plate mesoderm and their precursors	Vodyanik et al. (2010)

This shows the importance of Aplnr signal pathway in mesoderm migration with a receptor-ligand relationship. Also, it can be said that for a successful animal pole directed migration, Ela ligand and the Aplnr are needed.

Similar results have obtained in mice. D'Aniello and colleuges reported the first evidence that Aplnr pathway is necessary at the beginning of vertebrae gastrulation to mediate migration of myocardial progenitors toward the anterior lateral plate mesoderm. Moreover, they offered a novel pathway in addition to the nodal signaling which is highly studied in developmental processes. The Cripto pathway that is induced by an extracellular protein Cripto, also named as teratocarcinoma derived growth factor 1 (TDGF1), is retained during the evolution of higher vertebrates. While Aplnr mRNA is defined both in the primitive streak and in adjacent mesoderm, Apelin peptide is expressed only in the primitive streak which is similar to Cripto pathway. Likewise, the expression of Aplnr and Apelin could not be detected in the posterior mesoderm of Cripto-KO embryos whereas Aplnr and Apelin ligand continued to be expressed in the extraembryonic tissue of the Cripto-KO embryos (D'Aniello et al. 2009). Thus, indicating that the expression of Aplnr and Apelin ligand in the embryonic mesoderm is crypto-dependent, whereas extraembryonic tissue is crypto-independent. The relationship of Aplnr signaling with the formation of mesoderm has been frequently investigated in terms of cardiac development. It has been discovered that the mutation in the Aplnr encoding gene during the gastrulation phase showed cardiovascular malfunctions, including an improper cardiac cycle, thinning in the myocardium of the mice, and an enlarged right ventricle (Kang et al. 2013). These results show that the Aplnr signal is one of the most important active signals in the embryonic period.

A study was performed by Vodyanik et al., they mimicked mesoderm formation *in vitro* using hESCs. Aplnr expressing cells were found in the mesoderm on day 3. Also, it has been reported that Aplnr+ cells showed the expression of lateral plate/extra-embryonic markers, Forkhead Box F-1 (FOXF-1), Iroquois-class Homeodomain Protein-3 (IRX-3), BMP-4, WNT-5A and Heart- and Neural Crest Derivatives-Expressed Protein-1,2 (HAND-1,2), but did not show paraxial mesoderm markers, Mesenchyme Homeobox-1 (MEOX1), Transcription Factor-15(TCF15), and Paired Box-3,7 (PAX3,7), and intermediate mesoderm markers, PAX2 and PAX8 (Vodyanik et al. 2010). Similarly, it was reported that Aplnr signaling induced mesoderm differentiation by inducing hESC *in vitro* and formed mature and functional cardiac-like cells (Wang et al. 2012). Another study displayed Aplnr signaling in angiogenesis. Wang and colleagues reported that in the presence of Ela, Aplnr activation induced vessel sprouting *in vitro* and its overexpression increased this sprouting, but when Aplnr was knocked down with small-interfering RNA (siRNA), branched points decreased in endothelial cells (Wang et al. 2015). Another study showed that, while Ela was expressed higher than Apelin in mid-gastrulation, Apelin expression has reached the highest level in later development. Thus, both peptides are responsible for angiogenesis at different developmental periods (Helker et al. 2015). Therefore, these outcomes elucidated that Aplnr activity is vital for cardiovascular development. Effect of Aplnr signalization in cell migration was performed in several studies (Scott et al. 2007; Doğan 2019). It was reported that Mixl1-expressing hESCs upregulated Aplnr expression and did not express E-cadherin, an epithelial marker (Yu et al. 2012). In the studies related to EMT, Aplnr was seen as an important pathway for EMT mediated migration signal. Therefore, it can be said that Aplnr signaling works with EMT during mesoderm formation. Altogether, it can be seen that Aplnr signaling has very important functions in the formation of the mesodermal layer along with its ligands. During mesoderm formation, a mutation in the Aplnr itself or signaling components causes developmental or even death of the embryo.

4 Conclusion

Apelin receptor (Aplnr) as a member of class A rhodopsin-like guanine nucleotide binding (G) protein coupled receptors, is an important molecular regulator in many tissues. Aplnr and two peptide ligands have been identified up to now. Modulation of the Aplnr by two peptide ligands which have different active isoforms and distinct cellular functions impair the identification of exact mechanisms during development and adulthood. Identification of exact molecular mechanisms and peptide-receptor interactions during different cellular events and at different time points are required to solve developmental regulation by Aplnr signaling. Although there are several published researches reporting the role of Apelin in adult and developmental processes, the recent peptide Elabela needs to be studied in detail. Embryonic stem cell studies might be promising to determine Aplnr signaling and components in detail during mammalian development in the near future.

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Epidermal Stem Cells in Regenerative Medicine

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Abstract

Stem cells present in the epidermis, and hair follicle, guarantee the conservation of adult skin maintenance and hair renewal, but they also play a pivotal role in wound repair and tissue regeneration. Adult stem cells present in the epidermis are also responsible for epidermis different layers' regeneration.

We here summarize the epidermal stem cells information in term of their central features in stem cells niche, their signalling pathways and their maintenance, and activation.

Keywords

Regenerative medicine · Stem cell · Wound repair

Abbreviations

BMP-4	bone morphogenetic protein-4
CEA	cultured epithelial autografts
ECM	extra-cellular matrix
iPSCs	induced pluripotent stem cells
JEB	junctional epidermolysis bullosa
MCSP	chondroitin sulfate proteoglycan
SCID	Severe Combined Immunodeficiency
TACs	transient amplifying cells

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1 Biology of the Skin

The skin is the large body organ as well as one of the most vital due to its pivotal role as a protecting structure beside numerous exterior agents and working also as a regulator of the body temperature (Kanitakis 2002).

Skin is constituted of three layers: hypodermis, the deepest one, dermis and the most superficial, the epidermis (Martinotti and Ranzato 2020). The most abundant cells present in the epidermis are the keratinocytes, while other cell types present are melanocytes, Merkel cells and Langerhans cells (Rognoni and Watt 2018).

The epidermis is normally constituted of some sheets that are classified agreeing to keratinocytes level and expression. Keratinocytes originate from stem cells present at basal level migrating from the lower layers as metabolic active cells and transforming into dead cells as they move towards the upper surface (Candi et al. 2005). On the contrary, the dermis structure derived from the extracellular matrix (ECM), formed by fibroblasts. In the dermis, there are also

endothelial cells of the vessels, nerve ending, hair follicles, and cells of the adipose tissue (Ranzato et al. 2013). The hypodermis is the deepest skin layer containing loose connective tissue, blood vessels, nerves and cells that stores fat.

2 Wound Repair Physiology

Wound repair is a well-orchestrated biological phenomenon occurring in all tissues and organs (Martinotti et al. 2012a). The ultimate aim of wound repair is to restore tissue integrity and homeostasis. The wound repair process encompasses a well-adjusted activity of epithelial cells and connective tissue as well as the involvement of vascular cells and inflammatory mediators. These mechanisms strongly require an ECM to assist the repair event. The process of wound repair is normally summarized as four, partially overlapping, parts: haemostasis, inflammation, proliferation, and maturation (Martinotti et al. 2012b).

Tissue repair is a normal process occurring in all tissues, but in difficult conditions, such as different types of burns or during diabetes, this orchestrated event is not sufficient to allow real management.

The skin displays remarkable repair potential thanks to the existence of several stem cells types, present in the skin and its appendages. Some authors have already demonstrated that skin stem cells, localized in the lower epidermis layer as well as in the bulge of follicle hair, are vital sources of new cells for renewal and skin repair (Yang et al. 2019).

3 Epidermal Stem Cell and Wound Repair

Stem cell can be divided in two groups: somatic stem cells and embryonic stem cells. Embryonic stem cells derived from the inner cell mass of blastocyst. They are pluripotent, so with the potential to originate progeny cells of the three germ layers (i.e. endoderm, mesoderm and ectoderm). Somatic stem cells are normally present in tissues or organs. These cells are typically multipotent but the majority of somatic stem cells are one-lineage limited.

In the idea to use potential regenerative abilities of embryonic stem cells, Guenou and co-workers (Guenou et al. 2009) demonstrated that human embryonic stem cells maintained in a medium with ascorbic acid and BMP4 (bone morphogenetic protein-4) could originate keratinocytes of the epidermal basal level, utilized to reconstitute epidermis.

The epidermis is a tissue that undergoes continuous renewal, considering keratinization and exfoliation as natural features of epidermis. This process relies on stem cells resident in the epidermis (Staniszewska et al. 2011).

Skin stem cells are somatic stem cells, but for the several types of skin cells have already been described (Shi et al. 2006).

Subgroups of skin stem cells are:

- *epidermal stem cells*, normally present in the lower layer of the epidermis. They can be divided into transient amplifying cells (TACs) and terminal-differentiated epidermal cells. Specific cell markers are CD71 and chondroitin sulfate proteoglycan (MCSP) (Suzuki and Senoo 2012). TACs divide fast, and they differentiate after some cell division rounds (Hsu et al. 2014). The TAC, or cells that enter the transit stage, are able to generate some differentiated cells, also during wound repair and tissue repair process (Rangel-Huerta and Maldonado 2017).
- *follicular stem cells*. The bulge of the hair follicle is a source of keratinocytes and hair follicles cells. These stem cells are capable of regenerating a number of skin structures including the hair sebaceous glands and follicles (Cha and Falanga 2007). Specific cell markers are CD34, Lgf5, K15, Sox9 (Jaks et al. 2008).
- *melanocyte stem cells*. Melanocyte stem cells originate TACs and differentiated melanocytes (Lang et al. 2013).

In the past decade, important advancement has been made in the identification of stem cells/

progenitors markers for their isolation and enrichment (Barrandon and Green 1987). Through different cell culture approaches, we know that epidermal keratinocytes represent a heterogeneous population for their clonogenicity (Barrandon and Green 1987). In fact, it is possible to recognize different colonies types, and in particular three types such as holoclones, paraclones, and meroclones. These types originate from single keratinocytes due on their proliferative potential.

Holoclones possess the highest growth potential, as well as self-renewing capabilities, giving origin to both meroclones and paraclones (Senoo et al. 2007). Meroclones contain a transitional cell type considered as reservoir of TACs and paraclones (Staniszewska et al. 2011). The passage from holoclone to meroclone to paraclone is described as "clonal conversion" and is usually not reversible (Ojeh et al. 2015).

Despite works regarding the phenotypic features of skin stem cells, the specific control differentiation and proliferation activities are still not completely described. The "stem cell niches," i.e. the microenvironment of stem cells, are fundamental in controlling the propagation, differentiation and migration of stem cells (Spradling et al. 2001). Some signalling pathways are involved and among them Wnt (wingless/integrated) and Notch signalling pathways play a pivotal role for the stem cell "niches" (Kretzschmar and Clevers 2017).

When the skin is wounded, the stem cell niche varies in term of cytokines production, cell signalling involved, ECM alterations and other stimuli, resulting in the stimulation of the regulatory network, including Wnt and Notch signalling pathways in the wound tissue (Zhang et al. 2018).

Generally, more are the remaining skin stem cells on the tissue damaged area, the faster the healing speed, and the less the development of the scar. However, how and which signalling pathways modulate the differentiation and the growth of skin stem cells remain still unclear as well as their relationship with tissue restoration processes and scar development.

4 Skin Stem Cell for Epithelial Repair

In intact skin, keratinocytes do not move. They migrate only vertically in the epidermis, being passively pushed by cells growing at the *stratum basale* and gradually differentiating to keratinocytes.

In response to wound, stem cells represent an important reservoir for wound re-epithelialization. They exhibit lateral migration and cells migrate laterally to cover the wound surface (Staniszewska et al. 2011).

Some studies have already established the epidermal stem cells survival in *in vitro* cell culture system (Ojeh et al. 2015). Dunnwald and co-workers (2001) used *in vitro* cell culture approach to discriminate in mouse three groups of epidermal cells: stem cells, TACs, and not-proliferative basal cells. In fact, only the stem cell population, when seeded with dermal fibroblasts on a collagen type I gel, can induce the formation and maintenance of a normal epidermis for up to 6 months.

Further indications derived from *in vitro* keratinocyte layers use, also known as cultured epithelial autografts (CEA), resulting from skin. In the best conditions, keratinocytes obtained from a 3-cm^2 skin biopsy, are maintained in *in vitro* conditions to produce large, multi-layered CEA after few weeks (usually 3 or 4) in culture (Green et al. 1979).

Epidermal stem cells show the ability to renew epidermis, and they represent a suitable instrument for genetic manipulation, demonstrating a novel treatment option. The group of prof. De Luca utilized genetic modified keratinocytes from patients with junctional epidermolysis bullosa (JEB) on a SCID (Severe Combined Immunodeficiency) mouse model for efficient skin propagation and renewal (Mavilio et al. 2006). The same group showed also that cultures from autologous transgenic keratinocyte are able to regenerate a whole functional epidermis on a seven-year-old child JEB (Hirsch et al. 2017). In this work, the authors by clonal tracing demonstrated that the growth of epidermis is not maintained by equipotent progenitors, but it relies

on a limited number of long-lived stem cells, noticed as holoclones. These holoclones were *in vitro* and *in vivo* able to widely self-renew and to induce precursors that refill terminally differentiated keratinocytes.

5 Induced Pluripotent Stem Cells

Adult differentiated somatic cells (e.g., fibroblasts, skin keratinocytes, as well as other cells) can be organized to produce induced pluripotent stem cells (iPSCs) with comparable features to embryonic stem cells (Aasen et al. 2008). The genetic reprogramming of adult somatic cells could be achieved using retroviral transduction of four transcription factors (c-Myc, Sox2, Klf4 and Oct-3/4) (Takahashi and Yamanaka 2006).

iPSCs have already been utilized to produce a varied range of differentiated cell varieties including melanocytes and keratinocytes (Aguiar et al. 2016; Ohta et al. 2013).

Some results suggest the possible utilization in iPSC-based therapy of skin stem cells (Bilousova and Roop 2014; Dinella et al. 2014). These cells may be successfully incorporated into skin scaffolds to create all cell varieties, components, and skin appendages for the handling of long-lasting wounds and other integumentary system disorders. However, notwithstanding experimental data supporting the positive effects of iPSCs, some safety issues are still unresolved, and they need to be addressed before any large use in a clinical setting. These unresolved issues comprise inefficient cell re-programming, related tumour risk expansion through retroviral vectors utilization, genetic instability, epigenetic memory taken from parent cells, and potential immunogenicity (Okano et al. 2013). However, iPSCs utilization overcomes any ethical and moral issues related to the manipulation of embryonic stem cells.

6 Conclusions

The research strongly needs to elucidate the biology and possible application of skin adult stem cells, to dissect related cellular pathways, and associated extracellular matrix constituents, in order to re-establish troubled skin homeostasis, thus helping in the future progress of more effective therapeutic approaches. Moreover, other basic knowledge as well as clinical trials are essential to promote iPSC-based therapy long-term effects and to offer safer and more effective opportunities for upcoming clinical uses.

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The Cellular and Molecular Patterns Involved in the Neural Differentiation of Adipose-Derived Stem Cells

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Abstract

Injuries to the nervous system cause serious problems among affected patients by preventing them from the possibility of living a normal life. As this tissue possesses a reduced capacity of self-regeneration currently, lots of different strategies are being developed in order to make the regeneration in the nervous system possible. Among them, tissue engineering and stem cell-based therapies are to date very exploded fields and tremendous progress has been made in this direction. As the two main components of the nervous system, react differently to injuries and behave different during disease, it is clear that two separate regeneration approaches have been taken into consideration during development of treatment. Special attention is constantly given to the potential of adipose-derived stem cells for this kind of application. Due to the fact that they present remarkable properties, they can easily be obtained and have demonstrated that are capable of engaging in neural and glial lineages, adipose-derived stem cells are promising tools for the field of nervous system regeneration. Moreover, new insights into epigenetic control and modifications during the differentiation of adipose-derived stem cells towards the neural liege could provide new methods to maximize the regeneration process. In this review, we summarize the current applications of adipose-derived stem cells for neural regeneration and discuss in-depth molecular patterns involved in the differentiation of adipose-derived stem cells in neuron-*like* cells and Schwann-*like* cells.

Keywords

Adipose-derived stem cells · Brain injuries · Epigenetic control · Nerve trauma · Neuron-*like* cells · Regeneration · Schwann cell

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Abbreviations

3’ UTR	3′ untranslated regions
AD	Alzheimer’s disease
ASCs	adipose-derived stem cells
ATF3	activating transcription factor 3
BDNF	brain-derived neurotrophic factor
b-FGF	basic fibroblastic growth factor

Ca^{2+}	calcium
cAMP	cyclic-adenosine-monophosphate
CGRP	calcitonin gene-related peptide
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CREB	cAMP responsive element binding protein
DNA	Deoxyribonucleic acid
DRG	dorsal root ganglia
Dsh	Disheveled
EGF	epidermal growth factor
Egr2/Krox20	early growth response 2 gene
EMC	extracellular matrix
ESCs	embryonic stem cells
GAP-43	growth cone associated proteins
GDNF	glial-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
HATs	histone acetyltransferases
HD	Huntington's disease
HDASCs	histone deacetylases
HGF	hepatic growth factor
IL-10	interleukin 10
IL-1α	interleukin 1 alpha
IL-6	interleukin 6
iPSCs	induced pluripotent stem cells
Lef1/Tcf	lymphocyte enhancer-binding factor/T cell factor
lncRNA	long non-coding RNAs
MAG	myelin-associated glycoproteins
MAP 2	microtubule-associated protein 2
miRNA	microRNAs
mRNA	messenger RNA
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	nerve growth factor
nm	nanometer
NSCs	neural stem cells
PD	Parkinson's disease
PDGF	platelet-derived growth factor
PGE2	prostaglandin E2
PI-3 K	phosphoinositide 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PNS	peripheral nervous system
qPCR	quantitative polymerase chain reaction
RA	retinoic acid
RISC	RNA-induced Silencing Complex
RNA	ribonucleic acid
SCs	Schwann cells
STAT3	activator of transcription
TE	Tissue engineering
TGF-β	transforming growth factor beta
TGS-6	tumor necrosis factor-inducible gene 6 protein
TNF-α	tumor necrosis factor alpha
TrkB	tropomyosin receptor kinase B
Tuj-1	neuron-specific class III beta-tubulin
VEGF	vascular endothelial growth factor
Wnt	Wingless-related integration site

1 Introduction

The central nervous system (CNS) is most likely to be the most complex organ in the human body. Injuries, such as traumatic brain injury and spinal cord injury, are known to lead to unpaired functioning of motor and cognitive behaviors. Lately, lots of efforts have been made in order to determine new methods that will help with such major injuries of the CNS. These efforts have two approaches: on one hand, to enhance neuroprotection via preventing further cell damage and axonal degeneration and on the other hand, to ensure neuronal regeneration trough establishing cell plasticity and axonal outgrowth (Shoichet et al. 2008). In contrast to the CNS that possesses a very low regenerative capacity, the renewal of damaged nerves in the peripheral nervous system (PNS) is most likely to occur by itself if the injury is not invasive (Huebner and Strittmatter 2009). In case of a serious injury to the peripheral nerves, the brain loses the ability to control several muscles in the body, therefore the patient suffers from loss in motor or sensory functions (Rosen et al. 2016). This kind of trauma represents a global clinical problem that affects the quality of patients' lives by causing a socio-economic burden (Gu et al. 2014). To overcome the slow regeneration process of peripheral nerves and to guarantee a full recovery, lots of strategies

have been lately proposed. Among them, autologous grafting is widely used although it has considering disadvantages, for instance, insufficient donors, incompatibility between donors, immune system issues or the possibility of sacrificing a healthy functioning nerve. Since this procedure does not deliver the expected result in the setting of peripheral nerve trauma (Kubiak et al. 2018), new and modern biological approaches are developed in order to overcome this challenge. Thus, tissue engineering (TE) combined with stem cell therapy holds a great promise in stimulating peripheral nerve regeneration.

Stem cells have emerged as a useful tool for regenerative medicine and TE applications more than a decade ago. Due to their outstanding characteristics, they are still widely used by researchers in the hope of finding the optimal treatment method for several severe diseases. The most preferred type of stem cells which are often chosen for different studies, are the embryonic stem cells (ESCs) or the induced pluripotent stem cells (iPSCs). Even if these stem cells can turn towards any desired lineage, they come with some disadvantages: ESCs, for instance, raise ethical issues, whereas iPSCs are genetically modified cells that can easily turn towards unwanted cell types, such as progressive cancer cells. Therefore, more and more experimental studies that involve the use of stem cells are realized with the help of adult mesenchymal stem cells. They have the potential to differentiate towards multiple lineages, such as osteogenic, adipogenic and chondrogenic lineages (Ignat et al. 2019). These cells can be found in several accessible places of the body such as the bone marrow or the adipose tissue. Thus, adipose-derived stem cells (ASCs) can be easily obtained by minimum invasive methods, isolated (Galateanu et al. 2012; Dinescu et al. 2013) and further expanded in culture. They also have proven to be effective in the differentiation towards non-mesodermal lineages, such as the ectoderm. Jahan-Abad et al. succeeded in obtaining neurospheres from ASCs after 6 days of specific induction. The expression of specific neural markers was detected in the obtained neurospheres (Jahan-Abad et al. 2017). Hence, ASCs together with an engineered biomaterial could be a solution towards the regeneration after crush or disease in the setting of nervous system injuries.

Epigenetic investigations and analyzes are a hot topic and currently, researchers are looking into the epigenetic dynamics in each matter they are interested in. Among them, DNA methylation, histone acetylation, and non-coding RNAs are the most studied and addressed. According to recent research (Wahane et al. 2019), it was shown that transcription factors that have the capacity to enhance a regeneration process may have to interact with chromatin regulators in order to be active in this function. A better understanding of the molecular functioning of the epigenetic processes during the differentiation of ASCs towards neuron-*like* cells may lead to finding new methods that could promote fully functional recovery after traumatic injuries in the nervous system.

2 Injuries to the Nervous System – The Cellular Response

During embryonic development, neural stem cells (NSCs) within the neural plate and the neural tube proliferate rapidly and give birth first to neurons and then to astrocytes and oligodendrocytes in the CNS. When the last stage of this process is completed, NSCs form a niche in specific brain areas (the subventricular zone of the lateral ventricles and the dentate gyrus in the hippocampus) where they remain dormant. In this way, these cells serve as a reservoir of fully available cells for replacement and regeneration purposes during lifetime (Andreotti et al. 2019). The PNS, on the other hand, will be generated by a subpopulation of cells, known as the neural crest cells which originate from the dorsal part of the neural tube. These cells will further engage in the formation of sensory neurons, satellite cells and Schwann Cells (SCs), among others. Motor neurons in the PNS will be raised as a result of the ventral polarization of the neural tube (Catala and Kubis 2013).

2.1 The Peripheral Nervous System Damage

Once an injury to the peripheral nerve has occurred, two axonal regions are to be observed: the proximal stump (which remains attached to the neuronal body) and the distal stump (Kubiak et al. 2018). Following injury, the neuronal body receives a series of action potentials that open the calcium channels. Therefore, Ca^{2+} ions can freely circulate in the injured axon. These events will lead to the initiation of signaling cascades that will influence the processes of protein synthesis to ensure neuron survival (Brushart 2011). As a consequence, the neuron will change his function from neurotransmission back to its growth and development stage. In this time, the neuron body controls the formation of growth cones at the end of the proximal axon stump, which further guides the axon back to the target tissue. In this stage of injury, the neuron produces massive quantities of neurotrophins (brain-derived neurotrophic factor – BDNF, glial-derived neurotrophic factor – GDNF), growth cone associated proteins (GAP-43) and neuropeptides (calcitonin gene-related peptide – CGRP) (Zochodne 2000; Caillaud et al. 2019) and represses the production of neurofilament synthesis and neurotransmitter release (Fu and Gordon 1997; Caillaud et al. 2019). During this, the main glial cell in the PNS, namely the SCs, together with macrophages, will clear up all myelin debris, as these can inhibit axonal regrowth. Next, SCs will dedifferentiate to a proliferative phenotype, migrate and build up the Bands of Brügner, which will serve as support and guidance for the proximal stump of the injured axon. After approximately 4 weeks the maturation of the regenerated axons occurs, which resides in axon myelination ensured by SCs and further muscle re-innervation (Caillaud et al. 2019). If the regeneration was successful, after 3 months the nerve fibers should appear normal and the body should regain its lost functions. Even so, not in all cases, the recovery is complete. When the injury is too deep and the nerve gap too large, the nerve does not have the capacity to regenerate on its own. In this case, the process would take more than 3 months and a full recovery is not guaranteed. Therefore, autologous grafting, cell-based therapies, and TE strategies are currently being developed in order to enhance slow regeneration during serious trauma in the PNS.

2.2 The Central Nervous System Damage

In the CNS, the regeneration does not occur like in the PNS due to the fact that the cells surrounding axons are different and respond distinctively in case of injuries. Whereas SCs in the PNS immediately react after an injury and start cleaning up axonal and myelin debris, the oligodendrocytes in the CNS do not have this capacity. As soon as the oligodendrocytes loose axon-contact they undergo programmed cell death, therefore the clearance of debris is poor. Thus, myelin remains among injured axons and inhibits any regeneration possibilities. This is one of the important factors that leads to regeneration failure in the CNS (Gordon 2016). Up to date, a huge challenge for researches remains the neuronal degeneration which leads to serious neurodegenerative disorders such as Parkinson's disease (PD), Alzheimer's disease (AD), and Huntington's disease (HD). Lots of cell-based therapies are now under research in order to find out which could be a better approach in order to restore damaged neurons. Among them, ASCs have gained serious research interest as they are potentially capable of differentiation towards neural lineages.

3 Adipose-Derived Stem Cells: A Promise for Nervous Tissue Engineering

ASCs are a class of adult mesenchymal stem cells found in fatty tissues all over a persons' body. Even though their differentiation capacity is not as complex as the one of ESCs or iPSCs, ASCs presented exceptional results when they were differentiated towards non-mesodermal lineages (Ferroni et al. 2012; Frese et al. 2016).

As mentioned before, the brain is equipped with a pool of available NSCs which will become active in case of injuries to help with the regeneration processes. Unfortunately, these cells are most likely to be insufficient in case of serious traumas or major neurodegenerative diseases. For this reason, other cell sources are investigated in order to overcome one of the biggest challenges science is facing: the full regeneration of the nervous system. In case of nerve damage in the PNS, all cells surrounding the nerve have to ensure the survival of the neuronal body and at the same time the regrowth of the affected axons back to its' target tissue. Therefore, as the CNS and PNS present such big differences in response to injuries, different approaches have to be considered when regeneration strategies are set up.

The biggest issue in terms of traumas are the degenerative diseases and the incapacity of the affected nervous tissue to self-regenerate. Therefore, many researchers focus on trying to find an external appropriate cell source (e.g. iPSCs, ASCs) that would overcome this impediment and regenerate the nervous tissue. As for the damaged nerves in the PNS, the proposed strategy is to ensure the neuron survival, through axonal regrowth.

3.1 Differentiation of Neurons from Adipose-Derived Stem Cells

Under specific conditions, ASCs have effectively proven that they have the potential to differentiate into neuron-*like* cells (Fig. 2). All kinds of protocols have been established to induce neuron differentiation of ASCs *in vitro*. The workflow of this procedure starts with isolating ASCs from fatty tissue obtained after liposuction and expanding them in culture. Later, the cells are treated with a specific cocktail of induction containing various growth factors that are known to stimulate differentiation. Chemical and growth factors that are known to induce engagement towards the neural lineage in ASCs are all-trans retinoic acid, some commercially available supplements, such as, supplement B27, supplement N2 and growth factors, like basic fibroblastic growth factor (b-FGF), nerve growth factor (NGF), epidermal growth factor (EGF) and neurotrophin 3 (Gao et al. 2019; Khademizadeh et al. 2019). Usually after this step, neurospheres are obtained, containing neural progenitor cells, which morphologically resemble neurons.

This, however, is not enough to prove the generation of neuron-*like* cells, as it has been reported that morphological changes may appear due to cell reduction and not to neural differentiation (Neuhuber et al. 2004; Bertani et al. 2005). Thus, the validation of neural marker expression by the differentiated cells is required. Markers such as vimentin and nestin have been detected also in undifferentiated ASCs (Jang et al. 2010), but once the engagement towards the neural lineage occurred, their expression decreased (Cardozo et al. 2012). Markers such as neuron-specific class III beta-tubulin (Tuj-1) and microtubule-associated protein 2 (MAP 2) are proof of a more advanced stage of neural differentiation (Cardozo et al. 2012). The last step in this process would be to finally state that the obtained cells are also functional, by assessment of neurotransmitter expression and electrophysiological analysis (Addae et al. 2012). So far, there are no reports that suggest the generation of fully functional neurons from ASCs but taking into consideration all the advances in the last decade, the great potential of ASCs is still not completely explored. Nowadays, the idea of transplantation of ASCs derived-neurospheres is explored continuously and seems to have great potential for future therapies in the setting of neurological disorders (Peng et al. 2019).

3.1.1 The Effect of Neurotrophins Secreted by Adipose-Derived Stem Cells

In neural tissue engineering the differentiation of ASCs is the one of the mechanisms believed to enhance repair in the damaged tissues. Several studies state that ASCs actually use their own paracrine machinery to enhance neural differentiation through the secretion of neurotrophic factors (Wei et al. 2009). In their native, undifferentiated state, ASCs can secrete tropic factors (Fig. 1) that have important roles in stimulating regeneration and controlling muscle atrophy (Santiago et al. 2009). Several studies indicated

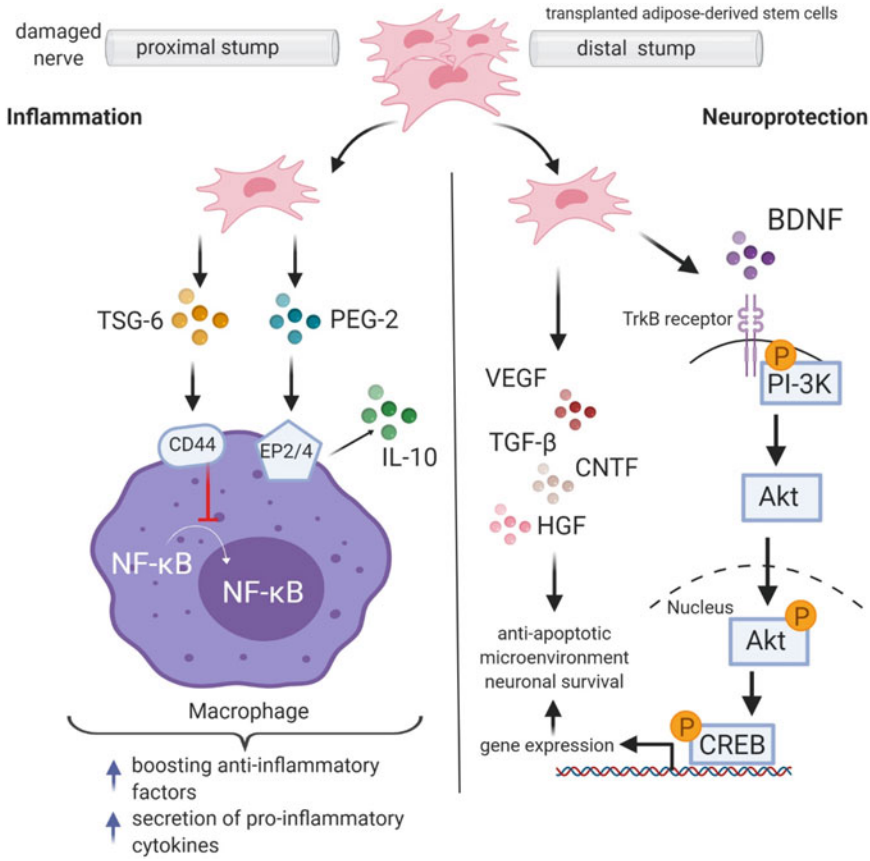

Fig. 1 The dual effect of ASCs derived growth factors and molecules during peripheral nerve regeneration. The anti-inflammatory effect of ASCs (left) relies in the secretion of TSG-6 and PGE-2 which act on macrophages. TSG-6 blocks the translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) to the nucleus, event which will decrease the secretion of pro-inflammatory factors such as interleukin 1 alpha (IL-1α) and tumor necrosis factor alpha (TNF-α). PGE-2 binds to EP2 and EP4 receptors on macrophages and determine phenotypic changes which will lead to interleukin 10 (IL-10) secretion. The neuroprotective role of ASCs (right) is established by secretion of a large pale of neurotrophies. BDNF molecules bind to tropomyosin receptor kinase B (TrkB) membrane receptor and triggering phosphoinositide 3-kinase (PI-3 K) signaling which is involved in ensuring neuronal survival. Similarly, other growth factors generate an anti-apoptotic microenvironment. (Figure created in BioRender.com)

the expression of factors BDNF, neurotrophin-4 and ciliary neurotrophic factor (CNTF) in ASCs, which are responsible for ensuring neuroprotection (Razavi et al. 2013, Moon et al. 2014). Moreover, during nerve injuries or degenerative diseases, apoptosis of neurons has to be prevented, representing an important step in tissue regeneration (Ekshyyan and Aw, 2004). Thus, ASCs have also been found to secret a series of factors that help prevent cellular death, such as vascular endothelial growth factor (VEGF), transforming growth factor β (TGF-β) and hepatic growth factor (HGF) (Rehman et al. 2004) which can establish a proper anti-apoptotic microenvironment. At this point it has to be mentioned that the glial cells of the two main components of the nervous system play different roles during traumatic events. In the CNS, the proliferation and activation of microglia results in neurodegeneration progress during traumatic brain injuries. Studies also proved that ASCs possess the capability of maintaining the microglia in their resting phase, therefore slowing down the degenerative events. Whereas in the

PNS such a cell type does not exist and all cell types fight for neuronal survival and axonal outgrowth during injuries (Jha et al. 2018).

3.1.2 Adipose-Derived Stem Cells Effect upon Inflammatory Microenvironment Regulation

Following an injury, the first response of the body is the inflammation process. In contrast to the CNS, where the regeneration rarely occurs, the PNS has the capacity of self-repair mostly due to macrophages, key cells in the regeneration process of peripheral nervous tissue (Liu et al. 2019). ASCs ability to govern the inflammatory events has been demonstrated in a different model, among them bowel inflammatory disease (Dave and Cominelli 2017), graft-versus-host disease (Zhang and Rosen 2018) and tissue regeneration (Zhang and Rosen 2018). Lately, it has been taken into consideration that native ASCs could also exhibit similar effects in case of sciatic nerve injuries (Marconi et al. 2013). In this case, ASCs at the injury site (Fig. 1) present the ability (1) to improve the microenvironment for neural regeneration by boosting anti-inflammatory factors (Bowles et al. 2017), (2) to prevent neural cell apoptosis (Marycz et al. 2017) and (3) to secrete a series of immunoregulatory factors such as tumor necrosis factor-inducible gene 6 protein (TGS-6) (Zhu et al. 2019) and prostaglandin E2 (PGE2) (Cui et al. 2007). Therefore, the evidence that ASCs could regulate the inflammatory response and differentiate to neural cell types is increasing, making these cells a perfect candidate for neural tissue engineering.

3.2 Differentiation of Neuroglia from Adipose-Derived Stem Cells

A complex development of many different types of glial cells, such as astrocytes, oligodendrocytes, SCs occurs during embryogenesis and adulthood. These processes are all known under the term gliogenesis. Glial cells in the CNS are responsible for the formation and maintenance of the blood-brain barrier and axon myelination, whereas SCs in the PNS ensure axon protection via myelination, segregate axons into bundles and clean axon debris in case of injuries. All of these cells have impressive roles throughout a lifetime, therefore any dysregulation or injuries that may appear can cause serious disturbance and wrong functioning. Due to the fact that there is no secure source of such cells in case of significant loss, researchers have developed a large pool of protocols in order to obtain glial cells from adult stem cells. Among them, ASCs were shown to present the capacity to generate glial-*like* cells, observation that opened up a whole new field of research.

3.2.1 Differentiation Towards PNS Main Glial Cell – The Schwann Cell

The SCs are the first cells to react in the setting of peripheral nerve injuries. These cells are responsible for supporting and ensuring axonal regeneration and remyelination. The first stage which occurs after an injury is known as the Wallerian degeneration, when SCs together with macrophages clean up axonal remains and myelin debris (Caillaud et al. 2019). This step is critical and needs to happen fast because the remaining myelin can inhibit the elongation of the injured axons. In the regeneration phase, these cells will start to secrete adhesion molecules, cytokines, and neurotrophic factors in order to create a permissive microenvironment for axonal regeneration (Jessen et al. 2015). All these events need to happen as quickly and as accurately as possible so that the axon can reach its target tissue and reinnervate the muscle. As stated before, the process of peripheral nerve regeneration is very slow. Therefore, recent cell-based strategies encourage the use of autologous SCs to enhance the regeneration (Levi et al. 2016). Even so, a big disadvantage comes with this approach, namely that a healthy nerve has to be harvested (Flores and Wang 2018) in order to obtain autologous SCs form the patient. This could further cause other complications in a patients' body. Thus, the use of ASCs has lately proven to be effective in the case of axonal outgrowth and myelination.

As far as *in vitro* studies show, numerous experiments have been carried out to prove the

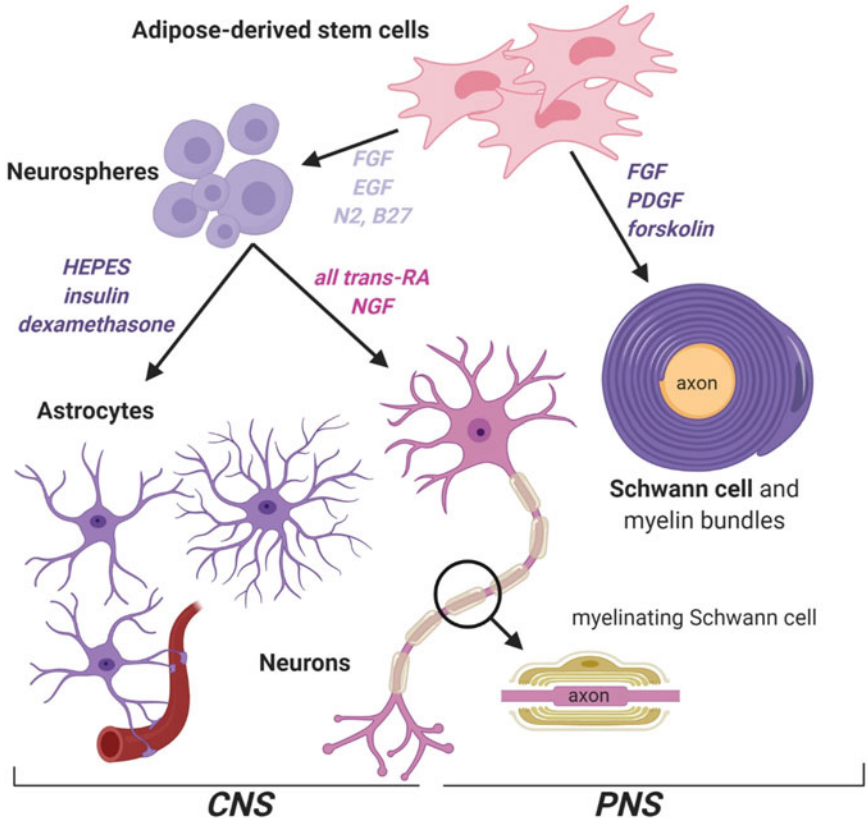

Fig. 2 Differentiation potential of ASCs towards cell types in the CNS and PNS under specific conditions. (Figure created in BioRender.com)

effective differentiation of ASCs to SCs-*like* (Fig. 2). Even though the obtained cells are more likely not fully functional SCs, they have proven excellent behavior for neurites outgrowth and myelination. Upon induction with complex factors such as b-FGF, platelet-derived growth factor (PDGF-AA/PDGF-BB), forskolin, neuregulin-1 and β-mercaptoethanol ASCs change their phenotype and partially their function into SCs-*like* cells. These factors have a role in mimicking the native environment of SCs. Therefore, ASCs undergo complex cellular and molecular changes so that in the end they gain SCs proprieties. Such a differentiation protocol was developed by Kingham et al. which then leads to obtaining SCs-*like* cells after 2 weeks of induction (Kingham et al. 2007). Xie et al. obtained differentiated human ASCs that presented unexpected morphological and functional resemblance to the genuine SCs. They're obtained SCs-*like* cells expressed glial markers such as S100 and glial fibrillary acidic protein (GFAP) (Xie et al. 2017). When put in co-culture with dorsal root ganglia (DRG) neurons (Xie et al. 2017) or with motor neurons from NG108-15 cell line (Kingham et al. 2007) SCs-*like* cells expressed neurotrophic factors NGF, BDNF, and GDNF and ensured neurite growth and axonal myelination. Therefore, it is important to co-culture SCs-*like* cells with neurons in order to validate their functionality. Other approaches suggest an indirect co-culture system between ASCs and SCs, leading to the differentiation of ASCs into SCs-*like* cells. Wei et al. developed a Millicell system in which ASCs became SCs-*like* cells and expressed typical glial

markers S100, GFAP and $P75^{NTR}$, demonstrating that the necessary environment can also be induced by the simple presence of native SCs (Wei et al. 2010). Besides this, it has been proven that SCs-*like* cells could also be obtained from neurospheres, which formed upon induction with specific culture media and a cocktail of growth factors (Radtke et al. 2009).

Observing the success of obtaining SC-*like* cells *in vitro*, researchers tested their theory and further made several efforts to prove that ASCs could also be as effective *in vivo*. Sun et al. engaged in a complex study where they used five different groups to bridge the sciatic defect for a period of 12 weeks: the hallowed group, which received a 10 nm long biodegradable chitin conduit, the group which received undifferentiated ASCs, the third which had pre-differentiated ASCs for 10 days, the forth pre-differentiated ASCs for 7 days and the last group received an autologous nerve graft. After performing several complex assessments, they concluded that the intermediate induction of ASCs has proven to be more effective for nerve regeneration *in vivo* than the traditional methods (Sun et al. 2018). On the other hand, Orbay et al. demonstrated that differentiated ASCs, as well as undifferentiated ASCs, could equally support nerve gap regeneration in the case of peripheral trauma (Orbay et al. 2011). Together these studies support the use of ASCs for further clinical trials in the setting of nerve regeneration and peripheral nerve trauma.

3.2.2 Differentiation Towards CNS Main Glial Cell – The Astrocyte

Astrocytes represent the largest portion of glial cells in the CNS. These complex cell types have various roles in the correct functioning of the brain. They are the first to react in case of injuries, ensure neuron nutrition, serve as a guide for NSCs in the process of migration, are a major component in the blood-brain barrier and are involved in ion transport and metabolic functions (Siracusa et al. 2019). The reduced capacity of regeneration in the CNS comes also from the fact that astrocytes themselves are difficult to regenerate, due to the low availability in NSCs. When injuries to the CNS occur, not only the neurons are affected, but also astrocytes, which serve as the main support for neurons. Therefore, these cell types have also to be taken into consideration in the setting of brain trauma. Current treatment methods suggest the use of cell transplantation, namely NSCs. Considering that the supply of these cells is very low and also impossible to obtain, recent advances propose the chemical induction of ASCs in order to obtain at first ASCs-derived neural stem/progenitor cells and then later on ASCs-derived astrocytes (Fig. 2) (Yuan et al. 2016).

This field is still not as explored as in the case of the PNS, due to major obstacles that have not been yet resolved, among them long induction time and low rate of survival (Ou et al. 2011). Even though ASCs-derived astrocytes presented typical electrophysiological characteristics as well as normal membrane potential and ultrastructure (Ou et al. 2011), the stress which the cells undergo during the differentiation is tremendous, leading some of them to apoptosis, as the autophagy fails to clear up all the damaged mitochondria (Yuan et al. 2016). As the role of autophagy in the differentiation of ASCs to astrocytes is still unclear, there is no stable explanation of these events happening in this manner. It may seem that the addition of an autophagy agonist could increase the intensity of the process and help with the differentiation efficiency (Sun et al. 2014), but still more research has to be carried out in order to obtain significant quantities of astrocytes, enough to be used in clinical trials.

4 Signaling Pathways Involved in the Neural Differentiation of ASCs

Together with all the factors that modulate this differentiation process, some signaling pathways play a major role in the generation of neuron-*like* cells form ASCs. Lately, lots of pathways were found to be implicated in this biological event, among them Wingless-related integration site (Wnt), Notch and cyclic-adenosine-monophosphate (cAMP) signaling pathways.

4.1 Wnt/β-Catenin Signaling Pathway

Wnt molecules are highly conserved glycoproteins which play important functions in a pale of cellular processes such as adhesion, proliferation, and differentiation (Visweswaran et al. 2015). The Wnt ligands which take part in this signaling cascade are hydrophobic proteins rich in cysteine and they bind to the frizzled receptor found cellular membrane, an event that initiates the signaling pathway (He et al. 2018). The signal is then further transmitted to intercellular proteins, Disheveled (Dsh), axin and β-catenin. Next, β-catenin is translocated to the nucleus where it forms a complex with lymphocyte enhancer-binding factor/T cell factor (Lef1/Tcf) which will cause the Sox family to induce expression of target genes (MacDonald et al. 2009). This pathway is not only important in the development of adult stem cells but also in the differentiation and proliferation of neural progenitor cells (Etheridge et al. 2004). For example, Wnt5a ligand was found to have activated neural differentiation in ASCs by binding to Frz3 and Frz 5 and directing axin/GSK-3b (Jang et al. 2015).

4.2 Notch Signaling Pathway

The Notch signaling pathways are activated when the transmembrane ligands (Jagged 1 and 2; Delta-like 1, 3 and 4) bind to the Notch receptors. Then, the intercellular Notch domain is released and will be translocated in the nucleus where he will form an active complex with RBPjκ/CBF leading to direct expression of Notch target genes (Fortini 2009). In the case of neural development in mammals, this signaling cascade has the role to maintain a set of undifferentiated precursors, while others are selected to continue the differentiation towards mature neurons (Gaiano and Fishell 2002; Cardozo et al. 2011). Cardozo et al. demonstrated that differentiated ASCs possess functional Notch signal which is down regulated after 14 days of neural induction, supporting the hypothesis that ASCs differentiation in nerve cells is a regulated mechanism, similar to the differentiation of neural stem cells into mature neurons (Cardozo et al. 2011).

4.3 Cyclic-Adenosine-Monophosphate Signaling Pathway

An intercellular messenger, which is actively involved in both the differentiation of ASCs to neuron-*like* cells and SCs-*like* is cAMP. When the G protein-coupled receptor is activated it induces membrane-anchored adenylyl cyclase will physiologically synthesize cAMP from adenosine-triphosphate. After a certain amount of cAMP accumulates in the cell cytoplasm, protein kinase A (PKA) will be activated and transported to the nucleus where it will ensure the phosphorylation of cAMP responsive element binding protein (CREB) transcription factor. The phosphorylated CREB and some other support factors will regulate the expression of several genes which have fundamental functions in neural cell survival, neuronal plasticity and long-term memory (Navarro et al. 2007; Makwana and Raivich 2005).

During nerve trauma it has been reported that levels of cAMP elevate in damaged neurons as a response to injuries, this leads to the activation of PKA and allows axons to bypass the growth inhibitory effect of myelin-associated glycoproteins (MAG) by inhibiting RhoA GTPase (Neumann et al. 2002). Subsequently, cAMP and PKA will overexpress arginase I by phosphorylating CREB, an event that will lead to the production of polyamines which also help diminish the inhibitory effect of MAG and myelin debris. All of these events promote axon elongation by assembling cytoskeleton proteins (Him et al. 2018; Hannila and Filbin 2008). In addition to CREB, PKA has been shown to act on the activating transcription factor 3 (ATF3, a cAMP-dependent transcription factor) as well as on the signal transducer and activator of transcription 3 (STAT3) to ensure polyamine synthesis

and subsequently axon elongation. Increased levels of cAMP induce il-6 expression that stimulates pro-regenerative genes (GAP43) via STAT3 (Knott et al. 2014).

cAMP signaling via PKA was also identified at the level of SCs during the regeneration phase in the PNS. In this case, cAMP activates PKA and acts on PI-3 K, which in turn will activate protein kinase B (PKB/Akt), resulting in proliferation of SCs (Him et al. 2018; Knott et al. 2014). Moreover, under the action of cAMP, SCs exhibit increased levels of myelin lipid galactocerebroside (O1), a marker present when SCs turn to their mature proliferating state (Bacallao and Monje 2015). The presence of cAMP also determines the expression of early growth response 2 gene (Krox-20/Egr2) which will stimulate the expression of a series of myelin-associated proteins and lipids, thus proving that cAMP is also actively involved in inducing the myelination phenotype of SCs at the end of the regeneration process (Reiprich et al. 2010).

As this signaling pathway play multiple functions during the regeneration phase, but also during development and neuronal survival, the addition of this element to the differentiation media of ASCs towards neurons or SCs could turned out to ensure satisfying results. A very popular product used in the differentiation of ASCs in neuron-*like* or SCs-*like* cells is forskolin, which is an activator of adenylyl-cyclase (Neirinckx et al. 2013). Jang et al. (2010) used a combined treatment of b-FGF and forskolin to trans-differentiate ASCs towards functional neuron-*like* cells. Differentiated cells were found positive for expression of several neural markers such as, nestin, MAP 2 and NeuN, demonstrating that these cells are candidates for developing into neurons in the setting of brain injuries. Concerning the PNS, ASCs can also be differentiated in SCs-*like* when treated with forskolin, PDGF and heregulin-β1. These obtained SCs-*like* differentiated from ASCs have shown to show significant proliferation *in vitro* (Fu et al. 2016) and enhanced nerve repair and regeneration when transplanted *in vivo* (Sun et al. 2018).

5 The Molecular Patterns Involved During Neural Differentiation of ASCs

In the past, the only debated changes which took place in ASCs during the neural differentiation were the cellular events. Lately, some serious attention is given to the molecular patterns, which seem to have a significant control in the generation of neuron-*like* cells form ASCs.

5.1 Non-coding Molecules Such as microRNAs (miRNA) and Long Non-coding RNAs (lncRNA)

These short nucleotide sequences have been continuously investigated in order to better understand their implication in the neural differentiation of ASCs. miRNAs (22–30 nucleotides) regulate gene expression by targeting messenger RNA (mRNA) molecules. After the pre-miRNA is formed in the nucleus, it is exported in the cytoplasm. Here it is maturated and assembled into RNA-induced Silencing Complex (RISC). In this complex, the mature miRNA will bind to the 3′ untranslated regions (3′ UTR) of the target mRNA and inhibit or promote gene expression (Wu et al. 2019).

LncRNAs are made of no more than 200 nucleotides and are key regulators of many important biological processes. These molecules usually bind to transcription factors and so their function is blocked, in this way lncRNAs regulate downstream gene transcription (Wu et al. 2019). Some studies have emphasized that a series of such non-coding molecules play significant roles in the differentiation of ASCs toward the neural lineage (Fig. 3c, d). Hu et al. evaluated the effect of retinoic acid and its function in the neural differentiation of ASCs. Deep sequencing methods revealed a complex profile of miRNA that regulates ASCs' neural differentiation. While miR-23a, miR-23b, miR24a, miR-22, and let-7a were found to be overexpressed in untreated ASCs, miR-222, miR-145, miR-124 a-5p were found to be overexpressed in retinoic acid-treated

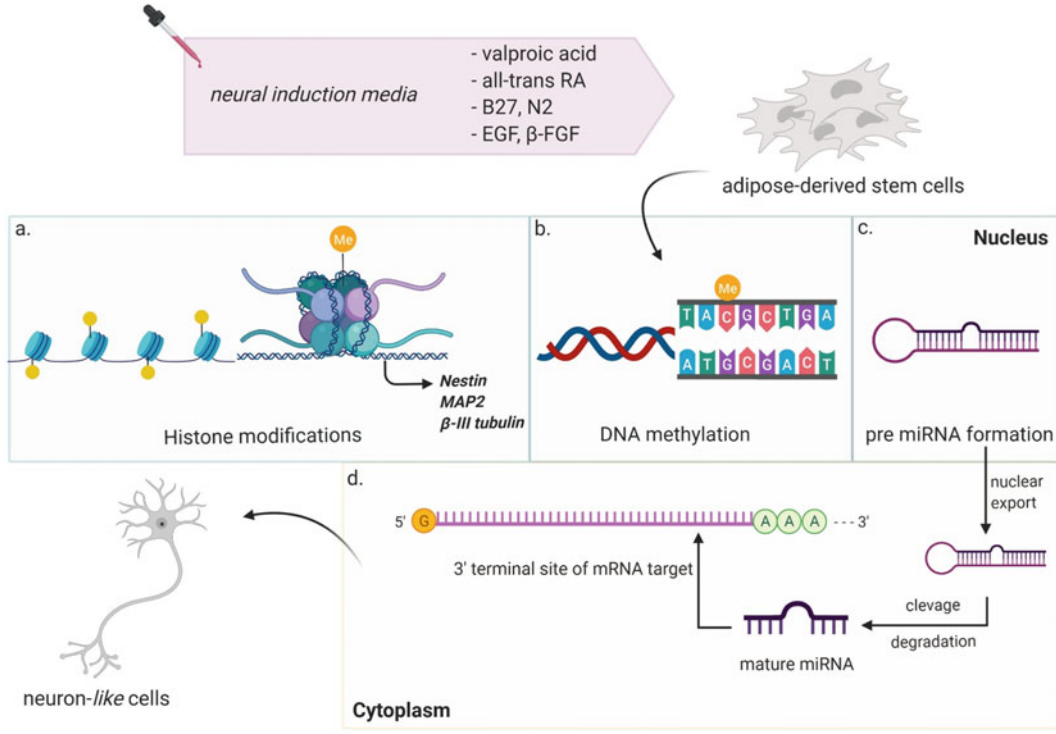

Fig. 3 Molecular events during the trans-differentiation of ASCs in neuron-*like* cells. (**a**) Histone modifications, (**b**) DNA methylation (**c**) miRNA biogenesis and (**d**) miRNA actions upon mRNA targets involved in the differentiation process (Figure created in BioRender.com)

ASCs. Their findings confirmed around 73 miRNAs in neural differentiated ASCs that were not detected in normal ASCs, suggesting that those molecules have specific functions in the nerve cell (Hu et al. 2017). Yang et al. found that miR-142-5p binds to RhoA and ROCK1 mRNA, therefore modulating the neural differentiation of ASCs through control over the RhoA/ROCK1 signaling pathway (Yang et al. 2018). Wu et al. identified a profile of 24 lncRNA and 738 mRNA which were up regulated and 37 lncRNA and 968 mRNAs which were down regulated during the neural induction of mesenchymal stem cells, suggesting their possible involvement in this biological process (Wu et al. 2015).

Another new and elegant approach that might be involved in the differentiation of ASCs could be controlled is the influence of extracellular vesicles. Among them, ***exosomes and their cargo*** turned out to present tremendous interest in this kind of application. By definition, exosomes are a family of extracellular nanovesicles that mediate intercellular communication through their content in proteins and miRNAs (Hong et al. 2019). The bioactive cargo of exosomes has the potential to trigger specific genes or induce epigenetic modifications that have essential functions in the cell, thus demonstrating to be a useful tool for cell-based regenerative therapies (Hayashi et al. 2017). There are more possible approaches in which exosome can act in the setting of the nervous system trauma: (i) to use ASC-derived exosomes in order to enhance proliferation in SCs or to promote neurite outgrowth in the neuron, both leading to regeneration in case of injuries or (ii) to use neuron-derived exosomes to promote the differentiation potential of ASCs in neuron-*like* cells. Recent work demonstrated the presence of BDNF, insulin-like growth factor-a (IGF-1), NGF and GDNF in ASCs-exosomes proving their potential for peripheral nerve trauma (Farinazzo et al. 2015). Roballo et al. evaluated

the potential of exosomes in the neuronal differentiation of ASCs, by co-culture between ASCs and neurons and analyzed the exosomal cargo. Finally, they transplanted the neuron-*like* cell *in vivo* with the purpose to prevent further neuronal degradation. The outcomes of this research were that neurons could induce the neural differentiation in ASCs, by exosomal transfer and the delivery of miR-9 and miR-132 and neuronal differentiated ASCs prevented neuron degeneration *in vivo* (Roballo et al. 2019).

5.2 Epigenetic Control

In the last decade, research has focused on exploring ASCs' differentiation potential towards neural lineages. Even though amazing progress has been made, no reports indicate obtaining fully functional mature neurons or glial cells derived from ASCs. Therefore, it is pivotal to first understand exactly which molecular changes occur during this event in order to make ASCs a more useful tool in the setting of nervous system disorders and traumas. Some of the key players in the determination of stem cell fate and differentiation are the *epigenetic factors*. Published research has indicated that epigenetic events are critical during gene expression in the event of stem cell differentiation (Ariff et al. 2012; Srinageshwar et al. 2016; Atlasi and Stunnenberg 2017). Epigenetic factors which have been found to be involved in this particular differentiation process of ASCs to neurons are DNA methylation and histone modifications (Atlasi and Stunnenberg 2017). Moreover, it seems that ASCs possess their *own epigenetic barrier*, which can only be crossed with the help of specific growth and transcription factors of neural cells (Encinas and Fitzsimons 2017; Luo et al. 2018). As this process brings major changes in gene expression and epigenetic profile, it is essential to study these changes in-depth in order to elucidate ASCs' true potential for nervous system regeneration (Fig. 3). Even so, the research for epigenetic control during this event is very limited and many processes remain still unknown.

5.2.1 Histone Post-transcriptional Modifications

Histone post-transcriptional modifications (Fig. 3a) include histone methylation, acetylation, and phosphorylation of the end sites of the histone core. The methylation process happens on the side chains of arginine and lysine and does not alter the charge of the histone protein (Bannister and Kouzarides 2011). The expression of the early neural markers such as Nestin, which also appears to be expressed in ASCs-derived neurons, appears to be regulated through this epigenetic mechanism (Boulland et al. 2013). Dynamic changes in histones H3K4, H3K9 and H3K27 methylation on nestin locus during neurogenic differentiation have been reported, suggesting ASCs potential to differentiate beyond mesodermal cell types (Boulland et al. 2013). Whereas, the dynamic reaction of phosphorylation occurs mostly in the N-terminal sites of histone tails, more specific on serine, threonine, and tyrosine. The most abundant histone modification is the acetylation of lysine which is regulated by the action of two big families of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs) (Bannister and Kouzarides 2011). The functional manner and the role of these modifications in ASCs neural differentiation are still largely unknown. So far, it has been reported that upon HDAC inhibitors (sodium butyrate, trichostatin A and valproic acid), ASCs differentiated into neuron-*like* cells as demonstrated through qPCR and Western blotting techniques. Simultaneously it was found out that most of the Wnt-related genes were found to be highly expressed after inhibitor treatment, indicating the activation of Wnt signaling pathways (Jang and Jeong 2018). Studies demonstrate that the addition of valproic acid to the differentiation media significantly increases the neural differentiation of mesenchymal stem cells by leading to the up-regulation of specific neural markers such as Tuj-1 and MAP 2 (Talwadekar et al. 2017). All brought together, it can be stated that further investigations are required in order to elucidate the true nature of the epigenetic control during neural differentiation of ASCs. A better

understanding of this phenomenon could lead to ground-breaking stem cell-based therapies for neural disorders and injuries.

5.2.2 DNA Methylation

DNA methylation (Fig. 3b) represents an epigenetic mark during which a methyl group is transferred to the C5 position of the cytosine ring of DNA. This whole reaction is catalyzed by DNA methyltransferase (Jin et al. 2011) and the DNA methylation site in the ASCs genome plays a crucial role in their further lineage commitment (Berdasco et al. 2012). Therefore, targeting these positions may significantly switch cells' fate towards neural commitment. Interestingly, Alexanian (2015) has induced neuronal differentiation in mesenchymal stem cells which not only were capable of turning back to adult stem cells, but also presented adipocyte morphology when exposed to adipogenic differentiation media. All these treatments have included chromatin-modifying agents, demonstrating that these agents can increase cells plasticity and lineage commitment of mesenchymal stem cells.

6 Tissue Engineering of the Nervous Tissue – Current Stage

The regeneration of the nervous system is a difficult process for there is yet no clear and validated strategy that may have satisfying results and fully functional recovery. The field of TE has emerged as a necessity due to the fact that the request for organ transplantation increases significantly year by year. The principle of TE relays in the combination of engineering and life science in the hope of developing natural biological substitutes that can guide injured tissue towards regeneration and function recovery. Thus, the three important elements that form a biological construct are an engineered scaffold, the presence of growth factor or other enhancing molecules and finally, the adequate cell type. However, TE is not the only method which is proposed for the regeneration of nervous tissues. Attempts have been also made with the help of cell-based therapies or by using more modern and new techniques, the development of organoids, in this case brain organoids. As the nervous system possesses an intrinsic regeneration capacity and a remarkable plasticity scientist seized the opportunity to create a large pale of biomaterials which should promote the regeneration potential of the nervous tissue (Khaing and Schmidt 2012). Over the last decades researchers have come to better understand micro-patterning techniques and mechanotechnology, therefore the desire to generate biomaterials for nervous tissue engineering grew rapidly. For the purpose of this type of regeneration, many different natural or synthetic components were used to fabricate the proper biomaterial in order to mimic the extracellular matrix (EMC) as good as possible. Based on several studies (Sell et al. 2010; Boni et al. 2018) natural-based polymers and composites have proven to have a better efficiency in replicating EMC components as they present biofriendly and biocompatible proprieties in contrast to synthetic materials which have limited outcomes in clinical trials. Moreover, the use of a natural derivate as the main component of the materials' structure is known to ensure low levels of cytotoxicity and a minimal immunogenic reaction after transplantation (Kriebel et al. 2017; Ai et al. 2014). Some of the most used materials in this direction are the biomaterials based on collagen, gelatin and silk. Altinova et al. tested a collagen-based material in rats that suffered from spinal cord injuries. Their results showed that these kinds of grafting in the setting of spinal cord injuries are a necessity for a better outcome of the regeneration process (Altinova et al. 2019). Besides the basic material, modern material fabrication methods include nanostructures as a second element to the biomaterial that may have a maximizing effect on the regeneration. Such structures include nanoparticles (gold, diamond or magnetic nanoparticles) (Paviolo and Stoddart 2015), carbon nanotubes (Olakowska et al. 2010) or graphene oxide (Wang et al. 2019). For

instance, the nanoparticles are considered to be an optimal drug delivery system that could transport specific factors deep in affected brain regions and to assist the regrowth of damaged neurons, as they have the capacity to cross the blood-brain barrier (Elzoghby et al. 2018; Binan et al. 2014). Therefore, the use of these kinds of scaffolds in combination with the use of stem cells could be a perfect tool for nervous tissue regeneration.

Due to the limitations of cell variety in scaffolds, other techniques were developed in order to examine specific disorder scenarios. Thus, the *in vitro* three-dimensional *organoids* have been proposed to provide more inside information about several particular mechanisms. Among them, also brain organoids have been developed as a model to study brain development, circuitry, neuronal structures and several degenerative disorders (Kawada et al. 2017).

7 Conclusions

To conclude, ASCs are a distinguished category of stem cells with unique abilities that hold great promise in elucidating many molecular mechanisms, with the final purpose of validating these cells as treatment for neural disease and nervous tissue regeneration. Therefore, ASCs have been lately widely explored in order to establish their use for nervous system clinical applications. Modern and new generation techniques have made it possible to observe the behavior of ASCs upon neural differentiation and get new information on the molecular events that take place during this complex process. The whole molecular machinery changes in ASCs upon several cues that induce neural differentiation, but there are lots of unknown factors that remain to be discovered. Even so, ASCs show so far great applicability for the field of CNS and PNS repair, regeneration and disease treatment. More efforts need to be made in order to therapeutically target several factors involved in the neural differentiation of ASCs in the setting of nerve trauma, spinal cord injuries or neurodegenerative diseases.

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Biomatrices for Heart Regeneration and Cardiac Tissue Modelling *In Vitro*

I. Kulvinskiene, R. Aldonyte, R. Miksiunas, A. Mobasheri, and Daiva Bironaite

Abstract

Cardiac muscle is the hardest working muscle in the body, pumping approximately 70 g of blood with every heartbeat, circulating 9500 l of blood daily and contracting over 3 billion times during the average human's life. Heart failure – a heterogeneous syndrome – is a major and increasing health care problem worldwide and a leading cause of hospitalization and morbidity in elderly. Adequate heart tissue regeneration in human is lacking. Challenges to engineer heart tissue and employ it in vitro or in regenerative medicine remain to be solved. First of all, cardiac tissue bioengineering requires robust and powerful cells capable of differentiating into cardiomyogenic lineages in combination with effective, safe and highly specialized biomaterials, hydrogels and/or scaffolds for recreating the native extracellular microenvironment. Advances in stem cell and biomaterial science already provided an increasing array of cell resources, their cultivation technologies and biomatrices for efficient and safe cardiac tissue reconstruction. In order to develop new cardiac tissue mimicking technologies *in vitro*, it is necessary to analyze the advantages and drawbacks of already established biosystems. Therefore, in this paper, we provide a comprehensive overview of recently employed cells, 2D and 3D biomatrices for cardiac tissue engineering and review the current state-of-the-art in this field as well as future directions.

Keywords

Biomaterials · Biomatrices · Cardiac muscle · Regenerative medicine · Remodeling · Tissue engineering

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Abbreviations

ADSC	adipose tissue-derived stem cells
AuNRs	albumin electrospun fibers and gold nanorods
BMP4	bone morphogenetic protein 4
CDC	cardiosphere-derived cells

CM	cardiomyocytes
CNT	carbon nanotubes
CPCs	cardiac progenitor cells
CPS	cardio progenitor spheres
CVD	cardiovascular diseases
EBs	embryonic bodies
ECM	extracellular matrix
ESCs	embryonic stem cells
GAG	glycosaminoglycan
G-CSF	granulocyte colony-stimulating factor
GSK3	glycogen synthase kinase 3
HF	heart failure
hFFs	human foreskin fibroblasts
HGF	hepatocyte growth factor
IC	intracoronary infusion
IGF-1	insulin-like growth factor
IM	intramyocardially
iPSC	induced pluripotent stem cells
LVEF	left ventricular ejection fraction
MEF	mouse embryonic fibroblast
MRI	magnetic resonance imaging
MSC	mesenchymal stem/stromal cells
Nrg	Neuregulin-1
PCL	polyε-caprolactone
PCL	poly-ε-caprolactone
PDGFRα	platelet-derived growth factor receptor-alpha
PFHy	polyethylene glycol-fibrinogen hydrogel
PGA	poly(glycolic acid) or poly-glycolide
pHLIP	pH low insertion peptide
PIPAAm	poly N-isopropylacrylamide
PLA	poly-lactic acid
PSC	pluripotent stem cells
RCVI	retrograde coronary venous infusion
Sca − 1	stem cell antigen-1
SDF-1	stromal-derived growth factor 1
TESI	transendocardial injection
TGF-β	transforming growth factor β
VEGF	vascular endothelial growth factor

1 Introduction

Cardiovascular diseases (CVD) are the primary cause of morbidity and mortality worldwide, with ischemic heart disease being the primary cause of death globally (Roth et al. 2017). One of the major CVD types, heart failure (HF), affects 26 million people worldwide including 7.5 million in North America and is increasing in prevalence (Ponikowski et al. 2014; Savarese and Lund 2017). Heart transplantation is very often the only treatment of choice for many patients with end-stage HF who remain symptomatic despite of optimal medical treatment. Moreover, the number of people with impaired myocardial function leading to heart failure is increasing and requires more and more heart transplants. The current imbalance between needs and possibilities for cardiac transplantation is partially due to the limited number of donors and graft rejections as the major risk on the one side, and growing transplantation capabilities in a clinical setting on the other. It is becoming increasingly clear that less invasive and more sophisticated and more targeted strategies are needed to repair and restore damaged myocardium.

The heart is very complex organ, composed from various types of the cells such as cardiomyocytes (CM), cardio fibroblasts (primary mesenchymal stromal cells), endothelial and perivascular cells and is mechanically and electro physiologically active. It has been suggested that cardiomyocytes comprise major part of mammalian heart (70–80%) (Tang et al. 2009). However, recent stereological analysis of histological human heart section suggested ratio between CM, mesenchymal cell and endothelial cell populations being 33%, 58% and 24% and estimated by flow cytometry – 33%, 43% and 24% (Bergmann et al. 2015). While it is agreed that cardiomyocytes account for approximately 25–30%, the composition of non-myocyte population estimated by immunohistochemistry still differ: endothelial cells over 60%, hematopoietic-derived cells 5–10%, and fibroblasts under 20% (Pinto et al. 2016). Such discrepancy of obtained results might be related to the great diversity of used tissue sources and estimation methods. Despite that, both types of the cells, myocyte and non-myocyte, respond to external physiological and pathological stimuli and cell-cell signals important for human heart functioning and regeneration. In addition, it should not be forgotten that the heart tissue is

highly electrically cell-cell coupled and mechanically active tissue that hardens therapeutic application of tissue engineering technologies.

The limited inherent regenerative capacity of heart tissue is a key factor resulting in progression of heart disease, mortality and also restricted abilities to heal or regenerate (Ong et al. 2017). Regenerative capacity of the human heart is around 1% at the age of 25, and it decreases to 0.45% at the age of 75 (Bergmann et al. 2009). Therefore, studies on locally delivered pro-regenerative molecules, cells and/or combined strategies incorporating cells, tissues and biomaterials are performed extensively worldwide. To successfully develop cardiac therapeutic interventions, 2D and especially 3D cardio modeling *in vitro* is crucially important before any *in vivo* studies are initiated. However, it is still challenging to develop novel multiparametric platforms mimicking 3D cardiac cell environment on biomatrices in combination with electrical, metabolic and mechanical stimuli for the research and therapeutic strategies (Qiao et al. 2019). Recapitulating the cardio microenvironment and cellular morphology, several significant challenges are particularly important: optimal support for cell attachment and alignment, proper distribution of loaded cells, relevant level of stiffness of biomatrices, biocompatibility and optimal their disintegration over the time (Schwach and Passier 2019; Mathur et al. 2016). Moreover, 3D tissue engineering technologies are not only investigated in laboratories, but were already used in the clinical setting, i.e. delivered stem cells to the failing and infarcted human hearts (Menasche et al. 2018).

This review focuses on 2D and 3D myocardial regeneration technologies that were recently designed for *in vitro* studies and for therapeutic strategies. The major scientific challenges in the field of cardiac tissue engineering and repair include selection of the cells with highest cardio regenerative potential and development of the cultivation techniques on bioactive hydrogels and scaffolds *in vitro* (Fig. 1).

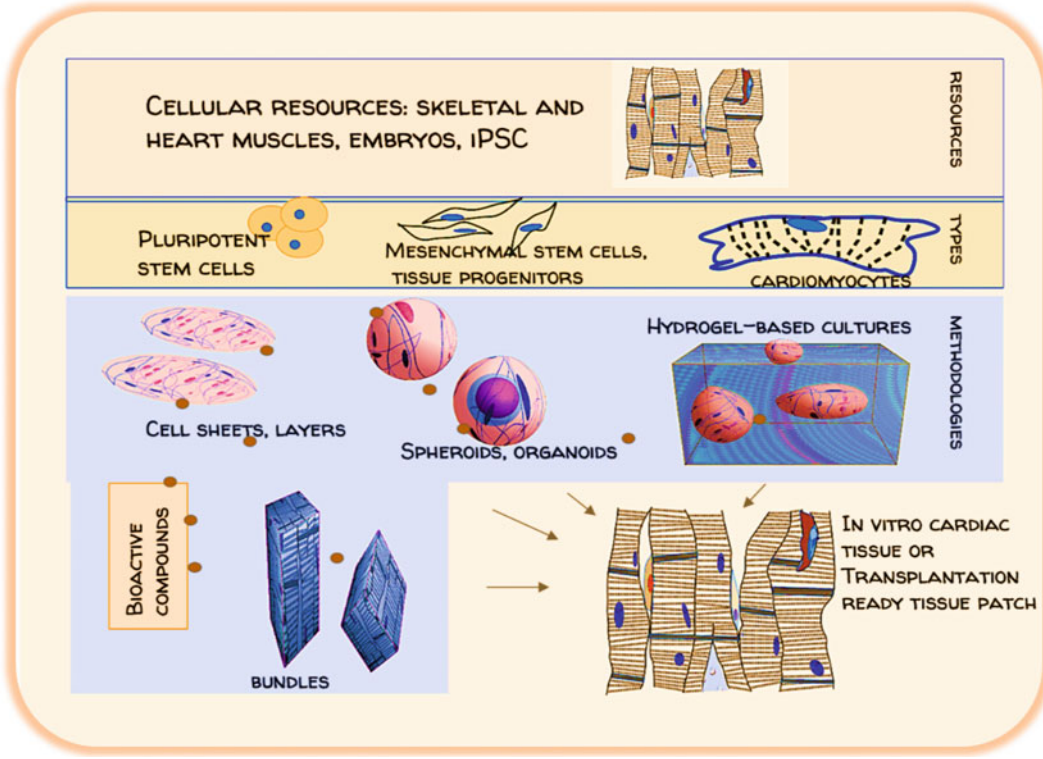

Fig. 1 Cells and technologies used for cardio-regenerative purposes

1.1 Heart Tissue-Derived Cells for Cardio Regeneration

Cardiac regeneration comprises a waste range of efforts to regenerate/repair irreversibly damaged heart muscle with cutting-edge science, including cell-based and cell-free strategies. Cellular content remains a major reparative tool used in restoration of damaged tissue's structures and functions. Cells are also essential component for *in vitro* heart tissue modeling. Many types of cells with cardio regenerative potential are being studied and already tested in clinical trials. Although functional cardiomyocytes can be grown from pluripotent stem cells, a strong enthusiasm to use progenitor cells of heart origin remains.

1.1.1 Heart Stem Cells

For many years heart self-regenerating capabilities were not recognized and cardiomyocytes were the only cells investigated in healthy and diseased hearts (Mitcheson et al. 1998). Later, several theories of cardiac regeneration emerged: it was shown that 4% of myocyte nuclei express Ki-67 and new CM may be derived from division of pre-existing CM (Beltrami et al. 2001; Senyo et al. 2013); from primary, mesenchymal type cells (Davis et al. 2010) or from stem cells residing in heart tissue (Bearzi et al. 2007). Unique markers for heart stem cells are still under investigation. So far, the researchers agreed that cardio stem cell population is heterogenic with dominating markers: c-Kit, stem cell antigen-1 (Sca-1); Islet-1 and platelet-derived growth factor receptor-alpha (PDGFRα+) (Ge et al. 2015). c-kit is a surface protein that acts as a receptor for stem cell factors triggering proliferation or differentiation (Marino et al. 2019; Foster et al. 2018). Mature cardiac myocytes do not possess c-kit, whereas the presence of c-kit on cardio stem cell surface suggests the ability of these cells to differentiate into several heart cell types (Ellison et al. 2013). For some period, the presence of c-kit on cardiac progenitor cells has been one of the main parameters used to define cardiac stem cells (Bearzi et al. 2007). However, it was shown that Isl1+ is also a marker of cardio progenitor cells capable of differentiation into three major cardiac cell types, i.e. CMs, endothelial cells and smooth muscle cells *in vitro* (Li et al. 2017). The combined technologies using mixture of mouse Isl1+ cardiac progenitor cells (CPCs) mixed with vehicle containing extracellular matrix (ECM) components fibrinogen and thrombin stimulated recovery of cardiac function in post-MI model (Wang et al. 2017). Cardiomyocytes, derived from the same mouse Isl1+ cardio progenitors spontaneously contracted and responded to β-adrenergic stimulation. The bone marrow MSC also comprise a category of cardiomyogenic, vasculogenic and/or fibrogenic c-kit-positive cells, whereas some c-kit-positive cells remain undifferentiated within the damaged heart (Czarna et al. 2017). The cardiomyogenic potential of c-kit positive cells was shown to be doubtful and so-called "String theory" (Keith and Bolli 2015). As endogenous c-kit positive cells within adult cardiac myocardium is negligibly small (Vicinanza et al. 2017), a large amount of biopsy tissue is required and long period of cultivation is required in order to derive clinically significant number of c-kit positive cells with doubtful heart regeneration result. All mentioned drawbacks forced research to find other cell candidates for cardiac tissue regeneration.

1.1.2 Cardio Progenitor Spheres-Derived Cells

Currently, another promising approach is gaining attention, i.e. employment of cardiac tissue-derived spontaneously self-assembling cardio-progenitor spheres (CPS) for obtaining large numbers of cardiosphere-derived cells (CDC) that can be transplanted (Marban et al. 2012). Cardio progenitor spheres (CPS) contain predifferentiated stem-like cells that grown in 3D and high density were able to differentiate to cardiomyocyte and endothelial phenotype cells trough the NOTCH and Wnt pathways (Vukusic et al. 2013). CPSs are identified as population of autologous cardiac progenitor cells, obtained by outgrowing from the biopsy material, expanding and forming multicellular spheroid bodies, and most importantly capable to regenerate human

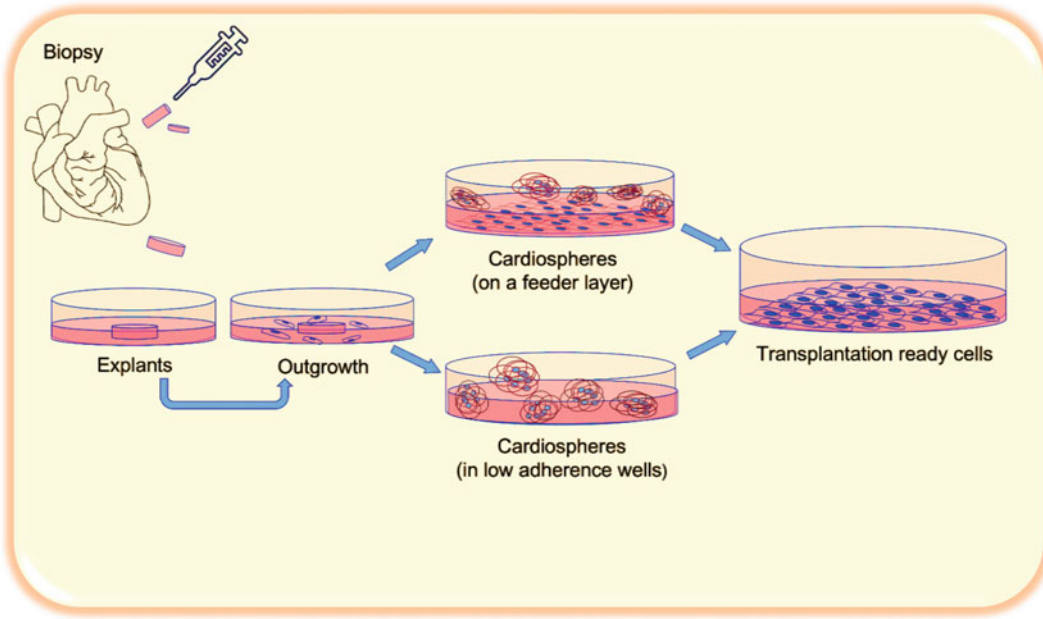

Fig. 2 Generation of heart tissue-derived cardiospheres and cardio-sphere-derived cells (CDC) used for transplantation

cardiomyocyte population (Fig. 2) (Davis et al. 2010).

CDC markers, beside c-kit, are more related to the adult mesenchymal stem cells (MSC), such as positive CD105 (the transforming growth factor-β subunit) and negative CD45 (a hematopoietic marker) (Ashur and Frishman 2018). CD105 positive canine CDC infused intravenously in a doxorubicin-induced mouse model of dilated cardiomyopathy improved cardiac function, decreased cardiac fibrosis and increased capillary density. Indeed, it was found that the autologous CDCs were able to better restore left ventricular ejection fraction (LVEF) than the MSCs in pigs with heart failure (Lee et al. 2011). Human heart biopsies-derived CDCs expressed gap-junction protein connexin 43 and possessed other adult MSC (also named as primary fibroblasts) markers such as CD105, CD90, CD73, endothelial cells markers (CD34, CD31), and were negative for hematopoietic markers (CD133, CD34, CD45) (Smith et al. 2007; Davis et al. 2009; Marban et al. 2012). Unlike mature CM, CDC cells express Ki67, a mitotic indicator located in the nucleus, and are neither striated nor rectangular.

When implanted into a rat heart, CDC demonstrated the ability to contract and expressed cardiac specific proteins (Marban et al. 2012). CDCs, derived from human endocardium biopsies, proliferate fast when cultured *ex vivo* and differentiate into endothelial cells, cardiac myocytes, smooth muscle cells and are considered to be one of the most promising autologous adult progenitor cells for cardiovascular therapy (Amini et al. 2017). It was shown that CDCs improve cardiac function, reduce scar size and promote viable myocardium in small and large animals with ischemic cardiomyopathy (Malliaras et al. 2013; Johnston et al. 2009; Lee et al. 2011; Smith et al. 2007). CDC improved cardiac contraction and mitochondrial capacity in a rat infarct model as compared to an infarcted heart injected with just a medium (Marunouchi et al. 2018). Human cardiac progenitor population secrete high level of grow factors such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF) and insulin-like growth factor (IGF)-1 and have not only direct but also paracrine effects on cardiac tissue preservation and angiogenesis (Chimenti et al. 2010). It has

been demonstrated in large animal models that CDCs inhibit proliferation of reactive lymphocytes and have potent immunosuppressive activity mediated via PGE2 (Dutton et al. 2018). Despite these promising results, some studies showed that CDCs did not improve heart function in rat heart failure model (Kasai-Brunswick et al. 2017) as well as in rat dilated cardiomyopathy model (Wang et al. 2019).

Altogether, the main advantages of cardio progenitor cells in cardiac tissue regeneration include their ability to form 3D structures and/or organoids, better differentiate to various types of heart cells in comparison to 2D cultures *in vitro* and *in vivo*, safety and possibility of autologous use. Even if CPSs hold promise for cardiac regeneration, more efficient methodologies for their expansion, identification and delivery are needed.

1.1.3 Cardio Progenitors-Based Clinical Trials

Clinical trials with cardio progenitors as well as with pluripotent cells, allogeneic cell products and artificially-modified cells belong to the second-generation of cardiac trials investigating little known cell types such as cardio sphere c-kit + progenitor cells and/or cardiosphere-derived cells (CDC) (Banerjee et al. 2018). CDC delivery to human heart following infarction has caused significantly improved ejection fraction and reduced cardiac remodeling (Cohen and Gaudette 2009). This was demonstrated in a human clinical trial called Cardiac Stem Cells in Patients with Ischemic Cardiomyopathy, SCIPIO (Bolli et al. 2011). The cardiac progenitor cells were isolated from the right atrial biopsies and expanded. These cells were then injected within the vascular bypass graft and left ventricular ejection fraction (LVEF) was used to measure cardiac function. After 1 year, LVEF improved by 8% and in following 4 years – by 12%. Though this success is modest, it demonstrates the ability of cardio sphere-derived cells to return mechanical function to a damaged human heart.

Another successful clinical trial CADUCEUS (Cardiosphere-Derived Cells For Heart Regeneration After Myocardial Infarction) showed cardiosphere-derived cells decreasing scar mass and regenerating viable myocardium in both animal and human models (Makkar et al. 2012). Cardiac MRI showed a significant improvement in right ventricle EF, cardiac tissue regeneration, and an overall clinical improvement in heart failure status. Another promising application of CDCs is the use of allogeneic cell therapy (Kapelios et al. 2016). The ALLSTAR trial looked at the effects of intracoronary allogeneic stem cell infusion on patients with an anterior myocardial infarction (MI) within the prior year (Chakravarty et al. 2017). Using cardiac magnetic resonance imaging (MRI), a 15% relative reduction in infarct size with a 4% improvement in EF has been demonstrated in the treatment group after 1 year. Patients with ischemic and nonischemic dilated cardiomyopathy in ongoing clinical trial (DYNAMIC) have been treated with allogeneic myocardium-derived progenitors CDC and safety and efficacy of intracoronary infusion of allogeneic CDCs in patients was observed (Chakravarty et al. 2019). However, CPS and CDC-based clinical trials also have some limitations, i.e. lack of suitable biomarkers, requires big size of human heart biopsy, high heterogeneity of population, limited cardiomyogenic differentiation and functional adaptation, long lasting isolation and expansion procedures (65 days and longer until clinical use, which is a relatively long period for patients waiting for treatment). Many clinical trials were done and knowledge and expertise are building up, but many more trials and investigations need to be done for a better cardio regeneration result.

1.2 Pluripotent Stem Cells

In order to develop biomimetic tissues for successful cardiac regeneration and for studies *in vitro* it is necessary to choose right type of the cells with most intense cardio regenerative properties. Multiple studies have shown that pluripotent stem cells (PSC), i.e. embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are the most efficiently self-renewing, indefinitely maintained and expanded in culture *in vitro* without losing pluripotent state cells that

produce daughter cells with the same properties of the progenitor cell and differentiate into multiple cell types of therapeutic interest of all three germ layers (Peischard et al. 2017; Abou-Saleh et al. 2018). Both types of pluripotent cells are cultivated in 3D aggregates that mimic the natural human embryo growth factor gradient and cell-cell interaction (Sachlos and Auguste 2008). Therefore, the pluripotent stem cell-derived cardiomyocytes bring promising significance for basic research, disease modeling, drug development and regenerative medicine.

1.2.1 Embryonic Stem Cells

ESC are pluripotent stem cells derived from the inner cell mass of early stage embryo (Martin 1981). Although human ESC are derived from embryos or fetal tissue, they are not embryos by themselves (from the National Institutes of Health Guidelines for Research Using Human Pluripotent Stem Cells). Initially, the cultivation of human ESC (hESC) cells on mouse embryonic fibroblast (MEF) feeders was important for saving hESC pluripotency (Xu et al. 2001). However, for the generation of the cardiac tissue, the combined technologies using chemical inducers, cardio ECM and/or secreted components incorporated into hydrogel matrices and pluripotent cell derived-CM gave most promising results. Animal studies revealed cardiac environment triggering differentiation of murine ESC into functional cardiomyocytes and mediators belonging to the transforming growth factor β (TGF-β) family TGF-beta and BMP2 up-regulated mRNA of mesodermal (Brachyury) and cardiac specific transcription factors (Nkx2.5, MEF2C) (Behfar et al. 2002). To induce cardiogenesis, human ESCs have been also treated with TGF group stimulus activin A and BMP-4 (Laflamme et al. 2007). The hESC-CMs, obtained by incubation of hESC with pro-survival cocktail (30-min heat shock followed by RPMI-B27 medium supplemented with IGF1 and cyclosporine A) and injected into monkey MI models showed favorable effects (Chong et al. 2014).

Comparing cardioregenerative effect of hESC-CMs and hESC derivatives on infarcted rats myocardium revealed the predifferentiated hESC-CMs form more stable cardiomyocyte grafts, better attenuate the pathologic remodeling process and show functional benefit on the heart compared to undifferentiated pluripotent stem cells (Caspi et al. 2007). The clinical application of ESC for the cardiovascular or heart tissue regeneration requires linearization of ESC. Recently produced clinical-grade hESC-derived cardiovascular progenitors supported their short- and medium-term safety properties used in patients with severe ischemic left ventricular dysfunction (Menasche et al. 2018). However, the employment of ESC in regenerative medicine brings limiting ethical and safety issues forcing to investigate new types of pluripotent stem cells such as induced pluripotent cells (iPSC).

1.2.2 Induced Pluripotent Stem Cells

The more than decade ago, introduction of pluripotent stem cells, generated from adult somatic cells, has revolutionized stem cell science as reviewed by the discoverers themselves (Yoshida and Yamanaka 2017). The main advantages of pluripotent stem cells, compared to the other types of stem cells, are their differentiation to specialized cell types of all three germ layers: endoderm (interior stomach lining, gastrointestinal tract, the lungs), mesoderm (muscle, bone, blood, urogenital), or ectoderm (epidermal tissues and nervous system), but not into extra-embryonic tissues like the placenta both *in vivo* and *in vitro*. iPSCs, opposite to ESC, has no ethical limitations, can be autologous and provide an excellent platform for customized diseases' modeling, including cardiac, and therapeutic drug discovery (Matsa et al. 2014).

Similar to ESC, iPSC can be used to regenerate cardiovascular system (Kattman et al. 2011). Mesoderm stage pluripotent cells expressing Flk-1/KDR and PDGF receptor alpha, Activin/Nodal and BMP4 signaling pathways potentially differentiate to cardiomyocytes (up to 60%). However, the need to optimize cardio differentiation conditions for individual human iPSC cultures in order to get the best functional response of heart tissue still exist (Kattman et al. 2011). Chemical mixture of bone morphogenetic protein 4 (BMP4), inhibitor of glycogen synthase

kinase 3 (GSK3) CHIR99021 and ascorbic acid was sufficient to rapidly convert monolayer-cultured human iPSCs into homogeneous cardiovascular progenitors (Cao et al. 2013). Beside the chemical cardio induction, 3D self-assembling iPSC-based cardiac tissue biomimetics were most promising for cardiac regeneration. Heart tissue strips engineered from human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes and hiPSC-derived endothelial cells and transplanted to infarcted guinea pig heart improved left ventricular function by 31% (Weinberger et al. 2016). Human iPSC-derived CM transplanted into infarcted guinea pig also created cardiomyocyte network, decreased scar size and improved left ventricle functioning (Castro et al. 2019). Human ESC and iPSC-derived CM, when injected into infarcted murine hearts, have improved myocardium functions significantly better than un-differentiated pluripotent cells mainly through the secreted cardiac specific paracrine factors (Tachibana et al. 2017; Oikonomopoulos et al. 2018).

Although pluripotent cell application belongs to the second generation of clinical trials, broad clinical application of iPSC has also some limitations related to spontaneous mutations, heterogeneity of population, feeder-layer growth conditions, incomplete integration within heart tissue and insufficient recovery of beating functions. Despite some promising data, the usage of human ESCs have critical ethical limitations and remains controversial, whereas clinical trials with iPSC-derived phenotypic cells are still in their infancy and genetic instability remain to be solved before iPSC enter clinical settings with uncompromised safety.

1.3 Mesenchymal Stem/Stromal/ Medicinal Signaling Cells

It is agreed that heart tissue is not a postmitotic organ, constitute of cardiomyocytes, fibroblasts (or mesenchymal stromal cells), endothelial cells and have some, albeit limited, regenerative capacity (Bergmann et al. 2015). Since the adult human heart has very limited number of cardiac progenitor cells (Simpson et al. 2012), the mesenchymal stem/stromal cells (MSCs), having fibroblasts morphology and phenotype, can be successfully used for cardio regenerative purposes (Jackson et al. 2017; Denu et al. 2016). The mesenchymal stem cells was suggested to name "multipotent mesenchymal stromal cells", adult mesenchymal" or "adult stromal" cells (Horwitz et al. 2005). Recently it was suggested to rename MSC to "medicinal signaling cells" mainly due to discovered their paracrine effects (Caplan 2017). Adult tissue-derived MSCs are capable of dividing, renewing themselves, and differentiating into mesodermal type cells such as osteoblasts, chondrocytes, myocytes and adipocytes (Pittenger et al. 1999). Traditional isolation of MSC from bone marrow (BM) or other tissues is based on isolation of multinuclear cells and their cultivation in plastic-adherent way thus separating MSC from hematopoietic cells (Gronthos et al. 2003). However, the colony-forming adult MSCs are a mixture of mesenchymal, progenitor cells, endothelial and other type of the cells that must be purified and/or populations with the best cardiomyogenic potential (such as Sca-1) isolated (Seshi et al. 2000; Siegel et al. 2012). The identification of MSCs according to the expression of specific surface agents also is facing difficulty to find unique and specific biomarkers (Maleki et al. 2014). MSCs exert paracrine and autocrine effects via secretion a variety of cytokines and growth factors able to suppress immune cells, inhibit fibrosis and apoptosis, enhance angiogenesis, stimulate mitosis and differentiation of tissue progenitors and stem cells (Caplan and Dennis 2006).

The regenerative capacity of the human heart is around 1% at the age of 25, and decreases to 0.45% at the age of 75 (Bergmann et al. 2009). The regenerative potential of post infarcted heart is further reduced due to loosing huge amount of cardiomyocytes and causes heart failure (Olivetti et al. 1991). Therefore, the MSCs are extensively studied in fundamental, pre-clinical and clinical cardiac studies as additional cell source for regeneration purposes. MSCs of different origin were studied in the context of regeneration of heart tissues, some of them, namely human BM-MSC,

adipose tissue-derived stem cells (ADSC) and dental pulp-derived MSC, were demonstrated to differentiate into cells with a cardiac phenotype expressing GATA4 and Nkx2.5 (Arminan et al. 2009). Adult smooth muscle (SM)-derived MSCs and ADSCs injected into the myocardium of nude mice 1 week after MI improved LVEF predominantly by reduction of infarct size and increasing vascularized granulation tissue in the border zone (Beitnes et al. 2012). However, despite improved LVEF and infarct size in nude rats, the human skeletal muscle-derived MSCs and ADSC did not transdifferentiate into cardiomyocytes or any vascular cells (Beitnes et al. 2012). Human amniotic epithelial cells and cord blood-derived MSCs were also successfully used to decrease areas of myocardial infarction in athymic nude rats (Fang et al. 2012). The combination of two types of stem cells, human BM-MSCs and c-kit(+) cardioprogenitors, were shown having better MI size reduction effect in Yorkshire swine hearts than individual cells (Williams et al. 2013).

Many studies suggest that MSCs are mitigating degeneration within myocardial infarction zone without actually directly replacing the lost tissue but rather by local modulatory effects. Such effects of MSC in myocardial infarction zone could be attributed to either paracrine factors, i.e. to numerous secreted growth factors, chemokines and cytokines that promote angiogenesis, prevent apoptosis and modulate inflammatory responses (Cunningham et al. 2018), or to cell-cell contact with or without fusing of cell membranes (Reinecke et al. 2000). The MSC secrete insulin-like growth factor (IGF) – an important growth factor not only for the MSC niche but also for the proliferation of cardiac myocytes in human embryo and in Zebrafish (Youssef et al. 2017; Huang et al. 2013). In addition, MSC secrete factors that are packed in exosomes and participate in cardiac repair. The growing body of evidence suggest exosomes, when isolated and applied clinically, are more safe, more efficient and with better therapeutic choice (Bagno et al. 2018). It is possible that the reduction of heart scar size, observed in MSC therapy, can be attributed to the induced myocyte proliferation and regulated Notch pathway (Xuan et al. 2020). Evidence suggests that some MSCs, i.e. monocyte subpopulation-derived MSCs (CD14+; CD45+; CD34+; type I collagen+), in the prolonged (21 d.) co-cultures with GFP-tagged cardiomyocytes induce generation of spontaneously beating human cardiomyocytes without cell fusion (Kodama et al. 2005). The anoxic pre-conditioning of MSCs used in the rat diabetic cardiomyopathy model also improved functioning of cardiomyocytes through the upregulation of Bcl-2/Bax ratio and inhibition of caspase-3 activation (Li et al. 2008). The bone marrow MSCs under hypoxic conditions secreted vascular endothelial factor (VEGF), which also benefit the native myocytes trough inhibition of miRNA-23a and miRNA-92a and suppression of cardiomyocyte apoptosis (Song et al. 2017). In addition, various types of injectable, fast gelling, and thermosensitive hydrogels have been tested to improve BM-MSC delivery to the injured rat heart sites in order to promote paracrine effects and tight muscle retention by grafted cells and to select most suitable for the grafting of cells into human heart and skeletal muscle tissues (Niu et al. 2019).

Many first-generation preclinical trials to treat acute or chronic MI have been performed with skeletal myoblasts, BM-MSC, peripheral blood mononuclear cells, ADSC, BM-MSC, embryonic endothelial cells, autologous unfractionated BM and other, whereas for the clinical trials the most popular were skeletal myoblasts, BM-MSC, ADSC and autologous unfractionated bone marrow with circulating MSC progenitors (Gathier et al. 2018; Sanganalmath and Bolli 2013). Nonrandomized clinical trials of skeletal myoblasts at the beginning gave encouraging results, but later on failed to confirm positive effects on the heart (Veltman et al. 2008). Adipose tissue, as well as BM-ADSC for a long time also have been attractive object of clinical trials possessing proangiogenic CD34+ MSC (Henry et al. 2017). Bone marrow MSC injected intracoronary have been also shown increased left ventricle ejection fraction (EF) in patients (Lipinski et al. 2007). Among most promising MSCs, umbilical cord-derived cells have been tested in patients with HF caused by ischemic

cardiomyopathy and improved left ventricle ejection fraction (LVEF) and walking test (Fang et al. 2016). In addition, improved left ventricular function, functional status of heart and improved quality of life have also been reported in patients treated with umbilical cord MSCs (Bartolucci et al. 2017). However, it is still not clear which type of adult MSCs is the best for cardiomyogenic differentiation and heart regeneration, however, due to the safety, availability, high proliferation and differentiation potential, immune modulatory and migration properties, autologous and allogenic bone marrow-derived MSCs seems to be the most promising type of MSCs in cardiac engineering (Guo et al. 2018). Recently, a second generation of clinical trials using cardiac progenitors and/or pluripotent cell predifferentiated CM in combination with 3D technologies are taking over.

2 2D or 3D Cardiac Tissue Engineering Techniques: Advantages and Disadvantages

While designing cell-based therapies for cardiac dysfunctions or *in vitro* testing systems, a crucial step is to select an appropriate tissue culture model for *in vitro* experiments. The first cell-based technology to regenerate failed heart was developed by directly injection of autologous cardiomyocytes into mouse heart failure model (Soonpaa et al. 1994). Later on, other types of autologous cells such as skeletal myoblasts, BM-MSC and other types have been cultivated in 2D, dissociated and used to regenerate human heart tissue after injection (Menasche et al. 2001; Wollert et al. 2004). In order to get better cardiomyogenic differentiation response, 2D surfaces are often modified by precoating with ECM components. Precoating of cell growth surfaces by collagen I or laminin proteins was more effective in promoting cardiomyocyte interaction with 2D surface and cardiomyocyte maturation while peptide molecules, i.e. cell-adhesive oligopeptide, arginine-glycine-aspartic acid (RGD) did not provide comparable effects (LaNasa and Bryant 2009). Similar findings confirmed that RGD and YIGSR peptides can promote the same degree of cellular adhesion as their native proteins; however, they were unable to promote the signaling required for normal expression of focal adhesion kinase and complete sarcomere formation in cardiac myocytes (Boateng et al. 2005). Surface precoating with fibronectin, vitronectin, and stromal-derived factor-1 was also more effective for CM maturity and even viability compared to RGD (Gandaglia et al. 2012). Additionally, the different stiffness and elasticity of cell culture matrix are also strongly involved in regulation of cell differentiation potential (Engler et al. 2006). Thus, precoating cell culture surfaces with various ECM proteins and/or regulation surface morphology might induce more effective 2D cardiomyogenic differentiation.

2D cultivated and dissociated cells can be injected into the damaged heart tissue either intramyocardially (IM) or intracoronary infusion (IC), intravenous delivery and transendocardial injection (TESI) via catheter (Fisher et al. 2015; Fiarresga et al. 2015). However, the all ways of stem cell injection into the heart have some drawbacks: IM injections are time-consuming, cells are rapidly wash-out and need special catheterization laboratory (van den Akker et al. 2017). IC or other type of injections are quick and easy to perform but coronary artery or vein systems are often damaged and might cause a risk of embolism (Freyman et al. 2006). The retrograde coronary venous infusion (RCVI) is considered to be more safe way to inject stem cells into the heart, however, the problems of control of grafted cells viability and integration remains. Therefore, 2D cell sheets of various cell types have been developed and used for cardiac tissue engineering as an alternative to dissociated cells and/or scaffolds. Such sheets were transplanted into patients' hearts in order to recover damaged or attenuated/compromised cardiac functions (Sekine et al. 2011). Moreover, self-beating and contracting 3D cardiac tissue was made by stacking multi-cellular sheets (Achilli et al. 2012). Human cardiac progenitor cells cultivated as sheets preserved their phenotype, multipotency, decreased apoptosis and better

improved heart regeneration compared to the regularly 2D cultivated cells (Forte et al. 2011). Altogether, cell-sheet stacking technology is among the most promising 2D cell cultivation technologies allowing to easily develop 3D tissue/organ engineering system *in vitro*.

Despite certain successful cardiomyogenic differentiation studies, 2D techniques do not fully mimic native environment of the human heart. Interestingly, one of the first engineered heart tissue was in 3D and contained chicken embryonic heart cells seeded in collagen I and producing beating and force-generating structures (Eschenhagen et al. 1997). Significant differences between 2D and 3D cardiac cultures were reported, including morphology of the cells, contraction ability, proliferation rate, presence of intercellular adhesion structures, organization of myofibrils, mitochondrial morphology and endoplasmic reticulum contents, cytoskeletal filaments, extracellular matrix distribution and expression of cardiac differentiation markers (Soares et al. 2012). Maturation of human ESC-derived CM on 3D fibrin-based cardiac patch compared to 2D monolayer cultures was also better (Zhang et al. 2013). Next organizational levels would be various cell types-containing organoids and, indeed, cardiomyocyte-, endothelial cells- and fibroblasts- containing cardio organoids exhibit good mechanical integration and can be regarded as a promising technology (Ong et al. 2017). In addition, heart tissue engineering in 3D requires several important choices: relevant substrate chemistry and structure, proper stiffness, required biocompatibility and disintegration over the time (Mathur et al. 2016). Such spatial environment-defined modulations of cellular functions are crucially important when designing studies for the investigation of cardiac drugs, growth factors and/or toxic substances. Although 3D cell culture systems, compared to the 2D systems, have been shown to better recreate conditions and microenvironment of heart tissue and are increasingly used in research and clinical settings, both cardiac models *in vitro* are facing the main problem – lack of complete cardiac cell maturation including cell morphology changes, electrophysiological, calcium stores and calcium-dependent excitation-contraction coupling measurements, contractile force, metabolic maturation, expression of myofilament proteins and other (Karbassi et al. 2020). An overview of differences between 2D and 3D cardiac culture technologies is summarized in the Table 1. Both, 2D and 3D technologies have some advantages and disadvantages and should be chosen depending on the research and/or therapeutic purposes.

3 Matrices for Cardiac Tissue Engineering and Repair Studies

3D cell culture methods for cardiac regeneration include a) scaffold-free, self-assembling 3D aggregates or spheroids; b) cell cultures within hydrogel matrices; c) cell cultures within synthetic scaffolds or decellularized native matrices.

3.1 Self-assembling 3D Aggregates

The term ‘spheroid’ refers to 3D, well-rounded cell aggregate consisting of one cell type (homospheres) or multiple cell types (heterospheres or organoids) that better mimic *in vivo* tissue physiology and are suitable for *in vitro* drug screening (Huebsch et al. 2016), cell generation, expansion and differentiation for regenerative purposes (Xu et al. 2016), direct therapeutic cell implantations (Zhang et al. 2018) and other applications. In order to form spheroids, cell suspension is transferred to specialized microenvironment, where cell–cell contacts and compact multicellular spheroid structures are established (Moshksayan et al. 2018). Self-assembling cell spheres do not need additional scaffolds and can be used to investigate various effects of cell-cell interactions that make them highly relevant for *in vitro* disease modelling, drug screening and therapeutic applications. Multicellular spheroids comprise inner sphere gradients according to the cell proliferation rate, polarity and certain gene expression. In addition, uneven drug or nutrition penetration across the layers differently affect

Table 1 Summarized characteristics of 2D and 3D cell-based technologies

Cell-based regeneration technologies	Advantages	Disadvantages	Citation
2D cell cultures	Easy cultivation and injection to heart; good cell number control. Easy cell assays *in vitro* and electrophysiological measurements (patch clamp, sharp electrodes)	Low amount and viability of grafted cells; difficult to control cell growth orientation and place; no pre-injection ECM; low differentiation and functional adaptation *in vivo*	Soonpaa et al. (1994), Menasche et al. (2001), Wollert et al. (2004), Fiarresga et al. (2015), van den Akker et al. (2017)
2D cell cultures on modified surfaces	Better cell attachment, viability, and differentiation potential compared to not modified surfaces. Easy cell assays and measurements *in vitro*	Difficult to control cell growth environment, still low grafted cell viability, amount and adaptation *in vivo*	LaNasa and Bryant (2009), Boateng et al. (2005), Gandaglia et al. (2012)
2D cell sheets	Better cell-cell contacts at the edges; preserved cell surface proteins; better cell survival and sheet adherence on the tissue; high cell density; better regeneration properties than dissociated 2D cultures, easy to identity cells in vitro	Complicated transplantation and cell number control; cells interact with ECM from one side	Zuppinger (2016), Hata et al. (2010)
3D cell sheet stacks	Less of apoptosis; presence of ECM; can make organoids from heterogenic cell populations; do not need scaffolds; high cell density. Cells interact with ECM in several dimensions; better tissue regeneration	Complicated transplantation; bad cell number control. Hard to identify cells *in vitro.* Problems with mechanical and electrophysiological tests	Edmondson et al. (2014), Forte et al. (2011)
3D self-assembling cultures	Better cell-cell contacts, cell junctions, cell surface proteins; better cell morphology and intracellular parameters; ability to make heterogenic cultures-based organoids	Possibility of coronary occlusion; uneven size, gradients of oxygenation and nutrition penetration in spheres. Problems with cell identification assays in vitro mechanical and electrophysiological tests	Eschenhagen et al. (1997), Huebsch et al. (2016), Xu et al. (2016), Davis et al. (2009) Moshksayan et al. (2018), Soares et al. (2012), Zhang et al. (2018)
3D cultures in hydrogels	Better cell sphere oxygenation, nutrition supplement; cell concentration; secretion of paracrine factors; ECM generation; co-cultures for organoids *in vitro*	Biocompatibility problems; problems with cell injection and into heart and assays *in vitro*, mechanical and electrophysiological tests	Li et al. (2016), Kang et al. (2017), Masumoto and Yamashita (2018), Koivisto et al. (2019), Ikonen et al. (2013)
3D cultures on scaffolds	Ability to modify cell growth surface; mechanical and electrical stimulation assays in vitro and better cell maturation	Week cell attachment, biodegradability and biocompatibility problems; transplantation problems to the heart; diffusion gradients of oxygen, nutrients and waste still present; problems with electrophysiological tests	Ravichandran et al. (2013), Amand and Esmaeili (2020), Macadangdang et al. (2014), Huang et al. (2018)

cell phenotype and functional capabilities (Davis et al. 2009). Moreover, cell–cell and cell-ECM interaction differ in 2D cultures and 3D spheroids, as well as between 3D spheroid cell layers strongly affecting cell susceptibility and properties (Soares et al. 2012).

Several well-established methods to form spheroids are known, including hanging drop cultures, spinner cultures and microfluidics, pellet cultures on low adherent surfaces and cell monolayer-fed spheroids (Achilli et al. 2012; Ong et al. 2017; Fennema et al. 2013) (Fig. 3).

The Hanging Drop (gravity-assisted cell assembly) method comprises formation of cellular aggregates in drops of cell culture medium hanging down from the surface, allowing for more precise control of spheroid size compared to other spheroid forming techniques (Fennema et al. 2013). However, culture medium supplementation in long-term hanging drop cultures may become challenging. Human bone marrow- and adipose tissue-derived mesenchymal stem cells, Isl1(+) cardiac progenitors derived from human embryonic stem cells (hESC-Isl1(+) cells) and undifferentiated human induced pluripotent cells (hiPSCs) were all subjected to hanging drops technique to generate scaffold free, 3D-like cardio tissue to enhance cellular engraftment and survival (Emmert et al. 2013). However, the hanging drop cardio tissue technology is not so popular for cardiomyogenic differentiation due to difficulty to change media for long-term cell cultivation. The novel hanging drop technology, i.e. pipe based microbioreactor setup was used to make heterogenic cardio spheres from cardiomyocytes, fibroblasts and endothelial cells that are promising 3D cardio tissue biomodels for the investigation cardio toxic drugs such as doxorubicin and other (Polonchuk et al. 2017).

Spinner Culture and Microfluidics Spheroids can be formed under dynamic culture conditions, i.e. in stirred bioreactors and microfluidic systems. This approach permits large-scale production of spheroids. Human pluripotent stem

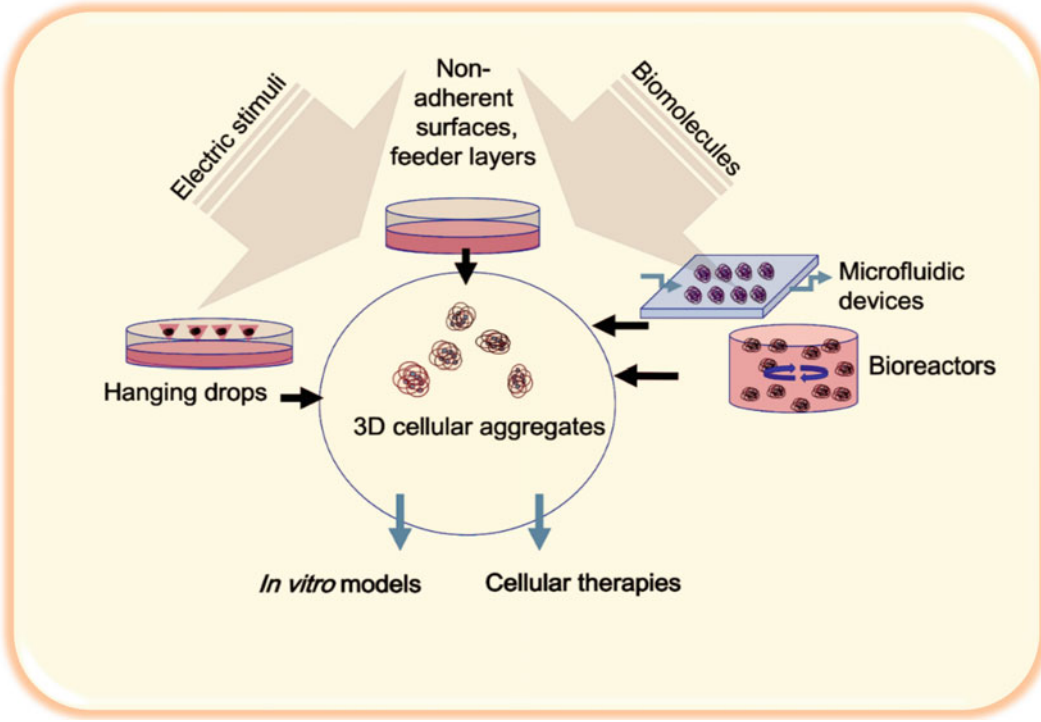

Fig. 3 Self-assembling cell technologies for cardio regeneration

cells were differentiated to ventricular-like cardiomyocytes using 100 mL spinner flasks and resulted in contracting embryonic bodies (EBs) with a heterogeneous size (350–600 mm in diameter) (Kempf et al. 2014). Pluripotent stem cell lines-derived CM were identified and cell aggregate size, agitation rate and other parameters were optimized reaching 80–99% efficiency of cardiac differentiation in bioreactor prototype for automation of cell manufacturing and drug testing assays (Chen et al. 2015). Human pluripotent stem cells were also successfully used to generate cardio spheroid-on-a-chip model using microfluidic channel slide system and Wnt pathway modulators. This system allowed to generate cardiac spheroids of uniform structure, each consisting of approximately 2500 cells (~250 μm in diameter) for further differentiation to functional cardiomyocytes (Christoffersson et al. 2018). However, the fluidic system-based production of CM can have some disadvantages: flow-induced shear stress effects as well as production of nonuniform size spheroids in bioreactors (Ting et al. 2014).

Low Adherent Surfaces Cellular aggregates may be produced by cultivation of cell suspension on commercially available ultra-low or non-adherent U or V shape bottom plates or surfaces, precoated with not adherent chemicals (Archer et al. 2018). It was shown that beating spheroids might be produced as early as in 3 days by mixing cardiomyocytes, endothelial cells, fibroblasts by 3D printing and culturing in media mixtures on commercially available low adhesion dishes (Ong et al. 2017). CM and/or cardiac fibroblasts can be used as building blocks to form larger microtissues by fusing homotypic and heterotypic spheroids and seeding them in low-binding wells, i.e. coated by agarose (Kim et al. 2018). Human cardiac microvascular endothelial cells, rat neonatal ventricular cardiomyocytes and human normal dermal fibroblasts and human coronary artery endothelial cells were cultured in low binding plates prior to the successful transplantation into rat hearts (Noguchi et al. 2016). Self-organization method has been also applied for long-term culturing of rat cardiomyocytes and fibroblasts on the laminin. Approximately 180 h after plating, the monolayer of cells began to detach from the periphery of the laminin substrate and contractile cardiac tissue started to form (Baar et al. 2005). Self-organized microtissues have been reported to express α and β-tropomyosin, but little or no SERCA2a expression was detected. Production of cardiomyocyte cultures from rat iPSC and rat EC cultivated on feeder-free surface coated with low binding agarose surface and stimulated by ascorbic acid and Wnt pathway modulators was also reported (Dahlmann et al. 2018).

Cell-Feeder-Layer-Based Spheres The potential of ESC and iPSC to differentiate into functional cardiomyocytes depends on growth conditions, various types of differentiation inducers and feeder layers. It was shown that highly contractile cardiomyocytes may be obtained from iPSC by growing them on human gingival fibroblasts as a feeder cells *in vitro (*Matsuda et al. 2018*)*. Feeder layer-based cardiomyocytes increased expression of troponin T (cTnT), decreased levels of pluripotent markers, exhibited robust sarcomere structure and significantly stronger contractility in comparison to feederless cardiomyocytes (Matsuda et al. 2018). Mouse embryonic fibroblasts (MEFs) and human foreskin fibroblasts (hFFs) also have been used as feeder layers for the cardiac differentiation of human EC and iPSC (Pekkanen-Mattila et al. 2012). However, certain feeder layers can contaminate human cells with animal cell components and impair cell homogeneity, induce spontaneous differentiation and negatively affect final cardio differentiation response.

3.2 Native Compound-Based 3D Matrices for Cardio Regeneration

Matrices for the 3D cardiac tissue engineering and regenerative purposes may be comprised of natural biomaterials such as collagen, fibronectin, chitosan, alginate, hyaluronic acid, and albumin

(Srinivasan and Sehgal 2010), synthetic fibers, like poly-ε-caprolactone (PCL), poly-lactic acid (PLA), poly-glycolide (PGA) (Jenkins and Little 2019; Mani et al. 2019; Zong et al. 2005) and their combinations (Ravichandran et al. 2013; de Pinho et al. 2019). In addition, decellularized native eukaryotic (Hata et al. 2010) as well as plant origin (Gershlak et al. 2017) matrices are also used as a scaffolds for cardio cell cultures. All types of scaffolds should be designed focusing on their biocompatibility, biodegradability and capabilities to be used in combination with conductive or other bioactive materials (Fig. 4).

In general, native compound-based scaffolds offer possibilities to recapitulate myocardial architecture, size and shape required for cell cultivation, high-precision surface topography and functionality. Increasingly rapid development of biomaterials provides growing number of fiber types that can be employed to make scaffolds as well as methods, including electrospinning, lithography, micro-photopatterning, melt-blowing and microfluidics adopted scaffolds (Amand and Esmaeili 2020; Macadangdang et al. 2014; Huang et al. 2018; Jenkins and Little 2019). Injectable type of hydrogel-based 3D technique is also popular mode of cardiac regeneration strategies *both in vivo* and *in vitro* and already has a therapeutic application. Hydrogels, similar to scaffolds, can be made from natural or synthetic polymers and/or their combination for tailored, tissue specific, applications.

The expertise on cell-loaded-hydrogel application, i.e. direct cell injection to the specific sites requiring treatment, comes from successful application in anti-tumor treatment (Liang et al. 2019). Many traditional hydrogels, that perform well in the *in vitro* studies, cannot be applied for minimally invasive catheter-based stem cell delivery to the heart tissue *in vivo* due to high viscosity and rapid gelation. To increase efficiency of functional engraftment of scaffolds/hydrogels based cells, the appropriate balance between cell type, their carriers and delivery methods should be taken into account.

Collagen Among natural biomaterials, collagen type I and III are the major heart ECM

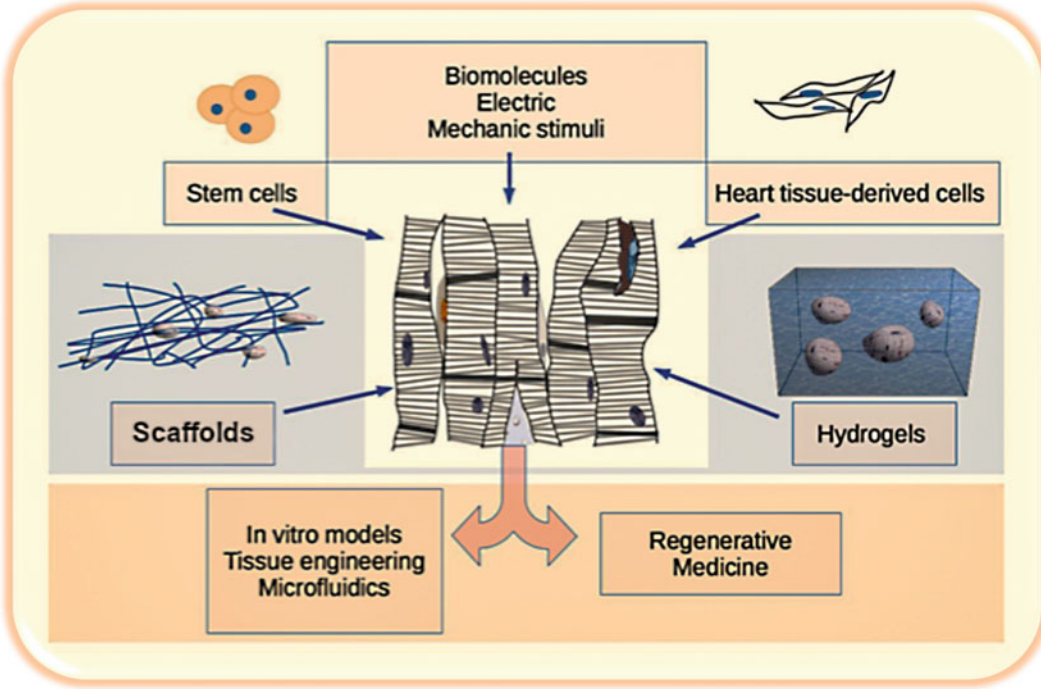

Fig. 4 Scaffolds- and hydrogels-based matrices for cardio regeneration

components used in heart tissue engineering with great biocompatibility, adhesiveness, porosity, mechanical support and integration needed for natural scaffolds (Srinivasan and Sehgal 2010). Collagen I is a main component of connective tissue and the most abundant protein in mammals (up to 25–30% of whole-body proteins), which mutations are strongly associated with human connective tissue disorders (Di Lullo et al. 2002). Collagen by itself may prevent cardiac remodeling, promote angiogenesis and inhibit cardiomyocyte apoptosis (Serpooshan et al. 2013). When combined with growth factors and cells collagen constructs are even more beneficial (Shi et al. 2011). It was shown that cardio progenitors cultured in 3D collagen/matrigel constrained hydrogels became readily mechanosensitive, had a rod-shaped morphology and oriented growth direction, increased expression of ECM components such as collagen I, collagen III, elastin and fibronectin and cardiomyogenic differentiation markers (van Marion et al. 2015). The neonatal rats heart-derived cells cultivated on collagen I-based hydrogels improved cardio cell differentiation and functioning of post-infarcted rat heart (Zimmermann et al. 2006). Furthermore, the addition of collagen I to Matrigel-based hydrogels enhanced matrix stiffness and maturation of H9 embryonic stem-cell line-derived ventricular cardiomyocytes (Edalat et al. 2019). Addition of collagen within synthetic scaffolds also gave positive effects to cardioregeneration (Ravichandran et al. 2013).

Fibronectin and its polymerization product fibrin is another attractive natural material due to good biocompatibility with the cells, availability from patient's circulation (autologous whenever needed), plasticity to combine with other polymers, incorporate small peptides and growth factors, and fibrin's neovascularization and cardio regeneration properties (Ardila et al. 2019; Xiong et al. 2011; Zhang et al. 2006). Fibronectin is one of ECM proteins responsible for cell adhesion, survival, migration and, importantly, differentiation (Jung et al. 2016). Among five different ECM proteins used in cardiac engineering: collagen I, fibronectin, gelatin, laminin and a gelatin/fibronectin mixture, fibronectin is the best in supporting cell adhesion and proliferation, whereas gelatin – in the cardiomyogenic differentiation (Choi et al. 2013). Human MSC grown on micropatterned prolonged fibronectin strips exhibited activated mechanotransduction pathways and improved cardiomyogenic differentiation (Tijore et al. 2015). Human ADSC were also able to differentiate into cardiac cells on fibrin as well as myoblasts injected with fibrin glue improved decreased infarct size (Bagheri-Hosseinabadi et al. 2017; Christman et al. 2004). The human iPSC-derived cardiac cell transferred from temperature responsive silicone gels to fibrin gel allowed to generate cardiac cell sheet-tissues with dynamically beating properties. The contractile force of such cardiac cell sheet-tissues was around 1mN and the mean force value per cross-sectional area of 3.3 mN/mm2 (Sasaki et al. 2018). Fibronectin is often substituted by RGD peptide that binds cell's cytoskeleton and thus promotes cell adhesion and differentiation just like full length protein does (Sondermeijer et al. 2008). However, the full-length fibronectin protein was shown having better effect on cardiomyogenic differentiation than its RGD fragment.

Laminin is a high a high-molecular weight (400–900 kDa) protein of the ECM. It is a major component of basal lamina and regulates cell adhesion, migration and differentiation trough different expression of laminin-binding integrins (Shibata et al. 2020). Preclinical studies have shown that human ADSC grown on 3D biodegradable microcarriers that could release growth factors in a prolonged manner undergo cardiomyogenic differentiation environment and subsequently improve heart function (Karam et al. 2015). Precoating of microcarriers with laminin stimulated the expression of cardiac markers more effectively in comparison to fibronectin (Karam et al. 2015). Replacement of full-length laminin with its fragment YIGSR (represents amino acids Tyr–Ile–Gly–Ser–Arg)

was also used with similar success (Boateng et al. 2005). Peptide-enriched scaffolds may promote contractility and functional capacity of the seeded cells even more efficiently than proteins do, without side effects and costs associated with proteins (Gandaglia et al. 2012). Scaffold modifications and their effects on the quality of engineered heart tissue are widely researched not only *in vitro* but *in vivo* as well (Yu et al. 2009, 2010).

Albumin is the most abundant plasma protein with molecular weight of 66.5 kDa. Solution of albumin has native globular conformation and need chemical modification for example with trifluoroethanol to become spinnable (Regev et al. 2010). Similar collagen and fibronectin, albumin is inexpensive and biocompatible. Three-dimensional cardiac patch fabricated from albumin fibers was more suitable for the generation of the functional cardiac tissue with higher beating rate and better contraction compared to synthetic poly(e-caprolactone) fibrous scaffolds (Fleischer et al. 2014). The multifunctional scaffolds were composed of electrospun albumin fibers that served as both a substrate and a passivation layer for cardiomyocytes seeded that organized into a functional cardiac tissue (Feiner et al. 2018). Cardiac cells were able to assembled into a functioning patch on nanocomposite scaffolds comprised of albumin electrospun fibers and gold nanorods (AuNRs) (Malki et al. 2018). The scaffolds were able to absorb light, change it to thermal energy and positively affect functioning of cardiac cells. Bovine serum albumin microbubbles incorporated in the polyethylene glycol-fibrinogen hydrogel (PFHy) scaffold improved H_2S release and favored the proliferation and differentiation of human Sca-1(pos) cardiac progenitor cells (hCPCs) (Mauretti et al. 2016). Such albumin microbubbles can be used as carriers of various bioactive compounds.

Hyaluronan (HA) also known as hyaluronic acid, is an anionic, nonsulfated glycosaminoglycan and is one of the most abundantly natural component of connective, epithelial and neural tissues (Garcia et al. 2019). HA, similarly to collagen, is one of the most abundant components of ECM and was shown to be involved in cardiac cell adhesion, healing of infarcted cardiac tissue and regulation of inflammatory responses in porcine model (Chen et al. 2014), rats (Chen et al. 2013) and in humans (Bonafe et al. 2014). Human CDC seeded onto hyaluronan and porcine gelatin hydrogels were tested in a mouse model of myocardial infarction and have improved cell retention, engraftment and efficacy in preclinical studies (Cheng et al. 2012). The pre-transplantational encapsulation of CDC cells stem cells into the HA-succinimidyl succinate hydrogels promoted cell viability and attachment, reduced cell apoptosis and improved angiogenesis (Chan et al. 2015). Chemical modifications of native materials for cardiac regeneration are widely popular. For the development of the heart tissue models *in vitro* native hydrogels are often modified by synthetic compounds, e.g. HA-based hydrogels might be enriched with synthetic ECM molecules for bioprinting techniques (Prestwich 2011), thiolated hyaluronic acid-based hydrogels might be crosslinked with poly(ethylene glycol) diacrylate for better stiffness (Young and Engler 2011). Pre-transplantational encapsulation of CDC into the HA succinimidyl succinate reduces cell loss during injection procedure due to apoptosis, improved cells attachment and engraftment in the target area (Chan et al. 2015). Hyaluronic acid can also promote heart tissue healing and regulate inflammation in infarcted myocardium (Abdalla et al. 2013).

Alginate a natural hydrophilic polysaccharide, is a component resembling glycol part of cardio ECM. Alginate can be obtained with wide range of molecular weight (32–400 kDa) and have two long chains (Kong et al. 2003). Alginate is mainly used as a drug or cell carrier and is one of the main components of injectable hydrogels. The first-in-man pilot study showed improved revascularization and functioning of pot-infarcted man heart after injection of alginate and calcium gluconate-based hydrogels (Frey et al. 2014). Patients with advanced chronic heart failure

were successfully treated with alginate-based hydrogels injected into the left ventricle heart muscle in addition to standard medical therapy and showed better results compared to patients receiving only standard medical therapy alone (Anker et al. 2015). The combination of alginate with graphene oxide showed high anti-oxidant activities of hydrogel and was used to decrease a shear stress during MSC transplantation into post infarcted hearts (Choe et al. 2019a). Alginate is also used as a matrix for cells that prevents cell emigration while supporting secretory phenotype (Karpov et al. 2019). The RGD peptide-modified alginate micro-hydrogels protected encapsulated hMSC against oxidative stress (Choe et al. 2019b). The conductive properties of injectable alginate-based hydrogels to restore cardio electrical impulses can be also improved by its combination of alginate with nontoxic polypyrrole (Ketabat et al. 2017).

Some of the natural scaffolds mentioned above are already in clinical trials and about to enter clinical practice, i.e. cell-loaded collagen patches significantly improved post myocardial thickness of the infarct scar and helped to normalize cardiac wall stress in injured regions in comparison to the cells only group (Chachques et al. 2007) or alginate scaffolds that are used to treat myocardium remodeling after infarction (Frey et al. 2014).

Chitosan is a glycosaminoglycan composed of alternating units of *N*-acetyl-d-galactosamine and d-glucuronic acid and was reported to be able to maintain high concentration of growth factors and strong cellular receptor adhesion due to its hydrophilicity providing long-term release of bioactive molecules within the injection sites (Hussain et al. 2013). Chitosan is the partial deacetylated cationic polymer derived from chitin, which is obtained from the shells of crabs and shrimp. Chitosan-based hydrogel also improved the microenvironment and enhanced the retention and engraftment of rat ADSC transplanted into infarcted rat hearts (Wang et al. 2014). Chitosan provides good hydrophilicity and cell adhesion properties (Amand and Esmaeili 2020; Hussain et al. 2013), whereas alginate and gelatin nanofibers provides superior cell motility, proliferation and ventricular cardiomyocyte maturation (Majidi et al. 2018). The adhesion and migration properties of cardiac cell co-cultures on chitosan scaffold were further improved by precoating the scaffold with fibronectin (Hussain et al. 2013) as well as by the addition of RGD fragments which increased adhesion of neonatal rat cardiomyocytes, prevented cell apoptosis and accelerated cardiac tissue regeneration (Shachar et al. 2011).

Combined-Type Scaffolds The contractile force of engineered cardio cells depends not only on cellular content within the scaffolds, cell alignment and/or maturation level, but also on the mechanical and electrical properties of used scaffold and good biocompatibility with the cells. To obtain such results with one type of scaffold material is impossible, therefore, increasing number of studies investigate combined types of scaffolds from various components. Pure synthetic polymeric scaffolds are usually stiffer, easier to process, they provide better properties needed for heart biomechanics and contraction studies *in vitro*, but they are less biocompatible and have low cell adherence properties compared to the natural scaffolds (Jenkins and Little 2019). In order to get artificial polymers more biocompatible with the cardiac cells, precoating of scaffolds with ECM proteins or their components are broadly used.

The most popular synthetic scaffold in cardiac field poly(ε-caprolactone) (PCL) scaffold made of spring-like structure and coated with fibronectin better mitigated mechanical properties of rat left ventricle-derived CM compared to the linear ones (Fleischer et al. 2013). For the better stimulation cardiomyogenic differentiation, PCL can be also mixed with the conductive carbon nano particles (Crowder et al. 2013). The aligned poly(-ε-caprolactone)/gelatin (PG) composite closer matched the requirements of native cardiac anisotropy than random PG scaffolds (Kai et al. 2011). PG fibers increased hydrophilicity and lower stiffness compared to electrospun PCL nanofibers. Humans MSC cultivated on 3D

aligned PCL scaffolds precoated with different ECM proteins revealed collagen coating to be the best for the cardiomyogenesis (Ghosh et al. 2019). Combination of two poly(L-lactic acid) (PLA) and PCL, scaffolds with gelatin and vascular endothelial growth factor improved cardiomyogenic differentiation of human mesenchymal stem cells (hMSCs) (Tian et al. 2013). Thermoformed PCL/gelatin/fibrinogen scaffolds, precoated with fibronectin and collagen IV were also used in order to develop human tissue-engineered vascular grafts (Ardila et al. 2019). The mixture of synthetic (poly(lactic-co-glycolic acid) (PLG) and natural proteins (gelatin, alpha elastin and/or fibrin) showed good cytocompatibility with myoblasts and bone marrow MSC for cardiomyogenic differentiation (Li et al. 2006). Mixture of poly(lactic-co-glycolic acid) (PLGA)/NCO-sP(EO-stat-PO) polymers can be used as carriers of cardioactive growth factor Neuregulin-1 (Nrg) regenerating rat heart after myocardial ischemia (Simon-Yarza et al. 2015).

Other types of composed scaffolds such as electrospun chitosan nanofibers with gelatin and polyurethane have been proposed to be suitable material for cardiac valves, i.e. endothelial cells grown on the scaffold withstood shear stress and showed good cell retention properties (Cynthia et al. 2010). It was also shown that different stiffness and Young's modulus of collagen I-laminated polyacrylamide gels can change cytoskeletal structure of fibroblasts, endothelial cells and neutrophils, i.e. softer than 1600 Pa scaffold prohibited expression of actin fibers (Yeung et al. 2005). The application of collagen-like synthetic self-assembling nanofibers hydrogels in combination with hyaluronic acid supported growth of human cardiomyocytes (Ikonen et al. 2013). In order to avoid biocompatibility problems, the direct injection of nanofiber gel with glycosaminoglycan (GAG) mimetic peptide in the post MI rodent heart also improved heart functioning (Rufaihah et al. 2017). The surface glycosylation such as sialo-glycoproteins, N-glycans and fucosylation are also being employed in hiPSCs cardio differentiation revealing the N-acetyl-D-glucosamine structures emerging on immature hiPSC-derived cardiomyocytes (Konze et al. 2017).

Altogether, different types of synthetic polymers in cardio regeneration have generated positive results only when used in combination with natural components.

Other Bioactive Components Bioactive compounds are used not only to promote cardiomyocytes' adhesion but also proliferation, differentiation and angiogenesis and can be incorporated into engineered scaffolds and hydrogel substrates. Variety of methods are used to introduce functional groups into the scaffolds and hydrogels, including chemical reactions, photo-induced grafting, plasma treatment, physical adsorption, bulk mixing in, molecular imprinting and combinations thereof (Tallawi et al. 2015). Biomolecules such as, growth factors, cytokines, chemokines, peptides and their combinations with scaffolds' surface topography, porosity and other morphological modifications thereof are used to improve cardiomyogenic differentiation. Growth factors are small, soluble, signaling molecules with specific locally supporting roles, receptors and distribution. They are important players in post-myocardial infarction healing, including granulocyte colony-stimulating factor (G-CSF) (Minatoguchi et al. 2004); stromal-derived growth factor (SDF-1) (Gong et al. 2019); leukemia inhibitory factor (Zou et al. 2003); insulin-like growth factor (IGF-1) (Musaro et al. 2004); members of TGF-β superfamily such as TGF-β1 and GDF11 (Su et al. 2019); vascular endothelial growth factor (VEGF) (Chen et al. 2006), erythropoietin (Calvillo et al. 2003), neuregulin-1 (Simon-Yarza et al. 2015). Many chemical compounds and their combinations are also used to induce cardiomyogenic differentiation such as 1H-pyrrole,2,2′-(phenylmethylene)bis compound named 31,002 (Kim et al. 2011); DNA methylase inhibitors (Gasiuniene et al. 2019); inhibitors of WNT pathway such as KY02111 (Minami et al. 2012), deletion of GSC-3β pathway (Gupte et al. 2020), ascorbic acid and other. However, the science of stem cells and tissue engineering still

drives the cardiac research to search for the best combination of bioactive compounds and scaffolds for better cell adhesion, proliferation and cardio differentiation properties.

3.3 Hydrogels and Scaffolds with Special Functions

Successful regeneration and engineering of cardiac tissue requires a functional scaffold to support cells and mimic heart tissues. Often modifications are essential for cell attachment, growth and differentiation. A combination of different techniques is often necessary for the achievement of optimal results.

3.3.1 pH Sensitive Hydrogels

It is known, that local pH in the area of myocardial injury is lower than in normal cardiac tissues due to the inflammation (Lin et al. 2005). Therefore, a hydrogel that is responsive to the specific pH can be employed while targeting infarction area. Based on these findings, several injectable and pH hydrogels suitable for the delivery of cellular content via myocardial injection has been developed and tested (Li et al. 2011). These hydrogels solidify at the lower pH at the infarcted heart sites but do not solidify in the blood stream. Thus, they can be injected via minimally invasive procedures. *N*-isopropylacrylamide, propylacrylic acid, methacrylate poly (ethylene oxide) methoxy ester or similar polymers might be used. In addition, hydrogels degrade in pH-dependent manner and their effect to the growth of cells and expression of cardiac markers, like cardiac troponin T, myosin heavy chain α, calcium channel CACNA1c, cardiac troponin I, and connexin 43, varies accordingly. Furthermore, another promising finding showed that the pH (low) insertion peptide (pHLIP) coated liposomes enable targeting cells in tissues with low pH (Sosunov et al. 2013). The pH sensitive liposomes were tested in regional and ischemic murine ischemia models revealing the pH sensitive compounds being promising in targeted delivery of cardio treatment strategies (Sosunov et al. 2013). Chitosan-based pH-sensitive injectable hydrogel with instant gelation can be also developed and improved myocardial regeneration via injected ADSC (Alimirzaei et al. 2017). The pH- and temperature-responsive random copolymer, poly(N-isopropylacrylamide-co-propylacrylic acid-co-butyl acrylate) can be used to carry fibroblast or other growth factor to the ischemia rat model improving therapeutic effects (Garbern et al. 2011).

3.3.2 Photo-Reactive Hydrogels

Another group of hydrogels that recently have been used for the cardiac regeneration are photo-reactive hydrogels. Kang et al. tested various photo crosslinked hydrogels composed of methacrylated gelatin/poly-ethylene glycol diacrylate/alginate for their effects on encapsulated human ADSCs, aortic valve interstitial cells and aortic valve sinus smooth muscle cells. Authors have reported that oxidative stress increased during photo crosslinking procedure, which resulted in lower cell viability (Kang et al. 2017). However, acellular cytocompatible photo-crosslinked hydrogels loaded with chemokines were employed to promote cellular immigration in the tissue and regeneration. Controlled and sustained release of SDF1 was demonstrated, controlled by density of crosslinking showing preserved functional activity of SDF1 (Schesny et al. 2014). This system can be used also as a drug or other compound delivery mean to the heart during treatment of cardiovascular ischemic disease (Schesny et al. 2014). In situ photopolymerizable PEGylated fibrinogen (PF) hydrogels also showed better maturation of human embryonic stem cells and rat neonatal cardiomyocytes (Shapira-Schweitzer et al. 2009). The employment of photo-reactive hydrogels in human cardiac tissue engineering brings a promise of tunable mechanical properties in a very precise location as reviewed by Choi et al. (Choi et al. 2019).

3.3.3 Temperature Responsive Hydrogels

Temperature responsive polymers were successfully used to prepare 2D and 3D cell sheets for

transplantation (Masumoto and Yamashita 2018). Pluripotent stem cell-derived cardiac tissue 3D sheets with several cardiac cell lineages were stacked and clues for functional vascular network were included. After transplantation of iPSC within temperature-responsive hydrogels resulted in successful development of vascular network and long-term survival of transplanted constructs (Masumoto and Yamashita 2018). Using poly (N-isopropylacrylamide) (PIPAAm), a temperature-responsive polymer, which changes the property of culture surface from hydrophilic to hydrophobic with the temperature change, iPSC cell-derived CM, endothelial and mural cells' sheets were created. These heterogenic cell sheets regenerated human myocardium and functionally recovered rat infarcted heart functions mainly by paracrine mechanisms (Masumoto et al. 2014). The combination of temperature responsive silicone with fibrin gels allowed to generate human iPSC-derived cardiac cell sheets with dynamically beating properties (Sasaki et al. 2018).

3D cardiac tissues were also fabricated by stacking multicellular sheets on thermo-responsive polymers that spontaneously contracted and synchronically pulsated. 3D heterogeneous cell sheets can be also used to fabricate capillary-like network making transplantation and other manipulations more feasible (Haraguchi et al. 2012). Recently, poly-L-lysine- or laminin-coated reverse thermal gels were used to engineer cardiac-like tissue. The biopolymers provided a liquid-based cardiac cells delivery vehicle, which transitioned into hydrogel matrix shortly after reaching body temperature and supported cardiomyocyte phenotype (Pena et al. 2016). The laminin-based reverse thermal gels showed better heart regeneration properties than poly-L-lysine-based. Moreover, thermosensitive and highly flexible poly(N-isopropylamide) (PNIPAAm)-based hydrogels can be used in static and dynamic stretching conditions (11–40 kPa) and improve cardiac differentiation of CDC (Li et al. 2016). Altogether, temperature responsive hydrogels enable growth and easy collection of homo- or heterogenic 3D cellular constructs while preserving cell-cell and cell-ECM proteins connections.

3.3.4 Electroconductive Hydrogels

Electroconductive hydrogels have very promising implications in heart tissue regeneration since rhythm generation, support and/or its regulation technologies are of special importance for proper functioning of heart biomatrices (Hosoyama et al. 2019). It has become increasingly clear, that not only muscle defect but also electrophysiological function of the heart tissue might be restored with some currently used hydrogels. The electrophysiological function of human iPS-derived cardiomyocytes grown attached to gelatin-gellan gum hydrogels recovered their spontaneous beating in 24 h (Koivisto et al. 2019). The beating is a crucial characteristic of the heart tissue and proper connection between the heart cells is critical moment for proper cell electrical signal conductance. Therefore, use of hydrogels with electroconductive properties could significantly improve beating properties of engineered tissues. Modified chitosan hydrogels with polyaniline, as an electroactive polymer, was used for cardiac and/or neuronal tissue engineering applications, i.e. for tissues that require biocompatibility and conductivity (Guo et al. 2011; Bidez et al. 2006). In another study, oligo(polyethylene glycol) fumarate was successfully modified by polypyrrole to improve the neuronal differentiation which application can be extended to the field of cardiac engineering (Runge et al. 2010). It was also shown that carbon nanotubes (CNT) incorporated into gelatin hydrogels increase electrical conductivity of the neonatal rat ventricular cardiomyocytes (Shin et al. 2013).

In a more recent report, CNT were embedded within gelatin/chitosan-based hydrogels to yield hybrid biomaterials with improved mechanical properties and electrical signals between neonatal rat ventricular cardiomyocytes (Pok et al. 2014). To improve conductivity, necessary for the cardiac cell maturation and functions, artificial scaffolds such as poly(glycerol sebacate):gelatin (PG) electrospun nanofibers combined with CNT increased fiber alignment and improved the electrical conductivity and toughness of the scaffolds and notably enhanced CM viability, retention and contractile activities, compared to those cultured on PG scaffold (Kharaziha et al. 2014).

CNT-based methacrylate anhydride hydrogels better supported functioning and growth of neonatal rat ventricular cardiomyocytes, helped to enhance the formation of cardiac tissue, induced upregulation of mechanical and electrical junction proteins and contraction of neonatal rat ventricular cardiomyocytes compared to collagen-based hydrogel (Sun et al. 2017). CNT can also be incorporated into gelatin methacryloyl hydrogels, resulting in improved cardiac differentiation of mouse embryoid bodies compared to hydrogel without CNT (Ahadian et al. 2016). Modern cardiac muscle engineering requires reproduction of the intricate coordination mechanisms for synchronized cardiac cell beating. Therefore, strategies are being increasingly focused upon the complex delivery of mechanical, electrical and biochemical cues to the engineered tissue. The biomimetic myocardium is expected to be contractile, active tissue with polarization and depolarization cycles and these criteria are driving design and employment of conductive materials in cardiac tissue engineering.

4 Conclusions

The extracellular environment that supports cells is crucial for proper development and function of tissues and organs. Recreation of optimal native extracellular matrix by cardiac tissue engineering is still in process. Undoubtedly, there is still a significant unmet necessity to identify the best cells types or populations suitable for cardiac therapies with long-term survival, full functional adaptation and clinical safety in order to successfully recover heart tissue. So far, the most promising stem cells for the cardioregenerative purposes *in vitro* and i*n vivo* seems to be cardio progenitors, pluripotent, and adult mesenchymal stem/stromal cells. As a rule, the different stem cell types require different cultivation protocols, scaffolds and compositions with carrying biomolecules in order to stimulate cardiomyogenic differentiation, production of ECM and heart tissue regeneration. This partially explains a huge variety of recently known biomatrices for cardio tissue engineering. However, most promising and safe cardio regenerative potential in humans seems to have cardiac progenitors predifferentiated to CM in combination with various hydrogels/scaffolds mimicking natural heart tissue environment. The iPSC and iPSC-derived have also promising results in model animal and *in vitro* systems. For clinical investigations iPSC needs additional safety studies.

Due to the strong contracting nature of the heart tissue, biomatrices used for cardioregeneration should be strong enough, elastic, biocompatible and biodegradable in order to survive electrical and mechanical stimuli. Data acquired until today demonstrate various types of customized hydrogels and scaffolds as attractive 3D cell carrier systems for *in vitro* cardio tissue engineering and therapeutic studies. The limitation of pure synthetic scaffolds is their lower biocompatibility, low cell surviving and provoked higher immunological response compared to the native biomaterials. On the other hand, the native, cardio ECM-based biomatrices, are expensive, quickly biodegradable and not strong enough for cardio tissue regeneration purposes. Therefore, the various combinations of synthetic scaffolds together native origin and/or bioactive components are increasingly applied in cardioregenerative studies. It is still difficult unanimously to say, which type of 3D system is the best for the cardiac regeneration purposes, however, data suggest that injectable native compound-based 3D hydrogels and/or smart, environment-responsive hydrogels better suits for therapeutic purposes, due to easy cell delivery way, whereas synthetic polymers-based scaffolds better suits for the investigation of the mechanical and electrical purposes of cardiomyocyte maturation and functioning both in vitro and animal models.

Altogether, most of the research *in vivo* have been done small animal models, while studies applying engineered myocardium therapies on larger primates and humans are still lacking. In addition, the more complex cardiac investigation systems including, mechanic, metabolic and electrical cell-based sensing systems are still required. Nevertheless, functional cell-loaded biomatrices

with therapeutically targeted characteristics still remain a major goal for cardiac tissue engineering.

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Dentin-Pulp Tissue Regeneration Approaches in Dentistry: An Overview and Current Trends

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Abstract

Conventional treatment approaches in irreversible pulpitis and apical periodontitis include the disinfection of the pulp space followed by filling with various materials, which is commonly known as the root canal treatment. Disadvantages including the loss of tooth vitality and defense mechanism against carious lesions, susceptibility to fractures, discoloration and microleakage led to the development of regenerative therapies for the dentin pulp-complex. The goal of dentin-pulp tissue regeneration is to reestablish the physiological pulp function such as pulp sensibility, pulp repair capability by mineralization and pulp immunity. Recent dentin-pulp tissue regeneration approaches can be divided into cell homing and cell transplantation. Cell based approaches include a suitable scaffold for the delivery of potent stem cells with or without bioactive molecules into the root canal system while cell homing is based on the recruitment of host endogenous stem cells from the resident tissue including periapical region or dental pulp. This review discusses the recent treatment modalities in dentin-pulp tissue regeneration through tissue engineering and current challenges and trends in this field of research.

Keywords

Dental pulp · Dentin · Dentistry · Mesenchymal stem cells · Regeneration · Tissue engineering

Abbreviations

3D	Three dimensional
ALP	Alkaline phosphatase
BMMSCs	Bone marrow derived mesenchymal stem cells
BMP	Bone morphogenic protein
DPSCs	Dental pulp stem cells
DSP	Dentin sialoprotein
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FGF	Fibroblast growth factor
G-CSF	Granulocyte-colony stimulating factor
IGF	Insulin growth factor
L-PRF	Leukocyte-platelet rich plasma
MSCs	Mesenchymal stem cells
MTA	Mineral trioxide aggregate
NF-κβ	Nuclear factor kappa beta
NGF	Nerve growth factor
PDGF	Platelet-derived growth factor

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PDL	Periodontal ligament
PDLSCs	Periodontal ligament stem cells
PRF	Platelet rich fibrin
PRP	Platelet rich plasma
SCAP	Stem cells from apical papilla
SDF-1	Stromal-derived factor-1
SHED	Stem cells from human exfoliated deciduous teeth
TGF-β	Transforming growth factor-beta
VEGF	Vascular endothelial growth factor

1 Introduction

Dental pulp is a soft tissue, whose main function is to create the dentin tissue and provide its biological and physiological continuity. Since the dental pulp is surrounded by dentin, its blood supply can only be provided from the root apex, and thereby it does not have good collateral vascularization compared to that of other soft tissues of the oral mucosa. Therefore, it is very sensitive to external stimuli and infection, and its capacity to eliminate inflammation is relatively low. The current approach in the treatment of pulp inflammation is the complete removal of the pulp tissue from the root canal followed by the mechanical debridement and chemical disinfection procedures, and then obturation of the root canals with thermoplastic filling materials.

The root canal treatment is a dental procedure with a high success rate (up to 98%); however, there are several drawbacks including the loss of tooth vitality and defense mechanism against carious lesions, susceptibility to fractures, discoloration and microleakage (Siqsueira 2001; Imura et al. 2007). Furthermore, complications due to iatrogenic factors during endodontic procedures such as fractured root canal instruments and perforation, may lead to the extraction of the tooth. Regarding immature teeth whose root development is not completed, although root canal treatment can be applied, root development cannot be completed after the treatment (Pace et al. 2007). In order to overcome such shortcomings of the current endodontic treatment approach, the preservation of the dentin-pulp complex may be the most plausable option. However, it is not possible to apply a preventive treatment when the pulp tissue is extensively exposed as a result of damage to the tooth, rendering the development of regenerative therapies for the dentin-pulp complex of crucial importance.

2 Inflammation in Dental Pulp

Unlike other soft tissues of the human body, dental pulp is enclosed by the dentin, which is a highly calcified and inextensible tissue (Giraud et al. 2018; Goldberg et al. 2015; Jeanneau et al. 2017). When the dental pulp is injured due to caries and physical trauma, an inflammatory reaction begins at the remaining healthy pulp tissue (da Rosa et al. 2018; Goldberg et al. 2015; Jeanneau et al. 2017). Inflammatory reactions with increased vascularization and blood flow at other tissues of the human body do not lead to serious consequences, whereas an inflammation in the pulp can lead to destruction and necrosis of the dental pulp. If inflammation continues, the resulting chronic inflammation prevents regenerative processes, and eventually pulp necrosis occurs (Giraud et al. 2018).

Odontoblasts are dentin-forming cells (also known as dentin-producing cells) located around the pulp that first encounter pathogens (da Rosa et al. 2018). Furthermore, odontoblasts are called immunocompetent cells that can regulate the inflammatory response (Chmilewsky et al. 2014b; Cooper et al. 2014). It has been reported that odontoblasts can detect pathogens at the dentin-pulp interface and release antimicrobial agents targeting the elimination of pathogens as well as autocrine and paracrine signaling factors such as proinflammatory chemokines and cytokines (di Nardo Di Maio et al. 2004; Farges et al. 2015; Veerayutthwilai et al. 2007).

Studies have shown that the inflammatory response developed against pathogens is locally regulated by the dentin-pulp complex (Giraud et al. 2018; Jeanneau et al. 2017). The first step of this localized defense system begins with odontoblasts and pulp cells under the dentin barrier expressing pattern recognition receptors

(Chang et al. 2005). Pattern recognition receptors contain Toll-like receptors that can recognize pathogen-associated molecular patterns on the surface of bacteria. After the recognition, pro-inflammatory cytokines are produced and pathways that launch the inflammatory cascade (Chang et al. 2005; Cooper et al. 2010), such as the nuclear factor kappa beta (NF-κβ) and p38 mitogen-activated protein kinases (p38 MAPK) are activated (Botero et al. 2010; Cvikl et al. 2016). After a number of inflammatory steps, leukocytes which eliminate pathogens and cell debris, are located at the inflammatory site (Giraud et al. 2019).

Mild to severe inflammation is observed at the dentin-pulp interface after the pulp tissue is exposed to pathogens or during and after dental operations. A recent review stated that inflammatory responses should be a prerequisite for dental tissue healing (Goldberg et al. 2015). As a result of the interactive balance between pro and anti-inflammatory responses, cell necrosis, apoptosis, resorption, calcification or vascularization can be observed (Shah et al. 2020). Low levels of proinflammatory signals trigger differentiation and mineralization, while high levels of proinflammatory signals accelerate the inflammatory response by allowing more inflammatory cells to be involved (Goldberg et al. 2015). Hence, low level of cytokine secretion enables mesenchymal stem cells to exhibit healing and anti-inflammatory functions, while high level of cytokine secretion may inhibit the involvement of stem cells (Li et al. 2014; Miller et al. 2008; Murdoch 2000).

Inflammation has been considered to be a mere undesirable effect in pulp healing for a long time (Goldberg et al. 2015). Today, it has been shown that inflammation and tissue regeneration are involved in an interaction in human body, which applies to the pulp tissue as well. The microenvironment of the inflamed pulp differs from that of the healthy pulp, and it releases various signals necessary to continue regeneration (Rombouts et al. 2016). Mild or moderate inflammation is shown to stimulate the regeneration, yet; severe or chronic inflammation can be detrimental to the pulp (da Rosa et al. 2018; Jeanneau et al. 2017).

Pulp fibroblasts are capable of the synthesis of all complement proteins that initiate pulp and nerve regeneration processes and act in the destruction of cariogenic bacteria (Chmilewsky et al. 2013, 2014b, 2016; Jeanneau et al. 2015). Traumatic injuries to the dentin-pulp complex, carious decay and biomaterial applications can prompt complement activation, which is involved in inflammation and regeneration processes (Chmilewsky et al. 2014a). Complement cascade involves formation of anaphylatoxins, C3a, C4a and C5a, which leads to the recruitment of inflammatory cells and the release of inflammatory mediators. These anaphylatoxins have been studied in regard to their applications in tissue regeneration. In particular, C5a shows the potential to stimulate pulp tissue regeneration (Giraud et al. 2018; Rombouts et al. 2016). C5a is capable of activating pulp progenitor cells to migrate towards injured site to induce the regeneration of the dentin-pulp complex (Chmilewsky et al. 2014a). It has also been shown that the pulp capping materials can regulate the C5a secretion (Giraud et al. 2018).

Deep cavity preparations or severe carious lesions, which disrupt the integrity of dentin-pulp complex, may impair odontoblasts, leading to their apoptosis (Mitsiadis et al. 2008). This stimulates dentin-pulp tissue repair through stimulation and proliferation of progenitor cells, which are recruited to the site of injury to differentiate towards odontoblast-like cells to replace injured dentin to reparative dentin tissue (Fitzgerald et al. 1990). Extra cellular matrix's components and growth factors have been shown to stimulate the migration of stem cells. In the dental pulp, transforming growth factor-beta (TGF-β1) promotes progenitor cell migration and odontoblast differentiation (Laurent et al. 2012). The schematic illustration dentin-pulp tissue regeneration via differantiation of the dental pulp stem cells (DPSCs) into odontoblast-like cells can be seen in Fig. 1. Studies have shown that the dentin matrix contains TGF-β1 and other bioactive molecules that include angiogenic growth factors (Roberts-Clark and Smith 2000). During dentin demineralization caused by carious lesions or acid administration, these molecules

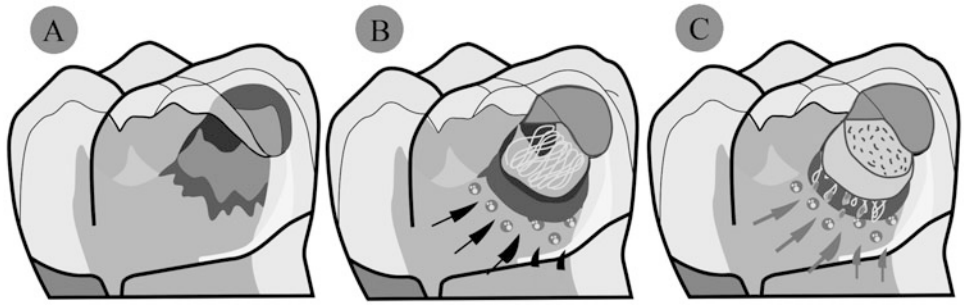

Fig. 1 Schematic illustration of the differantiation of DPSCs into odontoblast-like cells to induce reparative dentinogenesis. (**a**) Injury to the dentin pulp complex due to severe carious lesions and destruction of odontoblasts. (**b**) Migration of progenitor cells to differentiate towards odontoblast-like cells. (**c**) The damaged dentin is replaced by a reparative dentin formed by newly differentiated odontoblast-like cells

are released into the pulp tissue, where they can mediate the dentin regeneration process. In this regard, pulp capping materials such as calcium hydroxide and mineral trioxide aggregate (MTA) have been shown to induce formation of reparative dentin by modulation of TGF-β1 secretion from pulp cells (Laurent et al. 2012). Dentin and pulp tissues are intensely exposed to pathogens and occlusal stresses due to their unique microenvironment, thereby, the pulp inflammation must be under control before regeneration of the dentin-pulp complex.

3 Tertiary Dentinogenesis

Regeneration is the formation of a physiologically similar dentin tissue, while repair is a new tissue formation that resembles the natural dentin-pulp complex at the histological level with the expected physiological functions (Murray et al. 2007; Smith et al. 2016). Although vital pulp therapies' primary goal is the complete regeneration of tissues lost by carious or traumatic injuries, current treatments are unlikely to be able to stimulate natural dentin formation (Smith et al. 2016). In this context, two types of tertiary dentin formation can be described as reactive and reparative dentinogenesis which include primary odontoblasts and dental pulp stem cells with the capability of differentiation towards odontoblast-like cells. If primary odontoblasts are present, this type of tertiary dentin is called "reactionary dentinogenesis" and it occurs after mild to moderate dentin damage. However, localized tissue injury can cause necrosis or apoptosis of primary odontoblasts, and the formation of tertiary dentin by odontoblast-like cells is then called "reparative dentinogenesis" (Cooper et al. 2014).

Tertiary dentinogenesis is the result of a balance between the inflammatory response and irreversible pulpitis (Simon et al. 2010). Dentin has a permeable structure due to dentinal tubules (da Rosa et al. 2018; Hanks et al. 1994; Pashley 1988), and after dental injuries, through the diffusion of various molecules including bioactive proteins, bacteria and toxins, inflammation can commence (da Rosa et al. 2018). Although a mild inflammation stimulates tertiary dentinogenesis, inflamed pulp may impair tertiary dentinogenesis through necrosis and apoptosis (Cooper et al. 2010).

Reparative dentinogenesis requires differentiation of DPSCs into odontoblast-like cells to induce dentinogenesis signals (Smith et al. 2012). In the dental pulp, mesenchymal stem cells are mostly found at the perivascular area and cell rich region of Hohl adjacent to the odontoblastic layer. These sites act as a source of odontoblast replacement. Odontogenesis involves induction of peripheral cells of the dental papilla to differentiate towards odontoblasts with the release of various growth factors from the inner enamel epithelium, such as FGF-1, TGF-β1 and TGF-β3 (da Rosa et al. 2018).

Regenerative dentin-pulp treatment strategies investigated the role of a molecular approach through the application of growth factors in pulp capping and pulpotomy studies. Bioactive dentin matrix proteins are shown to enhance tertiary dentinogenesis with fewer initial inflammatory responses, TGF-β1and BMP-7 constituting the most extensively studied molecules (da Rosa et al. 2017). Although many studies have evaluated the effects of individual molecules, differentiation processes include interactions between various factors and their synergistic impact can differ significantly from their individual effects. Efforts in designing the correct combinations these molecules, as well as investigating their dynamics, can benefit the development of treatments focusing on tertiary dentinogenesis (Piva et al. 2014).

Considering the significant advances in the understanding of the cellular and molecular aspects of the dental pulp and dentin formation process, current approaches regarding regeneration of dentin-pulp complex are promising. The application of tissue engineering principles, using scaffolds loaded with multipotent stem cells and bioactive molecules to regenerate desired dental tissue might be the ideal treatment method as the preclinical studies are translated into prospective clinical treatments.

4 Dentin-Pulp Complex Regeneration

Regenerative endodontic treatment is defined as the biological-based processes that enable replacement of damaged structures of the dentin-pulp complex (Murray et al. 2007). The main purpose behind the regenerative endodontic treatment is to regain normal physiological features such as innate immunity, tertiary dentinogenesis, sense of occlusal pressure and pain that the necrotic pulp does not have. Regaining such properties to the teeth would eliminate the disadvantages of conventional endodontic treatment and increase the long-term success rate, thus allowing patients to retain their natural dentition for longer periods. In this context, it is necessary to regenerate the dentin-pulp complex with its normal physiological functions, not just retain the vitality of the dental pulp. This can only be achieved through tissue engineering applications as opposed to conventional treatment. Tissue engineering is a multidisciplinary and continuously expanding application area that deals with tissues damaged by congenital disorders, cancer, trauma, infection, inflammation, iatrogenic injuries or other conditions to restore structures and physiology (Reddi 1998; Nakashima and Reddi 2003). Tissue engineering consists of three elements called tissue engineering triad, which generally include stem cells, scaffolds, and bioactive molecules (Alsberg et al. 2001; Nakashima 2005).

4.1 Stem Cells

Stem cells are undifferentiated clonogenic cells with self-renewal capability which can differentiate into various lineages (Rao 2004). Mesenchymal stem cells (MSCs) reside in adult tissues and can be recruited upon injury for tissue healing and regeneration. Ever since their first isolation from bone marrow (Friedenstein et al. 1970), MSCs can be isolated from a variety of tissues including dental tissue, adipose tissue and umbilical chord (Ding et al. 2011). Dental stem cell sources have gained popularity due to high accesibility, ease of cell isolation and reduced ethical concerns as these tissues can be obtained during routine dental procedures (Ercal et al. 2018).

Dental pulp is the first dental tissue to be used as a stem cell source, which showed the ability to form a dentin-pulp complex-like structure containing a well-mineralized tubule matrix *in vivo* (Gronthos et al. 2000). This discovery boosted subsequent studies in more MSC sources of dental origin, as well as investigations in stem cell mediated dentin-pulp regeneration.

Other dental MSCs have been identified and characterized: stem cells from human exfoliated deciduous teeth (SHED) (Miura et al. 2003), periodontal ligament stem cells (PDLSCs) (Seo et al. 2004), dental follicle stem cells (DFSCs) (Morsczeck et al. 2005), and stem cells from

apical papilla (SCAP) (Sonoyama et al. 2006, 2008). First isolated by Miura et al. in 2003, SHED showed higher proliferation rate than DPSCs and bone marrow derived mesenchymal stem cells (BMMSCs) but were unable to regenerate a distinct dentin-pulp complex unlike DPSCs, rendering DPSCs a more favorable source for odontogenic differentiation (Miura et al. 2003). First isolated from third molars, PDLSCs have been reported to differentiate into cementoblast-like cells due to their expression of higher levels of tendon-specific transcription factors compared to DSPCs and BMMSCs (Seo et al. 2004). Although mineral deposition is observed in PDLSCs comparable to that of DSPCs and BMMSCs, newly formed tissue resembles cementum-PDL complex rather than dentin-pulp complex (Seo et al. 2004). First described by Sonoyama et al. in 2008, SCAP is isolated from the apical papilla of immature permanent teeth which evolve into the dental pulp. SCAP can transform into odontoblast-like cells capable of forming primary dentin. Proliferation rate and cell migration activity of SCAP were found to be higher than DPSCs isolated from the same tooth (Sonoyama et al. 2008).

In regard to dentin-pulp tissue regeneration approaches, the most suitable stem cell sources are DPSCs, SHED and SCAP as they are either derived from dental pulp tissue, including DPSCs and SHED, or from the pulp precursor, SCAP (Huang 2011). In terms of proliferation capacity, SHED is found to exhibit a higher proliferative activity and express increased levels of bFGF, which is involved in angiogenesis, compared to that of DPSCs and BMMSCs (Kunimatsu et al. 2018). Angiogenic properties of these cells are essential since newly formed dental pulp tissue requires sufficient vascularization considering the small size of the root apex. SCAP and DPSCs have shown to express various angiogenic proteins such as vascular endothelial growth factor (VEGF), insulin-like growth factor binding protein 3, angiopoietin-1 (Hilkens et al. 2014). However, the same authors showed that *in vivo* transplantation of DPSCs and/or SCAP embedded in a hydrogel containing 3D hydroxyapatite scaffolds exhibit less vascularization in the newly formed pulp-like tissue compared to control groups with only hydrogel. The authors suggested the role of transplantation period and the microenvironemt on the secretion of angiogenic factors in this unexpected outcome as well as the hard-tissue forming capacity of these cells, which can avert angiogenesis as they undergo osteo/odontogenic differentiation (Hilkens et al. 2017). The selection of a proper scaffold is as critical as the differentiation potential of the stem cell sources to achieve the desired regeneration considering the impact of its properties on the microenvironment.

4.2 Scaffold

Tremendous efforts in biomaterial sciences contributed to the enhancement of regenerative dentistry by moving preclinical studies to clinical applications. Scaffold is one of the three basic elements of the tissue engineering. It acts as a three-dimensional structural skeleton supporting the organization and vascularization of cells (Demarco et al. 2011; Nakashima and Akamine 2005). The material and physiomechanical properties of scaffolds have critical effects on stem cells and tissue regeneration (Barnes et al. 2007; Demarco et al. 2010; Graziano et al. 2008; Yang et al. 2001). In addition, the scaffold design determines the microenvironment that enables efficient delivery of cells and bioactive molecules. Scaffold is an imitation of the extracellular matrix, which should contain growth factors to help proliferation and differentiation, nutrients, and antibiotics to prevent bacterial growth (Karande et al. 2004; Oringer 2002; Tabata 2005). In order to provide these features, it should have sufficient porosity and pore width (Sachlos and Czernuszka 2003). The scaffold must also be biocompatible, and have optimal physical and mechanical properties. One of the most important features of a scaffold is that it should be bioresorbable or biodegradable; not require additional operation after fulfilling its purpose (Nakashima 2005; Smith 2001).

Natural polymers offer a favorable environment for tissue engineering, while allowing

partially controlled release of bioactive molecules (Galler et al. 2011c; Khayat et al. 2017; Yang et al. 2012). In contrast, production processes are challenging, and can trigger immune responses and consists pathogens (Galler et al. 2011c; Wang et al. 2010a). Natural polymers can originate from animals, algae, bacteria, plants, and host tissues (Chen et al. 2017; Galler et al. 2011c; Yang et al. 2012). Generally, decellularized host-derived/xenogeneic extracellular matrix (ECM) scaffolds are preferred for natural polymers in tissue engineering (Chen et al. 2017; Khayat et al. 2017).

Synthetic polymers can be designed in various geometries and porosities according to the application site, and their properties such as degredation rate, wettability and mechanical properties can be modified. It is much easier to add bioactive molecules to the structure of synthetic polymers that attract cells or are required in tissue engineering (Galler et al. 2011c). Synthetic polymers are divided into two as solid wall and nanofibrous structure according to their structure. Scaffolds with nanofibrous architecture were found to be more favourable than solid walled scaffolds in terms of cellular attachment, proliferation, differentiation and mineralization (Lee et al. 2012; Wang et al. 2010a, 2011).

Hydrogels are preferred due to their high biocompatibility and uniform encapsulation features (Galler et al. 2011c; Karakaya and Ulusoy). Gel formation dynamics, crosslink density, mechanical properties of the material, and degredation rates can be controlled. Synthetic hydrogels commonly used in dental pulp regeneration are polyethylene glycol (PEG) based hydrogels and self-assembling peptide hydrogels. Hybrid hydrogels such as fibrin/PEG aim to overcome shortcomings like rapid degredation of fibrin and is able to form a vascularized dental pulp-like connective tissue (Galler et al. 2011a).

Bioceramics are biocompatible, biodegradable and osteoconductive materials, which stimulate mineralization. On the other hand, porosity is difficult to form in these materials because of their fragile structure, and their workability is insufficient (Galler et al. 2011c; Zheng et al. 2011). Various indications are present in dentistry for the use of bioceramics including root repair, apical fill, perforation and bone defect treatment. Bioceramics can be divided into two as phosphate-based and silicate-based (Karakaya and Ulusoy 2018). Tricalcium silicates are currently considered to be the preferred materials for the treatment of vital pulp, because histological studies show that they stimulate dentin-pulp tissue regeneration with no pulp inflammation (Khayat et al. 2017). Silicate bioceramics, as opposed to calcium phosphate bioceramics, can have enhanced physicochemical and biological properties with improved bone regeneration capabilities (Wu and Chang 2013).

In dentin-pulp tissue regeneration, considering the complex root anatomy and limited space for pulp tissue, injectable biomaterials can be more advantageous due to the ease of clinical handling, and transplantation of cells into the root canals (Fukushima et al. 2019). Natural scaffolds including collagen, ECM, chitosan, and hyaluronic acid in the presence of stem cells show some kind of tissue formation with or without bioactive molecules while the lack of cells result in no tissue formation. Synthetic and hybrid scaffolds overcome the shortcomings of natural scaffolds but hybrid scaffolds are unable to form pulp-like tissue without stem cell presence while synthethic scaffolds require growth factors combined with stem cells in addition to difficulties in attracting host stem cells (Fukushima et al. 2019).

4.3 Bioactive Molecules

Bioactive molecules are another indispensable element of the tissue engineering. It is known that bioactive molecules recruit cells necessary for the tissue regeneration with chemotactic effects and induce their proliferation and differentiation. Bioactive molecules can be delivered exogenously to the regeneration area in addition to be supplied endogenously by demineralization of the dentin. It has been reported that 17% ethylenediaminetetraacetic acid (EDTA) liberates bioactive molecules trapped in primary and secondary dentin (Becerra et al. 2014; Galler et al. 2011b, 2015; Martin et al. 2014). These

molecules can be applied alone or in different combinations.

Transforming growth factor-β superfamily is among the most important growth factors in bioactive molecules. TGF-β has three isoforms, TGF-β1, TGF-β2 and TGF-β3. TGF-β1 is effective in primary odontoblastic differentiation and tertiary dentinogenesis (Cassidy et al. 1997). TGF-β3 induces ectopic mineralization in pulp tissue and increases osteocalcin and type1 collagen levels (Huojia et al. 2005). BMPs, members of the transforming growth factor-β superfamily, are involved in many biological activities including cell proliferation and differentiation. BMP-2, a growth factor approved for clinical use by the American Food and Drug Administration has shown to induce osteo/odontogenic differentiation and *in vivo* bone formation. The addition of BMP-2 to cell medium of DPSCs showed improved odontogenic differention as indicated by increased DSPP, DMP-1, MMP20 and Phex expression and osteo-dentin matrix tissue formation with vascularization *in vivo* (Atalayin et al. 2016).

Blood-derived growth factors such as platelet-derived growth factor (PDGF), TGF-β, FGF-2, VEGF, and insulin growth factor (IGF) are found in blood clots. VEGF is regarded as a dominant signaling protein involved in lymphangiogenesis, vasculogenesis, and angiogenesis (Holmes et al. 2007). Aksel and Huang (2017), showed that the delivery of VEGF to DPSCs in the early proliferating stage enhances osteo/odontogenic differentiation *in vitro* when combined with a continious application of BMP-2. As VEGF can also inhibit BMP-2 expression leading to an undesired less bone formation (Schönmeyr et al. 2009), determination of the most appropiate timing in growth factor addition during differentation cascades is of critical importance in the resultant tissue formation.

IGF-1 is an essential peptide for new bone formation and mineralization. IGF-1 is a multifunctional peptide, which promotes osteogenic proliferation and differentiation of DPSCs and SCAPs, and promotes alkaline phosphatase production (Alkharobi et al. 2016; Feng et al. 2014; Wang et al. 2011). IGF-1 is needed for maximum growth during development.

Resolvines (RvE1 and RvD1) that provide anti-inflammatory effects are molecules derived from omega-3 polyinsatured fatty acids that provide anti-inflammatory effects (Serhan and Petasis 2011; Serhan et al. 2000). Considering the negative effects of prolonged inflammation on stem cells, resolvins may be favorable in the treatment of pulpal inflammation, and the preservation or regeneration of a vital dental pulp. RvE1 down-regulates NF-κβ, allowing macrophages in M1 stage to shift to M2 stage, thereby resolving initial inflamatory responses (Goldberg 2019). In spite of this, although the inflammatory resolving effect of resolvins is known, its effectiveness in dentin-pulp complex regeneration should be investigated further.

Wnt signaling molecule family plays critical roles in normal morphogenesis of oral tissues including taste buds, taste papillae and teeth. Wnt proteins are short-range ligands that activate canonical or the Wnt/β-catenin pathway and non-canonical signaling pathways (Liu and Millar 2010). Cell migration, proliferation and differentiation during tooth development is known to be regulated by Wnt3, Wnt4, Wnt7b and Wnt10b, which are expressed in the dental epithelium and mesenchyme (Wang et al. 2014). A study demonstrated that upon injury, pulp cells are shown to differentiate into secretory odontoblasts responding to an amplified Wtn stimulus, a liposome-reconstituted Wnt3a, resulting in enhanced dentin regeneration (Hunter et al. 2015). Furthermore, Wnt3a and BMP-9 is shown to synergistically induce alkaline phosphatase (ALP) activity in immortalized SCAP and form a highly mineralized trabecular bone, indicating the importance of the crosstalk between Wnt and BMP signaling in osteo/odontogenic differentation (Zhang et al. 2015a). However, Wnt1, a member of Wnt/β-catenin signaling pathway, inhibits ALP activity and interferes with the differentiation of DPSCs into odontoblasts which might indicate its involvement in preserving MSCs in their undifferentiated state (Scheller et al. 2008). Nonetheless, as the Wnt/β-catenin is involved in tooth initiation, approaches in manipulation of this signalling pathway for promotion of dentin-pulp complex must be explored in future studies.

5 Cell-Based Pulp-Dentin Complex Regeneration

Cell-based dentin-pulp complex regeneration is primarily based on the transplantation of exogenous stem cells into the disinfected root cavity. Stem cells can be obtained from the host (autologous) or can be obtained from different individuals (allogenic). These cells can be transplanted directly or expanded in cultures so that they can reach sufficient numbers under laboratory conditions. In the regeneration of cell-based dentin-pulp complex, the main goal is to produce a new dentin-pulp complex covering the root canals and pulp chamber, which has the capability of dentin formation. Various studies investigated cell-based approach to regenerate dentin-pulp complex over the last decade (Table 1).

5.1 De Novo Regeneration of Dentin-Pulp Complex

Approach for the regeneration of lost pulp tissue in the emptied root canal space is called *de novo* regeneration. Many studies in regenerative dentistry are focused on revascularization of the inflamed tooth with incomplete root formation. Although there are promising case studies, in which periapical inflammation is resolved and pulp-like and dentin-like tissues are formed, revascularization is indicated for tooth with open apice and incomplete root development (Banchs and Trope 2004; Ding et al. 2009; Nosrat et al. 2011; Peng et al. 2017). Considering the regenerative treatment of fully developed teeth, cell-based approaches should be developed. *de novo* regeneration approaches focus on stem cell sources including DPSCs, SCAP and SHED. Schematic illustration of the cell-based *de novo* regeneration approaches can be seen in Fig. 2.

Huang et al. (2010) isolated DPSCs and SCAP and inserted them on a poly-D, L-lactide/glycolide scaffold to perform *de novo* regeneration in empty root canals for the first time. The prepared root canal fragments were subcutaneously transplanted into immunocompromised-SCID mice. As a result of histological analysis after 3–4 months, the root canal cavity was filled with pulp-like tissue and a good vascularization was established compared to scaffold alone groups which displayed fibrous tissue formation. Moreover, new dentin-like mineralized tissue formation by newly formed odontoblast-like cells was observed on the existing dentin wall located at the cavity periphery. Autologous transplantation of CD105-positive dental pulp cells with stromal-derived factor-1 (SDF-1) in adult canine teeth after pulpectomy demonstrated complete pulp regeneration indicating the possibility to regenerate the entire pulp tissue for the first time (Iohara et al. 2011). Another canine *in vivo* study investigated dentin-pulp tissue regeneration in immature teeth with autologous DPSCs embedded in a chitosan hydrogel scaffold combined with various growth factors in an induced apical periodontitis model. Radiographic and histopathological evidence indicated significantly greater root lenghtening, higher apical closure and presence of a pulp-like tissue with stem cell groups whereas scaffolds loaded with only growth factors showed no soft tissue formation (el Ashiry et al. 2018). An *in vivo* study in swine included delivery of autologous or allogeneic DPSCs in hyaluronic acid or collagen gel into the root canals subjected to total pulpectomy and canal enlargement procedures, which were then closed with collagen or tricalcium phosphate/hydroxyapatite with MTA cement. Results indicated orthotopic pulp regeneration with dentin-like mineral tissue deposition and newly formed dentinal tubules, although newly formed hard tissue lacked natural dentin structure which is difficult to obtain in regeneration (Zhu et al. 2018). Studies indicate the necessity of the stem cell use to achieve a viable vascularized pulp tissue regeneration.

Succeeding promising *in vivo* data, clinical studies are initiated to translate these findings into daily practice. In a recent clinical pilot study with 5 cases, *de novo* regeneration was attempted in a tooth with irreversible pulpitis and no apical lesion (Nakashima and Iohara 2017; Nakashima et al. 2017). Mobilized DSPCs

Table 1 Cell-based approach studies (*De novo* regeneration)

Author	Cell	Method	Scaffold/ bioactive molecule	Outcomes
Iohara et al. (2011)	DPSCs	*In vivo*	Collagen	Similar mRNA expression patterns in the regenerated pulp compared to that of normal pulp
				Presence of pulp-like tissue, odontoblast-like cells attached to dentinal wall extending into dentin tubules
				Neurogenesis and angiogenesis shown by immunostaining and complete regeneration of whole pulp after pulpectomy in mature teeth
Suzuki et al. (2011)	DPSCs	*In vivo*	Collagen gel SDF1, FGF-2, BMP-7	SDF1 and FGF-2 can recruit DPSCs into a 3D collagen scaffold whereas BMP-7 has minor effect on cell recruitment
				BMP-7 is effective in inducing mineralization in cell culture of DPSCs
				FGF-2 adsorbed collagen scaffold resulted in re-cellularization and tissue integration in human teeth implanted into the dorsum of the rats
Zhu et al. (2012)	DPSCs	*In vivo*	PRP	All canals filled with blood clot showed new vital tissue formation
				DPSCs and/or PRP transplantation groups indicated no improvement in new tissue formation compared to blood clot alone groups
Wang et al. (2013)	DPSCs	*In vivo*	Gelfoam	Formation of pulp-like tissues containing blood vessels and dentin-like tissue and thickening of dentinal wall in groups with DPSCs loaded gelfoam
Dissanayaka et al. (2014)	DPSCs	*In vivo*	No scaffold Prevascularized with HUVEC	Prevascularized microtissue spheroids of DPSCs can regenerate vascular dental pulp-like tissue in tooth-root slices
Zhu et al. (2014)	DPSCs	*In vivo*	PRP	Newly formed tissue is shown to be mainly cemental, bone and periodontal-like fibrous tissue instead of dentin-pulp tissue as indicated by positive staining for tartrate-resistant acid phosphatase, bone sialoprotein, periostin and osteocalcin
Chen et al. (2015)	DPSCs	*In vivo*	PRF	PRF promotes the proliferation and induces osteo/odontogenic differentiation of DPSCs
				Vascularized pulp-like tissue is formed and intracanal walls of root show deposition of regenerated dentin with DPSC cell sheets of DPSCs and PRF
Dissanayaka et al. (2015)	DPSCs	*In vivo*	Peptide hydrogel (PuraMatrix™) HUVEC co-culture	DPSCs promote remodeling of capillary structure established by HUVECs
				Hydrogel/cell constructs show partial regeneration of pulp-like tissue in root canals up to the middle to lower third
Hatab et al. (2015)	DPSCs	Clinical case study	Chitosan	Autologous transplantation of DPSCs in chitosan into the root canals shows pulp-like tissue as indicated by the presence of odontoblast-like cells aligning newly formed dentin-like tissue

(continued)

Table 1 (continued)

Author	Cell	Method	Scaffold/ bioactive molecule	Outcomes
Na et al. (2016)	SCAPs	*In vivo*	3D Scaffold-free stem-cell sheet-derived pellet	Scaffold-free stem-cell sheet-derived pellet transplantation in human treated dentin matrix fragments indicate presence of a vascularized pulp-like tissue and dentin-like tissue deposition
Hilkens et al. (2017)	DPSCs SCAP	*In vivo*	3D-printed hydroxyapatite Hydrogel	Constructs loaded with stem cells display pulp-like tissue with vascularization with a distinctively lower collagen content compared to dental pulp tissue
				DPSC+SCAP constructs show lower vascularization than negative control transplants due to osteo/odontogenic differentiation
Nakashima et al. (2017)	Human mobilized DPSCs	Clinical study	Atelocollagen G-CSF	Human MDPSCs are safe and efficacious for complete pulp regeneration in humans.
				Continued thickening of radicular walls.
				Electric pulp tester for evaluation of the sensibility of teeth: Positive response in 4 cases out of the 5 cases.
Xuan et al. (2018)	Autologous SHED	Clinical trial	–	Stem cell implantation to immature permanent traumatized teeth shows pulp regeneration, increased root length and reduced width of the apical foramen
Zhu et al. (2018)	DPSCs SCAP	*In vivo*	Collagen TE Hyaluronic acid	Autologous DPSCs transplantation shows vascularized pulp-like tissue in root canal space and new dentin-like tissue deposition along root canal walls in swine
el Ashiry (2018)	DPSCs	*In vivo*	Chitosan hydrogel VEGF FGF-2 PDGF NGF BMP-7	DPSCs transplants with growth factors in immature teeth show pulp-like tissue formation, greater root lengthening and higher apical closure compared to scaffolds loaded with only growth factors in apical periodontitis
Meza et al. (2019)	DPSCs	Case study	L-PRF	Autologous DPSCs transplantation combined with L-PRF in the treatment of irreversible pulpitis of a mature tooth shows healthy periapical bone, dentin bridge formation in the middle third of the root, calcification of the canal in the apical third and delayed response to thermal stimuli after 36 months follow-up
Brizuela et al. (2020)	Encapsulated human umbilical cord MSCs	Randomized clinical trial	Plasma derived biomaterial	After treatment in mature teeth with apical lesions, increased positive pulp response is observed after 12 months of follow up

3D three dimensional, *BMP* bone morphogenic protein, *FGF* fibroblast growth factor, *DPSCs* dental pulp stem cells, *G-CSF* granulocyte-colony stimulating factor, *L-PRF* leukocyte-platelet rich plasma, *MSCs* mesenchymal stem cells, *NGF* nerve growth factor, *PDGF* platelet-derived growth factor, *PRF* platelet rich fibrin, *PRP* platelet rich plasma, *SCAP* stem cells from apical papilla, *SDF-1* stromal-derived factor-1, *SHED* stem cells from human exfoliated deciduous teeth, *VEGF* vascular endothelial growth factor

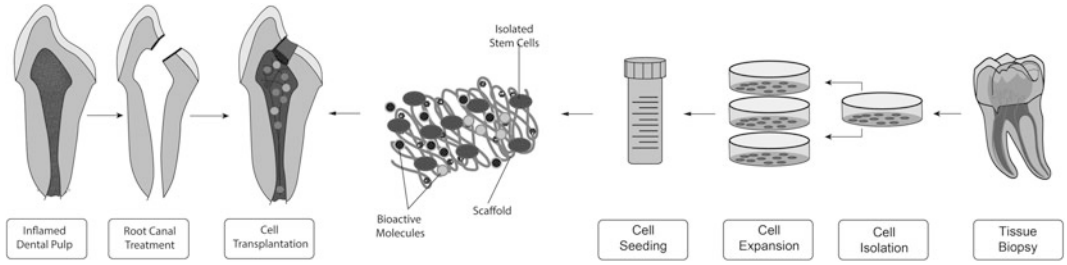

Fig. 2 Illustration of cell-based *de novo* approach for dentin-pulp tissue regeneration. This approach is based on the transplantation of isolated cells to the disinfected root canal system after cell harvesting by tissue biopsy. The isolated stem cells are expanded under laboratory conditions and transplanted to the previously disinfected root canal space in combination with scaffolds and bioactive molecules

isolated from the patient's discarded tooth were transplanted into the empty root canal of the disinfected tooth and were followed for 24 weeks. After 4 weeks, the teeth responded positively to the electrical pulp test. In the magnetic resonance imaging (MRI) and cone beam computed tomography (CBCT) examination performed 24 weeks later, dental pulp tissue and functional dentin formation were observed similar to untreated control. As a result, researchers commented that human mobilized DPSCs are safe and efficacious for complete pulp regeneration. A recent and the only human clinical controlled study on treatment of injured permanent immature teeth with autologous decidous DPSCs, showed regeneration of the dental pulp as well as increased root length and reduced width of the apical foramen 12 months post-treatment (Xuan et al. 2018). Even at the follow-up of 24 months, the regenerated pulp tissue was still viable and no complications were noted, rendering this a promising approach to treat immature teeth with pulp trauma.

Regarding the necessity to regenerate the entire pulp tissue in cases with irreversible pulpitis, the availability of viable and multipotent MSCs in inflamed dental pulp might be critical in the treatment approach. Studies comparing the DPSCs and SHED harvested from normal and inflamed pulp tissue revealed that the diseased pulp contains cells with similar proliferation and differentiation potential to that of normal tissue (Alongi et al. 2010; Yu et al. 2014). With the aim to ameliorate properties of cells of inflamed pulp, application of FGF-2 to MSCs isolated from inflamed pulp tissue during ex vivo expansion was investigated. This study demonstrated enhanced proliferation and migration potential, increased formation of dentin-like material *in vivo* but showed reduced differentiation potential *in vitro* (Kim et al. 2014). However, an investigation on immunomodulatory properties of inflamed pulp deciduous tissue indicated that these cells do not posess typical MSC charateristics and show lower immunsupression properties compared to that of healthy tissue (Yazid et al. 2014). Although the use of inflamed pulp tissue for regeneration approaches might prove to be beneficial, more research is necessary before considering inflamed tissue as a reliable stem cell source for dentin-pulp tissue regeneration.

De novo regeneration is a promising method, but there are a number of question marks. The entire root canal system must be disinfected in order to perform the procedures of *de novo* regeneration. However, the root canal system contains many complex anatomical formations such as istmus, lateral canals and apical delta. Presence of residual bacteria in these formations may lead to persistent infections, which risk the *de novo* regeneration. As the conventional endodontic therapy is currently focused on the disinfection of persistent infections, developments in this regard will also contribute to *de novo* regeneration procedures. On the other hand, the root canal structure preferred in studies has a wide and simple anatomy, so it is an issue to perform *de novo* regeneration in teeth with complex anatomy.

6 Cell-Free Dentin-Pulp Complex Regeneration

Cell homing is a tissue regeneration approach based on recruitment of host stem cells into the defect site (Fig. 3). Concerning this appoach in dentin-pulp tissue regeneration, the scaffolds containing bioactive molecules are aimed to induce the chemotaxis, proliferation and differentiation of host endogenous stem cells from resident tissue including periapical region or dental pulp (Eramo et al. 2018; Kim et al. 2013; Mao et al. 2012). In the first study of cell-homing based dentin-pulp tissue regeneration, Kim et al. combined VEGF, FGF-2, PDGF with basal nerve growth factor (NGF) and BMP-7, and reported that after implantation into endodontically treated root canals, the entire cavity was regenerated with dentin-pulp-like tissue (Kim et al. 2010). Recent cell-free investigations in dentin-pulp tissue regeneration are listed in Table 2.

Host endogenous cells involved in this process are residual DPSCs, periapical tissue orginated cells such as SCAP and BMMSCs. Therefore, angiogenic and odontogenic differentiation capacity of these cells can play a key role in the treatment outcomes of this approach. In clinical scenarios with pulpitis where vital tissue still remains, host endogenous stem cells can still remain viable. A method for distinction between remaining healthy and damaged pulp tissue might serve beneficial for upcoming treatment modalities whereas in cases with pulp necrosis, promotion in recruitment of stem cells from periapical tissues and blood stream is necessary (Galler et al. 2014).

An important stem cell source in this regard is SCAP that can survive due to collateral blood supply during pulp necrosis (Yang et al. 2016). On the other hand, BMMSCs which are associated with the dentin-pulp complex, may be another source of cells (Yang et al. 2016; Zhang et al. 2015b). Ostby's study on the importance of blood clot in wound healing can be considered as the first study to evaluate the local regeneration of pulp tissue in the root canal system (Ostby 1961). In this study, hemorrhage was attempted by laceration of periapical tissues through overenstrumentation with endodontic files following aseptic access to the root canal system and pulp removal. As a result of the study, it was reported that the apical inflammation caused by overinstrumantation resolves in 2 weeks and the clot in the root canal system is replaced by granulation tissue, and then fibrous tissue; however, dentin resorption was observed and no formation of new dentin was reported. Later on, it was shown that the revascularization in the root canal is important for root development, as indicated by the studies in the field of dental traumatology (Galler 2016; Skoglund et al. 1978). Today, this treatment modality is adopted by the American

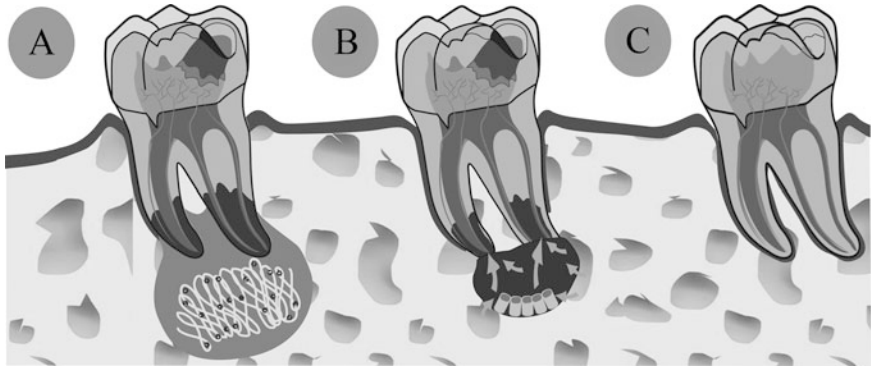

Fig. 3 (**a**) Host endogenous cells involved in the cell homing process are residual DPSCs, SCAP and BMMSCs. SCAP and BMMSCs are located at the apical region of the tooth and can survive due to collateral blood supply during pulp necrosis. (**b**) Recruitment of host stem cells from periapical tissues into the defect site. (**c**) Efficient regeneration of the dentin-pulp complex by cell homing

Table 2 Cell-free approach studies

Author	Method	Scaffold/bioactive molecule	Outcome
Torabinejad et al. (2015)	*In vivo*	Blood clot/Gelfoam PRP	Bone-like and cementum-like hard tissue ingrowth in pulp space
			Root lengthening was not detected
			No difference between PRP and blood clot/Gelfoam groups in hard tissue formation or intracanal inflammation
Priya et al. (2016)	Case study	PRP	The use of PRP injection in avulsed mature tooth showed internal and external root resorption after 6 months with periapical radiolucency
			Antibiotic placement into root canal resolved periapical radiolucency at the 9 and 12 month radiographs with a positive response to pulp vitality tests
Alagl et al. (2017)	Clinical study	PRP	All treated immature teeth were asymptomatic and showed a decrease in periapical lesion size and increased bone density
			No difference between test and blood clot control groups except for increased root length in PRP groups
Palma et al. (2017)	*In vivo*	Chitosan hydrogel	Significantly more mineralized tissue formation inside root canal in blood clot groups
			New dentin formation in isolated areas of blood clot groups
			The use of chitosan scaffolds did not enhance regeneration
Shivashankar et al. (2017)	Triple blind randomized clinical trial	PRP PRF	PRP was superior than PRF and blood clot in periapical wound healing after 6 months of follow-up
			No significant difference between groups in clinical and radiographic examination criteria
Alqahtani et al. (2018)	*In vitro*	Decellularized ECM	Decellularized pulp ECM showed CD-31+ and DSP + pulp-like tissue formation in pulp space and deposition of mineralized tissue within the dental pulp
	In vivo		
el Kalla et al. (2019)	*In vivo*	PRF	No significant difference in apical closure and new vital tissue formation in PRF groups and control groups (calcium hydroxide treatment) in contaminated immature teeth
			In non-contaminated root canals, cementoid tissue is formed in PRF treatment while fibrous tissue is seen in calcium hydroxide treatment
El Nasser et al. (2019)	*In vivo*	L-PRF	Enhanced vascularization and increased number of odontoblast-like cells in L-PRF treated groups compared to blood clot after 1 months
Nosrat et al. (2019)	Clinical study	Collagen-hydroxyapatite scaffold (SynOss Putty)	SynOss Putty + blood showed mineralized tissue formation inside root canal while only SynOss Putty resulted in intracanal and periapical inflammation
Rizk et al. (2019)	Randomized controlled clinical trial	PRP	Significant increase in root length, periapical bone density and decreased apical diameter in groups treated with PRP compared to control groups with blood clot after 12 months
			No response to pulp sensibility tests
Tomer et al. (2019)	Case study	PRF and biodentine	PRF can be used for the treatment of immature permanent teeth with periapical lesion, as part of a regenerative endodontic procedures.

(continued)

Table 2 (continued)

Author	Method	Scaffold/bioactive molecule	Outcome
Ulusoy et al. (2019)	Prospective randomized trial	PRP PRF Platelet pellet	All treated immature teeth displayed similar outcomes in terms of periapical healing, root development and response to pulp sensitivity tests
			Root and root canal area was greater in blood clot groups than those of PRF, PRP and platelet pellet groups in radiographic examination
Mittal and Parashar (2019)	Clinical study	PRF Collagen Placentrex Chitosan	12 months after procedures, all groups demonstrated improvement without a significant difference in parameters including periapical healing, root lengthening and apical closure
			PRF and collagen scaffolds performed better in dentinal wall thickening
Alexander et al. (2020)	*In vivo*	Collagen-hydroxyapatite scaffold (SynOss Putty)	In groups treated with blood clot, mineralized tissue formation is observed in root canals while SynOss putty yields no regenerated tissue
Kandemir Demirci et al. (2020)	Case series	PRF	Treatment of immature permanent teeth with PRF indicated resolution of periapical lesions but no response to pulp sensibility tests after 36 months of follow-up
Yoshpe et al. (2020)	Case series	PRF	PRF treatment in necrotic immature teeth showed cessation of replacement resorption

DSP dentin sialoprotein, *ECM* extracellular matrix, *PRF* platelet rich fibrin, *PRP* platelet rich plasma, *L-PRF* leukocyte-platelet rich plasma

Dental Association since 2011, as the practice of evoked bleeding, also known as apical revascularization.

Revascularization has positive results in apical healing, mineralized tissue formation and completion of root development (Bose et al. 2009; Ding et al. 2009; Jeeruphan et al. 2012; Shah et al. 2008; Torabinejad and Turman 2011). MSCs can be recruited as a result of bleeding from the root canal from periapical tissues, and growth factors within the clot are important for local regeneration (Lovelace et al. 2011; Nosrat et al. 2012; Shah et al. 2008). Thus, intracanal bleeding provides the stem cell, scaffold, bioactive molecules required for the tissue engineering triad (Lovelace et al. 2011; Nosrat et al. 2012; Wigler et al. 2013). An investigation on MSCs gene expression in blood collected by intracanal evoked bleeding technique from mature teeth revealed upregulation of CD73, CD90, CD105 and CD146 while CD45, a negative marker for MSCs, was significantly downregulated, indicating the delivery of MSCs into root canal by this method (Chrepa et al. 2015). Schematic illustration of revascularization procedure can be seen in Fig. 4. Another *in vivo* study showed that establishing a blood clot after pulpectomy displays similar new tissue formation in root canal compared to transplantation of DPSCs and/or platelet rich plasma (PRP) histologically (Zhu et al. 2012), rendering this approach a viable alternative as it involves no time consuming cell isolation procedures. Various studies investigate PRP injection into the pulp space due to its physiological concentration of growth factors to enhance homing of host stem cells (Alagl et al. 2017; Priya et al. 2016; Rizk et al. 2019; Shivashankar et al. 2017; Torabinejad et al. 2015; Ulusoy et al. 2019). However, there is no consensus on treatment protocols as the results vary in such investigations. The use of PRP in immature necrotic teeth show similar clinical and radiographic outcomes with blood clot, in periapical healing, root development and response to pulp sensitivity tests (Shivashankar et al. 2017; Ulusoy et al. 2019). On the other hand, in another randomized clinical trial, significant changes were noted in periapical bone

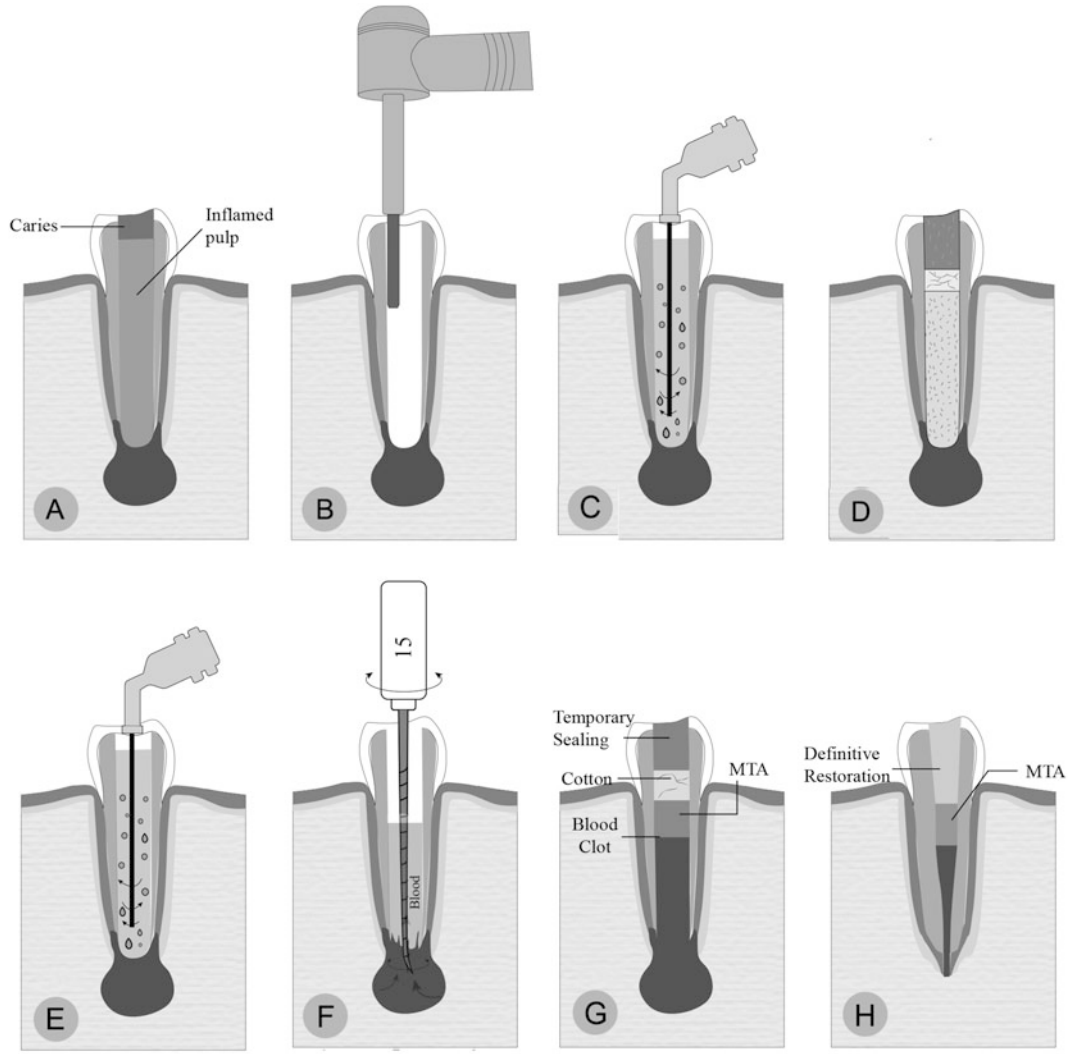

Fig. 4 Revascularization procedure of the inflamed tooth with incomplete root formation. (**a**) Inflamed tooth with open apices. (**b**) Endodontic access cavity preparation. (**c**) Irrigation of the root canal space using low concentration of NaOCl (less than 3%), followed by 17% EDTA. (**d**) Intracanal medicament (calcium hydroxide) placement with temporary filling material. E) After confirming the resolution of inflammation, root canal system is irrigated with 17% EDTA followed by sterile saline. (**f**) Over instrumentation with precurved file to promote bleeding and formation of the blood clot. (**g**) Pulp capping materials such as Biodentine or MTA are placed over the blood clot. (**h**) Root develepment with apical closure and maintenance of viable pulp tissue are achieved

density, root length and apical diameter for PRP treated teeth (Rizk et al. 2019). As the trials show comparable results, disadvantages including blood withdrawal and requirement of additional equipment must be considered when preferring PRP whereas blood clot method obtained by provoked bleeding includes risks such as progressive deposition of hard tissue or continious obliteration of root canals (Ulusoy et al. 2019).

Although pulp vitalization and resolution of apical inflammation are achieved as a result of revascularization, there are still question marks if the revascularized tissue can perform the functions of the dentin-pulp complex. It has been reported that cementum aposition, ectopic bone formation, and fibrous tissue can be detected in the revascularized root canal, but dentin-pulp complex cannot be mentioned (Martin et al. 2013;

Wang et al. 2010b). The formation of mineralized tissue inside newly formed pulp-like tissue is attributed to the differentiation of migrated PDLSC into cementoblasts, which can be prevented by the addition of cementogenesis inhibitor factors in pulp regeneration approaches (Alqahtani et al. 2018).

The residual DPSCs in the inflamed pulp tissue, which is reported to have similar MSC characteristics as the DPSCs in healthy pulp tissue, might serve as a potential source in dentin-pulp regeneration (Alongi et al. 2010). However, Chang et al. (2013) reported that NF-κβ had an inhibitory effect on the osteogenic differentiation of MSCs. In another study, it was stated that DPSCs lost their differentiation and mineralization abilities due to exposure to TNF-alpha, an inflammatory cytokine (Boyle et al. 2014). In order to increase the success of the local regeneration of the dentin-pulp complex, vital tissues need to be preserved as much as possible (Wigler et al. 2013). Therefore, it is expected that both the antimicrobial agents used in resolving the inflammation and the restorative materials used to provide coronal sealing should have a minimum cytotoxic effect on the viability and the proliferation of stem cells and fibroblasts (Thibodeau and Trope 2007). In conventional endodontic therapy, NaOCl is used as an irrigation solution in concentrations ranging from 0.5% to 6% (Kontakiotis et al. 2015). Studies have reported that NaOCl at a concentration above 3% shows cytotoxic effects on SCAP and interferes with the cell adhesion to the dentin surface (Chang et al. 2001; Martin et al. 2014; Ring et al. 2008). On the other hand, 17% EDTA application liberates bioactive molecules such as growth factors, which are fossilized in dentin tissue after primary and secondary dentinogenesis (Becerra et al. 2014; Galler et al. 2011b, 2015; Martin et al. 2014). For this reason, it is beneficial to irrigate with a 1.5% NaOCl solution, and 17% EDTA cam be used as the final irrigation agent for the protection of SCAP and the secretion of bioactive molecules from dentin.

Local regeneration using DPSCs is quite similar to conventional pulp capping therapies. Previous studies have reported that some bioactive molecules, such as BMPs, induce healing and reparative responses in inflamed pulp tissue (Nakamura et al. 2006; Rutherford and Gu 2000; Six et al. 2002). However, the application of only bioactive molecules without the use of a scaffold induces only reparative dentin synthesis, and is no different than vital pulp treatments such as pulp capping. Nakashima (1994) used inactivated demineralized dentin matrix as a natural scaffold, and found that BMPs increased the production of new dentin by inducing differentiation of pulp cells to odontoblasts. Also, the importance of blood supply for the success of regeneration cannot be ignored. Local regeneration of the dentin-pulp complex can occur in two situations: teeth with a pulp exposure as a result of carious dentin, where healthy pulp tissue is partially preserved, and teeth with open apicies, which root development is incomplete. Studies have reported that the healing rate decreases with a foramen apex width of less than 1.5 mm, whereas revascularization of the pulp cannot be achieved in those narrower than 1 mm (Huang et al. 2013).

In summary, local regeneration capacities of bone and dentin tissues are quite different from each other. Bone has a morrow space with stem cells responsible for new bone regeneration. Unfortunately, dentin does not have morrow space and regeneration is completely dependent on the condition of the pulp tissue. On the other side, revascularized pulpal tissue obtained using periodontal ligament and alveolar tissues is not similar to healthy pulp tissue. Therefore, preserving healthy pulp tissue as much as possible is very important for dentin tissue regeneration. The amount of the remaining healthy pulp tissue required for local regeneration is unknown. In cases where there is not enough healthy pulp tissue, vascularization or *de novo* regeneration approaches can be preferred for regeneration of the dentin-pulp complex.

7 Challenges and Future Perspectives

Today, a wide array of studies are carried out with various bioactive molecules, stem cells and scaffold combinations in order to provide the ideal

microenvironment for regeneration of dentin-pulp complex. There are promising results from these studies, but it is still difficult to translate these findings into the clinical practice. As opposed to immense *in vitro* and *in vivo* evidence, clinical data is scarce. Although evidence indicates the transplantation of stem cells for pulpal regeneration a promising approach, publication bias and inconsistency in study outcomes pose a serious question regarding the success of this treatment modality (Fawzy El-Sayed et al. 2019). The only recent clinical study have shown that autologous transplantation of MSCs promote complete pulp regeneration and tooth recovery after 24 months. However, the follow-up period is still short to render this approach superior compared to the success of conventional treatment methods. Furthermore, there is still a lack of evidence on possible side effects of stem cell transplantation in pulp regeneration as the mechanisms regarding the regeneration process of the dental pulp in such treatment approaches remain unelucidated.

Regarding technical difficulties of cell-based approaches in dentin-pulp tissue regeneration, the ex vivo cell expansion period is time consuming considering the necessary steps including tooth extraction, extirpation of the pulp tissue, ex vivo cell expansion, selection of MSC populations, and the transfer and storage of the transplants. There is still the immune reaction problem in cell culture due to the presence of animal proteins in growth media or even in the use of allogeneic human serum. Autologous use of serum can eliminate such risks but the availability of necessary amounts to expand sufficient number of cells is limited. These processes are prone to contamination and the cost-efficiency is debateable.

Another issue in cell-based treatment modalities of dentin-pulp tissue regeneration is the availability of a suitable and affordable stem cell source. Although there is a wide use of umbilical cord stem cell banking, dental stem cell banking is still on the onset. Many dental stem cell banking services accept stem cells from decidous teeth enabling the use of only SHED and DPSCs (Zeitlin 2020), excluding promising dental stem cell sources including apical papilla, periodontal ligament and dental follicle. As there is a lack of studies on allogeneic transplantation of cells, current dental stem cell banking cannot wholly benefit adult or elderly patient population requiring pulpal regeneration. In addition, the efficiency of regenerative therapies in aged recipients is questionable. The regeneration of dental pulp was shown to be poor after autologous transplantation of aged DPSCs compared to that of the young DPSCs (Iohara et al. 2014), indicating the impact of reduced proliferation and differentiation abilities and age dependent host microenvironment.

A major drawback in the majority of the published studies is regarding their conduction in conditions not relevant to most probable and common clinical scenarios such as infected root canals and apical periodontitis. Ideal conditions were sought where cell transplants were applied to immature teeth with large apical foramina with removed healthy pulp tissue. Studies on permanent teeth with narrow canal openings are necessary as this is the most likely clinical scenario suffering from loss of pulp tissue in need of whole pulp regeneration. Pulp tissue usually has limited blood supply due to the presence of only one apical foramen in most cases, therefore enhancement strategies regarding angiogenesis are necessary.

Considering the cause of pulp tissue loss, infected root canal and apical periodontitis pose a risk to the success in dentin-pulp tissue regeneration when using cell/scaffold mediated pulp regeneration. Establishment of a sterile environment for regeneration is essential in survival and differentiation of stem cells towards formation of a healthy new dentin-pulp tissue. As the root canal system is complex with variations, remaining bacteria in persistent infections and possible toxic effects of disinfectant agents to stem cells might alter the necessary microenvironment for the stem cells to contribute to *de novo* regeneration process.

In conclusion, stem cell-based *de novo* dentin-pulp tissue regeneration shows promising results though further investigations are necessary to regenerate a higher quality dentin and pulp tissue and to overcome difficulties such as mineralized tissue formation in the pulp, root canal

calcification and regaining a physiologically functioning pulp. Steps to reduce the cost and the time consuming process of cell culture are necessary to propagate this approach to clinical practice.

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Conflict of Interest The authors declare no potential conflict of interest.

Ethical Approval The authors declare that this article does not contain any studies with human participants or animals.

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Stem Cell Culture Under Simulated Microgravity

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Abstract

Challenging environment of space causes several pivotal alterations in living systems, especially due to microgravity. The possibility of simulating microgravity by ground-based systems provides research opportunities that may lead to the understanding of *in vitro* biological effects of microgravity by eliminating the challenges inherent to spaceflight experiments. Stem cells are one of the most prominent cell types, due to their self-renewal and differentiation capabilities. Research on stem cells under simulated microgravity has generated many important findings, enlightening the impact of microgravity on molecular and cellular processes of stem cells with varying potencies. Simulation techniques including clinostat, random positioning machine, rotating wall vessel and magnetic levitation-based systems have improved our knowledge on the effects of microgravity on morphology, migration, proliferation and differentiation of stem cells. Clarification of the mechanisms underlying such changes offers exciting potential for various applications such as identification of putative therapeutic targets to modulate stem cell function and stem cell based regenerative medicine.

Keywords

In vitro model · Simulated microgravity · Stem cells

Abbreviations

Ad-hMSC	human adipose-derived mesenchymal stem cell
BDNF	brain derived-neurotrophic factor
bFGF	basic fibroblast growth factor
BMOL	bipotential murine oval liver
BM	bone marrow
cMSC	cranial bone derived-mesenchymal stem cell
CBSC	cord blood stem cells
GDNF	glial-cell derived-neurotrophic factor
GDNF	glial cell line-derived neurotrophic factor
hDPSC	human dental pulp stem cell
HSCs	hematopoetic stem cells
HGF	hepatocyte growth factor
hEpSCs	human epidermal stem cells
mBMSC	mouse bone marrow stem cell
MG	(simulated) microgravity
NG	normal gravity

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Rab-BMSC	rabbit bone marrow stem cell
RCCS	rotating cell culture system
RWV	rotating wall vessel
SSCs	spermatogonial stem cells
TGF-β	transforming growth factor
VEGF	vascular endothelial growth factor

1 Introduction

Space missions induce many changes in human health and physiology, and among many factors changes in gravitational loading is one of the major contributors to these changes (Garrett-Bakelman et al. 2019). Mechanical forces, including those governed by gravitational loading, act as regulatory signals that effect morphology and function of tissues (Benjamin and Hillen 2003; Buravkova et al. 2008; Ozcivici et al. 2010a). Therefore, lack of gravity causes several health problems such as bone loss (Vico et al. 2000), muscle atrophy (Fitts et al. 2001), cardiovascular deconditioning (Aubert et al. 2005) and immune system dysregulation (Crucian et al. 2008). Biomedical implications of gravitational alterations highlight the importance of microgravity research to ensure the possibility of extension of space flights duration for future missions and potential colonization efforts in space bodies with different gravity fields compared to that of earth.

Consistent with tissue-level changes, microgravity is known to induce structural and functional changes in various cell types (Buravkova et al. 2004; Kacena et al. 2003). For instance, even though immune cells in circulation were found to be increased in number during spaceflights (Mills et al. 2001), microgravity weakens host defense by decreasing phagocytic capacity of these immune cells (Kaur et al. 2005). As another example, in bone tissue microgravity reduces the number and function of osteoblasts involved in bone formation (Hughes-Fulford and Lewis 1996), reducing new bone formation as well as loss of regulation over bone resorption by osteoclasts (Nabavi et al. 2011), resulting in net bone loss. Although studies focusing on mature cells provide valuable biological information on adaptations to microgravity, tissue level implications of long-term spaceflight infer deterioration of different magnitudes (Buravkova 2010). An important aspect of long-term health considerations for space-flight focus on augmenting recovery through regeneration (Blaber et al. 2014). Stem cells with their vast capacity for self-renewal and differentiation carry a great regenerative potential for tissues (Roobrouck et al. 2008) and therefore they are considered as strong therapeutic candidates for space biomedicine.

Spaceflight technologies allow life sciences laboratory setups in orbit for the execution of *in situ* microgravity experiments, both *in vivo* (Sandonà et al. 2012) and *in vitro* (Tamma et al. 2009). However, opportunities on space-based experimentation are rare and expensive (Delikoyun et al. 2019a), highlighting the importance of earth-based models for microgravity research. It is possible to simulate reduced weight-bearing (Wagner et al. 2010) or complete lack of weight-bearing (Judex et al. 2013; Judex et al. 2016; Ozcivici et al. 2007; Ozcivici and Judex 2014; Ozcivici et al. 2014) in rodents however, these models are mostly specific to testing hypotheses on the musculoskeletal tissues (Morey-Holton and Globus 2002). Alternatively, several microgravity simulation devices have been developed to study molecular and cellular level adaptations *in vitro*. Here, we reviewed the available literature for the operating principals and findings of *in vitro* microgravity simulation devices with a specific emphasis on stem cells.

2 Earth-Based *In Vitro* Techniques That Simulate the Microgravity

In order to simulate effects of microgravity, that is also depicted as weightlessness to define the situation where a net sum of all forces is zero (cell surface binding, hydrostatic pressure etc.) (Anken 2013), several physical techniques were translated and developed into laboratory devices. These devices are (1) Clinostat; (2) Random

positioning machine (RPM); (3) Rotating wall vessel (RWV); and (4) Magnetic levitation-based systems.

2.1 Clinostat

Clinostat devices (Fig. 1) rotate samples (i.e. cells) perpendicular to the gravitational vector in order to counteract and negate gravitational acceleration on them (Briegleb 1967; Eiermann et al. 2013). These devices can reduce gravitational pull on samples to an average of 10^{-3} g (1 g = 9.81 m/s^2). Clinostat systems were initially designed with slow rotation frequencies (<10 rpm, rpm: revolutions per minute) to study the gravity-related effects on the growth of plant specimens (Dedolph and Dipert 1971; Hasenstein et al. 2015). The system was then upgraded to reach faster rotational velocities to nullify the gravity vector on smaller objects including cells (Briegleb 1992). Basically, cells in the fast-rotating clinostat system move on circular paths and the diameter of this path decreases as the rotation speed increases and finally relative liquid motion is canceled out in the system. This phenomenon occurs because the radially transferred velocity of the chamber wall and in turn cells in the device begin to rotate around their own center with their surrounding liquid boundary layers. In this condition, cell sedimentation stops and the gravitational pull becomes imperceptible by the cells (Dedolph and Dipert 1971; Klaus 2001).

Clinostats may be in different configurations depending on the number of rotation axes as 2D clinostats or 3D clinostats. Clinostats with the configuration providing rotation in one rotation axis are commonly referred as 2D clinostats (Eiermann et al. 2013; Shang et al. 2013) and sometimes as 1D clinostats (Uddin et al. 2013; Uddin and Qin 2013). 2D clinostats were mainly designed to expose cell suspensions to the simulated microgravity condition (Klaus et al. 1998), but then adapted for the culture of adherent cells (Eiermann et al. 2013). Most of the *in vitro* stem cell culture microgravity studies have been performed in adherent culture condition with varied designs of clinostats; such as a horizontally rotating sealed culture chamber with a medium chamber and a gas-permeable membrane (Chen et al. 2015) or horizontally rotating coverslips inserted into the fixture of chambers (Xue et al. 2017). Although clinostats offer simplicity in application, its rotating nature is an important limitation in design. Smaller centrifugal acceleration is generated on the cells located closer to the center of rotation and in order to minimize variations for accurate force balance, the diameter of the chamber should be kept minimal (~ a few mms), limiting clinostat working volume to accommodate samples of various sizes (Briegleb 1992; Grimm et al. 2014). Another clinostat design, referred as

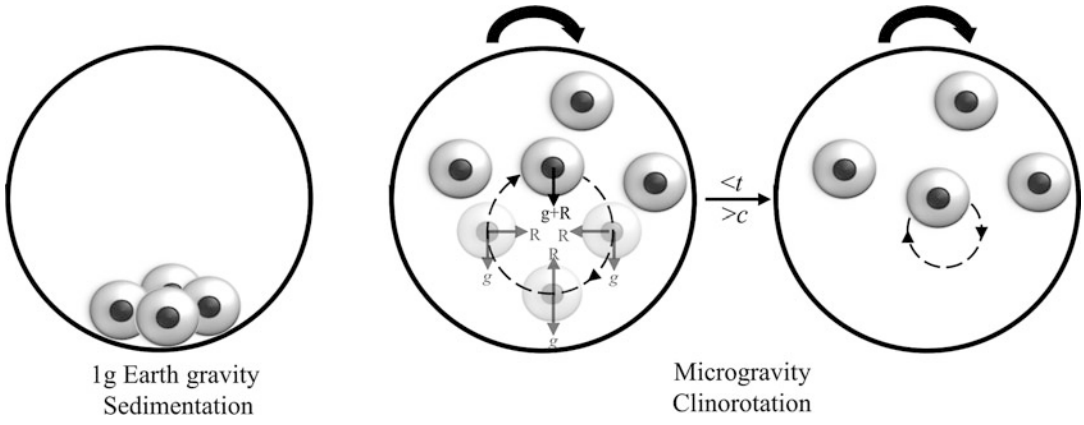

Fig. 1 Principle of the fast-rotating clinostat system. Cells in the clinostat follow a circular trajectory. Increase in the frequency of rotation (*t*) will be resulted in a decrease in perimeter of circular path (*c*) and microgravity is simulated when the cells eventually begin to rotate around its own axis. g: gravity vector, R: radial force

3D clinostat, has two rotation axes in a gimbal mount with an operation of constant direction of rotation (Hoson et al. 1997), offers a larger working capacity than single-axis clinostats (Hauslage et al. 2017). Despite the limited number of studies reporting stem cell culture in 3D clinostats, the effects of microgravity created with 3D clinostats on stem cells have been reported on different culture conditions; monolayer culture (Otsuka et al. 2018) and scaffold-based three-dimensional (3D) culture (Nishikawa et al. 2005). Approximately one third of the reports investigating the effect of microgravity on stem cells using Earth-based *in vitro* techniques were performed with various forms of clinostats.

2.2 Random Positioning Machines (RPM)

In addition to horizontal rotation, it is possible to add a vertical rotation component in clinorotation-based devices to create a different magnitude of the outward acceleration for enhancing the resultant mechanical field (Hoson et al. 1997). Random positioning machine (RPM) rotates samples around two rotational axes in a gimbal mount similar to 3D clinostats. Unlike 3D clinostats however, RPMs operate with changing not only the velocity but also the direction of rotation randomly for constant reorientation of the gravity vector (Wuest et al. 2015). The region for the most effective microgravity is the center of the two rotation axes, this limits the preferred sample volume for RPMs (Grimm et al. 2014). Centrifugal acceleration of RPMs depends not only on rotation velocity and distance from the center of rotation, but also position of the sample in space and time due to the resultant force of two axes (Fig. 2). Therefore, these devices provide a better microgravity environment than clinostats, especially for larger samples.

Originally introduced for plant-based studies (van Loon 2007), RPMs have been adapted for environmental requirements (i.e. temperature, humidity and CO_2) of mammalian cell cultures. Currently RPMs are available with desktop configurations (Borst and van Loon 2009) and majority of stem cell related RPM studies were conducted on such devices (Gershovich et al. 2012; Ratushnyy et al. 2018). In alternative RPM designs, it has been shown that it is possible to increase the working volume greatly up to 14 L (Wuest et al. 2014), or algorithm based controlling of the motion to reach partial gravity rather than complete weightlessness (Benavides Damm et al. 2014).

2.3 Rotating Wall Vessel (RWV)

The rotating wall vessel (RWV) platform was developed and commercialized by NASA

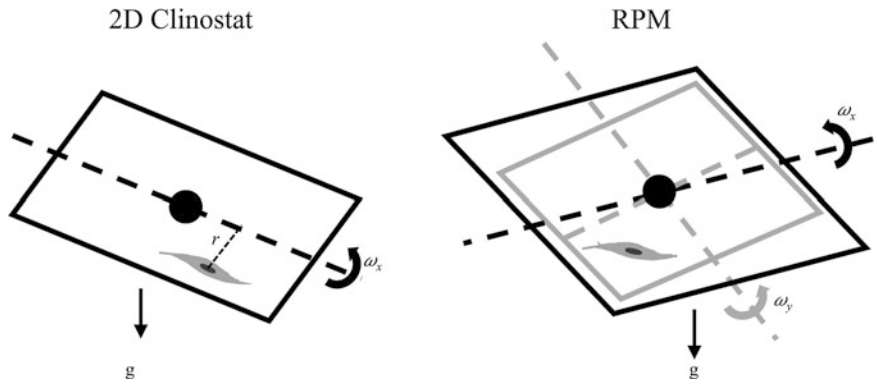

Fig. 2 Working principles of the 2D clinostat (left) and RPM (right). Acceleration in a clinostat rotating around one axis is time independent and determined by the rotation velocity (ω_x) and distance of the cell from the center of rotation (r), whereas acceleration in a RPM, that is rotating around two perpendicular axes, depends on time and position of the sample in space as well as the rotation velocities (ω_x, ω_y)

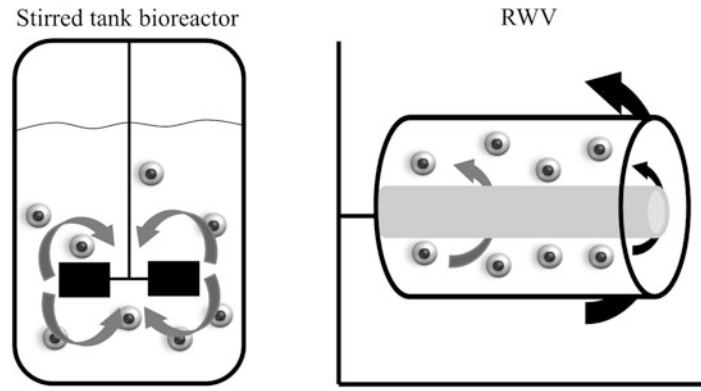

Fig. 3 Flow pattern in a bioreactor with internal mixer and a RWV (right). Grey arrows show flow pattern in the bioreactors. Unlike the stirred tank bioreactor, RWV minimizes turbulence owing to not requiring a mixer for transfer, and provides better formation and growth of 3D aggregates

(National Aeronautics and Space Administration) to simulate microgravity for cell culture applications (Schwarz et al. 1992). RWV is basically a specialized clinostat that transfer rotational velocity onto the cells in fully fluid-filled vessel, allowing them to fall through circular paths (Klaus 2001). Unlike clinostats however, RWVs provide oxygenation by a coaxial tubular oxygenator, that aims to provide sufficient cell movement for an efficient nutrient, oxygen and waste transport and an enhanced cell-cell contact (Schwarz et al. 1992). RWV system (Fig. 3) ensures minimized turbulence in the flow by virtue of eliminating the need to use the internal mixer for transport and it makes the system suitable for *in vivo*-like 3D aggregate formation and growth (Goodwin et al. 1993), however on this system cells cannot be cultured as monolayers on glass slides unlike the clinostat. Instead, for adherent cell culture polymer microcarriers should be used as solid support materials (Gao et al. 1997).

Almost half of the studies investigating the effect of microgravity on stem cells have been performed with RWVs. Commercial RWV systems allow tunable velocities and for oxygenation they can be equipped with different types of gas transfer membranes; high-aspect ratio vessels (HARV, volume up to 50 mL), and/or autoclavable slow turning lateral vessels (STLV, volume up to 500 mL). Stem cells can be cultured in RWVs in different forms, such as pellets (Luo et al. 2011), adherent on scaffolds (Koç et al. 2008) or on microcarriers (Sheyn et al. 2010), in encapsulated form (Hwang et al. 2009) or in organoid form (Mattei et al. 2018).

2.4 Levitation by Negative Magnetophoresis

Application of mechanical rotation to create microgravity inherently creates additional mechanical forces on cells, such as shear stress vectors based on fluid motion (Leguy et al. 2011) but the acceptable limits for rotational speed and thus these mechanical forces have still not been fully elucidated. As an alternative, microgravity of cells may also be achieved by compensating the gravitational vector by a counteracting external force.

Diamagnetic materials such as water, cells and proteins tend to move away from strong magnetic fields to weaker ones (Beaugnon and Tournier 1991b; Zhao et al. 2016). Using this principle, non-living (Beaugnon and Tournier 1991a) and living (Berry and Geim 1997) objects were successfully levitated under strong magnetic fields. Living structures show homogeneity in terms of diamagnetic property and therefore the magnetic force acts homogeneously on the entire structure at the molecular level, providing a proper microgravity (Schenck 1992).

In principle, the magnitude of the magnetic force acting on the object is directly proportional to the magnetic susceptibility difference between the object and the surrounding environment, and

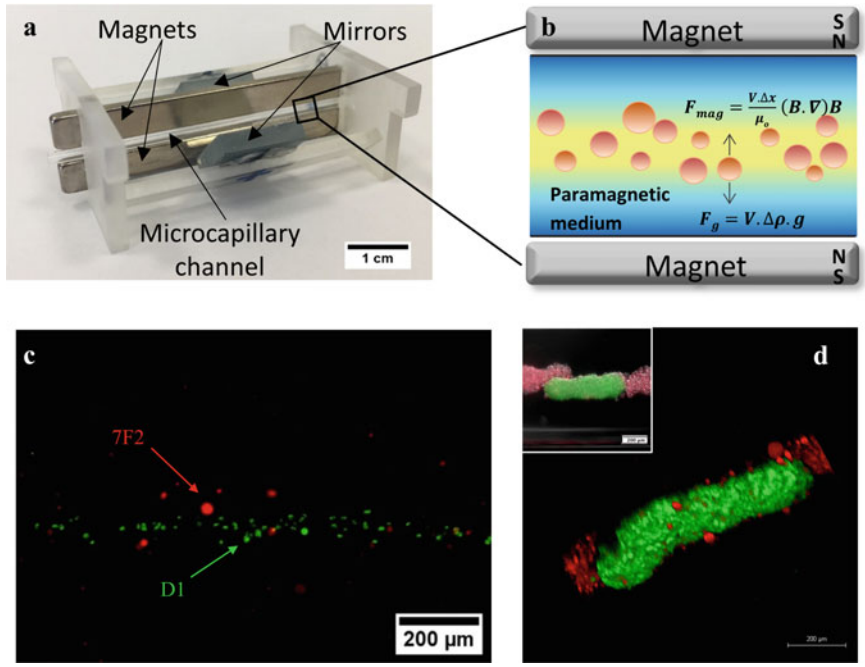

Fig. 4 Magnetic levitation of the cells in a paramagnetic medium. (**a**) Structure of the magnetic levitation device composed of two neodymium magnets, a microcapillary channel and two mirrors placed at 45°. (**b**) Forces acting on cells at the equilibrium position in the device, where F_{mag}: magnetic force, F_b: buoyancy force, V: cell volume, $\Delta\chi$: magnetic susceptibility difference between the paramagnetic medium and the cell, μ_0: permeability of free space, B: magnetic induction, $\Delta\rho$: density difference between the paramagnetic medium and the cell, g: gravitational acceleration. (**c**) Levitated quiescent D1 ORL UVA cells (mouse bone marrow mesenchymal stem cells, green) and adipogenic differentiated 7F2 cells (mouse bone marrow osteoblasts, red). Reprinted from Sarigil et al. (2019b). (**d**) Confocal and conventional fluorescence microscopy (upper left) image showing self-assembled coculture clusters formed with magnetic levitation (100 mM Gd-BT-DO3A). MDA-MB-231^{dsRed} (human breast cancer cells) cells were seeded onto D1 ORL UVA^{eGFP} clusters formed with magnetic levitation and (total 50,000 cells in the device). Reprinted from Anil-Inevi et al. (2018). Scale bars: 200 µm

magnitude of the magnetic field gradient (Yaman et al. 2018). In order to levitate diamagnetic objects, there is a need for stable substantially large magnetic field gradient; for example, superconducting magnets with large magnetic field intensity (i.e. ~ 12 Tesla) that were used to investigate the behavior of stem cells in microgravity (Meng et al. 2011; Shi et al. 2010). It is also possible to levitate objects with a moderate magnetic field gradient (<1 Tesla) in a medium that provides a high magnetic susceptibility difference (Mirica et al. 2009), and this principle can be applied to living cells in a microfluidic system consisting of two permanent magnets with the same poles facing each other (Tasoglu et al. 2015). This magnetic levitation system (Anil-Inevi et al. 2019b), that was initially used for cell separation based on single-cell density (Delikoyun et al. 2019b; Durmus et al. 2015; Sarigil et al. 2019a; Sarigil et al. 2019b), can also be used to apply microgravity condition on stem cell culture (Anil-Inevi et al. 2019a; Anil-Inevi et al. 2018) (Fig. 4). Although levitation approach is novel and at the initial stages in development, the system offers many advantages such as real time monitoring and rotation free application.

3 *In Vitro* Culture of Stem Cells in Simulated Microgravity

Studies on stem cell biology during microgravity exposure were conducted in various studies, which are reviewed below and summarized (Tables 1, 2, 3 and 4) based on the source of the stem cell used.

Table 1 Applications of microgravity simulation techniques for embryonic stem cell culture

Culture Method	Strategy	Cell type	Culture period in MG	Results	Reference
2D clinostat	Pipette method	Mouse ESCs	3 days (60 rpm)	Disruption of genes expression especially in cytoskeleton and changes in the cardimyogenesis process	Shinde et al. (2016)
3D clinostat	LIF-free cell culture	Mouse ESCs	7 days (10^{-3} G)	Cell number ↑	Kawahara et al. (2009).
3D clinostat	Cell culture	Mouse ESCs	up to 7 days (10^{-3} G)	Cell number ↓ (5 days)	Wang et al. (2011)
				Adhesion rate ↓ (8 h)	
				Apoptosis ↑ (2 days)	
				Delayed DNA repair	
Rotating microgravity bioreactor	Hepatic cell differentiation on biodegradable scaffolds	Mouse ESCs	14 days (35 rpm)	Hepatic differentiation: Albumin ↑ α-fetoprotein ↑ Cytokeratin 18 ↑ Transthyretin ↑ Glucose-6-phosphatase ↑	Wang et al. (2012)
Rotating microgravity bioreactor	Alginate encapsulation and cell culture	Murine ESCs	After undifferentiated 3 days, cultured up to 21 days (25 rpm)	More realistic 3D structure and functional bone tissues Metabolic profile during culture in RWV: Glucose ↓ Lactate ↑ Ammonia ↑ PH ↓ Oct4 ↓ Osteogenic differentiation: OSX ↑ Cbfa-1/RUNX2 ↑	Hwang et al. (2009)

Table 2 Applications of microgravity simulation techniques for hematopoietic stem cell culture

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
RWV	Cell culture	HSCs from human umbilical cord blood	8 days (6 rpm)	Total cell number ↑	Liu et al. (2006)
				$CD34^+$ cells ↑	
RWV	Cell culture	HSCs from bone marrow of male mice	7 days (30 rpm)	Number of progenitor cells ↓	McAuliffe et al. (1999)
				High-proliferative potential colony-forming cells ↑	
RWV	Cell culture	Human BM $CD34^+$ cells	4–6 days (8 rpm)	Rate to exit G_0/G_1 phases of cell cycle ↓	Plett et al. (2001)
				Degree of hematopoietic potential ↑	
RWV	Cell culture	Human BM $CD34^+$ cells	2 to 18 days (short term and long term), 10–12 rpm	F actin expression ↓	Plett et al. (2004)
				Migration to SDF-1α ↓	
				Cell-cycle progression ↓	
				Erythroid differentiation ↓	
RWV	Cell culture + growth factors and microcarrier beads	Human umbilical cord blood stem cells (CBSCs)	14 days (gradually increase from 8 to 20 rpm in 48 h)	Cellular proliferation with 3D tissue-like aggregates ↑	Chiu et al. (2005)

Table 3 Applications of microgravity simulation techniques for culture of other stem cell types

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
Rotary bioreactor	Cell culture on Cytodex-3 micro-carriers	Human epidermal stem cells (hEpSCs)	10–15 days (12 rpm-1 day, 22 rpm 14 day)	Proliferation ↑	Lei et al. (2011)
				Viability ↑	
				Higher percentage of ki67 positive cells	
				Low expression of involucrin	
Rotating cell culture system (RCCS)	Cell culture with glial cell line-derived neurotrophic factor (GDNF) growth factor and Sertoli cell feeder	Mouse spermatogonial stem cells (SSCs)	14 days (10–12 rpm)	Proliferation ↑	Zhang et al. (2014)
				Aggregates are similar to native in vivo cells	
				mRNA expression of Oct-4, GFRα1 and Bcl6b ↑	
3D clinostat	Cell culture	Bipotential Murine Oval Liver (BMOL) stem cells	2 h (10^{-3} G)	Proliferation ↑	Majumder et al. (2011)
				Induce hepatic differentiation	
				Downregulation of Notch1, upregulation of BMP4	

3.1 Embryonic Stem Cells

Studies investigating the effects of microgravity on embryonic stem cells (ESCs) are not as common compared to other types of stem cells. Most of the studies involving ESCs focused on using microgravity as a biotechnological tool for ESC expansion and differentiation. A study performed with 2D clinostats indicated that while microgravity had no effect on the distribution of ESCs in different cell cycle phases, it disrupted cytoskeletal gene expression and altered the process of cardiomyogenesis of ESCs (Shinde et al. 2016). Another study performed using 3D clinostats revealed that the need for feeder layer, serum and Leukemia-inhibitory factor (LIF) in the conventional method to prevent the mouse ESCs to spontaneously differentiate was eliminated by 3D clinostat culture (Kawahara et al. 2009). In another clinostat based study, ESC pluripotency markers Oct-4 and Nanog as well as ALP were found to be unaffected from 7 days of microgravity exposure (Wang et al. 2011). This study also suggested that, like 2D clinostats, with 3D clinostat-based microgravity exposure cells showed no significant alteration in the cell cycle. However, mouse ESCs showed sensitivity to microgravity with increased apoptosis and delayed DNA repair processes.

In addition to basic cell biology, effect of microgravity in tissue engineering applications was also examined with ESCs. Hepatic cell differentiation and expansion on polymeric (non-aligned poly-L-lactic acid – poly-glycolic acid) scaffolds was achieved from mouse ESCs under induced microgravity using RWV (Wang et al. 2012). Alginate encapsulated mouse ESCs were also exposed to microgravity during osteogenesis, showing enhanced osteogenic differentiation in the absence of embryoid body formation (Hwang et al. 2009). Though number of ESC based studies are limited for earth-based microgravity applications, generation of novel pluripotency inducing pipelines (Okita et al. 2007; Yu et al. 2007) that are free of ethical considerations are expected to help addressing hypotheses regarding developmental pathways during microgravity.

3.2 Adult Stem Cells

3.2.1 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are present in various tissues such as bone marrow, umbilical cord, adipose tissue, dental pulp and they have the capability of differentiation into cells of mesenchymal origin such as bone, fat, cartilage, muscle and tendons (Ding et al. 2011; Pittenger et al. 1999). With their ease of handling and high capacity for differentiation, MSCs were studied extensively under microgravity to model spaceflight. For example, the relationship between adipogenesis and osteoblastogenesis in bone marrow is crucial for bone health (Abdallah 2017; Muruganandan et al. 2009) and lack of mechanical loading such as spaceflight, bed-rest and immobilization can enhance adipogenic differentiation in bone marrow and inhibit bone formation (Arfat et al. 2014; Nishikawa et al. 2005; Ozcivici et al. 2010a, b; Uddin and Qin 2013). Furthermore, MSC derived osteoblasts are important regulators of bone resorbing osteoclasts (Zhang et al. 2017), implying that microgravity not only may have bone formation suppressive effects, but also in turn inhibitory control over bone resorption process performed by osteoclasts (Chatani et al. 2015).

Earlier studies on clinostat-based microgravity showed that proliferation rate of the human MSCs (hMSCs) decreased and more flattened cells were observed by exposure to microgravity during 10 days in comparison to normal gravity (Merzlikina et al. 2004). Consistent with hMSCs, proliferation rate of rat bone marrow stem cells (rBMSCs) was inhibited and the response to growth factors such as IGF-1, EGF and bFGF was decreased by simulated microgravity via clinostat (Dai et al. 2007). This study also demonstrated that microgravity disturbed gene expression by upregulating negative regulators of cell cycle and proliferation and down-regulating cytoskeletal and osteogenic genes. Similarly, proliferation of rBMSCs was also shown to be suppressed with microgravity applied with clinorotation for 7 days (Huang et al. 2009). Prolonged exposure of hBMSCs to microgravity for 20 days with clinostat revealed similar inhibition of cell proliferation as well as decreased expression of cell adhesion molecules with increased migration (Gershovich and Buravkova 2007). Moreover, it was reported that the changes of proliferation in MSCs of murine origin were related with the arrested cell cycle in G2/M phases and increased apoptosis after 48 h clinorotation treatment (Yan et al. 2015). Another study that applied simulated microgravity to primary mouse MSCs showed reduced cell proliferation and deteriorating cellular ultrastructure, which can be reversed by daily application of low intensity mechanical vibrations at 90 Hz (Touchstone et al. 2019), an exercise mimetic that was shown to be osteogenic (Demiray and Ozcivici 2015) and anti-adipogenic (Baskan et al. 2017) on mouse bone marrow MSCs previously.

Aside from cell proliferation and cell cycle, clinorotation systems were also used to investigate the effect of microgravity to osteogenic, adipogenic, cardiomyogenic, endothelial and neuronal commitment of rBMSCs (Chen et al. 2011, 2016; Chen et al. 2015; Huang et al. 2009; Uddin and Qin 2013; Xue et al. 2017; Yan et al. 2015; Zhang et al. 2013). As an example of effects of microgravity on osteogenic commitment, it was shown that expression of osteogenic markers such as Cbfa1, ALP and COL1A1 by rBMSCs cultured in induction medium was decreased via clinostat (Huang et al. 2009). In discordance, another study reported that microgravity exposure by clinostat decreased ALP expression at 3 days and increased ALP expression at 10 days for rBMSCs (Xue et al. 2017). Simulated microgravity also reduced expression of other osteogenic markers such as Runx2, OSX, OCN and OPN in MSCs derived from different origins (i.e. adipose tissue, bone marrow) (Chen et al. 2015; Uddin and Qin 2013). The suppressive effects of microgravity on osteogenic commitment can be reversed using external mechanical sources. For example, daily application (20 min/day for 5 days) of low intensity pulsed ultrasound to microgravity exposed adipose derived hMSCs increased ALP, Runx2, OSX, RANKL and decreased OPG gene

expression (Uddin and Qin 2013). Mechanistic studies linked the decrease in the expression of PDZ-binding motif (TAZ) gene to reduced expressions of Runx2 and ALP expression during microgravity and it was shown that TAZ activation by using lysophosphatidic acid (LPA) retained osteogenic differentiation of MSCs under microgravity (Chen et al. 2015). In a follow-up study, inhibition of osteogenic differentiation during microgravity was further linked to the depolymerization of F-actin related to F-actin-TAZ pathway (Chen et al. 2016). Adipogenic differentiation of stem cells was also addressed under simulated microgravity. For example, expression of adipogenic marker PPARγ was shown to be increased under microgravity (via clinostat) with or without adipogenic inducers in the culture media (Huang et al. 2009). Also, similar to that shown in ALP during osteogenesis, increased exposure time to microgravity exhibited reversed effect in adipogenic differentiation decreasing PPARγ expression at 10 days of exposure (Xue et al. 2017).

The effects of simulated microgravity were also tested on neuronal and endothelial differentiation of the MSCs by using clinostat system. Neuronal markers such as MAP-2, TH and CHAT were shown to be upregulated in rBMSCs under microgravity with an increase in secretion of select neurophins, such as BDNF, CNTF (Chen et al. 2011) remarking the therapeutic potential of simulated microgravity for neural tissue engineering applications. Increase in short-term (72 h) microgravity treatment and decrease in long-term (10 days) treatment were observed for ability of MSCs to differentiate into endothelial lineage based on endothelium-specific marker vWF (Xue et al. 2017). Furthermore, another study confirmed that vWF expression increased during culture with VEGF stimulation for 12 days when cells were pre-treated with a 72-h microgravity condition (Zhang et al. 2013). In that study, active level of RhoA marker was associated with lineage commitment during microgravity for adipogenic, endothelial, neural and osteogenic cell lines by regulating the cytoskeleton. In another study, it was demonstrated that exposure of rBMSCs to microgravity for 3 days inhibited cardiomyogenic differentiation evidenced by specific markers including cTnT, GATA4 and β-MHC (Huang et al. 2009).

Clinostat system based on seeding cells on coverslips enables detection of morphological changes in MSCs when they are exposed to microgravity. For example, hMSC had more flattened morphology once they were exposed to microgravity simulated by clinostat system (Merzlikina et al. 2004), and in contrast rBMSCs appeared to have more rounded morphology after exposure to microgravity for 3 days (Chen et al. 2011; Zhang et al. 2013). Cytoskeleton is responsible for cell morphology and structure in addition to other functions (Fletcher and Mullins 2010) and microgravity may induce changes in cytoskeletal components based on exposure time. For example, rBMSCs cultured for 3 days under microgravity revealed that gene expression of cytoskeletal components such as actin and Cofilin1 was downregulated (Dai et al. 2007). When microgravity exposure time was prolonged to 10 days, microtubules (Chen et al. 2011) and microfilaments (Zhang et al. 2013) were shown to be re-organised. RhoA activity that is associated with cytoskeleton regulation showed similar trend and the decreased activity of RhoA after a short microgravity exposure was increased when exposure time was prolonged to 10 days (Xue et al. 2017; Zhang et al. 2013). In addition to cytoskeletal components, migration capacity of rBMSC was inhibited during microgravity based on inhibition of actin reorganization (Mao et al. 2016).

Because of the limitation of 2D clinostats that cannot completely remove the gravity vector due to constant rotation in one direction, 3D clinostats are preferable for the proper simulation of microgravity based on multidirectional rotation. Human BMSCs that were cultured in 3D clinostat system showed increased cell proliferation during microgravity exposure (Yuge et al. 2006) in discordance with 2D clinostat (Gershovich and Buravkova 2007). The inconsistencies of these results may be caused by differences in mechanical environment caused by respective simulation techniques. 3D clinostats were also utilized to determine whether microgravity culture may

augment therapeutic potential of MSCs in cell-based therapy strategies. Exposure to microgravity condition of mouse (Yuge et al. 2010) and rat (Mitsuhara et al. 2013) BMSCs has been shown to increase CXCR4 expression, which is an important factor of cell migration. In human BMSCs (Otsuka et al. 2018), HGF and TGF-β expression associated with cell migration and proliferation have been increased. These studies demonstrated that functional recovery was enhanced by transplantation of MSCs after microgravity culture, suggesting that this technique can be advantageous for cell-based therapies, with increased potential of neuroprotective and anti-inflammatory gene expression.

Another alternative tool for microgravity simulation is RWV bioreactors, tested especially on osteogenic and adipogenic differentiation of MSCs. When the expression of osteogenic (ALP, COL1A1, Runx2, OC, COL1, ON, BMP-2) (Li et al. 2015; Meyers et al. 2005; Meyers et al. 2004; Sheyn et al. 2010; Sun et al. 2008; Zayzafoon et al. 2004; Zheng et al. 2007) and adipogenic (PPARɣ, Adipsin, Leptin, Glut4) (Meyers et al. 2005; Sheyn et al. 2010; Sun et al. 2008; Zayzafoon et al. 2004; Zheng et al. 2007) specific genes and proteins were tested, in general simulated microgravity suppressed the osteogenic differentiation and induced adipogenic differentiation. Similar to clinostat studies, in RWV cultures adipogenic differentiation potential and lipid accumulation of MSCs were increased even if the cell culture was performed without adipogenic inducers (Li et al. 2015; Sun et al. 2008). Moreover decreased expression of osteogenic markers such as Runx2, OC, COL1, ON in hMSCs was not reversible even when the cells were removed from microgravity condition (Zayzafoon et al. 2004). In contrast to the studies demonstrated the decrease in osteogenic differentiation under microgravity conditions, another study reported increased ALP activity of rBMSCs cultured on 3D bovine bones during microgravity (Jin et al. 2010). Furthermore, to investigate potential molecular mechanisms, it has been shown that overexpression of active RhoA, which is a positive regulator of osteogenesis and a negative regulator of adipogenesis, restored stress fibers and the expression of osteogenic markers and suppressed adipogenic gene expression in hMSCs cultured in microgravity (Meyers et al. 2005). On the other hand, the telomerase activity which is known to be related with lineage commitment of stem cells (He et al. 2018; Kang et al. 2004) was found to decrease during microgravity (Sun et al. 2008). Effects of microgravity on MSC commitment to other mesenchymal lineages such as chondrocytes, were also addressed with RWV on rabbit bone marrow stem cells (rabBMSCs) (Wu et al. 2013), human mesenchmal stem cells (hMSCs) (Mayer-Wagner et al. 2014) and human adipose derived stem cells (Ad-hMSCs) (Weiss et al. 2017; Yu et al. 2011). It was shown that rabBMSCs had upregulated the expression of chondrogenesis specific markers, such as collagen II, aggrecan and SOX-9 during pellet culture in RWV (Luo et al. 2011; Wu et al. 2013). In contrast, another study suggested an opposite effect showing that microgravity downregulated the expression of COL10A1, COL2A1 and aggrecan in hMSCs (Mayer-Wagner et al. 2014).

RPM is one of the most realistic simulators of microgravity and it was largely applied to test effects of microgravity on cytoskeleton rearrangements and differentiation of stem cells. It was shown that RPM culture affected dimensional structures, position and amount of actin filaments very quickly (30 min), and organization of actin cytoskeleton was partially or completely restored at 120th h of exposure in hMSCs (Gershovich et al. 2009). Besides, the number of detached cells, which increased in the first 48 h, decreased after 120 h, possibly due to alterations in proteins related with cell-cell and cell-matrix interactions such as vinculin and integrins (Carisey and Ballestrem 2011; Ratushnyy and Buravkova 2017). In accordance with these findings, expression of structural and regulatory genes interacting with actin cytoskeleton, α-actin, γ-actin, b-tubulin, cofilin, and small GTPase RhoA was altered after 48 h of microgravity exposure, and restoration of expression levels was observed after 120 h (Gershovich et al. 2012). Analysis of genes related to differentiation of stem cells revealed that prolonged RPM

exposure (10–20 days) showed an increased expression of adipogenic marker (PPARγ) and decreased osteogenic marker genes (ALPL, OMD). It was also reported that inhibition of osteogenic differentiation and induction of adipogenic differentiation were accompanied by the up-regulation of genes specific for tumorigenesis in the later stages of RPM exposure (14 days) (Li et al. 2019). Another study showed that as a result of the microgravity exposure on human ad-MSCs for 96 h, angiogenesis-related genes were up-regulated as well as genes associated with tumorigenesis (Ratushnyy et al. 2018). The effect of microgravity on stem cell morphology and viability was tested in rBMSC with RPM and it was seen that simulated microgravity (48 h) caused a decrease in cell area, an increase in cell aspect ratio and a reduction in viability (Monfaredi et al. 2017).

Environmental conditions in ground-based simulators can affect cellular properties. It was shown that oxygen concentration in RPM systems had a key role on cell adhesion, and signaling in hMSCs. (Versari et al. 2013). Besides, shear stress as a result of rotation in RWV bioreactors can affect osteogenic differentiation and mineralization (Grellier et al. 2009; Yeatts and Fisher 2011). The techniques working with rotation principle requires the adaptation of cells to the physical effects caused by rotation speed and fluid properties such as viscosity and density. A system that can cope with these challenges is magnetic levitation which is a novel technique for simulation of microgravity (Qian et al. 2009). Although the general interest towards this system has been increasing for the last decade, their use for testing stem cells is still rather limited. In a study that applied large gradient high magnetic field (LGHMF) to create a force against gravitational force on the cells and thus to simulate microgravity, it was shown that a 6-h treatment disrupted cytoskeletal filaments, and extending the treatment time (48 h) resulted in the death of almost all cells (Shi et al. 2010). In addition, by applying LGHMF-induced microgravity (6 h) at different stages of osteogenic induction process, postponement of treatment stage was shown to reduce the intensity of osteogenesis suppression. It was concluded that this treatment affects the initiation stage of osteogenesis. Levels of signaling molecules involved in osteogenic differentiation, such as ERK, FAK and their phosphorylated forms, except ERK, were decreased for treatments in all stages. Another result noted in the study is that the adipogenesis-related gene, PPARγ2, was not detected in any condition. Unlike studies reporting that clinorotation-induced microgravity had no effect on apoptosis of rat MSCs (Dai et al. 2007; Wang et al. 2014), a study examining the effect of LGHMF-induced microgravity on apoptosis showed that microgravity treatment (12 h) induced apoptosis associated with increased expression of p53 in hMSCs (Meng et al. 2011). Also, F-actin fibers disappeared mostly whereas α-tubulin was reorganized above nucleus, and nuclei shifted from center to one side of the cells under microgravity.

As 3D *in vitro* cultures play a key role in examining *in vivo*-like living structures (Anil-Inevi et al. 2020; Fatehullah et al. 2016; Yildiz-Ozturk et al. 2017), they can also be adapted to systems that simulate microgravity. As an example, it was observed that simulated microgravity by RWV enhanced chondro-induction and chondrogenesis in scaffold-based (type I collagen) coculture of MSCs and meniscus cells (Weiss et al. 2017). Another example is that RWV culture of the bone constructs resulted in better repair of the bone defects once transplanted compared to the static flask culture (Jin et al. 2010). In addition, magnetic levitation system can also be utilized for 3D cultures for tissue engineering studies. It was demonstrated that magnetic levitation could be used for microgravity culture and density measurement of MSCs (Anil-Inevi et al. 2019b). A recent study showed that magnetic levitation system enabled self-assembly of MSCs with label- and scaffold-free application under microgravity environment (Anil-Inevi et al. 2018). Also, this microgravity environment induced by diamagnetic levitation was capable of the detection of MSCs based on their densities (Sarigil et al. 2019b).

3.2.2 Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are active regulators and participants of hematopoiesis and

they can be affected by microgravity conditions, causing anemia and hematopoietic disorders in astronauts exposed to the microgravity (Wang et al. 2019). These abnormalities related to spaceflight lead to research to understand the mechanosensitivity and mechanoresponse of HSCs to the microgravity condition in physiological and molecular basis. A study aimed to understand early hematopoiesis observed in space flights showed that treatment of mice HSCs with a 7-day RWV culture decreased the total number of progenitor cells, while increasing the high-proliferative potential colony-forming cells compared to standard flask culture (McAuliffe et al. 1999). For human HSCs, simulated microgravity via RWV bioreactor caused the cells to exit G_0/G_1 phases at a slower rate and maintain higher hematopoietic potential shown by the ability to initiate and maintain secondary long-term suspension, compared to cells cultured in 1 g (Plett et al. 2001). Another study on human HSCs showed that RWV culture resulted in a significant reduction of migration to stromal cell–derived factor 1 (SDF-1 α), accompanied by decreased levels of F-actin and cell cyclin proteins. It was also reported that RWV culture inhibited overall progenitor proliferation and erythroid differentiation (Plett et al. 2004). In contrast, long-term RWV culture (for more than 8 days) provided a more suitable environment for expansion of hematopoietic stem cells collected from human umbilical cord blood compared to standard flask culture (Liu et al. 2006). Besides, human umbilical cord blood stem cells (CBSCs) showed increased proliferation as 3D tissue-like aggregates and developed vascular tubular assemblies in RWV culture (Chiu et al. 2005).

3.2.3 Other Types of Stem Cells

In addition to stem cells types classified and mentioned above, other stem cell types were also studied under microgravity conditions for several tissue engineering applications. A study aimed at providing better culture conditions for skin tissue engineering applications revealed that rotating cell culture system provided enhanced proliferation and viability for human epidermal stem cells (hEpSCs) and they were able to form multilayer 3D epidermis structures (Lei et al. 2011). Another study examined effects of the simulated microgravity on differentiation of the bipotential murine oval liver (BMOL) stem cells (Majumder et al. 2011). It was reported that prolonged culture (240 min) of BMOL stem cells in simulated microgravity condition (via 3D clinostat) increased cell death, while short exposure time (up to 2 h) did not have a negative effect on cell viability. Moreover, exposure of BMOL stem cells to microgravity condition for 2 h was alone able to induce the differentiation of stem cells to hepatocytes within 2–3 days, interplaying with BMP4/Notch1 signaling. Finally, a study that applied simulated microgravity on spermatogonial stem cells which are a subset of primitive germ cells, also showed that cell culture in a rotating cell culture system enhanced proliferation of these cells (Zhang et al. 2014). Furthermore, after simulated microgravity treatment the cells maintained clone-forming capacity and differentiation ability, and were able to differentiate into round spermatids with flagella. These results have been suggesting that the technique may be used to restore fertility for cancer patients after chemotherapy or irradiation.

4 Future Outlook

Increasing interest in long-term space flights has led to growing interest in understanding the functional and structural changes in living systems under microgravity condition. Especially, microgravity-triggered changes in stem cells have gained remarkable attention due to their potency. Real spaceflight experiments, which provide wide opportunities to study *in vitro* and *in vivo* biological effects of microgravity, are challenging due to limited availability, high cost and complexity of experimental condition. Although there are available ground-based simulating systems to investigate microgravity-related *in vivo* effects such as rodent hindlimb unloading models, it is not possible to apply them for each type of tissue and to use for research at the cellular level. Ground-based simulated microgravity experiments for *in vitro*

Table 4 Applications of microgravity simulation techniques for mesenchymal stem cells

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
2D Clinostat	Cell culture	hBMSCs	1–10 h	Morphological changes: Flattened cells	Merzlikina et al. (2004)
				Proliferation rate ↓	
2D Clinostat	Stem cells seeding on coverslips transferred to polyethylene (PE) culture bag	rBMSCs	30 rpm, 1–4 days	Proliferation: p-ERK1/2 ↓ Akt ↓ Response to growth factors (IGF-I, EGF, bFGF) ↓ Apoptosis →	Dai et al. (2007)
				Cytoskeleton gene expression: Actin filaments ↓ Cofilin1 ↓	
				Osteogenic differentiaition: Cbfa1 ↓	
				Gene expression profile: Among 413 expressed genes; 207 genes were down-regulated 206 genes were up-regulated Mostly genes acting negative regulation of cell cycle and cell proliferation were up-regulated, osteoblast differentiation related-genes were down-regulated (look reference for detail)	
2D Clinostat	Cell culture	hBMSCs	6 rpm, 20 days	Proliferation rate ↓ (between 2.5 and 6.5 fold depending on passage)	Gershovich and Buravkova (2007)
				Migration activity ↑	
				Immunophenotypic analysis (CD34, CD45, CD54, CD106, CD105, CD90 and class 1 HLA) with or without osteogenic induction medium	
2D Clinostat	Coverslips transferred to PE culture bag	rBMSCs	30 rpm, 1, 3, 5 or 7 days	Reorganization of microfilaments Proliferation ↓	Huang et al. (2009)
				Osteogenic differentiation: With and without inducer ALP, Cbfa1, COL1A1↓	
				Cardiomyogenic differentiation: With and without inducer cTnT, β-MHC, GATA4↓	
				Adipogenic differentiation: With and without inducer PPARɣ↑	
				p-ERK1/2 ↓	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
2D Clinostat	Coverslips inserted into chamber	rBMSCs	30 rpm, 4 h, 72 h, 7 days, 10 days and 14 days	Cytoskeletal reorganization Ratio of length/width ↓ (round-shaped cell morphology)	Chen et al. (2011)
				Neuronal differentiation: MAP-2, TH, CHAT ↑	
				Neurotrophins: BDNF ↑ CNTF ↑ NGF ↓	
				Action potential: Membrane input resistance ↑ Membrane capacitance ↑	
2D Clinostat	Coverslips transferred into chamber	rBMSCs	30 rpm, 4 h, 72 h and 10 days	Morphological changes: Ratio of length/width ↓ (round-shaped cell)	Zhang et al. (2013)
				Cytoskeletal reorganization: Decreased microfilament and cytoskeletal tension in 4 h and 72 h Recovered microflament and cytoskeletal tension in 10 days Activation of RhoA ↓ in 72 h (80%, compared with 4 h) ↑ in 10 days (tenfold, compared with 72 h)	
				Differentiation capability: Flk-1 → for 4 h ↑ (1.71- fold) for 72 h – The most proper exposure time ↑ (1.28-fold) for 10 days	
				Endothelial differentiation: Removing MG after 72 h exposure and stimulation with VEGF for 12 days Flk-1 ↑ (1.46-fold compared with NG) vWF ↑ (1.31-fold compared with NG) Capillary formation ↑	
2D Clinostat[a]	Opticell	Ad-hMSCs	15 rpm, 5 days	Osteogenic differentiation: Runx2, ALP, OSX (osterix) ↓ RANKL ↓ OPG ↑ Collagen content (type I and III) ↓ Calcification ↓	Uddin and Qin (2013)
				Appliying low intensity pulsed ultrasound (LIPUS):	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
				ALP ↑ (compared with MG and NG-u) Runx2 ↑ (compared with MG and NG-u) OSX ↑ (compared with MG and NG-u) RNAKL ↑ (compared with MG and NG-u) OPG ↑ (compared with NG-u) OPG ↓ (compared with MG and NG) Collagen content ↓ (compared with NG-u) Collagen content ↑ (compared with MG and NG-u)	
2D Clinostat	Coverslips transferred into chamber	BMSCs	30 rpm, 48 h	Cell proportion in G1 phase ↓ (0.04-fold, similar between the two groups) in S phase ↓ (0.22-fold) in G2 phase ↑ (1.0-fold) in comparison with control Apoptosis ↑	Yan et al. (2015)
				Cell proliferation: PCNA ↓	
				Osteogenic differentiation with inducer (BMP2) ALP, Cbfa1, SATB2 ↓ Hoxa2 ↑	
				Overexpression of SATB2 SATB2 ↑ ALP ↑ (in comparison with that without SATB2 overexpression)	
2D Clinostat	Flat chamber	rBMSCs	10 rpm, 48 h	Osteogenic differentiation: ALP, OPN, OCN, Runx2 ↓	Chen et al. (2015)
				Adipogenic differentiation: PPARɣ ↑	
				TAZ ↓	
				TAZ ↑ with LPA (lysophosphatidic acid) correspondingly Runx2 ↑ with LPA	
2D Clinostat	Flat chamber	rBMSCs	10 rpm, 48 h	Morphological changes: F-actin reorganization (thinner and disordered) Fractal dimension ↓	Chen et al. (2016)
				ALP activity ↓ Runx2 ↓ TAZ nuclear aggregation (TAZ activation) ↓	
				Jasplakinolide or LPA induction in MG:	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
				ALP activity ↑ Runx2 ↑	
2D Clinostat	Flat chamber	rBMSCs	10 rpm, 24 h	Migration ↓ F-actin remodeling (thicker) Cell stiffness ↑ (Young's modulus↑) Rock-F-actin pathway	Mao et al. (2016)
				Restoring gravity: Cell stiffness and migration -could not recover totally	
2D	Coverslips transferred into chamber	rBMSCs	30 x g 72 h and 10 days	Decreased microtubule formation and α-actin filaments at 72 h and reestablished microtubeles and actin filaments at 10 days	Xue et al. (2017)
				Activation of RhoA ↓ in 72 h (83%, compared with 4 h) ↑ in 10 days (nine-fold, compared with 72 h)	
				Neuronal differentiation: MAP-2 ↑ at 72 h MAP-2 ↓ at 10 days (compared with 72 h)	
				Osteogenic differentiation: ALP ↓ at 72 h ALP ↑ at 10 days (compared with 72 h and NG)	
				Endothelial differentiation: vWF ↑ at 72 h vWF ↓ at 10 days (compared with 72 h and NG)	
				Adipogenic differentiation: PPARγ ↑ at 72 h PPARγ ↓ at 10 days (compared with 72 h and NG)	
3D Clinostat	Porous calcium hidroxyapatite ceramic blocks	rBMSCs	10^{-3} G, 2 weeks	ALP activity ↓	Nishikawa et al. (2005)
				ECM formation ↓	
				Total protein content →	
				Proliferation →	
3D Clinostat	Culture flask	hBMSCs	10^{-3} G, 5 rpm, 7 days	Proliferation rate ↑ (13-fold in a week) Cell proliferation in NG - 6.8% (d1), 8.3% (d3), 24.7% (d7) in MG – 23.5% (d1), 48.7% (d3), 85.5% (d7)	Yuge et al. (2006)
				Chondrogenic differentiation	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
				Aggregan: undetectable Collagen II: undetectable Telomere length → Telomerase activity: undetectable	
3D Clinostat	Opticell	mBMSCs	10^{-3} G, 7 days	Morphological changes in growth and neural induction medium →	Yuge et al. (2010)
				In growth medium: Oct-4 → NF-H no expression	
				In neural induction medium: Oct-4 ↑ (compared to NG in a week) NF-H no expression	
				Neural differentiation: MAP-2 ↓ NF-H ↓	
				CXCR4→ After transplantation CXCR4 ↑	
3D Clinostat	Opticell	rBMSCs	10^{-3} G, 7 days	Morphological changes: Round-shaped cell	Mitsuhara et al. (2013)
				Migration ↑ Oct-4, CXCR4 ↑	
				Functional recovery ↑ NGF, BDNF →	
3D Clinostat	Coverslips inserted into chamber	rBMSCs	30 rpm, 48 h, 72 h and 120 h	Ratio of width/length ↑ in all time duration (compared to NG) Ratio of width/length ↓ in 120 h (compared to 72 h) Apoptosis →	Wang et al. (2014)
				Pluripotency: Oct-4 ↑ in all time durations (compared to NG) Oct-4 → in 120 h (compared to 72 h)	
				Endothelial differentiation: vWF ↑ CD31 ↑	
3D Clinostat	Culture flask tranferred into Gravite®	h-cMSCs	10^{-3} G, 5 days	Differentiation potential (adipogenic, osteogenic and neural) →	Otsuka et al. (2018)
				Neurotrophic and anti-inflammatory factors: HGF, TGF-β ↑ BDNF, bFGF, GDNF, VEGF →	
				Motor functional improvements after transplantation ↑	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
RWV	Plastic microcarrier beads	hBMSCs	7 days +30 days (readaption) (MG-E)	During MG Actin→	Zayzafoon et al. (2004)
				Osteogenic differentiation: Runx2, ALP, OC, COL1, ON ↓	
				Adipogenic differentiation: PPARγ, Adipsin, Leptin, Glut4↑	
				Removing MG Runx2, OC, COL1, ON ↓ ALP ↑	
				PPARγ, Adipsin, Leptin, Glut4↑	
RWV	Polystyrene microcarrier beads	hMSCs	Readjusted rotation regarding to growing constructs, 7 days	Osteogenic differentiation: Runx2, ALP, COL1 ↓	Meyers et al. (2004)
				İntegrin↑ FAK↓ PYK2↓ GTP-Ras↓ p-ERK1/2 ↓ Akt →	
RWV	Polystyrene microcarrier beads	hMSCs Transduction of hMSCs by adenoviral vector, RhoA-V14	Readjusted rotation regarding to growing constructs, 7 days	F-actin↓ G-actin↑ GTP-RhoA↓ p-cofilin↓	Meyers et al. (2005)
				Osteogenic differentiation: Runx2, ALP, COL1 ↓	
				Adipogenic differentiation under MG: Glut4 ↑ Lipid accumulation ↑	
				Introduction of an adenoviral construct expressing constitutively active RhoA: Runx2, ALP, COL1 ↑ Leptin, Glut4↓	
RWV	Plastic microcarrier beads	hBMSCs	16 rpm, 14 days	ALP↓	Zheng et al. (2007)
				Adipogenic potential ↑	
RWV	Microcarrier beads	rBMSCs	15 rpm, 5 days/ 7 days	Osteogenic differentiation under MG: BMP-2 ↓	Sun et al. (2008)
				Osteogenic differentiation (by removing MG for 4 days): ALP activity and OC concentration↓	
				Adipogenic differentiation under MG: PPARγ2, adipocyte number ↑ (without inducer)	
				Telomerase activity ↓	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
RWV	Mineralized polymer foams	rBMSCs	20 rpm, 28 days	Feasibility of mineralized PLGA scaffolds for cell adhesion, cell viability and osteogenic differentiation	Koç et al. (2008)
RWV	Gelatin microcarrier	hBMSCs	16 rpm, 7 days	Cell viability → (compared with static condition) Adipogenic differentiation Lipid accumulation↑	Sheyn et al. (2010)
				Osteogenic differentiation ALP activity →	
				Gene expression (>1,250 genes) in MG: Adipogenesis-related genes mostly upregulated Osteogenesis- and chondrogenesis-related genes mostly downregulated (look reference for detail)	
RWV	Ceramic bovine bone blocks	rBMSC	Readjusted rotation regarding to growing constructs, 15 days	3D bone constructs Cellularity of blocks ↑ (compared to static culture) New bone formation; thicker in MG condition	Jin et al. (2010)
				ALP activity ↑	
RWV	Pellet culture	rabBMSC	6.5 rpm, 7 days	Size of pellets ↑ Cell proliferation ↑	Luo et al. (2011)
				Chondrogenic differentiation: S-GAG ↑ Collagen II ↑ Aggregan ↑ SOX-9 ↑ Aggregan/collagen ↑	
				Cell culture with TGF-β1 in MG (compared to condition without TGF-β1 in MG): Cell proliferation, S-GAG, Collagen II, Aggregan, SOX-9 ↑ Aggregan/collagen ↓	
RWV	Opticell	Ad-hMSC (hADSC)	Rotation speed varied between 11-25 rpm, 21 days	Size and weight of pellets ↑	Yu et al. (2011)
				Chondrogenic differentiation: COL2A1 ↑ COL10A1 → Aggrecan ↑ SOX-9 ↑	
				p-p38 ↑	
RWV	Pellete culture	Rab-BMSC	20 rpm, 2 weeks	Chondrogenic differentiation: Collagen II ↑ Aggrecan ↑	Wu et al. (2013)
				Proteoglycan ↑	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
RWV	Pellet culture	hMSC	Rotation speed varied between 4.2-7.2 rpm, last 7 days of 21 days differentiation	Chondrogenic differentiation: COL2A1 ↓ COL10A1 ↓ COL2A1/COL10A1 ↓ Aggrecan ↓ SOX-9 →	Mayer-Wagner et al. (2014)
RWV	Microcarrier beads	Mice-BMSC	10 rpm, 7 days	PPARɣ ↑ (without inducer) Runx2 ↓ TAZ mRNA → TAZ protein expression ↓ Localization of TAZ and Runx2: In nuclei in NG ↓ localization in nuclei in MG	Li et al. (2015)
RWV	PLGA scaffold	hDPSC	Rotation speed varied between 15-25 rpm, 3 days	Proliferation potential ↑ (for 72 h) Regulator proteins of the G1/S phase transition p27, p21 ↓ Cdk2, cyclin A, cyclin E ↑ Actin ↓ Tubulin ↓ Migration ↓ Cell adhesion: ITGA6, ITGAV, ITGB1, LAMB1, TNC ↑ MMP13 ↓	He et al. (2016)
RWV	Type I collagen scaffolds	Ad-hMSC	20–30 rpm, 21 days	Coculture study (mesenchymal stem cells and meniscus cells) Cartilaginous matrix formation ↑ Chondrogenesis: GAG ↑ Aggrecan ↑ COL10A1 ↑ COL2A1 → COL1A2 → SOX-9 → GREM1 ↓ MMP-13 ↑	Weiss et al. (2017)
RPM	Culture flask	hBMSC	30 min, 6 h, 24 h, 48 h, 120 h	Reorganization of actin cytoskeleton Redistribution of focal adhesion sites Expression of adhesion receptors: CD29 → CD49b, CD49d ↑ CD106 ↓ Effect of passage number to CD54 expression	Gershovich et al. (2009)

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
				CD54 ↓ at passage 7 (compared with static condition, SC) CD54 ↑ at passage 8 (compared with SC) CD54 ↓ at passage 13 (compared with SC)	
RPM	Culture flask	hBMSC	10^{-3} G, 30 min, 6 h, 24 h, 48 h, 120 h	α-actin ↑ at 48 h β-actin → γ-actin ↓ at 48 h β-tubulin ↑ at 48 h and 120 h vinculin ↓ at 120h cofilin ↑ at 48h small GTP-ase RhoA (RhoA)↑ at 48h Rho-kinase(ROCK1) →	Gershovich et al. (2012)
				Expression of 84 genes: ↑ in 30 genes ↓ in 24 genes (look reference for detail)	
RPM	Opticell	Ad-hMSC	10^{-4} G, 14 days	Effect of oxygen concentration in SMG (look reference for detail)	Versari et al. (2013)
RPM	Culture flask	rBMSC	30 rpm, 12, 24 and 48 h	Cell morphology: Cell area: 12 h →, 24 h ↓, 48 h ↓ Cell aspect ratio: 12 h →, 24 h ↑, 48 h ↑ Cell rotation angle: 12 h →, 24 h →, 48 h →	Monfaredi et al. (2017)
				Cell viability: 12 h ↓, 24 h ↓, 48 h ↓	
RPM	Culture flask	Ad-hMSC	96 h	Expression of focal adhesion genes	Ratushnyy and Buravkova (2017)
				Changed expression levels of integrins, FAK-mediated signaling, G-protein-mediated signaling, integrin-associated signaling, AKT/PI3 signaling, caveolins, and actin binding proteins	
RPM	Culture flask	Ad-hMSC	10^{-4} G, 96 h	Angiogenesis-related gene and protein expression;	Ratushnyy et al. (2018)
				Genes: BDNF, CXCL1, VEGF-c, DKK1, FGF5, GDF10, VEGF-a ↑	
				Proteins: Serpin E1, Serpin F1, IGFBP-3, IL-8, VEGF ↑ TIMP-1 ↓ PTX3 and TSP-1 →	

(continued)

Table 4 (continued)

Culture method	Strategy	Cell type	Culture period in MG	Results	Reference
				Capillary-like tube formation ↑ Wound healing ↑	
RPM	Culture flask	hBMSC	0.1–10 rpm, 0 days, 2 days, 7 days, 14 days	Altered expression profiles of different genes:	Li et al. (2019)
				837 genes at day 2	
				399 genes at day 7	
				894 genes at day 14 (look reference for detail)	
RPM	Culture flask	Ad-hMSC	53–65 deg./s, 6, 24, 48, and 96 h	Cell viability →	Ratushnyy et al. (2019)
Levitation	Glass slide	hBMSC	0 g (magnetic field intensity: 12 tesla), 6, 12, 24 and 48 h	Actin filaments and tubulin ↓ Critically cell deaths at 48 h	Shi et al. (2010)
				6 h exposure in early stages of induction: Osteogenic differentiation: ALP, Runx2 ↓ Adipogenic differentiation: PPARɣ2 not detected Leptin not detected	
				Integrin, FAK → p-FAK, ERK, p-ERK ↓	
				6 h exposure in late stage of induction: ALP, Runx2 → Integrin, FAK, p-FAK, ERK, p-ERK →	
Levitation	Coverslip	hBMSC	0 g (magnetic field intensity: 12 T), 12 h	Proliferation ↓	Meng et al. (2011)
				Cell viability ↓	
				Apoptosis rate ↑	
				Caspase-3/7 activity ↑	
				p53 ↑	
Levitation	Contact-free	mBMSC	0 g (magnetic field intensity: < 1 T), 24 and 48 h	Self-assembly 3D cell culture	Anil-Inevi et al. (2018)
				Coculture of mesenchymal stem cells and cancer cells	
Levitation	Contact-free	mBMSC	0 g (magnetic field intensity: < 1 T), 10 min	Density and cell size measurement	Sarigil et al. (2019b)

↑ increasing
↓ decreasing
→ no significant difference
[a]This method was mentioned as 1D clinostat in the study
NG-u: ultrasound exposure under NG

investigations have been applied to overcome these limitations for more than 20 years. The existing systems are notably useful for researches at the cellular level, however, there is still a need for technological innovations and improvements to generate and monitor more complex structures with an *in vivo* microenvironment. These ground-based systems that are equipped with state-of-the-art devices for better monitoring and testing, may expand the knowledge in the field of gravitational biology. Technical evolution of devices to simulate microgravity and testing cells for new and partially unexplored scenarios may advance our understanding on molecular mechanisms underlying the alterations in stem cells under microgravity condition and on possibilities of managing them.

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Smart Polymeric Systems: A Biomedical Viewpoint

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Abstract

Stimuli-responsive polymers (SRPs) are a recent innovative approach that has numerous biomedical applications. These smart polymers can be used in various industrial and medical areas. Smart polymeric hydrogels are a new class of biomedical materials that have attracted much attention in recent years. These hydrogels change their properties in response to alterations in the chemical, physical, or biochemical properties of their environment, and can be used for many biomedical applications. In this review, we discuss these novel materials with a focus on temperature-responsive polymers as the most applicable type of SRPs. Different types of these polymeric systems and their thermodynamics, as well as challenges for their clinical translation, their combination with other biosystems such as liposomes, and their applications are presented.

Keywords

Smart polymers · Stimuli-responsive polymers · Temperature-responsive polymers

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Abbreviations

APS	Ammonium persulfate
PDEAAM	Poly(N,N-diethylacrylamide)
PDMAEMA	Poly(N,N-dimethylaminoethyl methacrylate)
PLGA	Poly(lactide-co-Glycolide) acid
PNIPAM	Poly(N-isopropylacrylamide)
PNIPMAM	Poly (N-isopropylmethacrylamide)
PNVIBA	Poly(vinylisobutyroamide)
PVCL	Poly(vinylcaprolactam)

1 Introduction

In recent years, there has been an increasing interest in stimuli-responsive (synthetic) polymers (SRPs) as a new class of biomaterials. These "stimuli-sensitive", "environmentally sensitive", "intelligent" or "smart" polymeric systems exhibit dramatic changes in their physical properties in response to small alterations in the

chemical, physical, or biochemical properties of their environment.

SRPs are generally categorized into three classes (physical, chemical or biomedical) based on the environmental stimuli. SRPs may also simultaneously respond to more than one special stimulus. Chemical and biomedical stimuli could influence the interaction between polymer chains as well as the interaction between polymer chains and solvent molecules. Similarly, physical stimuli impact molecular interactions and phase transitions of these systems (Kuckling et al. 2012). Each class of SRPs has different geometries and dimensions that include thin films, bulk materials, microgels, nanogels, and particles. Various structures and dimensions affect smart polymer properties (Kuckling et al. 2012). The applications of smart systems depend on the synthesis process, type of stimuli, structure, and architecture. These materials have been used as sensors, microfluidics elements, and semiconductors. Smart polymeric particles are a type of hydrogel that are potentially suitable candidates for biomedical applications. Drug delivery systems, biosensors, and smart scaffolds for tissue engineering, including cell sheet engineering, biomimetic devices, regenerative medicine and biomaterials for tissue engineering are examples of biomedical applications for SRPs. Nanotechnology has also assisted these novel systems and provides many other new applications and technological choices. Here, we review the basic aspects of SRPs and subsequently describe temperature-responsive polymer systems and their hybrid systems. We also discuss the biomedical applications and major barriers to future clinical use of these materials.

1.1 Physical Stimuli

Physical stimuli include temperature, ionic strength, solvent type, electromagnetic radiation (UV and light), magnetic and electric fields, sonic radiation, and mechanical deformations such as stress and strain (Roy et al. 2010). Temperature, light, and electric field alter the structural properties of polymeric systems (Cabane et al. 2012). Although physical stimuli are highly controllable and widely used in research, biological considerations limit their applications (Cabane et al. 2012). For example, the wavelength of the laser to stimulate photo-responsive polymers should be tuned to the near-infrared part of the electromagnetic spectrum rather than visible light to minimize the possibility of destruction and penetrate the deeper tissue layers. Electric field-responsive polymers can be obtained from a combination of liquid crystalline materials and electrically responsive polymers, which are conducting materials (Kuckling et al. 2012). Magnetic fields and ultrasound are suitable for the systems that are entrapped or encapsulated in a polymer (Cabane et al. 2012).

Shape memory, electroactive and color changing polymers, as well as some microgels are examples of physical stimuli-responsive systems. Flexible injection needles, drug delivery systems, new sensors and actuators, artificial muscles, elements of microfluidic devices, and micro-lens technology are examples of biomedical applications of physical stimuli-responsive systems. Physical-responsive polymers include poly(N-isopropylacrylamide) (PNIPAM) (Cheng et al. 2016; Hathaway et al. 2015), poly (N-isopropylmethacrylamide) (PNIPMAM) (Wei et al. 2017), poly(N,N-dimethylaminoethyl methacrylate) (PDMAEMA), poly(acrylic acid) (PAAC) (Zhao et al. 2011a), poly (N-(2-hydroxypropyl)-methacrylamide-lactate) (PHPMAm) (Korth et al. 2006; Ruiz-Hernandez et al. 2014), polythiophene (PT) (Valderrama-Garcia et al. 2016), and sulfonated-polystyrene (PSS) (Al-Sabagh et al. 2018). Antigen-responsive polymers have been developed based on physical stimuli. Different noncovalent interactions (electrostatic and hydrophobic), van der Waals forces, and hydrogen bonding (Roy et al. 2010) are factors that bind antigens and antibodies together. Among these novel stimuli-responsive smart hydrogels, temperature-responsive polymers have attracted considerable attention in nanobioechnology researches because variations in temperature are relatively easy to record and control both *in vitro* and *in vivo* (Hoffman 2013). These types of smart

hydrogels will be discussed in greater detail in the following sections.

1.2 Chemical Stimuli

Chemical stimuli such as pH, specific ions and chemical agents impact molecular interactions between solvents and polymers (Liu and Urban 2010). pH, an important chemical stimuli, is one of the fundamental parameters for biomedical applications that can be used for a specific tissue or in a cellular compartment to achieve a direct response. Attachment of specific ions, acidic or basic moieties to the backbone of a polymer is the main factor for pH responsive polymers. Indeed, pH-responsive mechanisms could be protonated or deprotonated by distribution of the charge over ionizable groups (carboxyl or amine) of the molecule (Cabane et al. 2012). These interactions, in turn, may alter the molecular structures of the chemical stimuli systems.

Dimensional changes, mobility and spatial limitations differ at the surfaces and boundaries of these polymeric hydrogels, which cause restricted mobility inside the network. Therefore, the type of polymer/copolymer chains and chemical-responsive polymer formulations determine how polymers respond to external or internal chemical stimuli. Greater challenges occur when mechanical integrity is needed for chemically cross-linked gels or solid polymeric networks. In order to obtain chemical-responsive polymeric systems for a certain application, it is essential to provide criteria such as designing a network that contains appropriate molecules (Liu and Urban 2010). Several chemical-stimuli responsive polymeric systems that have been used for biomedical applications include PAAC (Xiong et al. 2011), poly(methacrylic acid-g-ethylene glycol) (PMAA-g-EG) (Ye et al. 2006), poly(ethyleneimine) (PEI) (Inoue and Anzai 2005), albumin (Wang et al. 2017), chitosan (Fathi et al. 2017), and gelatin (Klotz et al. 2016). In most chemical-responsive polymeric systems, chemical stimuli are restricted to the creation or destruction of different non-covalent interactions such as hydrogen bonding, hydrophobic and electrostatic interactions, and binding of moieties and chemical groups to the polymer backbone such as osmotic pressure and acid-base reactions. However, in some other systems, more severe changes in the polymer structure such as pendant cross-linking groups and reversible/irreversible bonding with the polymeric backbone can be observed.

Other examples for chemical stimuli responsive systems are the chemical conjugation of the antibody/antigen to a polymeric system or the use of antigen-antibody couples as reversible cross-linkers inside a hydrogel network (Roy et al. 2010).

1.3 Biomedical Stimuli

Biological-dependent stimuli typically include biochemical agents such as analytes and biomacromolecules, affinity ligands, glucose, glutathione, enzyme, receptors, antigens, and redox/thiol groups (Roy et al. 2010; Takashi et al. 2002). Similar to chemical stimuli, biomedical stimuli alter the interaction between polymer chains and solvent molecules or polymer chains (Kuckling et al. 2012). These interactions commonly occur between nucleic acids, proteins or polypeptides chains, their surroundings, or polymer chains (Roy et al. 2010).

Biomacromolecules respond to the presence of specific biochemical agents by changing their molecular structures or their degree of self-assembly (Roy et al. 2010). However, this mechanism may not precisely work in the presence of multiple common functional groups or under *in vivo* situations because of the presence of unwanted reactions *in vivo*, which makes the control of these reactions challenging. Therefore, functional biomedical-responsive polymers have been studied in an attempt to reduce unwanted biomedical reactions (Theato 2008).

The strategies for synthesis of biomedical-responsive polymers such as glucose-sensitive hydrogels, enzymatically degradable hydrogels, and antigen-sensitive hydrogels should be investigated with respect to the applications and types of these materials (Takashi et al. 2002).

Polymers responsive to glucose, enzymes, and inflammation are examples of biological-dependent stimuli systems (Cabane et al. 2012). Engineered glucose sensitive polymers have attracted significant attention due to their applications for diabetic patients (Roy et al. 2010).

Glucose-sensitive polymeric systems are beneficial for the development of self-regulated insulin systems. These systems have the ability to sense and convert analytes into a physical/chemical change (Zhang et al. 2019; Rege et al. 2017).

Different types of glucose-sensitive polymeric systems use various sensing mechanisms. For instance, glucose-responsive systems that are based on glucose-glucose oxidase (GOx) rely on the GOx-catalyzed reaction of glucose with oxygen whereas glucose-responsive systems that are based on concanavalin A (ConA) exploit the competitive binding of glucose with glycopolymer-lectin complexes (Roy et al. 2010). The former systems are not based on direct interaction between glucose and the smart polymer. In these systems, the byproducts produced by enzymatic oxidation of glucose (gluconic acid and H_2O_2) could lead to the polymer's response. This enzymatic reaction (the action of GOx on the glucose molecule) is extremely specific; consequently, incorporation of a proper smart polymer that can respond to these small molecules within several unwanted reactions can indirectly lead to a glucose-responsive system.

Various biomedical-responsive systems such as GOx-conjugated PAAC (Chu 2011), PAAC and PAAC-grafted polyvinylidene fluoride (PVDF) (Chu et al. 2004), GOx conjugated chitosan (Qiu and Park 2001), DEXS/chitosan (Cabane et al. 2012), and PMAA-graft-EG (Huang et al. 2008) have been proposed.

2 Temperature-Responsive Polymers

Among the physical stimuli-responsive polymeric systems, temperature-responsive polymers have attracted considerable attention because temperature is relatively easier to recognize and control both *in vitro* and *in vivo* (Kuckling et al. 2012). Their mechanisms of action are explained by two behaviors, lower critical solution temperature (LCST) and upper critical solution temperature (UCST) (Milichovsky 2010). These materials have two stable thermodynamic states and a transition temperature, which is called the critical temperature (T_{cr}) or temperature of volume phase transition (TVPT). This reversible phase transition is based on several different intermolecular forces such as van der Waals and hydrophobic interactions as well as hydrogen bonding (Schmaljohann 2006).

Below the LCST, hydrogen-bonding interactions are dominant and polymeric chains are hydrophilic. Above the LCST, hydrophobic interactions are dominant and a reversible transition occurs from the coil to the globule (soluble chains to an insoluble state). Otherwise, systems which become soluble by increases in temperature show UCST behavior (Milichovsky 2010). Although certain types of polymeric systems show both LCST and UCST behaviors, they are not generally used for biomedical applications. LCST, or the solubility of a polymeric system in an aqueous solution, depends on the temperature, molecular weight, concentration of the co-solvent or co-polymer, surfactants, salts, and additives (Schmaljohann 2006). However, for certain polymer chains, the coil to globule transitions can be thermodynamically controlled by regulating polymer formulation or the process parameters. In recent years, synthesis, characterization and thermodynamics of many temperature-responsive polymers have been reported (Škvarla et al. 2014). We intend to discuss some aspects of temperature-responsive polymers in detail**.**

2.1 Thermodynamics

Thus far, most temperature-responsive polymers have shown LCST behavior (Milichovsky 2010). As mentioned earlier, structural changes in polymeric systems cause the polymer response. Hence, some physico-chemical requirements such as appropriate spatial and energetic system properties are essential and should be precisely

investigated and designed to obtain proper responses to both external and internal stimuli. Maintaining the mechanical integrity in the presence of chemically/physically cross-linked gels and degree of restrictions on the mobility of polymeric chains are other crucial parameters. Characteristics such as surface chemistry of polymeric gels as well as solid networks considerably differ for various stimuli-responsive polymeric systems (Liu and Urban 2010).

Liu et al. (Liu and Urban 2010) reported a relationship between equilibrium energy, the corresponding stimuli energy input, and physical/chemical responses for different states of temperature-responsive systems, including solutions, surfaces and interfaces, as well as gels and solids. Figure 1 shows a schematic of the mechanism of action and the equilibrium energy for stimuli-responsive polymeric solutions. According to the literature, stimuli-responsive transition in solutions has two energy minima. A certain amount of energy (ΔE_{SR}) is essential for transition from a stable state, A, to B. For the temperature-sensitive polymer with LCST behavior, the corresponding energy (ΔE_{SR}) of this endothermic peak is provided by increasing the temperature from an ambient temperature to above the LCST.

Below LCST, *delta Gibbs free energy is negative and* hydrogen-bonding interactions between the polymer chains and solvent molecules cause the formation of a homogenous mixing phase. Above LCST, enthalpic energy (ΔH) overcomes the entropy (ΔS), which results in unfavorable *Gibbs* free energy of the entire system ($\Delta G > 0$) and a phase separation occurs (Maeda 1966). The particle or aggregate size decreases during the hydrophilic coil to hydrophobic globule transition (Makhaeva et al. 2002).

Hydrophilic domains, along with more polar or ionic co-monomers and additional attractive polymer-water interactions, could increase LCST, whereas the presence of hydrophobic domains, less polar or hydrophobic monomers and more polymer–polymer interactions, could decrease LCST (Joshi et al. 2013). The structural changes in hydration layers (SCHL) theory was proposed to explain the hydration bonding concept and interaction mechanism in these hydrated systems. The SCHL theory is based on dipole orientation of water molecules. Two possible basic orientations around the hydrophilic sub-microdomains are assumed – orientation with the hydrogen or oxygen atoms of water molecules to the sub-microdomain with proton acceptor or proton donor activities, respectively. In terms of the water molecules' orientations, an intermolecular field is formed among hydrogen bonds that dissipates within the bulk system and reaches zero. This effect leads to the force action between domains of phase interfaces. If the orientations of the water molecules equal each of the interacting domains, repulsive hydration forces are formed, and hydration de-bonding is

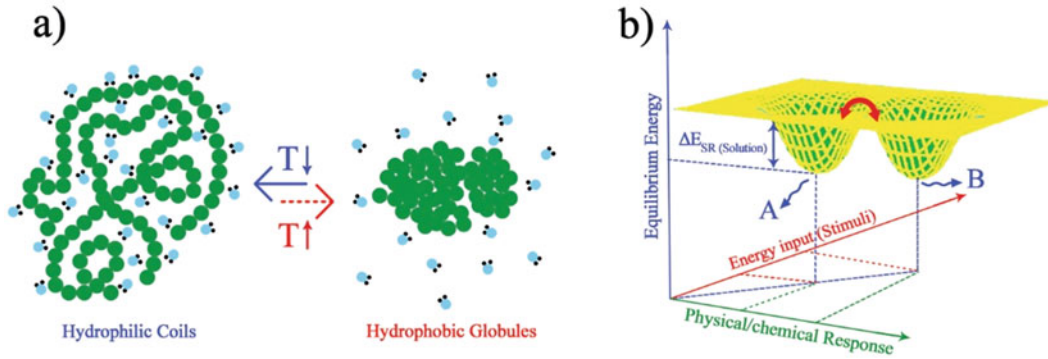

Fig. 1 Schematic of the mechanism of action for temperature-responsive polymer with LCST behavior (**a**) and equilibrium energy for a stimuli-responsive polymer and its physical/chemical response in polymeric solutions (**b**)

overcome. However, for the opposite orientation, attractive forces are formed, and the hydration bonding system dominates.

According to this theory, hydrogen donor groups and molecules (secondary and primary amino groups), amphoteric groups, and molecules (partially primary amino groups) as well as H-acceptor groups (tertiary amino groups) can form hydrogen bonds with water molecules. Under the same conditions, repulsive forces are effective over a greater distance, whereas attractive forces overcome at short distances (approximately smaller than 4 nm). These attractive forces are stronger than repulsive forces at the shortest distances (Milichovsky 2010).

2.2 Lower Critical Solution Temperature (LCST)-Based Polymers

Until now, different temperature-sensitive polymers have been used for biomedical applications, each of which has its own advantages and disadvantages. For certain biomedical applications, we need to develop specific chemical functionalities or copolymers. Numerous researchers have investigated synthesis processes and thermodynamics of smart systems (Clark and Lipson 2012). The rate of reversible LCST transition, as a switching mechanism, is important to prepare these smart systems. This is particularly important for smart drug delivery purposes as the rate of this transition can alter the drug release kinetics and impact its biological effects.

Poly(N-alkyl substituted acrylamides) and poly(N-vinylalkylamides) are the main classes of temperature-sensitive polymers under consideration since their LCST values approximate the temperature of the human *body*. A number of polymers have different transition temperatures. For example, the LCST of poly(N-vinyl piperidine) is in the range of 4–5 °C (Chan et al. 2013).

Numerous attempts have been made to use temperature-sensitive polymer systems for drug delivery. The entrapment and release of drugs and biological molecules such as proteins, growth factors, and enzymes in smart hydrogels, especially thermo-responsive polymers, could be of interest for various drug delivery and biotechnological applications. For instance, thermo-responsive polymers that contain a certain drug could be injected or given orally and due to local conditions (pH and temperature values) could release the drug according to its own release profile (Milasinovic et al. 2010). A comprehensive review on responsive hydrogels for controlled drug delivery applications is given elsewhere (Naddaf et al. 2010).

Doxorubicin delivery by different formulations of temperature-sensitive polymeric systems has been reported for cancer therapy. Doxorubicin can covalently couple to the hydrophobic core of temperature-sensitive polymers. Since the necrotic areas of tumor lesions have temperatures above 37 °C, smart polymers that use different types of copolymers that have an LCST of around 40–42 °C have been successfully synthesized and include polyethylene glycol (PEG)-b-polymethylmethacrylate (*PMMA*), P(NIPAM)-b-PAAC, P(NIPAM)-co-acrylic acid (*AA*)-co-HEMA)-g-Polycaprolactone (*PCL*), poly(ethylene glycol)-b-poly [N-(2-hydroxypropyl)-methacrylamide-lactate], and poly(N-vinyl piperidine) (Ruiz-Hernandez et al. 2014). Table 1 shows the most important SRPs in biomedicine.

2.2.1 Poly(N-Isopropylmethacrylamide) (PNIPMAM)

PNIPMAM can be obtained through radical precipitation copolymerization or emulsion polymerization of N-isopropylmethacrylamide and N-(3-aminopropyl) methacrylamide hydrochloride (APMH). PNIPMAM hydrogels that contain high primary amine concentrations can be generated by optimizing salinity of the reaction or using ammonium persulfate (APS) as an anionic initiator. PNIPMAM is used in drug delivery, biosensors, tissue regeneration, and chemical separations (Hu et al. 2010).

2.2.2 Poly(N-Ethyl-N-Methylacrylamide)

Poly(N-ethyl-N-methylacrylamide) is another polymer with an LCST around 56 °C (Kuckling

Table 1 Some important stimuli-responsive polymers (SRPs)

No.	Polymer	Stimuli	References
1	Poly(N-isopropylmethacrylamide) (PNIPMAM)	Temperature	Hu et al. (2010)
2	Poly(N-ethyl-N-methylacrylamide)	Temperature	Hazot et al. (2002), Kuckling et al. (2012)
3	Poly (N,N-diethylacrylamide) (PDEAAM)	Glucose	Scherzinger et al. (2010, Reinelt et al. (2014)
4	Poly(N,N-dimethylaminoethyl methacrylate) (PDMAEMA)	Temperature	Traitel et al. (2000)
5	Poly(vinylcaprolactam) (PVCL)	Temperature	Rao et al. (2016), Pich et al. (2006)
6	Poly (vinylisobutyroamide)	Temperature	Yamamoto et al. (2003)
7	Poly(N-isopropylacrylamide) (PNIPAM)	Temperature pH	Joshi et al. (2013), Naddaf et al. (2010), Trongsatitkul and Budhlall (2013)

et al. 2012). These particles are synthesized through the polymerization of N-ethyl methacrylamide. Ethylene glycol dimethacrylate and potassium persulfate have been used as hydrophobic cross-linkers and an initiator of this stable and monosized thermally sensitive hydrophilic microgel, respectively. The mentioned polymerization reaction was performed at a high temperature, and it has been reported that the yield of reaction for synthesis could be controlled by the type and concentration of the cross-linker (Hazot et al. 2002).

2.2.3 Poly(N,N-Diethylacrylamide) (PDEAAM)

The LCST of poly(N,N-diethylacrylamide) (PDEAAM) is around 32 °C (Kuckling et al. 2012) and it can be applied to control synthesis. It is used as a block copolymer (Scherzinger et al. 2010). Copolymers and block copolymers are usually used to design and synthesize new polymeric systems that have necessary and novel properties. Various amounts of the different copolymers can be used in polymer formulation to obtain a temperature-responsive polymer with a certain LCST.

In the case of PDEAAM, functional hydrophobic end-groups are achieved using thiol-functionalized 4-alkylphenols. Various cloud point values or LSCT could be also obtained based on the degree of polymerization and concentration of the methylated β-cyclodextrin (RAMEB-CD) (Reinelt et al. 2014).

2.2.4 Poly(N,N-Dimethylaminoethyl Methacrylate) (PDMAEMA)

The LCST of PDMAEMA is approximately 50 °C and it can simultaneously respond to pH and temperature. PDMAEMA has shorter hydrophobic alkyl chains compared to poly(N, N-diethyl aminoethyl methacrylate) (PDEAEMA) and at high pH values it shows stronger hydrophobic interactions and forms a hyper-coiled conformation (Kuckling et al. 2012).

Traitel et al. synthesized a glucose-responsive insulin-controlled release system based on the poly(2-hydroxyethyl methacrylate-co-*N*,*N*-dimethylaminoethyl methacrylate) (HEMA-co-DMAEMA) hydrogel. The morphology of this polymer was modified by changing the cross-linking agent along with GOx and catalase, as well as insulin. They observed that these hydrogels were stable in water without a cross-linking agent. The sensitivity of this system to pH and glucose was higher than the chemically cross-linked hydrogels (Traitel et al. 2000).

2.2.5 Poly(Vinylcaprolactam) (PVCL)

The PVCL microgel has a LCST in the range of 32–34 °C and it exhibits temperature and pH-sensitive properties. Increasing the amount of vinylimidazole (VIm) as a functional group in the PVCL hydrogel structure results in an increased degree of swelling of the hydrogel under acidic conditions, and the volume phase transition temperature shifts to higher temperatures. The sedimentation behavior of

microgels are pH dependent, which makes this system suitable for controlled particle separation (Pich et al. 2006). PNVCL is an important temperature-sensitive polymer and is similar to PIPA. Advantages of PNVCL compared to PNIPAM include rapid coil to global transition. Moreover, PNVCL is both temperature and pH sensitive and can be used for various biomedical applications (Rao et al. 2016).

2.2.6 Poly(Vinylisobutyroamide) (PNVIBA)

Poly(vinylisobutyroamide) (PNVIBA) is similar to PNIPAM in terms of chemical composition, but the presence of the reversed amide linkage results in a higher LCST (up to 39 °C). Hydrolyzed copolymers that contain N-vinylformamide (NVF) and N-vinylisobutyroamide (NVIBA) exhibit dual temperature and pH responsiveness. The LCST of this system increases with the hydrophilic component of NVF in the copolymer structure. Poly Vinylamine-co-NVIBA) could be obtained by selective hydrolysis of the NVF units in poly (NVF-co-NVIBA) As mentioned before, various cloud point values or LSCT could be obtained with respect to the degree of polymerization and the presence of NVF and NVIBA (Yamamoto et al. 2003).

2.2.7 Poly(N-Isopropylacrylamide) (PNIPAM)

PNIPAM is the most important thermally responsive polymer. There have been numerous studies that used this smart polymer for biomedical applications (Chan et al. 2013). PNIPAM exhibits LCST behavior and has a sharp transition around physiological temperature (Adibfar et al. 2018). This reversible transition is a switching mechanism for the drug delivery application of PNIPAM. This polymer has attracted extensive attention because of its capability to be used in different tissue engineering applications (Lima et al. 2016; Marquardt and Heilshorn 2016; Xu et al. 2016). PNIPAM has been considered as a carrier of nucleic acids (Ozdemir et al. 2008), drugs (Rejinold et al. 2014; Sudhakar et al. 2017), and proteins (Joshi et al. 2013; Naddaf et al. 2010; Trongsatitkul and Budhlall 2013). A few studies have assessed delivery of growth factors via PNIPAM (Ertan et al. 2013; Garbern et al. 2010). PNIPAM microspheres combined with other materials such as poly Poly(Lactide-co-Glycolide) acid (PLGA) and magnetic particles have been used to deliver biomacromolecules (Cheng et al. 2016; Dionigi et al. 2014; Tombácz et al. 2015). For example, magnetic nanoparticles (MNPs) coated with poly (N-isopropylacrylamide-acrylamide-chitosan) (PAC), which is simultaneously sensitive to magnetic fields, temperature, and pH have been developed for cancer drug delivery and the culture of human dermal fibroblasts and normal prostate epithelial cells (Khan and Tanaka 2017). Nevertheless, exact drug release kinetics and its corresponding biological effects have yet to be determined.

3 Applications

Until now, the results of numerous investigations of smart polymers along with several reviews have emphasized their potential use in biomedical applications (Alexander et al. 2014) (Schmaljohann 2006), including cell sheet engineering (Patel and Zhang 2013), drug delivery (Blackburn et al. 2009; Choi et al. 2002; Lee et al. 2008; Rejinold et al. 2014), biosensors (Kim et al. 2005, 2007; Zhang et al. 2006), tissue regeneration (Adibfar et al. 2018; Ertan et al. 2013; Joshi et al. 2013), and chemical separations (Grabstain and Bianco-Peled 2003). These materials can be combined with a variety of bioactive molecules such as proteins, nucleic acids, small molecules, and carbohydrates. Bioactive molecules are added to the smart polymeric systems through physical mixing, chemical conjugation or complexation reactions (Alexander et al. 2014). Despite the potential beneficial characteristics of smart polymeric systems, challenges exist for their biomedical applications and future clinical translation (Hoffman 2013). We intend to discuss some of these drawbacks in Sect. 4.

3.1 Cell Sheet Engineering

During the last few years, cell sheet engineering has been known as an innovative method for cell-based therapy. Viable and transplantable cell sheets for different tissue engineering applications can be fabricated by cell sheet harvest technology. Most cell sheet studies use temperature-sensitive systems to detach the cell sheet; however, it has been shown that pH and magnetic responsive systems have the potential for cell sheet harvesting (Wang et al. 2011; Patel and Zhang 2013). Plasma polymerization, electron beam, UV irradiation, solvent cast, and other fabrications approaches have been used to produce novel temperature-responsive surfaces for cell sheet engineering. This technology has a potential application as an engineered tissue (Tang et al. 2012).

3.2 Drug Delivery

Targeted and controlled drug delivery offers an appropriate strategy for cancer chemotherapy and numerous other medical applications. These new approaches have overcome the severe toxic side effects of conventional drug delivery methods. Until now, different smart polymers have been tested for delivery of drugs and macromolecules as therapeutic agents (Tang et al. 2018). Functionalized stimuli-responsive nanocarriers or the combination of these smart materials with other nanoparticles such as MNPs or liposomes have superior specificity for targeted and controlled drug delivery and might be responsive to multiple stimuli. In recent years, the use of numerous stimuli-responsive systems as drug delivery carriers have been investigated (Karimi et al. 2016; Ertan et al. 2013; Chen et al. 2012).

3.3 Biosensors

Stimuli-responsive biosensors are a new detection technique that can improve clinical diagnosis and treatment of various cancers and other diseases. These complex biosensor should be more sensitive for diagnostic applications. Functionalization of responsive polymers, surface modifications and the combination of stimuli-responsive biosensors with the proper materials (special copolymers) could improve precision and accuracy of the obtained data (Wang et al. 2018).

3.4 Tissue Engineering

SRPs can be used as tissue engineering scaffolds or to provide proper tissue conditions such as protein or growth factor release into a special tissue (Sood et al. 2016). These types of novel smart biomaterials respond to external stimuli and have the potential for tissue regeneration (Khan and Tanaka 2017).

Calejo et al. (2012) prepared a thermos-responsive polymer from the combination of ethyl (hydroxyethyl) cellulose (EHEC) and arginine-based surfactants, and evaluated its rheological and turbidity properties. These systems are appropriate candidates for injection into the human body, as they can be either a liquid or a gel form at low and high temperatures, respectively. Toxicity studies with HeLa cells have shown that these thermos-responsive hydrogels are suitable for human use because of the low toxicity of the precursors. More recently, we have synthesized VEGF loaded PNIPAM nanoparticles and a VEGF-PNIPAM nanoparticle loaded collagen hydrogel as VEGF delivery systems to increase vascularization in bone tissue engineering. The biological effects of the released VEGF on human bone marrow derived mesenchymal stem cells (hBMSCs) were studied *in vitro*. The results showed that the prepared thermos-responsive hydrogel appeared to be a promising approach that could be applied to deliver biological molecules for tissue engineering applications (Adibfar et al. 2018).

Ertan et al. (Ertan et al. 2013) studied the effects of double growth factor release from PNIPAM particles on cartilage tissue engineering. They compared the release of IGF-I and TGF–b1 from PNIPAM and PLGA nanoparticles. It was observed that double growth factor

delivery yielded better results than single growth factor or growth factor-free applications in terms of collagen type II and aggrecan expression. In addition, samples that carried both nanoparticles had the best cartilage differentiation results on the scaffolds.

Smart biomaterials have also been used for patterned cell seeding and co-culture (Yamato et al. 2002), new cartilage tissue formation (Khan and Tanaka 2017), control of osteoblast adhesion and proliferation (Stile et al. 2003), protein/DNA intercellular delivery (Stayton et al. 2005), scaffolds for tissue engineering (Khan et al. 2009), fibroblast growth and proliferation (Kyle et al. 2012), and controlled drug and cell delivery (Zhao et al. 2011b).

4 Hybrid or Complex Systems

In recent years, the development of hybrid or composite biomaterials to attain superior and more beneficial properties compared with single composition has attracted much attention. Composite systems have better function and greater characteristics. Combinations of natural and synthetic polymers, as well as SRPs, with various other materials such as liposomes have been proposed to prepare new composite hydrogels that have increased their potential and applications (Leach 2008).

A temperature-responsive chitosan-g-poly (N-isopropylacrylamide) nanosystem has been developed by ionic cross-linking for delivery of curcumin. According to the obtained results, LCST increased with the increase in the chitosan grafted polymer. This nanocarrier above LCST has shown interesting properties such as prominent curcumin release and a specific toxicity on cancerous cells. This thermoresponsive system is biocompatible and biodegradable, which makes it a powerful nanovehicle for curcumin drug delivery (Rejinold et al. 2011).

Magnetic nanohydrogels are produced by hybridization of chitosan with poly (N-isopropylacrylamide) and iron oxide (Fe_3O_4) MNPs. Chitosan serves as a cross-linker agent during the free radical polymerization reaction. More importantly, chitosan can control the growth of the poly (N-isopropylacrylamide)-chitosan based nanohydrogel. LCST would increase by increasing the chitosan weight ratio in the hybrid nanohydrogels. Encapsulation of Fe_3O_4 into this system caused an increase in LCST. Additionally, this magnetic nanohydrogel exhibited excellent cytocompatibility. Hence, it could be utilized as a potential candidate for hyperthermia treatment of cancer and for targeted drug delivery (Jaiswal et al. 2010). Other studies for hybridization of chitosan (Qi et al. 2014) or other natural polymers such as collagen (Adibfar et al. 2018) have been conducted.

Collagen is another natural polymer that has been used to design smart complex materials. The poor mechanical strength of collagen can be enhanced via chemical modification and cross-linking reactions. The combination of collagen with a temperature-responsive polymer such as PNIPAM can improve the mechanical properties of collagen. Addition of various concentrations of collagen could led to a different hydrogels that have shear moduli (102–105 Pa) compatible to soft tissues (Barnes et al. 2016).

PNIPAM and dansyl group-labeled pentapeptide (Gly-Arg-Lys-Phe-Gly-dansyl) were successfully conjugated through atom transfer radical polymerization. An average molar mass of 30,400 g/mol and molar mass dispersity of 1.14 were obtained. In an aqueous solution, increasing the temperature resulted in a phase transition and mesoglobule (spherical nanoparticles) formation. The external layer of the mesoglobule was formed by hydrophilic peptides. Incorporation of these peptides into the structure led to cleavage of the peptide sequence. Consequently, these systems are good candidates for peptide release applications (Trzebicka et al. 2013).

Another thermo-sensitive polymeric system that included alginate-graft-poly (N-isopropylacrylamide) (ALG-g-PNIPAM) and β-cyclodextrin (β-CD) was developed for the controlled release of 5-fluorouracil (5-FU). This thermo-sensitive polymeric system had hollow sphere structures and an LCST in the range of 35–37 °C. Due to the hydrophilic cavities, it had

high drug loading efficiency. Loading content and drug release kinetics depend on formulation and the initial composition of the mixtures, temperature, and pH. Acidic conditions or increasing temperatures above the LCST boost the drug release kinetics. These novel hollow particles are more convenient for intelligent drug delivery due to their good biocompatibility (Li et al. 2016).

A highly porous PNIPAM/hydroxyapatite (HAp) composite was produced by electrochemical polymerization for use in bone tissue regeneration. The swelling behavior could be controlled through the amount of HAp in the synthesized PNIPAM-HAp scaffolds. The antibiotic oxacillin was loaded into the scaffolds against *Staphylococcus aureus* (*S. aureus)* and *Pseudomonas aeruginosa (P. aeruginosa).* The results of this study demonstrated that the combination of PNIPAM and calcium phosphates is a promising technique to prepare non-toxic and biocompatible PNIPAM-HAp scaffolds for bone tissue engineering (Ribeiro et al. 2017).

Grafting PNIPAM on a microcarrier surface for cell culture applications has been developed using grafting-to, grafting-from and grafting-through techniques, along with non-covalent adsorption and seeded polymerization techniques. Microcarriers could release the cells by temperature adjustments in these systems. The PNIPAM grafting density might affect the molecular weight and molecular architecture of the whole system. Consequently, the efficiency of cell behavior such as cell attachment/detachment of the mentioned production techniques differ and can be optimized according to the applications. It is believed that cell culture systems based on a temperature-responsive microcarrier would be the next generation of large-scale cell culture approaches in biomedical applications (Zhang et al. 2015).

Liposomes are another example of a stimuli-sensitive system. Enzymes, light or pH could be selected as triggers in stimuli-sensitive liposomal systems. These systems possess the advantages of both smart and liposomal systems, and are important smart drug carriers (Liu et al. 2016). Although it is believed that these novel smart stimuli-responsive polymeric hydrogels may display better potentiality in future clinical applications, successful *in vivo* applications of these materials remain a challenge.

5 Challenges for Clinical Translation

There are several concerns in terms of stimuli-responsive polymeric systems for biomedical applications. Cellular toxicity of the smart polymers is the most controversial issue in this area (Hoffman 2013). Until now, the cyto- and genotoxicity of different smart polymers and co-polymers have been examined in different mammalian cell lines. Although many smart systems are composed of non-degradable, toxic acrylamide or acrylic acid polymers such as PNIPAM and PPAAc, no significant cyto- and genotoxicities have been observed in the necessary range for certain biomedical applications (Naha et al. 2010). For instance, no significant cyto- and genotoxicities ($p \leq 0.05$) were detected in HaCaT and SW480 cells for PNIPAM nanoparticles after 96 h over concentration ranges from 25 to 1000 and 12.5 to 800 mg/l, respectively. It was demonstrated that the PNIPAM nanoparticles internalized in the mammalian cells and localized in the lysosomes. A non-ecotoxicological response of PNIPAM nanoparticles has been reported (Naha et al. 2010). Other studies have also confirmed that PNIPAM is non-toxic for different cell lines (Némethy et al. 2013; Kjoniksen et al. 2014; Wadajkar et al. 2008). Although these studies have investigated the wide range of PNIPAM nanoparticles, higher concentrations (especially 10 mg/mL) are not pharmacologically relevant (Wadajkar et al. 2008).

It should be taken into consideration that the NIPAM precursor is toxic, whereas the polymeric PNIPAM is not. Consequently, the remaining linear PNIPAM chains that result from destruction of the network structure through biodegradable cross-linkages are too large to be absorbed by living organisms and may not have toxic effects (Zhang et al. 2004). Good blood compatibility along with lack of cytotoxicity has been

observed for PNIPAM-g-poly(N-isopropylacrylamide-co-styrene) microspheres (PNNS-MSs). Grafting other materials such as styrene that have better hydrophilicity onto the PNIPAM structure would improve its biocompatibility (Zhu et al. 2013). It has been shown that PEGylated PNIPAM nanoparticles did not have any toxicity effects on J774 and L929 cell lines up to a concentration of 2 mg/ml. Meanwhile, no interactions were observed when nanoparticles were incubated with blood cells, which suggested their hemocompatibility (Gulati et al. 2010).

It should be mentioned that further considerations are needed to clarify the toxicity of these smart materials in the nanoscale dimension. Hazards and different aspects of toxicological human health risk assessments of engineered nanoparticles have been investigated (Radad et al. 2012). It has been reported that these polymers are not biodegradable and not readily excreted via the kidneys after drug delivery. As a result, they would tend to accumulate in the body. Thus, they have not been tested in clinical trials (Hoffman 2013).

The data presented here would suggest that these smart materials are biocompatible with mammalian cells; hence, they can be used in biomedicine applications. However, further studies are required to overcome these challenges.

6 Concluding Remarks and Future Trends

Smart polymers and their composites are potential candidates for many biomedical applications. These materials have superior properties and can be used either embedded in or attached to structural materials. They can be used in normal and cancerous tissues. Various smart polymers and different types of stimuli-sensitive polymers have been used for cell sheet engineering, drug delivery, biosensors, tissue regeneration, and chemical separations.

Maintaining safety without losing sensitivity to stimuli changes and biological properties during the treatment process, fabricating smart materials sensitive to multiple stimuli, designing proper copolymer systems to sense small variation of multiple stimuli as fast as possible, and scale-up of the process and production as well as purification in a cost-effective way along with efficient *in vivo* outcome are challenges to be overcome. These items could be divided into the rational design, fabrication, and process development, as well as discovery steps. Controlling the response of these biomaterials after implantation in the body is challenging and has not been clearly elucidated (Khan and Tanaka 2017).

In the future, smart polymers will play critical roles in cutting edge science. Additional studies are needed before these novel smart polymers that have certain transition temperatures and pH levels can be developed and introduced commercially.

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Acute Lung Injury: Disease Modelling and the Therapeutic Potential of Stem Cells

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Abstract

Acute lung injury (ALI) is a severe clinical condition with high morbidity and mortality that usually results in the development of multiple organ dysfunction. The complex pathophysiology of ALI seems to provide a wide range of targets that offer numerous therapeutic options. However, despite extensive studies of ALI pathophysiology and treatment, no effective pharmacotherapy is available. Increasing evidence from both preclinical and clinical studies supports the preventive and therapeutic effects of mesenchymal stem cells (MSCs) for treating ALI. As cell-based therapy poses the risk of occlusion in microvasculature or unregulated growth, MSC-derived extracellular vesicles (MSC-EVs) have been extensively studied as a new therapeutic strategy for non-cell based therapy. It is widely accepted that the therapeutic properties of MSCs are derived from soluble factors with paracrine or endocrine effects, and EVs are among the most important paracrine or endocrine vehicles that can deliver various soluble factors with a similar phenotype as the parent cell. Therapeutic effects of MSCs have been reported for various delivery approaches, diverse doses, multiple origins, and different times of administration, and MSC-EVs treatment may include but is not limited to these choices. The mechanisms by which MSCs and MSC-EVs may contribute to ALI treatment remain elusive and need further exploration. This review provides an overview of preclinical studies that support the application of MSC-EVs for treating ALI, and it discusses emerging opportunities and their associated challenges.

Keywords

Acute lung injury · Animal model · Cell-free therapy · Extracellular vesicles · Intratracheal delivery · Mesenchymal stem cells · Pathophysiology

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Abbreviations

AEC I	Type I alveolar epithelial cells
AEC II	Type II alveolar epithelial cells
ALI	Acute lung injury
Amφ	Alveolar macrophages
ARDS	Acute respiratory distress syndrome
ATS	American thoracic society
BALF	Broncho-alveolar lavage fluid
DMSO	Dimethyl sulphoxide
EBD	Evans blue dye
ELISA	Enzyme-linked immunosorbent assay
ESCRT	Endosomal sorting complex required for transport
H&E	Hematoxylin and Eosin
hAD-MSCs	Human adipose-derived MSCs
hBM-MSCs	Human bone marrow-derived MSCs
HLA	Human leukocyte antigen
hMens-MSCs	Human menstrual blood-derived MSCs
hUC-MSCs	Huma umbilical cord-derived MSCs
i.t.	Intratracheal
i.v.	Intravenous
LPS	Lipopolysaccharide
MFGE8	Milk fat globule-EGF factor 8 protein
MHC	Major histocompatibility complex
MISEV	Minimal information of studies of extracellular vesicles
MPO	Myeloperoxidase
MSC-EVs	MSC-derived extracellular vesicles
MSCs	Mesenchymal stem cells
PARDS	Pediatric ARDS
PDCD61P	Programmed cell death 6 interacting protein
PMN	Polymorphonuclear
qRT-PCR	Qualitative reverse transcriptase polymerase chain reaction
TSG101	Tumor susceptibility gene 101 protein
TSPAN29	Tetraspanin 29
VILI	Ventilator-induced lung injury

1 Introduction

1.1 Acute Lung Injury

Acute lung injury (ALI) or its clinical manifestation, acute respiratory distress syndrome (ARDS), is an acute inflammatory lung injury that usually is responsible for high morbidity and mortality as well as the development of multiple organ dysfunction. ARDS was first proposed in 1967 (Ashbaugh et al. 1967); "A" originally was the abbreviation for adult, but it was later changed to acute. As our understanding of the condition grew, the definition changed from the American-European Consensus Conference Committee definition (Bernard et al. 1994) to the Berlin definition (Force et al. 2012; Ferguson et al. 2012). The latter classifies the severity of the condition from mild to severe. In addition, the pediatric ARDS (PARDS) definition was developed by the Pediatric Acute Lung Injury Consensus Conference in 2015 (Pediatric Acute Lung Injury Consensus Conference Group 2015). Although tremendous progress has been made both in therapy and nursing over the last half century, ALI is still a significant source of morbidity, mortality, and financial burden.

Over three million patients suffer from ARDS every year, and they constitute more than 10% of patients of intensive care units. Moreover, ARDS is likely to be underreported in low-income countries, as it is under-recognized even in high-income countries (Thompson et al. 2017; Villar et al. 2016). Bellani et al. (2016) studied 29,144 patients from 459 intensive care units in 50 countries across 5 continents and found that clinical recognition rates ranged from 51.3% for mild ARDS to 78.5% for severe ARDS, and the condition appeared to be a public health problem globally, with a very high mortality of approximately 40%. Even patients who survive from ALI are at high risk for long-term poor quality of life (Herridge et al. 2016; Biehl et al. 2015). Children are no exception, as another international study that involved 23,280 patients from 145 pediatric intensive care units in 27 countries found that PARDS occurs in approximately 3% of patients

but results in ~17% mortality (Khemani et al. 2019).

To date, there has been no comprehensive epidemiology study of ARDS in China, but the incidence, mortality, and risk factors for ARDS and PARDS in China are thought to be similar to those in Europe and the United States based on several relatively regional studies, which suggest that the annual number of cases in China is more than 670,000 patients (Song et al. 2014). However, health emergency related ALI is not included. During twenty-first century, there are three outbreaks of coronavirus infection around the world, including SARS (Severe Acute Respiratory Syndrome) in 2002, 10 years later with MERS (Middle East Respiratory Syndrome) in 2012 and more recently from December 2019 with COVID19 (Corona Virus Disease). So far, there is no principle to follow for the therapy of COVID19 especially for severe patients because of absence of efficacious drugs and vaccines for SARS and MERS until now. Under the support of WHO (World Health Organization), several specific treatments were under investigation and would be tested through clinical trials, and cell therapy was included.

ALI is a public health problem and common complication in critically ill patient groups, with significantly high mortality and poor outcome. No effective pharmacotherapy exists, so it is necessary to further investigate its pathophysiology and try to find more effective treatments. The goals of this review are to summarize evidence from preclinical studies that supports more efficient therapy for ALI and to discuss emerging opportunities and their associated challenges. The use of mesenchymal stem cells (MSCs) has the potential to treat a variety of diseases, and MSC-derived extracellular vesicles (MSC-EVs) could be a future novel treatment strategy for pulmonary inflammatory disease via intratracheal delivery.

1.2 Pathogenesis of ALI

ALI originates from multiple factors, including direct and indirect lung injury (Table 1). Once triggered by infectious, chemical, or mechanical insult, the complex interaction between the immune system and the alveolar-capillary barrier gives rise to the pathophysiology of ALI (Lee et al. 2019). In addition, genetic studies in Chinese populations identified some genetic risk factors that might increase the development of ARDS, such as Toll-interleukin 1 receptor domain-containing adapter protein (Song et al. 2010) and the tumor necrosis factor receptor-associated factor 6 gene (Song et al. 2012). ALI is also a serious perioperative complication with crucial mortality and morbidity, and there are limited treatments available beyond conservative respiratory support (Jin et al. 2017).

Figure 1 shows the pathogenesis of ALI, including the exudative, proliferation, and fibrotic phases, and the difference between healthy and ALI alveoli (Thompson et al. 2017). The exudative phase usually takes place within 24 h of the

Table 1 Conditions associated with ALI

Direct lung injury insults	Indirect lung injury insults
Pneumonia[a]	Sepsis[a]
Gastric aspiration[a]	Major trauma
Pulmonary contusion	Non-cardiogenic shock
Pulmonary embolism	Pancreatitis
Inhalation injury	Severe burns
Near drowning	Multiple transfusion or transfusion-associated acute lung injury
	Cardiopulmonary bypass surgery
	Reperfusion edema after lung transplantation or embolectomy
	Drug overdose
	Genetic risk factors

[a]Pneumonia, gastric aspiration, and sepsis are the top three main triggers of ALI in recent clinical conditions

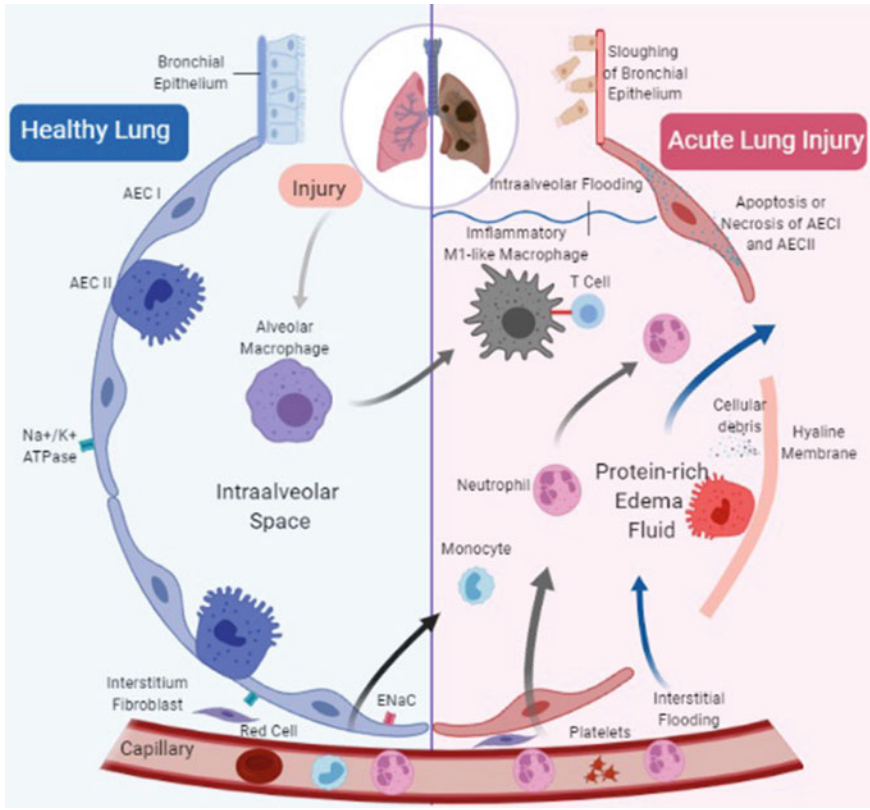

Fig. 1 The difference between a healthy and ALI alveolus. The hallmarks of ALI are disruption of alveolar-capillary barriers, recruitment of pro-inflammatory cells, formation of hyaline membranes, and flooding of protein-rich edema within the interstitium and alveolus. Injury begins with the disruption of alveolar-capillary integrity by either direct or indirect insults. Initially, resident alveolar macrophages are activated and polarized into M1-like macrophages, which secrete pro-inflammatory factors that contribute to recruitment of neutrophils and monocytes to facilitate and maintain inflammation and tissue injury. Extensive damage to the alveolar epithelium directly increases the permeability of alveolar-capillary barriers, and apoptosis of AEC II weakens pulmonary surfactant secretion and alveolar fluid clearance, which aggravate protein-rich edema fluid in the interstitium and alveolus

occurrence of insult; it is characterized by diffuse alveolar damage and represents innate cell-mediated damage of the alveolar endothelial and epithelial barriers and accumulation of protein-rich edema fluid in the interstitium and alveolus. Resident alveolar macrophages (Amφ) recognize microbial components or tissue injury via pattern recognition receptor signaling, which leads to NFκB-dependent polarization of Amφ into M1-like macrophages and the initiation of the exudative phase. M1-like macrophages secrete pro-inflammatory cytokines and chemokines that contribute to accumulation of neutrophils and monocytes as well as to activation of alveolar epithelial cells and effector T cells, which promote and maintain inflammation and tissue injury (Aggarwal et al. 2014). Activated neutrophils contribute to lung injury by releasing pre-formed inflammatory mediators, reactive oxygen species, and proteinases and by the formation of neutrophil extracellular traps and highly injurious histones. The injured and activated endothelium and epithelium initiate tumor necrosis factor-mediated expression of tissue factor, which results in coagulation of dysregulated intra-vascular and intra-alveolar, platelet aggregation, micro-thrombi formation, and hyaline membrane formation. Extensive damage to the alveolar epithelium also leads to the loss of alveolar ion channels and weakens the

osmotic pressure for alveolar fluid clearance, which further promotes alveolar flooding. Endothelial activation and microvascular injury further facilitate alveolar-capillary barrier disruption as well as interstitial and intra-alveolar flooding in ALI. Alveolar flooding and collapse result in severely compromised gas diffusion and hypoxemia.

Impaired and extensive epithelial injury results in the proliferative phase of ALI, which is essential for host survival and is characterized by the transient expansion of resident fibroblasts, the formation of a provisional matrix, and proliferation of airway progenitor cells and differentiation from type II alveolar epithelial cells (AEC II) to type I alveolar epithelial cells (AEC I) (Vaughan et al. 2015). In adult humans, this phase usually occurs between 3 and 7 days following respiratory failure, whereas the timing was reported to be 1 week after injury for experimental animals (Matute-Bello et al. 2011; Beasley 2010). When epithelial integrity has been rebuilt, alveolar edema is reabsorbed and alveolar architecture and function are restored.

The final fibrotic phase does not occur in all ALI patients, but evidence suggests that this phase is related to prolonged mechanical ventilation and increased mortality. Patients in this phase are substantially related to the demand of mechanical ventilation, the development of interstitial and intra-alveolar fibrosis results from extensive basement membrane damage and inadequate or delayed re-epithelialization.

1.3 The Status of ALI Treatment

Lung disease research has shown that ALI is a syndrome characterized by substantial heterogeneity (Thompson et al. 2017). The complex pathophysiology of ALI seems to provide a wide range of targets that offer numerous therapeutic options, but to date there is no available pharmacotherapy based on the pathophysiology of ALI. Currently, ALI treatment is limited to primarily supportive care approaches, such as lung-protective ventilation (Beitler et al. 2016), the fluid conservative strategy (National Heart and B.I.A.R.D.S.C.T. Network 2006), and prone positioning (Fan et al. 2017). Unfortunately, supportive therapies for ARDS focus only on preventing further lung injury rather than actively accelerating tissue repair, and this is why the treatment effectiveness is so limited. In addition, current evidence from practice and research indicates that there is no safe tidal volume or airway pressure for ALI patients. Because the aerated lung volume decreases during the course of the disease, even normal tidal volumes delivered with airway pressure may induce regional overstretch, leading to further epithelium activation or injury and inflammation amplification. For patients who suffer from moderate-to-severe ARDS, ventilation while in the prone position is closely associated with decreased mortality, and this is currently recommended in clinical practice (Fan et al. 2017). Unfortunately, no pharmacologic therapy has been shown to decrease ARDS either short-term or long-term mortality. Therefore, new approaches to develop feasible therapies for ALI are needed.

2 Animal Models to Study ALI

2.1 Experimental Animal Models of ALI

Animal studies provide an experimental scenario that allows investigators to study underlying pathophysiological mechanisms and search for therapeutic approaches before translating them into humans. A good animal model should share similar anatomy and responses with humans so that it can be used to predict the feasibility of a therapeutic approach and provide a bridge from bench to bedside. However, no animal model can perfectly duplicate all human features when exposed to stimuli or treatments, and the ALI animal model is no exception. As recommended in the official documents of the American Thoracic Society (ATS), at least three of four main features of ALI should be present in an ALI animal model, including histological evidence of tissue injury, alteration of the alveolar capillary barrier, inflammatory response, and physiological

dysfunction, which are specifically described in Table 2 (Matute-Bello et al. 2011). ATS documents also indicate that it is not necessary to establish a fully developed ALI animal model. In this regard, we previously summarized cellular mechanisms underlying lung regeneration and repair, and analyzed the role of stem cells both in small and large animal models (Yahaya 2012).

2.2 Evaluation of Common ALI Animal Models

For preclinical studies, numerous methods to develop animal models for ALI have been reported, including endotoxin (Wang et al. 2018; Zhu et al. 2017; Tang et al. 2017), bacteria (Monsel et al. 2015), ventilator (Hayes et al. 2015; Islam et al. 2019), and cecal ligation and puncture (Wang et al. 2015; Condor et al. 2016). According to these studies, neutrophils play an important role in the inflammatory response in ALI development in animal models, both for small and large animal.

Most ruminants, including goats and sheep, have segmented lungs, which means that many macrophages circulate in pulmonary vessels and that their pulmonary circulation tends to be sensitive to intravenous injection with endotoxin. Several studies reported that small doses of endotoxin induced increased pulmonary hypertension in these animals, and previous studies showed that smoke inhalation injury (Lange et al. 2012; Rehberg et al. 2013; Halim et al. 2019) and brushing injury (Yahaya et al. 2011; Kardia et al. 2018). Infiltration and accumulation of neutrophils were also reported to be the major feature in large animal models for ALI (Lange et al. 2012), but large animals are prone to microbial infection, so intravascular macrophages in these animals are easily augmented via stimulation of the local inflammatory reaction in response to microbe invasion.

In contrast, smaller animals and humans have fewer intravascular macrophages. Compared with large animals, small animals such as mice, rats, and rabbits are widely bred and very economical in terms of expenses. Numerous studies indicated that endotoxin-induced ALI in mice resulted in prominent inflammatory cell infiltration in the alveolar spaces, including neutrophils and macrophages, as well as interstitial edema and intra-alveolar septal thickening with fibrin and collagen deposition (Chen et al. 2014; Liou et al. 2017). The rat model showed a similar pattern of ALI characteristics following exposure to toxic chemicals such as sodium nitrate and naphthalene (Uriarte et al. 2013; Zhang et al. 2016). Moreover, activated neutrophils were found to play a key role in initiating the inflammatory processes involved in the formation of hemorrhage or alveolar damage (Wang et al. 2018; Zhu et al. 2017; Zhang et al. 2018). Murine lungs rarely develop hyaline membranes following ALI (Matute-Bello et al. 2011), whereas hyaline membranes in rabbit ALI models usually appear during the early exudative phase of ALI (Cao et al. 2012), which is consistent with the features in ALI patients. Moreover, gene sequence comparison analysis demonstrated that the rabbit shared a higher homology with the human leukocyte antigen (HLA) genes than mouse and rat, thus rabbit tissue is less likely to result in immune rejection of allotransplantation (Marche et al. 1985). Therefore, rabbits are commonly used in implantation and tissue engineering studies. However, the greater availability of specific reagents and genetically modified mice and rats make them popular for animal models.

The official ATS workshop report recommends the features and measurements of experimental ALI animal models and also describes the difference between ALI patients and several common ALI animal models in detail (Matute-Bello et al. 2011). Given the high frequency of use, we briefly summarized LPS, ventilator, and live bacteria-induced lung injury in Table 2. Almost all of the "very relevant" criteria are present in the top three most common animal models, so they can be used to further investigate the more efficient therapeutic approaches to treating ALI. The LPS-induced ALI mouse model is commonly used as a model of human ALI associated with severe pneumonia or sepsis because of its high efficiency. Intratracheal (i.t.) and intravenous (i.v.) delivery are commonly

Table 2 Presence of "very relevant" criteria in the top three common animal models of ALI

Human patient		Measurement	Notes	LPS	VILI	Live bacteria
Histological evidence of tissue injury	Accumulation of neutrophils in the alveolar/interstitial space	H&E staining	Hyaline membranes are rarely observed in murine models	+	+	+
	Formation of hyaline membranes			+	+	+
	Proteinaceous debris in alveolar space			+	+	+
	Thickening of the alveolar wall			+	+	+
	Injury by a standardized histology score			+	+	+
Alteration of the alveolar capillary barrier	Increased extravascular lung water content	Wet-to-dry ratios	More errors for very small lungs	+	+	+
	Accumulation of protein/tracer in airspaces/extravascular space	EBD	Intravenous injection in advance	+	+	+
	Total BAL protein concentration	BALF-total protein concentration, IgM	Technical challenges and difficult to standardize	+	+	+
	BAL concentration of high molecular weight proteins			+	+	+
	(Micro-)vascular filtration coefficient (K_f)	Under machine testing	Only for isolated perfused lung	(+)	+	(+)
Inflammatory response	BAL total neutrophil counts	BALF-cytospin, Wright-Giemsa staining	Neutrophil number and percentage	+	+	+
	Lung MPO activity	ELISA kits or colorimetric assay	Cell-free BALF or whole lung homogenates	+	+	+
	Concentrations of cytokines	qRT-PCR or ELISA kits	mRNA or protein expression	+	(+)	+
Physiological dysfunction	Hypoxemia	Under machine testing	Equipment limits	+	+	+
	Increased alveolar-arterial oxygen difference			+	+	+

Notes: *LPS* lipopolysaccharide, *VILI* ventilator-induced lung injury, *BALF* broncho-alveolar lavage fluid, *EBD* Evans blue dye, *MPO* myeloperoxidase, *RT-PCR* reverse transcriptase polymerase chain reaction, *H&E* Hematoxylin and eosin, *ELISA* enzyme-linked immunosorbent assay

+, the criterion was present in virtually all studies using this model

(+) the criterion was present in the majority of studies using this model

used to induce ALI in animal models in preclinical studies, but there are some differences between these two delivery approaches. The former is pulmonary administration, and the ALI model reveals how the alveolar epithelium structure in the lungs is injured, including by polymorphonuclear (PMN) cell infiltration in intra-alveolar areas, diffuse alveolar edema, and mild changes in epithelial permeability. Use of i.v. delivery shows how the vascular endothelium structure in the lungs is injured, such as via PMN cell accumulation in capillaries and the interstitium with mild infiltration in intra-alveolar areas, presence of protein-rich alveolar edema, and mild changes in epithelial permeability. Just like in human patients, i.t. and i.v. delivery in animal models mimic direct and indirect insult, respectively. However, these animal models usually heal with few areas of fibrosis remaining, so investigators should adjust the time points in

preclinical studies, especially in therapeutic research (Lopes-Pacheco et al. 2019).

3 Stem Cell Therapy for ALI

Cell therapies are new potential treatments that aim to repair injured tissue and mitigate inflammation via regeneration by virtue of their multipotency as well as the release of and regulation by their soluble bioactive factors. In recent years, preclinical studies have shown the potential of cell therapies in treating lung diseases and critical illness, and they are likely to provide novel therapeutic candidates for general ARDS patients. In this context, our group has previously established an aerosol-based cell therapy using AECs for the treatment of ALI models, both in vivo and in vitro. The results indicated that AEC delivery remarkably accelerated the repair and regeneration of the respiratory airway (Kardia et al. 2017, 2018). Currently, adult stem cells have been regarded as a promising approach for ALI because of their ability to alleviate the major pathologies underlying ALI (Zhu et al. 2013). Stem cell therapy for ALI disease is recognized as a promising option not only for controlling symptoms but also for its potential benefit as a curative treatment regimen.

3.1 Cell Therapy Using MSCs

To date, MSCs may offer the best choice for clinical trials due to their multi-lineage differentiation capability, potent ability to modulate the inflammation process and immune system, diverse sources, ease of harvesting, and extensive preclinical studies (Kim and Park 2017). MSCs are non-hematopoietic multipotent stem cells derived from a variety of tissues such as bone marrow, adipose tissue, umbilical cord, placenta, dental pulp of deciduous baby teeth, menstrual blood, and several organs including the liver, spleen, and lung (Samsonraj et al. 2017). In vitro functional studies indicate multiple physiological roles of MSCs related to their heterogeneity and tissue location of origin (Sacchetti et al. 2016; Klimczak and Kozlowska 2016; Heo et al. 2016). The International Society of Cellular Therapy has defined MSCs as cells having these criteria: (1) They adhere to a plastic surface under standard tissue culture conditions; (2) they express certain cell surface markers, such as CD73, CD90, and CD105, but they must not express other markers, including CD45, CD34, CD14 or CD11b, CD79a, CD19, and HLA-DR; and (3) they are able to differentiate into osteoblasts, adipocytes, and chondroblasts under standard in vitro conditions (Dominici et al. 2006). Numerous studies have demonstrated the therapeutic potential of MSCs in multiple diseases, especially for tissue injury and degenerative and immunological diseases.

Halim et al. (Halim et al. 2019) previously investigated the effects of MSC treatment on asthma-related airway inflammation via aerosolization delivery, and the results demonstrated that MSC treatment relieved airway inflammation and reversed airway remodeling. Additionally, MSCs likely are able to elude clearance by the host immune system through a variety of mechanisms, including low expression of MHC I and II proteins and lack of the T-cell costimulatory molecules, CD80 and CD86; thus, they often are referred to as being ‘immuno-privileged’ (Lee et al. 2011). Past studies provide a powerful basis for exploring innovative approaches for the treatment of inflammatory diseases.

Several pilot clinical trials were conducted by research institutes or hospitals from all over the world, and they can be tracked on ‘clinical trial.gov’ (Table 3). The aim of most of the clinical trials was to assess the safety and efficiency of MSCs in patients with ALI/ARDS. Only three early-stage clinical trials have been completed with updated results, and they demonstrated that one dose of MSCs with intravenous delivery was safe for moderate to severe ARDS patients (Zheng et al. 2014; Wilson et al. 2015; Matthay et al. 2019). However, there are multiple challenges for evaluation of treatment efficiency, as dosage, time interval, delivery route, and illness severity must be considered and compared between MSC-treated and placebo groups. Currently, the optimum therapeutic dosage of MSCs

Table 3 Clinical trials of MSC-based therapies for ALI/ARDS patients

No.	ID	Phase	Treatment	Intervention	Enrollment	Follow-up	Status	Results	Country	References
1	NCT01902082	I	hAD-MSCs	1 million cells/kg, single dose, i.v.	12 (6/6)	28 days	Completed	MSC administration is safe and well tolerated, but significant difference on clinical outcome was weak	China	Zheng et al. (2014)
2	NCT01775774	I	hBM-MSCs	Dose-escalation: 1/5/10 million cells/kg, single dose, i.v.	9 (3/3/3)	12 months	Completed	All MSC dose levels were well tolerated, with no infusion-related adverse events	USA	Wilson et al. (2015)
3	NCT02097641	II a	hBM-MSCs	10 million cells/kg, single dose, i.v.	60 (40/20)	12 months	Completed	A trend for improvement in oxygenation index was observed in the MSC group, but it was not significant.	USA	Matthay et al. (2019) Extension of NCT01775774
4	NCT02804945	II	hBM-MSCs	3 million cells/kg, single dose, i.v.	20	60 days	Completed	No results posted	USA	No reference available
5	NCT02611609	I/II	MultiStem	Low/high dose, no details	36	12 months	Completed	No results posted	USA/UK	No reference available
6	NCT03608592	Not applicable	hUC-MSCs	1 million cells/kg, single dose, i.v.	26	60 days	Recruiting	No results posted	China	No reference available
7	NCT02444455	I/II	hUC-MSCs	0.5 million/kg, once daily for 3 days. i.v.	20	14 days	Unknown	No results posted	China	No reference available
8	NCT02095444	I/II	hMens-MSCs	10 million cells/kg, twice a week for 2 weeks, i.v.	20	14 days	Unknown	No results posted	China	No reference available
9	NCT02112500	II	hBM-MSCs	i.v., no details	10	28 days	Unknown	No results posted	Korea	No reference available
10	NCT03042143	I/II	hUC-MSCs	Dose-escalation: 100/200/400 million cells/patient, single dose, i.v.	75	28 days	Recruiting	No results posted	UK	No reference available
11	NCT03807804	II	hBM-MSCs (MultiStem HLCM051)	900 million cells/patient, single dose, i.v.	30	28 days	Recruiting	No results posted	Japan	No reference available

(continued)

Table 3 (continued)

No.	ID	Phase	Treatment	Intervention	Enrollment	Follow-up	Status	Results	Country	References
12	NCT02215811	I	hBM-MSCs	Not reported	10	12 months	Unknown	No results posted	Sweden	No reference available
13	NCT03552848	Not applicable	hUC-MSCs	1 million cells/kg, once every 4 days for 4 times, i.v.	60 (30/30)	24 months	Recruiting	No results posted	China	No reference available
14	NCT03818854	II b	hBM-MSCs	10 million cells/kg, single dose, i.v.	120 (60/60)	60 days	Not yet recruiting	No results posted	USA	Extension of NCT01775774 & NCT02097,641

All information was taken from: https://clinicaltrials.gov/

Notes: *MSCs* mesenchymal stem cells, *BM* bone marrow-derived, *UC* umbilical cord-derived, *AD* adipose-derived, *Mens* menstrual blood-derived, *h* human, *i.v.* intravenous

for treating lung diseases is unknown. In addition, and perhaps more importantly, we do not know whether it is necessary to deliver multiple doses of MSCs to treat advanced ALI animal models or ARDS patients.

Although the precise therapeutic mechanisms by which MSCs alleviate ALI remain unclear, a number of important insights from recent preclinical studies have emerged (Fig. 2), and they include but are not limited to cell-to-cell interactions and secretion of soluble factors, such as growth factors, matrix proteins, cytokines and extracellular vesicles, as well as through mitochondrial transfer (Lee et al. 2019; Lopes-Pacheco et al. 2019; Zhu et al. 2013; Abraham and Krasnodembskaya 2019). MSCs have been proven to play crucial roles in anti-inflammatory and anti-apoptotic activities, to facilitate epithelial and endothelial cell restoration, and to increase microbial and alveolar fluid clearance, resulting in the improvement of lung and distal organ injury as well as survival (Lopes-Pacheco et al. 2019; Xiang et al. 2017; Pedrazza et al. 2017; Morrison et al. 2017; Ren et al. 2018).

In support of these findings, Halim et al. (Halim et al. 2018) demonstrated that MSC-secreted proteins facilitated airway epithelial repair by stimulating the regenerative ability and endogenous reparation of lung cells, and most of proteins were extracellular proteins. In addition, MSCs have been demonstrated to alleviate LPS-induced ALI through downregulation of miR-142a-5p, which mediates autophagy of pulmonary endothelial cells by increasing Beclin-1 protein (Zhou and You 2016). Additionally, the NF-κB, MAPK, and STAT3 signaling pathways are all thought to be involved in the effects of MSC treatment in the ALI animal model, but more studies are needed to elucidate the therapeutic mechanism. So far, our group has already explored the feasibility of cell therapy both in chronic (Halim et al. 2019) and acute lung disease (Kardia et al. 2018); the results were consistent with each other, which showed

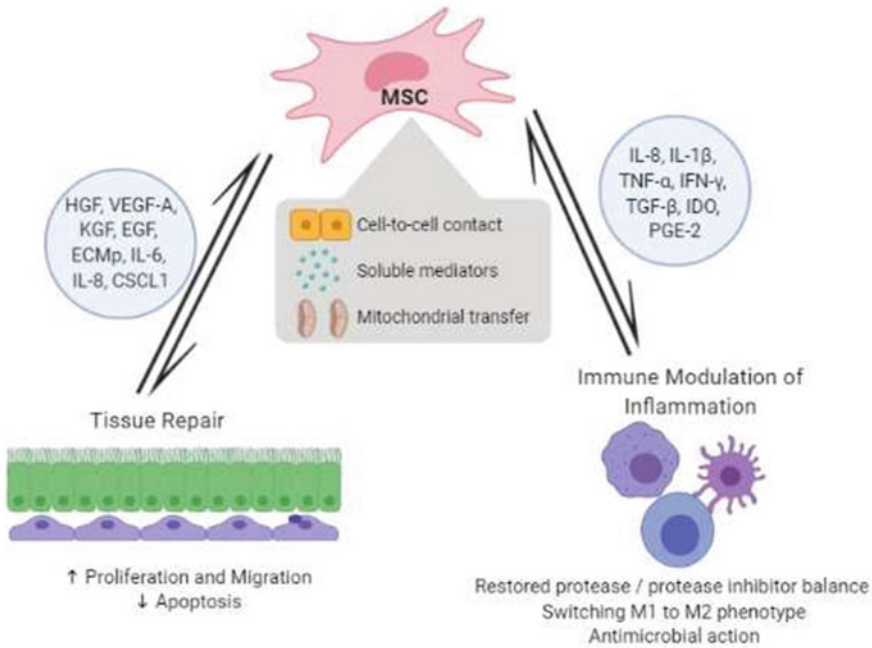

Fig. 2 Mechanisms underlying the modulation of inflammation and lung tissue repair by MSCs in ALI. MSCs have been proven to facilitate tissue repair and relieve inflammation by cell-to-cell contact, mitochondrial transfer, and paracrine or endocrine soluble factors, including growth factors, anti-inflammatory cytokines, and chemokines, as well as EVs

that MSCs or AECs reduced inflammation of the lung and airways and facilitated lung regeneration. In summary, all of these studies provided essential knowledge and data to support the therapeutic potential of MSCs to treat ALI conditions.

Nonetheless, cell-based therapy poses the risk of occlusion in microvasculature and unregulated growth in vivo. Among these concerns, the first is the risk of tumor formation. Additionally, extensive ex-vivo expansion are required for sufficient cell numbers in clinical protocols. Controversies about the stability of human derived MSCs (hMSCs) highlight the need to address hMSC stability in long-term cultures before use in clinical treatment (Bernardo et al. 2007; Meza-Zepeda et al. 2008; Rosland et al. 2009). A numbers of studies indicated that hMSCs may contribute to cancer development and progression either by acting as cancer-initiating cells or through interactions with stromal elements (Herberts et al. 2011). Lee and Hong (2017) demonstrated that MSCs have the ability to accelerate tumor growth due to their ability to migrate and home to the tumor site and alter its microenvironment, and they also can produce cytokines that stimulate tumor growth. However, although MSCs have the ability to induce tumor growth, there is no evidence from MSC clinical trials showing the involvement of MSCs in tumor development. Further research is needed before MSCs can be considered as a safe candidate for clinical treatment in patients.

Another issue related to MSC use is dose optimization in terms of number of cells in a single dose for testing in both preclinical and clinical experiments. High doses of MSCs are associated with several safety concerns; for example, a high dose by i.v. delivery could induce pulmonary embolism. Finally, MSCs are live cells, so particular care must be taken in their storage and transportation. DMSO is required as a preservative for MSC cryopreservation, and the process of cryopreserving and thawing reduce the viability of MSCs, which could have an adverse effect on their therapeutic efficacy in patients (Matthay et al. 2019). In view of these issues, there is an urgent need to find a safer cell-free therapeutic approach. It is widely accepted that the paracrine effects of MSCs are due mainly to mediation by extracellular vesicles (EVs) secretion, so the therapeutic potential of MSC-derived EVs is being actively explored as an alternative approach to MSC-based treatments.

3.2 MSC-EVs as a Potential Therapeutic for ALI

Initially, the therapeutic effects of MSCs were thought to derive from their engraftment in the injury site and regeneration afterwards. However, subsequent experimental evidence demonstrated that most MSCs administered get trapped in capillary networks and are transient in injury sites, which indicates that engraftment plays little role in therapeutic action (Eggenhofer et al. 2014). Subsequent studies demonstrated that the therapeutic properties of MSCs are derived from soluble factors with paracrine or endocrine effects, including growth factors, anti-inflammatory cytokines, and antimicrobial peptides, which can facilitate alveolar epithelial proliferation and stabilize the alveolar-capillary barrier, regulate the inflammatory microenvironment, and enhance alveolar fluid clearance and decrease infection (Lee et al. 2011). These findings provided a sufficient theoretical basis for the usage of novel cell-free therapies. MSC-EVs would be one of the most compelling alternatives for cell-free therapy because of their lower risk of allogenic immune rejection, accessible preservation, and higher stability compared with MSCs. In addition, EVs can carry micro and messenger RNAs as well as lipids, proteins, and even organelles, which can be used to regulate the behavior of target cells and shift gene expression (Yáñez-Mó et al. 2015). EVs also can bypass the blood-brain barrier by transcytosis through the endothelial layers to deliver cargo biomolecules to the brain parenchyma (Chen et al. 2016). The therapeutic application of MSC-EVs remains promising, and recent studies have underlined the new potential role of EVs as a paracrine of endocrine vehicle to deliver multiple soluble factors with a similar phenotype as the parent cell (Zhu et al. 2014).

Additionally, compared to their parent cells, EVs can be safely stored without losing function.

EVs were first clearly described by Pan and Johnstone (1983). Initially, EVs were thought to be a disposal mechanism by which cells eliminate unwanted proteins and other molecules. Subsequent research showed that EV secretions are important mediators of cell-to-cell communication that is involved in normal physiological process but also plays a crucial role in the development and progression of diseases. EVs are classified based on their cellular origin, biological function, and biogenesis, and the three main classes recognized currently are exosomes, microvesicles, and apoptotic bodies (Table 4) (El Andaloussi et al. 2013). According to the guidelines from the International Society for Extracellular Vesicles' minimal information for studies of extracellular vesicles 2018 (MISEV2018), EVs can be characterized by four distinct aspects (Théry et al. 2018). The first is quantification of EVs, both in terms of number of cell sources and the amount of EVs from a given number of cells; measurements can include the total levels of protein, lipids, or RNA. This aspect suggests that it would be informative to analyze at least one membrane bound (CD63, CD9, or CD81) and one cytosolic protein (TSG101 or ALIX) in EVs. The secondly aspect is the protein composition, specific markers in proteins, and non-protein components of EVs, and these can be analyzed using Western blotting or PCR (Hartjes et al. 2019). Third, single vesicle analysis of EVs can be conducted using visualization techniques such as transmission electron microscopy (Shao et al. 2018). Finally, other EV-associated components can be evaluated by topology analysis.

Importantly, Ratajczak et al. (2012) reported that EVs secreted by stem cells contributed to their maintenance and plasticity; in other words, stem cell-derived EVs play a critical role in tissue regeneration after injury. For example, EVs from MSCs have been used to stimulate tissue repair following cardiovascular (Lai et al. 2011), kidney (Bruno et al. 2016; Song et al. 2017) and lung (Lee et al. 2011; Hayes et al. 2012; Monsel et al. 2016) injury. On the other hand, the effect of EVs on regulation of the immune response depends on the status of particular immune cells, as they might trigger adaptive immune responses or suppress inflammation in a tolerogenic manner (Robbins and Morelli 2014). Such wide-raging cellular and biological functions indicate that MSC-EVs, in virtue of their pleiotropic signaling, may have innate therapeutic potential for regenerative medicine and immunotherapy. Moreover, Phinney et al. (2015) reported that there are functionally active mitochondria and numerous miRNAs in MSC-EVs. This is an important finding because ALI always clinically results in multiple organ dysfunction syndrome, which is

Table 4 Classification and characterization of EVs

Types	Origin	Size	Content	Markers
Exosomes	Endolysosomal pathway; intraluminal budding of multivesicular bodies and fusion of multivesicular body with cell membrane	40–120 nm	mRNA, miRNA, and other non-coding RNAs; cytoplasmic and membrane proteins including receptors and MHC molecules	Tetraspanins (TSPAN29 and TSPAN30), ESCRT components, PDCD61P, TSG101, flotillin, MFGE8
Microvesicles	Cell surface; outward budding of cell membrane	50–1,000 nm	mRNA, miRNA, non-coding RNAs, cytoplasmic proteins, and membrane proteins, including receptors	Integrins, selectins, CD40 ligand
Apoptotic bodies	Cell surface; outward blebbing of apoptotic cell membrane	500–2000 nm	Nuclear fractions, cell organelles	Extensive amounts of phosphatidylserine

Notes: *ESCRT* endosomal sorting complex required for transport, *MFGE8* milk fat globule-EGF factor 8 protein, *PDCD61P* programmed cell death 6 interacting protein (also known as ALIX), *TSG101* tumor susceptibility gene 101 protein, *TSPAN29* tetraspanin 29

associated with mitochondrial dysfunction. Therefore, mitochondria-targeted strategies are increasingly being explored as a promising therapeutic approach for treating lung injury, and MSC-EV-mediated mitochondria transfer is one of the most exciting among them (Agrawal and Mabalirajan 2015).

Although preclinical studies of the therapeutic use of MSCs-EVs in ALI are still in their infancy, MSCs-EVs are thought to be as powerful as their parent cells in promoting remission in ALI and other inflammatory lung disease models because they pass their cargo to recipient cells, thus facilitating the therapeutic benefits. The application potential of EVs versus intact live cells is significant because: (1) they have no iatrogenic tumor risk because of their non-self-replicating property; (2) they can be stored at −80 °C without DMSO and without loss of biological activity; (3) they offer potential for multiple doses or a higher single dose without significantly affecting the patient's hemodynamic or respiratory variables; and (4) they do not express MHC antigens so they can be used for allogeneic transplantation. However, utilization of MSC-EVs will require large-scale production and standardization, which pose issues concerning identification, characterization, and quantification.

3.3 Routes of Cell Delivery and Their Therapeutic Impacts

The therapeutic benefits of MSCs for ALI summarized all relevant articles from 2007 to 2019 for both natural and modified/preconditioned MSCs, and it demonstrated that many ALI models involved LPS by i.t. and i.v. challenge (Lopes-Pacheco et al. 2019). The preclinical studies of MSCs in ALI used various delivery approaches (systemic or local), diverse doses, multiple origins (bone marrow, umbilical cord, menstrual blood, adipose, or other tissues), and different schedules of administration (before or after challenge). However, bone marrow and umbilical cord are the more common sources of MSCs, and umbilical cord-derived MSCs are currently most popular for clinical application due to their accessibility and lack of ethical concerns (Li et al. 2012; Sun et al. 2011).

Several studies demonstrated the therapeutic efficacy and mechanism of action of human MSCs in a mouse ALI model induced by i.t. administration of LPS; i.v. delivery at 4 h (Zhang et al. 2018; He et al. 2015; Xu et al. 2018; Liu et al. 2018) and 6 h (Ren et al. 2018) after LPS challenge; or i.t. delivery at 4 h post-LPS challenge (Wang et al. 2018; Ionescu et al. 2012). In these studies, MSCs reduced alveolar inflammation and edema by decreasing the influx of inflammatory cells and total protein levels in the endotoxin-damaged alveolus. In addition, the therapeutic effects of the MSCs were comparable regardless of route of administration. Zhu et al. (2014) used the endotoxin-induced ALI mouse model to explore the therapeutic potential of MSC-EVs in ALI and reported that MSC-EVs decreased the influx of total inflammatory cells into the lung by 36% and the influx of neutrophils by 73%. Similarly, in another mouse model of hypoxia-induced pulmonary hypertension, i.v. injection of MSCs-EVs resulted in delayed pulmonary influx of macrophages and reduced production of pro-inflammatory mediators compared to injection of mouse lung fibroblast-derived EVs (Lee et al. 2012).

MSC administration has been performed via either systemic or local routes in experimental models. Systemic administration (e.g., i.v.) is readily available in clinical practice and provides wide distribution throughout the whole body, but it also results in cell waste along the route. In contrast, local administration (e.g., i.t.) delivers cells to the lung directly, so this is the more straightforward route for treating lung disease. However, i.t. intubation is a more difficult technique, especially for small animals such as mice and rats, whereas the i.v. route is more easily accessible for animals. Although the pulmonary first-pass effect has been detected with i.v. administration (Fischer et al. 2009), which results from cell retention in the lung, this effect may be beneficial for lung repair.

4 Conclusions

In conclusion, ALI remains a severe clinical condition with high morbidity and mortality, both for adults and children, but no effective pharmacotherapy exists. However, MSC-EV therapy is likely to provide a promising option not only for controlling symptoms but also for its potential benefit as a curative treatment regimen. EVs can be readily isolated from MSCs from various sources, and MSC-EVs have shown prominent therapeutic benefits in a range of ALI animal models. MSC-EVs also are considered to be non-immunogenic and break through the blood-brain barrier, so the therapeutic potency of MSC-EVs may be even better compared with their parental cells.

However, the dose, route, and time points of MSC-EV treatments vary substantially based on different preclinical animal studies, and the optimal treatment remains to be determined. Among the various delivery routes, intratracheal instillation seems to be the most straightforward approach to enhancing bacteria clearance, but for practical reasons it may not be feasible to instill MSCs or MSC-EVs for ALI patients (e.g., those who are hypoxemic). Thus, we need to balance efficiency and utility. Intratracheal intubation is the key technique for the pulmonary-induced ALI animal model, and it must be verified before real animal experiments begin. Moreover, many issues need to be addressed before translation of MSC-EVs to clinical trials, including the standardization of MSC-EVs collection, appropriate assessments for MSC-EVs in ALI, and whether we need to extract one or two components from EVs. Nevertheless, MSC-EVs have great therapeutic potential for treating ALI, and this cell-free therapy should be studied further.

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COVID-19 and Mesenchymal Stem Cell Treatment; Mystery or Not

Tunc Akkoc

Abstract

On December 31, 2019, novel SARS-CoV2 spread from Wuhan China to more than 200 territories around world and the World Health Organization declared a COVID-19 pandemic on January 30, 2020. At this time there is no particular therapy, drug or vaccine available to deal with COVID-19. Today actual data indicates that about 17% of closed COVID-19 cases died. Health care professionals, ministry of health in countries and the public are trying to read the runes to see when the COVID-19 pandemic will be over. Although mild cases of COVID-19 can be controlled with antiviral, anti-inflammatory and immunomodulatory treatment, severe cases may need intensive care unit support and ventilation. Cytokine storms cause high inflammatory responses and pneumonia in severe cases. Mesenchymal stem cells are immunomodulatory and they have regenerative capacity. In this sense, mesenchymal stem cells may improve the patient's clinical and immunological response to COVID-19.

Keywords

COVID-19 · Cytokine storm · Mesenchymal stem cells · Multiorgan failure · SARS CoV-2

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Abbreviations

ACE2	angiotensin-converting enzyme 2
ARDS	acute respiratory distress syndrome
CLR	C-type lectin receptor
CoV	Corona viruses
COVID-19	Coronavirus Disease 2019
CP	convalescent plasma
CRP	C-reactive protein
CRS	cytokine release syndrome
CT	Chest computerized tomography
GGOs	ground glass opacities
HCQ	Hydroxychloroquine
HIV	human immunodeficient virus
Ig	Immunoglobulin
IL	Interleukin
IP-10 or CXCL10	human interferon-inducible protein 10
MCP-1	monocyte chemoattractant protein-1
MERS-CoV	middle East respiratory syndrome-coronavirus
MIP-lα	macrophage inflammatory protein-1 alpha
MOD	multiorgan dysfunction
N	nucleocapsid protein
NOD	Nod-like receptor
PAMPs	pathogen associated molecular patterns
Rt-PCR	reverse transcriptase-polymerase chain reaction

S	spike protein
SARS-CoV	severe acute respiratory syndrome coronavirus
TNF-α	tumor necrosis factor alpha

1 Introduction

As of May 9, 2020, 4,032,763 million cases of Coronavirus disease 2019 (COVID-19) have been reported worldwide. A total of 212 countries and territories are affected around the world. Within closed cases, about 1,399,718 patients recovered (83%) and 276,677 died (17%) (worldometers.info). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a novel coronavirus, is the responsible causative virus for the infection and it spread worldwide dramatically in a short time. On January 30, 2020, the World Health Organization (WHO) declared the outbreak to be a Public Health Emergency of International Concern (PHEIC). Subsequently, on March 11th, it was declared a pandemic as it had spread to 113 countries (who.int).

COVID-19 shows complexity with different clinical outcomes. Patients may remain asymptomatic or show mild to severe symptoms up to pneumonia or not. The most important symptoms are fever, fatigue and dry cough. Other accompanying symptoms include vomiting, nausea, dyspnea, chest tightness, myalgia and diarrhea (Dong et al. 2020). With findings of viral pneumonia images and multiple bilateral ground-glass opacities on chest computerized tomography (CT) scans, mechanical ventilation and intensive care unit (ICU) support may be needed. In the advanced stage with cytokine storm and lymphopenia, multiorgan and systemic clinical manifestations such as sepsis, septic shock, and multiorgan dysfunction (MOD) syndrome and acute respiratory distress syndrome (ARDS) are an inevitable ending (Wu and McGoogan 2020; Wang et al. 2020a). Still there is no specialized therapeutic options for SARS-CoV-2 and new innovations are urgently required. Most of the patients benefit clinically from many known drugs which are used commonly as antiviral, immunomodulator and anti-inflammatory agents (Kruse 2020). But we still need a way to understand the viral properties and specific target points to stop this SARS-CoV-2 s from entering tissue, replicating in cells and surviving in the human body.

Currently, there is no defined antiviral treatment option for active COVID-19 and also specific vaccine development will take more than 6 months looking on the bright side. Recent studies of cellular therapies with mesenchymal stem cells for COVID-19 indicate good prognosis for patients who are in the middle of cytokine storm and require mechanical ventilation (Bari et al. 2020). This review focuses on recent therapeutic approaches for COVID-19 and mesenchymal stem cells as an probable option.

2 Coronaviruses

The coronavirus family belongs to Nidovirales and involves positive sense, enveloped, extraordinarily large single-stranded RNA viruses 26–32 kilobases in length and they are characterized by club-like spikes that project from their surface (Fehr and Perlman 2015). The origin of CoVs displays differences such as birds, livestock, and also mammals like camels, bats, palm civets, mice, dogs and cats. Phylogenetically, the Coronaviridae family further divides into two subfamilies of Letovirinae and Orthocoronavirinae.

There are four *Orthocoronavirinae* genera including alpha-, beta-, gamma- and deltacoronavisuses. Alpha- and betacoronaviruses transmit disease only in mammals, whereas gamma- and deltacoronaviruses infect avian species and sometimes mammals (Cui et al. 2019). There are seven coronavirus species that cause disease in humans. Alphacoronaviruses (229E and NL63) and betacoronaviruses (OC43 and HKU1) infect humans and cause the common cold with mild symptoms (Corman et al. 2018). Other CoVs (SARS-CoV, Middle East Respiratory Syndrome (MERS) CoV and SARS-CoV2) are all betacoronaviruses and a major threat for human health. SARS-CoV was responsible for

the first CoVs outbreak in China from November 2002 to January 2004 (Zhong et al. 2003). SARS-CoV first originated in bats and intermediate transmission was suggested to occur via bats to palm civet (Guan et al. 2003). Although viral transmission from animals to humans is still debated, direct contact with the intermediary host might be a possible route. Once SARS-CoV is transmitted to humans then nosocomial transmission is defined from person to person (Guan et al. 2003). MERS-CoV was responsible for the second outbreak of CoVs in 2012 in Saudi Arabia and remained regional. The origin of MERS-CoV is bats and the intermediate host was the dromedary camel in the Arabian Peninsula (Chowell et al. 2015). Nosocomial transmission is main transmission route for this virus. SARS-CoV and MERS-CoV induce life-threatening severe pneumonia in patients (Wang et al. 2018).

SARS-CoV-2 was described recently as the seventh Coronavirus that infects human and is globally referred as novel-HCoV-19 (Zhou et al. 2020). The first data about human infection caused by SARS-CoV-2 emerged in Wuhan, Hubei province, China in December 2019. HcoV-19 was isolated and the genomic structure was identified in China between 7 and 10 Jan (who.int) (Virological.org.). The clinical picture of patients infected with SARS-CoV-2 substantially resembles viral pneumonia and the disease was named as Coronavirus Disease 2019 (COVID-19) (Virological.org.). Although viral transmission history from animals to human is still a mystery, early cases were reported around Hunan Seafood market where several wild animals such as bats are on sale as well as aquatic animals. After first transmission of the virus into humans, rapid transmission occurred between humans via droplets, contact and fomites which further leads to pneumonia diagnosed with CT scan or chest X-ray (Chan et al. 2020). The most common symptoms of COVID-19 include fever, cough, and fatigue. The incubation period is approximately 3–7 days. One of the prominent features of SARS-CoV-2 is more efficient tendency to infect the human lung and higher duplication time than SARS-CoV. Indeed SARS-CoV-2 replicates 3.20-fold faster than SARS-CoV in infected lung tissue (Chu et al. 2020). Genetically SARS-CoV-2 is constructed on four structural proteins as spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins. Spread of the virus is accomplished by high affinity of S proteins to angiotensin-converting enzyme 2 (ACE2) receptors that are expressed in human organs, principally in lung alveolar epithelial cells and enterocytes of the small intestine (Peiris et al. 2003).

COVID-19 patients show diversity in clinical manifestations from asymptomatic to mild and severe symptoms accompanied with or without pneumonia. This wide range of clinical tableau may be related to viral load, advanced age, immune response of individuals and comorbid diseases such as hypertension, coronary disease, diabetes and renal disease (Zhang 2020). Based on recent research, varying laboratory results such as negative or positive SARS-CoV-2 real-time reverse transcriptase-polymerase chain reaction (RT-PCR), total cell blood count, C-reactive protein (CRP), serum amyloid A (SAA), and procalcitonin (PCT) and radiological images such as CT are present. Also, clinical and demographic data such as symptoms (fever, cough, sore throat, chest tightness, shortness of breath, loss of appetite, myalgia, fatigue, diarrhea, dizziness), age and gender show diversity within patients (Dong et al. 2020).

3 Immune Response to SARS-CoV-2

Actually, we have limited data about SARS-CoV-2 and the immune response of the COVID-19 patients so far. To understand the mechanism of the disease and immune reaction against SARS-CoV-2, it is better to analyze the pathway which the virus follows. Briefly, after reaching the airway epithelium, SARS-CoV-2 binds to epithelial ACE-2 with high tendency via its S protein. Viral entrance is the first step and this is followed by replication of virus with the help of the host genome. Then the virus is released from the host cell leaving damage. So, it continues to spread to

other cells that express ACE-2. During all these stages, the innate and adaptive immune responses are active against the virus. Some data basically reported increased total neutrophils, reduced lymphocyte count, enhanced serum IL-6 levels and increased CRP levels in the disease state (Wu et al. 2020; Huang et al. 2020). COVID-19 severity seems to be correlated with increased leucocytes, neutrophils, procalcitonin, serum ferritin and CRP and decreased lymphocyte and monocyte numbers (Qin et al. 2020). Although most of those values are in the normal range except for lymphocytes, serum ferritin, CRP, and procalcitonin they tend to increase compared to non-severe conditions. For lymphocytes, while the number of total $CD3^+CD4^+$T cell decreases in severe cases under normal range, the percent ratio of naïve Th cells ($CD3^+CD4^+CD45RA^+$) to Th cells increases in severe cases and percent ratio of $CD28^+$ Ts cells ($CD3^+CD8^+CD28^+$) to Th cells decreases in severe cases compared to non-severe subjects. Also, T reg levels are under normal range in severe cases (Qin et al. 2020). A healthy and coordinated immune response is essential to SARS-CoV-2 as for all viral infections, we have some limited input to understand the immune recovery process to this special virus. This data clearly shows the need for more studies in order to speculate further.

The innate immune response to RNA viruses such as SARS-CoV-2 starts with recognition of pathogen-associated molecular patterns (PAMPs) as viral genomic RNAs by pathogen recognition receptors (PRR) such as TLR8, TLR7, RIG-I-like receptors (RLR), NOD-like receptors (NOD), and C-type lectin-like receptor (CLRs) (Li et al. 2020). Activation of innate immune system with PAMP-PRR stimulates production of inflammatory cytokines such as tumor necrosis factor (TNF-a and IL-1) and chemokines like CCL2 and CXCL8. Besides these, the preventive type I IFNs response is suppressed in SARS-CoV-2 (Rokni et al. 2020).

Anti-viral immunoglobulin M (IgM), IgG, and IgA antibodies neutralize antibodies. Detection of SARS-CoV-2 specific neutralizing antibodies is helpful to confirm developed immune response against COVID-19. Indeed, sometimes the RT-PCR results for SARS-CoV-2 fall on the negative side, so it is important to clarify the virus has affected patients with antibody levels for epidemiological and surveillance studies. Recent data also declare that the first antibody specific to SARS-CoV-2 is IgA and it increases up into 3 weeks and persists about 38 days while IgM is still at moderate levels (Padoan et al. 2020). So mucosal immune responses, characterized first by the production of secretory IgA, are established, then systemic antibodies occur later (Zhao et al. 2020).

4 Current Therapeutic Approach

Many protocols have been recommended to cure COVID-19 from countries that are globally affected. Up to now, there is no conductive therapies available or protective vaccines. But nowadays therapies focus on treatments that already exist in the medical agenda which is specific to viral infections and pneumonia. So antiviral agents, immunomodulatory and anti-inflammatory agents are taken off the shelf. Also, other approaches like vaccines specific for SARS-CoV-2 for active immunization (Amanat and Krammer 2020), convalescent plasma (CP) for neutralizing the virus for passive immunization (Roback and Guarner 2020) and mesenchymal stem cell therapies to suppress cytokine storm and to prevent tissue damage and enhance alveolar tissue regeneration (Golchin et al. 2020) are still current issues.

Antiviral agents which are specific normally to treatment of human immunodeficient virus (HIV), hepatitis and flu symptoms are in the treatment protocol for COVID-19 patients. Among antivirals; lopinavir/ritonavir (Cao et al. 2020a), remdesivir (Wang et al. 2020b) and favipiravir (Khambholja and Asudani 2020) ameliorate the clinical circumstances of patients.

Clinical and laboratory observations of COVID-19 patients clearly show disturbed immune activity and increased inflammatory process, especially in the lungs. Current immunomodulatory and anti-inflammatory treatment approaches are also medicinal. Many trials have

been carried out about hydroxychloroquine (HCQ) as a treatment option for COVID-19 patients. A recent systemic review and meta-analysis (Sarma et al. 2020) showed some benefits like body temperature and cough days with HCQ but less improvement per case was observed for radiological progression of the lung. Also, days spent in hospital reduce with HCQ treatment (Gautret et al. 2020). The combination therapy of HCQ with azithromycin turned PCR positivity to negative and looks helpful and safe (Saleh et al. 2020). Many COVID-19 patients are highly affected by the cytokine storm and their progression worsens. Anticytokine therapy seems to be effective to control the inflammatory response by controlling the cytokine release syndrome (CRS). For this purpose, tocilizumab, an interleukin-6 (IL-6) receptor antagonist, was used for COVID-19 patients. Although some conflicting results were found (Radbel et al. 2020), tocilizumab will be useful in the early stages of COVID-19 and prevents the hyperactivation pathway, oxygen requirements, CT lesions, alleviates CRP levels and average hospitalization days (Xu et al. 2020).

5 Convalescent Plasma Therapy

German scientists in 1893 described serotherapy to treat patients who suffered from diphtheria. This is the first declared trial with plasma from animals immunized against diphtheria to cure patients stricken with diphtheria (Dunmire). Plasma therapy contains plasma from early actively immunized or convalescent patients. First person to person transferal of convalescent plasma therapy was used around 1917–1918 during the A/H1N1 Spanish flu (Luke et al. 2006) and about 100 years later from 2005–2015 during the A/H5N1 flu epidemics (Zhou et al. 2007), the 2009–2010 A/H1N12009p flu epidemics (Hung et al. 2011) and around 2013–2015 during the Ebola virus outbreak in West Africa (Burnouf and Seghatchian 2014). Since the outbreak of COVID-19 has rapidly spread globally, viral specific therapy protocols or medications cannot be found. But old-fashioned CP therapy turned the light on for severe COVID-19 patients. Until now there are numerous practices which have been performed and are encouraging. One of these is CP derived from convalescent patients and transfused to 10 severe patients who need maximal supportive in intensive care and also receive antiviral treatment. The results of therapy with CP indicates that it is well tolerated, clinical symptoms improved with increased oxyhemoglobin saturation within 3 days, increased absolute lymphocyte counts, normalized CRP levels and neutralized viremia (Duan et al. 2020). Another descriptive study also supports the effectiveness of CP therapy for COVID-19 (Ye et al. 2020). They transfused CP to 6 patients and 5 of them had obviated ground glass opacities (GGOs). Additionally, CP purified intravenous globulin (IVIg) also assists COVID-19. At the time of initiation of respiratory distress syndrome, high-dose intravenous globulin (IVIg) was used intravenously for 3 COVID-19 patients in China and resulted in convincing clinical and radiographic recovery (Cao et al. 2020b). In order to transfer CP infusion into practice, some important limitations should be considered. Both antibody and neutralization activity should be tested in convalescent patients' plasma. Previous to CP collection, host eligibility and donor qualifications must be debated. For safe use of CP in the SARS-CoV2 pandemic, we still need time to determine optimal dose and intervals for infusion with controlled clinical trials.

6 Vaccine

More than 100 centers around the world are focused on the urgent need for a vaccine against COVID-19 as a prophylactic and preventive approach (Gao et al. 2020) (Eyal et al. 2020). With the sequencing of SARS-CoV-2, different approaches have been used including small molecules targeting RNA polymerase, 3C-like protease, and RNA endonuclease. Among them, mRNA-127 (NCT04283461) is a synthetic strand of mRNA which encodes the viral spike protein was developed by Moderna and phase I trials are ongoing. The University of Oxford created a

non-replicating adenovirus vector and the genetic sequence of the S protein of SARS-CoV-2 in a vaccine named ChAdOx nCoV-19. This non-replicating adenoviral virus may also be safe for children and has also entered phase I/II clinical trials (NCT04324606). Other trials area bout stabilizing subunit vaccines and nanoparticle-based vaccines. It is believed that a vaccine target for COVID-19 would save many lives in this current pandemic. But indeed, we need time to develop a safe and effective vaccine based on bioethical issues and clinical trials and also risks and benefits should be considered.

7 Mesenchymal Stem Cells

It is now well known that SARS-CoV-2 induces a scary cytokine storm especially in the lungs with the early response of proinflammatory cytokines such as TNF-α, IL-6 and IL1 β. Also, other inflammatory cytokines like IL-2, IL-7, GSCF, IP10, MCP1 and MIP1α are involved in the process. Those cytokines lead to dramatic edema, air exchange dysfunction, acute respiratory distress syndrome, acute cardiac injury, secondary infection and catastrophic effects on tissues and organs which may lead to death (Huang et al. 2020).

MSCs are non-specialized multipotent cells that have self-renewal and differentiation capacity into diverse cell types such as adipose, chondrocyte and osteocyte cells. Mesenchymal stem cells (MSCs) are multipotent cells and can be isolated from several sources mainly from bone marrow, peripheral blood, adipose tissue (abdominal fat, bucal fat and infrapatellar fat pad), human placental tissue, umbilical cord, Wharton's jelly and dental tissues and further they have the ability to differentiate into various lineages of cell types (Al-Nbaheen et al. 2013). MSCs have high capacity to modulate immune responses. Basically, they suppress T cell proliferation and regulate the balance of Th1 (autoimmunity related)/Th2 (allergic disease related). On the other hand, they regulate the immune response with T regulatory cells and control antigen presentation of dendritic cells (Akkoc 2019; Duman et al. 2019; Genc et al. 2019). All of these modulatory effects are important to control cytokine storm disease. Further MSCs are involved in regenerative processes for damaged tissues and organs. Patients with COVID-19 pneumonia are faced with severely destroyed alveolar lung tissue and this leads to dysfunction of the lung which will end in death. After the intravenous transplantation of MSCs, cells accumulate in the lung and are activated because of the proinflammatory stimulus of the environment. Once MSCs are stimulated with proinflammatory cytokines, they become immunomodulatory cells and are further programmed to protect alveolar epithelial cells, prevent pulmonary fibrosis, and reverse lung dysfunction. However, currently, there are no approved MSC-based therapies for COVID-19 patients but there are some trials about MSC and COVID-19 ongoing.

Pathogenesis and current therapeutic approach. The role of MSCs are summarized

Prostaglandin E2 (PGE2), transforming growth factor (TGF)-β, indoleamine 2,3-dioxygenase (IDO), nitric oxide (NO), hepatocyte growth factor (HGF)

ClinicalTrials.gov Search Results 05/05/2020. Clinical trials conducted with MSC therapy for COVID-19

The Asian lineage avian influenza A (H7N9) virus, with outbreak reported between 2013–2017 in China, shares similar clinical outcomes like ARDS, lung failure, pneumonia and further multi-organ dysfunction in severe cases. The first clinical study related to MSC transplantation for H7N9 infected patients with ARDS ended up with lower mortality compared to the control group. In that study allogenic menstrual blood-derived MSCs were transplanted to 17 patients with H7N9-induced ARDS, while 44 patients with the same criteria were included as a control group. MSC therapy reduced CRP, procalcitonin, serum creatinine, and creatine kinase levels. Mortality seemed to be lower in the MSC treated group (17.6%) compared to controls (54.5%) (Chen et al. 2020). Regarding clinical observations, there are high similarities for ARDS and lung failure and further multi-organ dysfunctions between H7N9 and COVID-19.

Thereby, MSC-based cellular therapy may be an encouraging alternative for COVID-19 in severe cases.

It was shown that the administration of ACE-2 negative MSCs (free from COVID-19) improved functional outcomes in COVID-19 patients. Regarding that study, patients enrolled with COVID-19 pneumonia (Seven SARS-CoV-2^+patients) and received $1x10^6$ MSC cells per kilogram of weight in 100 ml saline intravenously. Two days after MSC transplantation they observed improved pulmonary functions and symptoms. MSC therapy lead to increased peripheral lymphocytes which normally decreased in severe cases. TNF-α and CRP are other signature markers of inflammation and they increased dramatically especially in severe COVID-19 cases. They showed clearly that MSCs reversed this and decreased TNF-α and CRP levels to the normal range. IL-10 is an important cytokine for immunomodulatory activity and suppresses the inflammatory response. MSC transplantation also enhances IL-10 levels and controls the cytokine storm. One of the attractive results is disappearance of overactivated cytokine-secreting immune cells of $CXCR3^+$ $CD4^+$ T cells, $CXCR3^+$ $CD8^+$ T cells and NK $CXCR3^+$. Additionally, $CD14^+$ $CD11c^+$ CD11bmid regulatory DC cell populations also increased dramatically (Leng et al. 2020). Similarly, Liang and colleagues transplanted 5×10^7 umbilical cord MSCs three times (every 3 days) intravenously to a 65-year-old female with COVID-19 pneumonia. After the second infusion, vital signs were improved, the trachea cannula was removed and serum bilirubin, C-reactive protein (CRP) and alanine transaminase/aspartate transaminase (ALT/AST) gradually reduced. Also, CT images improved (Bing et al. 2020). All these results indicate the safety and treatment options for MSC in COVID-19. Beside MSCs themselves, MSC-secretomes may also function in immunomodulation and silence the cytokine storm in COVID-19 disease (Bari et al. 2020). In this regard, two Chinese clinical trials recently appeared on http://www.clinicaltrials.gov (last access: 01/04/2020) investigating inhaled secretome for the treatment of COVID-19 pneumonia (NCT04276987) and its tolerance in healthy volunteers (NCT04313647).

As of May 6, 2020, 33 clinical trials are being conducted with MSCs therapy for COVID-19. All of them are summarized in. When we researched these trials, researchers chose different sources of MSCs such as Wharton's jelly, umbilical cord, adipose tissue, dental pulp and company-produced MSC. After completion of these studies, we will be able to obtain scientific results about the therapeutic intervention of MSC in COVID-19 disorder. Also, a lot of questions about therapy for COVID-19 with MSCs regarding the route of administration, dose, source of stem cells, safety of use and clinical outcomes, as well as ethical issues, will be clarified.

8 Conclusion

SARS CoV2 has spread to more than two hundred countries and territories. It has become a pandemic and affects people mostly with chronic disease, comorbidities and older age. Patients suffer from pneumonia which leads to ARDS and sepsis with multi organ dysfunction in severe cases. Although there are no confirmed treatment protocols, mild patients benefit from antiviral, immunomodulatory and anti-inflammatory therapies. But in severe cases, the situation worsens. Severe pneumonia and ARDS are real clinical pictures because of the inflammatory cytokine storm. After this, patients need intensive care unit support and ventilation. Recently, the literature showed that convalescent plasma infusion helps to control viremia. We also need remarkable time to develop a vaccine that is specific to COVID-19. Studies in recent years clearly showed the beneficial effect of mesenchymal stem cells for autoimmune and allergic disease, as well as regenerative and immunomodulatory impact. Since COVID-19 is noted for cytokine storm and high inflammation in lungs, MSC seems to be a treatment option. Although there are limited studies to that end, clinical and laboratory improvements with MSCs were declared. There are about 33 clinical trials ongoing to date. Additional studies about stem cell-based

therapies in larger groups of COVID-19 patients are needed to further validate this therapeutic intervention.

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PCDH19 Pathogenic Variants in Males: Expanding the Phenotypic Spectrum

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Abstract

Protocadherin-19 (*PCDH19*) pathogenic variants cause an infantile onset epilepsy syndrome called Girls Clustering Epilepsy due to the vast majority of affected individuals being female. This syndromic name was developed to foster early recognition and diagnosis in infancy. It has, however, sparked debate, as, there are rare males with postzygotic somatic, and therefore, mosaic, *PCDH19* pathogenic variants with similar clinical features to females. Conversely, "transmitting" males with germline inherited *PCDH19* variants are considered asymptomatic. To date, there has been no standardized neuropsychiatric assessment of males with *PCDH19* pathogenic variants. Here, we studied 15 males with *PCDH19* pathogenic variants (nine mosaic and six transmitting) aged 2 to 70 years. Our families completed a survey including standardized clinical assessments: Social Responsiveness Scale, Strengths and Difficulties Questionnaire, Behavior Rating Inventory of Executive Function, and Dimensional Obsessive-Compulsive Scale. We identified neuropsychiatric abnormalities in two males with germline *PCDH19* possibly pathogenic variants. One had a prior history of a severe encephalopathic illness, which may have been unrelated. We also describe a non-penetrant somatic mosaic male with mosaicism confirmed in blood, but not identified in skin fibroblasts. Our data suggest that transmitting hemizygous

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males are generally unaffected, in contrast to males with postzygotic somatic mosaic variants who show a similar neuropsychiatric profile to females who are naturally mosaic, due to X-chromosome inactivation. The penetrance of *PCDH19* pathogenic variants has been estimated to be 80%. Like females, not all mosaic males are affected. From our small sample, we estimate that males with mosaic *PCHD19* pathogenic variants have a penetrance of 85%. With these insights into the male phenotypic spectrum of *PCDH19* epilepsy, we propose the new term Clustering Epilepsy (CE). Both affected females and males typically present with infantile onset of clusters of seizures.

Keywords

Epilepsy · Mosaic · Neuropsychiatric · Pathogenic variant · PCDH19

Abbreviations

ADHD	attention-deficit hyperactivity disorder
ASD	autism spectrum disorder
BRIEF	Behavior Rating Inventory of Executive Functioning
CE	clustering epilepsy
DNA	deoxyribose nucleic acid
DOCS	Dimensional Obsessive-Compulsive Scale
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, 5th edition
EEG	electroencephalogram
EQ	Epilepsy Questionnaire
FIRES	febrile infection-related epilepsy syndrome
ID	intellectual disability
OCD	obsessive-compulsive disorder
PCDH19	protocadherin-19
SDQ	Strengths and Difficulties Questionnaire extended version
SRS-2	Social Responsiveness Scale, second edition

1 Introduction

Pathogenic variants in *PCDH19*, encoding Protocadherin-19, cause an infantile onset epilepsy syndrome called Girls Clustering Epilepsy, as it typically presents in girls with clusters of seizures. Affected females often have intellectual disability (ID), executive dysfunction, and a range of psychiatric disorders including autism spectrum disorder (ASD), obsessive-compulsive disorder (OCD), and attention-deficit hyperactivity disorder (ADHD) (Juberg and Hellman 1971; Kolc et al. 2019; Scheffer et al. 2008). Mosaicism describes an individual with two or more populations of cells and can develop at any point following fertilization (Vadlamudi et al. 2010). With regard to *PCDH19* epilepsy, all females are effectively mosaic due to inactivation of one of their two X chromosomes, resulting in some cells expressing wild-type *PCDH19* and some expressing variant *PCDH19*. Males are typically hemizygous having only one X chromosome, however, when they have a postzygotic variant of *PCDH19* they also become mosaic and are phenotypically similar to affected females. Twelve males with postzygotic somatic *PCDH19* variants (de Lange et al. 2017; Depienne et al. 2009; Liu et al. 2019; Perez et al. 2017; Terracciano et al. 2016; Thiffault et al. 2016) and one male with Klinefelter syndrome and a heterozygous *PCDH19* pathogenic variant (Romasko et al. 2018) have been identified with a similar phenotype to females. Although the reported clinical features of mosaic males are similar to affected females (Kolc et al. 2020), there has been no standardized neuropsychiatric assessment of males with *PCDH19* pathogenic variants.

Males who inherit a germline loss-of-function *PCDH19* variant are generally asymptomatic and referred to as "transmitting" males (Fabisiak and Erickson 1990; Juberg and Hellman 1971; Ryan et al. 1997; Scheffer et al. 2008). However, there have been four transmitting males reported with neuropsychiatric abnormalities (Van Harssel et al. 2013; Piton et al. 2011; Tarpey et al. 2009). One

male with High Functioning ASD (previously known as Asperger's syndrome), inherited his probably damaging missense variant from his asymptomatic mother (Van Harssel et al. 2013), another male with ASD had unknown inheritance of his probably damaging missense variant (Piton et al. 2011), and two males with ID (Tarpey et al. 2009). With such a small number of reported males, it is unclear if these clinical features are caused by the germline *PCDH19* variant. Here, we report the phenotypic profile of 15 mosaic and transmitting males with *PCDH19* pathogenic variants using standardized assessments conducted in conjunction with our PCDH19 survey (Kolc et al. 2020).

2 Method

2.1 Participants

Our 15 male participants were ascertained through the international PCDH19 survey (Kolc et al. 2020). This cohort comprised 9 mosaic males (#1, #2, #3, #4, #5, #6, #7, #8, #9), with an age range of 2 to 41 years (*Median* = 5.00, $M = 9.67$, $SD = 12.2$) and 6 transmitting males (#10, #11, #12, #13, #14, #15), with an age range of 16–70 years (*Median* = 46.00, $M = 44.0$, $SD = 21.5$).). Participants were included if they had a confirmed likely pathogenic *PCDH19* variant. Two transmitting males were recruited directly through the community (#10 & #11), while the remaining four transmitting males (#12, #13, #14, & #15) were ascertained as they were the fathers of affected females. English speaking participants were from Ireland (#1), Argentina (#2), Republic of Moldova (#3), United States (#4, #10, & #14), United Kingdom (#5), Israel (#8), Denmark (#9 & #13), Netherlands (#11), and Australia (#12). Three participants from Italy (#6, #7, & #15) spoke Italian and no English. Thirteen were Caucasian, with the remaining two Hispanic (#4) or Africana (#6). Variant pathogenicity was determined by gnomAD (http://gnomad.broadinstitute.org/) frequency and *in silico* assessment CADDv1.3, including PolyPhen2, GPP, MutPred, MutationAssessor, PROVEAN, and SIFT (Choi and Chan 2015; Kircher et al. 2014; Li et al. 2009; Reva et al. 2011; Traynelis et al. 2017; Vaser et al. 2016) through the web-based ANNOVAR (http://wannovar.wglab.org/).

2.2 PCDH19 Survey

The PCDH19 survey was developed and scored as previously described (Kolc et al. 2020) and incorporated the following: Social Responsiveness Scale, second edition (SRS-2) (Constantino and Gruber 2012) for the assessment of ASD-related symptoms, Strengths and Difficulties Questionnaire extended version (SDQ) (Goodman 1997) for the assessment of ADHD-related symptoms, Behavior Rating Inventory of Executive Functioning (BRIEF) (Gioia et al. 2000) for the assessment of executive dysfunction, and Dimensional Obsessive-Compulsive Scale (DOCS) (Abramowitz et al. 2010) for the assessment of OCD-related symptoms. Demographic, development, variant, medication, and seizure information was captured using the Epilepsy Questionnaire (EQ) (Kolc et al. 2020). Participants or parents responding on behalf of their child or adult child with ID were contacted to clarify or obtain additional information. Where possible, the treating clinician was also consulted to verify reported information. Italian translation of the EQ, survey scripts, and study material were performed and checked by either a professional translator or by an individual familiar with GCE and fluent in the relevant languages. Published authorized translations of the SRS-2, BRIEF, and DOCS were utilized. License agreements were obtained to reproduce assessments in an online format. Electronic informed consent was obtained from all participants or their parents or legal guardians in the case of minors or those with ID.

3 Results

3.1 Males with Mosaic PCDH19 Pathogenic Variants

Five of the nine mosaic males (#2, #3, #5, #6, #8) harbored novel *PCDH19* variants (Table 1). These included two nonsense (p.Glu574*; p.Tyr516*) and three missense (p.Arg198Pro, p.Asp230His, p.Asn340Lys) variants affecting the extracellular domain, which is a highly conserved region of the PCDH19 protein (Depienne et al. 2011). All five variants were predicted to be pathogenic or likely pathogenic by *in silico* assessment and comparison with ClinVar (Table 1 and Supplementary Table 1). The remaining variants were previously published and included: nonsense (1, individual #1), missense (2, individuals #7 and #9), and splice-site (1, individual #4) *PCDH19* pathogenic or likely pathogenic variants. Genetic segregation showed that the *PCDH19* variant arose *de novo* in eight males and the relevant information was not available for one individual (#8; Table 1).

Seizures presented in clusters for eight of the nine mosaic males. For the eight mosaic males with seizures, the median age at seizure onset was 10 months. One male (#8) experienced seizure onset at 8 years of age. This is the latest seizure onset recorded for an individual with PCDH19 CE. His seizures occurred in clusters for an average of 1 day, with approximately 5 seizures per day in a cluster, according to parent report. His seizures remain ongoing at 12 years of age. The mosaic nature of this variant was confirmed in saliva and predicted to be pathogenic.

One mosaic male (#9) had never had a seizure and no prior neuropsychiatric diagnoses. This male harbors a missense variant (c.1240G > A; p.Glu414Lys) that was transmitted to his daughter, who is affected (#28 in (Kolc et al. 2020)). We tested the blood DNA of this male and detected the *PCDH19* variant allele in approximately the same ratio as the wild-type allele, that is 50:50. The daughter of this male displayed the typical features associated with *PCDH19* pathogenic variants, with seizure onset at 8 months of age, normal early development, autistic features, and mild ID. This variant is recurrent and has been reported in a female with seizures between 14 months and 5 years of age, aggression, and ID (Liu et al. 2017). The variant was inherited from an asymptomatic mother and was predicted to be deleterious/likely pathogenic by three independent *in silico* tools (Liu et al. 2017).

3.2 Transmitting Males with Germline PCDH19 Pathogenic Variants

Six males with inherited *PCDH19* variants were included. Four scored in the normal range on all neuropsychiatric measures (Table 1). Two males had neuropsychiatric abnormalities. The first male (#11) was a 23-year-old with mild ID, ASD, and impulsive behavior. His parent reported first behavioural concerns at 12 months. He had no seizures. His parent and physician report that, despite his severe ASD, he has well-developed language skills. Our assessment revealed a global executive composite score that was greater than 99% of scores from the standardization sample of 18–29 year-olds, indicative of an executive functions deficit. Although all domains were affected, the shift domain was especially elevated. Such inflexibility and rigidity are common in ASD and would therefore be expected given his severe ASD. Scores on the SRS-2 supported the presence of ASD, with a total score in the moderate range. Elevation was also observed in the two DSM-5 equivalent domains; social communication and interaction and restricted interests and repetitive behavior. This male was reported to have a *de novo* frameshift variant (c.2341dup; p.I781Nfs*3). This recurrent variant has been previously reported in a female with refractory seizure clusters and attention-deficit features (Cappelletti et al. 2015; Marini et al. 2012).

The second male was 16 years old. The following medical information was gained from parental report. He was a healthy boy with normal development until 9.5 years of age when he developed febrile infection-related epilepsy syndrome

Table 1 Clinical characteristics of *PCDH19* males

	General		Seizures						Neuropsychiatric comorbidities									PCDH19 Variant				
Pt.	*Age*	*Zygosity*	*Onset*	*SE*	*Clusters*	*Isolated*	*Hosp.*	*ICU*	*ID*	*ED*	*EP*	*CoP*	*ADHD*	*PP*	*SD*	*ASD*	*OCD*	*cDNA; Protein*	*Type (loc)*	*Inheritance*	*Novel*	*In silico Prediction & ClinVar*
1	2.5y	Mosaic	22 m	–	+	–	Once	Yes	No	No	Av	Mild	Mild	Sev	Sev	No	NC	c.2656C > T; p. Arg886*	Non (CP)	*De novo*	–	Pathogenic (Depienne et al. 2011; Van Harssel et al. 2013)
2	2y	Mosaic	8 m	–	+	–	Twice	No	No	No	Av	Sev	Mild	Av	Mild	No	NC	c.1720G > T; p. Glu574*	Non (EC6)	*De novo*	+	Pathogenic
3	5y	Mosaic	5 m	–	+	+	5+	Yes	Sev	Sev	Sev	Mod	Mod	Sev	Sev	Sev	NC	c.593G > C; p. Arg198Pro	Miss (EC2)	*De novo*	+	Likely pathogenic
4	10y	Mosaic	11 m	–	+	–	5+	No	Sev	Sev	Av	Mild	Mod	Sev	Sev	Sev	NC	c.2147 + 2 T > C; p.?	(ex/Int1)	*De novo*	–	Pathogenic (Perez et al. 2017)
5	5y	Mosaic	8 m	–	+	–	5+	Yes	No	No	Av	Av	Mild	Mod	Sev	Mild	NC	c.688G > C; p. Asp230His	Miss (EC2)	*De novo*	+	Likely pathogenic
6	2.5y	Mosaic	5 m	–	+	–	5+	No	Mild	Mod	Av	Av	Sev	Mod	Sev	Sev	NC	c.1020 T > A; p. Asn340Lys	Miss (EC3)	*De novo*	+	Likely pathogenic
7	7y	Mosaic	10 m	–	+	–	5+	Yes	No	No	Av	Av	Av	Av	Mild	No	NC	c.1352C > T; p. Pro451Leu	Miss (EC4)	*De novo*	–	Likely pathogenic (Terracciano et al. 2016)
8	12y	Mosaic	96 m	–	+	+	5+	No	Bord	Mod	Mod	Av	Av	Sev	Sev	Mod	NC	c.1548C > A; p. Tyr516*	Non (EC5)	Unknown	+	Pathogenic
9	41y	Mosaic	NA	NA	NA	NA	NA	NA	No	No	NC	NC	NC	NC	NC	Mod	No	c.1240G > A; p. Glu414Lys	Miss (EC4)	*De novo*	–	Likely pathogenic (Liu et al. 2017)
10	16y	Hemi	114 m	–	+	+	Once	Yes	No	No	Mod	Av	Av	Mod	Av	Mild	NC	c.1672G > C; p. Asp558His	Miss (EC5)	Maternal	–	Likely pathogenic (Lindy et al. 2018)
11	23y	Hemi	NA	NA	NA	NA	NA	NA	Mild	Sev	NC	NC	NC	NC	NC	Mod	NC	c.2341dup; p. Ile781Asnfs*3	F/S (CP)	Maternal	–	Pathogenic (Cappelletti et al. 2015; Marini et al. 2012)
12	63y	Hemi	NA	NA	NA	NA	NA	NA	No	No	NC	NC	NC	NC	NC	No	No	c.1671C > G; p. Asn557Lys	Miss (EC5)	Unknown	–	Pathogenic (Dibbens et al. 2008; Hynes et al. 2010)
13	70y	Hemi	NA	NA	NA	NA	NA	NA	No	No	NC	NC	NC	NC	NC	No	[a]No	c.370G > A; p. Asp124Asn	Miss (EC1)	Maternal	–	Likely pathogenic (Liu et al. 2017)
14	41y	Hemi	NA	NA	NA	NA	NA	NA	No	No	NC	NC	NC	NC	NC	No	No	c.1683_1696del; p.Val562Thrfs*4	F/S (EC5)	Unknown	–	Pathogenic (Lindy et al. 2018)

(continued)

Table 1 (continued)

	General		Seizures						Neuropsychiatric comorbidities									PCDH19 Variant				
Pt.	*Age*	*Zygosity*	*Onset*	*SE*	*Clusters*	*Isolated*	*Hosp.*	*ICU*	*ID*	*ED*	*EP*	*CoP*	*ADHD*	*PP*	*SD*	*ASD*	*OCD*	*cDNA; Protein*	*Type (loc)*	*Inheritance*	*Novel*	*In silico Prediction & ClinVar*
15	51y	Hemi	NA	NA	NA	NA	NA	NA	No	No	NC	NC	NC	NC	NC	No	No	c.1463 T > A; p. Val488Asp	Miss (EC5)	Unknown	+	Likely pathogenic

Abbreviations: *ADHD* attention-deficit hyperactivity disorder, *ASD* autism spectrum disorder, *Av* average, *Bord* borderline, *CoP* conduct problems, *CP* cytoplasmic domain, *EC* extracellular domain, *ED* executive dysfunction, *EP* emotional problems, *Ex* exon, *F/S* frameshift, *Hemi* hemizygous, *Hosp.* hospitalizations, *ICU* Intensive Care Unit admissions, *ID* intellectual disability, *Int* intron, *isolated* single seizures, *loc* location, *m* months, *Miss* missense, *Mod* moderate, *NA* not applicable, *NC* not covered, *Non* nonsense, *NP* not provided, *OCD* obsessive compulsive disorder, *PP* peer problems, *Pt* participant, *SD* social deficits, *SE* status epilepticus, *Sev* severe, + present, − absent

[a]OCD score just below the clinical threshold (18 or higher)

(FIRES) (Van Baalen et al. 2010). Following a prodromal illness he presented with rapidly escalating tonic-clonic seizures requiring a phenobarbital induced coma for a month. Serial MRI scans showed progressive atrophy. Investigations for infectious and autoimmune etiologies were negative. Following the acute illness, he was left with motor and cognitive neurological sequelae, as well as pharmaco-resistant focal epilepsy. It took more than a year for him to regain walking and he remains ataxic with foot drop. He has almost monthly focal seizures consisting of a visual aura which can progress to a bilateral tonic-clonic seizure despite felbamate and phenytoin. EEGs showed multifocal epileptiform discharges. Our neuropsychiatric assessment found normal executive function, mild ASD symptoms, and no other behavioral disturbances. This profile is consistent with a diagnosis of FIRES and may not relate to the *PCDH19* variant. This male was reported to have a missense *PCDH19* variant (c.1672 G > C; p.Asp558His) inherited from his unaffected mother. The variant is located in the calcium-binding domain (important for protein structure/folding). There is a previous report of this variant as likely pathogenic in a commercial gene company series (GeneDx) so this may be the same individual (Lindy et al. 2018).

3.3 Neuropsychiatric Profile

Executive dysfunction occurred in 44% (4/9) mosaic males and one transmitting male (#11). All five individuals had a diagnosis of ID. As the SDQ is only available to individuals 18 years or younger, results from this measure were obtained for 8/9 mosaic and 1/6 transmitting males. This one transmitting male scored in the moderate range for emotional and peer problems and in the average range for all other measures. Emotional problems were recorded for 25% (2/8), conduct problems for 50% (4/8), inattention/hyperactivity for 75% (6/8), peer problems for 75% (6/8), and social deficits were recorded for all eight mosaic males. Sixty seven percent of mosaic males (6/9) including the non-penetrant male (#9) and two transmitting males (#10, #11) scored in the ASD clinical range. DOCS scores were all in the normal range, however, one male (#13) attained a total score just below the clinical threshold for OCD. Elevation in the harm, thoughts, and order domains were reported by this individual. The remaining transmitting males (#12, #14, and #15) scored within the normal range on all relevant measures. The neuropsychiatric profile of our mosaic males was similar to affected females (Fig. 1) (Kolc et al. 2020).

Our mosaic male (#9) without seizures had an SRS-2 score in the moderate range. Scores in this range indicate deficiencies in reciprocal social behavior that are clinically significant and lead to substantial interference with everyday social interactions. This finding was supported by an elevated score on the BRIEF shift domain and a slightly elevated score on the DOCS order domain, which indicates concerns in the area of mental flexibility and a need for order/symmetry, respectively. Sanger sequencing of blood-derived DNA showed somatic mosaicism of his *PCDH19* variant (c.1240G > A), however, *PCDH19* mosaicism could not be detected in fibroblast-derived DNA (Fig. 2).

4 Discussion

We compared males with somatic and germline *PCDH19* pathogenic variants and systematically describe their phenotypic spectrum. The clinical features of the mosaic males in our cohort concur with previous case reports (Depienne et al. 2009; Terracciano et al. 2016; Thiffault et al. 2016). We describe four new penetrant cases, expanding the number of reported *PCDH19* mosaic male cases to 17. We show that mosaic males have a neuropsychiatric profile that includes executive dysfunction, ADHD, and ASD; a clinical profile comparable to that reported for females who are naturally mosaic (Kolc et al. 2020). We also systematically assessed males with germline *PCDH19* variants. Previous reports are limited, however, germline males are generally asymptomatic apart from four reported with ASD (Piton et al. 2011; Van Harssel et al. 2013) and

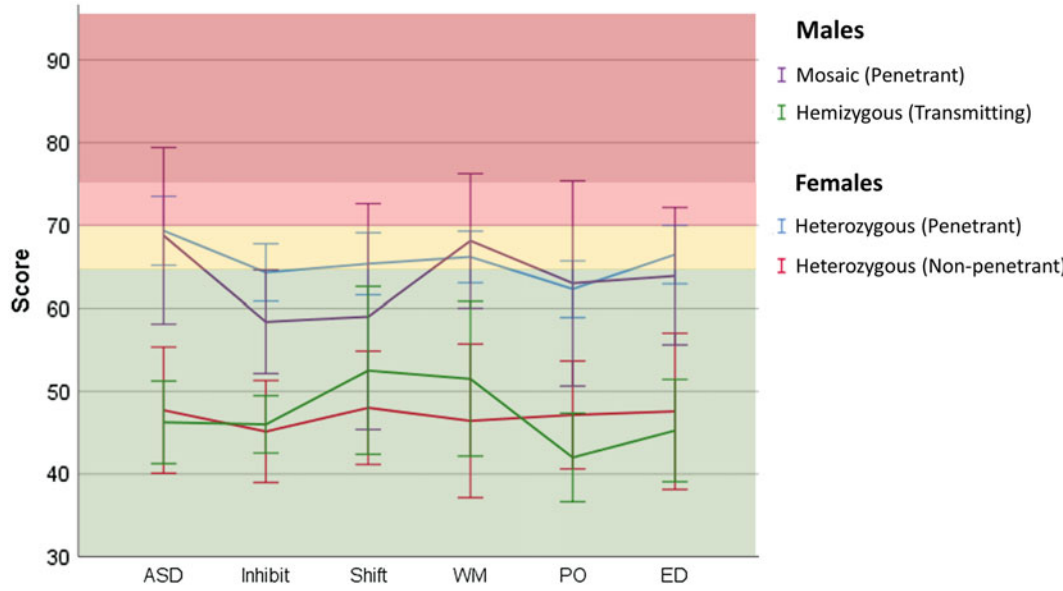

Fig. 1 Neuropsychiatric profile of males with pathogenic *PCDH19* variants based on average (±2 SEM) SRS-2 total and BRIEF total and subscale *t* scores. Males compared to the females from our previous publication (Kolc et al. 2020) illustrating the similar clinical profile between mosaic (penetrant) males and heterozygous (penetrant) females. Hemizygous (transmitting) males) and heterozygous (non-penetrant) females exhibit a clinical profile within the normal range. Green shaded region represents the normal range; yellow, mildly elevated; orange, moderately elevated; and red represents severely elevated *t* scores. *ASD* autism spectrum disorder, *ED* executive dysfunction, *PO* plan/organize, *WM* working memory

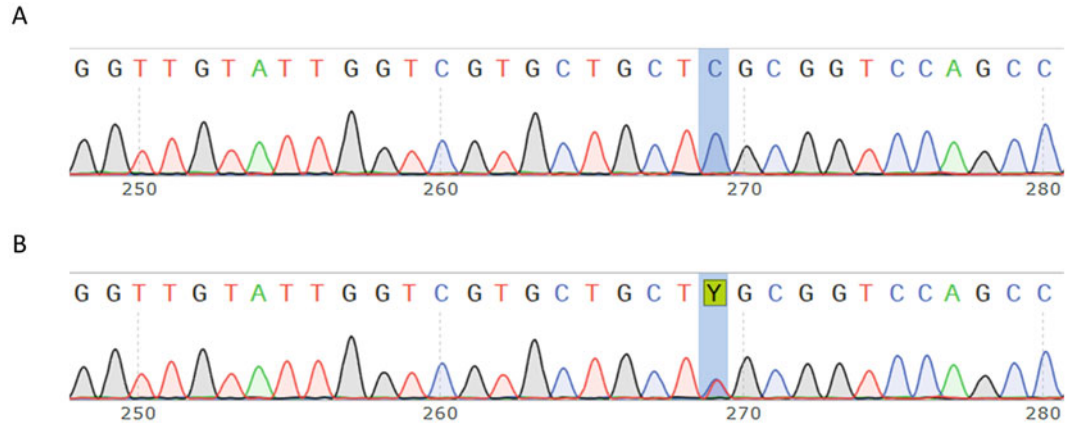

Fig. 2 gDNA Sanger sequence illustrating the A. absence of mosaicism in skin fibroblasts and B. presence of mosaicism in blood for the non-penetrant mosaic male (#9). Sequences were generated using a reverse primer (CGTATATGTCGCCTGAGTT)

ID (Tarpey et al. 2009). Here we describe two males with germline *PCDH19* variants and neuropsychiatric abnormalities. However, one had FIRES at 9.5 years, an acute encephalopathy with status epilepticus and fever which presents in previously normal children (Van Baalen et al. 2010). Most patients have cognitive, motor, and behavioral sequelae and epilepsy. Based on molecular grounds alone, there is compelling evidence for his *PCDH19* variant. However, when

his clinical profile and family history are considered, it is more likely that FIRES explains the neuropsychiatric abnormalities we detected, and they are unrelated to his *PCDH19* variant. The clinical distinction between FIRES and CE can be subtle, the key differences being the later seizure onset, male predominance, and viral illness preceding onset in FIRES (Specchio et al. 2011). We believe the hallmark features of PCDH19 CE in males to be threefold: (1) seizure clusters, often triggered by fever; (2) seizure onset before 2 years of age; and (3) comorbid social deficits.

We also describe a *PCDH19* mosaic male with moderate ASD but no seizures. It is possible that his autistic features are due to other genetic or environmental factors; however, it may also be due to neuronal *PCDH19* mosaicism. To our knowledge, there have only been two non-penetrant mosaic males reported (Liu et al. 2019). *PCDH19* somatic mosaicism was confirmed in multiple tissues for both individuals. Interestingly, their skin fibroblasts were not tested. Based on cellular interference, it was proposed that asymptomatic mosaic males have skewed neuronal mosaicism in favour of either wild-type or variant allele. Given that we were unable to identify mosaicism in the skin fibroblasts of our mosaic male, it is possible that a low level of mosaicism was present that was not detectable via Sanger Sequencing. Future studies should utilize more sensitive techniques (i.e., microdroplet PCR) for detection of *PCDH19* mosaicism. The penetrance of *PCDH19* variants in females has been estimated to be 80% (Kolc et al. 2019). There has been no estimate with respect to mosaic males. With the addition of our non-penetrant mosaic male, there have been three non-penetrant males reported in the literature. With our four additional penetrant males, there have been 17 penetrant mosaic males out of the 20 mosaic males described. *PCDH19* pathogenic or likely pathogenic variant penetrance is difficult to estimate accurately as it ultimately depends on the overall number of the patients identified and their ascertainment. Notwithstanding these limitations, we estimate the penetrance of *PCDH19* variants in mosaic males to be 85%.

4.1 Conclusion

While *PCDH19* variants predominantly affect females, published and our current evidence suggest that *PCDH19* variants also result in a spectrum of phenotypes in males, albeit less frequently. The phenotypic profile for somatic mosaic males resembles that of heterozygous affected females. We identified neuropsychiatric abnormalities in two males with germline *PCDH19* variants, however, the *PCDH19* variant in each case is likely serendipitous and our data suggest that transmitting males are generally unaffected. We also describe a non-penetrant somatic mosaic male with mosaicism confirmed in blood, but not identified in skin fibroblasts. We suggest microdroplet PCR for more sensitive detection of *PCDH19* somatic mosaicism.

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Conflict of Interest Ingrid Scheffer serves/has served on the editorial boards of the *Annals of Neurology, Neurology* and *Epileptic Disorders*; may accrue future revenue on pending patent WO61/010176 (filed: 2008): Therapeutic Compound; has a patent for *SCN1A* testing held by Bionomics Inc. and licensed to various diagnostic companies; she has a patent molecular diagnostic/theranostic target for benign familial infantile epilepsy (BFIE) [PRRT2] 2011904493 & 2012900190 and PCT/AU2012/001321 (TECH ID:2012–009) with royalties paid. She has served on scientific advisory boards for UCB, Eisai, GlaxoSmithKline, BioMarin, Nutricia, Rogcon and Xenon Pharmaceuticals; has received speaker honoraria from GlaxoSmithKline, Athena Diagnostics, UCB, BioMarin, Biocodex and Eisai; has received funding for travel from Athena Diagnostics, UCB, Biocodex, GlaxoSmithKline, Biomarin and Eisai. She receives/has received research support from the National Health and Medical Research Council of Australia, Health Research Council of New Zealand, CURE, March of Dimes and NIH/NINDS.

The remaining authors declare no conflict of interest.

Ethical Approval The project was approved by the University of Adelaide Human Research Ethics Committee (H-2016-184).

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