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Kursad Turksen *Editor*

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Stem Cells in Development and Disease

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Stem Cells in Development and Disease

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Preface

This next volume in the cell biology and translational medicine series continues to address the topic of stem cells in both normal and pathological situations. The volume includes such topics as the use of decellularized extracellular matrix in tissue transplantation, scaffolds for tissue engineering. It also includes new insights into animal models, exosomes in the context of their role not only in lineage progression in normal development and aging but also in what goes awry that leads to disease states.

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

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

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

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

Ottawa, ON, Canada — Kursad Turksen

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Conventional and Emerging Markers in Stem Cell Isolation and Characterization

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Abstract

Stem cells have emerged as a promising source of cell-based therapy in regenerative medicine with several stem cell-based products currently in clinical trials. Despite the immense therapeutic potential, their isolation from some of the emerging sources and their characterization has been naïve owing to the lack of standard markers for the same. Some biomarkers have now been well established for the isolation and characterization of stem cells. However, there are emerging markers that can be used in addition to these conventional markers or independent of them to establish the identity of the stem cells. In this review, an attempt has been made to describe a few conventionally used markers and emerging markers for the identification, isolation and characterization of stem cells from various niches across the three germ layer origins.

Keywords

Conventional markers · Emerging markers · Epidermal SCs markers · MSC markers · NSC markers · Stem cell markers

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Abbreviations

ALCAM	Activated Leucocyte-Cell Adhesion Molecule
ALP	Alkaline Phosphatase
ALPP	Alkaline Phosphatase Protein
AMP	Adenosine MonoPhosphate
bFGF	basic Fibroblast Growth Factor
Bmi1	B-cell specific Moloney murine leukemia virus integration site 1
BMP	Bone Morphogenic Protein
CAMK2N1	Calcium/Calmodulin dependant protein Kinase II Inhibitor 1
CBC	Cycling Crypt Base Columnar
CFU – GEMM	Colony Forming Unit – Granulocytes, Erythrocytes, Macrophages, Megakaryocytes
CFU – S	Spleen Colony Forming Unit
CFU assay	Colony Forming Unit assay
CKB	Creatine Kinase B Type
CNS	Central Nervous System
CoL1A1	Collagen Type 1 alpha 1
COL8A1	Collagen Type VIII Alpha 1
CPCs	Cardiac progenitor cells
CRYaB	Crystalline – aB
CRYAB	Crystalline alpha B
CSCs	Cardiac Stem cells
DKK1	Dickkopf WNT signaling pathway inhibitor 1
erbB2	Erb-B2 Receptor Tyrosine Kinase 2

FGFR4	Fibroblast Growth Factor Receptor – 4
Flk1	Fetal Liver Kinase 1
Gata5	GATA binding protein 5
GPI	Glycosyl – phosphatidylinositol
hBMSCs	Human Bone Marrow Stem Cells
HER-2	Human Epidermal Growth Factor Receptor 2
HMG	High Mobility Group
Hopx	Homeodomain only protein Homo Sapiens
HPP – CFU	High Proliferative Potential Colony Forming Cell
ISCT	International Society for Cellular Therapy
Isl1	Islet 1
Kdr	Kinase Insert Domain Receptor
Lgr5	Leucine Rich Repeat containing G Protein-Coupled Receptor 5
Lrig1	Leucine Rich Repeats and Immunoglobulin-Like Domains Protein 1
MAP 2	Microtubule associated protein 2
MAPK	Mitogen Activated Protein Kinase pathway
MCAM	Melanoma Cell Adhesion Molecule
MDR1	Multi Drug Resistant gene 1
MSCs	Mesenchymal Stem Cells
Myocd	Myocardin
NeuN	Neuronal Nuclei
NK cells	Natural Killer cells
NSCs	Neural Stem Cells
OCN	Osteo Calcin
PNS	Peripheral Nervous System
POU family	Pit-Oct-Unc family
PSA-NCAM	Poly-sialylated Neuronal Cell Adhesion Molecule
RUNX2	Runt-related Transcription Factor 2
SB-10	Sleeping Beauty Transposon system 10
SH3	Src – homology 3
SSEA-1	Stage Specific Embryonic Antigens – 1
STRO – 1	Stromal Cell Surface marker
TA	Transiently Amplifying
Tert	Telomerase Reverse Transcriptase
TGF – β R complex	Transforming Growth Factor Beta Receptor Complex
TPO	Thyroid Peroxidase

1 Introduction

Stem cells are undifferentiated cells that have the ability to self-renew and the potential to terminally differentiate into cells of one or more lineages (Kafienah et al. 2006). Although stem cells isolated from the inner cell mass of the blastocyst have the potential to differentiate into many cell types barring the trophoblast (Thomson et al. 1998), the technical complexities and ethical concerns associated with this source has led scientific fraternity across the globe to look for alternative sources of stem cells (Freed 2002).

Bone marrow derived mesenchymal stem cells (MSCs) were the first stem cells which were widely used for transplantation, identified and isolated as a source of multipotent adult stem cells. These cells had the capacity to differentiate into cell lineages like adipocytes, chondrocytes, and osteocytes (Friedenstein 1966). With bone marrow being established as an eminent source of adult stem cells, additional sources of mesenchymal stem cells were identified in the later years from 2004 till 2012 (Joannides et al. 2004; Laino et al. 2005; Yen et al. 2005; Meng et al. 2007; Haranova et al. 2011; Hassiotou et al. 2012; Lee et al. 2015; Macrin et al. 2017) and were compared against bone marrow as the gold standard (Baksh et al. 2007; Xie et al. 2012; Kao et al. 2015).

At the initial stages of stem cell research, the identity and the potency of the stem cells were determined by means of *in vivo* colony forming assays like spleen colony forming assay (CFU-S) and *in vitro* assays like colony-forming unit (CFU) assay, burst-forming unit erythroid (BFU-E) assay, granulocyte, erythroid, megakaryocyte, Colony Forming Unit – Granulocyte, Erythrocyte, Macrophage, Megakaryocyte (CFU-GEMM) assay, CFU

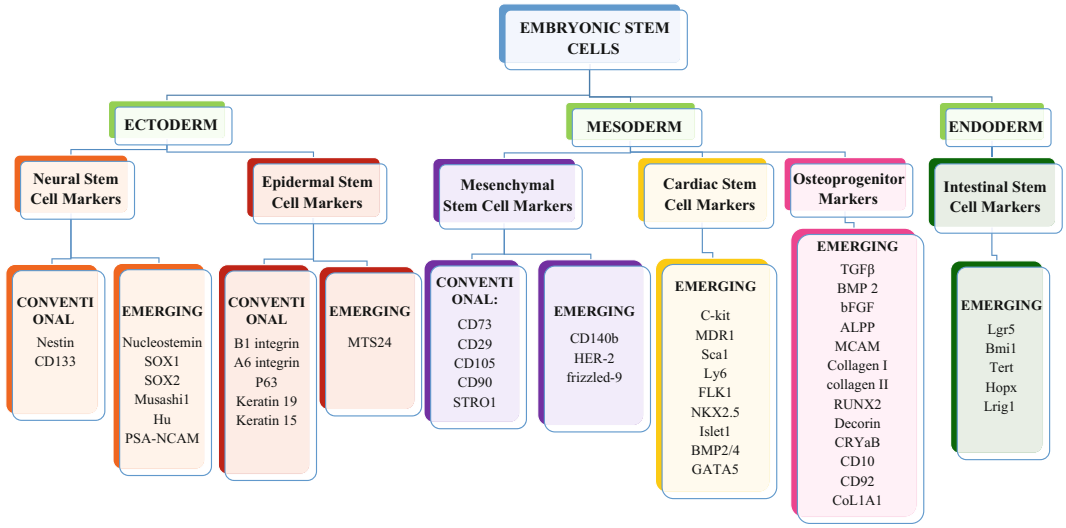

Fig. 1 A flow chart depicting the stem cell markers for characterizing stem cells that are precursors to cells of different lineages

granulocyte-macrophage (CFU-GM) assay and high proliferative potential colony forming cell (HPP-CFU) assay (Moore 1991). These stem cells possess ability to differentiate into multiple cell lineages by including growth factors and cytokines (Pittenger et al. 2004; Dominici et al. 2006; Muller et al. 2007). For characterising these stem cells, refined methods were reported in which the predominant one being the identification of markers expressed by stem cells (Dominici et al. 2006; Phinney and Prockop 2007; Ka et al. 2014).

The characterization of stem cells based on the presence of markers requires affirmation of their conformation to the standards of both the positive and the negative markers (Moore 1991). Positive markers are those proteins that are expected to be expressed on a particular stem cell population (Christensen and Weissman 2001; Potten et al. 2003), whereas negative markers refer to those proteins that are expected to be absent or present in very low levels in a particular stem cell population (Osawa et al. 1996). For instance, nestin positive cells i.e., the expression of nestin on cell surface is representative of neural stem cells (NSCs) (Cregan et al. 2007) suggesting that nestin is a postive marker for NSCs. Similarly, cells that do not express CD34 marker have been categorized as mesenchymal stem cells (MSCs), suggesting CD34 to be a negative marker for MSCs (Pittenger et al. 1999). A combination of these positive and negative markers have been standardized for the characterization of stem cell population of various niches (Moore 1991; Amoh et al. 2005). For instance, the minimum accepted standard for characterization of stem cells as MSCs is the coexpression of the markers CD73, CD90, CD105 (Dominici et al. 2006; Phinney and Prockop 2007; Ka et al. 2014).

Of the different types of markers, CD markers were the initial markers identified and used to characterize different cell types and later several newly identified proteins have emerged as stem cell markers that are representative of specific stem cell populations (Barker et al. 2007). This review describes a few conventional and emerging markers that have been reported for use in the identification, isolation and characterization of stem cells from sources arising from any of the three germ layers. (Fig. 1)

2 Conventional Markers

Conventional markers in this review refer to the genes and their proteins used by the scientists for identification and isolation of stem cells. As stem

cells are functionally defined, these conventional markers must be linked with the cell's function. In general, these conventional markers are of two types: cell surface markers and intracellular markers. Conventional markers, as selected by the authors, comprise of those stem cell markers that have been reported before the year 2000 and have been largely accepted as a standard for the characterization of stem cells of a particular niche. Markers of stem cells from various niches that cover all the three germ layers have been reported.

2.1 Neural Stem Cell (NSC) Markers

Neural stem cells constitute a pool of stem cells, present in the brain, having the ability to differentiate into neurons. NSCs, which are ectodermal in origin, are the potential source of stem cells used for replacing degenerated neurons in neurodegenerative diseases. Initially, when biomarker based characterization had not surfaced, the standard method used to isolate NSCs was to dissect out a part from any region of Central Nervous System (CNS) containing a dividing cell population and expose the cells to a dose of mitogens on a matrix (Gage 2000). However, with the identification of specified cell-surface markers and intracellular markers the method of identification and isolation of NSCs have transformed for the better. One of the earlier established markers for the characterization of neural stem cells was Nestin, a class VI intermediate filament protein transiently expressed in NSCs with downregulation during differentiation (Lendahl et al. 1990). Furthermore, Nestin endows the stem cells with survival and self-renewal capacities (Park et al. 2010) and has been considered as a predominant marker not only for NSCs but also in other cells of Central Nervous System (CNS) and Peripheral Nervous System (PNS) (Lendahl et al. 1990; Clarke et al. 1994), glioma cells (Sugawara et al. 2002) and muscle cells (Zimmerman et al. 1994). For these reasons, Nestin has been considered as a poor biomarker that cannot be used as a unique marker for the characterization of NSCs (Kornblum and Geschwind 2001). Also, CD133 or Prominin-1 is another cell-surface marker expressed in a variety of brain cells and is associated with brain tumors. It is also found expressed on the surface of NSCs and thus has been widely used to isolate NSCs from brain (Corti et al. 2007). This is due to its involvement in the mechanisms influencing cell polarity, juxtacrine comunications and migration (Zhang et al. 2008).

Recent reports showed that neuronal marker – Neuron specific class III beta-tubulin (Tuj1) was used for the identification of neural stem cells and they were stained with Microtubule associated protein 2 (MAP 2) and Neuronal Nuclei (NeuN) markers that were specific for mature neurons (Pan et al. 2018). There are also other neural markers including neurofilament and β-tubulin III used for the neural stem cell identification (Wu et al. 2013). Though there are reports showing the specific markers for identification of neural stem cells, CD-15 – a stage specific embryonic antigen-1 (Pruszak et al. 2009) and CD24 were also used as markers of NSCs (Yuan et al. 2011; Kim et al. 2015).

2.2 Epidermal Stem Cell Markers

Another major type of stem cells of the ectodermal origin is the epidermal stem cells. Although the identification of epidermal stem cells using molecular markers is not very promising, few markers have been found to be expressed on these cells facilitating the identification and isolation of these stem cells. β1 integrin, which confers adhesiveness to the epidermal cells, is essential for keratinocytes to maintain their stemness through the mitogen-activated protein kinase (MAPK) pathway (Zhu et al. 1999). Additionally, α6 integrin expression is also considered as a marker due to its association with the anchorage and long-term proliferative capacity of the basal cells (Li et al. 1998).

P63 is another traditional marker, which is a transcription factor belonging to the same family that includes p53 and p73 genes, reported to be necessary for the regenerative potential of epithelial cells (McKeon et al. 1999). It has been reported to be abundantly expressed by the epidermal clonal population of stem cells and

there is a downregulation in their transiently amplifying (TA) clones. This can distinguish these stem cells from their TA progeny in the squamous stratified epithelia (Pellegrini et al. 2001; Senoo et al. 2007).

Keratin 19, Keratin 15 (Janes et al. 2002) and β catenin (Zhu and Watt 1999) are other intracellular proteins associated with epidermal stem cell characterization, but these cannot be used in isolation for characterization.

2.3 Mesenchymal Stem Cell (MSC) Markers

When MSCs derived from bone marrow emerged as a novel source of stem cells, laboratories across the world developed methods to isolate and expand the MSCs. However, the lack of universally accepted standards to define this cell population caused much ambiguity and made the comparison of data from various laboratories difficult. This issue was addressed by the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy (ISCT), which set forth some criteria to identify MSCs. Besides the plastic adherence property and multilineage differentiation potential of MSCs, the differential expression of specific surface antigens was also advocated. (Dominici et al. 2006). The presence and the absence of some of these positive markers and negative markers are conventionally considered to be the minimum standard to identify the cells as MSCs.

CD73, also known as ecto-5′-nucleotidase is a glycosyl-phosphatidylinositol (GPI) linked cell surface enzyme that serves to convert Adenosine MonoPhosphate (AMP) to adenosine and is predominantly used as a marker for lymphocyte differentiation (Resta et al. 1998; Colgan et al. 2006). This was first identified by Pittenger et al., on mesenchymal stem cell population isolated from bone marrow as SRC Homology 3 (SH3) (Pittenger 1999). Later on, immunoprecipitation studies using SH3 antibody revealed that SH3 was an epitope present on CD73 (Barry et al. 2001). CD73 is reported to have various functions including its involvement in BM stromal interactions (Barry et al. 2001). Also, along with CD29, it is known to modulate the migratory capacity of MSCs by reducing the migration, thus, enabling the cells to get entrapped at a fracture site to fulfill their regenerative functions (Ode et al. 2011). Furthermore, CD73 expression is acquired by natural killer (NK) cells upon its interaction with MSCs and these CD73-positive NK cells can regulate NK cell activation in either autocrine or paracrine manner (Chatterjee et al. 2014).

CD105, also known as endoglin, is a type I membrane glycoprotein and is a component of transforming growth factor receptor (TGF-βR) complex. It is known to play a role in cardiovascular development (Sanz-Rodriguez et al. 2004), angiogenesis (Duff et al. 2003) and also cancer (O'Connor et al. 2007). It was first identified on MSCs, similar to CD73, as Src Homologue 2 (SH2)-a component of the receptor complex of transforming growth factor – beta (TGF-β) involving in cell proliferation, differentiation and migration (Pittenger 1999) which was later recognized as an epitope of CD105 (endoglin) (Barry et al. 1999). Besides MSC population, it is also a marker for hematopoietic stem/progenitor cells (Pierelli et al. 2001).

CD90 (Cluster of Differentiation 90), also known as Thy1 (cell surface antigen), is a Glycosylphosphatidylinositol (GPI) – linked cell surface protein belonging to the Ig superfamily and originally discovered as a thymocyte antigen. It is speculated to play roles in cell-cell and cell-matrix interactions (Rege 2006) and adherence of CD34-positive hematopoietic cells (Craig et al. 1993). CD90 expression has been identified in varied cell populations like hematopoietic stem cells (Craig et al. 1993), fibroblasts and endothelial cells (Saalbach et al. 1999).

Stromal Cell Surface Marker (**STRO-1**), the murine IgM monoclonal antibody produced from an immunization with a population of human CD34+ bone marrow cells, was found to identify a cell surface antigen expressed by stromal elements in human bone marrow (Simmons and Torok-Storb 1991). A STRO-1 positive enriched subset of marrow cells was reported to be capable of differentiating into multiple mesenchymal

lineages including hematopoiesis-supportive stromal cells with a vascular smooth muscle-like phenotype, adipocytes, osteoblasts and chondrocytes (Gronthos et al. 1994; Liu et al. 1999; Dennis et al. 2002).

3 Emerging Markers

With the popularization of characterization based on protein markers expressed on the cell surface and/or found in the intracellular space, several proteins have been recently tested for their use as markers for stemness. The emerging markers that have been reported post 2000 as markers for stemness of cells from various niches that cover all the three germ layers have been discussed here. Some of these markers include those that have been repurposed in the sense that the markers that have been established in one source are also being used as marker for stem cells from a different niche.

3.1 Neural Stem Cell (NSC) Markers

Neural stem cells (NSCs) are unique cells endowed with self-renewing, multipotent potential, responsible for the generation of main phenotypes of the nervous system. (De Filippis and Binda 2012)

Although conventional markers like prominin-1 (CD133) and nestin have been widely used for isolation of human NSCs, it was found that the integrin subunits α6 and β1 are highly expressed by human neural precursors and make suitable markers for the prospective isolation of NSCs (Hall et al. 2006).

The Pit-Oct-Unc (POU) family of transcription factors SRY-box transcription factor 1 (SOX1) and SOX2, the plasma membrane proteins Fibroblast Growth Factor Receptor −4 (FGFR4) and Stage Specific Embryonic Antigens −1 (SSEA-1) also have emerged as NSC markers in the recent times, all of which are highly expressed in the multipotent NSCs (Cai et al. 2002) (Capela and Temple 2002). **SOX1**, which is expressed exclusively in the CNS, is suggested to function as one of the earliest markers for neural fate decision of embryonic stem cells and marks the proliferating progenitors residing in the neural tube (Muñoz et al. 2012). The proliferation of stem cells that are positive for **SOX2**, an High Mobility Group (HMG) box transcription factor (Graham et al. 2003; Ellis et al. 2005), is reported to generate neural precursors along with cells identical to themselves (Suh et al. 2007). Graham et al., associated the inhibition of this gene with delamination of cells from the ventricular zone and exit from cell cycle (Graham et al. 2003), thus stating the importance of SOX2 in maintaining progenitor identity.

Furthermore, Musashi, Nestin and Nucleostemin also have been identified as NSC markers that are especially useful in their isolation (Cai et al. 2003b). Musashi1 is an evolutionarily conserved RNA-binding protein that is important for cell fate determination, maintenance of the stem-cell state and differentiation, found to be localized to the neuron cell bodies (perikarya) of CNS stem-like cells and non-oligodendroglial progenitor cells (Kaneko et al. 2000). Besides the nervous system, it is a known marker for intestinal stem cells (Potten et al. 2003). Nucleostemin is a nucleolar protein that is found predominantly associated with the proliferation of rat neural and embryonic stem cells and is emerging as a proliferative marker for human neural stem cells. It has also been reported that NS is necessary for the proliferative activity of both normal and cancerous cell lines (Kavyasudha et al. 2018).

Poly-sialylated Neuronal Cell Adhesion Molecule (PSA-NCAM) is another emerging marker of neuronal development and synaptogenesis that is expressed not only during the prenatal life but also adulthood (Quartu et al. 2008), and it is reported to be important for synaptic-plasticity (Muller et al. 1996) and also contributes to the early development of adult neurogenesis (Seki 2002).

Other membrane markers for mature neural cell types, such as O4 for oligodendrocytes and CD44 for astrocytes, can also be used to separate NSCs from more mature mixed populations. There are also reports showing successful isolation of multipotent NSCs using a negative selection strategy, in which cells not expressing

epitopes commonly expressed in lineage-restricted cells. These negative selection markers include CD271, CD44 and CD184 (Cai et al. 2003b; Yuan et al. 2011) (Vishwakarma et al. 2014).

3.2 Epidermal Markers

Stem cells (SCs) residing in the epidermis and hair follicles not only ensure the maintenance of adult skin homeostasis and hair regeneration, but they also participate in the repair of the epidermis after injuries (Blanpain and Fuchs 2006).

Like other adult stem cells, those that reside in the skin are critical in tissue homeostasis and wound healing. Epidermal stem cells, multipotent skin stem cells thought to reside within the hair follicle, are reported to be generally quiescent but could also be stimulated to proliferate and differentiate into the specialized cells that compose a hair follicle, thus facilitating in regenerative wound healing. Epidermal stem cells have been characterized *in vitro* for skin graft purposes and are known to express Integrin alpha 6/CD49f and the general stem cell marker, CD34 (Blanpain and Fuchs 2006, 2009).

Although markers like Tumour protein (p63), Keratin 19 (K19), β-catenin and α6 integrin have been suggested as putative epidermal stem cell markers, β1 integrin has emerged as the most promising epidermal marker that has been especially useful in the isolation of subpopulation of keratinocytes that are rich in stemness (Watt 1998; Reiisi et al. 2016).

However, researchers are on the lookout to identify additional stem cell markers for the isolation of skin or epidermal stem cells to obtain greater target specificity than at present. (Watt 1998)

MTS24 is an another cell-surface marker that identifies uncharacterized population of hair follicle keratinocytes located between the bulge and the sebaceous glands. MTS24 reactivity is first detected in the early stages of hair follicle development, and is increased during hair growth. MTS24-positive keratinocytes are distinct from the epidermal stem cells located in the bulge. Results suggest that the MTS24-positive keratinocytes represent an important new committed progenitor or stem cell compartment within the hair follicle (Nijhof et al. 2006).

3.3 Cardiac Stem Cell Markers

Cardiac stem cells, that track its origin to the mesodermal lineage, are thought to be a quiescent, heart-resident population of stem cells that can reenter the cell cycle following injuries such as acute myocardial infarction. Cardiac stem cells have self-renewal capabilities *in vitro* and the ability to differentiate *in vivo* into all three major cardiac cell types viz., cardiomyocytes, vascular smooth muscle cells and endothelial cells. For these reasons, considerable efforts have been made to identify specific markers of stem cells with cardiomyogenic potential (Beltrami et al. 2003; Cai et al. 2003a; Passier et al. 2008; Segers and Lee 2008; Laflamme and Murry 2011). Among the various markers employed to identify resident CSCs, c-kit has played a prominent role (Torella et al. 2006; Anversa et al. 2013). Some of the emerging markers for the cardiac stem cell population include c-kit/CD117, Multi Drug Resistant gene 1 (MDR1), and Spinocerebellar ataxia type 1 (Sca-1) cells (Urbanek et al. 2003). *In vitro* identification of a class of human c-kit-positive cardiac cells that possess the fundamental properties of stem cells – self-renewal, clonogenicity and multipotency – has been reported (Bearzi et al. 2007). Sca-1/Ly6-negative, (Lineage-negative) (Lin-), Sca-1-positive subset of cell population is found to display a mesenchymal profile, characterized by a limited ability to generate cardiomyocytes *in vitro* and *in vivo*, even after injury. Although, in other organs, Sca-1 expression is mainly observed on mesoderm-derived cells and not restricted to stem/progenitor cell populations, CPCs (Cardiac progenitor cells) have been isolated based on the expression of surface markers including Sca-1 and c-Kit suggesting the role of Sca-1 as an emerging CSC marker (Valente et al. 2014). Sca-1 has been suspected to be an essential component in the promotion of CSC proliferation and survival,

resulting in direct facilitation of early engraftment, with a possible indirect effect exerted on late cardiovascular differentiation after CSC transplantation (Tateishi et al. 2007). Markers for the embryonic stem cell derived cardiac progenitor cell population were found to initially express genetic markers representing all the three lineages viz., cardiomyocytes, vascular smooth muscle cells and endothelial cells, which included markers like c-kit, Fetal Liver Kinase 1 (Flk1) (also known as Kinase Insert Domain Receptor (kdr) and Homeodomain factor (Nkx2.5), but not brachyury, and were subsequently found to express Islet-1 (Isl1). Other cardiac proteins that are expressed along with Nkx2.5 include Myocardin (Myocd), Bone Morphogenic Protein (BMP2/4) and GATA binding protein 5 (Gata5) (Qsfdvstps et al. 2008).

FLK-1 (Fetal Liver Kinase 1), also known as Kinase Insert Domain Receptor (Kdr), an flt-related receptor tyrosine kinase is an early marker for endothelial precursors. The expression of FLK1, an early marker of lateral mesoderm, where cardio genesis occurs, aids to characterize and isolate cardiac stem/progenitor cells. Reports have confirmed that FLK1, especially FLK1 + CD31-, Cadherin-, is a feasible marker for detecting cardiac stem/progenitor cells (Iida et al. 2005).

NKX2.5, a homeobox transcription factor required for ventricular cardiogenic differentiation, is one of the factors reported to be expressed in developing embryonic cardiac regions and could be used to delineate CPCs (Lints et al. 1993; Raffin et al. 2000). It is one of the transcription factors of the heart primordium that is known to be expressed most strongly in cardiomyocytes of the heart tube during early cardiac development (Lyons et al. 1995) and is recognized as an early marker for cardiac cell differentiation (Lints et al. 1993; Jamali et al. 2001). Experiments suggesting the close correlation between the expression of the Nkx2.5 and FLK1 genes have been reported, implying the plausibility of the two proteins as markers in the detection cardiac stem/progenitor cells (Iida et al. 2005).

Islet1 (Isl1) is a LIM homeodomain protein expressed in distinct subdomains of the heart and in diverse cardiovascular lineages, and has been reported to have a critical role in cardiac progenitors of the second heart field (Sun et al. 2007). Isl1 expression and lineage tracing of Isl1-expressing progenitors have demonstrated that Isl1 is a marker for a distinct population of undifferentiated cardiac progenitors that give rise to the cardiac segments that were found to be missing in Isl1 mutants. The prominence of Isl1 in cardio-genesis has confirmed its position as a marker for undifferentiated cardiac progenitor state (Cai et al. 2003a).

3.4 Osteoprogenitor Markers

Another important stem cell niche of the mesodermal origin is the bone. Certain genes that have served as predominant osteoprogenitor markers include Transforming Growth Factor β (TGFβ), Bone Morphogenic Protein 2 (BMP-2), and basic Fibroblast Growth Factor (bFGF) or gremlin 1, Alkaline Phosphatase Protein (ALPP), Melanoma Cell Adhesion Molecule (MCAM), collagen I, collagen II, Runt-related Transcription Factor 2 (RUNX2), decorin, Thyroid Peroxidase (TPO) are also used (Worthley et al. 2015). Putative osteoprogenitor marker genes include Calcium/Calmodulin dependant protein Kinase II Inhibitor 1 (CAMK2N1), Collagen Type VIII Alpha 1 (COL8A1), Creatine Kinase B Type (CKB), Crystalline alpha B (CRYAB), and Dickkopf WNT signaling pathway inhibitor 1 (DKK1), which are expressed at high levels in osteogenic cells (Ng et al. 2008).

Although multiple relevant markers like Alkaline Phosphatase (ALP), Runt-related Transcription Factor 2 (RUNX2) and Osteo Calcin (OCN) were known, no single surface marker or panel of markers was distinctly known to identify the osteoprogenitor stem cell population until 2014 (Phillips et al. 2014) (Guo et al. 2015). The surface markers CD10 and CD92 have been reported to demonstrate significantly increased expression in hBMSCs differentiated towards the osteogenic and adipogenic lineages along with a slight increase in CD10 expression correlated with chondrogenic differentiation suggesting the use

of these proteins as markers for osteogenic stem cells identification (Granéli et al. 2014).

Crystalline – aB (CRYaB), an intracellular protein and a small heat shock protein belonging to the alpha family, which is composed of two gene products alpha-A (acidic) and alpha-B (basic) proteins, is also a potential novel osteogenic marker (Granéli et al. 2014). The gene expression of CRYaB has been shown to be significantly regulated in the early stages of the chondrogenic differentiation of the ATDC-5 chondroprogenitor cell line (Chang et al. 2013; Granéli et al. 2014).

Collagen Type 1 alpha 1 (CoL1A1) is another protein known to be an early marker of osteoprogenitor cells whose maximum expression level was reported at day 21 of differentiation in adult mouse (Jikkoa et al. 1999).

Cell surface markers like activated leucocyte-cell adhesion molecule (ALCAM), similar to Sleeping Beauty Transposon system 10 (SB-10), (Torella et al. 2006) and STRO-1 (Urbanek et al. 2003; Bearzi et al. 2007) also have been identified to be useful in detecting the earliest stages of the osteoblast lineage in bone marrow stromal cell (MSC) cultures (Kalajzic et al. 2002).

3.5 Mesenchymal Stem Cell (MSC) Markers

MSCs derived from Bone Marrow (BM-MSCs) are postnatal stem cells capable of self-renewing and differentiating into osteoblasts, chondrocytes, adipocytes and neural cells that express a panel of key conventional markers such as CD10, CD13, CD29, CD73, CD90, CD105, CD271, CD146, Oct4, STRO-1, and SSEA4 and emerging markers include CD140b, Human Epidermal Growth Factor Receptor 2 (HER-2)/Erb-B2 Receptor Tyrosine Kinase 2 (erbB2) (CD340), and frizzled-9 (CD349) (Urbanek et al. 2003).

3.6 Intestinal Stem Cell Markers

The intestinal epithelium is the most rapidly self-renewing tissue in adult mammals. (Barker et al. 2007). The G-protein coupled receptor Leucine Rich Repeat containing G Protein-Coupled Receptor 5 (Lgr5) and polycomb group protein B-cell specific Moloney murine leukemia virus integration site 1 (Bmi1) are two recently described molecular markers of self-renewing and multipotent adult stem cell populations residing in the crypt of the small intestine, capable of supporting regeneration of the intestinal epithelium. (Yan et al. 2012).

An important stem cell niche of the endodermal origin includes the stem cells in the intestinal crypts that are currently defined by the cycling crypt base columnar (CBC) cells and quiescent '+4' cells (Muñoz et al. 2012; Yan et al. 2012).

Lgr5 (Leucine rich repeat containing receptor) is an emerging intestinal stem cell marker that is being used to identify multipotent stem cells in the intestine (Muñoz et al. 2012).

Additionally, stem cells of the small intestinal crypt – the quiescent/'+4' cell have been reported to be negative for B-cell specific Moloney murine leukemia virus integration site 1 (Bmi1), Telomerase Reverse Transcriptase (Tert), Homeodomain only protein Homo Sapiens (Hopx) and Leucine Rich Repeats and Immunoglobulin-Like Domains Protein 1 (Lrig1) implying their use as negative stem cell markers for intestinal stem cells (Montgomery et al. 2011; Lin and Scott 2012; Powell et al. 2013; Takeda et al. 2013).

4 Conclusion

Recent stem cell biology research from across the world is focusing on identifying novel sources of stem cells and isolation and identification of the stem cells from these sources. This research has been driven by the immense therapeutic potential that stem cells hold for several degenerative ailments. Although there are several conventional markers that facilitate the isolation of stem cells from different niches, newer sources have posed newer challenges in this process. For this reason, standardization of markers to identify and characterize stem cells that are being isolated from emerging sources has become indispensable. In this regard, several reports have suggested

various new markers that enable the isolation of stem cells from various sources. Newer markers are being identified and standardized across the world. With the identification of each new marker, the naivety of the stem cell population is being cleared, paving way for the revelation of a better perspective about the newly identified cell population. This facilitates us to avoid misidentification and misrepresentation of cell populations. Furthermore, some of the conventional marker expressions overlap in several cell populations. For all these reasons, it is important to identify innovative markers that are specific to various stem cell niches, thus aiding easier identification and isolation of adult stem cells while being distinctive about different cell populations.

Conflict of Interest The authors declare no conflict of interest.

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Therapeutic Potential of Adipose Stem Cells

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Abstract

Adipose stem cells (ASCs) have gained attention in the fields of stem cells regenerative medicine due to their multifaceted therapeutic capabilities. Promising preclinical evidence of ASCs has supported the substantial interest in the use of these cells as therapy for human disease. ASCs are an adult stem cell resident in adipose tissue with the potential to differentiation along mesenchymal lineages. They also are known to be recruited to sites of inflammation where they exhibit strong immunomodulatory capabilities to promote wound healing and regeneration. ASCs can be isolated from adipose tissue at a relatively high yield compared to their mesenchymal cell counterparts: bone marrow-derived mesenchymal stem cells (BM-MSCs). Like BM-MSCs, ASCs are easily culture expanded and have a reduced immunogenicity or are perhaps immune privileged, making them attractive options for cellular therapy. Additionally, the heterogeneous cellular product obtained after digestion of adipose tissue, called the stromal vascular fraction (SVF), contains ASCs and several populations of stromal and immune cells. Both the SVF and culture expanded ASCs have the potential to be therapeutic in various diseases. This review will focus on the preclinical and clinical evidence of SVF and ASCs, which make them potential candidates for therapy in regenerative medicine and inflammatory disease processes.

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Keywords
Adipose stem cells · Mesenchymal stem cells · Regenerative medicine · Stem cell therapy · Stromal vascular fraction

Abbreviations

AD	Alzheimers disease
ALS	Amyotrophic Lateral Sclerosis
APP	Amyloid precursor protein
ASCs	Adipose stem cells
Aβ	Amyloid-beta
BM-MSCs	Bone marrow-derived mesenchymal stem cells
BrdU	Bromodeoxyuridine
CNS	Central nervous system
EAE	Experimental autoimmune encephalomyelitis
GM-CSF	Granulocyes-macrophage colony stimulating factor
IHD	Ischemic heart disease
LVEF	Left ventricular ejection fraction
MI	Myocardial infarctions
MS	Multiple sclerosis
MWM	Morris water maze
PD	Parkinson's disease
PLGA	poly(lactic-co-glycolic) acid
PVA	Poly(vinyl alcohol)
RA	Rheumatoid arthritis
SNpc	Substantia nigra pars compacta
SVF	Stromal vascular fraction
T1DM	Type 1 diabetes mellitus
Th	$CD4^+$ helper T
Tregs	regulatory T cells
WT	Wall thickness
ALS	Amyotrophic lateral sclerosis

1 Introduction

Stem cells are capable of asymmetric cell division promoting self-renewal and multi-lineage differentiation potential (Verfaillie 2002). Due to the controversy over the use of embryonic stem cells, recent interest has grown surrounding the potential of adult stem cells. Adult stem cells have been identified in most adult organs and tissues including bone, cartilage, skeletal muscle and adipose tissue (Gage 2000; Reyes and Verfaillie 2001; Jankowski et al. 2002; Gimble and Guilak 2003; Herrera et al. 2006; Barker et al. 2007). Although adult stem cells have a lesser capacity for self-renewal and differentiation than embryonic stem cells, there is a rapidly growing body of evidence that suggests that the therapeutic potential of adult stem cells is much more significant than was once thought.

Adipose tissue serves as a reservoir for mesenchymal stem cells, which we will refer to as adipose stem cells (ASCs) in this review. Adipose tissue is a multifaceted organ with many functions including endocrine functions with the secretion of various adipokines, structural and lipid storage functions and an immunology function as there are many immune cells and immunomodulatory cells, namely ASCs, resident in adipose tissue. ASCs can differentiate into mature cells of the adipogenic, osteogenic, chondrogenic, and myogenic lineages (Gimble et al. 2007). Adipose tissue is harvested and enzymatically digested to isolate the stromal vascular fraction (SVF). SVF is composed of many cell types: ASCs (15–30%), endothelial cells (10–20%), pericytes (3–5%), and immune cells (25–45%) (Bourin et al. 2013; Lee et al. 2013). After SVF isolation, the heterogeneous cell composition can be cultured, and the plastic adherence capacity of ASCs allows for the acquisition of homogenous populations of ASCs. Human ASCs can be phenotypically identified as $CD45^-CD235a^-CD31^-CD34^+$ and cultured ASCs can be phenotypically identified as $CD13^+CD73^+CD90^+CD105^+CD31^-CD45^-CD235a^-$ (Bourin et al. 2013). ASCs do not express human leukocyte antigens including HLA-DR molecules and co-stimulatory molecules that are important for immune recognition of "self" versus "non-self" that would trigger significant immune responses, making ASCs potential candidates for not only autologous therapy but possibly allogeneic therapy (Gimble et al. 2007; DelaRosa et al. 2012).

Adipose tissue confers an advantage because stem cell yield from is 100–500 times higher per tissue volume than from bone marrow rendering

ASCs a more attractive candidate for therapeutic use (D'Andrea et al. 2008; Zhang et al. 2013). Harvesting adipose via liposuction can yield large quantities of fat tissue with minimal risk to the patient. This procedure is very commonly performed, but the removed adipose tissue/lipoaspirate is discarded as medical waste (DelaRosa et al. 2012; Zhang et al. 2013). The American Society of Plastic Surgeons 2015 annual report registered a total of 222,051 liposuction procedures in 2015 alone https://plasticsurgery.org 2017. Thus, there is a vast number of potential ASCs going to waste.

Many reasons that make ASCs such an attractive cell type for regenerative medicine applications are: their multipotent potential for regenerative uses; ease of isolation; plentiful source; either autologous or allogeneic use; and their innate ability induce angiogenic traits. ASCs have been demonstrated to mediate robust anti-inflammatory and immunomodulatory effects, which have led to various preclinical studies and clinical trials to investigate therapeutic efficacy. Tissue inflammation activates ASCs to produce anti-inflammatory cytokines and angiogenic factors (Zuk et al. 2001; Gimble et al. 2007; Bourin et al. 2013). The immunomodulatory function of ASCs results in an environment where ongoing inflammation is minimized, and a regenerative environment is promoted to restore homeostasis. The regenerative and anti-inflammatory potential of these cells have led to studies using these stem cells across various disciplines, examples of which are covered in this chapter.

2 ASCs in Regenerative Medicine

2.1 ASCs as Therapy for Cardiac Disease

ASCs are multipotent and retain the ability to differentiation into mesodermal tissues. The potential to regenerate cardiomyocytes with ASCs has drawn substantial research attention due to the increasing incidence of myocardial infarctions (MI) and the poor prognosis associated with scar tissue formation in the myocardium post-MI (Nian et al. 2004; Gnecchi et al. 2008; Segers and Lee 2008; Aguirre et al. 2013; Le and Chong 2016).

Ischemic heart disease (IHD) is the leading cause of death worldwide. Much of the burden of IHD is due to the inability of the cardiac tissue to regenerate after cardiac events. Instead, infarctions lead to an inflammatory response, which ultimately results in necrosis of the infarct zone, rendering the region unable to participate in electrical conduction or cardiac muscle contraction. Currently, there are no effective ways to regenerate cardiac muscle tissue. ASCs have been studied for their ability to regenerate cardiac tissue and/or improve cardiac function in multiple model systems. The first study to demonstrate mesenchymal stem cells' ability to differentiate into cardiomyocytes by Rangappa et al. used rabbit ASCs (Rangappa et al. 2003). Another group demonstrated differentiation of human ASCs after exposure to rat cardiomyocyte proteins (Gaustad et al. 2004). These studies examined structural and functional capacities of cardiomyocytes to characterize differentiation; however, no *in vivo* experiments were preformed.

Valina et al. used a porcine model to show that intracoronary administration of autologous ASCs 15 min after reperfusion improved various cardiac functions post-infarction (Valina et al. 2007). The results showed significantly greater capillary density in the infarct border zone, wall thickness (WT) and left ventricular ejection fraction (LVEF), all in the ASC-treated group compared to the control 30 days post-procedure. A rat model was also used to assess the benefits of ASCs in the setting of chronic heart failure. The ASC-treated group showed a significantly improved LVEF and reduced infarct area (Mazo et al. 2008).

A Phase 1/2 clinical trial has also investigated the safety of SVF in patients with post-MI IHD. A total of 28 patients participated in the study. After noticing similar results as in the aforementioned studies (significantly increased LVEF and increased WT) and better patient performance in the 6-min walk test at the 3-, 6- and 12-month follow up compared to baseline (before cell

transplantation), with no significant adverse effects, the authors concluded that the intramyocardial injection of 30 million cells was safe and efficacious (Comella et al. 2016). Despite a limited number of patients tracked at the various time points and lack of a control group, these results provide some support that SVF may benefit patients with decreased ventricular performance due to MI-induced scarred cardiac tissue.

2.2 ASCs and Biomaterials

ASCs represent a cell source that has the potential to transform the field of tissue engineering and regenerative medicine. Seeding biomaterials, like poly(lactic-co-glycolic) acid (PLGA), Poly(vinyl alcohol) PVA, decellularized extracellular matrix or chitosan with ASCs has been used to assist in healing and regeneration of muscle, cartilage, functional fat tissue, tendon, and bone (D'Andrea et al. 2008; Dufrane et al. 2015; Vaicik et al. 2015; Choi et al. 2016; Bjorninen et al. 2017; Dufrane 2017; Farnebo et al. 2017).

Once implanted, one of the most important considerations for any tissue engineering product is the supply of nutrients to the scaffold and removal of cellular waste from the scaffold, either through transport through the biomaterials via diffusion and/or by encouraging neovascularization within the tissues. In the field of tissue engineering a major roadblock is blood vessel formation, especially for thick or dense materials. Substantial research is required to overcome these barriers by utilizing the innate proangiogenic traits of ASCs to induce blood vessel formation to supply engineered tissues with the necessary route for nutrients and waste transport.

2.2.1 ASC and Biomaterials for Angiogenesis

Harnessing and enhancing the angiogenic traits of ASC using biomaterials is of interest to the regenerative medicine field. It was demonstrated that the ASC encapsulated in larger PLGA spheroids were found to upregulate angiogenic growth factors and adipogenesis in vitro, and allowing of the scaffolds to recapitulate significant vascular ingrowth in vivo in a nude mouse model (Zhang et al. 2017). Similarly, other groups have also been working towards capitalizing on the angiogenic and/or adipogenic capabilities of ASC in tissue engineering application (Vaicik et al. 2015; Miyamoto et al. 2017). ASC have also been encapsulated in thermosensitive hydrogels, like chitosan/ gelatin mixtures, to create an injectable for therapeutic angiogenesis for ischemic materials by allowing for more prolonged survival of dissociated ASC (Cheng et al. 2017). Utilizing materials like these with pre-seeded ASC, it has been found to increase angiogenic growth factor concentration in the growth media *in vitro*, allowing for a more considerable amount of tubule formation in the hydrogel when co-cultured encapsulated with endothelial cells. Data from *in vivo* studies demonstrate higher densities of capillaries were found when applying the encapsulated ASCs in a chick embryo chorioallantoic membrane assay (Cheng et al. 2017). Like adipose tissue, other soft tissue applications have benefitted from ASCs. Choi et al. used an elastin-like polypeptide matrix with ASCs where the hydrogel would coagulate in the wound site. This is beneficial because it could mold to any wound shape, enabled retention of the ASCs at the wound site to promote regeneration, and activated wound clotting to promote faster regeneration (Choi et al. 2016). Conductive biomaterials were used with ASCs to help stimulate vascular smooth muscle repair. These scaffolds with stimulation improved ASC viability and differentiation towards smooth muscles cells improving their utility in vascular tissue engineering applications. Electrical stimulation systems may be a means to enhance differentiation for tissue engineering applications (Bjorninen et al. 2017). Alternatively, decellularized porcine small intestinal submucosa with human ASCs have been shown to be an effective biological scaffold for hernia repair in rat models. Treating the scaffold with fibronectin before ASC seeding improved ASC attachment and histology demonstrated the presence of the stem cells in the scaffold up to 1 month post-op

(Klinger et al. 2016). The retention of stem cells in these living scaffolds makes them attractive candidates for long-term sustainable regenerative therapies.

3 ASCs as Immunomodulators

3.1 ASCs as Therapy for Autoimmune Diseases

Lymphocytes are integral cells of the immune system. More specifically, $CD4^+$ helper T (Th) cells can be divided into subsets that distinguish the effector cells, Th1, and Th2 cells, and the regulatory T cells (Tregs) that maintain the balance between autoimmunity and immune tolerance, respectively. These effector T cells play a critical role in promoting autoimmune diseases, especially Th1 cells, which have been further delineated to include Th17 cells. Secreted pro-inflammatory cytokines by Th1 and Th17 cells perpetuate antigen-specific responses. During autoimmunity, Th1 and Th17 aberrantly recognize self-antigens and drive an immune response that propagates a cascade of pathologic events (Skapenko et al. 2005; Gonzalez et al. 2009; Zhou et al. 2011).

The immunomodulatory capacities of ASCs have been investigated to regulate the Th1/ Th2 balance and promote Tregs to restore immune tolerance in autoimmune diseases. ASCs secrete anti-inflammatory cytokine interleukin-10 (IL-10) that enhances Tregs' activity, which responds by further secreting and amplifying IL-10 signaling (Chaudhry et al. 2011; Park et al. 2015). Tregs and associated IL-10 attenuate the activities of Th1 and Th17, which, in turn, reduce the recruitment of additional pro-inflammatory immune cells to sites of pathology (Skapenko et al. 2005; Chaudhry et al. 2011). These anti-inflammatory and immunomodulatory effects of ASCs have been demonstrated in several preclinical models of autoimmune diseases.

3.1.1 Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune disease against integral components of the central nervous system (CNS) that leads to neurodegeneration and inflammation. Using the murine experimental autoimmune encephalomyelitis (EAE) model of MS, ASCs have demonstrated attenuation of disease that leads to comprehensive improvements. Treatment with ASC before the onset of EAE led to amelioration of the disease course by robust immunomodulation that countered Th1-mediated pathology. Collectively, infusion of ASCs resulted in reductions in tissue damage, and cellular infiltrates and preservation of myelin in the CNS, which ameliorated symptoms of this disease (Constantin et al. 2009; Riordan et al. 2009; Semon et al. 2013, 2014).

3.1.2 Rheumatoid Arthritis

Another common autoimmune disease, rheumatoid arthritis (RA), is characterized by Th1-mediated tissue damage and inflammation within joints. Th17 cells have also been correlated with the production of granulocyte-macrophage-colony stimulating factor (GM-CSF) that leads to the recruitment and subsequent infiltration of cells causing an inflammatory milieu and tissue damage (De Bari 2015; Lopez-Santalla et al. 2015). Using a mouse model of RA, treatment with ASCs diminished this pathogenic signaling while increased the Tregs. The production of IL-10 and generation of antigen-specific Tregs was attributed to treatment with ASC, which suggests re-establishment of immune tolerance. These studies demonstrate the immunomodulatory potency that attenuated pathogenic processes and countered autoimmunity that led to reduced incidence and severity of experimental arthritis (Constantin et al. 2009; Gonzalez et al. 2009; Zhou et al. 2011; Lopez-Santalla et al. 2015).

3.1.3 Type 1 Diabetes Mellitus

Type 1 diabetes mellitus (T1DM) is caused by autoimmune destruction of insulin-producing cells of the pancreas that results in hyperglycemia and abnormal glucose metabolism due to insulin deficiency (Lin et al. 2015). In a preclinical model of T1DM, intravenous administration of ASCs reportedly reduced fasting blood glucose levels, increased expression of insulin protein, and

suppressed islet injury (Li et al. 2012). Another interesting approach using ASCs for treatment of T1DM led to promising results. ASCs were transduced to express insulin and subsequently transplanted into the pancreas of T1DM animals. By harnessing the anti-inflammatory effects and the production of insulin, treatment with transduced ASCs lowered blood glucose levels and decreased glucose tolerance while improving the overall appearance of the animals (Lin et al. 2009).

3.2 ASCs in Neurodegenerative Diseases

Neurodegenerative diseases involve several pathophysiological mechanisms beyond the loss of neurons that determine the course and severity of illness, including neuroinflammation, mitochondrial dysfunction, and protein aggregation. While the brain is thought to be immune privileged there are several cell types that mediate debris clearance and regulate the environment of the CNS. Microglia are tissue-resident macrophages, and whether polarized to the classical pro-inflammatory or alternative anti-inflammatory activation states, can fight against or contribute to the hallmarks of neurodegenerative pathology. ASCs have been gaining attention as therapeutic candidates due to their ability to secrete neurotrophic and immunomodulatory mediators, restore mitochondrial function, promote neurogenesis, modulate glial activation states, enhance protein clearance, and fight neuroinflammation in neurodegenerative pathologies. The following section will summarize recent findings on the efficacy of ASCs in treating three distinct neurodegenerative diseases: Alzheimer's disease, Parkinson's disease, and Amyotrophic Lateral Sclerosis (ALS).

3.2.1 Alzheimer's Disease

Alzheimer's disease (AD), the most common cause of dementia amongst the aging population, is characterized by amyloid-beta (Aβ) plaques and neurofibrillary tangles, limbic system neurodegeneration, and progressive cognitive decline (Tanzi 2013). Using a mouse model of familial early-onset AD, ASCs were tested for their preventive and therapeutic effects by administering therapy at pre- and post-symptomatic time points. Both groups showed improved performance on the Morris Water Maze (MWM) task, a significant reduction in Aβ plaque formation in the cortex, and reduced protein levels of Aβ and amyloid precursor protein (APP) with enhanced levels of Aβ-degrading enzymes (Kim et al. 2012). In another AD mouse model, ASC treatment led to increased secretion of anti-inflammatory cytokines, enhanced expression of Aβ-degrading enzymes, and improved performance on learning and memory tasks (Ma et al. 2013). Additionally, mice showed increased brain levels of the anti-inflammatory cytokine IL-10, which polarizes microglia towards the alternative activation phenotype, and several angiogenic and neurotrophic factors (Kim et al. 2012; Ma et al. 2013; Hu et al. 2015).

Other studies demonstrated that ASCs might attenuate symptoms of AD by promoting neurogenesis. Intracerebral injection of ASCs gave rise to significantly higher numbers of newly generated bromodeoxyuridine (BrdU)-positive cells than vehicle-treated controls in both the dentate gyrus and subventricular zone, some of which differentiated into mature neurons (Yan et al. 2014). One proposed mechanism for this enhanced neurogenesis is the leptin secreted from ASCs, as this hormone alone has been shown to promote neurogenesis and reduce neurodegeneration in an AD mouse model (Perez-Gonzalez et al. 2011). Together, these studies highlight the capacity of ASCs to promote enhanced clearance of harmful protein aggregates, stimulate neurogenesis, and modulate the neuroinflammatory environment by secreting immunomodulatory cytokines in Alzheimer's disease models.

3.2.2 Parkinson's Disease

Parkinson's disease (PD) is the second most common cause of neurodegeneration and is characterized by progressive dopaminergic neuronal loss in the substantia nigra pars compacta (SNpc) and progressively worsening motor

symptoms. ASCs have been investigated in PD animal models to determine if and how they may attenuate neurodegenerative damage. In a rat model of PD, ASCs injected directly into the SNpc exerted neuroprotective effects via enhanced secretion of soluble growth factors (McCoy et al. 2008). Additionally, ASC treated rats had significantly decreased numbers of activated microglia in the lesioned brain areas compared to untreated controls, suggesting that these soluble factors are also impacting the neuroinflammatory aspects of PD (McCoy et al. 2008). ASCs also enhanced both acute and long-term neurogenesis in rat models of PD, which correlated with amplified secretion of anti-inflammatory cytokines and brain-derived neurotrophic factor (BDNF) (Schwerk et al. 2015). Despite this observed benefit, ASCs were unable to protect dopaminergic neurons from acute damage, and the newly created neurons are not able to functionally replace lost dopaminergic neurons (Schwerk et al. 2015). However, in a mouse model of PD long-term dopaminergic cell survival was significantly higher with ASC treatment (Choi et al. 2015).

3.2.3 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a debilitating neurodegenerative disease characterized by the rapid and progressive loss of upper and lower motor neurons, muscle wasting, and death for most patients within 5 years of diagnosis (Rowland 2001). In recent years ASC therapies have been investigated in animal models for their potential benefit in ALS, with some successes. In ALS mouse models, both single and repeated daily injections of ASCs resulted in preserved motor neuron survival, delayed disease progression, and fewer reactive astrocytes in the spinal cord that can contribute to neuronal cell death (Marconi et al. 2013; Fontanilla et al. 2015). Similar results were seen when ASCs were given before symptom onset, suggesting ASCs may have preventive capability (Kim et al. 2014). The injected ASCs persisted in the CNS in their undifferentiated state, indicating that the therapeutic benefit resulted from soluble factors secreted by the transplanted cells rather than engraftment and differentiation (Kim et al. 2014). Several lines of evidence further support the notion that ASCs exert their beneficial effects via secretion of paracrine factors. ASCs co-cultured with ALS mouse astrocytes demonstrates increased production of angiogenic and neurotrophic factors, upregulation of a critical suppressor of glutamate excitotoxicity and inhibition of apoptotic signals (Rothstein et al. 1995; Rossi et al. 2008; Gu et al. 2010). When neural stem cells from ALS mice are co-cultured with ASC-derived exosomes, small vesicles secreted from the cells, aberrant protein aggregation was suppressed while imbalanced mitochondrial protein levels were restored (Lee et al. 2016).

4 Clinical Applications of ASCs

4.1 Clinical Trials

To report on completed and ongoing clinical trials studying the utility of ASCs in the treatment of human disease, information was collected from the database http://clinicaltrails.gov/ 2017. The parameters of this search included studies using "adipose stem cells" and/or "stromal vascular fraction" that were ongoing or completed and excluded trials that had been withdrawn, were terminated, were not yet recruiting, or had unknown status. The details of the clinical studies were evaluated to include reports of using the stromal/stem progenitors from adipose tissue and/or SVF, excluding duplicates and studies of other mesenchymal stem cells, i.e., BM-MSCs. To date, there have been 165 clinical trials involving ASCs and/or SVF in the treatment of human disease. These studies have taken place in various fields with most of the trials focused orthopedic applications ($n = 39$) followed by autoimmune disorders ($n = 26$). Figure 1 details the areas in which ASCs are administered as a potential therapy in clinical trials found from the search. These clinical trials are conducted around the world with the majority taking place in the United States ($n = 54$) followed by South Korea ($n = 25$) and Spain ($n = 21$).

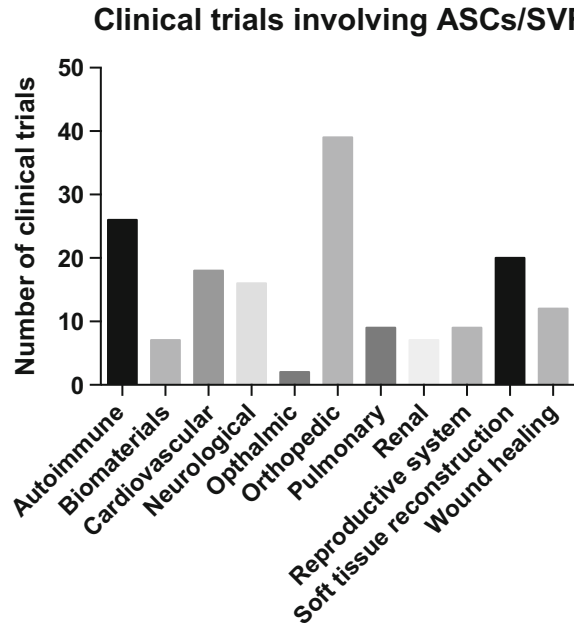

Fig. 1 Clinical trials utilizing ASC or SVF. The distribution of medical areas in which clinical trials utilizing ASC or SVF have been or are being conducted with the highest occurrence of trials in orthopedics

5 Future Directions

There has been growing evidence in vitro and in vivo suggesting the potential role of ASCs as therapy for numerous disease processes. ASCs constitute a seemingly attractive option for clinical treatment. Adipose tissue is abundant and readily procured with minimal risk to patients. ASCs obtained through lipoaspirate are easily expanded. Additionally, these stem cells are immune privileged, meaning allogeneic cells could potentially be used for cellular therapy (though autologous cells are preferred). While a large number of studies exist, more preclinical and clinical evidence is needed to determine if ASCs will meet the expectations of their utility to treat diseases with limited/inadequate current therapeutic options. In the continued work with these cells, it will be essential to study the appropriate timing of treatment in various diseases, the optimal method of delivery whether it be local or systemic, as well as the number of stem cells needed for therapeutic efficacy. The field has come a long way, and there is evidence supporting the use of ASCs and SVF in the clinic. However, it is essential to address these gaps as we move forward to harness the full potential of ASC therapy.

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Decellularized Extracellular Matrix as a Potent Natural Biomaterial for Regenerative Medicine

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Abstract

Decellularization technique is a favorable method used to fabricate natural and tissue-like scaffolds. This technique is important because of its remarkable ability to perfectly mimic the natural extracellular matrix (ECM). ECM-based scaffolds/hydrogels provide structural support for cell differentiation and maturation. Therefore, novel natural-based bioinks, ECM-based hydrogels, and particulate forms of the ECM provide promising strategies for whole organ regeneration. Despite its efficacious characteristics, removal of residual detergent and the presence of various protocols make this technique challenging for scientists and regenerative medicine-related programs. This chapter reviews the most effective physical, chemical, and enzymatic protocols used to remove the cellular components and their challenges. We discuss the applications of decellularized ECM (dECM) in tissue engineering and regenerative medicine with an emphasis on hard tissues.

Keywords

Bioink · Decellularization · Decellularized ECM · ECM mimicry · Tissue engineering

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Abbreviations

AF	Annulus fibrosus
ASCs	Adipose stem cells
BdECM	Bladder decellularized ECM
BMSCs	Bone marrow MSCs
cECM	Cardiac muscle ECM
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate
dDP-ECM	Decellularized dental pulp ECM
dECM	Decellularized ECM
DVN	Decellularized vascular network
ECM	Extracellular matrix

FPSCs	Fat pad-derived stem cells
GAGs	Glycosaminoglycans
hMSCs	Human mesenchymal stem cells
NTIRE	Non-thermal irreversible electroporation
PAA	Peracetic acid
PDCs	Periosteum-derived cells
PLGA	Poly (lactic-co-glycolic acid)
PRP	Platelet-rich plasma
SDC	Sodium deoxycholate
SDS	Sodium dodecyl sulfate
sECM	skeletal muscle ECM
ToF-SIMS	Time of flight secondary ion mass spectroscopy
TX-100	Triton X-100
UBM	Urinary bladder matrix
WdECM	Wharton's jelly dECM

1 Introduction

The extracellular matrix (ECM) provides structural support and controls cellular behavior. Although the major components of the ECM are conserved among various tissues, each tissue ECM contains several specific and unique compositions. Therefore, mimicking the biological and structural arrangements of each ECM provides an appropriate network to generate related organs and overcome tissue donor leakage. An excellent scaffold for engineered tissue would be natural ECM derived from the target tissue. However, it is difficult to accurately mimic native ECM because of the versatility in functions, multiple compositions, and dynamic nature of native ECM. Tissue engineering is a promising strategy for development of artificial organs and tissue regeneration, which has used decellularization to create tissue-like scaffolds. The decellularization process used to isolate the ECM of a tissue is a desirable procedure for fabrication of natural and tissue-like scaffolds.

Thus far, decellularization has been used to derive scaffolds from the skin (Brouki Milan et al. 2019), fat (Morissette Martin et al. 2018), pericardium, heart (Sesli et al. 2018), skeletal muscle (Naik et al. 2019), and liver (Willemse et al. 2020) tissues for both in vivo and in vitro experiments. These scaffolds have assisted with regeneration or formation of site-appropriate tissues when used as biomaterials in vivo. In some cases, the tissue-specific effects of these scaffolds were observed on cellular behaviors (Fernandez-Perez and Ahearne 2019). Recently, hydrogels that originated from decellularized tissues have emerged as an ideal network to provide a local reservoir of growth factors, and structural and biochemical cues for cellular regrowth. Hydrogels are injectable biomaterials that enable on-demand personalized three-dimensional (3D) scaffold fabrication with 3D-bioprinting and bio-plotting. Various organ-derived decellularized ECM (dECM) bioinks have been created from different tissues such as muscles, cartilage, the heart, liver, skin, and adipose tissue, as the most biomimetic bioinks; however, they suffer from limitations attributed to decellularization (Dzobo et al. 2019). Here, we describe the chemical, physical, and enzymatic treatments used to generate ECM that has the highest similarity to native ECM, with a major focus on hard tissues. We discuss how researchers could balance removal of potentially immunogenic components and maintain indispensable ECM components that are necessary for proper cell function. In addition, we review recent advances using dECM in tissue engineering and regenerative medicine.

2 Natural Extracellular Matrix (ECM), a Naïve Version of ECM Versus Decellularized ECM (dECM)

The ECM composition of each tissue and organ are determined by its resident cells, which are distinct in number and nature. The ECM is comprised of various molecules that have specific structural and biological properties which determine the final fate of the cells. These molecules consist of various structural and functional proteins, glycoproteins, and glycosaminoglycans (GAGs) organized in a unique ultrastructure. Collagen, especially type I, a variety of proteoglycans

(e.g., decorin), GAGs (e.g., heparin, heparan sulfate, chondroitin sulfate, and hyaluronic acid), and adhesion molecules (e.g., fibronectin) are some of the ECM molecules (Dzamba and Desimone 2018). Matrix molecules fill the spaces between cells and their junctions provide 3D structural support for the surrounding cells. These tissue-specific networks modulate cell migration, biomechanical force transmission, and mechanical behaviors of tissues (Jansen et al. 2017).

The ECM plays a crucial function in fetal development and determination of stem/progenitor cell fate, and influences cellular shape, survival, and proliferation. The physical features of the ECM such as topography, insolubility, rigidity, and porosity originate from its matrix composition and determines the mechanical behavior of each tissue. More precisely, integrin and the direction of collagen fibers, particularly in anchorage-dependent cells, modulate intra- and extracellular signals (Aiyelabegan and Sadroddiny 2017).

This unique and natural microenvironment has attracted the attention of researchers who aim to fill the present gap in biomaterial science. Specific ECM-based substrates that consist of unique ECM elements have been used in a wide range of preclinical and clinical applications (Hinderer et al. 2016; Masaeli et al. 2017; Giobbe et al. 2019). The ECM has been extracted from various tissues, including the skin, heart valves, nerves, blood vessels, skeletal muscles, ligaments, tendons, urinary bladder, submucosa of the small intestine (Stapleton et al.), and liver (Hoshiba et al. 2016). Nature, orientation, and the quantity of matrix molecules differ from tissue to tissue and are the fundamental factors used to specify the scaffold.

Given the complexity of the ECM, it is almost impossible to completely mimic this unique 3D structure. Therefore, decellularization is an alternative approach to obtain ECM from natural tissues. The aim of decellularization is to maximize removal of cellular components while minimizing loss and damage to the ECM (Xing et al. 2015). The decrease in immunogenicity of decellularized tissues makes them suitable for allogenic use. dECM is a natural scaffold that provides mechanical, structural, biological, and biochemical cues for cell proliferation, adhesion, migration, and differentiation. Both biocompatibility and a natural structure, as inherent features of dECM, represent off-the-shelf biomaterials that have favorably mimicked the extracellular niche (Gao et al. 2017). Although dECM preserves native bioactive proteins and molecules, scientists are looking for a permanent dECM that has broad applications in multiple tissues and solve the problem of inaccessibility, complexity, compactness or laxity that is present in some tissues. For example, Wharton's jelly ECM has been used as a permanent dECM for articular cartilage regeneration. Wharton's jelly dECM (WdECM) is an ideal candidate due to the type and levels of some of the chondrogenic growth factors such as IGF-I and TGF-β. Research results have indicated that WdECM has a unique function and performance compared with cartilage tissue (Xiao et al. 2017). Since skeletal muscle ECM (sECM) is similar to cardiac muscle ECM (cECM) in terms of structure and mechanical properties, the use of decellularized-sECM as a scaffold has been suggested for myocardial tissue engineering. Cell adhesion, proliferation, and cardiac differentiation potency were compared between the decellularized-cECM and decellularized-sECM. Upregulation of cardiac-specific markers α-MHC, MLC2v, and ANP at day 16 confirmed the higher cardiac differentiation potency of sECM. sECM is superior to cECM because it can be easily harvested, which enables a replacement for cECMs with low immunogenicity and excellent biocompatibility (Hong et al. 2018). These features of dECM make it a naïve scaffold that can be functionalized with different moieties and has the potential to be implanted as a permanent ECM in various tissues.

3 Versatility of Decellularization Protocols

The main goal of decellularization protocols is to eliminate most cellular ingredients in order to

reduce immunological responses and preserve physiochemical properties of the ECM. Various parameters, such as tissue thickness, density, and cellularity can be effective in selection of an appropriate decellularization protocol. The decellularization procedure may affect the composition and ultrastructure of ECM biomolecules, and these alterations adjust the cell behaviors during recellularization. It is highly desirable to minimize the deterioration of the ECM during decellularization and preserve an acceptable balance between cellular elimination and retention of ECM components.

Traditionally, the processes of decellularization can be categorized into physical, chemical, and enzymatic methods. It is essential to select a proper method or the correct chemicals to decellularize a distinct tissue to attain perfect decellularization and preserve an intact ECM. The type and the rigidity of the tissue or organ has tremendous impact on the decellularization outcome. It is challenging to obtain dECM from hard tissues, such as bones and biphasic tissues (osteochondral tissues).

The detailed process and methods described in the literature combine several of these major techniques to improve the efficacy of decellularization and simultaneously reduce undesirable effects on the ECM ultrastructure. An overview of some commonly used chemical, enzymatic, and physical agents, including their efficiency in the decellularization process and preservation of extracellular tissue components is provided (Fig. 1).

3.1 The Efficiency of Physical Methods for Decellularization

Some studies have evaluated the use of physical methods for decellularization rather than chemical agents, which have adverse effects. Hung et al. combined freeze-drying and sonication during the defreezing process to remove cellular components from larynx tissue. The drying was performed to eliminate ice produced during the freezing process and assist with sonication treatment after freezing. The main advantage of this method was the propensity of tissue to absorb fluids during rehydration after freeze-drying. Moreover, this technique might be used to promote the infusion of other decellularization reagents such as detergents and enzyme solutions (Hung et al. 2013). Multiple freeze-thaw cycles have also been used to decellularize tissues (Burk et al. 2013). Although freeze-thaw cycles make negligible disruptions in the ECM structure and the mechanical properties of tissues, they are insufficient for complete cell removal. It has been suggested that this procedure can be modified by the addition of other chemical or enzymatic decellularization protocols (Hung et al. 2013).

Sonication, mechanical agitation, direct pressure, and non-thermal irreversible electroporation (NTIRE) are other physical methods used for tissue decellularization. These methods have been simultaneously employed with enzymatic or chemical treatments to accelerate cell lysis and removal of cellular debris.

3.2 The Efficiency of Chemical Methods for Decellularization

Different types of chemical agents that used for decellularization include detergents (ionic, nonionic, or zwitterionic), acids, and bases. These agents generally lyse and disrupt the cell membranes, resulting in removal of cells and genetic materials.

White et al. used time of flight secondary ion mass spectroscopy (ToF-SIMS) to analyze detergent-based decellularization. The increased sensitivity of ToF-SIMS enabled them to assess the differences in the composition of porcine urinary bladder matrix (UBM) following treatment with sodium dodecyl sulfate (SDS), Triton X-100 (TX-100), sodium deoxycholate (SDC), peracetic acid (PAA), and 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS). Scaffolds treated with TX-100 and SDC preserved the complex fiber network of native UBM samples; however, treatment with CHAPS, SDS, and PAA changed collagen fiber organization. ToF-SIMS analysis found residual detergent pieces in the UBM scaffolds treated

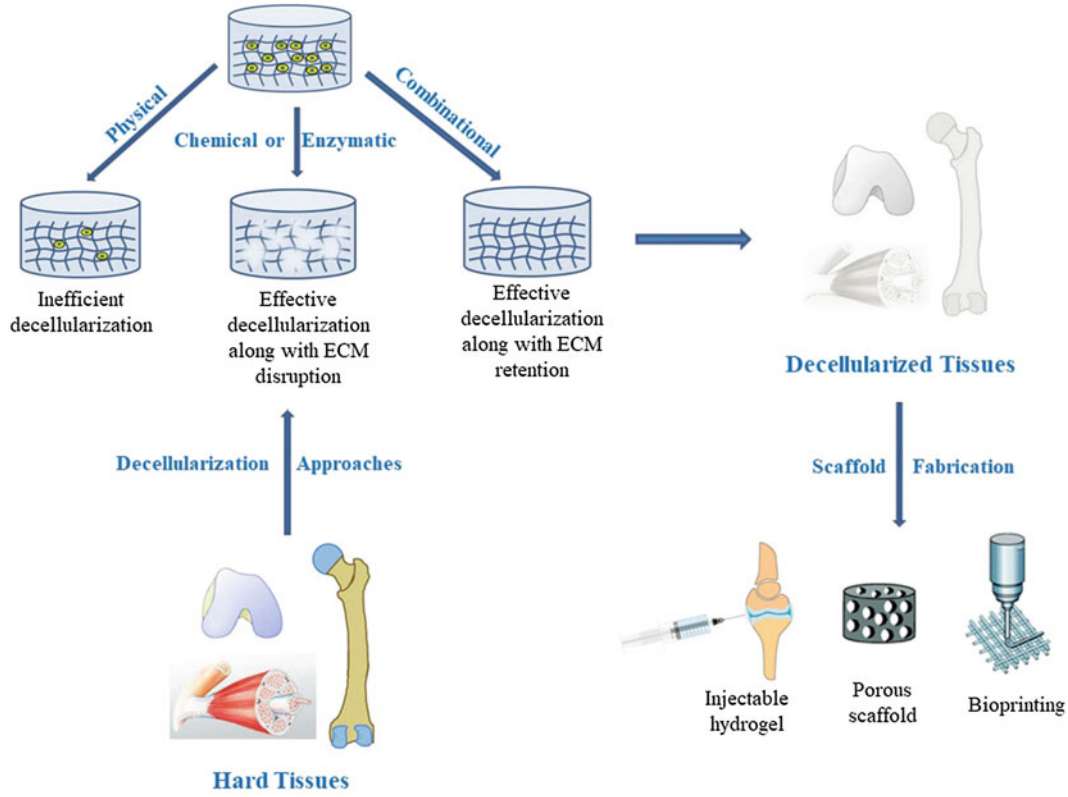

Fig. 1 Schematic representation of decellularization approaches and application of decellularized matrix-based materials for hard tissue engineering. Decellularization approaches can be classified into three categories: Physical with inefficient decellularization, chemical or enzymatic with effective decellularization along with ECM disruption and combinational approaches which lead to effective decellularization and ECM retention. Decellularized tissues can be manipulated to create various types of engineered scaffolds such as injectable hydrogels or bio-printed constructs

with SDC, SDS, and TX-100. Particularly, more intense deprotonated molecular ions were recorded in UBM samples treated with either SDC or TX-100 in comparison with the other detergent treatments. Samples treated with SDS exhibited the highest molecular ion intensity, which was concentration-dependent. CHAPS detergent was not detected due to its neutral charge, and might have been eliminated by washing. Furthermore, this zwitterionic detergent is highly susceptible to fragmentation. This study indicated the capability of ToF-SIMS to find cellular nuclei and membrane segments in biological scaffolds. ToF-SIMS analysis showed the existence of choline, phosphocholine, and glycerophosphocholine ions, which are crucial for the structural integrity of cell membranes in native, PAA, and CHAPS treated UBM. Higher intensities of phosphate groups, the main feature of cell nuclei, were observed in in the PAA and CHAPS treated UBM in comparison with TX-100, SDC, and SDS treated samples. Although the intensity of the phosphocholine in UBM treated with PAA was identical with the native sample, the phosphate group was less intense in this group when compared with native tissue. This finding showed that PAA was more efficient in elimination of cell nuclear components than cell membrane ingredients. The presence of phosphate and phosphocholine ions, which are indicative of cellular remnants, provides the criteria for an efficient decellularization process. Particularly, UBM samples treated with PAA or CHAPS did not

completely remove the cellular components as demonstrated by the presence of residual cellular components. Treatments with 1% SDS or SDC were the most effective for complete decellularization due to the high efficiency of these ionic detergents. However, decreased intensities of phosphate and phosphocholine ions showed that treatment with 0.1% SDS and non-ionic TX-100 were not as efficient as the higher concentration ionic detergents (White et al. 2017).

Chen et al. developed a novel, cost-effective, easily reproducible decellularization process based on acid treatments for porcine menisci tissue. This method was developed to avoid any surfactant treatment or enzymatic digestion. The researchers used different concentrations of acetic acid, formic acid, and PAA within a 2–12 h incubation time. The results indicated that acetic acid treatment for 2 h caused a significant reduction in collagen content (Chen et al. 2015a). Dong et al. previously demonstrated that decellularization of bovine pericardium with acetic acid resulted in severe destruction of collagen and, therefore, reduced tissue strength despite retention of the majority of the GAG (Dong et al. 2009). Treatment with PAA for 2 h showed a small effect on decellularization of the menisci, while 15% PAA treatment for 10 h resulted in reductions of DNA (4.68%), collagen (44.98%), and GAG (19.04%). PAA was unable to infiltrate throughout the menisci within a short period of time due to the dense architecture of the tissue. However, prolonged processing with PAA led to deterioration of the ECM.

Treatment with formic acid for 2 h resulted in efficient menisci decellularization but preserved a substantial portion of the ECM. Formic acid induced swelling in the menisci and exposed the embedded chondrocytes because of its high capability for infiltration through the dense meniscal structure. The amount of DNA within the dry menisci tissue was 37.20 ng/mg after 2 h treatment with formic acid. In this study, formic acid treatment was introduced as an economical, efficient, and convenient procedure for decellularization of meniscus tissue. This acellular scaffold promoted the chondrocyte phenotype and chondrogenic differentiation of human mesenchymal stem cells (hMSCs) (Chen et al. 2015a, b).

Although chemical approaches are more efficient than physical treatment in decellularization, their function mainly depends on maintenance of the ECM structure, which is critical for repair of targeted tissue.

3.3 Combined Chemical and Physical Methods for Decellularization

Physical methods facilitate tissue decellularization but they are insufficient for complete decellularization, especially in tissues that have complicated structures. Chemical and enzymatic treatments are more effective in removing cellular components such as nuclear residues and cytoplasmic proteins from dense tissues. On the other hand, these treatments may damage the main components of the ECM (collagen, GAGs, and growth factors). Therefore, a combination of decellularization methods have been used to develop an acellular scaffold to reconstruct target tissues (Woods and Gratzer 2005; Faulk et al. 2014).

Farag et al. used static and perfusion systems to assess different protocols for the decellularization of human periodontal ligament cell sheets. They utilized an electrospun polycaprolactone membrane to reinforce the cell sheet during decellularization. Assessments of various protocols showed that treatment with ammonium hydroxide, TX-100, and DNase resulted in effective DNA removal, preserved the integrity of the ECM, and retained growth factors (Farag et al. 2018). Researchers have reported the benefits of the perfusion technique in terms of economics, practicality, and less complicated features, all of which could be important for future commercialization. Therefore, the nature of the chemicals, detergents, and enzymes used during the decellularization determine the maintenance and integrity of the ECM ultrastructure.

The combination of freeze-thaw cycles with chemical reagents has been extensively used to

enhance the efficiency of the decellularization process for hard tissues. Burk et al. evaluated the decellularization of equine superficial and deep digital flexor tendons after treatment by repeated cycles of freeze-thawing and two chemical detergents, TX-100 and SDS. Four different protocols that included TX-100 and SDS treatment alone and the combination of freeze-thaw cycles with either TX-100 or SDS were evaluated. The results demonstrated that the freeze-thaw cycles could improve the efficiency of the decellularization process. However freeze-thaw cycles alone could not adequately remove DNA. The combination of freeze-thaw cycles with detergent led to increased cell and DNA removal compared to the groups that were only treated by detergent. Freeze-thaw cycles combined with TX-100 facilitated cell distribution into the scaffold (Burk et al. 2013). In a similar study, Youngstrom et al. developed a protocol based on the combination of treatment with 2% SDS detergent, a freeze/thaw cycle, trypsinization, DNase-I digestion, and ethanol sterilization to remove cellular components from equine flexor digitorum superficialis tendons. The authors reported that their protocol efficiently removed cellular components and also preserved the biochemical composition, ultrastructure, and mechanical properties of the decellularized tissue (Youngstrom et al. 2013).

Lu et al. compared seven methods for decellularization of ECM derived from a 3D cell culture. These researchers cultured human bone marrow MSCs in PLGA (poly(lactic-co-glycolic acid)) templates for 5 days to allow the ECM to deposit around the PLGA substrate. After construction of a cell-ECM-PLGA complex, they removed the cellular components. dECM scaffolds were prepared after treatment with seven decellularization methods: freeze-thaw cycling; ammonium hydroxide; osmotic shock; combined freeze-thaw cycling with either ammonium hydroxide or osmotic shock; detergent (TX-100) extraction; and detergent extraction with osmotic shock (Lu et al. 2012). A comparison of these methods showed that combined freeze-thaw cycling with ammonium hydroxide and detergent extraction along with osmotic shock (treatment with 1.5 M KCl) efficiently removed the cellular components. These two methods preserved the gross appearance and microstructure of the ECM and induced mild host responses following subcutaneous implantation in mice. Freeze-thaw cycling alone was ineffective for cell removal even after ten cycles. Ammonium hydroxide alone could not properly decellularize the scaffold because of the dense architecture of the 3D matrix that prevented its infiltration into the scaffolds. However, the combination of freeze-thaw cycling with ammonium hydroxide resulted in efficient removal of the cellular components. In agreement with these results, freeze-thaw cycling combined with detergent (SDS) and nuclease (DNase and RNase) treatment led to optimal decellularization of porcine meniscus (Stapleton et al. 2008). Although TX-100 and osmotic shock (1.5 M KCl) alone did not successfully remove the cellular components, the combination of these methods efficiently decellularized the scaffold.

A similar study was conducted to optimize the decellularization process for ECM generated by MC3T3-E1 pre-osteoblast cells. dECM obtained via three freeze-thaw cycles and treatment by TX-100 provided a suitable niche for proliferation and stemness preservation of bone marrow MSCs (BMSCs). The decellularization procedure almost entirely eliminated the DNA; however, both the architecture and protein distribution of the ECM were properly retained without loss of bioactivities in the BMSCs culture. The results demonstrated a positive effect of TX-100 for permeabilization of the cell membrane and assisted in efficacious cell disruption induced by the freeze-thaw cycles. The dECM had minimum DNA content with high integrity as evidenced by morphology, protein content, and distribution analysis, which resulted in biological function of the BMSCs. The complex protein profile of dECM was analyzed by mass spectrometry. Proteomic analysis has identified a total of 178 proteins that participate in numerous cellular activities and signaling pathways. For example, three ECM proteins (Wnt2, Wnt5a, and Wnt6) were recognized in the dECM. These ECM proteins might play crucial roles in

signaling pathways that adjust the stem cell pluripotency (Li et al. 2019).

The results of these studies show that physical and chemical approaches can be combined to complement each other for efficient tissue decellularization. Although physical methods are moderate approaches that retain the ultrastructure of tissues, they fail to completely remove the immunogenic component of these tissues. On the other hand, decreased concentrations of chemical agents alone may not remove all cellular remnants. Thus, the combination of the two approaches in a multistep procedure can yield a synergistic effect for proper decellularization.

3.4 Combination of Chemical and Enzymatic Methods for Decellularization

Chemical approaches, with the assistance of enzymatic treatments can efficiently eliminate undesirable cellular and genetic constituents of the ECM. In order to achieve a convenient decellularization protocol for porcine annulus fibrosus (AF) (Kajbafzadeh et al.) tissue, Xu et al. investigated three different agents for the decellularization process. TX-100, as a non-ionic detergent, SDS, an anionic detergent, and trypsin, an enzymatic agent, were used to decellularize the AF tissue (Xu et al. 2014). This tissue is a multilamellar fibrocartilaginous structure mainly composed of collagen and proteoglycans. In this tissue, proteoglycan-rich matrix encompasses the collagen fibers in an orderly arrangement (Hickey and Hukins 1980). All three agents effectively decellularized the tissue and retained collagen. However, trypsin caused detachment of the matrix proteins from proteoglycans and led to a reduction in GAG content. Trypsin slightly deteriorated the concentric lamellar structure. SDS is an anionic detergent with a negatively charged head-group that can change the native conformation of proteins by disrupting non-covalent interactions. No significant differences were detected in mechanical properties of ultimate stress, elastic modulus, and toughness between the TX-100, trypsin, and control treatments; however, these parameters were lower in the SDS-treated group than the control group. The mechanical properties of specimens treated with SDS were lower than natural tissue due to the destructive effect of SDS, which disturbed the structure of the collagen fibers. Among these three agents, TX-100 effectively preserved the major ECM components, concentric lamellar structure, and tensile mechanical properties of the decellularized tissue. The proper biocompatibility of TX-100 makes it a promising candidate for efficient decellularization of AF tissue.

In order to decellularize porcine articular cartilage grafts, Luo et al. developed a decellularization protocol that consisted of several chemical reagents, including SDS and nucleases. The decellularized cartilage explants were seeded with human infrapatellar fat pad-derived stem cells (FPSCs) and cultured in chondrogenic medium under static or dynamic conditions for 10 days. For decellularization, the researchers first applied a hypotonic buffer that contained Tris-HCl, EDTA, KCl, $MgCl_2$, and dithiotheritol (DTT), followed by two additional treatment cycles with a hypotonic buffer supplemented with 0.5% SDS. The presence of KCl, $MgCl_2$, and DTT improved the solubility of the cell membrane proteins and antigens in the decellularization solution and accelerated their removal. Then, hyaluronidase treatment was performed to eliminate hyaluronic acid and proteoglycan trapped within the cartilage ECM. According to Luo et al., this treatment eliminated DNA and subsequently repopulated the scaffold. The researchers believed that this treatment could be used to develop a more porous decellularized matrix. Subsequently, the cartilage disks were incubated in hypotonic buffer that contained DNase I and RNase A (25 U/ml), and subsequently treated with 0.5 M NaOH to digest small collagen fibers and increase the porosity of the decellularized matrix. This protocol significantly reduced the DNA content of the cartilage tissues and resulted in a substantial decrease in sulfated glycosaminoglycan (sGAG). The reduction in sGAG contents associated with the use of hyaluronidase enzyme in the decellularization

procedure led to a significant decrease in the equilibrium modulus of the tissue (Luo et al. 2016).

A similar study was performed by Kheir et al. A decellularization protocol that included freeze/thaw cycles, repetitive treatment with hypotonic Tris buffer and 0.1% SDS, along with nuclease treatment and disinfection with PAA was developed. This protocol removed about 99% of DNA from cartilage tissue within 6 days (Kheir et al. 2011). In agreement with these results, Elder et al. developed a protocol that contained 2% SDS with a short incubation period (8 h), which resulted in only a 40% reduction in DNA content of the cartilage tissue (Elder et al. 2010). In these studies, both the collagen content and tissue organization were properly retained. However, SDS treatment in the absence of hyaluronidase caused significant reduction of sGAG in articular cartilage. Subsequently, removal of sGAG from the articular cartilage led to a substantial reduction in the mechanical properties of the tissue. Therefore, it is essential to develop a protocol that can efficiently decellularize cartilage tissue.

3.5 The Combination of Physical, Chemical, and Enzymatic Methods for Decellularization

A decellularization protocol that combines chemical, physical, and enzymatic treatments is an ideal option for cellular removal and ECM retention. Whitlock et al. decellularized human Achilles tendons after freeze-drying, followed by treatment with hypotonic aqueous solutions, trypsin digestion, and processing with the combination of an oxidizing agent (PAA) and a detergent (TX-100). This protocol effectively removed infectious viral material and produced a decellularized, architecturally modified, cytocompatible scaffold that could promote cell infiltration in vivo, yet retain tensile properties similar to native tissue. The oxidative and enzymatic treatments were effective for increasing porosity and removing infectious particles and donor cellular material during scaffold production, and eventually led to increased cell infiltration in vivo (Whitlock et al. 2012).

In an attempt to develop a biologic scaffold using a rabbit decellularized periosteum (D-periosteum) for bone tissue engineering, Chen et al. combined physical, chemical, and enzymatic treatments. For physical processing, multiple freeze-thaw cycles were performed, which led to efficient lysis of periosteum-derived cells (PDCs) and minimal disruption of the physicomechanical properties of the ECM, along with modification in diffusion of decellularization solutions throughout the tissue and the elimination of cellular components.

In order to solubilize the cell membrane, they used two chemical detergents (TX-100 [non--ionic] and SDS [ionic]). Although SDS was more efficient than TX-100 for cell elimination, it was more destructive to the ECM ultrastructure. In order to diminish undesirable immunological responses, DNase I was used to completely eliminate the DNA fragments. The decellularized tissue had a higher swelling ratio in comparison to native tissue because of the increased porosity that was created by the removal of cells and movement of the solution within the tissues. The increase in swelling ratio might improve cell infiltration through the scaffold.

PDCs could adhere, proliferate, and infiltrate into the D-periosteum in vitro. The host response to the D-periosteum scaffold was evaluated by subcutaneous implantation in rabbits during 28 days. D-periosteum did not represent an intense immunogenic response; rather, there was low-grade inflammation, scarce scaffold degradation, and thin fibrous encapsulation. These results demonstrated that D-periosteum could be a biocompatible scaffold for bone tissue engineering (Chen et al. 2015b).

4 Challenges Faced by Emerging Decellularization Technique

The advantages and disadvantages of several procedures currently used in the decellularization process were previously discussed.

Notably, most of the decellularization agents and protocols cannot completely preserve the native 3D architecture of the ECM and might result in changes in its composition, arrangement, biological activity, and mechanical properties. Therefore, creating optimal protocols to minimize these undesirable effects and keep native ECM for clinical use is a major challenge of the decellularization process.

The ultimate goal of each decellularization protocol is to eliminate all cellular substances without negatively affecting the composition, mechanical integrity, and biological activity of the remnants of the ECM and successfully extract an ***intact version of the ECM***. It is critical to obtain pure and intact ECM from the desired organs to achieve complete regeneration (DeQuach et al. 2010; Frantz et al. 2010). However, nearly all approaches have resulted in damaged ECM organization or arrangement (Rana et al. 2017; Somuncu 2019).

Effectiveness, efficiency, and safety of decellularization depends on the quality of cell/antigen removal and preservation of structural proteins. Extraction of the ECM from entire mammalian tissues needs several processing steps, which strongly alter the ECM structure and subsequently impact the host response. Each step changes the architecture and integrity of the dECM and affects the mechanical and biological properties of the extracted ECM. Despite the various protocols that have been used to fully decellularize the dermis, urinary bladder, skeletal muscle, and adipose tissue, no protocol has fully eliminated all of the cellular components and antigens, and preserved the most crucial components (GAGs and collagens) of the ECM. Nevertheless, these protocoles does not encompass any organs and tissues. For example, there are a limited protocols for decellularization of hard and dense tissues like bones, cartilage, and teeth. This limitation is particularly noted for the tendon-bone interface. Four different tissues with different matrix densities such as bone, calcified fibrocartilage, fibrocartilage, and tendon are located in a small zone (1 mm) of the tendon-bone interface. This feature is a major obstacle for efficient cell removal. Therefore, specific tissue characteristics, matrix density, cell density, and type of detergent must be considered to achieve the most effective method. A few protocols have been developed for tendon-bone interface decellularization. Bronstein et al. used a specified protocol that could roughly preserve the biomechanical properties of the flexor tendon–bone interface. The combination of physical (ultrasonication) and chemical (PAA, EDTA, and SDS) treatment resulted in a decellularized multi-tissue scaffold (Bronstein et al. 2013). Xu et al. estimated the various physical procedures and chemical treatments to create an effective, time-saving, and repetitive protocol to decellularize whole fibrocartilage enthesis ECM. DNA analysis and biochemical characterization of the resultant ECM confirmed that the matrix structure and biomechanical features were maintained. This suggested protocol could decellularize bone, tendon, and its interface region (Xu et al. 2017).

The removal of detergent from decellularized tissues is another issue that affects the functionality of prepared cell-free ECM in clinical settings. Detergents are amphipathic molecules composed of a hydrophilic head group and a hydrophobic chain. These characteristics enable them to emulate, destruct, and insert themselves into cell membranes. The penetration rate and lysis capability of detergents differ, and consequently make it difficult to exclude the residual surfactant from decellularized tissues and cells (Daugs et al. 2017). The remaining detergents that have high penetration capability infiltrate through the cell membrane and disrupt the basement membrane complex in decellularized tissues. This might denature the native ECM structure and negatively affect the cell-ECM scaffold interaction (Faulk et al. 2014). To address this issue, Farrokhi et al. compared the effectiveness of a detergent-free method with a detergent-based method. The results indicated that the detergent-free methods because of their effectiveness, safety and minimal disruption to native ECM would be excellent alternatives and prevail over detergent-based challenges (Farrokhi et al. 2018). Aside from detergent-based protocols, enzyme-based methods have a disruptive effect on the ECM

and basement membrane component. Enzymes trapped in the ECM network substantially reduce the ECM content of elastin and GAGs over time and may diminish recellularization (Kim et al. 2016; Naso and Gandaglia 2018). Accordingly, there is a need to develop methods that concurrently accelerate both the decellularization and recellularization processes.

5 Potential Opportunities and Possibilities

Despite limitations and weaknesses, numerous organs such as the lungs, esophagus, urinary bladder, kidneys, cornea, and trachea, as well as heart valves and blood vessels have been successfully regenerated by using dECM. In the next section, we discuss advances attributed to the decellularization technique.

5.1 Extracellular Matrix (ECM)-Based Bioink

The regeneration of complex solid organs such as the heart, liver, or kidneys is faced with various difficulties due to the lack of suitable materials that can mimic the 3D structure of tissues. dECM is one of the promising materials that can generate complicated tissues. Researchers have the capability to prepare gel-like shear-thinning bioink from these cell-free ECM. The combination of bioprinting technology and the dECM bioink is an optimistic approach to generate on-demand tissue-like scaffolds. ECM hydrogels are composed of functional, structural, and signaling molecules such as collagen, laminin, fibronectin, GAGs, and growth factors that can be preserved in dECM. Therefore, organ-derived bioinks provide a biomaterial that exhibit the highest degree of similarity to native tissue (Chameettachal et al. 2019).

dECM of the skin (Ahn et al. 2017), urinary bladder, small intestine (Choudhury et al. 2018), heart (Jang et al. 2016), cartilage (Pati et al. 2014), and muscle (Choi et al. 2016) have been successfully bioprinted. Unlike soft tissues, which are easily decellularized and solubilized to form bioink, preparation of solubilized bioink from hard tissues faces distinct challenges. Among the hard tissues, cartilage dECM has been bioprinted. Pati and colleagues have decellularized porcine hyaline cartilage and solubilized the obtained dECM. Preservation of type II collagen and GAGs in dECM directed MSCs into chondrocyte lineage (Pati et al. 2014). The major challenge in hard tissue derived-ECM bioinks that restricts application of the hard tissue decellularized bioink is the complete maintenance of dECM.

Regardless, dECM has distinct advantages. The presence of collagen and fibronectin in dECM makes the gelation process more responsive to temperature alterations and triggers the formation of a crosslinked network. This feature leads to the crosslinking of bioink without the presence of a crosslinker. Moreover, the concentration of collagen and other fibrous molecules in dECM-derived bioink, due to their physical interaction and meshwork nature, could directly enhance bioink printability (Wang et al. 2017; Osidak et al. 2019). Ahn et al. have introduced a novel heating system based on this dECM bioink feature. An intrinsic characteristic of skin-derived dECM led to the construction of an integrated scaffold that had improved fidelity and maintained cell viability (Ahn et al. 2017).

5.2 Particulate Form of the Extracellular Matrix (ECM)

The application of powder-like biomaterial and biodegradable material has been proven in various tissues (Choi et al. 2009). Different biomaterials in the form of particles, powders, and granules have been successfully used as fillers, stabilizers, leaching agents, and bioactive factors to accelerate tissue regeneration (Sheikh et al. 2015; Iulian Antoniac et al. 2017). Recently, decellularized tissue-derived particles and powders have captured more attention due to their ease of access, high efficiency, and reservoir of signaling cues, bioactive peptides, and matrix metalloproteinases (Brown and Badylak 2014; Blaudez et al. 2019).

ECM powders and particles can be packed, sprayed, or solubilized, and minimally invasive techniques can be used to efficiently deliver them to irregular defects. ECM powder can be used to optimize synthetic substances that have poor biocompatibility in 3D bioprinting (Edgar et al. 2018). Bioactivity, accessibility, higher efficiency, and lower invasive intervention are distinct features of ECM powders.

The powder-like demineralized bone matrix has widely been used in periodontal and orthopedic regeneration to take advantage of the distinct capability of the bone matrix (Maddox et al. 2000; Tsai et al. 2002). Nevertheless, disadvantages include the lack of osteogenicity and osteoconductivity, and residual cellular debris make dECM powder-like materials a better alternative matrix for hard tissue regeneration (Sawkins et al. 2013). The results from a number of investigations have confirmed the remarkable capability of the powder and particulate forms of the ECM in various tissues. Penolazi and co-workers used powder and a purified form of urinary bladder dECM (BdECM) to evaluate its capability to induce osteogenic differentiation of Wharton's Jelly derived mesenchymal stem cells (WMSCs). BdECM could successfully maintain cell morphology and viability, and induce typical osteoblastic markers (Penolazzi et al. 2012). Soucy et al. have suggested that particulate ECM can be a biological substitute to improve cardiac function and increase cell proliferation when injected into left ventricular assist devices (Soucy et al. 2015). Human adipose tissues that contain adipose stem cells (ASCs) have increased application in stem cell delivery. Choi et al. reported that ECM powder-derived adipose tissue is an ideal carrier for adipose tissue engineering. The findings of an in vivo study that used cell-seeded ECM powder showed excellent tissue regeneration without any necrosis or fibrous tissue after 8 weeks (Choi et al. 2009). Maintenance of the avascularity feature of tissues such as cornea and cartilage is necessary to avoid de-differentiation of stem cells into unwanted lineages. Choi et al. have utilized chondrocyte-derived ECM powder to investigate its angiogenic inhibitory effect on rabbit cornea. The anti-angiogenic properties of chondrocyte-derived ECM are allocated to type II collagen-derived N-terminal propeptide (PIIBNP), chondroitin sulfate, and GAGs components. Both in vivo and in vitro analysis confirmed that chondrocyte-derived ECM efficiently hindered neovascularization (Choi et al. 2014). These data confirmed that ECM powder could highly support a 3D stem cell culture and maintain specific characteristics of the desired tissue.

The particulate form of dECM offers a promising approach that can be used in hard tissue engineering and regenerative medicine. Particulate meniscal ECM has been utilized to overcome its inadequate intrinsic regeneration capacity. Mobini et al. reported a novel and effective method for decellularization and micronization of the meniscus. The resultant powder-like meniscus was combined with platelet-rich plasma (PRP) and analyzed ex vivo in a canine model. A long-term culture confirmed both cellular infiltration and proliferation of the dECM+PRP scaffold, which suggested that the micronized meniscus ECM could be a novel candidate treatment for full-thickness meniscal defects (Monibi et al. 2016). Zahiri and co-workers have reported that cartilage decellularized nanoparticles could be an adequate biomolecule delivery system in chondrogenesis. Their results indicated that cartilage dECM nanoparticles clearly improved chondrogenic differentiation of human chondrocytes (Zahiri et al. 2018).

These studies confirmed the efficacy of decellularization and highlighted that the powder-like ECM preserved the inherent features of ECM. Ease of extraction, increased accessibility, and reproducibility make the particulate form of dECM more applicable for commercial and clinical use compared to its solubilized form. Regardless of the final product, there are no standard guidelines and processes for powder fabrication and treatments. The application of this promising scaffold is limited by the lack of a standardized protocol. Therefore, developing this protocol for each tissue would offer the same ECM compositions after processing and enable decellularization to be used in the clinical setting.

5.3 Mimicking the Vascular System

Complete regeneration of tissues and organs is restricted because of the lack of vascularization. Various techniques and investigations have attempted to generate vascularized constructs (Huling et al. 2016; Datta et al. 2017; Kant and Coulombe 2018; Esser et al. 2019). Utilization and stimulation of pre-existing vascular networks render the most beneficial strategy to recapitulate the vascular pattern and trigger the angiogenesis process (Rancy et al. 2019). Vascularized grafts and implants have the advantages of presenting the vasculature bed and are under consideration, especially in bone tissue engineering (Jiang et al. 2018). The most promising result that has used the vascularized bed was achieved by seeding endothelial cells directly into decellularized scaffolds, which took advantage of the channels that remained from the pre-existing vascular network (Pellegata et al. 2018). Therefore, decellularization offers a simple solution for the vascularization issue.

An intact vascular network is more important for whole organ decellularization. In order to maintain the vascular network, two prominent components of the ECM, laminin and fibronectin, must be preserved. Laminin and fibronectin both play critical roles in cell adhesion and differentiation (Schwarzbauer 1991; Li et al. 2002; Kajbafzadeh et al. 2019). In order to have an intact vascular bed, it is crucial to tightly control the decellularization method. For instance, the utilization of an ionic detergent destroys the ECM ultrastructure. A decrease in GAG alters the viscoelastic features of the ECM scaffold, which is a significant factor in decellularized vein functionality (Cheng et al. 2019).

It is a challenge to maintain the decellularized vascular network (DVN) and prevent clotting or clogging of the implanted DVN. Re-endothelialization of the DVN before the implantation process by seeding endothelial cells on the vessel wall is a promising technique (Zambon et al. 2018). The vascular corrosion casting method has been refined in an attempt to examine both the morphology and architecture of recellularized blood vessels and capillaries (Bagetti Filho et al. 2008). Creation of a method to preserve increased ECM components (mainly collagen and GAGs) could allow a more intact DVN (Lin et al. 2018). Despite the difficulties, DVN offers a reliable vascular bed to facilitate the recellularization of targeted tissue, especially in highly vascularized organs.

Regeneration of hard and dynamic tissues like dentin and bone are highly dependent on the vascular network due to their cell turnover. Unlike bone, dentin is avascular and nutrition for odontoblasts is provided by blood vessels located in pulp tissue (Florencio-Silva et al. 2015; Bedran-Russo et al. 2017). The dental pulp, which is a highly vascularized connective tissue in the center of the tooth, regulates the dentin-pulp complex through the dentinal tubules. Typical therapeutic approaches to cure infected dentin lead to elicit pulp tissue, expansion of apical periodontitis, and eventual tooth loss.

Decellularized dental pulp ECM (dDP-ECM) can extend the therapeutic strategy of tooth regeneration by providing an intact vascularized scaffold. An in vivo study on a canine root canal confirmed both the feasibility and superiority of dDP-ECM in tooth regeneration (Alqahtani et al. 2018). Another study has shown regeneration of the pulp vascular network by xenograft dDP-ECM. The expression of matrix proteins like collagen-IV and laminin, which play a leading role in blood vessel formation, were substantially upregulated (Hu et al. 2017). These findings have shown the clinical use of dECM for tissue regeneration.

6 Conclusion and Outlooks

dECM is a biocompatible and versatile scaffold that includes essential components such as structural proteins and GAGs; hence, it regulates cell behaviors and tissue regeneration. Numerous studies have demonstrated the biological advantages of dECM biomaterials for tissue engineering. Decellularization could reduce the immunogenicity of allogenic or xenogeneic tissues by removing the cellular components. It

may be useful in tissue remodeling because of improvements in cell invasion and repopulation following removal of cellular debris. Different parameters such as tissue thickness, density, and cellularity could influence the efficiency of decellularization protocols.

Given the advantages of dECM biomaterials in tissue engineering applications, tremendous developments have been made in the past few decades in pre-clinical and clinical applications. However, numerous challenges remain. One of the most important steps following cell removal is proper recellularization of the dECM by using both the appropriate cell type and an efficient method. It is important to note that all changes of the ECM scaffolds may influence the cell behaviors during recellularization. On the other hand, currently, there is no defined mechanism for the interaction of ECM with cells and in the in vivo microenvironments. Further studies in this area would be of benefit. Furthermore, more attention should be paid to the immunological responses of dECM biomaterials in vivo. It may be necessary to optimize and improve the current decellularization protocols to achieve more biocompatible and functional scaffolds for tissue regeneration. Recent technologies that include the development of bioprinting could provide suitable microenvironments for cell growth and tissue regeneration, which would be physically and biologically optimized. Thus, a promising future exists for dECM biomaterials in the field of tissue engineering and regenerative medicine.

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Stem Cell Based Exosomes: Are They Effective in Disease or Health?

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Abstract

Exosomes are nano-sized vesicles involved in intercellular communication via delivery of molecules including lipids, nucleic acids, proteins, or other cellular components to distant or neighboring sites. Their ability to pass biological barriers, stability in physiological fluids without degradation, and distinctive affinity to target cells make exosomes very remarkable therapeutic vehicles. Virus-based approaches are some of the most widely used gene therapy methods; however, there are many issues need to be clarified such as high immunogenicity. Using of the exosomes procures the functional transfer of their cargo with minimal intervention from the immune system and it has been reported to be secure and well-tolerated. When the regenerative medicine is taken into consideration, stem cell-based approaches have been aimed to utilize but the general efficacy and safety profile of stem cell therapy has still not been enlightened. At this point, stem cell-derived exosomes exhibit a way to procure cell-free regenerative medicine with their unique characteristics. Exosomes are considered as appropriate and highly stable biological nanovectors taking part in a wide variety of healthy and pathological processes for advanced targeted therapies. However, there are still crucial obstacles to achieve efficient isolation of large amount of specific and pure exosomes. Thus, large-scale exosome production under good manufacturing practice is required. The purpose of this review is to focus on stem cell-based exosomes for gene delivery and to introduce synthetic exosome-mimics as a potential alternative in the field of targeted gene therapies. Further, we aim to highlight the biobanking and large-scale manufacturing methods of exosomes.

Keywords

Biobanking · Exosome mimics · Exosomes · Gene delivery · Manufacturing · Stem cells

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Abbreviations

AAV	Adeno-associated virus
ALIX	Apoptosis linked gene 2-interacting protein X
AV	Adenovirus vector
BBB	Blood brain barrier
CSC	Cancer stem cell
dsDNA	double-stranded DNA
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth receptor
EMT	Epithelial-mesenchymal transition
ESC	Embryonic stem cell
ESCRT	Endosomal Sorting Complex Required for Transport
EV	Extracellular vesicle
exo-AAV	exosomes-enveloped viral vector
FBS	Fetal bovine serum
GFP	Green fluorescent protein
GMP	Good manufacturing practice
HIF-1α	hypoxia-inducible factor-1α
HSC	Hematopoietic stem cell
iPSC	Induced pluripotent stem cell
MSC	Mesenchymal stem cell
MTX	Methotrexate
MV	Micro-vesicle
NK Cell	Natural killer cell
PLGA	Poly Lactic-co-Glycolic Acid
ssDNA	single-stranded DNA
TGF-β	Transforming growth factor beta
Th	T helper
TRAIL	Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand
TSG101	Tumor susceptibility gene 101 protein

1 Introduction

Exosomes are one sub-population of extracellular vesicles (EVs) released from a majority of cell types in the organism and isolated from a wide number of body fluids such as semen, urine, breast milk, saliva, amniotic fluid, sputum, and cerebrospinal fluid (Ela et al. 2013). EVs are classified into three subsets based on their morphology and cellular origin; namely, exosomes, micro-vesicles (MVs), and apoptotic bodies. Exosomes are nano-sized membrane vesicles (30–100 nm in diameter), which originate from the endosomal pathway and released to the extracellular space by a process of exocytosis (Fig. 1) (Ratajczak and Ratajczak 2017; Colombo et al. 2014). In addition to their specific morphology, protein and lipid compounds in their structures are used in the determination of exosomes. Owing to their endosomal origin, each exosome harbors membrane transport and fusion proteins (Rab GTPases, Annexins, flotillin, Endosomal Sorting Complex Required for Transport (ESCRT) 0, I, II and III.), heat shock proteins (Hsc70, Hsp90), tetraspannins (CD9, CD63, CD81, CD82), proteins take part in multivesicular body biogenesis (Apoptosis linked gene 2-interacting protein X (ALIX), Tumor susceptibility gene 101 protein (TSG101)), as well as phospholipases and lipid-related proteins (Subra et al. 2010; Conde-Vancells et al. 2008). In spite of utilizing these proteins as positive markers, there is wide variety among exosomes released from different sources. Apart from these well-known membrane-associated proteins, over 4400 various proteins have been defined in relation with exosomes, probably serving as cargo for intercellular communication (Mathivanan and Simpson 2009). Besides proteins, exosomes are covered by phospholipid bilayer and they contain abundant certain raft-related lipids such as ceramide, cholesterol, other sphingolipids, and phospoglycerides with long and saturated fatty-acyl chains (Trajkovic et al. 2008; Skotland et al. 2017; Raposo and Stoorvogel 2013). This lipid composition is in charge of their unique rigidity. The importance for ceramide in budding of exosome vesicles into multivesicular bodies has been shown (Trajkovic et al. 2008). It has been recently reported that exosomes have saccharide groups on their outer surface and these groups are enriched in α-2,6 sialic acid, mannose, polylactosamine, and complex N-linked glycans (Batista et al. 2011). Furthermore, metabolic enzymes, growth factors and cytokines are also detected inside exosomes. Besides the aforementioned structures, exosomes also harbor different variants of nucleic acids such as mRNAs

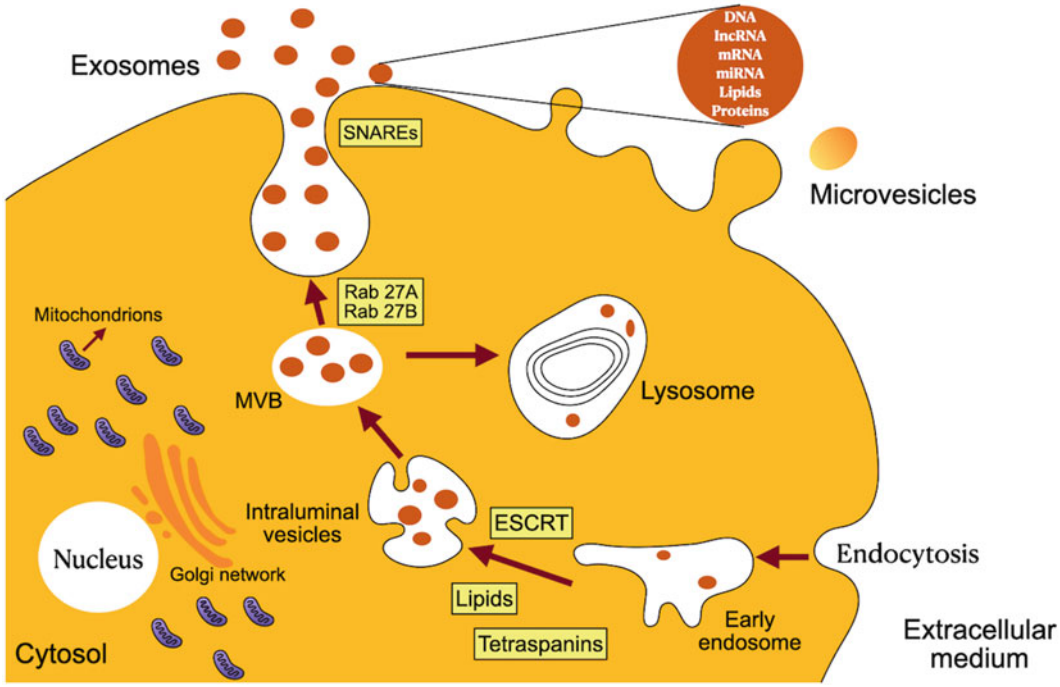

Fig. 1 Schematic presentation of stem cell-derived exosomes biogenesis and secretion. Exosomes are formed as ILVs by budding into early endosomes and MVBs. Many molecules take part in the formation of ILVs, such as lipids (such as ceramide), the ESCRT system and tetraspanins. MVBs can be fusion with lysosomes or fusion with the plasma membrane. Many Rab proteins have been indicated to participate in the transfer of MVBs to the plasma membrane and in exosome secretion. Moreover, SNAREs probably have a role in fusion of these MVBs with the plasma membrane. Other types of secreted vesicles bud directly from the plasma membrane, and are frequently called microvesicles. Abbreviations: *ILV* Intraluminal vesicle, *MVB* Multivesicular body, *ESCRT* Endosomal sorting complex required for transport, *SNARE* Soluble N-ethylmaleimide-sensitive factor attachment protein receptor

and miRNAs (mi-214, mi-29a) (Waldenstrom and Ronquist 2014).

As each exosome has a particular cargo which is related to the parental cell, exosomes retain the molecular signature of the cell origin. They broadly express protein and RNA factors distinctive to their originating cell. For example, MHC class-II molecules, CD80 and CD86 are expressed by exosomes released from dendritic cells (Robbins and Morelli 2014). Additionally, the expression profiles of exosomes have been exploited to elucidate the situations of the parental cell, changing in relation with a pathogenic condition. It has been shown that tumor and healthy cells secreted various populations of exosomes. For instance, exosomes secreted by papillary thyroid tumors overexpress miR-146b and miR-155 (Lee et al. 2015a, b). Therefore, exosomes have become a burgeoning area as a potential prognostic and diagnostic indicator for diseases ranging from cancers to neurodegenerative disorders. Formerly, it was considered that exosomes just released the undesirable or toxic materials for the cell (De Toro et al. 2015). On the other hand, today those vesicles are thought as natural signaling carriers and they are considered to have a crucial role for the intercellular neighboring and remote communication (Shahabipour et al. 2017). The capability of cross biological barriers including the cytoplasmic membrane and blood brain barrier is the one of the most beneficial properties of exosomes (Ha et al. 2016). This situation enables us to transport specific therapies to targeted cell types or distant sites

such as central nervous system. Besides their role in cellular crosstalk, they are involved in the transport of small molecules between cells, which usually regulate a wide range of functions associated with immunity. Exosomes have been implicated as important structures in the development of organs or in some diseases, such as cardiovascular pathologies (Sancho-Albero et al. 2020). Owing to their critical role in the cellular cross-talk, exosomes are also fundamental for the interactions among tumor cells and their niche and therefore they participate in cancer development stages, such as evasion of the immune system, development of tumor niches, supporting of angiogenesis and proliferation of tumor cells (Kahlert and Kalluri 2013). There has been a growing attention towards exosomes rather than synthetic delivery systems which have critical drawbacks, such as increasing the blood circulation time, evading the immune system (Raemdonck et al. 2014; O'Loughlin et al. 2012). As exosomes are inherently exist in body fluids, they are stable in the physiological environment. Moreover, they are less immunogenic and detrimental in comparison to its synthetic options and due to their membrane lipids and proteins, they can provide cargo to certain targeted cells. Lastly, they can be kept for extended periods. All these mentioned significant features make exosomes convenient, ideal and feasible biological nano-vectors to deliver nucleic acids, proteins, drugs, or nanoparticles.

In this review, we will focus on stem cell-derived exosomes as a therapeutic carrier and cutting-edge combinations of genetic materials, viruses and engineered tools together with exosomes in the field of targeted gene therapies. Moreover, biobanking and manufacturing methods of exosomes will be emphasized in the present review.

2 Gene Delivery Systems

Gene therapy is the unprecedented method that utilizes the gene to hamper or ameliorate any diseases. The way of gene therapy may enable clinicians to treat a disease by inserting a gene into a specific cell instead of performing surgery or using drugs. During the history of gene therapy, there are some important milestones (Fig. 2). The gene delivery systems are formed by three components: gene expression system that modulates the function of a gene in the targeted cell, a gene that codes a certain therapeutic protein, and a gene delivery system that regulates the delivery of the gene expression to particular location (Han et al. 2000). The efficient gene delivery system needs the foreign genetic material to remain stable in the host cells (Mali 2013).

To date, several gene therapy delivery methods have been developed: viral vector gene delivery systems and non-viral vector gene delivery systems (gene delivery systems based on cationic polymers, gene delivery systems based on polysaccharides, gene delivery systems based on poly(L-lysine), and gene delivery systems based on poly(ethylenimine)s). Currently, viral vectors, such as adenoviral vectors, retroviruses, lentiviruses, adeno-associated viruses (AAVs), poxviruses, are the primary carriers of nucleic acids used in gene therapy approaches to the certain sites, particularly *in vivo* (Table 1). But there are some drawbacks regarding virus-based vectors such as high immunogenicity and some organ related limitations. Using liposomes or polymer-based vectors to provide a wide hydrophilic lumen for the packaging of the virus has been presented in the literature. But high cytotoxicity of this approach and its difficulty make it unpreferable (Wang et al. 2019). Initially, adenovirus vectors (AVs) were a promising way regarding therapeutically in gene delivery *in vivo* with high efficiency due to their high transduction effectivity and absence of integration into the host genome (Wold and Toth 2013). But many issues still need to be enlightened to obtain better long-term patient results. The high host immune response still causes a serious concern; indeed, changeable expression of receptor proteins on target cells and an elevated prevalence of anti-AV vector immunity in the humans have been noted during preclinical and clinical researches (Atasheva and Shayakhmetov 2016; Gregory et al. 2011). Moreover, the inclination of AV vectors to be hijacked in the liver after systemic

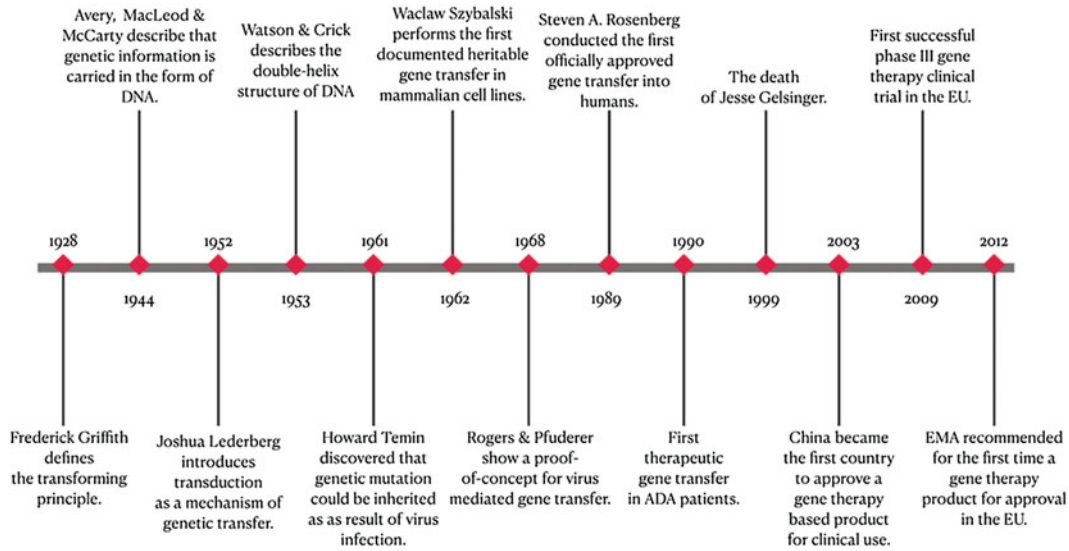

Fig. 2 Some crucial milestones of gene therapy

implementation hinders effective transgene transduction, giving rise to hepatotoxicity and death (Shayakhmetov et al. 2004). So far, although AVs continue being improved for various clinical administrations, including vaccine efforts, anticancer therapeutics, and neurological diseases, several researchers have concentrated toward the improvement of emerging viral vectors that would combine low genotoxic impacts and potential clinical immunogenicity with highly influential deliveries. Currently, AAV vectors are the most frequently used viral vectors at gene therapy (Wright 2008; Drouin and Agbandje-McKenna 2013). AAV vectors surround a linear single-stranded DNA (ssDNA) genome of roughly 4.7 kilobases (kb); moreover, these vectors harbor two open reading frames coding the structural Cap (capsid) and nonstructural Rep (replication) proteins, which are interchanged with exogenous DNA of choice. When they are delivered, the ssDNA genome is turned into double-stranded DNA (dsDNA) by the host cell. This stage is conducted by two cis-acting nucleotide inverted terminal repeats having 145 nucleotides in length, permitting the integration, packaging, and replication of the viral genome (Buning and Srivastava 2019). A new generation of recombinant AAV, which is lack of viral DNA, has lately been engineered able to traverse the cell membrane, where they could eventually deliver their cargos into the nucleus of a target cell (Penaud-Budloo et al. 2018). To date, AAVs have been applied for the treatments of hereditary blindness and spinal muscular atrophy (Gene therapy's next installment 2019; Bainbridge et al. 2015). Other clinical trials are continuing to evaluate the AAV for other disorders; but like AV vectors, it has been reported that symptoms of autoimmune response were encountered (Vandamme et al. 2017; Clement and Grieger 2016). Furthermore, their limited genome packaging capacity (~4.5 kb) is the other primary hindrance (Chamberlain et al. 2016).

Previous studies of the potential use of retroviruses for gene therapy began in the 1980s. Retroviral vectors were first implemented to treat monogenic diseases caused by a defect in a specific gene (Blaese et al. 1995; Bordignon et al. 1995). The crucial property of retroviruses is that they contain a preferential area for genome integration. Adverse effects of gene therapy caused by insertional mutagenesis were named "genotoxicity". Lentiviruses are much better vectors for gene therapy regarding genotoxicity. One of their advantages is the absence of preferential integration site (Cattoglio et al. 2010).

Table 1 Primary delivery methods of gene therapy products

Transgene delivery method	Size of inserted transgene, kb	Time of transgene expression	Preferred way of administration	Immunogenicity	Safety	Main field of application
Adenoviral vectors	Up to 30	Short (days)	Subcutaneous, intramural, local	High	Low; systematic administration can lead to a systemic inflammatory response, lethal case described	Vaccines, oncolytic viruses
Lentiviruses	Up to 10	Life-long	*Ex vivo* transduction of stem cells	Low	Acceptable in case of lethal diseases; risk of insertional oncogenesis	Correction of inherited genetic defects, mainly of hematopoietic system
Retroviruses	Up to 10	Life-long	*Ex vivo* transduction of stem cells	Low	Unacceptably low; high risk of insertional oncogenesis	
Adeno-associated viruses	Up to 4	Long (months, years maybe life-long)	Intramuscular	Low	High	Correction of inherited genetic defects, first therapy of polyetiological diseases
Poxviruses	Up to 20	Short (days)	Subcutaneous, local	High	Relatively high, tested on hundreds of thousands of people during the vaccination program; when using nonmodified vaccinia virus severe side effects are possible	Vaccines, oncolytic viruses
RNA viruses	Up to 2	Short (days)	Subcutaneous, intramural, local	Medium	Not enough data for the estimation; depends on each vector	Oncolytic viruses, niche applications
Plasmids	Up to 10	Short (days, weeks)	Gene gun, liposomes	Low	High	Vaccines
RNA	Up to 10	Short (days)	Gene gun, liposomes	Low	High	Therapy of polyetiological diseases

Experimental data reported that lentiviral vectors stimulated oncogenesis considerably less frequently than retroviral vectors (Montini et al. 2006).

The investigations on exosomes are ongoing to reach valuable information in terms of their intrinsic features in controlling complex intracellular pathways (Sharma et al. 2019; Agarwal et al. 2020). Counter to AAV vectors, using of the exosomes procures the functional transfer of their cargo with minimal intervention from the immune system and it has been proven to be secure and well-tolerated (Vlassov et al. 2012). A crucial side to emphasize consists of

the status of exosomes to mediate the propagation of disease related proteins participate in neurodegenerative disorders (Lim and Lee 2017). Due to their unique functions, exosomes provide improved transfection competence in gene therapy (Firquet et al. 2015). When those reasons are taken into consideration, the advantages and novelty of the combination of viral carrier ways together with exosomes are remarkable (van der Grein et al. 2018; Yao et al. 2018). Viruses are able to participate in the exosomes biogenesis pathway and integrate its viral RNA genome, miRNAs, mRNAs and proteins. After the unification, biologically active viral components would be delivered by those exosomes-enveloped viral vectors (exo-AAVs or vexosomes) from infected cells to the distant unaffected cells (Longatti 2015). Exo-AAV can also be designed to exhibit targeting peptides on their surfaces to allow improved deliveries to the target areas. In the literature, it has been shown that exo-AAVs have displayed an enhancement of transduction profiles in various AAV serotype *in vitro* and *in vivo* conditions (Frank 2010; Hudry et al. 2018). It has been noted that an improvement of the transgene expression originating from two different AAV serotypes (AAV6 and AAV 9) encapsulated with exosomes limited mostly to oligodendrocytes and neurons (Orefice et al. 2019). Clinical researches have explained that the trigger of neurodegenerative disorders could appear in a focus of genetically altered cells and spread from one site of the central nervous system to another (Frank 2010). There is a remarkable point in terms of optimizing the exo-AAV as an effective therapeutic gene delivery. The perpetual expansion of engineered optical fiber-based endoscopes allowed real-time visualization to track the exo-AAV spread into the brain, indicating it more widespread in the opposite hemisphere than conventional AAVs following intracerebral injections (Orefice et al. 2019). This study emphasizes the potential of utilizing exo-AAVs for gene delivery, especially to address the hardship of diffusion restrictions related with large fragments of DNA to arrive central nervous system cells distant from the intervention site. Encapsulated viral carriers with exosomes also propose notable benefits, both in decreasing the number of injections necessary for achieving spreading into a desirable brain area and delivering the optimal dose required to obtain the target concentration. All these mentioned properties make this type of EVs a promising delivery approach *in vivo*. Although, miRNAs and siRNAs, so called small RNAs, have been successfully loaded into EVs for various delivery implementations, the possible using of various vesicles based on their content, size, biogenesis, and secretion pathway to load and transfer foreign DNA is still relatively unclarified (Zhang et al. 2020; Zhao et al. 2020).

3 Stem Cells and Their Exosomes

Stem cells, which have the ability of self-renewal and can differentiate into any cell type, are undifferentiated cells of the human body. Asymmetric stem cells division is resulting in two unequal daughter cells: a committed progenitor and one new stem cell can enable stem cells to self-renew and create cellular diversity while maintaining a steady number of stem cells, therefore undesirable depletion or overgrowth of the stem cell population can be precluded and it helps maintain tissue homeostasis (Aguilar-Gallardo and Simon 2013). Stem cells are present in both adult cells and embryos. There are numerous steps of specialization. Totipotent stem cells, which have the highest differentiation potency, are able to form the whole organism. Zygote exemplifies totipotent stem cell regarding its characteristics. Pluripotent stem cells create all germ layers without extraembryonic structures. The pluripotency is a duration, commencing from entirely pluripotent cells such as embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSC) and ending up with less potent cells – multi-, oligo- or unipotent cells. Multipotent stem cells or adult stem cells have a more limited differentiation scope, but they are able to specialize in separate cells of specific cell lineages (Zakrzewski et al. 2019). Additionally, stem cells can be categorized into five subgroups based on their origin: ESCs, adult stem cells such as mesenchymal stem cells

(MSCs) and hematopoietic stem cells (HSCs), fetal stem cells, iPSCs and cancer stem cells (CSCs) (Kolios and Moodley 2013). CSCs are described as a small subgroup of cancer cells that form a population of self-sustaining cells with the specific ability to give rise to the heterogeneous lineages of cancer cells involving the tumor. These cells must demonstrate three major features including having a strong potential to generate the tumor from a limited number of cells, showing self-renewal *in-vivo* and having a differentiation capacity to cause heterogeneous progenitors (Chen 2009).

CSC-derived exosomes impact tumor microenvironment. Indeed, CSC-released exosomes are able to regulate cross-talk among other malignant cells or modify healthy surrounding cells to support immune tumor escape, tumor growth and metastasis. Many studies reported that CSC-derived exosomes have a crucial impact on tumor progression (Lindoso et al. 2017). EVs derived by CSCs may enable to create a convenient microenvironment for cancer development locally and to support distant metastatic niche formation. It has been shown that cancer exosomes play an important role in induction of chemo-resistance, tumor vascularization, remodeling of extracellular matrix (ECM), and epithelial-mesenchymal transition (EMT) with an improved migration, invasion, and metastasis (Corcoran et al. 2012; Chen et al. 2014; Sung et al. 2015). Moreover, EVs also take part in the intercellular communication between cancer cells and niche cells, like fibroblasts, that release EVs to provide invasiveness and chemo-resistance to cancer cells (Santi et al. 2015). Exosome-mediated exRNA and protein transfers have been defined and used as diagnostic markers. Especially, miRNA-mediated responses have been comprehensively studied. It has been shown that miRNAs in exosomes derived from lung cancer (miR-100-5p), breast cancer (miR-100, miR-222, miR30a, miR-17), and ovarian cancer (miR-21) induce chemo-resistance (Richards et al. 2017; Yeung et al. 2016). It has been discussed that cancer EVs may support the angiogenesis by stimulating the pro-angiogenic miRNAs like miR-155, miR-210, and miR-494 which are regulated by the hypoxia-inducible factor (HIF)-1α (Zhou et al. 2018). Recently, it has been shown that the formation of a pre-metastatic niche is supported by oncogenic miRNAs which has been transferred from cancer cells exosomes (Costa-Silva et al. 2015). Interestingly, exosomes which are released by CSCs, contribute to the tumor immune-escape response by the activation of tumor-related suppressor myeloid cells, macrophages, and the suppression of natural killer (NK) cell activity (Ludwig et al. 2017; Chow et al. 2014; Chalmin et al. 2010). It has been also reported by many researchers that cancer EVs play an immunosuppressive role in T cells by expressing Programmed Death Ligand-1, TGF-β, the Fas ligand, and the Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand (TRAIL) (Chen et al. 2018; Yen et al. 2017; Abusamra et al. 2005).

As other stem cells, HSCs are able to generate EVs including MVs and exosomes. It has been reported that among the cargo molecules of HSCs-released exosomes many miRNAs and anti-apoptotic and pro-angiogenic proteins are offered to be the mediators of heart regeneration, frequently via neovascularization (Seeger et al. 2013). However, the definitive evidence of the cardioprotective effects of exosomes and MVs derived from HSCs is still unclear in the literature. Besides, EVs generated in HSC-derived cells, particularly endothelial progenitor cells and dendritic cells, have been demonstrated to procure direct cardioprotective influences in cardiovascular diseases (Vasa et al. 2001; Liu et al. 2016). It has been shown that malign HSCs are able to run away from the immune system by various mechanisms such as, impairment in lymphocyte T-reg, downregulating target antigens on their surface and excretion of immunosuppressive substances (Vinay et al. 2015). TGF-β1 is one of the major cytokines which alleviate the function of immune cells such as lymphocyte T cytotoxic and helper (Kehrl et al. 2014; Kulkarni et al. 1993). Recently, it has been demonstrated that leukemic exosomes have an immunosuppressive impact that damage the function of immune system cells. The study conducted by Szczepanski et al. revealed that higher levels of TGF-β1 have

been indicated in acute myeloid leukemia patient exosomes, thus expression of NKG2D on NK cells is decreased and anti-leukemic features of NK cells are interfered (Szczepanski et al. 2011). Another study has highlighted that lymphoma and leukemia cells secrete malignant exosomes with high amounts of NKG2D ligands which inhibit the anti-leukemic features of NK cells (Xiao et al. 2012). Additionally, exosomes in chronic myelogenous leukemia patients harbor TGF-β1 which restrain the proliferation and function of many immune cells. Exosomal TGF-β1 in patients with malignant solid tumors are able to alleviate the function of lymphocyte T cytotoxic (Clayton et al. 2007).

MSCs, which are stromal cells, possess multipotent capacity to self-renewal and show multilineage differentiation. MSCs are frequently applied as a source of cellular therapy owing to their powerful immunosuppressive and regenerative impacts (Galipeau and Sensebe 2018). It has been formerly offered that MSCs display their therapeutic influence by migrating to areas of engrafting, injury, and interacting with other cells following infusion. Recently several *in vivo* researches showed that the therapeutic benefit of MSCs is mostly conducted by the paracrine secretion of a wide list of cytokines, chemokines, and growth factors (Wang et al. 2015). However, these pathways are not determined completely and still under research. In spite of their useful therapeutic impacts, MSCs have various drawbacks including ectopic tissue formation, immune reactions by the host, infusional toxicities given rise by the large cells physically trapped in the lung vasculature, and some concerns in terms of safety profile of the cells regarding tumor formation (Fennema et al. 2018; Jeong et al. 2011).

Recently, it has been demonstrated that MSCs secrete many EVs, including exosomes and MVs which may behave as paracrine mediators among MSCs and target cells (Mendt et al. 2018). It has been also reported that MSC-derived exosomes can replicate MSCs biologically and may serve as an alternative to entire cell therapy (Bagno et al. 2018; Lou et al. 2017). The utilization of exosomes may provide significant benefits over their cellular counterparts owing to lower immunogenicity, a higher safety profile, and the inadequacy to directly generate tumors (Liew et al. 2017). In comparison with the relatively extensive MSCs (30–60 um in diameter), nanosized exosomes, are able to migrate effectively to the target site after infusion without getting trapped in the lung microvasculature (Borger et al. 2017). Likewise, exosomes secreted from other cells, MSC-derived exosomes take part in cellular cross-talk and carry proteins, microRNA and mRNA into desired cells (Heldring et al. 2015). So far, more than 850 unprecedented gene structures and 150 miRNAs have been defined in MSC-secreted exosomes (Lai et al. 2012). In the literature, it has been indicated that the function and phenotype of MSC-released exosomes may be different depending on the source of MSCs (Borger et al. 2017). There are explicit discrepancies in tRNA species between MSC-secreted exosomes isolated from human adipose tissue and bone marrow regarding the differentiation condition of MSCs (Baglio et al. 2015). Furthermore, the source of MSCs has been demonstrated to affect the biological influences of MSC-derived exosomes (Borger et al. 2017). Human MSCs derived from bone marrow, endometrium and adipose tissues have been compared in a rat model of myocardial infarction. The outcomes confirmed the highest cardio-protection by endometrial MSCs relative to adipose and bone marrow derived MSCs (Wang et al. 2017). These data offer that innate variations of MSC-secreted exosomes owing to their original source may have a key role in their clinical influence. Along with their intrinsic features, MSC-derived exosomes are convenient vectors to carry and deliver drugs, therapeutic genes, RNA, or enzymes to targeted areas. It has been shown that MSC-derived exosomes are able to preserve their cargo towards degradation and enable their intracellular uptake by endocytosis (Bagno et al. 2018). Lately, it has been demonstrated that exosomes display intrinsic homing abilities similar to their parental cell type. Thus, MSC-derived exosomes may reflect an optimum delivery system to regulate processes in certain target cells. Furthermore, similar to

their parental cells, the outer surface of exosomes could be altered or modified in order to improve cell-free based treatment approaches (Yang et al. 2018).

Although MSC-derived exosomes have been reported in pre-clinical studies to be secure, the clinical use of MSC-derived exosomes is limited. There is a challenge to transfer MSC-derived exosome-based therapies from the preclinical works to the clinical practice. Obtaining of the optimal MSC culture conditions and protocols for exosome generation, isolation and storage are some primary issues to be addressed (Squillaro et al. 2016). It has been reported that MSC-derived exosomes effectually suppress autoimmunity and hamper the onset of the disease in designed mouse models with type 1 diabetes and experimental autoimmune uveoretinitis (Ezquer et al. 2012; Zhao et al. 2012). Outcomes showed that MSC-derived exosomes restrain development of T helper 1 (Th1) and Th17 cells improving the balance among Th1 and Th2 immunological responses. The clinical trial conducted by Nassar et al. revealed that MSC-derived exosomes enhanced urinary albumin creatinine ratio and eGFRs, as well as remarkable decreases in creatinine and blood urea nitrogen at 1 year in patients with chronic kidney disease. Additionally, the patients displayed a remarkable soar in plasma levels of IL-10 and TGF-β with persistent substantial decreases in TNF- α (Nassar et al. 2016). In another ongoing clinical trial is assessing the security and efficiency of exosomes obtained from cord tissue derived MSCs to support healing of refractory and wide macular holes in the eye. Previous preclinical research reported that systemic implementation of MSCs decreased the inflammatory response and restricted the damage in a laser-injured retina model by regulation of the intraocular microenvironment in a paracrine attitude (Jiang et al. 2014). The experimental study indicated that transplantation of both MSCs and/or their exosomes decreased retinal injury and blocked apoptosis caused by laser injury partly by the downregulation of monocyte chemoattractant protein-1 (MCP-1) (Yu et al. 2016). These outcomes offer that MSCs and MSC-secreted exosomes may enhance the vision function following refractory macular hole surgeries. Lastly, it has been revealed that MSC-derived exosomes loaded with miR-124 supported neurovascular healing following stroke, cured brain injury, and hindered post-ischemic immunosuppression in mice (Yang et al. 2017). As a result, clinical implementation of MSC-derived exosomes is a promising approach to treat many diseases.

3.1 Exosomes or Stem Cells?

In recent years, regenerative medicine has aimed to utilize human stem cells to cure tissue damages. The use of MSCs, ESCs, and iPSCs has a promising way regarding differentiation and proliferation to heal human tissue. Stem cells release various molecules including cytokines, growth factors, and EVs, in a paracrine way that lead to their therapeutic impacts (Thery 2011). However, these pathways are not elucidated entirely and still under research. In spite of their useful therapeutic impacts, stem cell based treatments have various drawbacks including ectopic tissue formation, immune reactions by the host, infusional toxicities given rise by the large cells physically trapped in the lung vasculature, and some concerns in terms of safety profile of the cells regarding tumor formation (Fennema et al. 2018; Jeong et al. 2011). The idea back in the early 2000s was that adult stem cells (MSCs) or progenitor cells may be administered to patients with diseases or damaged sites in order to treat them. It has been considered that by injecting stem cells into the body, these cells would replace the patient's unhealthy cells. But the general efficacy and safety profile of stem cell therapy has still not be enlightened (Marks et al. 2017). Although it looks like there are some benefits, cellular therapy for clinical regenerative applications has approval issues (Cuende et al. 2018). Today, only the use of blood-forming stem cells for patients with particular blood production diseases has received Food and Drug Administration approval in the clinical practice.

Exosomes harbor umpteen various types of products and this feature makes them extremely encouraging in the field of regenerative medicine.

Their bilayer lipid membranes have certain marker proteins that describe them particularly to specific cells. Therefore, exosomes have a crucial impact on cell-to-cell communication (De Jong et al. 2014). A broad diversity of molecules can be carried via exosomes, such as RNA, specific proteins, and miRNA. Moreover, many studies reported that horizontal transfer of protein and mRNA take place by exosomal machinery and the genetic data delivered successfully translated into the convenient proteins (Ratajczak et al. 2006; Neviani and Fabbri 2015). It has been shown that endothelial progenitor cells-released MVs are able to protect the kidney from ischemic conditions by packaging miRNA which is accountable for stimulating regenerative pathways in the kidney (Cantaluppi et al. 2012). These studies demonstrate the power of exosomes in regenerative interventions.

In spite of many shortcomings in terms of manufacturing exosomes, recently there are many benefits and promise in clinical applications. Due to their stability in physiological conditions such as pH or temperature, and multidimensional packaging, exosomes are appropriate candidates for therapeutic medicine and stem cell-derived exosomes exhibit a way to procure cell-free regenerative medicine. Their specific and unique markers, such as tetraspanins, flotillin, ALIX, enable exosomes with a cell specific manner to uptake and unload their cargo. This distinctive affinity to target cell makes exosomes very potent carriers to deliver miRNA, drugs, protein, nanoparticles, and so forth in the body fluids without being degraded. Moreover, exosomes are less immunogenic and toxic compared to synthetic delivery vectors. Additionally, they are able to pass through the blood brain barrier (BBB) and reach certain areas. Despite further required evidence, some authors reported that exosomes may pass through the BBB via active endocytosis mechanisms (Thery 2011; Druzhkova and Yakovlev 2018). Further, peripheral EVs may lead to alterations in BBB's features by interaction with the barrier. A recent study showed that zebrafish neurons-derived exosomes are able to control BBB integrity via miR-132 (Zhao and Zlokovic 2017).

Up to date, 91 ongoing and completed clinical trials are present about exosome-based applications. Three primary sources, namely MSCs, dendritic cells, and patient-derived tumor cells, of getting exosomes, are subjected to clinical trials. As a diagnostic and prognostic marker for many cancer types such as oropharyngeal cancer, sarcoma, and clear cell renal cell carcinoma, exosomes are promising tools. Moreover, their therapeutic impacts on various diseases are remarkable. Immature dendritic cell-derived exosomes have been utilized for non-small cell lung cancer and melanoma, for which the outcomes of reliance are similar, however in case of non-small lung cancer, MAGE-specific T-cell responses have been defined (Morse et al. 2005). To improve T-cell stimulation, dendritic cell maturation method has been laid out for non-small cell lung cancer patients.

Due to tumor antigens, such as carcinoembryonic antigen, could be derived from a patient with cancer, ascites-derived exosomes from patients were obtained. Well-tolerance and safety in phase I trial have been revealed, and a tumor-related antitumor cytotoxic T lymphocyte response has been detected in the ascites-derived exosomes plus granulocyte-macrophage colony-stimulating factor group (Dai et al. 2008). In addition to carrying tumor antigen, exosomes harboring chemo drug or siRNA have been utilized in the cancer treatment. There are two clinical trials (NCT02657460 and NCT01854866) applying chemo drug for the treatment of malignant pleural effusion. Methotrexate (MTX) and cisplatin has been used respectively as the anticancer drugs. The survival ratio has been found higher when MTX has been applied. KraG12D siRNA has been used as another anticancer drug type to treat patients with metastatic pancreas cancer (NCT03608631).

Consequently, exosomes are proper and highly stable biological nano-vectors taking part in a wide scope of healthy and pathological processes, for advanced targeted therapies.

4 Synthetic Exosome-Mimics

Pure populations of exosome could be obtained from exosome-releasing cells but these exosomes have oncogenic and immunogenic potential. Furthermore, EVs have complicated roles in health and disease conditions, including delivery of pathogens and disease-related proteins between cells, which are still not enlightened entirely (Saa et al. 2014). Lastly, there is a limitedness regarding the number of naturally secreted exosomes. These issues are the major obstacles for translation of naturally secreted exosomes to the clinic applications. Artificial exosome-mimics may help to overcome these limitations. Synthetic lipid vesicles or liposomes which have a spherical phospholipid bilayer pattern approximately 100 nm in diameter, can act as a primitive structure for engineered exosomes (Kooijmans et al. 2012). Liposomes could be loaded with exosome related constituents and therapeutic molecules such as nucleic acids, recombinant proteins, and synthetic drugs (Malam et al. 2009). The lipid content of liposomes can be modified. Phosphatidylserine which improves the stability of exosomes and regulate dendritic cell maturation, is one of the proper substances to imitate naturally occurring exosomes (Chen et al. 2004). Along with phosphatidylserine, cholesterol may improve cation-induced fusion (Shavnin et al. 1988). Therapeutics based on liposomes and nanoparticles have been approved for clinical practice (Fenske and Cullis 2008). A great number of engineered exosome-mimics can be produced through two methods: cell extrusion or polymer nanoparticles coated with cell membranes. Firstly, exosome-mimetic nanovesicles are fabricated via extruding cells method. Differently from liposomes, these nanovesicles loaded with doxorubicin have been targeted to tumors and displayed anti-tumor impacts like exosomes *in vivo (*Jang et al. 2013*)*. Moreover, green fluorescent protein (GFP)-silencing siRNA loaded into monocyte-secreted nanovesicles by electroporation could be caught by endothelial cells and ultimately knocked down GFP (Lunavat et al. 2016). Nanovesicles produced by extruding fibroblasts transfected with shRNA could also be utilized as a vector to deliver functionally active miRNAs to targeted cell groups. The other group exploited multiple microchannels to break down murine ESCs into membrane-bound nanovesicles with a size of 60–120 nm in diameter (Jo et al. 2014a). These nanovesicles could be included in fibroblasts to deliver endogenous proteins and RNAs of stem cells to fibroblasts. Additionally, the same researchers improved a device that can produce large-scale nanovesicles utilizing centrifugal force in order to extrude cells through 10 um and 5 um filters (Jo et al. 2014b).

Lately, cell-membrane coated nanoparticles have been a burgeoning area regarding drug delivery with benefits of stability, immune-compatibility and targeting ability (Cao et al. 2016). Anti-tumor drug sTRAIL has been delivered by MSC membrane-produced nanoparticles to tumor site and inhibited tumor development *in vivo,* while sTRAIL with liposome did not have any anti-tumor impact (Furman et al. 2013). Cancer cell membrane-cloaked nanoparticles displayed homotypic targeting to pathological tissue with immune-compatibility, thus may behave as a possible vector for anti-tumor therapeutic delivery (Fang et al. 2014). Recently, it has been reported that cell membrane-coated Poly Lactic-co-Glycolic Acid (PLGA) microparticles may have protective impacts and serve as exosome-mimics for drug therapy in myocardial infarction (Tang et al. 2017).

5 Banking of Exosomes

Biobanks can be described as establishments where high-quality biospecimens are obtained, processed, and conserved by long-term storage for future researches of clinical applications (Shaw et al. 2014). The emerge of patient or disease specific therapies requires high-quality human biospecimens with proper clinical interpretation, particularly in complicated diseases such as cancer, cardiovascular,

neurodegenerative, and metabolic changes in which sample heterogeneity and patient-related responses usually complicate the development of sensitivity therapeutic interventions (Natasha et al. 2014). In the burgeoning area of EVs studies, exosomes have been defined as a prognostic biomarker, diagnostic tool, drug vectors, and therapeutic targets (Vader et al. 2014; Lai et al. 2013; Properzi et al. 2013). But there is a lack of consensus on isolation procedures and meticulous criteria to characterize exosomes. When biobanking of exosomes is taking into consideration, some questions in terms of isolation, characterization and storage of exosomes need to be addressed and then biobanking of exosomes may take part in this growing area. The possibility of storing exosomes in biobanks can provide a beneficial way for several clinical interventions as well as for research aims.

5.1 Exosome Isolation and Characterization Methods

Different protocols have been defined to isolate and enrich exosomes from supernatants and some body fluids (Muller et al. 2014; Zlotogorski-Hurvitz et al. 2015). Mostly, centrifugation of the body fluid or the cell culture supernatant at 2000 g in order to eliminate the dead cells is effective and then centrifugation at 10,000 g to eliminate non-exosomal vesicles like MVs and the cellular debris has been reported. Later, a 200 nm filter, which segregates all larger particles of size over 200 nm, is used to pass through the supernatant involving exosomes. This step is continued by any well-accepted separation methods like immune-affinity sorting utilizing antibodies to the exosomal surface markers; ultracentrifugation at 100,000 g; size exclusion chromatography; isopycnic centrifugation; ultrafiltration of polymer-based precipitation (Abramowicz et al. 2016). Ultracentrifugation approach is the most commonly used method and proper for isolation of large volumes of the sample at low cost (Greening et al. 2015). Density gradient centrifugation utilizes a gradient medium like iodixanol or sucrose to segregate vesicles relying on their floatation densities. This technique is thought as a 'gold standard' approach for isolation of exosomes and combination of these two methods is indicated to provide high-grade quality exosomal samples for proteomic analysis (Abramowicz et al. 2016). Size exclusion chromatography provides high purity outputs with minimal protein contamination, but insufficient sample yield and sparseness of the final sample are the drawbacks of this method (Lobb et al. 2015). The simplest method to isolate exosome is the polymer-based precipitation technique in which exosomes are incubated with polymers such as polyethylene glycol followed at low-speed centrifugation. The mentioned method provides an adequate throughput of exosomes but possible contamination of non-exosomal particles jeopardizes the purity of sample (Zlotogorski-Hurvitz et al. 2015). The most specific way to isolate exosomes and even subgroups of exosomes is immuno-affinity separation. A diversity of platforms such as ELISA plates, microfluidic devices, modified chromatography columns, and immune beads have been improved to catch exosomes by targeting their surface biomarkers (Greening et al. 2015; Li et al. 2017).

Various properties for the characterization of exosomes have been suggested based on their contents or physical features. As exosomes cannot be detected via conventional microscopic approaches, electron microscopy techniques including scanning electron microscopy, whole mount electron microscopy, transmission electron microscopy, and electron tomography are utilized (Peterson et al. 2015; Fertig et al. 2014; Gyorgy et al. 2011). In comparison with conventional methods, electron microscopy techniques are more formidable and laborious. Alternatively, particle tracking analysis may be used but this morphological analysis does not procure functional data of the isolated exosome-fraction (Mehdiani et al. 2015). Along with CD9, CD63, and CD81, tetraspanin markers, other markers such as TSG 101, Annexins, of exosome content have been proposed as functional markers (Peterson et al. 2015; Taylor and Shah 2015). But currently there is no consensus regarding functional specific markers. Typically, functionality is assessed by measuring exosome binding,

delivery, and release of their cargo to targeted cells. Fluorescently labeling exosomes via internal fusion proteins or via tracking dyes are achieved through live tracking, flow cytometry, or other types of fluorescence-based imaging methods. Notedly, the PKH67 uptake dye assay was applied to display the capacity of exosomes obtained from the plasma samples and preserved at -20 °C for over 30 days in order to fuse with LIM 1215 colorectal cancer cells (Kalra et al. 2013). ELISA-based techniques and Acetyl-CoA cholinesterase colorimetric methods are utilized for total protein measurements. However, these approaches measure overall functions which harbor contribution from various EV types. Therefore, the outcomes regarding function are estimated roughly (Savina et al. 2002; Gupta and Knowlton 2007).

5.2 Exosomes Storage Conditions

So far, ideal conditions for isolating or storing exosomes are not defined strictly. There is no explicit data in terms of influence of anticoagulants in the obtaining and storage of exosomes, neither optimal storage period, temperature, thawing conditions, freezing-thaw cycles, or other storage factors have been assessed in detailed. It was reported that when exosomes were stored at −80 °C, there was an advantage regarding the stability of exosomes in comparison with other degrees (Kalra et al. 2013). However, this outcome may vary for exosomes obtained from various sources and /or specific isolation methods. Moreover, the kind of lipid content of exosomes (Llorente et al. 2013; Record et al. 2014), which is source-dependent as well, is anticipated to play a role in ideal cryopreservation procedures, soaring an additional hardship for biobanking of exosomes.

6 Manufacturing Exosomes

Paracrine-like activity of exosomes and their usage as a shuttle of nucleic acid or proteins in the therapeutic interventions make a demand in the large-scale manufacturing of purified exosomes (El Harane et al. 2018; Zhang et al. 2015). There are still remarkable obstacles to the effective and potent isolation of wide quantities of specific and pure exosomes due to the lack of understanding of the relationship among exosome functions and characteristics. Therefore, exosome production under good manufacturing practice (GMP) are required. Generally, current exosome isolation and purification methods has proven to be challenging and isolation of a specific subpopulation of exosomes is even more grueling (Cvjetkovic et al. 2014; Witwer et al. 2013). No centrifugation approach provides a great number of highly purified EVs or distinct exosomes from other subtypes of EVs. Moreover, it is hard to distinguish among various types of exosomes via centrifugation methods. Besides, microfluidic techniques are considered very efficient on exosome isolation and analysis. But the drawback of this method is insufficient capacity for large-scale manufacturing aims (Thery et al. 2006).

Several cultured cells will release exosomes utilizing standard cell culture media and T-flasks. As yield of cells, the production of exosomes is related to the ability to generate wide quantities of cells in ways that do not change distinct cell attitude and properties (Isasi et al. 2016). The possibility for alterations in cellular characteristics in the course of technical transfer such as equipment change and scale-up, should be taken into consideration. The consequences of alterations in culture environmental parameters such as mass transfer, pH gradient, and hydrodynamic force, upon the exosome product are not entirely found out (Brindley et al. 2011). In scaling up, some primary lines display more decreased proliferative capacity, restricted ultimate culture dimension, duration, amount of generating masses or reproducibility (Chen et al. 2011a). Serum-free medium are usually not recommended because several of the cultured cells for exosome investigations are anchorage-dependent and to some degree serum supplementation are necessary. Further proteomic analysis revealed that the serum-free exosomes harbor different component levels in comparison with fetal bovine serum (FBS) exosomes. Whereas stress-related proteins and reactive oxygen species were found in serum-free exosomes, FBS exosomes

exhibited higher levels of RNA-processing proteins. The serum-free media seems to induce alterations in the composition and function of the exosomes (Li et al. 2015). However, there is a concern regarding use of FBS: contamination of bovine exosomes. In order to hinder this situation, exosome-depleted versions of FBS may be utilized.

Stem cells secrete many crucial growth factors and stem cell conditioned medium is usually essential for effective stem cell culture *in vitro*. The lack of source for wide volumes of conditioned medium for exosome manufacturing from stem cells is another formidable factor. Accordingly, the possibilities for generating large number of stem cell-conditioned medium with which to support significant scale-up investigations on exosome manufacturing are limited (Colao et al. 2018). Moreover, it also reported that the expression of distinct exogenous proteins in cultured cells may affect the properties and type of exosomes (Whitford and Guterstam 2019).

Currently, state of art approaches, which improve culture surface area, such as hollow-fiber reactors, culture in fixed-bed or microcarriers in stirred-tank reactors have been focused on (Panchalingam et al. 2015). Along with large-scale cell production demands, it is mandatory to regulate environmental parameters such as pH, temperature, and cell's phenotype (Chen et al. 2011b). Large-scale mass exosome manufacturing is achieved in such formats like using many of large flasks, wide fixed-bed bioreactors, multiple stacked array multilayer culture flasks, stirred-tank bioreactors employing microcarriers or continuous production in perfusion reactors (Colao et al. 2018). These modern approaches exist for improvement of mass manufacturing and a clinically-related exosome production format.

Owing to the poor ability of in vitro exosome production, the present scale-up of standard batch-mode manufacturing could harbor hundreds of flasks or a remarkable investment in the more complicated and more costly multilayer flask systems. Drawbacks of these methods include the cost of culture expansion prior to the main production stage or the extra cost and timing of beginning of the production phase in serum-modified or serum-adjusted medium. Secondly, stirred-tank bioreactor cultures using microcarriers have been defined that there are not too many difficulties associated with this upstream production method. There are several scale factors and fundamental environmental conditions differentiating impeller-based bioreactor culture and small-scale flask culture. Gas mass transfer differences, cell-to-microcarrier binding, and the hydrodynamic forces created in agitation and sparging are some of the factors that cause concerns regarding production efficiency and culture progression (Chen et al. 2011b). Thirdly, perfusion-based production systems may overcome some of these concerns and hamper the additional processes and restrictions related to microcarriers. These approaches can not only promote culture over a prolonged period but also can concentrate exosomes in a membrane separated compartment, facilitating feeding and harvest. The cell containing side of the perfusion apparatus also promotes the sorting of growth factors, permitting a severe decrease in additional serum, factors or conditioned medium (Wen et al. 2011). For even larger mass production, many porous 3D scaffolds and fiber-based packed and fixed-bed bioreactors are now available (Habibi et al. 2020). Hollow-fiber perfusion bioreactors have been utilized in cell culture for years and their use in mass manufacturing of exosomes have been reported (Watson et al. 2016). These reactors are able to support wide quantities of cells at high densities in perpetual culture mode without splitting the cells and maintain larger secreted products such as exosomes.

Several various productions platforms and modes exist and they are laid out to produce different types of exosomes for investigations and clinical applications. Standard exosome isolation, characterization techniques have not been established entirely. Further tailoring and standardization for specific exosome subgroups, within the larger-scale exosome production, is required.

7 Conclusion

As mediators of crosstalk among cells, EVs can be exploited for therapeutic and diagnostic goals. Whereas naturally secreted exosomes may have beneficial impacts on definite diseases, targeted exosomes loaded with therapeutic molecules may make outcomes optimal. Biological molecules, such as recombinant proteins, siRNA and miRNA, are hard to be delivered intracellularly without the use of a carrier. EV obtaining and characterization methods have become more common in clinical implementations. But the complicated structure, the changeable composition and oncogenic, immunogenic potential of exosomes may restrict their applications. A potential alternative is the advancement of exosome mimetics which may improve stability, targeting, immunogenicity, and uptake. Taken together, with the improvement of gene delivery tools, the ongoing advancement of exosome-based carrier methods will thrive the targeted gene treatments as accessible therapies for grueling diseases in the future. Bioproduction of exosomes for preclinical tests, diagnostic and therapeutic purposes continue to be discovered. Further standardized and potent approaches regarding large-scale manufacturing of exosomes under GMP and storage conditions are required to transfer exosomes to the clinical practice.

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Interests of Exosomes in Bone and Periodontal Regeneration: A Systematic Review

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Abstract

Periodontitis is an infectious inflammatory disease characterized by clinical attachment loss and tooth supporting tissue destruction. As exosomes demonstrated pro-regenerative ability, their use in periodontal treatment has been suggested. The aim of this systematic review is to gather and summarize the most recent data regarding exosomes to determine their potential impact in bone and periodontal regeneration. Electronic databases (Pubmed, Web of Science) were searched up to February 2020. Studies assessing the impact of exosomes administration in experimental bone and periodontal defects have been identified according to PRISMA guidelines. Among the 183 identified articles, 16 met the inclusion criteria and were included in this systematic review. Experimental bone defects were mainly surgically induced with a dental bur or distraction tools. All studies considered bone healing after exosomes administration as the primary outcome. Results showed that mesenchymal stem cells derived exosomes administration promoted bone healing and neovascularization. Nevertheless, a dose-effect relationship was observed. Exosomes administration appears to promote significantly the bone healing and periodontal regeneration. However, only a limited number of studies have been carried out so far and the optimized protocols in this context need to be evaluated.

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Keywords

Bone healing · Cell-free · Exosomes · Periodontal regeneration · Periodontitis · Wound healing

Abbreviations

APSC	Apical papilla stem cell
BMC	Bone marrow cell
BMMSC	Bone marrow mesenchymal stem cell
DFSC	Dental follicle stem cell
DMOG	Dimethyloxaloyglycine
DPSC	Dental pulp stem cell
EMD	Enamel matrix derivative
EPC	Endothelial progenitor cell
Exos	Exosomes
GMSC	Gingiva-derived stem cell
GTR	Guided tissue regeneration
HA	Hydroxyapatite
hASC	Human adipose mesenchymal stem cell
hBSC	Human bone stem cell
hiPSC	Mesenchymal stem cells derived from human induced pluripotent stem cell
hiPS-MSC	Human induced pluripotent stem cell-derived mesenchymal stem cell
hMSC	Human mesenchymal stem cell
HSP	Heat shock protein
hucMSC	Hypoxic mesenchymal stem cell from human umbilic cord
HUVEC	Human umbilical vein endothelial cell
Micro-CT	Micro-computed tomography
MSC	Mesenchymal stem cell
MVBs	Multivesicular bodies
PBS	Phosphate-buffered saline
PC	Periosteum-derived cell
PDGF	Platelet derived growth factor
PDL	Periodontal ligament
PDLSC	Periodontal ligament stem cell
PLGA	Poly(lactic-co-glycolic acid)
PSC	Perivascular stem cell
SHED	Stem cell from human exfoliated deciduous teeth
SMC	Sinus mucosa cell
TLR	Toll-like receptor
uMSC	Umbilical cord mesenchymal stem cell
β-TCP	Tricalcium phosphate β

1 Introduction

Periodontitis is an infectious inflammatory disease caused by oral dysbiosis that affects 50% of adults and is more prevalent in men (Kinane et al. 2017; Eke et al. 2015). Periodontitis leads to the destruction of tooth supporting periodontal structures such as alveolar bone, periodontal ligament and cementum (Kinane et al. 2017). This disease is considered the sixth most widespread disease in the world and is the leading cause of tooth loss. Global economic burden owing to high grade periodontitis alone has been estimated to be 54 billion USD each year (Tonetti et al. 2017).

Periodontal treatment mainly consists of non-surgical scaling and root planing or surgical approach in severe cases with adjuvant therapy such as antibiotics, antiseptics or other therapeutics to optimize the treatment outcomes (Graziani et al. 2017). However, in most of the cases, only a repair of the degraded tissue is achieved resulting in the appearance of a long junctional epithelium, consequently, compromising an *ad integrum* regeneration (Caton et al. 2018) (Fig. 1). In the last decade, periodontal surgery has not seen much evolution in terms of techniques and materials used. According to the type of defect and the amount of remaining bone walls, different regenerative procedures with several types of biomaterials such as enamel matrix derivative (EMD), bone graft materials or membranes have been proposed (Cortellini and Tonetti 2015). Indeed, EMD induces and promotes periodontal regeneration in intrabony containing, deep and narrow defects (Cortellini and Tonetti 2015) which is 2.46 times more likely to achieve a clinical attachment level (CAL) gain greater

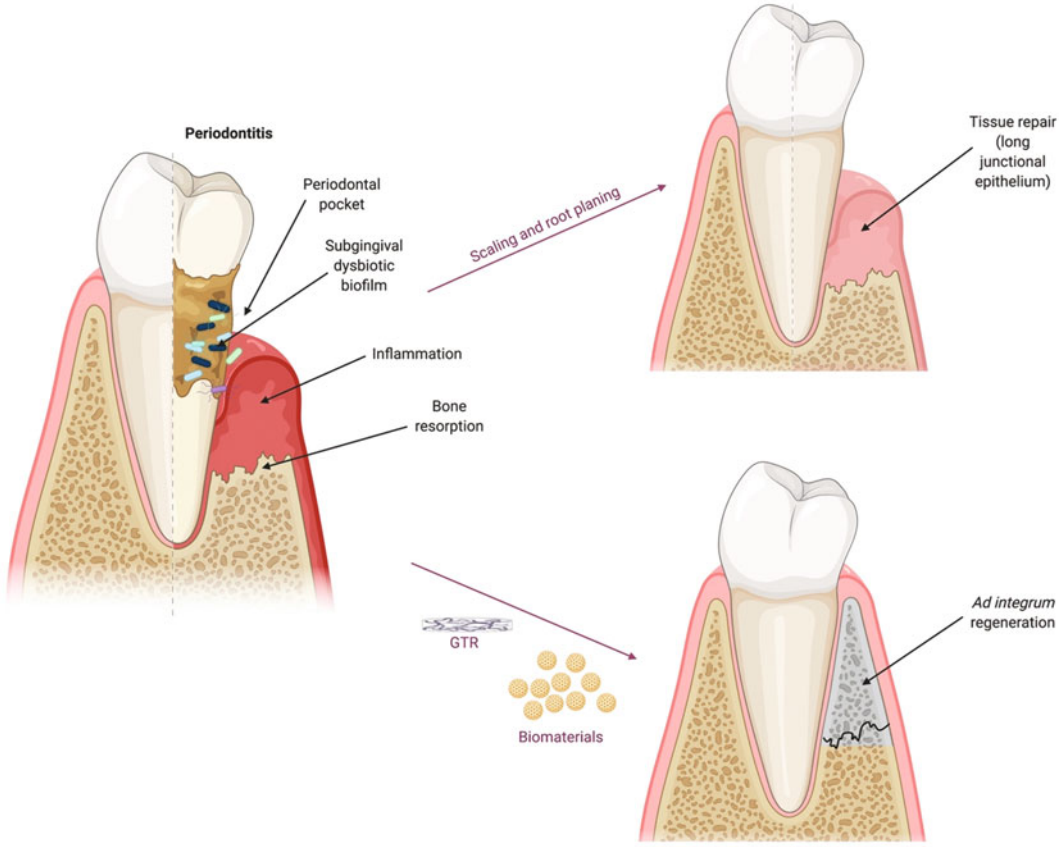

Fig. 1 Periodontal destruction associated with periodontitis. Current therapeutic strategies including scaling and root planing will lead to tissue repair through the establishment of a long junctional epithelium. Use of guided tissue regeneration (GTR) and specific bioactive materials will induce an *ad integrum* regeneration of the periodontium

than 3 mm in periodontal lesions of 22° than in periodontal lesions of 36° (Tsitoura et al. 2004). In the absence of bone walls or in wide defects, autologous or heterologous bone graft can provide space provision and blood clot stability. The bone graft acts as a scaffold while enhancing osteo-conductivity and osteo-inductivity (Trombelli and Farina 2008; Rosen et al. 2000). At long term, the results of such therapeutic strategies are stable over the years provided the patients have a rigorous follow-up (Nygaard-Østby et al. 2010; Sculean et al. 2006; Pretzl et al. 2009; Petit et al. 2019).

Despite the success of these methods, innovative periodontal regenerative techniques are still under development and the use of stems cells and growth factors has been suggested in order to stimulate host regenerative potential. Indeed, 22 studies concerning periodontal regeneration induced by stem cells were identified in a recent systematic review (Tassi et al. 2017). Such studies demonstrated that the use of mesenchymal stem cells (MSCs) may provide beneficial effect in the context of periodontal regeneration. Notably, several MSCs harvested in oral cavity have been tested for their pro-regenerative properties with interesting results. It includes dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), gingiva-derived stem cells (GMSCs), stem cells from human exfoliated decidual stem cells (SHED), dental follicle stem cells (DFSCs) and apical papilla stem cells (APSCs)(Yang et al.

2020). Nevertheless, other strategies have been proposed comprising the use of growth factors aiming to activate the healing process. Earlier, Nevins et al., showed an induction of periodontal regeneration by Platelet Derived Growth Factor (PDGF) treatment in localized defects (Nevins et al. 2013). Additionally, Lynch et al. demonstrated the efficacy of an aqueous gel functionalized with PDGF and Insulin Growth Factor (IGF) in periodontal regeneration (Lynch et al. 1989). Other studies have exhibited the possible positive effects of other growth factors such as Fibroblast Growth Factor (FGF), PDGF, IGF, Transforming Growth Factor (TGF), Epidermal Growth Factor (EGF) in periodontal wound healing and regeneration (Caffesse and Quiñones 1993). However, the use of stem cells and growth factors in dental practice is still debated. For instance, their use is prohibited in Europe due to potential risk related to malignant transformation and/or uncontrolled differentiation emphasizing the need of cell-free therapy (Volponi et al. 2010; Grayson et al. 2015).

The potential of exosomes in the context of tissue regeneration has been tested. Exosomes are microvesicles ranging from 40 to 100 μm released by cells in both physiological and pathological conditions after fusion of the multivesicular bodies (MVBs) with the plasma membrane (Carretero-González et al. 2018; Skotland et al. 2017). Exosomes are composed of a complex lipid bilayer membrane (Carretero-González et al. 2018) formed by proteins, nucleic acids, lipids and other metabolites (Skotland et al. 2017). These vesicles contain different molecules including mRNA, miRNA and proteins, and play a role in intercellular communication (Carretero-González et al. 2018). However, they can be rendered a cell-derived therapeutic carrier for efficient drug delivery as demonstrated with anti-cancerous agents (Carretero-González et al. 2018).

In a similar manner, exosomes can be used as a therapeutic tool to carry active molecule for the treatment of periodontitis, therefore, the aim of this systematic review was to identify the pro-regenerative effects of exosomes administration in several osseous lesion models.

2 Materials and Methods

2.1 Focused Question

In this systematic review, the authors addressed the following question:

- Does exosomes administration induce a pro-regenerative effect in bone and periodontal defects?

2.2 Screening and Selection Criteria

Two blinded independent reviewers performed an electronic literature search of the Pubmed and Web of Science databases up to February 2020 and reported according to the preferred items for systematic reviews (PRISMA) guidelines (PRISMA-P Group et al. 2015). Two different following search terms, filters, and combinations were used in the search using Boolean operators and an asterisk symbol (*) as truncation to identify papers using MesH, keywords and other free terms: ((exosome) AND (regeneration) AND (bone)) and also ((exosome) AND (bone regeneration OR periodontal regeneration OR periodontal healing OR bone healing) AND (in vitro OR in vivo)). The titles and abstracts of identified articles were screened by two reviewers and were categorized as suitable or not for inclusion in this review. Later, a careful assessment of the full text was performed for each study and disagreements between reviewers were resolved after discussion.

2.3 Eligibility Criteria

In this systematic review, the following criteria were used to determine the eligibility of a study for inclusion: (1) use of exosomes, (2) periodontal or bone defect, (3) *in vivo* study. Meta-analysis, reviews, and studies not focused on bone or periodontal regeneration induced by exosomes administration were excluded.

2.4 Outcomes

Quantitative and qualitative measurements relative to bone, cement and periodontal ligament regeneration were collected from the included studies.

3 Results

3.1 Study Selection

The flow diagram of the literature search has been described in Fig. 2. Briefly, following an initial search by querying selected databases (Medline/Pubmed and Web of Sciences), 183 articles were identified. 100 other articles were further identified after a manual search in the articles' references. After elimination of duplicates, 164 articles were screened. Based on the titles and the abstracts, 143 records were excluded. Among the 21 articles selected for the full-text screening, only 16 studies were included for the review. Three studies were excluded as they evaluated the *in vivo* effect of stem cells preconditioned by exosomes, another one for not being focused on the effect of exosomes and one due to the absence of a control group.

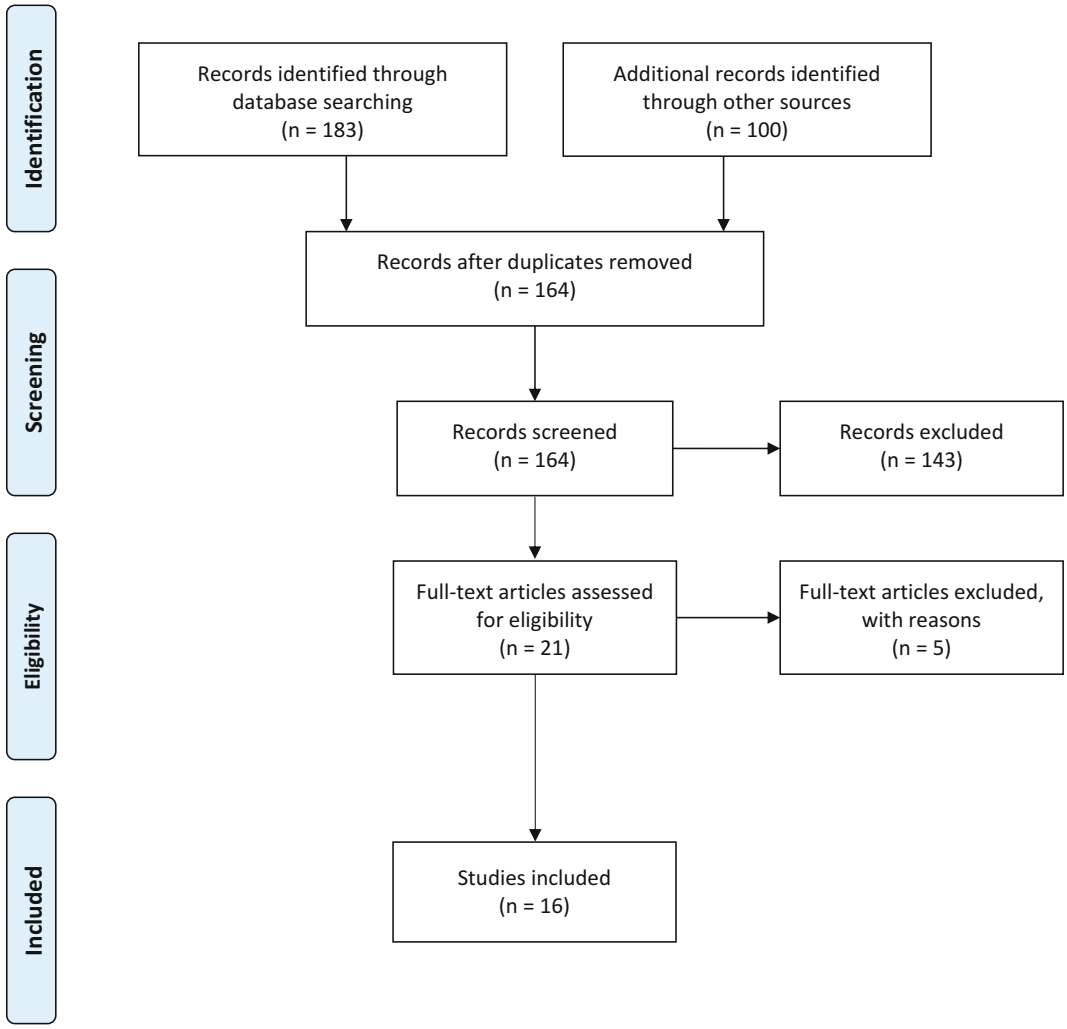

Fig. 2 Flow diagram of the literature search

3.2 Studies Characteristics

All the included studies were published between 2016 and 2020 and were performed in rodents. Among the 16 studies included, only one (Chew et al. 2019) focused on periodontal regeneration, while the remaining 15 focused on bone regeneration. Three studies used Wistar rats aged 10–12 weeks old (Takeuchi et al. 2019; Zhang et al. 2019, 2020) (Tables 1 and 2) and 7 studies used Sprague-Dawley rats (adults, 8 and 10 weeks old) (Chew et al. 2019; Chen et al. 2019a; Jia et al. 2019; Liang et al. 2019; Sun et al. 2019; Wu et al. 2019; Zhang et al. 2016) (Tables 1, 2, 3 and 4). One study was performed in osteopenic 12-weeks-old female Sprague-Dawley rats (Qi et al. 2016) (Table 1). Four studies were performed on mice, 1 study used 5-weeks-old male BALB/C mice (Li et al. 2018) and 2 studies used 10-weeks-old to 3-months-old C57BL/6 mice. Another study used CD9−/− mice in addition to C57BL/6 mice (Furuta et al. 2016; Luo et al. 2019; Xu et al. 2019) (Tables 1 and 2). One study used 10–12-weeks-old mice without specifying the strain (Liu et al. 2020) (Table 2).

3.3 Induction of the Periodontal or Bony Defect

Several tools and techniques were used to induce a bone defect, mainly in calvaria and long bone. Calvarial defects were created with a dental bur which was used to create a critical size defect of 5 mm (Takeuchi et al. 2019; Chen et al. 2019a; Liang et al. 2019; Zhang et al. 2016; Qi et al. 2016) (Table 1) except in 2 studies where a smaller bone defect (diameter of 1.8 mm or 4 mm) was created (Li et al. 2018; Xu et al. 2019).

For long bone fracture models, fractures were created by bender devices or scissors (Furuta et al. 2016; Luo et al. 2019; Liu et al. 2020) (Table 2). One study induced a bone defect by drilling in the diaphysis (Sun et al. 2019). In the alveolar model, a defect of 4×2×1.5 mm(Tonetti et al. 2017) was created with a bur at the buccal aspect of mandibular bone in molar area (Wu et al. 2019) (Table 3). Finally, in one study, a periodontal defect was developed by drilling the bone on the mesial aspect of the first molar resulting in a periodontal defect of 2×2×1.5 mm (Chew et al. 2019) (Table 4).

3.4 Exosomes Sources

Exosomes were isolated from different sources. Most of them were from stem cells, progenitor cells and stromal cells (Takeuchi et al. 2019; Zhang et al. 2019, 2020; Chen et al. 2019a; Jia et al. 2019; Liang et al. 2019; Wu et al. 2019; Zhang et al. 2016; Qi et al. 2016; Li et al. 2018; Furuta et al. 2016; Luo et al. 2019; Xu et al. 2019; Liu et al. 2020) (Tables 1, 2 and 3). However, 1 study used non-progenitor cells such as sinus mucosa-derived cells (SMCs) and periosteum-derived cells (PCs) (Sun et al. 2019) (Table 2).

3.5 Mode of Exosomes Administration and Dose

Exosomes were administered directly in the defect in association with β-TCP particles in 3 studies (Wu et al. 2019; Zhang et al. 2016; Qi et al. 2016) (Tables 1 and 3), with hydrogel in 2 studies (Zhang et al. 2019; Chen et al. 2019a) (Tables 1 and 2), with collagen sponge in 2 studies (Chew et al. 2019; Takeuchi et al. 2019) (Tables 1 and 4), and with hydroxyapatite (HA), Bio-Oss® Collagen or PLGA respectively (Liang et al. 2019; Sun et al. 2019; Li et al. 2018). In 5 other studies, exosomes were directly injected in the defect without a scaffold (Zhang et al. 2020; Jia et al. 2019; Furuta et al. 2016; Xu et al. 2019; Liu et al. 2020). Lastly, one study involved aptamer-functionalized exosomes injection in the tail vein (Luo et al. 2019). Moreover, a wide range of concentrations were used among studies (Tables 1, 2, 3 and 4).

Table 1 Interest of exosomes in calvarial defect

Authors	Animal type	# of animals	Origin of exosomes	Exosomes dose	Extraction method	Scaffold and administration mode	Results
Takeuchi et al. (2019)	10-week-old male Wistar rats	4 groups (n = 6/group)	hMSCs	30 µg on the membrane	Ultracentrifugation	Atelocollagen sponges (Terudermis1, Olympus Terumo Bio-materials Corp., Tokyo, Japan) loaded with exosomes and implanted in the defect.	Exosomes group showed statistically higher newly formed bone area (NFBA) ($p < 0,01$) compared to defect alone, PBS and exosomes conjugated with VEGF inhibitor.
					Characterized with: CD9, CD63, CD81		No difference between exosomes group and MSC-conditioned media (MSC-CM).
							Exosomes can be used as bioactive agents for bone regeneration.
Liang et al. (2019)	Adult male Sprague-Dawley rats weighing 250–300 g	3 groups (n = 10/group)	hBSCs	100 µg in 200 µL of PBS	Ultracentrifugation	Classical porous hydroxyapatite (HA) scaffold (5 mm diameter, 2 mm depth, average pore size of 500 µm, 75% porosity) loaded with exosomes + PBS by injection on the HA in the defect.	Bone volume/total volume (BV/TV) ratio in DMOG-MSC-Exosomes group higher than MSC-Exo ($p < 0.05$) and MSC-Exo better than control ($p < 0.05$).
					Characterized with: CD9, CD63, TSG101, GM130		DMOG-MSC-Exosomes promote neovascularization and enhance bone regeneration.
Xu et al. (2019)	10-week-old male C57BL/6 J mice	3 groups (n = 4/group)	Perivascular stem cells (PSC)	PSC-extracellular vesicles (PSC-EV) 1 or 2.5 µg total dose	Ultracentrifugation	None	Bone volume was significantly higher for PSC-EV 1 µg ($p < 0.05$) and for PSC-EV 2.5 µg ($p < 0.01$) compare to control group.
						Subcutaneous injection every 3 days during	Perivascular EVs positively regulates

(continued)

Table 1 (continued)

Authors	Animal type	# of animals	Origin of exosomes	Exosomes dose	Extraction method	Scaffold and administration mode	Results
					Characterized with: CD9, CD63, and CD81	4 weeks at the defect site.	bone defect repair by inducing proliferation, migration, and osteogenic differentiation of osteoprogenitor cells.
Zhang et al. (2016)	Sprague-Dawley rats weighing 250-300 g	3 groups (n = 6/ group)	Human-induced pluripotent stem cell-derived mesenchymal stem cells (hiPS-MSCs)	5×10^{11} or 1×10^{12} particles/mL of exosomes (100 μL) or an equal volume of exosome diluent (PBS) were blotted onto each β-TCP scaffold	Ultracentrifugation Characterized with: CD9, CD63, and CD81	Classical porous tricalcium phosphate (β-TCP) scaffold (5 mm diameter, 2mm depth) (Bio-lu Biomaterials Co. Ltd., Shanghai) loaded with exosomes and implanted in the defect.	Exosomes 1×10^{12} particles/mL group is significantly better compared with β-TCP group ($p < 0.05$), and better compared with the exosomes 5×10^{11} particles/mL group ($p < 0.05$) in term of BV/TV, bone mineral density (BMD) and newly formed bone area. Dose-dependent relationship. Increase in new bone formation in exosomes groups as compared to the control.
Qi et al. (2016)	Mature female Sprague Dawley rats (12-weeks-old, weighing 250–300 g) osteopenic animals	27 total, 3 groups (n = 9/ group)	MSCs derived from human induced pluripotent stem cells (hiPSCs)	100 μg and 200 μg	Ultracentrifugation & ultrafiltration Characterized with: CD9, CD63, CD81	β-TCP scaffolds (5 mm diameter, 2 mm depth) 500 μm pore size and 75% porosity, loaded with exosomes and implanted in the defect.β-TCP scaffolds (5 mm diameter, 2 mm depth) 500 μm pore size and 75% porosity, loaded with exosomes	BV/TV and BMD significantly Higher ($p < 0.05$) in exosomes 200 μg compare to exosomes 100 μg and control group. Exosomes 100 μg significantly Higher ($p < 0.05$) compare to control group. Dose-effect relationship found.

						and implanted in the defect.	Exosomes stimulated dramatically bone regeneration and angiogenesis.
Chen et al. (2019a)	Male Sprague Dawley rats	36 total, 3 groups (n = 12/ group)	Exosomes derived from miR-375-overexpressing human adipose mesenchymal stem cells (hASCs)	Hydrogel loaded with negative control (NC) – exosomes (50 μg/mL) or hydrogel loaded with exosomes (miR-375) (50 μg/mL).	Exosomes were extracted from supernatants of hASCs by differential centrifugation and filtration steps	Hydrogel (Glycosan biosystems), loaded with exosomes and implanted in the defect.	BV/TV differed significantly ($p < 0,01$) (blank< hydrogel< exosomes (NC) < exosomes miR-375; and exosomes (NC) < exosomes miR-375 ($p < 0,05$)). BMD differed significantly ($p < 0,01$) (hydrogel< exosomes (NC) < exosomes miR-375), but no difference between blank and hydrogel.
	Weighing 250–300 g				Characterized with: CD9, CD63, β-tubulin and histone 1		Exosomes derived from miR-375-overexpressing hASCs promoted bone regeneration.
Li et al. (2018)	5-weeks-old male BALB/C mice	3 groups (n = 8/ group)	Human adipose-derived stem cells (hASCs)	1 μg/μL exosomes solution (250 μL/ scaffold)	Ultracentrifugation	4 mm diameter and 2 mm high cylindrical PLGA (Shandong, China) with polydopamine (pDA) coating = PLGA/pDA loaded with exosomes and implanted in the defect.	Compared to groups without exosomes, significant new bone volume (NBV) in PLGA/pDA-Exosomes group than PLGA/pDA group and PLGA alone group ($p < 0.01$).
					Characterized with: CD63, CD9		PLGA/pDA scaffolds with exosomes promotes bone regeneration.

Table 2 Interest of exosomes in long bone fracture model

Authors	Animal type	# of animals	Origin of exosomes	Exosomes dose	Extraction method	Scaffold and administration mode	Results
Liu et al. (2020)	Mice	3 groups (n = 8/group)	Hypoxic	200 µg precipited in 200 µL	Ultracentrifugation and filtration	None	For callus volume/ total volume (CV/TV), exosomes statistically better compared to PBS ($p < 0,05$), hypo-exosomes better than exosomes ($p < 0,01$), hypo-exosomes better than PBS ($p < 0,001$).
			MSCs from human umbilic cord (HucMSCs)		Characterized with: TSG101, CD9, CD63, and CD81	Subcutaneous injection of exosomes right after the fracture at the fracture site.	Increase of CV/TV for hypo-exosomes and exosomes compare to PBS.
Sun et al. (2019)	8-weeks-old male Sprague Dawley rats weighing 250 g	3 groups (n = 12/group)	Sinus mucosa and periosteum-derived cells (SMC and PC)	200 µg of SMC-/PC-exosomes in 100 µL of PBS	Ultracentrifugation	BioOss® Collagen (Geistlich Pharmaceutical, Wolhusen, Switzerland) loaded with exosomes and implanted in the defect.	BV/TV
					Characterized with: CD63, CD81 and anti-betatubulin		Significantly better at 3 and 6 weeks for PC and SMC-exosomes groups compared to control group ($p < 0.05$). No difference between PC and SMC-exosomes.
Furuta et al. (2016)	C57BL/6 wild-type and CD9−/− mice between 17 and 19 weeks-old	77	Human bone marrow derived MSCs	100 µL of exosomes, conditioned media (CM), or exosomes-free conditioned media (CM-exosomes) injected at 1 and 8 days following fracture	Ultracentrifugation	None	Average period for bone union was significantly different ($p < 0.05$) with quicker healing time exosomes group and CM groups compared to CM-exosomes and control group, in CD9−/− mice. In WT mice, bone union period was significantly shorter in CM group and exosomes group compared with the control and CM-exosomes groups.
					Characterized with: CD9 and CD81	Subcutaneous injection of exosomes at day 1 and 8 after the fracture at the fracture site.	

Zhang et al. (2019)	12-weeks-old male Wistar rats weighing 400-450 g	48 total, 3 groups	Umbilical cord mesenchymal stem cells (uMSCs)	100 µg/mL	Ultracentrifugation Characterized with: CD9, CD63 and CD81	HyStem-HP hydrogel (Catalog: GS315, Glycosan Biosystems, Salt Lake City, UT, USA) loaded with exosomes and implanted in the defect.	Increased expression of VEGF in exosomes group compare to control group ($p < 0.01$). BV/TV statistically higher for exosomes group compare to PBS. Enhanced healing and angiogenesis in exosomes group.
Jia et al. (2019)	Sprague-Dawley rats	68 total, 4 groups (n = 17/group)	Endothelial progenitor cells (EPC)	1×10^{10} EPC-exosome (1) or 1×10^{11} EPC-exosomes (2)	Ultracentrifugation Characterized with: CD9, Alix, and TSG101	None Subcutaneous injection of exosomes right after the fracture at the fracture site.	At 2 and 4 weeks BV/TV was higher in exosomes (2) and EPCs groups ($p < 0.001$) compare to PBS. Exosomes (1) was higher at 2 weeks ($p < 0.05$) and at 4 weeks ($p < 0.01$) compare to PBS. Exosomes accelerate bone regeneration during distraction osteogenesis by stimulating angiogenesis.
Luo et al. (2019)	3-months-old C57BL/6 female mice and 3-months-old C57BL/6 male	For osteoporosis model, females mice were divided in 4 groups (µCT analysis (n = 6/group) and for quantitative analysis of the intensity for the positive staining areas (n = 4/group)). For fracture model males mice were divided in 3 groups for the (n = 4/group)	Bone marrow stromal cells (STs) were used to isolate their secreted exosomes (STExosomes).	100 µg of DIR- labeled STExosomes and an equal amount free STExosomes or DIR dye.	Ultracentrifugation	None	Acceleration of bone healing fracture (non-significant between PBS and STExosomes, but significantly higher for STExosomes-apt> STExosomes > PBS ($p < 0.01$)).
			STExosomes were conjugated with an aptamer (STExosomes-Apt)	The aptamer conjugated STExosomes (STExosomes-Apt)	Characterized with: CD63, CD81 and TSG101	Injection of exosomes in the tail vein once per week during 2 months.	For osteoporosis: BV/TV significantly higher ($p < 0.05$) comparing STExosomes-apt> ovariectomy (OVX) mice + PBS > OVX+ STExosomes but non-significant with sham + PBS.

(continued)

Table 2 (continued)

Authors	Animal type	# of animals	Origin of exosomes	Exosomes dose	Extraction method	Scaffold and administration mode	Results
				100 μg were dissolved in 100 μL PBS			Intravenous injection of the STExosomes-Aptamer complex enhances bone mass in OVX mice and accelerates bone healing in a femur fracture mouse model.
Zhang et al. (2020)	12-weeks-old mature male Wistar rats weighing 250-300 g	60 total, 3 groups (n = 20/group)	Bone marrow mesenchymal stem cells (BMMSCs) from the femurs of 3-weeks-old Wistar rats	100 μL of each: PBS, complete medium without exosomes and exosomes (10^{10} particles)	Ultracentrifugation	None	BV/TV was significantly higher in exosome group than in the control and in exosome-depleted CM (CM-exosomes) groups on week 20 ($p < 0.01$).
					Characterized with: CD9, CD63, and CD81	Subcutaneous injection of exosomes at the fracture site every week from the 8th to the 20th week.	Fracture gaps in the control and CM-exosomes groups became clearer as bone vanished, but in the exosomes group new bone was formed (data taken at 8 weeks, 14 weeks, 20 weeks).
							Exosomes accelerate proliferation and migration of endothelial cells and osteoblasts, which promotes angiogenesis and osteogenesis to enhance fracture healing.

Table 3 Interest of exosomes in bone alveolar regeneration

Authors	Animal type	# of animals	Origin of exosomes	Exosomes dose	Extraction method	Scaffold and administration mode	Results
Wu et al. (2019)	8-weeks-old male Sprague-Dawley rats	30 total, 3 groups (n = 10/group)	Stem cells from human exfoliated deciduous teeth (SHEDs) – immature MSC population	100 μg of exosomes combined with 5 mg of β-TCP	Ultracentrifugation	β-TCP (Biolu Biomaterials Co., Ltd., Shanghai) less than 1 mm diameter, loaded with exosomes and implanted in the defect.	BV/TV ratio in the exosomes group was 0.943 which was larger than the β-TCP (0.768) or the control group (0.659).
					Characterized with: CD9, CD81, TS101		BV/TV: Control< β-TCP ($p < 0.05$)
							β-TCP < exosomes ($p < 0.01$), control< exosomes ($p < 0.01$).
							Exosomes associated with β-TCP enhance osteogenesis and angiogenesis which contribute to alveolar bone regeneration.

Table 4 Interest of exosomes in periodontal regeneration

Authors	Animal type	# of animals	Origin of exosomes	Exosomes dose	Extraction method	Scaffold and administration mode	Results
Chew et al. (2019)	10-weeks-old male Sprague-Dawley rats weighing 289-413 g	18 total, 3 groups (n = 12/group (left right))	MSCs	40 μg	Tangential flow filtration	Collagen sponges (CS) (HealiAid®, Maxigen biotech Inc. Taoyuan City, Taiwan), loaded with exosomes and implanted in the defect.	At 2 weeks, significantly higher percentage of BV/TV in the exosomes group compared to the control group (p = 0.025)
					Characterized with: CD81, ALIX, TSG101		The exosomes group was significantly higher at 4 weeks compared to the control group (p = 0.010) and untreated group (0.007).
							Significant improvements in bone gap from 2 to 4 weeks for exosomes group (p = 0.025).
							Exosomes enhanced periodontal regeneration including bone and periodontal ligament.

3.6 Timing and Administration of Exosomes

In 2 studies, single exosomes administration was performed percutaneously after the bone defect creation (Jia et al. 2019; Liu et al. 2020) (Table 2). In 4 other studies, exosomes injection recurred with differences in timing, ranging from every 3 days to every week with a 1-week to 12-weeks duration (Zhang et al. 2020; Furuta et al. 2016; Luo et al. 2019; Xu et al. 2019) (Tables 1 and 2). In 9 studies, exosomes were implanted on a scaffold and placed into the defect (Chew et al. 2019; Takeuchi et al. 2019; Zhang et al. 2019; Chen et al. 2019a; Liang et al. 2019; Wu et al. 2019; Zhang et al. 2016; Qi et al. 2016; Li et al. 2018) (Tables 1, 2, 3 and 4). In addition to using a scaffold to deliver exosomes at the lesion site, one study injected a similar dose of exosomes every 2 weeks at the surface of the defect area (Sun et al. 2019) (Table 2).

3.7 Bone Healing

Most of the studies used micro-CT and histological analysis in order to compare and evaluate bone healing (Chew et al. 2019; Takeuchi et al. 2019; Zhang et al. 2019, 2020; Chen et al. 2019a; Jia et al. 2019; Liang et al. 2019; Sun et al. 2019; Wu et al. 2019; Zhang et al. 2016; Qi et al. 2016; Li et al. 2018; Furuta et al. 2016; Luo et al. 2019;

Xu et al. 2019; Liu et al. 2020). All studies showed an improvement in terms of bone healing in the test groups (groups treated with exosomes) when compared to the control group. Interestingly, studies demonstrated the differential effect obtained according to different pretreatments or types of exosomes. Indeed, Takeuchi et al. found better results in MSCs exosomes treated groups in comparison to the angiogenesis inhibitor associated MSCs exosomes treated group (Takeuchi et al. 2019). In another study, regular MSCs exosomes were compared to MSCs exosomes preconditioned with a low dose of dimethyloxaloylglycine (DMOG) which showed that bone volume/total volume (BV/TV) was higher in the DMOG-MSC-exosomes group (Liang et al. 2019) (Table 1). Similarly, enriched exosomes with miR-375 induced a significantly improved bone regeneration compared to that in the control group (Chen et al. 2019a) (Table 1). Liu et al. compared exosomes derived from MSCs under hypoxia (Hypo-Exos) to exosomes derived from MSCs under normoxia (Exos) and demonstrated that Hypo-Exos had a greater effect on bone fracture healing than Exos (Liu et al. 2020) (Table 2). Furthermore, Luo et al. conjugated bone stromal cells (ST)-derived exosomes with a BMSC-specific aptamer to target bone and found a significant acceleration in bone healing with their use upon comparison with regular ST-exosomes (Luo et al. 2019) (Table 2). Four studies used different exosomes concentrations among study groups (Jia et al. 2019; Zhang et al. 2016; Qi et al. 2016; Xu et al. 2019) (Tables 1 and 2). Interestingly, three of them demonstrated a correlation between exosomes dose and bone healing (Jia et al. 2019; Zhang et al. 2016; Qi et al. 2016) (Tables 1 and 2).

3.8 Exosomes Role in Angiogenesis

Nine studies demonstrated a link between exosomes use and increase of angiogenesis (Takeuchi et al. 2019; Zhang et al. 2019, 2020; Jia et al. 2019; Liang et al. 2019; Wu et al. 2019; Qi et al. 2016; Furuta et al. 2016; Liu et al. 2020) (Tables 1, 2 and 3). In some studies, the exosomes were derived from human bone marrow stem cells (hBMCs) and human mesenchymal stem cells (hMSCs) (Takeuchi et al. 2019; Liang et al. 2019; Furuta et al. 2016) (Tables 1 and 2). Moreover, given their high proliferation and migration capacities, their low immunogenicity, and their easy collection, 2 studies used stem cells from human umbilical cord (Zhang et al. 2019; Liu et al. 2020), while the others used endothelial progenitor cells (EPCs), human induced pluripotent stem cells (hIPSCs), stem cells from human exfoliated deciduous teeth (SHEDs) and rat bone marrow stem cells (BMCs) respectively (Zhang et al. 2020; Jia et al. 2019; Wu et al. 2019; Qi et al. 2016) (Tables 1, 2 and 3). In order to detect the progression of angiogenesis, all of the studies used CD31 immunohistochemical marker (Takeuchi et al. 2019; Zhang et al. 2019, 2020; Jia et al. 2019; Liang et al. 2019; Wu et al. 2019; Qi et al. 2016; Liu et al. 2020) (Tables 1, 2 and 3). Furthermore, most of the studies used micro-computed tomography to detect the formation, or the volume increase of vessels (Zhang et al. 2019, 2020; Jia et al. 2019; Liang et al. 2019; Qi et al. 2016; Liu et al. 2020) (Tables 1 and 2). All the studies found either new vessels formation or an augmentation in vessels volume (Zhang et al. 2019, 2020; Jia et al. 2019; Liang et al. 2019; Qi et al. 2016; Liu et al. 2020) (Tables 1 and 2). Concerning the different markers targeted, all studies which used angiogenic markers have shown an increase in vessels in test groups (Takeuchi et al. 2019; Zhang et al. 2019, 2020; Jia et al. 2019; Liang et al. 2019; Wu et al. 2019; Qi et al. 2016; Furuta et al. 2016; Liu et al. 2020) (Tables 1, 2 and 3).

3.9 Exosomes Role in Cells Proliferation and Migration

Most of the studies demonstrated a link between exosomes use and cells migration and/or proliferation (Chew et al. 2019; Takeuchi et al. 2019; Zhang et al. 2019, 2020; Jia et al. 2019; Liang et al. 2019; Sun et al. 2019; Wu et al. 2019; Zhang et al. 2016; Qi et al. 2016; Li et al. 2018; Xu et al. 2019; Liu et al. 2020) (Tables 1, 2, 3 and 4). 5 studies demonstrated a positive effect of exosomes on migration and proliferation of

human umbilical vein endothelial cells (HUVECs) (Zhang et al. 2019, 2020; Liang et al. 2019; Wu et al. 2019; Liu et al. 2020) (Tables 1, 2 and 3), 5 others on MSCs and BMCs (Takeuchi et al. 2019; Sun et al. 2019; Zhang et al. 2016; Qi et al. 2016; Li et al. 2018) (Tables 1 and 2), and 3 on osteoblastic cells (Xu et al. 2019) (Table 1), endothelial cells (Jia et al. 2019) (Table 2) and on periodontal ligament (PDL) cells (Chew et al. 2019) (Table 4). Analysis of cellular migration has been assessed by transwell migration assay (Chew et al. 2019; Takeuchi et al. 2019; Sun et al. 2019; Wu et al. 2019; Li et al. 2018; Liu et al. 2020) or scratch wound healing assay (Zhang et al. 2019, 2020; Jia et al. 2019; Liang et al. 2019; Zhang et al. 2016; Xu et al. 2019), and proliferation has been assessed by cell counting (Jia et al. 2019; Liang et al. 2019; Sun et al. 2019; Wu et al. 2019; Zhang et al. 2016; Qi et al. 2016; Li et al. 2018; Liu et al. 2020) and by immunofluorescence (Chew et al. 2019; Zhang et al. 2019, 2020; Xu et al. 2019) (Tables 1, 2 and 4).

4 Discussion

This systematic review demonstrated the potential interest in the utilization of exosomes in bone and periodontal regeneration. However, it has been shown that the outcomes in periodontal and bone regeneration are dose-dependent and that some exosomes pre-treatment can lead to enhanced healing outcome.

Stem cells therapy was developed to treat oral diseases especially periodontal diseases. (Mimeault and Batra 2008; Rajabzadeh et al. 2019). In 2004, Kawaguchi et al. used autologous bone marrow MSCs to treat experimental class III defects in beagle dogs. Such treatment procedure was associated with a significant increase in the percentage of new cementum length and new bone area in the test group (stem cells + atelocollagen) when compared to the control group (atelocollagen alone) (Kawaguchi et al. 2004). Such improved healing was also found in another study assessing the combination of MSCs and other scaffold such as anorganic bovine bone mineral (ABBM) in comparison with ABBM alone in a surgically and ligature-induced intrabony defects in a dog model (Paknejad et al. 2015). As mentioned previously, MSCs from the oral cavity that are easily harvested are also of great interest (Yang et al. 2020; Yu et al. 2015). Their use in association with specific scaffolds such as multi-layered PDL-derived cell sheets has been evaluated and has demonstrated positive outcomes, for instance, in 3-walls periodontal defects (Iwata et al. 2009).

Besides their strong pro-regenerative properties, MSCs display also anti-inflammatory and immunomodulatory effects through the inhibition of the secretion of cytokines such as TNF-α, IFN-γ, and IL-1β (Du et al. 2014).

Despite such promising results, stem cell therapy presents some limitations due to the costs but also due to potential safety concerns as they may promote tumor growth and metastasis (Volarevic et al. 2018). Accordingly, alternatives have been proposed. Indeed, the use of exosomes is a new-found technique which has been already tested in early-stage lung cancer diagnosis (Shin et al. 2020) and several other diseases (Palanisamy et al. 2010) as a diagnostic tool. Shin et al. have used exosomes as tools for detecting early-stage lung cancer, with high accuracy without resorting to specific biomarkers (Shin et al. 2020). Furthermore, exosomes can also be found in the saliva, a procedure that has notable advantages such as its noninvasive nature, no risk of hemorrhage, good patient compliance, a content similar to plasma and its ease of collection (Han et al. 2018). However, only a limited amount of clinical data is available so far. In animal models, the use of exosomes was studied in organs after ischemia-reperfusion injury (Huang et al. 2017), and it has been shown that exosomes provided a powerful cardio-protection partly through heat shock protein HSP-70 and toll-like receptor TLR-4 pathway. In cutaneous regeneration, exosomes have proven their efficacy during each phase of healing by delivering various molecules such as RNAs, including mRNA and miRNAs, trophic factors and functional proteins (Wu et al. 2018). Moreover, this review has indicated that exosomes are also studied in bone and periodontal regeneration (Chew et al. 2019). The improvement in bone and periodontal regeneration is partly explained by

the activation of angiogenesis/vasculogenesis through Wnt/β-catenin pathway (Zhang et al. 2015). Furthermore, the promotion of cell migration and proliferation by exosomes has also been demonstrated, such process playing a role in the tissue regeneration (Qin et al. 2016).

Surprisingly, the effects of exosomes on inflammatory state have not been elaborated in the selected articles but some authors demonstrated that exosomes can play both positive and negative roles in the regulation of inflammatory cascade (Pivoraitė et al. 2015; Wang et al. 2019). Pivoraitė et al. demonstrated that dental pulp exosomes can exert strong anti-inflammatory effects through carrageenan-induced edema inhibition, comparable to the effects of glucocorticoids. These effects may be due to Annexin A1 and 15d-PGJ2, both of which are found on the exosomes surface (Pivoraitė et al. 2015). Moreover, Wang et al. concluded that exosomes can have either pro- or anti-inflammatory effects, depending on the carried component (Wang et al. 2019).

To enhance the utility of exosome application and optimization of the healing outcome, exosome source, condition, and concentration should be the focus of future studies. In this review, it is evident that varying exosome conditions (Takeuchi et al. 2019; Zhang et al. 2019; Chen et al. 2019a; Liang et al. 2019; Luo et al. 2019; Liu et al. 2020) and concentrations (Jia et al. 2019; Zhang et al. 2016; Qi et al. 2016; Xu et al. 2019) can have different effects on bone healing, however, significant evidence is yet to be confirmed for exosome source variations (Zhang et al. 2019; Sun et al. 2019). Furthermore, origin of exosomes is another major determinant of exosome action due to certain origin-specific proteins and markers found on the exosome surface(Luan et al. 2017; Willms et al. 2016). While surface proteins and markers help to determine the origin of an exosome, only some of them are specific to certain cell types. The list of surface proteins, markers and RNAs carried by exosomes according to species and cell origin is available on exocarta.org, a database that could help determine the optimal exosome source for different therapies.

Exosome functionalization is another interesting strategy where exosomes can be loaded with therapeutic proteins, molecules, RNA, or imaging molecules using different functionalization techniques and, therefore, act as a tool to transport therapeutic products to for tissue-targeted delivery (Luan et al. 2017). For instance, Munagala et al. used exosomes to encapsulate chemotherapeutic and chemo-preventive agents against lung cancer and demonstrated that these functionalized exosomes had an anti-tumoral effect *in vivo (*Munagala et al. 2016*)*. Another study loaded curcumin inside peptide-conjugated-exosomes and showed that the conjugated-exosomes targeted desired tissues more easily and had a higher anti-inflammatory effect than conventional exosomes (Tian et al. 2018).

Exosomes demonstrate a great therapeutic potential owing to their capacity to enhance regeneration of different tissues (Chew et al. 2019; Huang et al. 2017; Wu et al. 2018). Their administration is considered a cell-free therapy which reduces the risk of malignant transformation that can be induced by stem cells or growth factors therapy (Volponi et al. 2010; Grayson et al. 2015). However, additional studies are needed to further establish the safety of such cell-free treatment application involving exosomes. In all identified studies (Chew et al. 2019; Takeuchi et al. 2019; Zhang et al. 2019, 2020; Chen et al. 2019a; Jia et al. 2019; Liang et al. 2019; Sun et al. 2019; Wu et al. 2019; Zhang et al. 2016; Qi et al. 2016; Li et al. 2018; Furuta et al. 2016; Luo et al. 2019; Xu et al. 2019; Liu et al. 2020), exosome isolation was performed after cell harvesting and culture. Some existing techniques allow exosome isolation directly from the blood of the patient or animal. These techniques open doors to new sampling techniques and allow a more convenient clinical use in the future (Shushkova et al. 2018).

In this review, we have highlighted different manners to treat bone or periodontal defects with exosomes. In addition to the several scaffolds described in this review such as β-TCP particles (Wu et al. 2019; Zhang et al. 2016; Qi et al. 2016) (Tables 1 and 3), hydrogel (Zhang et al. 2019; Chen et al. 2019a) (Tables 1 and 2), collagen sponge (Chew et al. 2019; Takeuchi et al. 2019) (Tables 1 and 4), hydroxyapatite (HA), Bio-Oss® Collagen or PLGA (Liang et al. 2019; Sun et al.

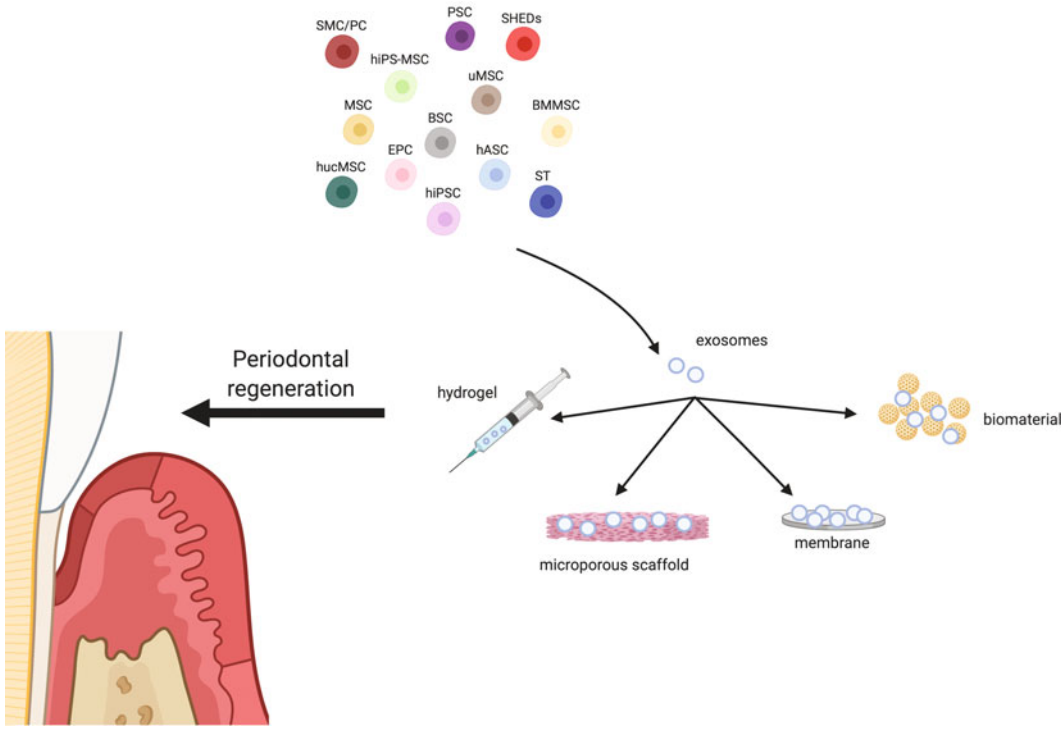

Fig. 3 Schematic representation of different exosomes sources and scaffolds potentially useful in periodontal regeneration

2019; Li et al. 2018), some studies also focus on other scaffolds such as titanium oxide nanotubes (Wei et al. 2019), 3D printed extracellular matrix/gelatin methacrylate scaffolds (Chen et al. 2019b) or protein nanocages (Cho et al. 2018).

As previously mentioned, a multitude of studies demonstrated the loading of biomaterials with different exosomes concentrations, which highlights the importance of exploring further the best exosomes scaffolds and concentrations to achieve an optimal regenerative outcome (Fig. 3).

It is note-worthy that this systematic review presents certain limitations. Herein, only one publication discusses exosome interest in periodontal defect. Moreover, these studies evaluated the effect of exosomes in different animal models, at different sites and different defects, which hinder the performance of a meta-analysis.

5 Conclusion

Exosomes use appears to be an innovative cell-free therapy with potential clinical application in the future, especially, in the treatment of periodontal defects, a therapy that presents ongoing challenges. Despite the limitations of this systematic review, it has been established that exosome therapy seems to enhance significantly the bone and periodontal regeneration. A dose-related effect has also been observed, however, no study has compared the influence of the source of exosomes on promotion of tissue healing and bone/periodontal regeneration. Further studies are required to determine more precisely their therapeutic perspectives.

Conflict of Interest The authors declare no conflict of interest.

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The Design and Application of an Appropriate Parkinson's Disease Animal Model in Regenerative Medicine

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Abstract

Objectives: Aging as an inevitable and complex physiological process occurs through a progressive decrease in the potential of tissue regeneration. Given the increasing global outbreak of aging and age-related disorders, it is important to control this phenomenon. Parkinson's disease (one of the age-related neurodegenerative and progressive disorders) resulted from predominant dopaminergic neurons deficiency. Usual Parkinson's disease treatments just can lead to symptomatically relieving. Recently, cell therapy and regenerative medicine a great promise in the treatment of several types of disorders including Parkinson's disease. Herein, before starting clinical trials, preclinical studies should be performed to answer some fundamental questions about the safety and efficacy of various treatments. Additionally, developing a well-designed and approved study is required to provide an appropriate animal model with strongly reliable validation methods. Hereupon, this review will discuss about the design

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and application of an appropriate Parkinson's disease animal model in regenerative medicine.

Evidence acquisition: In order to conduct the present review, numbers of Parkinson's disease preclinical studies, as well as literatures related to the animal modeling, were considered.

Results: Appropriate animal models which approved by related authorize committees should have a high similarity to humans from anatomical, physiological, behavioral, and genetic characteristics view of point.

Conclusion: It is concluded that animal studies before starting clinical trials have an important role in answering the crucial questions about the various treatments safety and efficacy. Therein, it is recommended that all of animal modeling stages be assessed by animal ethics and welfare guidelines and also evaluated by different validation tests. However, it is better to find some alternatives to replacement, refinement, and, reduction of animals. Nowadays, some novel technologies such as using imaging methods have been introduced.

Keywords

Animal welfare · Parkinson's disease · Regenerative medicine · Research design · Validation

Abbreviations

6-OHDA	6-Hydroxydopamine
APDM	Mobility Lab System
BBB	Blood-Brain Barrier
CSF	Cerebrospinal Fluid
DA	Dopaminergic
DBS	Deep Brain Stimulation
dMRI	diffusion-weighted MRI
ICLAS	International Council on Laboratory Animal Science
L-DOPA	3, 4-Dihydroxy-L-Phenylalanine
ML	Magnetic Resonance Imaging
MPTP	1-Methyl-4-Phenyl-1, 2,3,6-Tetrahydropyridine
OIE	World Organization for Animal Health
PD	Parkinson's disease
PET	Positron Emission Tomography
PQ	Paraquat
RM	Regenerative Medicine
SAM	StepWatch3
SN	Substantia Nigra
TBM	Tensor-Based Morphometry
VBM	Voxel-Based Morphometry
WHO	World Health Organization

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1 Introduction

Aging reflect growing, developing, maturing, and all changes in the period of life which reveal particular physical and mental signs (Reeve et al. 2014; Buczak-Stec et al. 2018). In the other words, aging is a normal biological process that happens inevitably and associated with gradual changing in the biochemical and physiological status of the cells, which can increase the risk of different kinds of diseases (Arking and Arking 2006; Sharma and Ebadi 2014). According to World Health Organization (WHO) estimation, between 2010 and 2050 the growth rate of the aged population will be increased especially in less developed countries. This increase will be associated with more illness and disability and it can impose a great impact on healthcare and social costs. Additionally, aged people are susceptible to multiple infections and frailty. Hence, a global effort to provide a healthy longevity is seriously needed (Reeve et al. 2014). In summary, aging can be considered as a risk factor for some disorders called age-related disease. Parkinson's disease (PD) is one of the most prevalent age-related neurodegenerative diseases (Sharma and Ebadi 2014). It is a voluntary movement disorder and comprised of complex symptoms such as tremor, bradykinesia, rigidity, and other motor and non-motor symptoms. Although, several treatment methods (such as multiple medications, deep brain stimulation, neurosurgical treatments, and etc.) have been developed, PD has been remained a progressive disorder and the advanced PD is still one of the most important challenges (Jankovic and Poewe 2012). Recently, stem cell-based therapy and regenerative medicine (RM) has been introduced as a novel and promising strategy for managing age-related disorders including PD (Goodarzi et al. 2015). However, there are multiple stages for translating basic researches and moving from bench to bedside. For instance, one of the most crucial stages in developing a safe and effective pharmaceutical or cell-based product is to design an appropriate preclinical study for demonstrating its safety and efficacy and applicability (Knoepfler 2015). Preclinical evaluation as a powerful tool has an important role in assessing various interventions for developing a validated and less invasive technique for clinical studies. In this regard, providing an appropriate and valid animal model seems to be critical in development of novel treatments including cell-based therapies (Knoepfler 2015). This review is going to describe different animal models with several validation methods in PD. In addition, advantages and disadvantages of different models and methods will be addressed.

2 Current Treatments for Parkinson's Disease

PD is a chronic neurodegenerative disease that comprise of motor and non-motor symptoms (Group, P.M.C 2014). The diagnosis of PD usually is based-on cardinal motor symptoms. In other words, motor symptoms are most important for diagnosis of early PD (Lane 2019). PD is a progressive disease in which there is no definitive cure for it and available treatments only can improve some of its symptoms. The main characteristic of PD is progressive degeneration of dopaminergic (DA) neurons. Dopamine is a monoaminergic transmitter which its neurons project to the corpus striatum (Fig. 1). In fact, it is a neuromodulator with an important role in CNS and its deficiency affects motor function, cognition, motivation, and etc. which cause neurological disorders. There are various types of dopamine receptors (D1, D2, D3, D4, and D5). These receptors have several differences including: different sequences, receptor structures and functions. Among the mentioned receptors, D2 has a pivotal role in control of motor functions and decrease of its expression cause locomotor impairment. Therefore, any treatment strategy which compensates this deficiency could be beneficial. Accordingly, 3, 4-dihydroxy-L-phenylalanine (L-DOPA) as a dopamine precursor has been considered as a gold standard treatment for PD. It can impose an effective role in diminishing of motor symptoms in the early stages of PD. However, its long-term application could be associated with adverse side effects (Lane 2019).

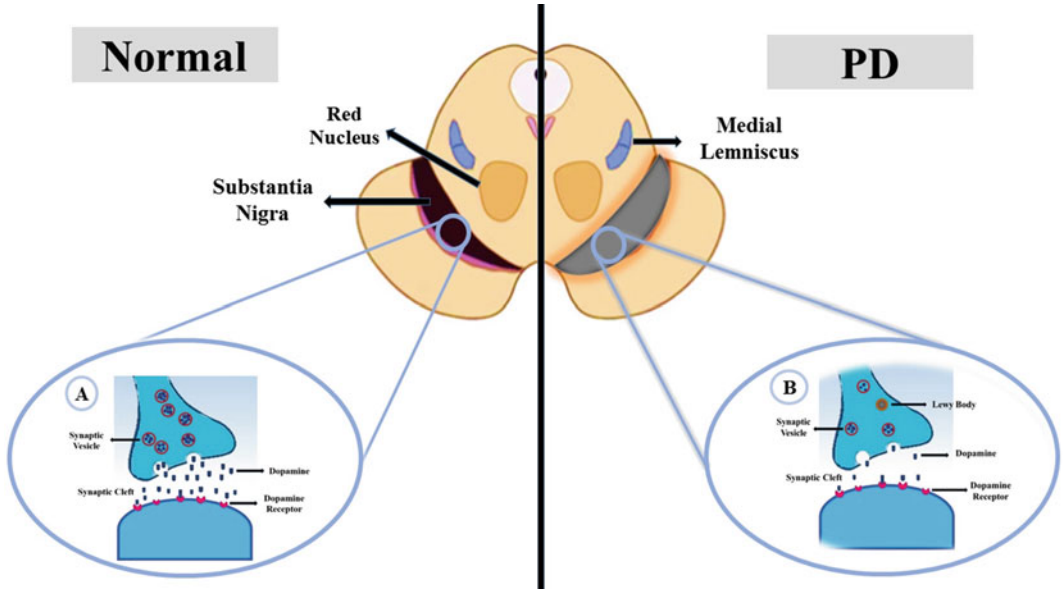

Fig. 1 Affected brain areas in Parkinson's disease. SN located in the midbrain contains DA neurons which regulates different critical functions. These dopamine-containing neurons have autonomous activity in normal condition (**a**). Degeneration of DA neurons leads to altered dopamine neurotransmitter levels which causes both motor and non-motor symptoms of PD (**b**). SN color loss and abnormal protein aggregations are the other hallmarks of this neurodegenerative disorder (Dickson 2018; Blesa et al. 2015). (PD: Parkinson's disease. *DA* Dopaminergic, *SN* Substantia Nigra)

Additionally, using of dopamine receptor agonists (e.g., pramipexole, apomorphine, piribedil) is another therapeutic tool for PD (Blandini and Armentero 2014). They stimulate dopaminergic receptors directly and can improve motor symptoms of PD. These agonists are less effective than l-DOPA but they cause continues stimulation on receptors and fewer side effects (Katunina et al. 2015). MAO-B and COMT inhibitors, anticholinergics, and amantidine are other examples of conventional treatments. In the past decades, some new therapeutic methods such as combination therapy, application of various dosages, advancement of drug delivery, exercise, deep brain stimulation (DBS) have been progressed (Fox et al. 2011). However, the current treatment strategies which generally try to postpone the PD symptoms in its early stage and improve motor complications such as motor fluctuations and dyskinesia seem to be crucial to achieve more constant benefits (Fox et al. 2011; Pahwa and Lyons 2014). Recently, cell therapy and RM reveal a great promising horizon to optimize the existing treatments through improving the DA neurons survival and providing a suitable supply of dopamine (Snyder and Olanow 2005; Goodarzi et al. 2014a). For instance, they could induce cell differentiation potency to provide specific types of dopaminergic neurons which can eliminate different side effects of the conventional treatments. In this regard, they could be considered as the appropriate alternatives, superior to current therapies (Sonntag et al. 2018).

3 Developmental Process of Regenerative Medicine

RM as a novel multidisciplinary technology deals with regenerating the damaged and dysfunctional cells, tissues, and organs to restore their normal structure and function. RM represents the new therapeutic methods which can revolutionize the treatment of incurable diseases like PD (Rahim et al. 2018a; Payab et al. 2018a; Ghodsi et al.

2012). Due to the unique regenerative properties of stem cells, they have a basic role in RM. For example cell replacement as a novel therapeutic approach in RM can be a notable achievement in RM field (Turksen 2018; Saberi et al. 2008, 2011; Ai et al. 2014; Goodarzi et al. 2014b). Fundamentally, prior to translation of basic researches to the clinic, their efficacy and safety should be evaluated according to related guidelines and regulations. In the process of RM, after scientific approaches, the hypotheses with regards to gap analysis are constructed. In the next step, these hypotheses are tested by preclinical studies (*in vitro* and *in vivo*). *In vitro* analyses provide evidences of efficiency and safety prior to translation into animals and humans. Moreover, *In vivo* investigations are the main part of preclinical studies. Animal studies can demonstrate the safety, efficacy, and potential side effects in a living organism. In the journey from bench to bedsides, general principles must be considered by scientists for instance: selection the appropriate animal model and cell type, duration of study, number of animals, the differences between animal model and human disease, animal ethics and welfare, and communication with regulatory bodies. Following this step and after appropriate toxicology, pharmacodynamics, and pharmacokinetic studies for cell-based products, the achievements in the preclinical phase can be translated to clinical trials (Okura and Matsuyama 2016). Finally, if the product or method has the overall criteria and is validated by related authorities and organizations it will be ready to use in a clinical manner (George 2011; Halme and Kessler 2006; Payab et al. 2018b).

4 Animal Study Design for Parkinson's Disease

Preclinical studies are key prerequisites of large scale clinical trials. Thus, it is important to choose the suitable animal species, considering the most anatomical, physiological, behavioral, and genetic similarities to human for *in vivo* studies (Potashkin et al. 2011). For instance, rodents and nonhuman primates (which can mimic many aspects of the pathophysiology of human neurodegenerative diseases) are used frequently in PD investigations. Due to probable breaks in model validation processes, some animal models cannot imitate the pathophysiology of PD. Accordingly, in some cases outcomes of such studies cannot be translated to the clinic (Potashkin et al. 2011). Hence, an animal model should meet a set of validation criteria to be appropriated for human pathology. On the other hand, the study design and ethics, animal selection, and model validation should be approved by related authorities and committees before starting the program. However, there are some ethical arguments against the animal experiments (Albus 2012). Herein, the '3Rs' rule (replacement, reduction, and refinement) is almost a universal and broad-acceptable tenet (Fig. 2). However, complete animal modeling replacement is still not feasible. Therefore, it is necessary to provide the appropriate and accurately validated animal models in accordance with the scientific standards and regulations. Additionally, development of fundamental infrastructures such as animal housing and husbandry to achieve standard validated animal models is important (Lahman et al. 2011; Fenwick et al. 2009; Festing and Wilkinson 2007).

5 Appropriate Animal Model

Animal modeling as a powerful tool is applied to mimic and predict the various pathophysiological human conditions to investigate multiple therapeutic approaches, especially when it is difficult to directly monitor in human. On the other hand, it also can provide some conditions of human disease processes and their pathogenesis (Xiong et al. 2017). Therefore, developing an appropriate animal model is the most important stage of designing an experimental study. According to Wessler's definition: an animal model is "a living organism with an inherited, naturally acquired, or induced pathological process that in one or more respects closely resembles the same phenomenon in man" (Ben-Hur et al. 2004; Yang et al. 2008). PD is the first neurological disorder that was modeled in an animal while it is a human specific

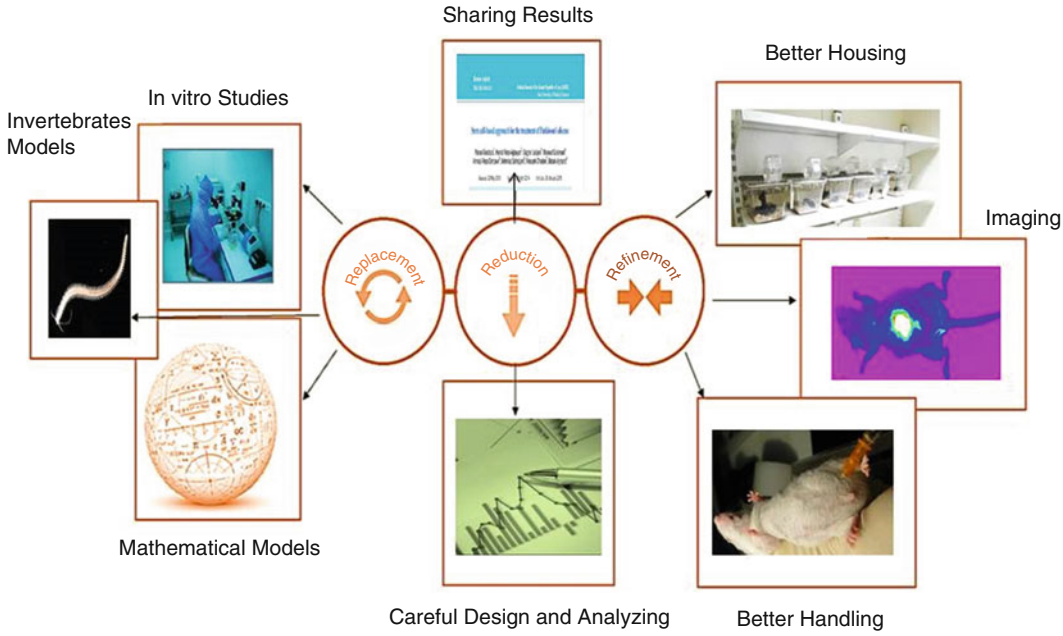

Fig. 2 The 3Rs rules. Replacement, Reduction, and Refinement are given a framework for implementing animal researches. Replacement refers to methods which replace the use of animals such as doing in vitro studies and using invertebrates, mathematical, and computational models. Reduction refers to methods which reduce the number of animals used per experiment such as sharing and publishing results and careful design and analyzing. Refinement refers to methods which decrease animal suffering and ameliorate welfare such as better animal housing, handling, and imaging (Lahman et al. 2011; Fenwick et al. 2009)

disease (Garcia-Ruiz and Espay 2017). Therefore, some additional considerations are required than other animal modeling strategies. On the other word, animal species which have the most physio-anatomical and genetic similarities with human are acceptable choices for PD modeling (Cai et al. 2009). Nowadays, several types of animals are commonly used which has advantages and disadvantages (Table 1). They ranged from single-celled microorganisms to the highest order of animals. Experimentally, specific characteristics such as sensitivity to specific chemical compound, physiological characteristics, availability, and type of disease make an animal species suitable for PD modeling. For instance, monkeys, the gold standard model of PD, mainly used for modeling of motor symptoms and behavioral studies due to their phylogenetically proximity to human species, while mice are suitable to model the molecular and cellular mechanism of PD (Tieu 2011; Kim et al. 2009). Additionally, non-mammalian organisms are used for investigation of gene expression pattern in PD most recent (Tansey and Goldberg 2010). Generally, an acceptable humanized animal model must be experimentally rational and cost-effective (Sonntag et al. 2007). Furthermore, the ideal model should represent DA neurons loss, Lewy body-like inclusions, and DA reduction in striatum to mimic most aspects of human PD (Halbach 2006).

6 Humanized Animal Modeling

Investigations on PD have benefited a lot from animal models. Two common approaches to provide PD animal models are toxin and genetic-based modeling (Table 2). Various known neurotoxins are used to target dopaminergic neurons either systemically or locally according to their ability to cross blood-brain barrier (BBB). ROS formation is the most common mechanism of neurotoxins. Perhaps reserpine was the first

Table 1 Commonly used animal species in modeling of Parkinson's disease. A wide range of animal species such as primates, rodents, mammals, fishes, and single-celled organisms can be used as the PD animal models. Each of them are suitable for studying different aspects of the disease. While single-celled microorganisms as well as small species usually are used for studying of molecular and cellular pathways, other animal species with more phylogenetic proximity to human are suitable choices for modeling of motor symptoms and behavioral studies

Animal Species	Advantages	Disadvantages
Monkeys	Exhibit behavioral and neuroanatomical similarities to the human condition	Usually represent an acute model
		Difficult availability
		Costly
	Showing a bilateral parkinsonian syndrome	Difficult handling
	Provide all stages of PD treatment research prior to starting clinical trials	
	Gold standard model	
Marmosets	Small new world primate	Difficult availability
	Relatively ease in handling	
	High reproductive efficiency	
	Specific cognitive and behavioral characters	
Mice	Generated data has led to a better understanding of molecular mechanisms	Difficult to develop a level of impairment equal to the human condition
	Genetically modifications	
	Useful for testing neuroprotective therapies	
	Useful to study of neuronal death process	
	Low cost	
	Common for MPTP (1-Methyl-4-Phenyl-1, 2,3,6-Tetrahydropyridine) model	
	Readily available	
	High reproductive efficiency	
Rats	Relatively low cost	Resistant to MPTP
	Readily available	Difficult to develop a level of impairment equal to the human condition
	Genetically malleable	
	High reproductive efficiency	
Cats, Dogs, Guinea pigs	Suitable for MPTM models	Difficult availability and handling
Sheep	Non-primate large animal model	Difficult handling
	Physiological parallel with human	
	Suitable for modeling of rare genetic disorder	Costly
	Appropriate for trial new treatments of rare human disease in animals	
Göttingen minipigs	Large gyrencephalic brain	Difficult availability and handling
	Suited for examination at conventional clinical scanning modalities	
	Allows to apply neurosurgical techniques	
	Possibility of use DBS and electrodes	
	Relatively low cost	
Zebrafish	Highly amenable to genetic alterations	No usage in study many of the clinical manifestations of the disease
	Small size	
	Short generation time	
	Low maintenance costs	
Goldfish	Being modeled with single dose of MPTP	No usage in study many of the clinical manifestations of the disease
	Relatively simple and low cost model	
	Accessible nervous system	
	Abbreviated BBB	

(continued)

Table 1 (continued)

Animal Species	Advantages	Disadvantages
Drosophila, Medaka fish, Caenorhabditis elegans, yeast, frogs	Useful for studying fundamental cellular processes involved with PD[a]	No usage in study many of the clinical manifestations of the disease
	Suitable for investigation of basic mechanism of PD	
	Rapid alternatives	
	Drosophila will allow the rapid characterization of enhancer and suppressor mutation	Lack of replicate the loss of neurons in the brain
	Drosophila is suitable for mitochondrial dysfunction model	

Potashkin et al. (2011), Jackson-Lewis et al. (2012), Blesa et al. (2012), Beal (2001), Okano et al. (2012) and Bretaud et al. (2004)

[a]Including: apoptosis, autophagy, oxidative stress, protein misfolding

PD Parkinson's disease

neurotoxin used to model the PD animals by Carlsson and Hilarp in the 1950s. They demonstrated that reduced catecholamine level in the brain due to reserpine toxicity can mimic the PD phenotype and L-DOPA alleviates the related symptoms partially (Cannon and Greenamyre 2010; Carlsson 2002). Temporary cell damage is a challenge in reserpine application. α-methyl-para-tyrosine is another pharmacological agent depletes the dopamine synthesis through inhibiting tyrosine hydroxylase, an enzyme of dopamine synthesis pathway, which shares the same limitation of impermanent neurodegeneration effect with reserpine. Concomitant reserpine and a-methyl-para-tyrosine application can prolong the duration of their neurodegeneration effects (Tieu 2011). MPTP has a lipophilic structure and this property helps MPTP to cross the BBB easily. After MPTP entrance to BBB and neurons, it metabolizes to MPP+ by monoamine oxidase-B enzyme. MPP+ as a potent mitochondrial complex 1 inhibitor can destroy the DA neurons. MPTP is considered as one of the popular systemic neurotoxins. MPTP-treated monkeys are the gold standard of PD animal models, however this model like the other models has its own pros and cons. Recently, some herbicides, pesticides, and fungicides like paraquat (PQ), rotenone, maneb, and ziram are applied as neurotoxins in PD animal modeling. Due to similar molecular structure between PQ and MPP+, it has the similar MPP+'s toxic effects. In addition, the PQ's neuron loss effect is controversial but recent researches have reported that its toxicity is dose dependent and DA neurons loss can be reached in high doses of PQ. 6-hydroxydopamine (6-OHDA), the oldest catecholamine neurotoxin, is another suitable choice for pharmacological studies. 6-OHDA cannot cross the BBB therefore it should be administered locally. Injection of 6-OHDA into the striatum, SN, or nigrostriatal tract induces DA neurons death. To avoid the severe adverse consequences of bilateral injection, 6-OHDA is injected unilaterally. In this approach the unlesioned hemisphere of animal can be left intact to serve as its own control. Recently, scientists have focused on the genetic pathogenesis of PD and novel discoveries in this area have provided an opportunity to develop genetic models. Mutations in α-syn and LRRK2 genes lead to autosomal dominant PD and mutations in PINK1/Parkin and DJ-1 genes lead to autosomal recessive PD. The noteworthy privilege of genetic models is their potential to represent the chronic nature of PD. α-syn a modulator protein in presynaptic terminals is the major protein of LBs. Some of the common applied genes in producing genetic-based PD models include SNCA, LRRK2, GBA, PRKN, PINK1, PARK7, VPS35, EIF4G1, DNAJC13, CHCHD2, UCH-L1 (Blesa et al. 2012; Cannon and Greenamyre 2010; Dauer and Przedborski 2003). Accordingly, several genetic-based studies are applied to investigating such crucial genes which can improve targeted therapies (Sardi

Table 2 Neurotoxin and genetic-based approaches in animal modeling for Parkinson's disease: Their methods, induced pathologies, and symptoms. Different modeling approaches cause PD through different mechanisms. Neurotoxin-based methods are classified according to the mod of action and their neurotoxic effects. Using PD-causing genes, it is possible to produce genetic-based PD models with the potential to represent the chronic nature of PD

Approach	Method of modeling	PD pathology	PD symptoms	Mode of action	Development of LBs[a] like inclusions	Additional points
Neurotoxin-based	6-OHDA	Non-progressive nigrostriatal dopaminergic neurons death	Quantifiable motor deficit	Induced stress oxidative in neural cells	No	Usable in both in vitro and in vivo screenings but frequent favorite animal is rat
			Akinesia			
			Rigidity			Requirement to intracerebral injection
			Tremor			
			Rotational behavior			Induction of acute effects
						Inducing degeneration of both dopaminergic and noradrenergic neurons
						Rare presence of parkinson's tremor in lesioned rodents with 6-OHDA
						Poor construct validity
	MPTP	Non-progressive nigral DA neurons death	Tremor	Mitochondrial complex I Inhibitor-	Rare	Systemic administration
			Rigidity			
			Akinesia			
			Postural instability	Reduced ATP/induced stress oxidative		Good construct validity
			Less evident of models motor deficits In rodent			
	Rotenone (herbicide and insecticide)	Progressive nigral DA neurons loss	Decreased motor activity	Non-selective mitochondrial complex I inhibitor	Yes	Frequent animal is rat
			Abnormal postures			Systemic administration
			Slowness of movement	Penetrated cell membrane		Low construct validity
				Induced oxidative stress		
	Paraquat (N,N -dimethyl-4-4-4 –bypiridinium/a herbicide)	Dose-dependent loss of DA neurons	Reduced motor activity	Oxidative stress	No	Systemic administration
						Low construct validity

(continued)

Table 2 (continued)

Approach	Method of modeling	PD pathology	PD symptoms	Mode of action	Development of LBs[a] like inclusions	Additional points
Genetic-based	Overexpression of α-syn	DA neurons loss	Progressive loss of motor function	Reduced the activity of tyrosine hydroxylase (TH)	Yes, but these inclusions are generally not seen in SN nucleus	Slight DA pathology
			DA responsive locomotor deficits	Granular intracytoplasmic inclusions		Good construct validity
			Some aspects of the pathology of dementia			
	α-syn-knockout (A53T)	Low DA neuron loss	Severe motor deficits	Enhanced α-syn aggregation	Yes, level of these inclusions are high in contrast to A30P mutated form	Slight DA pathology
						Good construct validity
	α-syn-knockout (A30P)	Low DA neuron loss	Less motor deficits	Enhanced α-syn aggregation	Yes, level of these inclusions are low in contrast to A53T mutated form	Slight DA pathology
						Good construct validity
	Mutated parkin	No or low nigral cell loss	Motor impairment is obvious in most cases of exon deleted	Mitochondrial dysfunction	No or in low levels	Slight DA pathology
						Good construct validity
	Mutated form of LRRK2	No or low levels of DA neurons loss (age-dependent neurodegeneration)	Few behavioral deficits	Autophagic and mitochondrial abnormalities	Generally not observed	Positive relation between the levels of LRRK2 and α-syn phosphorylation and aggregation in PD brains
			Different levels of motor impairment dependent on its mutation form	Enhancing α-syn aggregation		
	Mutated form of PINK1	No or low nigral cell loss	No or low motor impairment	Mitochondrial dysfunction	No or in low levels	Slight DA pathology
						Good construct validity
	Mutated form of DJ-1	No or low nigral cell loss	Motor impairment is depend on the exon deleted region	DJ-1 accumulation in mitochondria/ oxidative stress	No or in low levels	Slight DA pathology
						Good construct validity

Blesa et al. (2012), Dauer and Przedborski (2003), Terzioglu and Galter (2008), Lim and Ng (2009) and Ramonet et al. (2011)

[a]Lewy Bodies

PD Parkinson's disease

et al. 2018). Generally, each animal model has advantages and disadvantages for example related toxin-based models are suitable options for pharmaceutical studies and toxicology, while genetic-based models are generally used to study the genetics effects on the pathogenicity of diseases. It is predicted that to achieve more appropriate animal models for PD future humanized animal models will be provided considering environmental and aging factors too.

7 Validation and Critical Evaluation of Provided Model

Laboratory animals which can provide models of different human diseases, have cause several breakthroughs in medical science. Accordingly, it reveals the importance of selecting appropriate animals and also modeling and research methods. Despite to several advantages of animal models, none of them can mimic all aspects of human diseases especially in neurodegenerative disorders with the coexistence of cognitive and motor impairment, such as PD (N Prasad 2017). Therefore, according to the main purpose of studies such as investigating the behavioral symptoms, molecular mechanisms and pathways, the best model of disease can be selected and developed. After this stage, selected appropriate model should pass various validation stages. Based-on the standards of developing animal models, there are different evaluation criteria and processes. Generally, validation method could be categorized to five main types; (1) internal validity, (2) face validity, (3) predictive validity, (4) construct validity, and (5) external validity (van der Staay et al. 2009). Basically, a well-designed PD animal model should possess the main pathological and behavioral hallmarks. Todays, several behavioral analysis methods are used by investigators in order to validate the induction of behavioral deficits, for example; locomotor activity test, catalepsy test, rearing test, stepping test, rotarod/accelerod test, and probabilistic learning test are commonly used. Further, some of the mentioned behavioral tests are used as the gold standard in different conditions. For example asymmetric circling behavior is the gold standard validation for the unilateral lesions (Bezard and Przedborski 2011). Despite the existing gold standard tests which provide desirable validation aspects, they are not strongly reliable. This limitation is resulted from different validation methods using by investigators and restricted comparison opportunity. On the other word, the mentioned techniques provide subjective results. Therefore, to overcome this limitation, developing alternative objective methods for tracking the PD-related biomarkers seems to be essential. Recently, new technology-based devices have been produced to record manifestations of PD with decrease effects of confounders on data management (Garcia-Ruiz and Espay 2017). These devices can provide objective results in both basic and clinical researches (Godinho et al. 2016; Heldman et al. 2017). They are divided in wearable and non-wearable devices. Some examples of these instruments are available in the market such as; Mobility Lab System (APDM), Physilog®, StepWatch3 (SAM), TriTracRT3, WiiBalanceBoard, and GAITRite®. Most of them can optimize validity, reliability, and sensitivity in addition to assessing various clinical parameters (Godinho et al. 2016; Heldman et al. 2017; Lopane et al. 2018). However, the incidence of motor symptoms of PD is associated with advanced stages of disease which is hard to manage. Therein, molecular genetics with a special focused on "OMICs" technologies can provide early diagnosis of disease using validated biomarkers of PD (Gilany et al. 2018; Rahim et al. 2018b). On the other words, it would be able to distinguish "at risk" people and provide a great opportunity for monitoring of disease progression results in improving PD management. Clinically, biomarkers are characteristics which objectively reflect the normal or pathological processes and pharmaceutical responses to specific interventions. They can be assessed through blood and cerebrospinal fluid (CSF) analysis and can be easily standardized because of its objective results. Biomarkers analysis is possible with a small sample size and a reasonable repeatability (Shi et al. 2011; Miller and O'Callaghan 2015). Therefore, they are considered as valuable translators of preclinical studies to clinic as they can be used as disease stage

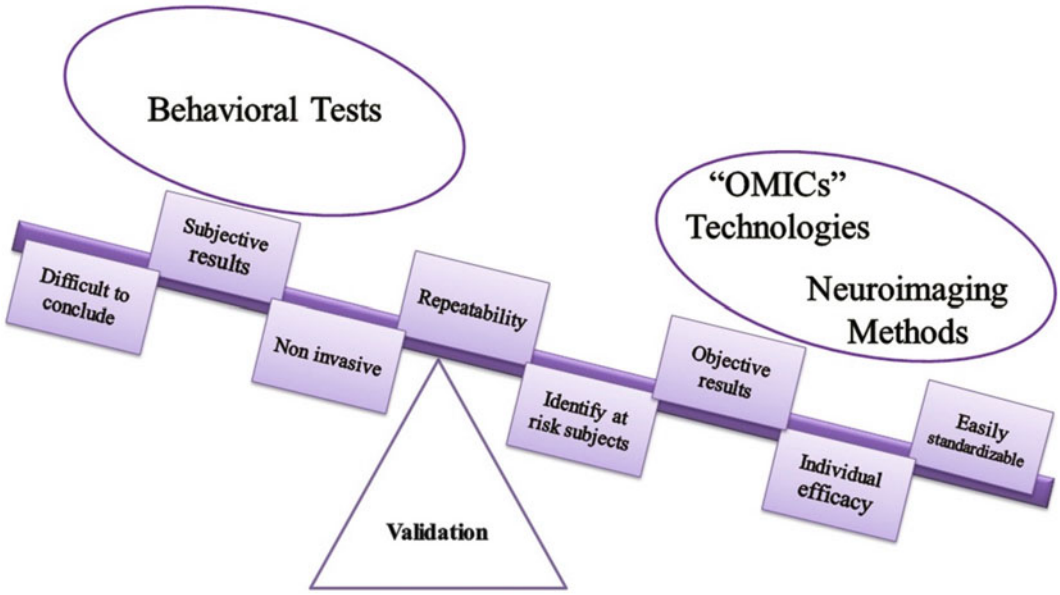

Fig. 3 Different validation methods in Parkinson's disease. A prepared PD model is evaluated using different validation methods such as behavioral tests, neuroimaging techniques, and biomarkers analysis. Each of them has their own advantages and disadvantages. Behavioral analysis methods present beneficial outcomes for validation of behavioral deficits; though, their results are subjective and difficult to conclude. However, some other validation methods like evaluating the physiological biomarkers and different neuroimaging techniques are more useful in the case of early diagnosis, monitor different stages of the disease, and personalized analysis. They represent more objective results which are easy to standardize while possess rapid outcomes (Bezard and Przedborski 2011; Shi et al. 2011; Yang et al. 2018)

specific markers to provide the possibility of assessing various drug effects in different stages of disease (Fig. 3). Interestingly, biomarkers are powerful tools in drug development for the proof of drug mechanisms, toxicity and dosage (Miller and O'Callaghan 2015). Furthermore, biomarker studies represent a remarkable impact on RM. Through the assessment of biomarkers in different stem cells secretome profile, potency and efficiency will be predictable and leads to optimized and efficient cell-based therapies (Mimeault et al. 2007; Kim et al. 2019). Overlay, PD as a neurodegenerative disease is a progressive disorder which resulted from the dysfunction of neural networks, alteration of neurotransmitters and receptors. Substantially, all of these procedures occur gradually and need to be monitored and recorded according to their chronicity. In this regard, more attention has been drawn to some novel strategies such as "OMICs" assessments and neuroimaging techniques in clinical diagnosis and medical management of PD.

8 Neuroimaging and Validation of Parkinson's Disease Model

Modern techniques of imaging have been applied to diagnosis of neurodegenerative disorders such as PD (Sarkaki et al. 2008). Neuroimaging methods are widespread, non-invasive, and cost-efficient tools for continuous evaluation of motor and non-motor impairments of PD. They are categorized to structural and functional groups to monitor different biomarkers and demonstrate the progression and changes of disease (Yang et al. 2018; Burciu and Vaillancourt 2018; De Micco et al. 2018). For instance, magnetic resonance imaging (MRI- T1-weighted and T2-weighted) as a structural technique is the most common used scan in PD. Additionally, voxel-based morphometry (VBM) is one of the widely used techniques in primates to distinguish differences between specific brain parts in different experimental groups, actually, it provides studying of focal differences in brain regions

using MRI (Okano et al. 2012). However, VBM techniques are failed to identify brain regional differences in PD models. Accordingly, tensor-based morphometry (TBM) technique have been introduced to overcome major weak points of VBM (Kielar et al. 2012). As another example, diffusion-weighted MRI (dMRI) is sensitive to changes in water molecules diffusion caused by microstructural tissue alterations (Burciu and Vaillancourt 2018; Mateos-Pérez et al. 2018; Cordes et al. 2018; Guimarães et al. 2018). 0ne of the clinical approved functional neuroimaging methods is positron emission tomography (PET), which can specify the cellular and molecular mechanisms, such as neurotransmitter and receptor changes (dopamine), protein aggregation (beta-amyloid), and etc. It will be easy to identify and predict different stages of PD using this technique (Brumberg et al. 2019; de Natale et al. 2018; Hammes et al. 2018). Recently, several supportive techniques such as machine learning (ML) algorithms have been added to the mentioned neuroimaging methods to provide a better prediction or assortment of broad-spectrum diseases using neuroimaging technique outcomes as input data (Katako et al. 2018; Aich et al. 2018; Cuadrado-Godia et al. 2018; Wan et al. 2019). Generally, deficiency of available and gold-standard method to reveal linear results is considered as a remarkable problem. Fortunately, approved guidelines and standards are available as most beneficial tools to upgrade the quality of animal study designs.

9 The Importance of Guidelines and Standards to Model Animals for Parkinson's Disease

Globally, non-human animal studies are progressively increased (Politis et al. 2010). Therefore, more concerns about ethical principles have been aroused. Interestingly, animal ethics has a long history in several cultures around the world which reveals the importance of animal rights (Szűcs et al. 2012). In different religions and cultures such as Christianity, Judaism, Islam, Hinduism, and Sikhism animal ethics and welfare has been considered. Universally, global guidelines and regulations on animal research are recommended by World Organization for Animal Health (OIE) and International Council on Laboratory Animal Science (ICLAS). Furthermore, several animal code of ethics have been established by ethical committees, for example in Iran, National Committee for Ethics in Biomedical Research authorizes ethical standards throughout the research centers, further examples are Regional Ethics Committees(Sweden), Institutional Animal Care (or Animal Care and Use) Committees(Canada, USA), and Animal Ethics Committees(Australia, New Zealand) which are consider different conditions of the animal life like; nutrition, habitat, and health. Basically, all stages of a standard experimental study should be assessed by ethics committees through the ethical and legal approved guidelines. An animal ethics committee has some mechanisms to balance cost and benefits of animal use for research. They are also trying to establish different guidelines and standards to protect laboratory animals that are used for either scientific or educational purposes and strongly restrict animal abuse and cruelty based-on 3Rs principles (Politis et al. 2010; Giles 1987; Council, N.R 2010). According to the practical guidelines; before the use of animals, all the stakeholders should be aware of the animal requirements, welfare, genetic characteristics, and breeding management to reduce the animal suffering, furthermore, they should study the research protocols carefully to diminishing unnecessary use of animals, and in the end of the study, they should euthanize animals through standard procedures (Touitou et al. 2004; Association, A. P 1986; Guillén et al. 2018; Green 2019). Since the animal models play a fundamental role in experimental biomedicine and considered as a cornerstone of biomedical researches, it is important to design and establish standard and ethical approved animal study to achieve more reliable data sources for biomedical researches (Chiorazzi et al. 2018).

10 Conclusion and Future Perspectives

Regarding to the worldwide development of aging, there is a need for the management of age-related problems and disorders such as PD (Reeve et al. 2014). Hereupon, several types of pharmacological and non- pharmacological treatments are applied for the management of PD. Recently, cellular-based therapies as a novel approach to PD can be useful through regeneration and replacement of dopaminergic neurons, and also symptomatic relieving. Therein, it is exceptionally essential to be cautious about new therapies (with different ranges from pharmacological to using cell based medicinal products) which will be brought to the bedside following safety and efficacy assessment. Accordingly, conducting toxicity, pharmacodynamic, and pharmacokinetic studies are needed as the preclinical evaluation for development of advanced therapeutic medicinal products (ATMPs) (Okura and Matsuyama 2016; Broichhausen et al. 2014). Additionally, animal models have been used for the assessment of advanced therapies and they have a fundamental role in all pathways of drug development. In the case of PD, identification of involved genes has shed light on the knowledge of the genetic pathogenesis of PD, the novel medications, and new animal modeling methods. On the other hand, the toxin-based animal modeling methods are relatively older than the genetic-based. Nowadays, due to several scientific evidences like feasibility and cost-effectiveness, the old approach is universally more acceptable. For example, MPTP-treatment is the gold standard of PD modeling. However, the future models can provide the combined properties of not only toxin and genetic-based models but also the environmental and aging factors (Dauer and Przedborski 2003; Schober 2004). Generally, finding an appropriate model with a suitable validation method is the most important stage in experimental studies. Accordingly, several validation strategies have been provided for different manifestations of PD. However, applying more reliable methods can result in perfect models. Therefore, more applicable validation methods with objective results have to be standardized. While, animal models are essential tools for experimental studies, it has some limitations that should be considered. According to ethical and legal principles, the use of animals for different research approaches should be justified. On the other word, despite the using of several animal models, its justification still is controversial. Thus different ethical declarations, guidelines, and codes have recommended some alternatives to replace animal models (Harriss et al. 2017). For this purpose, novel technologies such as microfluidics and "OMICs" should be developed. Moreover, progression in computer simulation models and synthetic replacements could provide reasonable alternatives for animal testing. Computer-based technology can simulate animal metabolism and its interaction with the environment. Moreover, it could optimize profitability with predicting of different outcomes. In the future, more development of computer models will be required for advanced experimental studies as the substitutes for animal models (Lennernas et al. 2017). Additionally, 'OMICs' technologies have presented advances in histopathological analysis through improving diagnosis procedures, shortening the period of time, and using small samples (Bennett 2010; Grafström et al. 2015). In summary, providing appropriate animal model and performing accurate validity in preclinical study is strongly recommended.

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Cell Therapy Targets for Autism Spectrum Disorders: Hopes, Challenges and Future Directions

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Abstract

Autism spectrum disorders as a group of pediatric neurodevelopmental diseases is a crucial part of the worldwide disabilities which have influence in communication skills, social interactions, and ability to understand the concepts. The precise pathophysiology of autism spectrum disorders due to the abundance of involved mechanisms is unknown. Some of these involved mechanisms are related to genetic factors, chronic neuro inflammation, mitochondrial dysfunction,

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oxidative stress, immune dysregulation, hormonal imbalance, and environmental factors. Current main treatments for autisms are behavioral, nutritional and medical therapies, however there is not definitive treatment approach. Therein, more novel therapies are still required to improve the symptoms. Several preclinical and clinical evidence were shown that stem cell therapy is a potential treatment option for autism spectrum disorders individuals. Considering the significant factors which can affect the outcome of stem cell therapeutic effects including stem cell types, route and dosage of administration, and mechanism of activity along with selecting best animal models can be very important in performing clinical trials.

Keywords
Autism spectrum disorders · Neuro inflammation · Neurological disorders · Regenerative medicine · Stem cell therapy

Abbreviations

ABA	Applied Behavior Analysis
ADHD	Attention Deficit Hyperactivity Disorder
ASD	Autism spectrum disorders
AUCB	Autologous Umbilical Cord Blood
BBB	Blood Brain Barrier
BDNF	Brain-Derived Neurotrophic Factor
BMMNCs	Bone marrow Mononuclear Stem cells
BTBR	Black and Tan, Brachyuric
CBMNCs	Cord Blood Mononuclear Cells
CDD	Childhood Disintegrate Disorders
CGI	Clinical Global Impression
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
DAT1	Dopamine Transporter gene 1
DRD4	Dopamine D4 Receptor gene
DSM-5	Diagnostic and Statistical Manual of Mental Disorders
ESCs	Embryonic Stem Cells
FIM	Functional Independence Measure
GI	Gastrointestinal
GRM	Glutamate metabotropic receptors
GSH	Glutathione
HSCs	Hematopoietic Stem Cells
ID	Intellectual Disability
IL-1	Inteleukin-1
IL-10	Interleukin 10
IL-12	Interleukin-12
IL-6	interleukin-6
iPSCs	induced Pluripotent Stem Cells
IQ	Intelligence Quotient
ISAA	Indian Scale for Assessment of Autism
MD	Mitochondrial Damage
MSCs	Mesenchymal Stem Cells
mtDNA	Mitochondrial DNA
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NHIS	National Health Interview Survey
NLGN	Neuroligin
NRXN	Neurexin
PCOS	Polycystic Ovary Syndrome
PDD-NOS	Pervasive Developmental Disorder Not Otherwise Specified
PRT	Pivotal Response Treatment
RTT	Rett syndrome
SSRIs	Selective Serotonin Reuptake Inhibitors
UCMSCs	Umbilical Cord-derived Mesenchymal Stem Cells
VEGF	Vascular Endothelial Growth Factor
VPA	Valproic Acid

1 Introduction

Autism spectrum disorders (ASDs) as a group of neurodevelopmental diseases which being more prevalent in boys than girls, affects about 14.5 per 1000 eight-year-old children over the past decades (Christensen et al. 2018; Ichim et al. 2007a). Indeed, ASD encompasses autism, Asperger's syndrome, Rett syndrome (RTT), and childhood disintegrate disorders (CDD). Problem in social interaction, repetitive or

restrictive behavior and problem with speech and impairment in communication can be introduced as three main clinical conditions of ASD (Christensen et al. 2018; Ichim et al. 2007a). More associated symptoms are aggression, compulsion, irritability, gastrointestinal (GI) issues and sleep disorders (Siniscalco et al. 2018a). Additionally, some abnormalities such as synaptic connectivity problems and disorganization of white matter lead to impaired communication skills in children (Sivanesan et al. 2017). Further, neural hypoperfusion, oxidative stress, mitochondrial dysfunction and dysregulation of immune system are some other pathophysiological mechanisms, however the exact pathophysiology of it remains unclear and it needs further investigations (Siniscalco et al. 2013a; Sharma et al. 2017a). Since genetic alterations are one of the causes of ASD, it is considered to be one of the most heritable diseases. Generally, based on the genetic point of view, there are two forms of ASD: the syndromic form and the non-syndromic form (de la Torre-Ubieta et al. 2016). On the other hand, ASD can be influenced by environmental factors including pre and post-natal exposures to infections and pesticides (Yang and Shcheglovitov 2019). Diagnosis of ASD is quite difficult as it is a heterogeneous disorder and the related symptoms are seen in other neurological and/or psychiatric conditions such as epilepsy, Attention deficit hyperactivity disorder (ADHD) and intellectual disability (ID) (Yang and Shcheglovitov 2019). Autism is generally diagnosed definitely around 24–36 months, but in some cases it may occur in adulthood as long as its severity varies and some children grow as independent adults (Ichim et al. 2007a). Using diagnostic and statistical manual of mental disorders, autism is determined and basically, it is diagnosed by observational and psychometric tests (Ichim et al. 2007a; Siniscalco et al. 2018a; Siniscalco et al. 2013a). Hence, current main treatments for autisms are behavioral (e.g. applied behavior analysis, speech therapy, social skills training), nutritional (e.g. use of vitamins and supplements) and medical (e.g. selective serotonin reuptake inhibitors (SSRIs) and antipsychotic drugs) therapies, however there is no gold standard approach (Ichim et al. 2007a; Siniscalco et al. 2018a). Therefore, novel therapies are still required to improve the symptoms of ASD. One of these new potential treatments is stem cell therapy and due to stem cell regenerative capability, it is a beneficial choice for neurologic disorders (Goodarzi et al. 2014, 2015, 2019a; Arjm et al. 2013; Rahim et al. 2018; Soleimani et al. 2016; Derakhshanrad et al. 2015; Larijani et al. 2019a; Rahim and Arjmand 2017) like neurologic impairment in ASD. Therefore, many preclinical and clinical studies have tested stem cell transplantation and indicated therapeutic promises. Further, paracrine effects and immune modulatory capacity of stem cells can influence the related abnormalities in ASD. Accordingly, various types of stem cells including embryonic stem cells (ESCs), fetal stem cells (FSCs), adult stem cells, and induced pluripotent stem cells (iPSCs) have been investigated (Siniscalco et al. 2018a; Ichim et al. 2007b).Generally this review will be focused on ASD and its known underlying mechanisms, the types and relevant investigations of stem cells and their targeted sites of action, and procedures for stem cell therapy in ASD.

2 Background and History of Autism

The term “autism” was first used at 1943, in the title of a clinical case report by Kanner: “Autistic Disturbances of Affective Contact”. It was about 11 children who had similar autistic symptoms to the present form of criteria. Before that, in 1900s, there were reports of childhood schizophrenia and childhood disintegrative disorder (Heller’s syndrome) and patients with similar conditions to autism (Evans 2013; Feinstein 2011). Nowadays, autism is known as one of the lifelong complex neurodevelopmental disorders and its symptoms appear in the first 3 years of life. It displays impairment in social and communication skills and cognitive functions (Siniscalco et al. 2018a; Evans 2013; Freitas et al. 2014, 2018; Eissa et al.

2018). Various etiologies can cause quite same behaviors so many disorders which show autistic features are grouped as ASD. It affects more than 1% of the general population and there are heterogeneity and variety in severity, functional disabilities and symptoms (Siniscalco et al. 2018a; Freitas et al. 2014). Classical autism, pervasive developmental disorder not otherwise specified (PDD-NOS), Asperger's syndrome, RTT and CDD are included in ASD group (Freitas et al. 2014). Not only autism causes higher mortality rate, but also most of the adults with autism have problems in personal and social life, however varying developmental processes have indicated insignificant autistic symptoms and normal social communication (Fein et al. 2013; Lai et al. 2014). Considering screening and early identification of ASD, there are different instruments to be used for children, adolescents, and adults. Although individuals with autisms were not diagnosed until 3–4 years, now toddlers are frequently identified, however it becomes hard in many cases (Lai et al. 2014). Accordingly, in order to diagnose ASD based on diagnostic and statistical manual of mental disorders (DSM-5) (one of the major diagnostic criteria for developmental disorders), individuals must meet following social communication/interaction criteria including deficits in reciprocating social or emotional interaction, severe problems in developing, maintaining and understanding relationship, and problem in nonverbal communication. Additionally, they must fulfill at least 2 of the 4 criteria for restricted and repetitive behaviors which include: repetitive or stereotyped speech, use of objects or motor movements, adherence to routines, resistance to change, restricted interests that have abnormal intensity of focus and hypo or hyper- reactivity to sensory input or unusual interests in sensory aspects of the environment. As it was mentioned before, some associated co -occurring conditions may be found related to ASD such as GI problems, immune dysregulation, anxiety, depression and aggressive behaviors (Evans 2013; Lai et al. 2014; DeFilippis and Wagner 2016). These conditions affect more than 70% of patients and it seems that they can persist into adolescence from childhood and the more they become, the worse the disability of patients will be (Lai et al. 2014). Despite its growing prevalence and investigations, there is no efficient therapy especially for problems of social communication and repetitive/restricted behavior (Eissa et al. 2018). Current quite applicable treatments can be divided in to behavioral therapy, pharmacological intervention, and complementary alternative medicine (DeFilippis and Wagner 2016). Among many psychosocial interventions which target core and associated symptoms of ASD, applied behavior analysis (ABA) and pivotal response treatment (PRT) are known as extensively studied methods (DeFilippis and Wagner 2016; Landa 2007). ABA is based on principles of operant conditioning and theories of learning. Intensive ABA therapy can lead to positive effects on features like intelligence quotient (IQ), intellectual and social functioning, language development and daily living skills (DeFilippis and Wagner 2016; Landa 2007). However, it needs a significant length of time and it is costly. PRT requires less time and it includes methods targeting specific skills and leads to generalized gains in areas such as joint attention (DeFilippis and Wagner 2016). In the category of pharmacological intervention, variety of medications are studied and some of them are used to control associated symptoms of ASD such as hyperactivity, anxiety, insomnia, inattention, irritability, aggression, repetitive behavior and self-injury (Eissa et al. 2018; DeFilippis and Wagner 2016). Several classes of drugs are under investigation in different phases of clinical trials and so far, risperidone and aripiprazole-as atypical antipsychotics- have been approved by FDA to improve behavioral symptoms (irritability) (Eissa et al. 2018; DeFilippis and Wagner 2016). Risperidone reduces repetitive behavior, aggression, irritability, anxiety and aripiprazole is able to decrease irritability, hyperactivity and stereotypy. Other examples of the medications that are being used including SSRIs, anti convulsants, and psychostimulants and their major therapeutic effects are described in

Table 1 Some of currently used medications for ASD, *SSRI* Selective serotonin reuptake inhibitors

Name of medication	Class	Effect
Risperidone	Atypical antipsychotics	Decrease in irritability, repetitive behavior, aggression, anxiety, depression, and nervousness. Increase in brain antioxidant activity
Aripiprazole	Atypical antipsychotics	Decrease in irritability, hyperactivity, and stereotypy
Clozapin	Atypical antipsychotics	Improvement in aggression and hyperactivity
Fluvaximine	SSRI	Improvement in compulsive repetitive behavior and aggression
Fluoxentine	SSRI	Decrease in repetitive and stereotyped behavior
Clomipramine	Tricyclic antidepressant	Improvement in compulsive behavior and anger
Methylphenidate	Psycho-stimulant	Improvement in attention. Hyperactivity, impulsivity, and social communication
Lamotrigine	Anticonvulsant	Improvement in whole autistic symptoms
Amantadine	Glutamate antagonist	Improvement in speech disturbance and hyperactivity

Table 1 (Eissa et al. 2018). In regard to complementary medicine, melatonin seems to be a safe option for sleep in children with ASD and has been studied in some trials. Omega-3 fatty acids and methyl B12 are other proposed alternative treatments (DeFilippis and Wagner 2016). After all, since the drugs and other available therapies are not adequately effective and helpful, there is still the need for finding a suitable, applicable and efficient treatment for ASD and further studies on etiology and underlying mechanisms of the disease are demanded.

3 Causes and Mechanisms of Autism

In general, the exact pathophysiology of ASD is remained unknown due to the abundance of involved mechanisms (Strunecka et al. 2018; Ansel et al. 2017). Indeed, subjects who are genetically susceptible to ASD in the face of environmental factors are more likely to develop various ASD affecting conditions such as hormonal imbalance, immune dysregulation, chronic neuroinflammation, mitochondrial dysfunction, and oxidative stress conditions (Fig. 1).

3.1 Genetic Factors

Investigations around the ASDs associated genes have shown some mutations through the common molecular pathways related to neurexin (NRXN), CACNA, glutamate metabotropic receptors (GRM), CNTN genes (Uzunova et al. 2014; Hadley et al. 2014; Almandil et al. 2019). Accordingly, it has been revealed that modifying the synaptic structure and function during the mutation of NRXN, related neuroligin (NLGN), and ProSAP/Shank gene families, can more increase the risk for ASDs. Indeed, these mutations can participate in transsynaptic pathways and excitatory synapses maturation in the central nervous system (CNS) (Almandil et al. 2019; Arons et al. 2012; Lin et al. 2016; Washbourne 2015). Moreover, alterations of genes involved in the roles of neurotransmitters such as dopamine transporter gene (DAT1) and dopamine D4 receptor gene (DRD4) may notably change the risk of autism (Almandil et al. 2019; Qian et al. 2003). On the other hand, based on different types of research studies, epigenetic variations (heritable modifications in gene expression (via molecular factors at regulatory regions of DNA) which occur without a variation of primary DNA sequence) can be considered as an essential cause of the alternations in the risk of autism (Wiśniowiecka-Kowalnik and

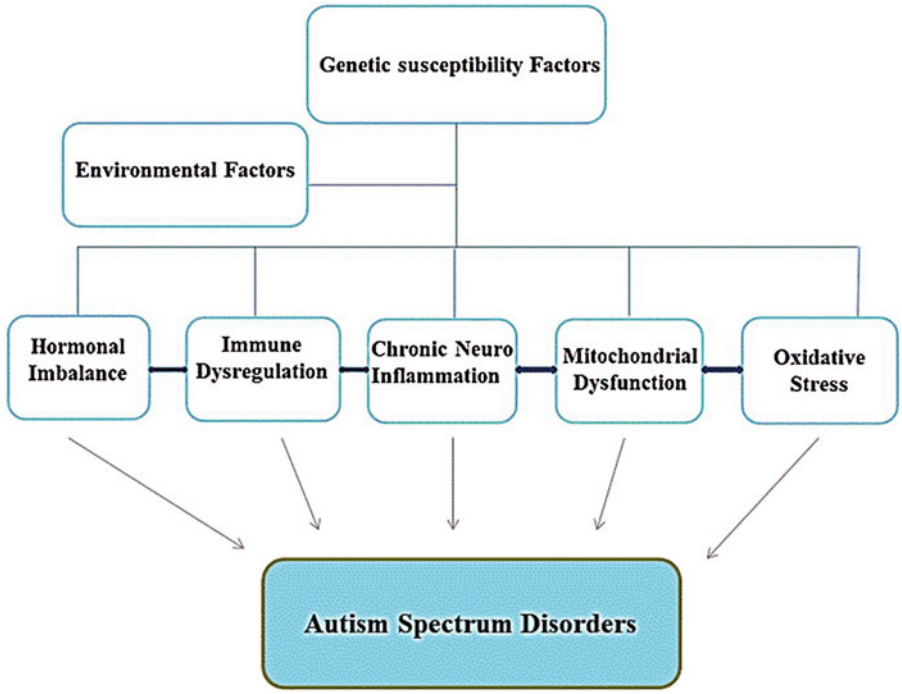

Fig. 1 Causes and mechanisms of autism spectrum disorders. Several basic physiological mechanisms that are related together including genetic susceptibility along with environmental factors and hormonal imbalance, immune dysregulation, chronic neuroinflammation, mitochondrial dysfunction, and oxidative stress conditions can cause ASD

Nowakowska 2019; Tremblay and Jiang 2019; Loke et al. 2015).

3.2 Chronic Neuro Inflammation

There is a growing interest to investigate about the role of the inflammation and immune system in the development of ASD. Therein, investigation summaries are indicated that the possible maternal viral infection occurred during the first trimester of gestation, can lead to acute immune activation and finally raise the risk of ASD in children. On the other hand, maternal anti-brain autoantibodies (which found in ~20% of mothers) lead to fetal brain development can bind to embryonic proteins and alter neural development pathways (Patterson 2011; Brucato et al. 2017; Fox et al. 2012; Jones and Van de Water 2019). Consequently, the immune profile of individuals with ASD specifically pro-inflammatory markers such as inteleukin-1(IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and interleukin-12 (IL-12) along with nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) expression and microglial and astrocytic activation are different from healthy people (Tonhajzerova et al. 2015; Siniscalco et al. 2018b). Generally, due to some of evidences the function of the immune system and inflammation has pathogenic effects through various mechanisms. All of these mechanisms can result in low-grade chronic inflammation as well as changes in the central nervous system and immune responses (Chen et al. 2017).

3.3 Mitochondrial Dysfunction

Mitochondrial dysfunction can be created by either primary mitochondrial damage (MD) (because of hereditary defects in

mitochondrial DNA (mtDNA) or deficiency of vital genes for the function of mitochondria) or secondary MD (which can be induced by genes that participate in hereditary non-mitochondrial diseases) (Nicolson 2014; Niyazov et al. 2016). A lot of studies were indicated that mitochondrial dysfunction has been tied to ASD (especially some forms of syndromic ASD) (Varga et al. 2018a; Frye and Rossignol 2011; Palmieri and Persico 2010). In general, neural tissue as one of the tissues with high-energy demands is more affected by mitochondrial dysfunction than others. Accordingly, in mitochondrial dysfunction conditions, neurodevelopmental diseases such as ASD, intellectual disability, and childhood epilepsy are more prevalent than others (Valenti et al. 2014; Patel et al. 2011). In this context, many biochemical modifications (including raised levels of lactate, carnitine, pyruvate, creatine kinase, alanine, and ammonia) detected in the serum of subjects with ASD and changed respiratory chain enzyme functions found in the autistic brain, indicating mitochondrial dysfunction (Rossignol and Frye 2012; Griffiths and Levy 2017; Varga et al. 2018b).

3.4 Oxidative Stress

According to several recent large-scale studies oxidative stress (damaging of proteins, lipids, and DNA along with variations in function of valuable enzymes in redox metabolism) is intended to be momentous in the etiology of ASD (Rose et al. 2012; Rossignol and Frye 2014; Yui et al. 2016; Mandic-Maravic et al. 2019). Herein, genetic variations in glutathione (GSH) -related pathways, a decrease in mitochondrial GSH (the important cellular antioxidant), and an increase in oxidative stress markers (specifically peripheral markers containing those found in blood and urine) have been reported in individuals with ASD. Also, more molecular evaluations have demonstrated that there is significant oxidation of protein, lipids and DNA in their brain tissue (Yui et al. 2016; Atkuri et al. 2009). On the other hand, it was shown that oxidative stress has been associated with the GI difficulties severity in ASD individuals (Dhaliwal et al. 2019).

3.5 Immune Dysregulation

Frequent immune-mediated comorbid health problems were indicated in ASD individuals. Additionally, based on National Health Interview Survey (NHIS), ASD children have a higher prevalence of most medical conditions defined in autoimmune areas than other children. Hereupon, they need higher health care use. Therein, investigations are remarked that immune dysfunction and dysregulation underlined the bulk of these comorbidities (Hughes et al. 2018a; Xu et al. 2018). Furthermore, rises in inflammatory cells and cytokines along with reduces regulatory T cells were observed in subjects with ASD (Ashwood et al. 2011; Hughes et al. 2018b).

3.6 Hormonal Imbalance

Investigating the role of endocrine factors and their effect on different stages of the neurodevelopmental process and understanding the possible importance of endocrine etiopathogenesis in ASD has shown that hormonal imbalance during pregnancy (such as polycystic ovary syndrome (PCOS)) can increase the risk of having a child with autism. Additionally, it is known that women with PCOS are more susceptible to obesity than healthy women while obesity through pregnancy has been independently connected to autism (De Leo et al. 2016; Long et al. 2019; Kosidou et al. 2016; Moosa et al. 2018). Generally, chemical messengers including different hormones, hormone-like substances, and neuropeptides, accompanied by neurotransmitters can promote the social behaviors encoding in the developing brain (Kovács 2004). Hence, any imbalance in this chemical transmission can lead to incomplete encoding and abnormal social behaviors as prominent features of ASD (Kovács 2004; Tareen and Kamboj 2012).

3.7 Environmental Factors

According to recent studies, about the half percentage of causes for increased autism risk are related to the influence of environmental factors including maternal smoking, most likely assisted reproductive technologies, vaccination, birth complications (associated with trauma or ischemia and hypoxia), heavy metals as environmental toxins (most famous inorganic mercury and lead), and etc. Indeed, they can influence the pathogenesis of ASD via epigenetic effects (Karimi et al. 2017; Emberti Gialloreti et al. 2019).

4 Stem Cells and Autism Spectrum Disorders

The advent of stem cell technology has provided exciting opportunities to treat a wide range of neurodegenerative disorders such as ASD. Hereupon, multiple factors including the types of stem cells, dosage and routes of administration, and frequency of transplantation can alter the outcome of stem cell therapy (Siniscalco et al. 2018a; Sharma et al. 2017b).

4.1 The Types of Applied Stem Cells for the Autism Treatment

According to regenerative potential of stem cells, various fetal and adult tissue-derived stem cells such as mesenchymal (MSCs) and hematopoietic (HSCs) stem cells along with ESCs and iPSCs are applied in the road of ASD treatment (Siniscalco et al. 2018a; Ichim et al. 2007b; Siniscalco et al. 2014). Until now, several preclinical and clinical investigations were demonstrated that using the bone marrow mononuclear stem cells (BMMNCs) and umbilical cord-derived mesenchymal stem cells (UCMSCs) for ASD are more common than other sources (Siniscalco et al. 2018a; Sharma et al. 2017b; Liu et al. 2019a). Herein, finding and choosing an appropriate route and dosage of stem cell administration for creating best treatment outcomes is of great importance. Additionally, it is clear that different types of stem cells play their roles through various mechanisms (Siniscalco et al. 2018a; Biehl and Russell 2009). Therein, studying these functional mechanisms can be helpful in selecting the best cell type for treatment.

4.2 Appropriate Dosage and Delivery Route of Stem Cells

Like selecting the type of stem cell to be used for individuals with autism, There are three main routs including intrathecal, intracerebral and intravenous transplantations (Sharma et al. 2017a; Liem NT et al. 2018). Using lumbar puncture for delivering the cells in intrathecal route is considered a minimally invasive method (Sharma et al. 2017a; Bakshi et al. 2004). However, it is known to be efficient as the injected cells can get to the target tissue through cerebrospinal fluid (CSF). On the other hand, intravenous route is the least invasive one but since the cells need to pass the blood brain barrier (BBB) and a considerable number of them do not reach the target site, it is less efficient (Sharma et al. 2017a; Bakshi et al. 2004). Although the direct delivery of cells is the most efficient one and the intraventricular (intracerebral) injection is favorable, it is risky and invasive for clinical application (Sharma et al. 2017a; Liem NT et al. 2018; Bakshi et al. 2004). It should be mentioned that to date, the clinical trials have used intrathecal, intravenous and even both forms of injections to apply cell therapy for ASD and it is claimed that they are safe, so a more precise examination of this issue is absolutely required (Siniscalco et al. 2018a; Bradstreet et al. 2014; Lv et al. 2013; Sharma et al. 2013a). Regarding appropriate dosage, which is another important part of therapy, there is a variety of cell sources and numbers in the clinical trials which studied stem cell therapy for ASDs (Siniscalco et al. 2018a). For instance, about $8.19 * 10^7$ autologous BMMNCs were used intrathecally in an open label study while in another trial, approximately $1*10^6$ /kg UCMSCs and $2*10^6$/kg cord blood mononuclear

cells (CBMNCs) were injected intravenously (Lv et al. 2013; Sharma et al. 2013a). Noticing this variety, determining the effective and optimal cell dosage is essential in order to find the best unified one for helping and treating patients (Siniscalco et al. 2018a). More examples of cell dosages and delivery routes can be found in Table 2.

4.3 Mechanism of Stem Cell Function

Since there are inflammatory abnormalities, imbalance in innate and adaptive immunity, and disruption of synapse function and plasticity in ASD subjects, stem cell therapy can be useful via some key-action mechanisms including immunomodulatory properties, paracrine effects, and differentiation ability (Siniscalco et al. 2013a; Liu et al. 2019b; Siniscalco et al. 2013b). In this context, stem cells can play their role within some potential mechanisms for synaptic function and plasticity modulation by secreting specific growth factors, maintaining synaptic plasticity, regenerating synaptic transmitter release, and combining into existing synaptic networks (Siniscalco et al. 2014; Sharma et al. 2013a). On the other hand, they can perform their act on the adaptive and innate immune system through inhibiting the maturation of dendritic cells, repressing the pro-inflammatory activities, supporting the regulatory T cells generation, polarizing macrophages towards the anti-inflammatory state, decreasing the proliferation and activation of B cells, passing the BBB and migrating to sites of inflammation, and suppressing the proliferation and cytotoxicity effects of NK cells (Siniscalco et al. 2014; Joel et al. 2019). Moreover, the differentiation abilities of injected cells into neural cells may contribute to ASD treatment (Siniscalco et al. 2018a).

4.4 Scientific Evidences

Among various studies investigating properties of stem cells to find new therapeutic options for different diseases, there are many studies exploring their effects on treating autism. Accordingly, there are a growing number of animal studies and clinical trials focusing on different features of each type of stem cells related to pathophysiology of ASD (Siniscalco et al. 2013a, 2018a).

4.4.1 Preclinical Investigations

As ASDs are about human behaviors, animal models cannot reproduce this condition thoroughly, yet. They can present information on the effect of stem cells on ASD- associated phenotypes. Animal models can be divided into drug-induced and mutagenesis generated models. For instance, valproic acid (VPA) maternal challenge in rodents can produce a valid autism model and administration of Propionic acid (PPA) results in autism similar abnormalities in rats. The exact genetic basis of autism is still unclear, so, some mutant mouse models are generated to develop associated mutations in patients to investigate mutations and alterations in the proteins related to autism such as shank, NRXN, NLGN, Mecp2,Foxps, Cntnap2,Ube3a, and Tsc (Tania et al. 2014). In this context, the Mecp2-null murine model underwent a transplantation of wild- type bone marrow, resulting in positive effects and improvements in Rett syndrome which suggested the bone marrow transplantation as a potential therapy for ASD (Tania et al. 2014; Derecki et al. 2012). Notably, an emerging animal model for investigating neurodevelopmental disorders like ASDs is Zebrafish. Zebrafish models can develop ASD associated symptoms and be potential useful models to study autism (Meshalkina et al. 2018). Additionally, an animal model which is commonly used is the black and tan brachyuric (BTBR) inbred mouse strain (Segal-Gavish et al. 2016). The mice indicate behavioral deficits and brain abnormalities and when human MSCs were transplanted into them, repetitive and social behaviors were improved and cognitive rigidity and stereotyped repetitive behaviors were decreased. However, there was no difference in anxiety-related behavior and the transplantation did not have any impact on gross motor activity. The human MSCs increased brain-

Table 2 Clinical trials on stem cell therapy in ASDs

Authors	Trial	Type of transplanted cells and number	Follow-up duration	Mode of transplantation	Number of participants	Scales	Results
Sharma et al. (2013a)	Open-label, proof of concept	$8.19*10^7$ autologous BMMNCs	26 months	Intrathecal	32	CGI	Safety
						ISAA	Improvement in behavior pattern, cognitive component, social relationship, emotional response, speech domain, balancing of brain metabolism
						FIM	
						Wee-FIM	
Lv et al. (2013)	Non-randomized, open-label, single center, phase I/II	$2*10^6$/kg CBMNCs $1*10^6$/kg UCMSCs	24 weeks	Intravenous and intrathecal	Control:14	CARS	Safety
					CBMNC:13	CGI	Improvement in behavior-body use- visual, emotional and intellectual response-nonverbal communication-activity level
					Cobination:9	ABC	
Bradstreet et la (2014)	Open- label pilot study	1.6 ml of $>30*10^6$/ mil fetal liver derived HSCs and 2.12 ± 0.49 ml of $>8.70*10^6$/ml fetal brain derived nucleated neuroprogenitor cells	12 months	Intravenous and subcutaneous	45	ATEC	Safety
						ABC	Improvements in speech, sociability, behavior, cognitive ability and immune system
Bansal et al. (2016)	Non-randomized open-label	Autologous BMMNCs	24 months	Intrathecal	10	ISAA	Safety
						Wee-FIM	Improvements in socializing ability, hyperactivity, meaningless play, confidence, motor skills, aggressive behavior
Dawson et al. (2017)	Single-center, phase I open- label	Target: $1–5 *10^7$ /kg AUCB cells, median TNC count: $2.6*10^7$/kg	12 months	Intravenous	25	VABS	Safety
						EOWPVT-4	Improvement in behavior, socialization, communication, nonverbal IQ
						CGI	
						PDDBI	
Chez et al. (2018)	Randomized, double-blinded, placebo-controlled, crossover	Mean AUCB TNC dose: $16.16*10^6$ / kg	49 weeks (cross over at 24 weeks)	Intravenous	29	ROWPVT-4	Safety
						EOWPVT-4	No significant efficacy
						CGI	
						VABS-II	
						SBFR	
						SBKN	

Liem NT et al. (2018)	Open-label un controlled	Mean autologous BMMNCs:19.3∗106/kg	6 months	Intrathecal	24	CARS	Safety
							Improvements in behavior, body use, visual response, fear or nervous score, taste, smell, touch score
Riordan et al. (2019)	Single-arm phase I/II	36∗106 UCMSCs (9∗106 viable) each infusion (Total:4 infusions)	89 weeks (1 year after the last dose)	Intravenous	20	CARS	Safety
						ATEC	Improvements in social communication, awareness and motor ability (in 8 subjects)

BMMNCs Bone marrow-derived mononuclear cells, *CBMNCs* Cord blood mononuclear cells, *UCMSCs* Umbilical cord-derived mesenchymal stem cells, *AUCB* Autologous umbilical cord blood, *HSCs* Hematopoietic stem cells, *TNC* Total nucleated cell, *CGI* Clinical global impression, *ISAA* Indian scale for assessment of autism, *FIM* Functional independence measure, *CARS* Childhood autism rating scale, *ABC* Aberrant behavior checklist, *ATEC* Autism treatment evaluation checklis, *VABS* Vineland adaptive behavior scales, *EOWPVT-4* Expressive one-word picture vocabulary test, fourth edition, *PDDBI* Pervasive developmental disorder behavior inventory, *ROWPVT-4* Receptive one word picture vocabulary test, fourth edition, *SBFR* Stanford-Binet fluid reasoning, *SBKN* Stanford-Binet Knowledge

derived neurotrophic factor (BDNF) levels and hippocampal neurogenesis (Siniscalco et al. 2018a; Segal-Gavish et al. 2016). In another study, human ASCs were transplanted into VPA-induced autism mouse model, resulting in decreased anxiety, increased social behaviors and motor coordination. They increased vascular endothelial growth factor (VEGF), interleukin 10 (IL-10) expression, PTEN, and p-AKT/AKT ratio in the brains of mouse models that can somehow explain the pathways and mechanisms of therapeutic effect of ASCs (Siniscalco et al. 2018a; Griffiths and Levy 2017). Studying ASD-derived stem cells as they show some different properties and gene expression, makes them another area of concern especially for autologous cell transplantation. For instance, maternal immune activation mouse models exhibited changes in HSCs differentiation (Siniscalco et al. 2018a; Hsiao et al. 2012). Therefore, more investigations on both these stem cells and application of different types of them for uncovering biologic mechanisms of autism are required (Siniscalco et al. 2018a).

4.4.2 Clinical Investigations

Evaluating the safety and efficacy of cellular therapy for ASDs is a fundamental step in the road of clinical application that is carried out through performing clinical trials (Siniscalco et al. 2018a). Accordingly, there are some clinical trials on using stem cells in autism; for instance, some trials used BMMNCs in years 2013, 2015, and 2018 (Liem NT et al. 2018; Sharma et al. 2013a; Bansal et al. 2016). In an open label proof- of – concept study in 2013, autologous BMMNCs which consists of HSCs, MSCs, multipotent adult and endothelial progenitors, were separated from aspirated bone marrow of each patient and transplanted. The patients underwent other forms of therapies including occupational therapy interventions, activities of daily living training, psychological intervention, speech therapy, and specific dietary recommendations. The core symptoms of autism were reported based on Indian scale for assessment of autism (ISAA), Clinical global impression (CGI) scale (indicates the effect of treatment and severity of the disease), Functional independence measure (FIM), and Wee-FIM scales. Along with some adverse events such as nausea, vomiting, aspiration, minimal increase in hyperactivity and seizures (in 3 patients), there were improvements in social relationships, cognitive aspects, speech and language patterns and also decrease in exaggerated and inappropriate emotions. Also, CGI scale showed improvement in disease severity and considering hypo perfusion in staple brain areas in ASDs, the metabolism of hypo metabolic areas increased probably because of improved function and oxygenation of the damaged neurons. Since cell therapy has the capacity of repairing damaged neural tissue, it is hypothesized that they can restore the specialized neural system with the help of their immunomodulatory activity and paracrine effects (Ichim et al. 2007a; Siniscalco et al. 2018a; Sharma et al. 2013a). In quite same time, there was a case report of autologous BMMNCs transplantation on 14-year-old boy followed for 1 year, resulting in decreased childhood autism rating scale (CARS) score, improved behavior, social interaction, attention and emotion. Also, the PET scan showed enhanced Fluoro-deoxyglucose (FDG) uptake in brain (Siniscalco et al. 2018a; Sharma et al. 2013b). Furthermore, there was a small study of bone marrow aspirate concentrate (BMAC) stem cells transplantation in patients in addition to occupational therapy, speech therapy, and psychological intervention and it was revealed that the intervention did better in younger patients and the severity of the disease affects the outcomes (Bansal et al. 2016). In another open label uncontrolled trial in 2015, same cells (BMMNCs) were injected for 2 times at baseline and 3 months after that in autistic children. Although no severe complications were reported and the safety was supported, some cases presented transient or prolonged increased hyperactivity. Improvements were reported in 19 patients (79.2%) and their total CARS scores became considerably lower, especially in some domains including body use, intellectual response, and visual response. However, no

change in three domains of relating to people, emotional response, and activity level (Liem NT et al. 2018). Surprisingly, Sharma et al. recently performed a same therapy on an a 25-year- old autistic male and injected MNCs separated from his bone marrow aspiration in lumbar region. The cell therapy was along with psychological intervention, special education occupational therapy and physiotherapy and he was followed for 6 months. There was no recording of adverse events yet his concentration, social interaction, memory, sitting tolerance, objects utilization and attention improved as well as his CARS, ISAA and FIM scores. He was developed regarding to daily activities like bathing independently. His hyperactivity decreased and as it was showed in F-FDG PET scan, the brain hypometabolism enhanced (Sharma et al. 2018).

In another phase I/II trial in 2011, the experimental group underwent CBMNC or combination of CBMNC and UCMSC transplantation and also rehabilitation therapy in order to investigate the safety and efficacy (Lv et al. 2013). The control group received only the rehabilitation therapy. Each experimental group received 4 cell transplantations as the CBMNC one had one intravenous and 3 following intrathecal transplantation and the combination group received 2 injections of each type. Interestingly, the combination group indicated better results which proposes that CBMNCs and UCMSCs act in a positive synergistic way. As it was mentioned before, two potential pathogenesis of autisms are immune dysregulation and cerebral hypoperfusion. Accordingly, MSCs have immuneregulatory properties, so based on recent investigations, UCMSCs may act as a controller in related pathologic mechanisms (Lv et al. 2013; Chen et al. 2010; Kaplan et al. 2011). Moreover, the angiogenesis capacity of $CD34^+$ cells which are enriched in CBMNCs has been proved, so it can be useful for hypoxia in brains of autistic patients (Siniscalco et al. 2018a; Lv et al. 2013). In 2017, a phase I/II clinical trial of allogenic UCMSCs with repeated dose of administration was performed. In this single-arm study, autistic children received UCMSCs every 12 weeks for 4 times and followed for 1 year after the last dose. The aim of this study was to evaluate the safety and effectiveness of repeating injections and also the relations between inflammatory properties and ADS symptoms (Riordan et al. 2019). It seems that the severity of autism is connected with inflammation and increased serum levels of macrophage-derived chemokine (MDC) and thymus activation-regulated chemokine (TARC) (Riordan et al. 2019; Al-Ayadhi and Mostafa 2013). Like previous studies, it can be said that no serious adverse events were reposted and those treatments related ones were resolved without using any medication. CARS and ATEC scores of 40% of subjects were reduced and most of them, indicated a decrease in MDC and TARC level. So, there might be a potential link between the symptoms and inflammation but it does not encompass all the improvements and changes found in the subjects. There was a variability in results as some showed improvements in social communication, awareness and motor ability but also an increase in anxiety and emotional liability were seen in some subjects. Using repeated dose of UCMSCs exhibited more efficient anti-inflammatory effect of them which affected TARC and MDC levels (Riordan et al. 2019). Additionally, the safety of the application of FSCs in ASD children was assessed in an open label pilot study. Two cell suspensions were used, one contained HSCs from fetal liver (administered intravenously) and another one neuroprogenitors of fetal brain (injected into the subcutaneous abdominal adipose tissue). After the transplantation, some positive effects such as improved appetite, eye contact, socialization, cognitive ability, and behaviors and no side-effects were observed. FSCs caused enhanced cell- mediated immunity due their immunomodulatory functions. FSCs can migrate to the spinal cord and brain through BBB and as it appeared that the permeability of the BBB is increased in autistic patients, they may potentially have a therapeutic impact on that. Moreover, they can exert paracrine effects to influence the tissues and probably, restore the neural tissues (Siniscalco et al. 2018a; Bradstreet et al. 2014). Unfortunately, the

results of experimenting Autologous umbilical cord blood (AUCB) (rich in HSCs) on ASD children do not comply with each other as in a phase I open-label study, the safety of the transplantation was assessed for 12 months and significant improvements in behavior, communication and socialization were reported (Siniscalco et al. 2018a; Dawson et al. 2017; Chez et al. 2018). However, the results of a placebo-controlled crossover study in 2016 showed the safety of this transplantation but there seems to be a minimal clinical effectiveness. In this form of study, (one group received placebo and the other one received cord blood and at 24 weeks, the first group received cord blood and the second group, received saline injection) subject acts as their control which is an advantage in comparison to the previous study (Chez et al. 2018). Therefore, more studies including a blinded investigation and a control group are needed to find out the exact impact of AUCB transplantation on autistic patients (Chez et al. 2018). Besides, electroencephalography (EEG) is known to be useful as a biomarker of treatment efficacy, so in the open-label study, EEG measures were explored to find whether they show any change after the transplantation. It appeared that the U-shaped power profile of autistic patients (increased power in delta, theta, and gamma and decreased power in alpha and beta) normalizes by 12-month post-transplant. In addition, EEG can be used as a predictive tool for improvement in social communication and treatment response (Murias et al. 2018). Another study was conducted as a part of the mentioned phase I trial to investigate the possible link between changes in white matter connectivity and improvements in behaviors. Differences in white matter developmental patterns are related to autism symptoms, so by using MRI and some other scales it was found that increased connectivity between the hippocampus and the left thalamus is associated with enhanced social skills. Also, it appeared that the mutual connectivity in the right hemisphere, between the frontal lobe and temporal poles increased as clinical and communication features improved. More areas of brain faced increased connectivity resulting in improvements in ASDs symptoms possibly due to decrease in neural inflammation by AUCB transplantation (Carpenter et al. 2019).

Currently, the number of clinical trials on stem cell transplantation for ASDs is not considered enough and also, their different results, methods, enrolled subjects and cell types do not develop a definitive claim that which type of stem cell can be used as a new treatment. Yet, there are some ongoing trials. To date, considering the present outcomes, this field is known as a promising therapeutic approach that need more investigations and large trials (Siniscalco et al. 2018a). A more classified version of some main details of mentioned trials is described in Table 2.

5 Conclusions and Future Research Directions

Since ASD as one of the pediatric neurological disorders is a major part of the worldwide disabilities by affecting communication skills and social interactions as well as the ability to understand the concepts, from early childhood, trying to resolve this social-health problem is a very critical issue (Geschwind 2009; Magnuson and Constantino 2011). In this respect, billions of dollars are spent on ASD investigations and treatments, yearly. In recent years, there are pieces of preclinical and clinical evidence that stem cell therapy is not only safe but also improves the behavior of ASD subjects. Therefore, stem cell therapy as a potential treatment option for ASD individuals has received more support. Considering the limitations along with promising sedative effects of cellular therapies in the treatment of ASD, more comprehensive research and large trials will be demanded to claim definite results (Siniscalco et al. 2018a; Ichim et al. 2007b). Herein, analyzing the factors affecting the outcome of stem cell therapeutic functions such as stem cell types, route and dosage of administration, and mechanism of activity is of great importance in conducting clinical trials (Siniscalco et al. 2018a; Sharma et al. 2013a;

Herberts et al. 2011). Additionally, future researches should be considered to employ modern radiological tools and OMICs technology (as new biomarker discovery tools) for tracking changes occurring at the cellular and molecular level after stem cell therapy (Arjmand 2019; Larijani et al. 2019b; Goodarzi et al. 2019b) in ASD subjects.

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Regenerative Medicine Perspectives in Polycystic Ovary Syndrome

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Abstract

Polycystic ovary syndrome (PCOS) is the most common gynecologic endocrine disorder in women between the ages of 15 and 40, with uncertain etiology. It is mostly presented with hyperandrogenism and insulin resistance along with a variety of comorbidities that significantly reduce a patient's quality of life. Many disturbed metabolic pathways are correlated with PCOS. Moreover, it is evident that there is a strong genetic factor for PCOS. Indeed, several altered gene expressions have been found in PCOS subjects, but the exact genetic origins are still unclear. The major treatment options such as pharmacological treatments are to improve the symptoms. In addition, surgical procedures (Bariatric surgery and assisted reproductive technologies) can be used to treat some of the patient's complications and reduce their severity.

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Generally, using pharmacological agents for a long period of time can increase the risk of adverse effects. Moreover, surgical options may have high-risk consequences. Herein, there is an undeniable need for a different multidisciplinary approach to PCOS. Regenerative medicine with the help of stem cells can develop a worthy alternative approach for the treatment of PCOS. Furthermore, animal models can provide valuable knowledge of genetic alterations and metabolic pathway disturbances in PCOS. They can also be used for testing novel treatments in pre-clinical stages. Therein, the current knowledge of PCOS and investigation about the potential role of regenerative medicine in developing new and more efficient treatments for PCOS are summarized here.

Keywords

Disease modeling · Polycystic ovary syndrome · Regenerative medicine · Stem cells transplantation

Abbreviations

ACTH	Adrenocorticotropic hormone
AE-PCOS	Androgen-excess-PCOS society
AMH	Antimullerian hormone
AR	Androgen receptor
ArKO	Aromatase knockout
ASCs	Adipose stem cells
BAT	Brown adipose tissue
BMI	Body mass index
BM-MSCs	Bone marrow-derived mesenchymal stem cells
BMSC	Bone marrow stem cell
BPA	Bisphenol A
COCP	Combined oral contraceptive pills
CRH	Corticotropin-releasing hormone
DEHP	Di-(2-ethylhexyl) phthalate
DGE	Differential gene expression
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulfate
DHT	Dihydrotestosterone
EB	Estradiol benzoate
ESCs	Embryonic stem cells
EV	Estradiol valerate
FAH	Functional adrenal hyperandrogenism
FSH	Follicle stimulating hormone
GnRH	Gonadotropin-releasing hormone
Gsdf	Gonadal soma derived factor
HDL	High-density lipoprotein
hESCs	Human embryonic stem cells
HSC	Hematopoietic stem cell
hUC-MSCs	Human umbilical cord blood mesenchymal stem cells
ICSI	Intracytoplasmic sperm injection
IL-6,8	Interlukin 6,8
iPCSs	Induced pluripotent stem cells
IUI	Artificial insemination
IVF	In vitro fertilization
IVM	Immature oocyte in vitro
LDL	Low-density lipoprotein
LH	Luteinizing hormone
MCR	Metabolic clearance rate
MDS	Meyelodysplastic syndrome
NC-CAH	Non-classical congenital adrenal hyperplasia
NGF	Nerve growth factor
NZO/HILt	New Zealand obese mice
OHSS	Ovarian hypersensitivity syndrome
PCO	Polycystic ovary syndrome
PCOM	Polycystic ovarian morphology
POF	Premature ovarian failure
POI	Primary ovarian insufficiency
PORs	Poor ovarian responders
PPARγ	Peroxisome proliferator-activated receptor gamma
SCID	Severe combined immunodeficient
T reg	Regulatory T cells
TBT	Tributyltin
TGF-β	Transforming growth factor β
TNF-α	Tumor necrosis factor α
UCP1	Uncoupling protein 1
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
ZFP423	Zinc finger protein 423

1 Introduction

PCOS is the most commonly recognized gynecologic endocrine disorder in women of reproductive age. Indeed, PCOS is considered a clinical syndrome in which several diagnostic criteria have been defined for it. In other words, based on various criteria, diagnoses can be confirmed or ruled out to select the best treatment option and the correct prevalence estimate can be reached. On the other hand, it has been suggested that PCOS is a lifelong condition presenting as early as prenatal stages, but there are no definitive statistics in younger ages as well as screening in infants (Bellver et al. 2018). PCOS presentation varies in individuals due to different extents of hormonal imbalance. It affects the patient's quality of life as it can cause obesity, insulin resistance, infertility, cardiovascular diseases, endometrial cancer, and psychological disorders. Aetiologically, a number of genes associated with PCOS have been discovered (Wolf et al. 2018). Variations in these genes mutations and epigenetics are a strong determinant in disease development. Herein, current available treatment options for PCOS are either pharmacological or surgical. Pharmacological therapies are divided into the following groups: Pharmacological treatments for ovulation induction (aromatase inhibitors, Metformin, and gonadotropins), for menstrual dysfunction (cyclic progestin or oral contraceptive), and for adrenogene-related symptoms (antiandrogens, oral contraceptive pills, and insulin sensitizer agents) which are often advised in combination. In general, all available therapies are insignificantly effective and have several adverse effects (Patel 2018). Hereupon, there is an immense need for multidisciplinary approaches to develop new and more effective treatments for PCOS. Regenerative medicine (RM) is a new field of research that can be a starting point for developing regenerative and personalized treatments for PCOS. Furthermore, it aims to replace the damaged tissues causing disease with de novo generated cells (Goodarzi et al. 2014, 2015, 2018a, b, 2019a; Larijani et al. 2015, 2019, 2020; Mao and Mooney 2015; Arjmand et al. 2017). In this respect, human pluripotent stem cells (hPSCs) can be differentiated into any cell type and therefore offer the potential of tissue engineering (Tabar and Studer 2014). Moreover, bone marrow stem cells (BMSC) have shown promising results in treating premature ovarian failure (POF). BMSC transplantation recovers menstrual cycles and improves ovarian reserve (He et al. 2018). RM and tissue engineering also offer the potential for drug development and testing. Generally, since there is not a clear understanding of PCOS pathophysiology and available treatments have some side effects, RM can help in understanding cellular mechanisms of PCOS pathophysiology and developing cellular treatments that are more efficient and have fewer side effects than the current treatment regimen. In this context, research on the potential role of RM in creating new and more powerful treatments for PCOS is reviewed here.

2 Polycystic Ovary Syndrome and Its Pathophysiology

PCOS was first introduced by Stein and Leventhal in 1935 as a combination of oligo/amenorrhea, polycystic ovary, hirsutism, obesity, and acne (Azziz and Adashi 2016; Rosenfield and Ehrmann 2016; Bani Mohammad and Majdi Seghinsara 2017). Later, some criteria were proposed in order like NIH criteria that include clinical and/or biochemical evidence of hyperandrogenism and evidence of oligo/anovulation. After a while, Rotterdam criteria-as the broadest one- added polycystic ovarian morphology (PCOM) by using ultrasound to the two previous criteria. Finally, in 2006 androgen-excess-PCOS society (AE-PCOS) criteria proposed presence of androgen excess accompanied by PCOM, oligomenorrhea, or both. It could help diagnose women with PCOS who do not have anovulatory symptoms (ovulatory PCOS) (Rosenfield and Ehrmann 2016; Bani Mohammad and Majdi Seghinsara 2017). Also, exclusion of other hyperandrogenism disorders such as

non-classical congenital adrenal hyperplasia (NC-CAH), hyperprolactinemia, endogenous secretory-related cancers, thyroid disorders, and Cushing's syndrome should be considered (Bani Mohammad and Majdi Seghinsara 2017). In adolescence, symptoms may be overlapped with physiological changes during maturity. Hereupon, it is difficult to definitively diagnose polycystic ovaries in adolescence (Rosenfield 2015). Besides, it seems that there are no definite diagnostic criteria for post- menopausal women (Rosenfield and Ehrmann 2016). Androgens -secreted mostly by gonads and adrenal glands – play an important role in physiological processes and generation of estrogen in women. They are controlled by the hypothalamic-pituitary-adrenal and hypothalamic-pituitary-ovary axis. In the first axis, CRH is produced by the hypothalamus and stimulates the secretion of ACTH by the pituitary and eventually, it makes adrenal glands generate androgens, glucocorticoids, and mineralocorticoids (Pasquali et al. 2011; Indran et al. 2016). On the other hand, GnRH from the hypothalamus increases secretion of LH, so the ovarian theca cells secrete androgens in response to LH. It should be noted that peripheral tissues such as adipose tissue, hair follicles, and genital skin are involved in the synthesis and circulation of androgens. For example, adipose tissue can convert androgens like testosterone and androstenedione to more potent agents (Indran et al. 2016). Among different types of androgens, androstenedione and testosterone are produced in the ovaries, adrenal glands, and peripheral tissue while DHEAS is almost just from the adrenal cortex (Pasquali et al. 2011). Under normal circumstances, the androgens production rate and their metabolic clearance rate (MCR) act in balance and have daily rhythm (Pasquali et al. 2011). In PCOS women production rate of androstenedione, and testosterone increases more than their MCR, and ovaries secrete steroids and androgens excessively as a result of gonadotropin stimulation which makes them hypersensitive to LH (Pasquali et al. 2011; Rosenfield and Ehrmann 2016). Another androgen-related dysfunction is functional adrenal hyperandrogenism (FAH) which can be seen in PCOS as a type of adrenal abnormality that results in DHEA hyper responsiveness and ACTH hypersensitivity (Rosenfield and Ehrmann 2016). Additionally, insulin resistance and the following hyperinsulinism is a contributing factor to hyperandrogenism in PCOS. Insulin help adrenals androgen secretions, ovarian stimulation, and LH adjustment. Therefore, hyperinsulinism and resistance to insulin can be major underlying causes of PCOS. Moreover, androgen excess triggers abdominal and visceral adiposity and their dysfunction that then leads to insulin resistance (Escobar-Morreale 2018). Another associated factor with hyperandrogenism and PCOS is increased sympathetic activity. It is demonstrated that the density of catecholaminergic nerve fibers and the production of nerve growth factor (NGF) (a marker of sympathetic activity) by the ovaries are enhanced in PCOS (Pasquali et al. 2011). Interestingly, it was reported that modulating sympathetic activity by physical exercise and electro-acupuncture decreases levels of androgens, estrogens, and sex steroid precursors and also improves menstrual cycles in these patients (Jedel et al. 2011; Pasquali et al. 2011). Furthermore, granulosa cells and folliculogenesis are abnormal in PCOS. In an unusual physiologic condition, primordial follicles grow to primary follicles and then maturation arrests in the antral phase. Hence, the number of small antral follicles increases as a feature of folliculogenesis dysfunction. It is attributed to hyperandrogenism and insulin excess (Rosenfield and Ehrmann 2016). The enhanced folliculogenesis leads to increased anti-Mullerian hormone (AMH) production which is secreted by granulosa cells and has a regulatory role in follicular development (Rosenfield and Ehrmann 2016; Bani Mohammad and Majdi Seghinsara 2017). In addition, granulosa cells are luteinized prematurely in PCOS women and they are hyper responsive to LH and follicle stimulating hormone (FSH) (Rosenfield and Ehrmann 2016). It is stated that there are several disturbances in biologic mechanisms like fatty- acid metabolism, oxidative metabolism, and inflammatory responses. Accordingly, ovarian hyper vascularity is due to increased vascular endothelial growth factor

(VEGF) levels that causes the presence of inflammatory cytokines and dense cortex of ovaries (Rosenfield and Ehrmann 2016). Generally, inflammation is said to be a key underlying factor in the pathogenesis of PCOS (Kalhori et al. 2018). Besides, oocyte gene expression is affected in PCOS and may increase the risk of pregnancy loss (Rosenfield and Ehrmann 2016).

3 Hereditary and Environmental Factors

PCOS has a complex trait along which genetic, epigenetic and environmental factors affect hyperandrogenism and insulin resistance (Rosenfield and Ehrmann 2016, Escobar-Morreale 2018). Accordingly, studies on twins have revealed that familial factors are quite strong in PCOS pathogenesis and it appears to have a variable penetrance and an autosomal dominant pattern of inheritance (Rosenfield and Ehrmann 2016). However, there is still a long way to identify the exact genetic causes of PCOS (7). Several gene variants, polymorphisms, and linkages have been studied. For example, changes in fibrillin 3 have been proposed for hyperplasia of ovarian stroma as they affect transforming growth factor β (TGF-β) signaling (Hatzirodos et al. 2011; Rosenfield and Ehrmann 2016). Also, DENND1A expression is enhanced in theca cells and it has become a significant locus for PCOS etiology although it needs more investigation (McAllister et al. 2014; Rosenfield and Ehrmann 2016). Considering epigenome, environmental insults which occur during the development of the fetus or child can lead to PCOS. For instance, congenital virilization and androgenization can cause PCOS features (Barnes et al. 1994; Rosenfield and Ehrmann 2016). Besides. It is said that insufficient fetal nutrition and low birth weight are associated with PCOS (Ibanez et al. 1998; Rosenfield and Ehrmann 2016). Similarly, some harms and conditions during pregnancy such as maternal diabetes, smoking, and hypertension may cause growth retardation which can then induce resistance to insulin, overweight, and hyperandrogenism (Escobar-Morreale 2018). In addition, risk factors related to the postnatal environment including insulin resistance, androgen excess, and excessive LH stimulation at puberty can promote the manifestation of susceptible traits (Rosenfield and Ehrmann 2016). Based on what was mentioned above, it should be noted that PCOS has a variety of phenotypes and is a heterogeneous disorder. It can develop without the presence of important factors like obesity or insulin resistance or it may not occur in women with extreme obesity or hyperinsulinism. Thus, it seems that the most essential intrinsic factor is hyperandrogenism and genetic factors can trigger it to manifest (Escobar-Morreale 2018). Unfortunately, the pathogenesis of PCOS is not fully understood yet and more precise studies are required.

4 PCOS Current Treatments

Although PCOS is described as a manifestation of symptoms which vary according to different guidelines, they mostly include hypergonadism and menstrual irregularities that cannot be otherwise explained. The presence of ultrasound evidence of PCOS a diagnostic criterion along with clinical symptoms can assistance to diagnose. However, its absence does not rule out the disease in the presence of the other two symptoms (El Hayek et al. 2016). PCOS can be associated with high risk of some comorbidities which strongly affected the patient's quality of life including endometrial cancer. Since the definitive mechanism of PCOS formation is not yet known, current treatments are prescribed to reduce clinical symptoms (e.g. menstrual disorders, infertility, and the hyper-endogenous symptoms) of the disease. The recommended first line of treatment is always lifestyle interaction, restricted calorie intake, exercise, creating healthy habits, giving up smoking and drinking that can improve PCOS comorbidities such as insulin resistance, excess serum androgen levels, hirsutism, acne, etc. Herein, some trials show that lowering body mass index (BMI) with diet, exercise or a combination of both is equally effective in ameliorating

PCOS comorbidities (Thomson et al. 2008; Nybacka et al. 2011). In different studies recommended exercise varies from moderate aerobic exercise to high-intensity resistant exercise or the combination of both, while all have proven effective there is still no specific recommendation about diet or exercise plan. The 2018 EB guideline suggests 150 min per week moderate-intensity exercise or 75 min of intense training per week (Teede et al. 2018). Pharmacological treatments for PCOS include combined oral contraceptive pills (COCP) (which can decrease free testosterone levels and help with symptoms associated with hormonal imbalance. However, it has been associated with insulin resistance development and adverse effects on the cardiovascular system and developing thrombosis), insulin sensitizers (such as metformin that decreases gluconeogenesis in the liver and increases glucose consumption in other tissues and inositol which can be very effective in combination with other medications to improve outcomes and ameliorate metabolic syndrome, irregular menstruation cycles, insulin resistance, and lipid profile), anti-obesity, anti-androgen agents, infertility treatments (including letrozole, Clomiphene Citrate, and Gonadotrophins) (Lidegaard et al. 2012; Ganie et al. 2013; El Hayek et al. 2016; Williams et al. 2016; Jin and Xie 2018; Teede et al. 2018). Furthermore, there are some invasive methods for PCOS treatment e.g. bariatric surgery (which can lead to lower BMI index and improve obesity in obese women who have not responded to the aforementioned therapies), assisted reproductive technics (ART) (including in vitro fertilization (IVF), immature oocyte in vitro (IVM), artificial insemination (IUI), and intracytoplasmic sperm injection (ICSI) which are costly techniques lead to ovarian hyperstimulation syndrome (OHSS)) (Mourad et al. 2017; Patel 2018; Teede et al. 2018). In general, all treatment strategies are symptom-oriented and only partially effective. Pharmacological options need to be taken for a long time and there are often multiple adverse effects. Invasive treatments are expensive and may cause serious complications. Therefore, there needs to be an effort to find treatments that can address all the pathologies of PCOS with minimum adverse and long-term therapeutic effects. Hereupon, RM and stem cell researches can be the means of developing a more effective treatment for PCOS. In this respect, also characterization of stem cells from PCOS subjects can be valuable. Indeed, the mentioned characterization can be useful in selecting the most appropriate sources for stem cell-based regeneration. Additionally, choosing the appropriate animal model to simulate PCOS and conduct preclinical studies can be very helpful in advancing regenerative medicine at the clinical level.

5 Animal Models of PCOS

In order to find an appropriate and effective treatment for any disease, some steps should be taken prior to the clinical application including in vitro and in vivo studies. Also, developing and selecting suitable animal models for in vivo studies is of great importance for preclinical evaluation of treatments and investigational products based on significant similarities between the animals and humans (Goodarzi et al. 2019b; Larijani et al. 2019). However, there are still some variations and the outcomes of animal studies do not always comply with the ones from studies on humans (Goodarzi et al. 2019b). Hence, validating the models can help reach the maximum quality of testing and as a result, there are various animal models for different kinds of diseases such as Alzheimer's disease, obesity, diabetes, ovarian failure (Steindler 2007; Gunawardana and Piston 2012; Liu et al. 2015; Sheikhansari et al. 2018; Goodarzi et al. 2019b; Larijani et al. 2019). In regard to RM, animal models are used to understand the underlying pathways of regeneration and eventually, find a new therapeutic option (Steindler 2007). For instance, mice and rats are widely used in stem cell therapy such as neural progenitor cells transplantation on NOD-severe combined immunodeficient (SCID) mice or MSC transplantation on MI rat models (Tang et al. 2005; Steindler 2007). Considering tissue transplantation, BAT was used in nude mice to treat type1 diabetes

(Gunawardana and Piston 2012). Also, different kinds of stem cells have been used in mice for POF (Sheikhansari et al. 2018). Therefore, it can be said that animal models in RM can facilitate the process of translating the investigational product in to the clinical application. In this context, there is a lack of a gold standard model for PCOS, so there are a variety of species including sheep, monkeys, and rats with their different benefits and relevance to humans (Indran et al. 2016). Developing models can be done through different mechanisms and phases but the hallmark of PCOS condition is hyperandrogenism, so increasing androgens like testosterone level is considered the main factor (Divyashree et al. 2019). Also, PCOS can be induced at different developmental stages of animals including prenatal, postnatal, prepubertal, and adult. For instance, dihydrotestosterone (DHT) can be used at prenatal, prepubertal and adult stages but letrozole (an aromatase inhibitor) is not used at the prenatal phase (Divyashree et al. 2019). Further, the main mechanisms of inducing PCOS are defined as hormonal intervention, genetic manipulation, environmental factors, and lifestyle (Fig. 1) (Divyashree et al. 2019).

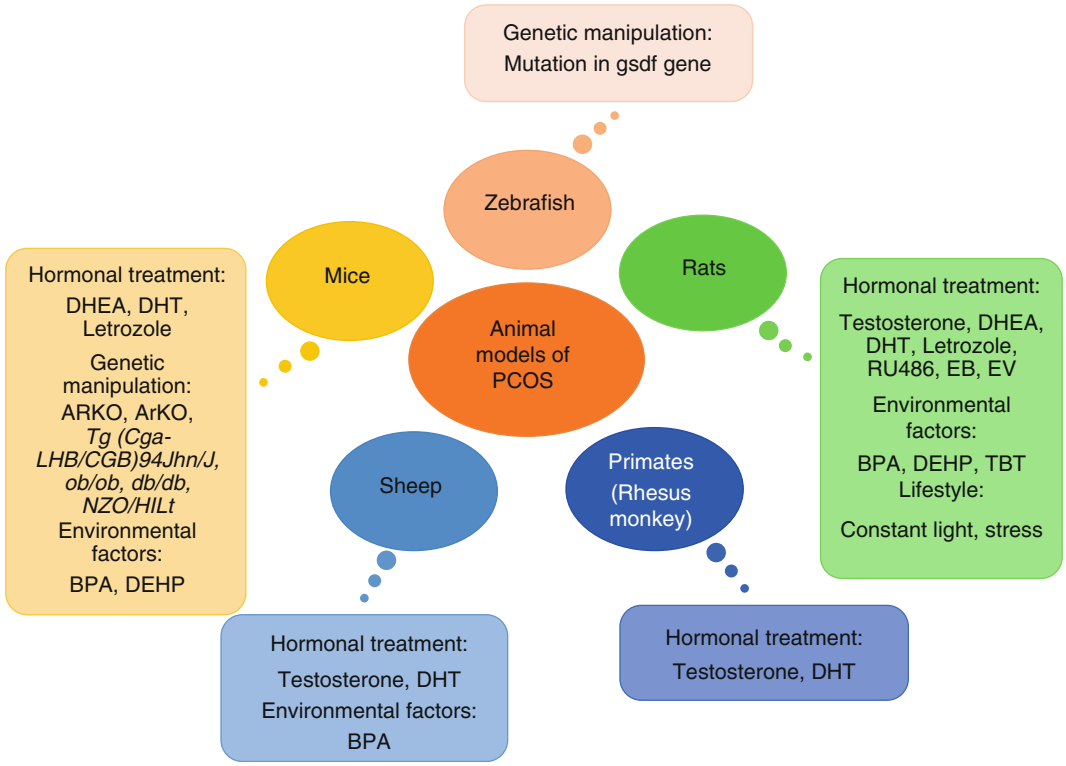

Fig. 1 Different mechanisms of generating animal models in PCOS. Animal models of polycystic ovary syndrome (PCOS) can be produced through different mechanisms including hormonal intervention (such as using androgens or progesterone receptor antagonists), genetic manipulation and mutations, environmental factors and lifestyle. In addition, the widely used models in this field are rats and mice, however, primates, sheep and recently zebrafish have been used. In this figure, some staple mechanisms of inducing each animal model are depicted (Paixão et al. 2017; Osuka et al. 2018; Ryu et al. 2019; Stener-Victorin et al. 2020). *DHT* Dihydrotestosterone, *DHEA* Dehydroepiandrosterone, *EV* Estradiol valerate, *EB* Estradiol benzoate, *ARKO* Androgen receptor knock out, *ArKO* Aromatase knockout, *gsdf* Gonadal soma derived factor, *BPA* Bisphenol A, *DEHP* Di-(2-ethylhexyl) phthalate, *TBT* Tributyltin, *NZO/HILt* New Zealand obese mice

6 Characteristics of Stem Cells in PCOS Patients

Since the exact underlying pathophysiology of PCOS and its characterization such as hyperinsulinism, infertility and obesity are not fully understood, attempts to identify these mechanisms have led to analyzing features of stem cells from PCOS patients. PCOS-derived stem cells provide a medium to study disease pathogenesis, gene mapping and therapeutic options with gene or stem cell therapy. Embryonic stem cells (ESCs) from PCOS subjects present normal morphologies and have the same developmental and differentiation ability as stem cells from normal control cells. In-vitro cultures of ESCs showed normal karyotype and gene expression profiles of gene markers of the three germ layers using immunohistochemistry. Induced pluripotent stem cells (iPSCs) derived from epithelial or endometrial cells of PCOS patients have the ability to differentiate into three germ layers and form different tissues and thus provide the opportunity to develop cell models to study PCOS and disease- specific pathogenesis. iPSCs derived from PCOS patients' epithelial stem cells have normal morphologies, characteristics, and karyotype to that of normal cells and can be induced to form adipocytes. PCOS-derived iPSCs have stronger potential and higher efficacy in differentiating into adipocytes (Yang et al. 2016). There are controversies regarding glucose consumption and insulin response in adipocytes differentiated from PCOS-derived iPSCs. One study states that adipocytes present significantly higher glucose consumption in the absence of insulin on day 27 of induction indicating higher insulin response-ability in the cells, however, another study reports no significant difference between PCOS and non-PCOS adipocytes in insulin response on day 21 of induction. In both studies, PCOS-derived adipocytes had significantly higher glucose consumption (Liu et al. 2015; Yang et al. 2016). Adipose stem cells (ASCs) of subcutaneous abdominal tissue of normal-weight PCOS patients exhibit significant differential gene expression (DGE) change in 120 genes compared to the control group. DGE showed altered expression in genes of both functional and canonical groups and primarily included androgen receptor function, lipid metabolism, extracellular matrix, and angiogenesis. Most significant changes in functional genes were in developmental disorders, embryonic development, and cellular movement genes; and in the canonical pathway group primarily included LXR/RXR activation, notch signaling and CDK5 signaling pathway. The expression of genes in the functional group was associated with fasting insulin and serum testosterone levels (Dumesic et al. 2019). ASCs in normal-weight women also exhibit strong pre-adipocyte commitment. Increased zinc finger protein 423 (ZFP423) and peroxisome proliferator-activated receptor gamma (PPARγ) protein expression showed a significant correlation with higher lipid content of adipocytes derived from ASCs, however, this gene expressions' correlation with serum testosterone levels were insignificant. Fasting plasma glucose levels exhibit a negative correlation with ZFP423, independent of serum testosterone levels (Fisch et al. 2018). A brief review of altered genomic function and characteristics in PCOS-derived stem cells provided in Table 1.

6.1 Differential Expression of Genes Responsible for Insulin Resistance and Obesity in Adipocytes Derived from Human ESCs of PCOS Subjects

NR0B2 is a gene that participates in many metabolic pathways such as adipocyte differentiation, fatty acid metabolism, glycometabolism, hepatocyte, and pancreatic islet cell transcription and is associated with obesity, fasting insulin levels, diabetes and intrauterine growth of the fetus. Differential expression of the NR0B2 gene was significantly higher in embryonic stem cells drive from PCOS patients compared to the control group, although much more evidence is needed to prove the role of NR0B2 in PCOS pathogenesis (Wang et al. 2014). DNA methylation analysis

Table 1 A brief review of differentially expressed genes in PCOS-derived stem cells (Chuang et al. 2015; Min et al. 2018, 2019; Sun and Pisarska 2019)

Cell type	Function	Genes
PCOS-derived iPSC	Androgen receptor function	FDX-R, CXCL2, ST3GAL5, IGFBP7
	Lipid synthesis	TAC1, BDNF, EDNRB, ST3GAL5
	Lipid accumulation	GULP1, RGS4
	Lipid metabolism	SCD, APOC1, NR0B1, PLTP, UGCG
	Extracellular matrix function and formation	CHI3L1, CRISPLD2, LM07, ITGA6
	Cardiovascular development	CXCL5, TNC, ST3GAL5, CTSB
	Inflammatory response	REG3A
	Cellular growth cycle	CGA, IGFBPL1
	Organ development	HAS2, CER1,IFI16, TBX5
	Mitochondrial biogenesis	PGC1-α, TFAM, NRF1
PCOS iPSC-derived granulosa cells	Cell adhesion	MLCP(reg), PKC-α, α-actinin, TGF1-β, TGFβ receptor
	Immune response	MHC11-α and β chain, PKC-α, LY75
	Neurophysiology process	NMDA receptor, PyK2(FAK2), B-Raf, GRB2, NSF, Dynein1, GABA-A receptor, dynamin
	Oxidative stress	TR10, PKC-α, MEK1 and2, cPKC

results on canonical pathways of iPSCs derived from granulosa cells of PCOS women compared with control group revealed altered expression of genes of CREB signaling pathway which is responsible in glucose and lipid metabolism, although it did not reach statistical significance which could be due to small sample size (Huang et al. 2019). PCOS-derived iPSCs present metabolic abnormalities and mitochondrial dysfunction compared to non-PCOS iPSCs. Gene ontology results of PCOS-derived iPSCs revealed altered expression of genes responsible for lipid and glucose metabolism along with decreased mitochondrial ability of respiration and glycolysis, although the number of mitochondrial copies and their biogenesis had increased (Min et al. 2018). Glucose metabolism-related genes was altered in favor of decreased glycolysis, glucose transportation and increased gluconeogenesis that can lead to insulin resistance and glucose intolerance.

6.2 Inflammatory and Oncogenic Potential

Endometrial mesenchymal stem cells of the obese or overweight PCOS women showed up-regulated expression of inflammatory and oncogenic genes independent of BMI in comparison to the obese or overweight control group. Interleukine-8 (IL-8) and ICAM1 up-regulation can induce a pro-inflammatory state and disrupt normal endometrial function. The most up-regulated oncogenic gene was the LCNZ gene which has a correlation with endometrial cancer, however, due to the small sample size and possible confounding factors, more studies are required to support this research (Piltonen et al. 2013).

6.3 Neuroendocrine Characteristics

Gene ontology revealed that genes associated with neurogenesis, endocrine differentiation, and low-density lipoprotein (LDL) particle binding process were down-regulated in PCOS-derived iPSCs. IPSCs derived from the endothelium of PCOS patients also revealed higher testosterone secretion than non- PCOS derived iPSCs, which is consistent with hypergonadism in PCOS. However, no significant difference was observed in estradiol secretion between the two groups. Increased GnRH and consequently LH levels in PCOS is mediated through GABAergic neurons. GABRA5 is a type of a GABA receptors that were found to have up-regulated gene expression

in PCOS-derived iPSCs, possibly resulting in both psychological and endocrine presentations of PCOS (Min et al. 2019). Neural stem cells differentiated from PCOS-derived iPSCs display normal morphology, neural progenitor markers expression and normal gene expression of pluripotency, however, they display decreased mitochondrial respiratory ability compared to the control group. The maximum respiratory function of mitochondria was decreased in PCOS-derived iPSCs.

7 Challenges in iPSC-Based Models and Therapies for PCOS

Recent advances in our knowledge about stem cells can be useful for understanding biological and molecular processes which causing dysfunction in cells. In this context, using IPSCs can be helpful to develop more efficient models and methods of cellular treatments (e.g. autologous transplantation) (Kanherkar et al. 2014). Even though iPSCs are a very powerful tool in RM for disease modeling, cell therapy and drug discovery, there are challenges that need to be addressed. Studies have shown that iPSCs might still be carrying epigenetic memory from the somatic tissue they were derived from. Since dermal cells are prevalently used as the source of iPSCs, there might be many genetic mutations due to mechanisms of aging like a high rate of cell turnover and increased exposure to ultraviolet light (Lo Sardo et al. 2017). Another area of concern in iPSC-based treatments is the tumorigenicity potential of transplanted cells (contain undifferentiated iPSCs). However, there are methods that purify cells and selectively cause death in undifferentiated iPSCs. There is no evidence of tumor formation in transplantations but these outcomes are unlikely to be reported. Herein, there is still more evidence needed to see if these methods are adequate for preventing tumors after transplantation (Hunt and Lako 2016).Immune rejection concerns are another major challenge in iPSC-based treatments. Since the use of immunosuppressants can cause many complications, there have been some new methods to escape immunity such as inactivation of both classes of MHC genes. These new methods can solve the rejection challenge of transplantation. Additionally, iPSC production is quite expensive which may not be affordable for many patients (Bravery 2015). On the other hand, the efficiency of this method for disease modeling depends on the nature of the disease. IPSC-based disease modeling is much more accurate when the disease is monogenic and has an early onset, whereas PCOS is a polygenic disorder and has an unclear onset. As the cells then spend a period of time in an in-vitro culture they are likely to develop mutations and genetic instability. The longer they are kept in culture the risk of mutation bursts increases (Doss and Sachinidis 2019). Another major challenge in iPSC-based PCOS treatment is the lack of cohort studies and clinical and pre-clinical research. Most literature in this field is based on a small sample size in a short period of time. Therein, more data is needed to make a conclusion about the use of iPSCs in PCOS disease modeling and treatment.

8 Regenerative Medicine in PCOS

Despite the advancements of current available treatments, in the attempt to find therapies that can restore the function or somehow replace the absent tissue or organ of the female reproductive system, RM has emerged as an alternative therapeutic option (Magalhaes and Atala 2019). Hence, tissue engineering, tissue and stem cell transplantation have been used in this field for different kinds of impairments in uterus, vagina, and ovaries (Magalhaes and Atala 2019). Noticing the disorders related to ovaries, several animal studies and some clinical trials have been performed to treat POF, primary ovarian insufficiency (POI) and poor ovarian responders(PORs) (Chen et al. 2018; Herraiz et al. 2019). For instance, POF induced by chemotherapy, human umbilical cord blood mesenchymal stem cells (hUC-MSCs) and bone marrow-derived mesenchymal stem cells (BM-MSCs) were administered in rats and mice respectively, resulting in promoting folliculogenesis,

improving ovarian function and endocrine system (Elfayomy et al. 2016; Song et al. 2016; Yoon 2019). Further, among clinical trials, an example is the transplantation of autologous BM-MSCs on 10 women with POF that led to resuming menstruation (in 20%) and one pregnancy following with healthy delivery (Edessy et al. 2016). About PCOS, there are a few animal studies and a case report in this context, which are summarized in this review.

8.1 Preclinical Investigations About RM in PCOS

8.1.1 Tissue Transplantation in PCOS Models

There are two main types of adipose tissue in humans and other mammals: white adipose tissue (WAT) and brown adipose tissue (BAT) (Payab et al. 2018). While the responsibility of WAT is storing energy, BAT maintains body temperature through thermogenesis and energy expenditure by uncoupling protein 1 (UCP1) and several secretory cytokines (Liu et al. 2015; Yuan et al. 2016). Also, it is revealed that increasing BAT mass or activating it can have a therapeutic effect on some metabolic disorders such as obesity and insulin resistance (diabetes) (Gunawardana and Piston 2012; Liu et al. 2015). On the other hand, as it was mentioned before, insulin resistance is a key etiological characteristic of PCOS and women with PCOS have low BAT activity, so BAT transplantation may have a beneficial role in improving related features of PCOS (Yuan et al. 2016; Shorakae et al. 2019). Accordingly, BAT was transplanted on DHEA-induced rats in a study in comparison with control, sham-operated and muscle transplanted groups. In addition to increased BAT activity and thermogenesis and improved insulin sensitivity, menstrual cyclicity and plasma LH level normalized in 70% of the BAT –transplanted group. In connection with ovarian histology and infertility, the thickness of theca cell layer was increased and corpora luteal number was decreased in sham and muscle transplanted groups while they were normal in the BAT transplanted group and the transplantation helped eats to give birth to a litter. In addition, it is reported that sympathetic tone is increased in the ovaries in women with PCOS, which is reduced after transplantation. Since BAT transplantation increases adiponectin level (which is attenuated in patients with PCOS), in order to find out the possible role of this hormone in BAT effects, recombinant adiponectin protein was injected daily in a PCOS rat. The results showed same changes such as increased endogenous BAT activity, improved glucose homeostasis, LH/FSH ratio, acyclicity, and ovarian phenotype. Therefore, adiponectin seems to be partly in charge of some of the BAT beneficial effects. As a result, BAT transplantation or possibly, enhancing BAT activity, might be an appropriate strategy to improve PCOS phenotypes (Yuan et al. 2016). As it was said that BAT activation may be beneficial in PCOS treatment, endogenous BAT in DHEA-induced PCOS rats was treated with rutin (a flavonoid) to test this hypothesis (Hu et al. 2017). It was observed that BAT activity and related genes and also the expression of lipid metabolism – related genes were increased in comparison with the PCOS group treated with DMSO. Moreover, there were improvements in insulin sensitivity and glucose homeostasis and serum adiponectin and high-density lipoprotein (HDL) levels were increased in the rutin group. Considering PCOS phenotypes, rutin treatment had a beneficial influence on LH/FSH ratio, acyclicity, the morphology of corpus luteum, ovarian steroidogenic enzymes, and fertilization ability (Hu et al. 2017). Generally, it was demonstrated that activating endogenous BAT, whether by transplantation or rutin treatment, improves PCOS and due to limitations and risks of transplantation in clinical application, using an activating compound might be more applicable and a novel therapeutic option in the clinic. However, the effect and safety of such procedures are to be investigated in humans (Hu et al. 2017).

8.1.2 Cell–Based Approaches in PCOS Models

Based on studies on the etiology of PCOS, inflammation and oxidative stress are suggested to be involved in the pathogenesis of PCOS

(Kalhori et al. 2018). Besides, obesity has inflammatory properties and inflammatory cytokines can trigger androgen production and follicle atresia (Xiong et al. 2011; Gunawardana and Piston 2012; Kalhori et al. 2018). Therefore, targeting the mentioned mechanisms can be a promising area. Accordingly, BM-MSCs have been used in the treatment of different inflammatory diseases due to their anti-oxidative, immunomodulatory and anti-apoptotic qualities (Singer and Caplan 2011; Lv et al. 2013; Kalhori et al. 2018). In addition, BM-MSCs transplantation is reported as being useful in treating damages and disorders of ovarian function (Fu et al. 2008; Kalhori et al. 2018). Hereupon, to investigate the potential beneficial effects of these cells on PCOS, BM-MSCs were injected in testosterone-induced PCOS mice (Kalhori et al. 2018). The transplanted group showed an increase in the volume of ovary and cortex and the number of corpus luteum and antral follicles to the control level when compared with the PCOS group. This result was probably because of testosterone reduction in the transplanted group. Further, hyperinsulinemia affects oocyte size and follicles and MSCs increase insulin sensitivity, so oocyte volume was increased in the BM-MSC group. Additionally, an anti-apoptotic feature of MSCs on granulosa cells caused an increase in zona pellucida thickness, which is reduced in PCOS. Similar to BAT transplantation, LH/FSH ratio decreased in the transplanted group and serum levels of inflammatory cytokines such as IL-6 and TNF-α were reduced (Kalhori et al. 2018). Referring to the inflammatory aspect of PCOS and immunomodulatory potential of MSCs, hUC-MSCs were used in DHEA- induced PCOS mice in contrast to the control and DHEA group treated with normal saline (Xie et al. 2019). Somehow similar to previous studies, MSC treatment reduced the number of cystic follicles, increased the number of corpus luteum and mature follicles and the normalized estrous cycle. Besides changes in the ovary, uterine tissue in PCOS mice was congested and full of a hydrocele that indicated an inflammatory state of the uterus. However, MSC transplantation led to the thinner endometrial epithelium and normal morphology of uterus. Accordingly, the expression of inflammatory and fibrosis factors was inhibited in ovarian tissue due to MSC transplantation. Analyzing immunity alteration has revealed that MSC could modulate macrophages decrease their infiltration into ovaries and uterus. Also, in regard to adaptive immunity MSC promotes regulatory T cells (T-reg) differentiation and inhibits B cell proliferation in contrast to the PCOS group treated with normal saline (Xie et al. 2019). In conclusion, MSC transplantation is reported to have significant curative effect on the wide range of PCOS phenotypes via its beneficial capacity (Xie et al. 2019). However, more studies on the safety and precise underlying mechanism of its therapeutic impacts are required. A simplified picture of the mentioned studies is illustrated in Fig. 2.

8.2 Clinical Advancements

Unlike POI or POF, there has been no clinical trial on using tissue or stem cell transplantation for PCOS so far. However, there is a case report of women with PCOS and myelodysplastic syndrome (MDS) in the form of refractory anemia who underwent mega chemotherapy and allogeneic hematopoietic stem cell (HSC) transplantation and delivered a full-term baby (Usnarska-Zubkiewicz et al. 2010). A 21-year-old woman was first hospitalized and PCOS (hirsutism, oligomenorrhea and irregular menstrual cycles) and refractory anemia were diagnosed in 2000. After 3 years, it was decided to perform HSC transplantation from her brother and then, she received different drugs before and after the transplantation. The patient lost 20 kg in peritransplantation period and examination in 2 years post intervention showed regular menstruation, resulting in a pregnancy with no anemia or other hematopoietic disturbances and no episode of hypertension or raised glucose level. Despite the fact that chemotherapy impairs ovarian function and production of sex hormones, the reproductive system in this patient remained quite normal. Also, 2 years after the delivery her hormonal findings exhibited normal testosterone level but

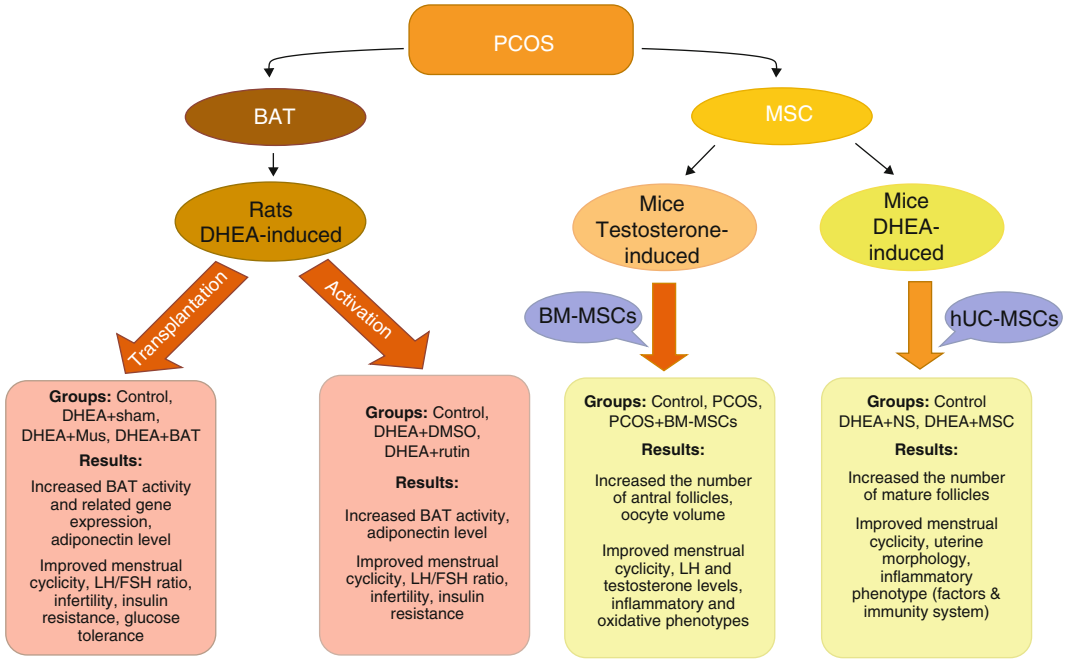

Fig. 2 Preclinical Studies on PCOS in The Field of Regenerative Medicine. In order to find new therapeutic options for polycystic ovary syndrome (PCOS), brown adipose tissue (BAT) and mesenchymal stem cells (MSCs) have been used in mice and rats resulting in promising outcomes. In addition, activation of endogenous BAT has been proposed as a less invasive way to control this disease. On the other hand, MSCs from different sources were used in mice and they were beneficial in improving the PCOS-like phenotype. In this figure, some examples of these studies, control and investigational groups, and a summary of their results are depicted (Yuan et al. 2016; Hu et al. 2017; Xie et al. 2019). DHEA: Dehydroepiandrosterone, BM-MSCs: Bone marrow mesenchymal stromal cells, *hUC-MSCs* Human umbilical cord derived MSCs, *Mus* Skeletal muscle-transplanted, *NS* Normal saline, *LH* Luteinizing hormone, *FSH* Follicle – stimulating hormone

there were irregular menstrual bleeding and relapse of obesity (Usnarska-Zubkiewicz et al. 2010).

9 Conclusion

Although PCOS and its comorbidities including insulin resistance and obesity are so common in women around the world, the current existing treatments cannot cure PCOS and its long term administration only alleviate symptoms while they have substantial complications. Hereupon, RM which has been used in similar disorders such as POI and POF has recently stepped into proposing novel therapeutic options for PCOS. Herein, in the current review brief summary of running treatments, experimental models of PCOS along with recent studies on BAT and MSC transplantation on animal models is provided. Also, a case report of pregnancy in women with MDS and PCOs following with HSC transplantation was introduced. In general, according to the bulk of studies, RM seems to be an astonishing alternative for treatment and control of PCOS phenotypes but further clinical explorations in a variety of regenerative medicine strategies for PCOS are demanded. In this context, considering that stem cells can control angiogenesis and inflammatory responses, stem cell application for PCOS individuals can be a unique approach in the road of chronic inflammation inhibition, metabolic abnormalities improvement, and ovarian androgen output reduction.

Accordingly, the well-known positive effects of MSCs, make them a potential therapeutic tool for PCOS cases. On the other hand, stem cells derived from PCOS subjects –specifically iPSCs- poses some different features with the normal ones. Therefore, this phenomenon should be noticed in order to use them either for transplantation or experimental studies on pathogenesis mechanisms. Indeed, they can be also used as a potential model to mimic the metabolic abnormalities of PCOS individuals. Therein, they can provide a suitable solution to reduce the use of animal models and related ethical concerns. In other words, they could directly or indirectly increase the animals' welfare and leading to 3R (Replacement, Reduction, and Refinement) rules enforcement.

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Opportunities and Challenges in Stem Cell Aging

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Abstract

Studying aging, as a physiological process that can cause various pathological phenotypes, has attracted lots of attention due to its increasing burden and prevalence. Therefore, understanding its mechanism to find novel therapeutic alternatives for age-related disorders such as neurodegenerative and cardiovascular diseases is essential. Stem cell senescence plays an important role in aging. In the context of the underlying pathways, mitochondrial dysfunction, epigenetic and genetic alterations, and other mechanisms have been studied and as a consequence, several rejuvenation strategies targeting these mechanisms like pharmaceutical interventions, genetic modification, and cellular reprogramming have been proposed. On the other hand, since stem cells have great potential for disease modeling, they have been useful for representing aging and its associated disorders. Accordingly, the main mechanisms of senescence in stem cells and promising ways of rejuvenation, along with some

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examples of stem cell models for aging are introduced and discussed. This review aims to prepare a comprehensive summary of the findings by focusing on the most recent ones to shine a light on this area of research.

Keywords

Aging · Model · Partial reprogramming · Rejuvenation · Stem cell

Abbreviations

5, 15 DPP	5, 15-Diphenylporphirine
ACC1	Acetyl-Coa Carboxylase
AD	Alzheimer Disease
ADSCs	Adipose Derived Mesenchymal Stem Cells
Atg7	Autophagy-Related Gene
BMMSCs	Bone Marrow-Derived Mscs
BMP5	Bone morphogenic Protein5
CoPP	Cobalt Protoporphyrin
CR	Caloric Restriction
CRP	C-reactive Protein
CYGB	Cytoglobulin
DDR	DNA Damage Response
DKK1	Dickkopf-1
DMOG	Dimethyloxalyglycine
ERCC1	Excision Repair Cross Complementing-Group1
FAPs	Fibro-Adipogenic Progenitors
FASN	Fatty Acid Synthase
FGF	Fibroblast Growth Factor
GDF11	Growth Differentiation Factor 11
Gnrh	Gonadotropin Releasing Hormone
GPDH	Glycerol-3-Phisphate Dehydrogenase
H2AX	H2A Histone Family Member X
hCPCs	Human Cardiac Progenitor Cells
HGPS	Hutchinson-Gilford Progeria Syndrome
HSCs	Hematopoietic Stem Cells
HSL	Hormone- Sensitive Lipase
HSP70	Heat Shock Protein 70
HSPCs	Hematopoietic Stem and Progenitor Cells
IGF1	Insulin-Like Growth Factor 1
IL	Interleukin
iPSCs	Induced Pluripotent Stem Cells
ISCs	Intestinal Stem Cells
JAK/STAT	Janus Kinase and Signal Transducer and Activator of Transcription
LVCP	Lateral Ventricle Choroid Plexus
MAPK	Mitogen-Activated Protein Kinase
miR	micro RNA
Mnsod	Manganese Superoxide Dismutase
MSCs	Mesenchymal Stem Cells
Mterc	Mouse Telomerase RNA Component
mTOR	Mammalian Target Of Rapamycin
NAC	N-acetyl-L-cysteine
NO	Nitric Oxide
NR	Nicotinamideriboside
Nrf2	Nuclear Factor Erythroid2-Related Factor2
NSCs	Neural Stem Cells
OCN	Osteocalcin
OPN	Osteopontin
PDIA3	Protein Disulfide-Isomerase A3
PGC1α	Pparg Coactivator 1 Alpha
RB	Retinoblastoma Protein
ROS	Reactive Oxygen Species
sFRP3	Soluble Frizzled-Related Protein3
SIRT	Sirtuin
SOD2	Superoxide Dismutase 2
SOX2	Sex Determining Region Y-Box 2
spry1	Sprouty1
SVZ	Subventricular Zone
sXBP1	Spliced X-Box Binding Protein 1
TBI	Total-Body Irradiation
Tert	Telomerase Reverse Transcriptase
TET	Ten-Eleven Translocation
Tet2	Ten Eleven Translocation Methylcytosine Dioxygenase2
TNFα	Tumor Necrosis Factor-α
TXNIP	Thioredoxin-Interacting Protein
Upr^{mt}	Unfolded Protein Response
WISP1	WNT1 Inducible Signaling Pathway Protein 1
β3-AR	β3-Adreno Receptor

1 Introduction

Epidemiological studies show that by 2050, 22% of the world population will be over 60 years of age (Kanasi et al. 2016). This increased life expectancy has led to aging and age-related disorders such as neurodegenerative and cardiovascular diseases, cancers, and aging frailty (López-Otín et al. 2013; Shetty et al. 2018; Goodarzi et al. 2019a; Larijani et al. 2019; Oliva et al. 2019; Baradaran-Rafii et al. 2020). Although these diseases have been treated and discussed as isolated disorders, there are recognized links between them and the aging process (Saeidimehr et al. 2016; Oliva et al. 2019). Accordingly, the field of geroscience is about finding the pathways and biology of this relationship which can contribute to preventing the development of such disorders through targeting the aging itself (Oliva et al. 2019; Picca et al. 2019). In that regard, the general cause of aging is the accumulation of cellular damage. In addition, aging is characterized by different hallmarks, including genomic instability, telomere attrition, epigenetic alterations and changes, impaired proteostasis, deregulated nutrient sensing (e.g. changes in insulin-like growth factor 1(IGF-1) signaling pathway), mitochondrial dysfunction, cellular senescence, changes in intercellular communication, stem cell exhaustion and the decline in the regenerative capacity. All of these hallmarks provoke the aging process and its phenotype (López-Otín et al. 2013; Picca et al. 2019). However, one of the most obvious ones, the stem cell aging, has become a novel theory that states aging happens partly due to the diminished regenerative potential of stem cells and impairment in their function as they grow old (Sharpless and DePinho 2007; López-Otín et al. 2013; Ahmed et al. 2017; Wang et al. 2019). On the other hand, aging impairs the therapeutic potential of stem cells (in-vitro cultured stem cells in several passages) or isolated stem cells from aged subjects exhibited diminished productivity and function (Sharpless and DePinho 2007; Ahmed et al. 2017). Therefore, in either case, the age-associated diseases may develop as a result of the deterioration of adult stem cells, such as neural stem cells (NSCs), mesenchymal stem cells(MSCs) and hematopoietic stem cells (HSCs) (Ahmed et al. 2017). Consequently, studying mechanisms and pathways involved in stem cell aging like mitochondrial dysfunction and changes in epigenetic regulation is essential in order to find new therapies for aging and aging-related diseases (Oh et al. 2014; Ahmed et al. 2017).

2 Stem Cell Aging

Stem cells are known as a population of cells characterized by their self-renewal capacity and the ability of differentiation to various cell types (Arjmand et al. 2017; Khorraminejad-Shirazi et al. 2018). Whether stem cell aging is the cause or consequence of aging is not yet fully understood, however, it has been demonstrated that stem cell exhaustion can lead to premature aging in mice. Inducing stem cell exhaustion with partial repetitive depletion of adult sex determining region Y-box 2 + (SOX2+) Cells caused more gray hair, reduced hair regrowth capacity and decreased epithelial cellularity in esophagus and trachea. The senescence associated beta-galactosidase activity was significantly higher in kidney frozen sections, after stem cell exhaustion. This funding suggests a systematic response to stem cell exhaustion since SOX2 is not expressed in kidney cells. In a longer exposure to repetitive depletion of SOX2+ adult cells, mice subjected to stem cell exhaustion had a progressive decline in spontaneous activity and exploratory behavior. It is proposed that adult stem cell depletion can lead to premature tissue aging by triggering a systematic response (Vilas et al. 2018). Hence, adult stem cell dysfunction is an essential factor of aging and its associated diseases, since adult stem cells (or somatic stem cells) that are found in each tissue type and organ, have a pivotal role in the maintenance of tissue homeostasis and regeneration (Ahmed et al. 2017; Ren et al. 2017). For instance, NSCs are responsible for generating different specific cells in the CNS

like neurons, astrocytes, oligodendrocytes, and ependymal cells (Ahmed et al. 2017; Conover and Todd 2017). As the age increases, their neurogenic capacity diminishes and some cognitive deficits occur as a consequence of aging, including neurodegenerative diseases (notable Alzheimer's disease (AD)), olfactory dysfunction, and spatial memory deficits (Ahmed et al. 2017; Conover and Todd 2017; Hou et al. 2019). Although there are reports of the persistent neurogenesis in adults, NSC aging and the decline in the proliferation ability have been indicated in many studies on animals and human (for example by investigating the in vitro culture of NSCs) and also, it is suggested that the loss of neurogenesis might be associated with the deeper quiescent state of stem cells and their activation defects (Daniele et al. 2016; Audesse and Webb 2020; Han et al. 2021). HSCs are another group of stem cells, which are susceptible to aging (Ahmed et al. 2017; Lee et al. 2019). They reside in the red bone marrow and continuously produce blood cells, including erythrocytes, immune cells, and platelets (Ahmed et al. 2017, Lee et al. 2019). With aging, cell-intrinsic/extrinsic factors cause HSCs to have a functional decline in repopulation capacity, defects in homing and mobilization and unbalanced differentiation (lineage skewing from lymphopoiesis toward myelopoiesis), which results in several types of immune diseases such as anemia, myeloid and lymphoid leukemia, and compromised adaptive immunity (Rossi et al. 2008; Kikushige and Miyamoto 2014; Lee et al. 2019). Therefore, rejuvenating aged HSCs can improve the quality of life for the elderly patients (Lee et al. 2019). MSCs contribute to the maintenance of the organs and differentiate to mesodermal derivative including chondrocytes, osteocytes, adipocytes, and myocytes (Goodarzi et al. 2015, 2018a, b, 2019b; Ahmed et al. 2017; Arjmand et al. 2019, 2020; Fafián-Labora et al. 2019; Baradaran-Rafii et al. 2020; Parhizkar Roudsari et al. 2020). They can be isolated from different organs as well as umbilical cord tissue and blood, but the most common sources are bone marrow-derived MSCs(BMMSCs) and adipose tissue-derived MSCs(ADSCs) (Ahmed et al. 2017). Due to their beneficial properties such as keeping tissue homeostasis and modulating inflammation, they have been used in many studies in order to find new therapeutic approaches. However, aging influences these properties and functions by senescence and its associated mechanisms such as oxidative stress (Yang 2018; Fafián-Labora et al. 2019). These changes can be divided into in-vitro and in-vivo behaviors. For instance, after in-vitro expansion, it was demonstrated that the self-renewal capacity and cell viability were decreased and immunosuppressive properties and differentiation potential (significantly in the case of chondrogenesis and osteogenesis) were reduced(Yang 2018, Fafián-Labora et al. 2019). Additionally, shifting in their morphology, genetic instability and changes in the expression of specific surface markers expression were found. In-vivo senescence of MSCs is characterized by reduced proliferative capacity, colony forming efficiency, and differentiation potential (adipogenesis, osteogenesis and chondrogenesis). Moreover, the number of isolated MSCs from the bone marrow aspiration declines with age (Yang 2018). Hence, the aging of MSCs seems to be detrimental in regard to their essential functions (Ahmed et al. 2017). Further, stem cells of skeletal muscle -called satellite cells -are responsible for sustaining the regeneration throughout life. Aging impacts the remarkable regenerative capacity of skeletal muscle as it is associated with a decline in the number and functionality of the satellite cells (García-Prat et al. 2013; Muñoz-Cánoves et al. 2020). Both environment alterations as an extrinsic factor and intrinsic changes such as genomic instability, metabolic defects, and drop in autophagy (which is important in senescence) contribute to satellite cell impairment. Therefore, old satellite cells generate insufficient progeny, which possess limited differentiation and myogenic potential, and accumulation of the altered progeny results in deterioration of the function and structure of the tissue (García-Prat et al. 2013; Fafián-Labora et al. 2019; Muñoz-Cánoves et al. 2020). Cardiovascular diseases, as one of the leading causes of mortality and morbidity, are affected by aging since intrinsic aging can cause progressive changes in the structure and functions

of the heart regardless of other risk factors like hypertension and diabetes (Dai et al. 2012). Some of the physiological changes of cardiac aging are the increase in left ventricle wall thickness, myxomatous degeneration of the valves and valvular sclerosis, which lead to increased prevalence of related diseases (Dai et al. 2012). The underlying mechanisms of these alterations include mitochondrial dysfunction, free radicals and reactive oxygen species (ROS), nutrient signaling, and cardiac stem cell aging (Sussman and Anversa 2004; Dai et al. 2012). Nowadays it is accepted that cardiac myocytes are generated after birth. One of the hypotheses of the underlying causes of the myocyte turnover is the resident stem cells. Cardiac stem cells play an important part in cardiac homeostasis and their senescence is thoroughly involved in the cardiac aging and heart failure, though its mechanisms are not fully understood (Anversa et al. 2005; Dai et al. 2012; Cesselli et al. 2018).It was found that the senescence of cardiac stem cells and the expression of some of the associated genes were increased in the aging mouse model and human hearts (as it was demonstrated in the in-vitro study of evaluating and comparing normal donor hearts and explanted hearts in end-stage heart failure) (Torella et al. 2004; Anversa et al. 2005; Cesselli et al. 2011). Also, the deletion of the pro-senescent gene p66shcin a diabetic mouse model can prevent age-related changes (Rota et al. 2006; Dai et al. 2012). Moreover, telomere shortening is another factor of senescence in cardiac progenitors and human cardiac stem cells (Cesselli et al. 2011; Dai et al. 2012). These data suggest that stimulation of cardiac stem cells can prevent the effects of aging on the heart, though more investigations are needed (Anversa et al. 2005; Dai et al. 2012).

3 Mechanisms of Aging in Stem Cells

As stem cells age, they lose their self-renewal and regenerative potential, thus contributing to aging-related tissue dysfunction. Understanding the mechanisms underlying stem cell aging can help us in the prevention and treatment of aging related symptoms, especially in reversible mechanisms. Factors and mechanisms involved in stem cell aging can be cell-intrinsic or cell-extrinsic that are essentially inter-dependents and interacting (de Haan and Lazare 2018). We can also categorize stem cell aging mechanisms into tissue specific stem cell mechanisms of aging and common mechanisms of aging. Common mechanisms of aging include genetic damage to nuclear and mitochondrial DNA, epigenetic aging related changes, cell cycle alterations, ROS and mitochondria dysfunction, protein homeostasis disruption, signaling pathway alterations or extrinsic and systematic changes (López-Otín et al. 2013). Accumulated DNA damage is a common factor in aging and premature aging diseases (Burtner and Kennedy 2010). The DNA replication system is not error-proof and through numerous replications, repair error can cause permanent damage in the form of accumulated mutations. The number of divisions stem cells have made correlates directly with the risk of cancer in the tissue (Tomasetti and Vogelstein 2015). The stem cell's quiescent state makes them more prone to DNA damage once they enter replicative phase because double strand breaks are more likely to be repaired with non-homologous ends joining rather than homologous recombination which is used by replicative cells and is less prone to errors (Kanaar et al. 2008). Repair error is not the only source of DNA damage, chemical agents such as ROS or UV-induced photoproducts and gamma radiation can cause defects on DNA (Han et al. 2014; Robinson et al. 2018). HSCs are one type of the stem cells with the highest rate of self-renewal that makes them more susceptible to DNA damage. Double strand breaks in the DNA tend to increase with age along with accumulated mutation and telomere attrition (Rübe et al. 2011). Accumulated DNA damage results in genomic instability. These DNA damages can cause cell death or senescence. These genotoxins do not just affect the DNA, but also other nucleophiles such as RNA and phospholipid (Miquel et al. 1980; López-Otín et al. 2013). Aging-associated epigenetic changes include alterations in DNA

methylation, histone modification, non-coding RNA, transcription factor binding and nucleosome positioning. One of the most prominent characteristics of epigenetic changes in cellular senescence is DNA methylation. DNA methylation level changes are associated with a cell's biological age and are among the most accurate biological age estimators. DNA-methylation changes happen regularly throughout cell cycle as an adaptive response; however, it can act as an aging accelerator. DNA-5 methyl cytosine (5-mc) is the most common type of DNA methylation that primarily prevents the binding of transcription factor to promoter region and therefore prevents gene expression. DNA methylation induces aging by loss of proteostasis, mitochondrial dysfunction, stem cell exhaustion and immunosenescence (Jiang and Guo 2020). Alterations in DNA-methylation patterns show a generalized hypo methylation along with locus hyper methylation in tumor suppressor genes. Altered histone methylation pattern along with global heterochromatin loss and redistribution increases transcriptional noise, chromosome instability and RNA processing aberrations (Tan et al. 2017). DNA methylation in aging not only disrupts DNA degradation, it also affects protein synthesis. Hyper methylation of the promoter region of r-RNA causes replication senescence in cells (Sanokawa-Akakura et al. 2019). Aging is associated with autophagic activity. The promoter regions of Atg5 and LC3 genes are hyper methylated in aged macrophages and therefore the expression of these autophagy proteins are down regulated. DNA methylation also affects other autophagy-related genes such as FOXO3a or Beclin-1 (Khalil et al. 2016; Jiang and Guo 2020). As another example, it was indicated that the deficiency of excision repair cross complementing-group1 (ERCC1), which is an endonuclease responsible for incision of the damaged strand of DNA in some of the repair pathways, promotes cellular senescence and apoptosis. Cultured ERCC1 depleted human fibroblast shoed that the senescence is p53-dependent and $Ercc1^{-/\Delta}$ mice showed greater levels of tumor necrosis factor-α (TNFα), a senescence-associated secretory phenotype (SASP) factor which induced apoptosis, and it is secreted by senescent cells. Also, the stem cell depletion was found via decreased expression of marker of epithelial, basal and hair follicle stem cells (p63,K14,Lgr6 respectively) that might be due to apoptosis and can be one of the main causes of premature aging in the ERCC1 deficient-mice (Kim et al. 2019). ROS production is a side effect of mitochondria's aerobic metabolism. It has been suggested that ROS production has a central role in cellular aging (Jung et al. 2016). Elevated ROS levels and irradiation in HSCs lead to P38 activation. P38 is a protein kinase that is involved in cell proliferation and differentiation, apoptosis and senescence. In bone marrow suppression disease like Myelodysplastic Syndromes, P38 activation has been reported. P38 inhibition in human umbilical cord blood can improve stem cell expansion and engraftment post transplantation (Zou et al. 2012). Moreover, it has been demonstrated that mammalian target of rapamycin complex1 (mTORC1) increases intestinal epithelial aging by enhancing p38 Mitogen-Activated Protein Kinase (MAPK)s signaling via p53 and p16. These kinases are involved in cell proliferation, differentiation and survival (Wagner and Nebreda 2009). Inhibition of P38 MAPK alleviated villus aging phenotypes (He et al. 2020). The mitochondrial theory of aging suggests mitochondria's ROS production damages mitochondrial DNA and subsequently affects mitochondrial respiration, leading to more ROS production and oxidative damage and creating a vicious cycle (Miquel et al. 1980). Since the introduction of this theory core principles are still approved, however, further studies led to a better understanding of ROS and oxidative damage's role in aging. Studies have suggested that ROS production may not decrease lifespan while higher ROS production leads to longevity. These studies have shown rather a protective role against stress signals for ROS. Findings in rodents and Caenorhabditis elegans (C.elegans)can show that the former observed association between elevated ROS levels and aging were due to the role of ROS in mediating stress responses to age- related damages rather than their primary role in aging (Hekimi et al.

2011; López-Otín et al. 2013). Besides, mitochondrial dysfunction can contribute to aging not only by producing ROS but also by biogenesis impairment and it can cause premature apoptosis. Mutation accumulation and damage instability are other sources of mitochondrial DNA damage; however, we now know that ROS is not the only factor in DNA instability and mutation (Jang and Van Remmen 2009; López-Otín et al. 2013). Furthermore, studies have revealed that nuclear genome instability by itself can induce senescence and raise ROS and oxidative damage in-vivo (Robinson et al. 2018). Telomere shortening has been associated with aging, longevity and aging-related diseases. Telomeres are terminal DNA structures of eukaryotic chromosomes that protect DNA from degradation, recombination and fusion by contributing to the pairing of homologous chromosomes and distinguishing true chromosomes ends from double-stranded-break DNA. With each cell division, telomeres shorten. The amount of this shortening is variable, possibly due to individual variations of telomere length, different methods of telomere length measurements along with cell type and age difference in telomere measurement studies. When telomere reaches a critical length, chromosome fusion increases and contributes to cellular senescence (Ishikawa et al. 2016; Zhu et al. 2019b). Mechanistic target of Rapamycin (m-TOR) is a serine-threonine kinase that integrates cellular and environmental signals to control cell growth, metabolism and proliferation. M-TOR induces anabolic processes while inhibiting autophagy and subsequently cell growth and proliferation. A wide set of stimuli, including hormones, growth factors, oxygen and amino acids can affect m-TOR function. The correlation of aging processes and hallmarks with m-TOR signaling pathway is very complex and not yet fully understood. M-TOR levels during aging can decrease or increase depending on sex or tissue. M-TOR also functions as an immunosuppressant that will make its inhibition effects on aging controversial, because of aging-associated inflammation or inflammaging. Despite all controversies, it is clear that m-TOR inhibition by pharmacological means, or caloric restriction increases lifespan, however, more studies are needed to discover m-TOR are related mechanisms of cellular aging (Mannick et al. 2014; Papadopoli et al. 2019). Environmental and extrinsic aging factors have been studied by exposing young stem cells to senescent milieus. CCL11 levels in blood increase with aging; this cytokine impairs NSC function and neurogenesis via CCR3 receptors. TGF-β also inhibits the satellite cell function and repair (Mendelsohn and Larrick 2011). Other pro-inflammatory factors also increase in blood with aging. Complement component 1q (C1q) induces senescent phenotype in muscle stem cells by accelerating Wnt signaling pathway which is associated with the impaired regeneration ability and enhanced fibrogenic response (Brack et al. 2007; Villeda et al. 2011; Naito et al. 2012). Low-grade chronic inflammation, caused by age-related immunologic changes, has been recently recognized as an important contributing factor in stem cell aging. Basal levels of pro-inflammatory chemokine such as interleukin-6(IL-6), TNF- α and C-reactive protein (CRP) increase in serum with aging, they are mostly known for causing myelopoiesis in HSCs, which is a characteristic of HSC aging. Furthermore, myeloid cells produce more inflammatory cytokines and cause an even further myeloid dominance. Inflammation also induces intracellular ROS production (Kovtonyuk et al. 2016; Hormaechea-Agulla et al. 2020). However, assessing microRNAs(miRNAs) involved in controlling the inflammation revealed that both pro and anti-inflammatory miRNAs were upregulated but the pro inflammatory phenotype could prevail over the anti- inflammatory type (Alicka et al. 2020). To be mentioned, there is growing evidence that the geometry, volume and density of extracellular space regulate neural stem cell proliferation and differentiation (Syková et al. 2002). In addition to common mechanisms of aging in all cells, each line of stem cells goes through different changes in aging. More details on specific mechanisms driving aging in specific tissue stem cells are provided in Table 1. Also,

Table 1 Mechanisms of aging in different types of stem cells. There are several kinds of pathways responsible for senescence of stem cells. Some examples of each category are shown in this table. For instance, some genetic alterations in HSCs, BMMsCs, and CSCs are depicted

	Type of stem cell											
	HSC		NSC		BMMSC		ADSC		MuSC		CSC	
Mechanism	Changes	Outcome	Changes	Outcome	Changes	Outcome	Changes	Outcome	Changes	Outcome	Changes	Outcome
Genetic and epigenetic alterations	NER and NHEJ down regulation	-susceptibility to DNA damage ↑ -self-renewal potential ↓	BRAF activation	-expression of acidic β-galactosidase, PAI-1, $P16^{INK4a}$ ↑ -down regulation of SOX2 (a NSC factor) -proliferation ↓ -senescence induction	-PPAR-γ expression ↑ -RUNX2 expression ↓	Osteogenesis ↓	P53,p16,p21, Casp-3,Casp-9 expression ↑	Cell cycle arrest, apoptosis ↑	-histone markers H3K27me3, H3K9me2 ↑ -histone level ↓	Repressive state of chromatin, loosing overall transcription potential	Telomere length ↓	-proliferation and clonal amplification ↓ -activating p53 -inducing autophagy to elicit the age-associated change in CPC fate
	Hypermethylation of DNMT1	-proliferation -altered differentiation	Exosomal microRNAs (miRNAs) in the cerebrospinal fluid ↓	-acceleration of ageing	FOXP1 expression ↓	$-P16^{INK4a}$ expression ↑ -impaired self renewal capacity and accelerated senescence	SIRT1, hTERT, RB1 expression ↓	Changes in cell cycle control and senescence regulation, telomere length, oxidative stress	High ROS levels -epigenetic derepression of the INK4A locus	-induction of the senescence-associated cell cycle inhibitor p16INK4a	$p16^{Ink4a}$ expression ↑	Blocking cell growth
	Hematopoietic cell-targeted deletion of the STAT3	-very shortened lifespan -dysfunctional mitochondrial function -dysregulated mitochondrial function -reactive oxygen species ↑ -blood phenotype with similarities to premature aging	Expression of *Sox2* ↓	-age-associated chronic inflammation -miR-133b-3p and miR-294, inhibition of TGF-β1-mediated EMT in HK2 cells	-the inhibitory effect of BM-MSC-MVs on TGF-β1-mediated HK2 cell EMT	-fibrosis of Aging renal tissues	PPAR-γ, CEBP-α, adipinectin, FABP4,LPL, GPDH, FASN, ACC1, HSL expression ↓	Adipogenic differentiation potential (slower response to adipogenic inducing factors) ↓	Decline in autophagy	Rewiring of rhythmic gene expression in old satellite cells -disruption in the daily rhythmic oscillation of autophagy genes	Bmi-1 down regulation	Defects in replication and chromatin structure

	Cyp2aaa9 knockdown	-defective HSC development -reduced Wnt/β-catenin activity	BubR1 expression ↓	-induces aneuploidy	microRNA-31a-5p (miR-31a-5p) ↑	-adipogenesis and aging phenotypes ↑ -osteogenesis and stemness ↓	PGC1α, UCP1 expression ↓	Losing brown-like features			Reduction in nuclear phospho-Akt and telomerase activity	Telomere shortening and uncapping in WT cardiac cells
	Activity of small RhoGTPase cdc42 ↑	-loss of polarity			-miR-384-5p ↑	- inhibited osteogenic differentiation -accelerated senescence - inhibited the expression of *Gli2*	ALP, Runx2, BMP2, OPN, OCN expression ↓	Osteogenic potential ↓			NS expression ↓	-lowered proliferation potential and shortened telomere length -up-regulation of p53 and p16
	Absence of P19 (INK4d)	-accelerated cell cycle exit -accumulation of DNA double strand breaks -apoptosis when cells progress to the S/G2-M stages of the cell cycle					CXCR4, CXCR7 expression ↓	Migration ability ↓				
							DNMT1, TET-2, TET-3 expression ↓	Changes(defects) in stem cell homeostasis and DNA methylation				
							-IL-8, TNF-α, IL-1β expression ↑ -miR-203b-3p, miR-21, miR-16-5p upregulation -TGF-β1 expression ↓	Inflammation and pro-inflammatory phenotype				
							GLUT-40, INSR expression ↓	Insulin resistance and reduced glucose homeostasis				

(continued)

Table 1 (continued)

	Type of stem cell											
	HSC		NSC		BMMSC		ADSC		MuSC		CSC	
Mechanism	Changes	Outcome	Changes	Outcome	Changes	Outcome	Changes	Outcome	Changes	Outcome	Changes	Outcome
Cell morphology, cell marker and cell cycle	-cell cycle activity ↓ - cell in G1 phase ↓	Cell division ↓	Transformatiof of α-type NSCs to Ω-type NSCs: α-Type NSCs ↓ Ω-type NSCs ↑	-multibranched morphology -proliferation and division ↓ -senescent phenotype	-sGAG production ↓	Chondrogenesis ↓	Cells in G0/G1 and G2/M phases ↑ Cells in S phase ↓ Nuclear diameter ↑	Decreased proliferation rate	G0 cell cycle arrest	Quiescence – to-senescence switch, impaired self-renewal capacity	-flattened morpholog -Stemness marker LIN28 ↓	Stemness potential ↓ and senescence
			Defective primary cilia	- age-related cognitive decline -brain tumor development	-CFU-F ↓ -$CD45^{low}CD271^{+}$ cells ↓	Number and proliferative capacity ↓			Inhibition of PERK	- prevents myofiber regeneration	NS silencing	- cell flattening -multinucleated cells -decreased S-phase progression
			Chronic inflammation	-induces the conversion of α-cells into Ω-cells (much lower probability of division even under a pro-activation challenge)	Surface marker VCAM-1 and CXCR4 expression ↓	Homing ability ↓					-accumulation of reactive oxygen species in aged CPCs	-modifications of the EphA2 receptor -loss of migratory ability
			CD44 transmembrane receptor ↑	Neural stem cell expansion and differentiation ↓							Loss of CPC self-renewal through increased asymmetric division	-pushes the stem cells toward lineage commitment -stem cell pool exhaustion
Cellular senescence biomarkers			-large lysosomes ↑ - active proteasomes ↓	-differences in their protein homeostasis network.	SA-β-gal activity ↑	Senescent BMMSCs	SA-β-gal activity ↑	Susceptibility to replicative senescence ↑	SA-β-gal activity ↑	Impaired and senescent satellite cells	SA-β-gal and DNA damage marker γH2AX ↑	- senescent CSCs - secretion of SASP factors ↑
Mitochondrial dysfunction	UPR^{mt} ↑	Regenerative capacity of HSCs ↓	Disruption of mitochondrial dynamics and chronic fragmented state	-ROS production ↑ - impaired self-renewal capacity of NSCs -depletion of stem cell pool			ROS production ↑	Induced cellular senescence	-ROS production ↑ -concentrations of ATP and NAD+ ↓	Cellular energy and mitochondrial function ↓	Mitochondrial integrity ↓	ROS production ↑

			Conditional deletion of alpha-ketoglutarate-dehydrogenase				SOD2 expression ↓	Mitochondrial dysfunction and cellular senescence	Deregulation of the UPRmt	Contributes to satellite cell senescence	ATF4, a transcription factor involved in the UPR response, increases the expression of Parkin	-ultimately favors the propagation of ROS from ER to the mitochondria
Protein homeostasis defects	Polycomb-group proteins manipulation (Bmi1)	Repopulating capacity of aged HSCs ↓	Lysosome function ↓	- protein accumulation ↑ - functional decline	-kynurenine accumulation	-bone loss induction -mRNA expression levels of CXCL12 ↓ - expression level of CXCR4 and ACKR3 ↓ -osteogenic gene RUNX2 expression ↓	Proteosome activity ↓	Accumulation of damaged proteins	Atg7 ablation	Autophagy ↓ Mitochondrial activity	Cardiac troponin T levels ↑	Susceptibility to injury ↑
			TGF-β1 ↑	- neurogenesis ↓ -apoptosis ↑ -proliferation of neural stem/ progenitor cells. ↓			sXBP1, PDIA3 expression ↑	Impaired ER protein folding, UPR activation, promoting apoptosis	Reduced amounts and/or function of β1-integrin and fibronectin	Decline in MuSC regeneration and homeostasis with aging		
Signaling pathways alterations	TGF-β signaling ↑	-Prolifeation ↓ - HSC quiescence	Wnt/β-catenin signaling pathway ↑	Cell viability and proliferation ↓	Downregulation of Ca_v 1.2 ↓	Compromised osteogenic capacity	Diminished osteogenic signal	Impedes osteoporosis-ameliorating potential of ADSC			SFRP1 expression ↑	Antagonizing canonical Wnt signaling
Extrinsic and systemic changes	Downregulation of KLF6	-impaired differentiation - increased colony-forming potential - changes in expression	CD8+ T-cell infiltration in NSC niches	-decline in activated NSC or neural progenitors - NSC proliferation impairment					Hypoxia	-upregulation of p53 and p21 -inhibited GG MuSC proliferation -decreased myogenic differentiation	Secretion of SASP factors (e.g., IL-6,IL-8, MMP-3, and PAI1)	Proliferation capacity ↓ and switching to senescence

(continued)

Table 1 (continued)

Mechanism	Type of stem cell											
	HSC		NSC		BMMSC		ADSC		MuSC		CSC	
	Changes	Outcome	Changes	Outcome	Changes	Outcome	Changes	Outcome	Changes	Outcome	Changes	Outcome
			IFN-Ɣ response ↑	- cell cycle dysfunction - NSC proliferation impairment					Increase in muscle stiffness	-YAP/TAZ-mediated pathogenic expression of matricellular proteins by fibroblasts -disrupting MuSC	Reduced cardiac growth reserve	Reduced cardiomyocyte turnover
			CCL 11 and ß2-microglobulin ↑	- differentiation into neurons ↓ - differentiation into astrocytes ↑							Ca^{2+} released from the ER during stress	-increases oxidative phosphorylation -ROS themselves can alter calcium homeostasis by regulating IP_3R and RyR function

MSCs Mesenchymal stem cells, *BMMSCs* Bone marrow-derived MSCs, *HSCs* Hematopoietic stem cells, *NSCs* Neural stem cells, *MuSCs* Skeletal muscle stem cells, *ADSC* Adipose tissue-derived MSCs, *CSCs* Cardiac stem cells, *DNMT1* DNA methyltransferase-1, UPR^{mt} Mitochondrial unfolded protein response, *TGF-β* Transforming growth factor beta, *IFN-Ɣ* Interferon gamma, *sGAG* Sulfated glycosaminoglycan, *FOXP1* Forkhead box P1, *RUNX2* Runt-related transcription factor 2, *CFU-F* Colony-forming unit fibroblast, *VCAM* Vascular cell adhesion molecule-1, *SA-β-gal* Senescence-Associated β- galactosidase, *SASP* Senescence-associated secretory phenotype, *IL* Interleukin, *MMP* Matrix metalloproteinase, *SOX2* Sex determining region Y-box 2, *PAI1* Plasminogen activator inhibitor1, *SFRP1* Secreted frizzled-related protein1, *H2AX* H2A histone family member X, *ROS* Reactive oxygen species, *Atg7* Autophagy-related gene 7, *NAD+* Nicotinamide adenine dinucleotide, *ATP* Adenosine triphosphate, *GLUT* Glucose transporter, *SOD2* Superoxide dismutase 2, *sXBP1* Spliced X-box binding protein 1, *PDIA3* Protein disulfide-isomerase A3, *ER* Endoplasmic reticulum, *UPR* Unfolded protein response, *miR* micro RNA, *TERT* Telomerase reverse transcriptase, *INSR* Insulin receptor, *PPAR* Peroxisome proliferator-activated receptor, *CEBPA* CCAT/ enhancer-binding protein, *FABP4* Fatty Acid-binding protein 4, *LPL* Lipoprotein lipase, *GPDH* Glycerol-3-phisphate dehydrogenase, *FASN* Fatty acid synthase, *ACC1* acetyl-CoA carboxylase, *HSL* Hormone- sensitive lipase, *OCN* Osteocalcin, *OPN* Osteopontin, *ALP* Alkaline phosphatase, *RUNX* Runt-related transcription factor, *TET* ten-eleven translocation, *PGC1α* Pparg coactivator 1 alpha, *SIRT* Sirtuin, *RB* Retinoblastoma protein, *CPC* cardiac progenitor cell, *EphA2* Ephrin A2, *RyR* ryanodine receptor, IP_3R inositol 1,4,5-trisphosphate receptors, *ATF4* Activating transcription factor 4, *UPR* unfolded protein response, *NER* Nucleotide excision repair, *NHEJ* Non-homologous end joining, *DNMT* DNA methyltransferases, *NS* Nucleostemin, *PERK* Protein kinase RNA-like endoplasmic reticulum kinase (Barber and Wilson 1980; Harris and Cotman 1985; Bjerrum and Borregaard 1989; Chambers et al. 2007; Nijnik et al. 2007; Raabe et al. 2011; Rübe et al. 2011; Florian et al. 2012; Liu et al. 2012; Mantel et al. 2012; Pineda et al. 2013; Wang et al. 2013; Beane et al. 2014; Hilpert et al. 2014; Sun et al. 2014; Hariharan et al. 2015; Li et al. 2015; Mohrin et al. 2015; Nguyen and Sussman 2015; Wang et al. 2015; Chen et al. 2016; Khacho et al. 2016; Larrick et al. 2016; Oh et al. 2016; Cesselli et al. 2017; Li et al. 2017; Liu et al. 2017; Nakamura et al. 2017; Zhang et al. 2017; Hwang and Brack 2018; Leeman et al. 2018; Maryanovich et al. 2018; Matsumoto et al. 2018; Sousa-Victor et al. 2018; Adelman et al. 2019; Álvarez-Satta et al. 2019; Cianflone et al. 2019; Dulken et al. 2019; Fei et al. 2019; Ganguly et al. 2019; Li et al. 2019; Lin et al. 2019; Lukjanenko et al. 2019; Martín-Suárez et al. 2019; Alicka et al. 2020; Cianflone et al. 2020; Elmansi et al. 2020; Mejia-Ramirez et al. 2020; Zhu et al. 2020)

some other examples of aging pathways which have rejuvenation strategies are introduced in the next subtitle and Fig. 1.

4 Rejuvenation Strategies

Rejuvenation has been considered a fascinating topic in many cultures and human history, which has become a challenge in the modern world (Ludke et al. 2014).Accordingly, as the aging of stem cells is one of the contributing factors of aging, defining strategies which reverse the aged stem cells, and rejuvenate them is a potential therapeutic approach (Ludke et al. 2014; Ahmed et al. 2017). Some of the proposed mechanisms for treating age-related dysfunctions in stem cells are summarized briefly.

4.1 Genetic Modification and DNA Damage

Accumulation of DNA damage has a critical role in the loss of aged-stem cell function, so increasing the repair pathways can prevent related defects (Wang et al. 2019).An example of this phenomenon is the deletion of Atm (which is a sensor for the DNA damage)in HSC, leading to increased ROS and decreased repopulation capacity. Accordingly, treating $ATM^{-/-}$ mice with antioxidant N-acetyl-L-cysteine (NAC) can restore the HSC function (Ito et al. 2006; Oh et al. 2014; Wang et al. 2019). In addition, alteration in gene expression is another factor of aging in stem cells. Moreover, the regulation of autophagy and protein homeostasis is essential for maintaining quiescence. In this context, overexpression of Atg7, which is involved in autophagy and the formation of autophagosomes, in satellite cells exhibited reduced senescence and restored regenerative capacity (Oh et al. 2014; García-Prat et al. 2016; Zhu et al. 2020). Further, heat shock protein 70 (HSP70), a chaperone protein and a key regulator of cellular defense, has a pivotal role in protecting tissues such as brain and skeletal muscle from aging and improving stem cell survival(Feng et al. 2014; Oh et al. 2014). In addition, it preserved the number of satellite cells in muscles of the transgenic mice with overexpression of HSP70 after immobilization (Miyabara et al. 2012). Besides, overexpression of Satb1, which organizes chromatin, and it was reduced in aged HSCs, can partially enhance the lymphopoietic potential (Satoh et al. 2013; Wahlestedt et al. 2015; Zhu et al. 2020). Moreover, one of the causes of genetic instability is mobile DNA elements called retro transposons. L1 retro transposons are reported to have a role in aging and their activity can be repressed by SIRT6, suggesting a therapeutic approach for slowing down the aging process (Oh et al. 2014; Van Meter et al. 2014). Also, in intestinal stem cells (ISCs) the epithelial homeostasis can be improved by the overexpression of Piwi which can reduce the expression of the related retro transposons and decrease apoptosis and DNA damage (Sousa-Victor et al. 2017; Wang et al. 2019). Further, the expression of a protein kinase named Pim-1 increases due to injury as it has anti-apoptotic and pro-proliferative actions, it can have a protective effect against myocardial infarction.Pim-1 expression in human cardiac progenitor cells (hCPCs) is decreased via aging (Kaur and Cai 2018). Accordingly, overexpression of Pim-1 in engineered hCPCs obtained from patients with heart failure and injected into the hearts of mice revealed enhanced cellular differentiation and engraftment, improved vasculature, and decreased infarct size (Mohsin et al. 2012). Besides, the other markers of aging in the heart are miRNAs as a post transcriptional regulation way .After myocardial infarction, the expression of miR-34 family increases, and inhibiting them improves systolic function, angiogenesis and increased Akt activity (Bernardo et al. 2012; Kaur and Cai 2018). In addition, downregulation of miR-29c in h BMMSCs could suppress p53-p21 and p18-pRB pathways resulting in inhibition of cellular senescence (Shang et al. 2016; Kaur and Cai 2018). It was reported that increased expression of miR-195deteriorates the regenerative ability of old BMMSCs since it targets and deactivates telomerase reverse transcriptase (Tert). Therefore, abrogating miR-195 contributes to Tert

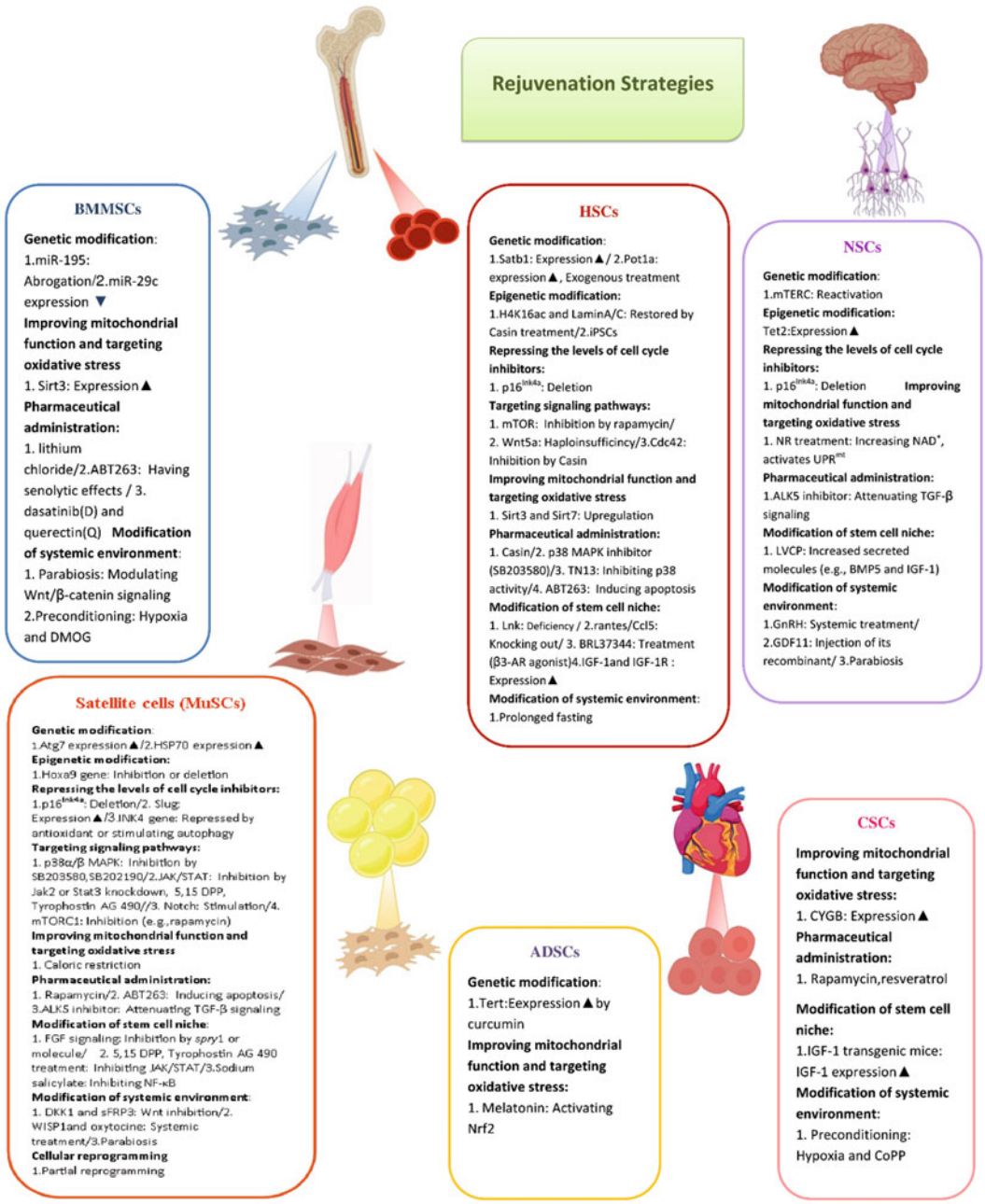

Fig. 1 Rejuvenation strategies for different types of stem cells. There are different kinds of strategies for rejuvenating adult stem cells including genetic and epigenetic modifications, targeting signaling pathways, improving mitochondrial function, and altering the extrinsic factors. For instance, overexpression of Tet2 in old NSCs, prevents regenerative decline and improves neurogenesis. Some examples of these methods are shown in this figure (Torella et al. 2004; Ito et al. 2006; Haider et al. 2008; Chakkalakal et al. 2012; Florian et al. 2012; Miyabara et al. 2012; Elabd et al. 2014; Oh et al. 2014; Price et al. 2014; Sinha et al. 2014; Baht et al. 2015; Chang et al. 2015; Wahlestedt et al. 2015; Yousef et al. 2015; Zhu et al. 2015; García-Prat et al. 2016; Khatiwala and Cai 2016; Ahmed et al. 2017; Neves et al. 2017; Grezella et al. 2018; Grigoryan et al. 2018; Kaur and Cai 2018; Egerman and Glass 2019; Fafián-Labora et al. 2019; Lin et al. 2019; Lukjanenko et al. 2019; Wang et al. 2019; Zhu et al. 2019a; Muñoz-Cánoves et al. 2020; Sarkar et al. 2020; Wu et al. 2020) *MSCs* Mesenchymal stem cells, *BMMSCs* Bone marrow-derived MSCs, *HSCs* Hematopoietic stem cells, *NSCs* Neural stem cells,

reactivation and re-lengthening of the telomere. In order to examine the functional effect, old MSCs with miR-195 inhibitors were transplanted into the infarcted heart of mice and caused improved cardiac function (left ventricular function) and reduced infarction size (Okada et al. 2016). Hence, miRNAs seem to be potential therapeutic targets for preventing aging, but more research is needed to find various related mechanisms in different stem cells. As was previously mentioned, reactivating telomerase or telomere lengthening can somehow reverse the senescent phenotype (Kaur and Cai 2018). Reactivating mouse telomerase RNA component (mTERC) in mice led to reverse neurodegeneration and neural stem cells restored their proliferative and neurogenic capacity (Jaskelioff et al. 2011; Oh et al. 2014; Wang et al. 2019). The shelterin complex is involved in the regulation of telomere length and its protection. Protection of telomeres1 (POT1) is one of the six subunit proteins of this complex and Pot1a in mice prevents DNA damage response (DDR) at telomeres. Pot1a is expressed higher in young HSCs and its overexpression increases the proliferative and self-renewal capacity. In addition, treating aged HSCs with exogenous Pot1a improved their activity and engraftment ability (Hosokawa et al. 2017; Wang et al. 2019). Recently, it was reported that curcumin increased the expression of Tert gene in ADSCs of rats. It improved the lifespan of stem cells and decreased the number of senescent cells and it had some antioxidant properties that can prevent oxidative stress- induced –apoptosis (Pirmoradi et al. 2018). Although upregulation of the telomeric pathway can be used in anti-aging mechanisms, it is also used by cancer cells. Therefore, it should be taken into account that such alterations in telomerase activity may result in malignancies and they should be studied and investigated more precisely (Oh et al. 2014).

4.2 Epigenetic Modification

The different epigenetic regulatory mechanisms such as DNA methylation and histone modification can alter the function of stem cells, their senescence, and their differentiation potential (Wang et al. 2019). For example, the expression of the gene homeobox A9(Hoxa9), a member of Hox genes that regulate stem cells during embryogenesis, is increased as a result of global and locus-specific changes in the epigenetic stress response in aged satellite cells. This overexpression induces senescence signaling pathways like JAK/STAT. Hereupon, deletion or inhibition of the Hoxa9 gene enhanced the myofiber regeneration in the injured muscle of aged mice, increased the regenerative capacity of aged satellite cells (Schwörer et al. 2016; Wang et al. 2019). Also, regarding the DNA demethylation, the level of the enzyme ten eleven translocation methylcytosine dioxygenase2 (Tet2), which catalysis the 5-hydroxymethylcytosine(5hmC) generation and regulates neurogenesis in NSCs and neural progenitor cells and neurogenic niche, is decreased in the aged hippocampus of mice. Hence, overexpression and

Fig. 1 (continued) *MuSCs* Skeletal muscle stem cells, *ADSC* Adipose tissue-derived MSCs, *CSCs* Cardiac stem cells, miR:micro RNA, *DMOG* Dimethyloxalyglycine, *ALK5* Activin receptor-like kinase 5, *TGF-β* Transforming growth factor β, *iPSCs* induced pluripotent stem cells, *POT1* Protection of telomeres1, *mTOR* Mammalian target of rapamycin, *β3-AR* β3-adreno receptor, *IGF1* Insulin-like growth factor 1, *mTERC* Mouse telomerase RNA component, Sirt Srtunin, *Tet2* Ten eleven translocation methylcytosine dioxygenase2, *NR* Nicotinamideriboside, *UPR^{mt}* Unfolded protein response, *LVCP* Lateral ventricle choroid plexus, *BMP5* Bone morphogenic protein5, *GnRH* Gonadotropin releasing hormone, *GDF11* Growth differentiation factor 11, *HSP70* Heat shock protein 70, *FGF* Fibroblast growth factor, *spry1* Sprouty1, *MAPK* Mitogen-activated protein kinase, *JAK/STAT* Janus kinase and signal transducer and activator of transcription, *5, 15 DPP* 5,15-diphenylporphirine, *DKK1* Dickkopf-1, *sFRP3* Soluble frizzled-related protein3, *WISP1* WNT1 inducible signaling pathway protein 1, *Tert* Telomerase reverse transcriptase, *Nrf2*:Nuclear factor erythroid2-related factor2, *CYGB* Cytoglobulin, *CoPP* Cobalt protoporphyrin

restoring Tet2 increased neurogenesis, prevented regenerative decline during aging, improved cognitive processes, and it seemed that it could promote rejuvenation via its associated hydroxymethylation (Schwörer et al. 2016, Wang et al. 2019). Another mechanism is histone modification in which acetylating histone tails results in a more open transcriptional state. Accordingly, the level of histone H4 lysine 16 acetylation (H4K16ac) was reported to be decreased during aging in HSCs. However, treatment with Casin which is a Rho GTPase cell division cycle52(Cdc42) inhibitor and it is mentioned in targeting signaling pathways, can restore the level and spatial distribution of H4K16ac in aged HSCs similar to young ones, accompanied by other rejuvenating functions (Florian et al. 2012; Wang et al. 2019; Zhu et al. 2020). Recently, it was found that other factors are also involved in histone modification of H4K16ac including chromosome 11 architecture which is affected by the nuclear envelope protein LaminA/C. Increasing Cdc42 activity is associated with repression of LaminA/C, deficiency of which causes premature aging in HSCs. So, it was found that Casin treatment and reducing the Cdc42 activity resulted in restoring chromosome 11 architecture and regulating nuclear volume and shape (Grigoryan et al. 2018). Besides, another approach in epigenetic modification is the application of iPSCs which are generated directly from adult somatic cells reprogrammed and reached an embryonic stem cell (ESC)-like state (Wahlestedt et al. 2013, 2015; Ahmed et al. 2017). In another study, iPSCs from fibroblasts of patients with Hutchinson-Gilford progeria syndrome (HGPS), a premature aging disorder, were so similar to the control group and their differentiated cells could recapitulate disease progression (although not entirely). (Chen et al. 2017). Also, iPSCs were used in different studies in order to model aging-associated disorders like Parkinson's and Alzheimer's diseases (Ahmed et al. 2017). Overall, this technique seems beneficial, though the efficacy of it in comparison to other strategies should be evaluated.

4.3 Repressing the Levels of Cell Cycle Inhibitors

There are several cell cycle inhibitors including $p16^{Ink4a}$, p53/p21,and $p19^{Arf}$ that can promote senescence by arresting the cell division.$p16^{Ink4a}$ is involved in stem cell self-renewal in different tissues and its expression increased in aging in stem cells like HSCs, MuSCs, and NSCs (D'Arcangelo et al. 2017; Wang et al. 2019). Aged HSCs from $p16^{Ink4a}$ knockout mice exhibited increased number and function, lower apoptosis frequency, and enhanced repopulation ability while deletion of $p16^{Ink4a}$ in young mice decreased the repopulation ability (Janzen et al. 2006). Furthermore, geriatric satellite cells are not able to maintain a normal quiescent state and their regenerative ability is impaired. Silencing and depression of $p16^{Ink4a}$ in old satellite cells can make them restore their quiescence and self-renewal capacity (Sousa-Victor et al. 2014). Moreover, old $p16^{Ink4a}$ deficient mice improved the decline in proliferation in the subventricular zone (SVZ) (but not in the dentate gyrus and enteric nervous system), neurogenesis in the olfactory bulb, and progenitors activity (Molofsky et al. 2006; Wang et al. 2019).Furthermore, Slug is a member of the zinc-finger transcription factors, which acts as a transcriptional repressor of p16. Recently, it was reported that skeletal muscles of slug deficient mice showed impaired regenerative potential, and its forced expression in cultured satellite cells led to suppressed expression of p16 and restored regenerative potential of satellite cells (Zhu et al. 2019b). Besides, it was found that ROS regulates the gene INK4 and autophagy reduced the p16 expression. Therefore, treatment with antioxidant or stimulating autophagy can repress INK4 locus and restore the regenerative capacity of satellite cells (García-Prat et al. 2016; Wang et al. 2019).

4.4 Targeting Signaling Pathways

Various signaling pathways are involved in stem cell functions and aging, so modulation of these

pathways can enhance the repair system of stem cells and tissues (Wang et al. 2019). For instance, the p38α/β MAPK pathway plays an important role in the proliferation capacity of satellite cells, their exit from quiescence, and their self-renewal. Over-activation of p38α/β MAPK, which is seen in aged satellite cells compared to young cells, impairs self-renewal capacity and disrupts asymmetric divisions, while inhibiting p38α/β MAPK by a specific inhibitor molecule (SB203580, SB202190) enhances the engraftment potential and self-renewal ability of aged satellite cells in culture (Bernet et al. 2014; Cosgrove et al. 2014; Neves et al. 2017; Wang et al. 2019). It has also been demonstrated that increased P38MAPK activity is associated with NSC senescence in-vitro and in-vivo. P38MAPK pharmacological inhibition resulted in NSC rejuvenation, however, the role of p38 in NSC senescence is integrated with many molecular pathways such as Wnt signaling pathway, EGF orinteractions with neurogenic niches (Moreno-Cugnon et al. 2020). It is estimated that P38α is responsible for around 50% of P38MAPK activity. Genetic deletion of P38α seems to reduce age-associated phenotype such as neuron-loss, neuro-inflammation, cognitive decline and NSC exhaustion (Moreno-Cugnon et al. 2019). Further, inhibition of JAK/STAT-a cytokine receptor pathway that increases with age- by knocking down Jak2 or Stat3 or pharmacological intervention improves engraftment in ex-vivo and enhances regenerative capacity after intramuscular delivery of the drugs (Price et al. 2014; Wang et al. 2019; Muñoz-Cánoves et al. 2020). On the contrary, the regeneration of aged muscle impairs as a consequence of the decrease in Delta/Notch signaling, so Notch signaling stimulation restores the proliferative ability of satellite cells (Carlson et al. 2009; Wang et al. 2019). Moreover, the activity of the mTOR pathway is found to be increased in aged HSCs. Accordingly, using its inhibitor, rapamycin, reduced HSC numbers(that were increased due to aging) and improved the reconstitution potential and self-renewal capacity (Wahlestedt et al. 2015; Wang et al. 2019). Also, inhibiting mTORC1 in MuSCs and epithelium stem cells (intestine of the fly and the trachea of the mouse) by using genetic manipulation or rapamycin can prevent the stem cell loss during aging and repeated episodes of regeneration (Haller et al. 2017; Wang et al. 2019). During aging, Wnt signaling shifts from canonical to non-canonical as the expression Wnt5a which is associated with canonical signaling increases and Wnt5a haploinsufficiency in mice reduces aging in HSCs (Florian et al. 2013). On the other hand, the activity of Cdc42 which regulates cell polarity is elevated in aged HSCs and results in loss of polarity and reduced reconstitution capacity (Florian et al. 2012, Neves, Sousa-Victor et al. 2017). It was demonstrated that Wnt5a pathway is associated with Cdc42, so the activity of Cdc42 was reduced in Wnt5a knockdown aged HSCs, which led to increased frequency of polarized cells and rejuvenated the aged HSCs. In addition, using Casin prevented the aging-related differentiation skewing (the myeloid bias) in aged HSCs treated with Wnt5a (Florian et al. 2013; Wahlestedt et al. 2015).

4.5 Improving Mitochondrial Function and Targeting Oxidative Stress

Aging of stem cells is associated with a decline in mitochondrial function, so improving its function is a potential target for rejuvenation (Oh et al. 2014; Kaur and Cai 2018). Sirt3 is responsible for regulating the acetylation of mitochondrial proteins and its expression decreases by aging, so upregulation of sirt3 could enhance the regenerative potential of HSCs (Brown et al. 2013; Zhu et al. 2020). Also, SIRT3 is demonstrated to increase cell survival, decrease cell apoptosis, and enhance the antioxidant activity of hBMMSCs by increasing the expression of manganese superoxide dismutase (MnSOD) catalase (Wang et al. 2014). Studies have shown that cytoglobulin (CYGB) is associated with nitric oxide (NO) metabolism and mitochondrial respiration. It is reported that overexpression of CYGB in human cardiac stem/progenitor cells (hCPCs) enhances cell survival, decreases apoptosis and generation of ROS by upregulating the NFκB/

iNOS pathway and NO production. So, it provides a new target for the effectiveness and efficiency of hCPCs for therapeutic use (Zhang et al. 2016; Kaur and Cai 2018). Due to the importance of oxidative stress and toxic metabolites in the process of aging, different ways of targeting them have gained attention, some of which were mentioned. Further, melatonin was reported to inhibit the senescence of canine ADSCs and improved their survival and efficacy after transplantation through activating nuclear factor erythroid2-related factor2(Nrf2), which regulates many antioxidant and detoxification enzymes and inhibiting NF-κB pathway and endoplasmic reticulum stress(ERS) (Fafián-Labora et al. 2019; Wang et al. 2019). Caloric restriction (CR) is known to have a preventive effect on the onset of aging in different species and short-term CR was able to enhance satellite cell functionality through increased mitochondrial content and oxygen consumption. CR not only influences mitochondrial function, but also has other effects on different pathways and mechanisms in stem cells like modulating DNA damage, autophagy induction, and SIRT and AMPK pathways (Oh et al. 2014; Muñoz-Cánoves et al. 2020). Besides, $NAD^+$is revealed to have a pivotal role in mitochondrial activity and SIRT1 is involved in its related pathway. Moreover, increasing the amount of NAD^+ in NSCs from aged mice by treatment with its precursor nicotinamide riboside (NR) increased proliferation and neurogenesis in the SVZ and dentate gyrus (Oh et al. 2014; Zhang et al. 2016). In addition, NR treatment in MuSCs activates unfolded protein response (UPR^{mt}) and induces prohibition proteins that resulted in an improvement in mitochondrial function, homeostasis, and also preventing senescence of MuSCs in the Mdx mice (model of Duchenne muscular dystrophy) (Zhang et al. 2016). Moreover, it was proposed that NAD precursor supplementation in HSCs could improve the function of mitochondria probably through the SIRT1 pathway (Moon et al. 2018).

4.6 Pharmaceutical Administration

As previously stated, there are different pharmacological drugs used in preventing aging and stem cell senescence. For instance, rapamycin, which inactivates m-TOR, and Resveratrol that activate AMPK and increases mitochondrial function were used on CSCs from explanted decompensated hearts (E-CSCs) of patients and this combination led to restoring the capacity of the stem cells to improve myocardial healing, increased their reparative ability in-vivo in mice and reduced their senescence. Despite the side effects of rapamycin, which need further research, it can be used in order to avoid genetic modifications, which might have detrimental effects (Avolio et al. 2014; Kaur and Cai 2018). Additionally, rapamycin re-established proliferation and enhanced autophagy in old satellite cells (García-Prat et al. 2016). Another change in signaling pathways occurred in a study increasing WNT/β-catenin pathway in MSCs. BMMSCs from cardiovascular patients were isolated and they exhibited reduced WNT/β –catenin signaling along with decreased proliferation and myogenic differentiation. Treating aged MSCs with lithium chloride caused recovered potential for myogenic differentiation as it increases the β-catenin bioavailability (Brunt et al. 2012; Kaur and Cai 2018). Moreover, Casin was said to be an inhibitor of Cdc42, so it can rejuvenate aged HSCs. Recently, it was demonstrated that aged HSCs treated with Casin reconstituted an immune system in T- and B- cell deficient $RAG^{-/-}$ mice similar to young animals (Leins et al. 2018; Zhu et al. 2020). In addition to rejuvenating the activity of p38 MAPK inhibitor (SB203580) in satellite cells, it can reduce the ROS levels in HSCs, so it restored the repopulation capacity and maintained quiescence of HSCs (Ito et al. 2006; Zhu et al. 2020). Similarly, a cell penetrating peptide-conjugating peptide derived from Thioredoxin-interacting protein (TXNIP) named TN13, can inhibit p38 activity and it was able to rejuvenate aged HSCs in-vitro and in-vivo as well

as increasing their homing ability (Jung et al. 2016; Zhu et al. 2020). Besides, another strategy in pharmacological intervention is clearing the senescent cells. Accumulation of senescent cells and expression of the complex SASP change the microenvironment and causes the development of aging and its related diseases (Wang et al. 2019; Zhu et al. 2020). Therefore, suppressing SASP and using senolytic drugs can be a potential therapeutic approach (Kaur and Cai 2018; Wang et al. 2019). There are different senolytics which were used in animal models and they have been found successful in reducing senescent cells and alleviating many senescence-related phenotypes (Shetty et al. 2018). Accordingly, some of these drugs are reported to be useful in the depletion of aged stem cells (Wang et al. 2019). For instance, ABT263 which induces apoptosis by inhibiting the anti-apoptotic proteins BCL-2 and BCL-xl, was able to kill aged HSCs and MuSCs after oral administration in mice. This agent acts selectively on senescent stem cells and attenuated total-body irradiation (TBI) associated premature aging of HSCs and rejuvenated them and also resulted in improved function of remaining HSCs and MuSCs. However, it has some toxic side effects, which should be taken into account in further human and animal studies (Chang et al. 2015; Wang et al. 2019; Zhu et al. 2020). Lately, ABT263 was revealed to have senolytic effects on aged human BMMSCs and decreased SA-β-gal staining but did not have a positive effect on telomere length or epigenetic rejuvenation (Grezella et al. 2018). In addition, dasatinib(D) and querectin(Q) were administered orally in old mice reduced the number of aged BMMSCs by interfering with apoptosis pathways along with improvement in cardiac function, exercise capacity, and carotid vascular reactivity.Additionally, in-vitro studies exhibited the death of senescent human preadipocytes by D (Zhu et al. 2015; Kaur and Cai 2018). Moreover, rapamycin has senolytic effects by reducing the pro inflammatory phenotype of senescent cells. This concept seems promising and various drugs have shown therapeutic effects by eliminating senescent cells(not specifically stem cells) but further research is required (Grezella et al. 2018; Wang et al. 2019).

4.7 Extrinsic Strategies

4.7.1 Modification of Stem Cell Niche

Alteration of the stem cell environment, including vasculature, innervating neural fibers, ECM, immune, somatic and stromal cells have a pivotal role in the rejuvenation of the aged stem cells (Wang et al. 2019). There are different examples of intervening approaches for rejuvenation via manipulating stem cell niches, some of which are shown in Fig. 1. For instance, the lack of Lnk, an adaptor protein and negative regulator of HSC homeostasis, which reduces signals of extrinsic cytokines, enhanced the self-renewal capacity and prevented the aging- related lineage bias in mice (Bersenev et al. 2012; Wahlestedt et al. 2015). Similarly, knocking out the inflammatory cytokine Rantes/C-C motif chemokine ligand 5(Ccl5) resulted in decreased m TOR activity, increased lymphoid lineages and engraftment potential (Ergen et al. 2012; Wang et al. 2019). Besides, the sympathetic nervous system (SNS) regulates the function of HSCs and using a sympathomimetic (β3-adreno receptor (AR) agonist, BRL37344) as a supplementation was able to rejuvenate senescent HSCs in mice (Maryanovich et al. 2018; Zhu et al. 2020). Additionally, chronic treatment of progeroid mice with this agonist decreased premature myelopoiesis and restored the megakaryocyte-HSC interaction (promoting quiescence of HSCs) (Ho et al. 2019; Zhu et al. 2020). Since vessel formation in the niche is reduced during aging, overexpression of Notch signaling by inhibiting a p16-dependent pathway can restore the endothelial cells and prolong their lifespan. It can be a new target for treating aging- associated vascular disorders (Kaur and Cai 2018, Wang et al. 2019). A circulating factor involved in cardiac disease and its recovery is IGF-1 that is responsible for stem cell mobilization and angiogenesis and its overexpression in transgenic BMMSCs caused reduced cell apoptosis and improved engraftment

in the infracted hearts of rats (Haider et al. 2008; Kaur and Cai 2018). Similarly, IGF-1 transgenic mice indicated increased nuclear phospho-AKt and telomerase activity and the overexpression of IGF-1 preserved CSCs and prevented their senescence (Torella et al. 2004). Regarding MuSCs aging, FGF-2 is known to be upregulated in the niche of aged satellites causing their depletion and diminished regenerative potential. So, inhibiting FGF signaling by overexpression of Sprouty1 (spry1) or inhibitory molecule can prevent this depletion (Chakkalakal et al. 2012; Wang et al. 2019). Moreover, it was found that the lateral ventricle choroid plexus (LVCP), a component of ventricular-subventricular zone (V-SVZ) and producer of CSF, is an important niche for NSCs during aging and affects their behavior. It has a role in colony formation and proliferation and its secreted factors like BMP5 and IGF-1 can improve the function of the aged NSCs, elucidating new insights into therapeutic approaches toward NSCs behavior (Silva-Vargas et al. 2016; Wang et al. 2019). Another factor in the stem cell niche is the oxygen level. It is reported that expansion of hCSCs in hypoxia led to improved engraftment in infarcted hearts in mice and culturing MSCs under hypoxic conditions is beneficial as it is similar to the physiologic condition of the bone marrow (Hu et al. 2008; Kaur and Cai 2018).

4.7.2 Modification of Systemic Environment

Not only is the stem cell niche altered during aging, but also circulatory signals, including hormones, secreted molecules from different tissues and immune cells are changed and change the function of stem cells (Oh et al. 2014; Wang et al. 2019). In contrast to Wnt signaling in the myogenic differentiation potential of MSCs, it can induce aging in MSCs by activating ROS and in satellite cells, canonical Wnt signaling can antagonize Notch signaling (Zhang et al. 2013a; Kaur and Cai 2018; Wang et al. 2019). So, injecting and incubating Wnt inhibitors, Dickkopf-1(DKK1), and soluble frizzled-related protein (sFRP3) caused reduced fibrosis and enhanced muscle regeneration in aged mice (Brack et al. 2007; Wang et al. 2019). On the other hand, fibro-adipogenicprogenitors (FAPs), a regulatory cell in MuSC niche, is impaired during aging and the secretion of the FAP-derived matricellular protein WNT1 inducible signaling pathway protein 1(WISP1) is reduced. Recently, systemic treatment with WISP1 was able to rescue skeletal muscle regeneration and restore the myogenic potential and function of MuSCs in old mice (Lukjanenko et al. 2019; Muñoz-Cánoves et al. 2020). Additionally, IkB kinase-b (IKKb)/Nf-κB have a role in the hypothalamus- developed –aging and they inhibit gonadotropin releasing hormone (GnRH). Therefore, in order to improve neurogenesis and cognitive ability, systemic treatment with GnRH, as a potential strategy, could decelerate aging (Zhang et al. 2013b; Wang et al. 2019). Intriguingly, oxytocin was reported to be beneficial and required for regeneration and homeostasis of muscle tissue. The plasma levels of this hormone and its receptor on MuSCs decline with age that reduces muscle regeneration. Accordingly, systemic administration of oxytocin can enhance the proliferation and myogenesis of MuSCs via activating MAPK/ERK pathway and it might be a potentially safe treatment for maintaining aged muscles and prevent aging (Elabd et al. 2014; Wang et al. 2019). Moreover, there are controversial data about the effects of circulating protein growth differentiation factor 11(GDF11), one of the members of the TGF-β family, the systemic level of which declines during aging. Injecting recombinant GDF11 in aged mice could increase the number of satellite cells and their regenerative capacity in addition to enhancing autophagy and improving exercise endurance (Sinha et al. 2014; Wang et al. 2019). However, several studies have reported a negative effect of GDF11 on satellite cells and muscle regeneration including inhibited differentiation of MuSCs and their reduced population, increased fibrosis, and wasting of skeletal muscle. Also, there are similar opposite findings of the GDF11effect on bone and cardiac muscle, so more studies are needed to find out the exact role of this factor on aging and its associated complications (Egerman and Glass 2019, Wang et al. 2019). On the other hand, data on the

injection of recombinant GDF11 have shown beneficial effects on neurogenesis and angiogenesis in old mice as a rejuvenating factor (Rochette and Malka 2019; Wang et al. 2019). An interesting approach in the category of systemic modulation is parabiosis which is about the anatomical joining of two animals. Heterochronic parabiosis is an experiment in which an old tissue is exposed to a young systemic environment (for instance a young and old mice are surgically linked) through creating a conjoined and shared blood circulation (Oh et al. 2014; Sinha et al. 2014; Ahmed et al. 2017; Kaur and Cai 2018). The signals from a young circulation influence the aging tissue and its function, for example, heterochronic parabioses in mice could upregulate Notch signaling and restore satellite cell activation (Sinha et al. 2014; Kaur and Cai 2018). Conboy et al. reported that parabiotic pairing between young and old mice increases the proliferation and regeneration rate in old mice both in-vitro and in-vivo. They established parabioisis between young and old mice (heterochronic parabiosis), two young mice and tow old ones. The muscle of their hindlimbs was injured and it was revealed that in contrast to old isochronic prabiosis, heterochronic parabiosis enhanced the regeneration of muscle in aged mice which was mainly doe to the activation resident progenitor cells of the old mice. Also, in heterochronic parabiosis the Notch ligand Delta was upregulated in aged satellite cells. Similarly, the aged satellite cells cultured in the presence of young mouse serum shoed up regulated Delta expression and enhanced proliferation (Conboy et al. 2005). Further, adult stem cells in the CNS produce new myelin sheaths as a regenerative process, which declines with aging. Heterochronic parabiosis in mice indicated that the remyelination was enhanced in old mice in addition to restored neurogenesis of aged stem cells (Katsimpardi et al. 2014; Oh et al. 2014; Ahmed et al. 2017). It should be mentioned that despite the promising outcomes, due to ambiguous and indefinite known effects of some factors like GDF11, this method is not completely safe and beneficial (Ahmed et al. 2017, Kaur and Cai 2018). Besides, prolonged fasting can protect cells from toxins and toxicity in mice and humans. In addition, it was demonstrated that it can rejuvenate HSCs since it reduced the circulating level of IGF-1 and activity of protein kinase A (PKA). Hence, it could promote self-renewal and increase lymphoid-biased lineage (Cheng et al. 2014; Wahlestedt et al. 2015; Zhu et al. 2020). Preconditioning is another way to improve survival and proliferation of stem cells after transplantation (especially for myocardial repair) and to provide a basis to prevent their senescence (Khatiwala and Cai 2016). It can be induced by pharmacological intervention, using anti-aging compounds and their associated molecular modification and in-vitro hypoxic shock. As it was mentioned before, hypoxia is beneficial for cell therapy to treat infracted heart. Preconditioning MSCs with hypoxia increased the expression of anti-apoptotic (Bcl-2) and pro-survival (NF-κB) proteins and enhanced angiogenesis after transplantation in the infarcted heart (Hu et al. 2008; Khatiwala and Cai 2016; Kaur and Cai 2018). Moreover, preconditioning of hCSCs with cobalt protoporphyrin (CoPP), which induces heme oxygenase-1(HO-1) and causes cellular protection, increased their survival, proliferation, and resistance to stress-induced apoptosis (Kaur and Cai 2018). Further, prolyl1 hydroxylase enzymes activate the pro-apoptotic pathways. Hence, using pharmacological agent Dimethyloxalyglycine (DMOG) for preconditioning BMMSCs resulted in increased cell survival, reduced infarct size, and enhanced cardiac function after transplantation into infarcted hearts of rats, mimicking the effects of hypoxia (Liu et al. 2014; Khatiwala and Cai 2016). A brief summary of these strategies and some other examples of each rejuvenation mechanism are exhibited in Fig. 1.

4.8 Cellular Reprogramming

Somatic cell nuclear transfer (SCNT) revealed that somatic cells could reacquire pluripotency and thus reverse all differentiation and age-associated characteristics. This method required somatic nucleus implantation in an enucleated oocyte. The molecular factors

initiating and affecting the differentiation process in this method are not fully understood (Han et al. 2015). The recent introduction of reprogramming factors facilitated the process of dedifferentiation and led to deriving iPSC from senescent somatic cells. It was proposed that reprogramming aged somatic or stem cells into iPSCs could reset the aging- related memory (Oh et al. 2014). For instance, hematopoietic stem and progenitor cells (HSPCs) were reprogrammed into iPSCs and then redifferentiated to HSCs via blastocyst complementation. The iPCS-derived HSCs exhibited young functional characteristics and did not resemble aged HSCs (Wahlestedt et al. 2013; Wahlestedt et al. 2015). IPSCs can be re-differentiated into young somatic cells that have lost the senescent phenotype (Takahashi and Yamanaka 2006; Singh and Newman 2018). The first, introduced reprogramming factors were Yamanaka factors or OSKM (Oct4, Sox2,Klf4, c-Myc), since then, more specific reprogramming factors have been introduced like NKx3 or Gata4, Mef2c, Tbx5 (GMT) for cardiomyocytes (Mai et al. 2018; Tani et al. 2018). In these methods, both age reprogramming and developmental reprogramming occur concomitantly. Partial reprogramming is a method in which the induction stops when cells are rejuvenated while still possessing their specialized phenotype and before the expression of pluripotency associated features (point of no return). In partial reprogramming epigenetic rejuvenation is the first step, preceding dedifferentiation. The most accurate indicator of rejuvenation in partial reprogramming is called the epigenetic clock that is based on the level of cytosine methylation at 353 CpG sites. With the transient cyclic induction of reprogramming factors in partial reprogramming senescence-associated gene expressions, DNA damage markers and ROS levels decrease without dedifferentiation, teratoma formation or iPCS detection (Mendelsohn et al. 2017). Since the introduction of partial reprogramming method, it has been implanted to rejuvenate senescent cells in-vivo, identifying stages of differentiation and directly reprogramming somatic cells into other differentiated cell types, bypassing the pluripotent stage (Tani et al. 2018; Hsu et al. 2019; Jasper 2020). Although, this method has not been as abundantly experimented in the field of senescent stem cell rejuvenation. Somatic aging stem cells can also be targeted for partial reprogramming and rejuvenation. The implantation of partial reprogramming on mouse-derived skeletal muscle stem cells improved the cells' myogenic potential and ability to differentiate into myotubes and improved single cell's ability to form colonies. Transient reprogramming also reduced the time of the first division and mitochondrial mass without any effect on the myogenic marker (MyoD) and the myogenic fate of stem cells. Rejuvenated stem cells showed higher potency to regenerate new tissue post-transplantation compared to aged stem cells and their regenerative potency was equal to young stem cells. Rejuvenated stem cells also produced a higher number of myofibers compared to aged stem cells and had higher cross-sectional area, even compared to the young untreated stem cells. No neoplastic lesions or teratomas were discovered on a three-month follow-up biopsy. On a second artificial injury, 60 days post-transplantation, rejuvenated stem cells still produced better results than both aged and young stem cells. These results revealed a new method to rejuvenate senescent stem cells and enhance their regeneration potential (Sarkar et al. 2020) . Cyclic in-vivo induction of reprogramming factors can induce expansion in muscle stem cells of old mice and improve muscle regeneration after injury (Ocampo et al. 2016).

5 Stem Cells' Potential to Model and Study Aging

Whether we consider aging an independent disease per se or a risk factor for most comorbidities, in all approaches to aging, the root cause is biological and molecular mechanisms that lead to cellular dysfunction and consequently to the associated diseases. Thus modeling aging in stem cells can significantly contribute to our understanding of cellular mechanisms of aging (Stern 2012; Costantino et al. 2016; Soenen et al. 2016; Fulop et al. 2019; Hou et al. 2019; Sarbacher and

Halper 2019). Different animal model organisms have been used in order to discover cellular aging mechanisms. Studies on rodent models have led to great discoveries on aging mechanism and possible preventive interventions. The effects of calorie restriction on aging and different mutations associated with longevity like the mutation in DNA helicase Wrn., that causes premature aging, have been studied on mice (Lombard et al. 2000). The relatively short lifespan of mice (compared to human), convenience of environmental control and low cost maintenance are the advantages of rodent models, however, since different strains show different prevalence of pathologies and lifespan, choosing the most suitable rodent models for each study is still a major obstacle. Furthermore, since these studies are usually conducted on a single strain, the results may not be fully representative of human population (Mitchell et al. 2015). Non-human primates have also been used to model and study aging. Their similarity to humans on many levels is an undeniable advantage; however, long lifespan, complicated and expensive maintenance, lower reproduction rate and potential of aggressive behavior are all drawbacks in using non-human primates as models to study aging (Mitchell et al. 2015). Fish, including several fish and killifish, have also been used to study aging. Short lifespan and high reproductive rate along with low cost maintenance are the advantages of using fish as animal models. Zebra fish has an exceptional regenerative potential that makes it an ideal model for regenerative experiments (Gut et al. 2017). There are also invertebrate models of aging which are mainly used in preclinical development of anti-aging drugs. C.elegans are mostly used in studying neurodegenerative and metabolic aspects of aging, since genes and pathways involved in neurological and metabolic disorders in humans have orthologues in C.elegans. The effects of anti-aging drugs such as Metformin and Rapamycin have been studied and on invertebrate animal models such as Drosophila Melanogaster (Folch et al. 2018). While studying aging in animal models provides invaluable information on systematic and cellular pathways of aging, there are still questions about molecular factors that induce aging, the relationship between systematic, cellular and nuclear aging, the rule of stem cell aging in the aging phenomenon as well as tissue specific ageing mechanisms. Answers to these questions are much easier and reliable found in using stem cells to model and study aging. Each tissue has its own reserve of stem cells, which are aging along with the rest of the cells. Since stem cell function is vital in tissue regeneration and longevity potential, discovering stem cell-specific pathways of aging can lead to preventing stem cell aging and therefore increase tissue's longevity. The study of tissue-specific aging is now possible, since the reprogramming method gives us a tool to produce tissues and model aging faster, easier and more precise than animal modeling. By using iPSCs we can simulate aging in any tissue and thus study molecular factors and biological pathways along with treatment strategies (Brunauer et al. 2017). Both neurodegenerative disorders and cognitive reserve decline are most prevalently associated with aging. With the iPSCs derived from human donors, studies so far have been able to discover numerous key features of neurodegenerative disorders, although, since iPSCs-derived neural cells have lost disease and aging-specific characteristics and need stressors to induce disease specific phenotype (Mertens et al. 2015). Direct reprogramming is a new method that facilitates studying aging on stem cell models with direct conversion of one specific cell type to another without going through the pluripotent state and thus; conserving aging characteristics such as accumulation of age-related DNA mutations, protein damage, mitochondrial nuclear and protein damage and epigenetic markers of aging (Chow and Herrup 2015; Mertens et al. 2015; Huh et al. 2016). Different methods have been experienced to induce direct reprogramming such as specific transcription factors and micro RNAs to convert the cells into any specific cell type (Yoo et al. 2011; Li et al. 2015; Mertens et al. 2018).The biggest obstacle in using stem cell direct reprogramming to model aging associated diseases is the high cost of this method (Mertens et al. 2018). Direct reprogramming of

fibroblast cells into cardiac cells have led to the discovery of many small molecules for direct cardiac reprogramming, including microRNAs that induce cardiac reprogramming both in-vivo and in-vitro. In this approach microRNA1, 133, 208 and 199 can induce direct reprogramming to cardiomyocytes in fibroblasts in-vitro. The significance of this method is that administration of microRNAs into ischemic cardiomyocytes leads to direct cardiac reprogramming in-vivo in mouse models (Jayawardena et al. 2012). Neurons derived from human fibroblasts with Alzheimer's show increased levels of Aβ 42, 38 and Tau levels, indicating that direct reprogramming can be used to generate mutation-specific neural cells for different diseases and use them to study disease mechanisms and drug discovery (Mitchell et al. 2015). Direct reprogramming has also been used to convert astrocytes and polydendrocytes into functional neurons in-vivo for brain injury with the help of retrovirus encoding Neuro-D1, a pro-neural transcription factor (Guo et al. 2014). These results elucidate the potential stem cell modeling has for modeling age-related diseases in-vitro to study their underlying mechanisms, explore reprogramming techniques and drug discovery. Afterwards, these information, techniques, specific transcription factor and small molecules can be used to treat diseases in-vivo (Traxler et al. 2019).

6 Conclusion

Aging is characterized by functional and regenerative potential decline tissue that could be partly due to stem cell aging. This phenomenon can be the underlying cause of many aging-related and degenerative disorders such as Alzheimer's. Aging and stem cell dysfunction are codependent, aging impairs stem cell's function and impaired stem cell function contributes to aging. Therefore, using stem cells to model and study aging-related disorders like Alzheimer's, can pave the way for discovering etiologies and develop more effective therapies for each etiology. Stem cells can also be used to generate cerebral organoids that mimic the brain structure and study all aspects of Alzheimer's (Machairaki 2020). Concerning cell-extrinsic and intrinsic pathways of aging, many molecular factors and genes have been identified. In addition to different animal models of aging, stem cells can be used as a helpful model per se, for instance using induced pluripotent stem cells (iPSCs) for neurodegenerative disorders due to their replicative and therapeutic capacities and stimulating aging to study the mechanisms, have led to understanding many important features of aging and the related diseases (Oh et al. 2014; Ahmed et al. 2017; Brunauer et al. 2017; Mertens et al. 2018; Ziff and Patani 2019). Using stem cells to model aging will solve many obstacles in animal models of aging, especially the debate of whether or not these results can be generalized to humans; however, the biggest problem in developing stem cell models of aging is the cost. Besides, a new method called direct reprogramming is introduced for modeling aging in stem cells, in which cells can be converted into another cell type without passing through the pluripotent stage while the age-related characteristics are maintained (Yoo et al. 2011; Li et al. 2015; Mertens et al. 2015). Although these techniques have some disadvantages, they are considered beneficial for answering the questions of aging mechanisms and can contribute to developing rejuvenation strategies both in-vivo and in-vitro (Mertens et al. 2018). Moreover, there are several proposed approaches in order to reverse the aging process or to treat the aging of stem cells including alteration of the tissue environment, telomere lengthening, different pharmacological interventions, cellular reprogramming and an emerging of age reprogramming (partial reprogramming) which is rejuvenating the old cells without de-differentiation (Ahmed et al. 2017; Shetty et al. 2018; Singh and Newman 2018; Wang et al. 2019). In this review, several kinds of pathophysiology and mechanisms of stem cell aging, their potential for studying aging and the anti-aging approaches along with the difference between aging of stem cells in different tissues and some examples of targeting aging n novel therapies were discussed. Based on

the studies reviewed in this paper, it seems that using stem cells in modeling aging and aging-related disease and developing mutation-based therapies holds a lot of promise for discovering new treatment strategies. Using partial reprogramming technique as a novel strategy to rejuvenate stem cells can pave the way for new aging therapies.

Conflict of Interest There is no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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Role of Tumor Specific *niche* in Colon Cancer Progression and Emerging Therapies by Targeting Tumor Microenvironment

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Abstract

Colorectal cancer is the third most common form of cancer worldwide leading to escalating mortality rates and mainly includes hereditary, sporadic and colitis-associated cancer development. The escalated mortality rates is due to the limited treatment options as this form of cancer is usually not easy to diagnose in its early stages and are highly invasive leading to rapid metastasis of the malignant cells to the neighbouring tissue. In order to combat this limitation several chemotherapeutic regimens are now being combined with targeted therapies after the knowledge acquired on the inevitable effects of the tumor microenvironment on the colon cancer growth and progress. The colon tumor *niche* mainly consists of a large mass of tumor cells along with various immune cells, inflammatory cells, tumor macrophages and fibroblasts that infiltrate the tumor as it is a site of predominant inflammation. Among cells of the microenvironment, mesenchymal stem cells (MSCs) exhibiting ability to evolve into cancer associated fibroblasts (CAFs) have recently generated a major interest in the field. The physiological state of the tumor microenvironment is closely connected to discrete steps of tumorigenesis. The colon cancer cells elicit various factors with their direct interaction with MSCs or via paracrine fashion, which modulate these cells to promote cancer instead of performing their innate function of abating cancer progression. This review intends to highlight the necessity to exploit the cellular landscape of tumor microenvironment of colon cancer and a detailed understanding of the interactions between tumor epithelial cells and their stromal/inflammatory elements will aid in future perspectives for designing therapeutic regimens targeting tumor microenvironment to improve the clinical outcome of colon cancer.

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Keywords
Colon cancer · Cytokines · Mesenchymal stem cells · Metastasis · microRNAs · Targeted therapy · Tumor *niche*

Abbreviations

CAFs	Cancer Associated, Fibroblasts
CC	Colon Cancer
CRC	Colo Rectal Carcinoma
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
EMT	Epithelial to Mesenchymal Transition
FAP	Fibroblast Activating Protein
HGF	Hepatocyte Growth Factor
MDSC	Myeloid Derived Suppressor Cells
MIF	Migration Inhibitory Factor.
MMP	Matrix Metalo Proteinases
MSCs	Mesenchymal Stem Cells
RANTES	Regulated on activation, Normal T-cell Expressed and Secreted
SCF	Stem Cell Factor
TAMs	Tumour Associated Macrophages
TME	Tumour Microenvironment
TNF	Tumour Necrosis Factor
VEGF	Vascular Endothelial Growth Factor

1 Introduction

Colon cancer (CC) initiates as a benign or a metastatic mass of cells at any given point in the lining of the colon or rectum which mainly includes hereditary, sporadic and colitis-associated cancer development. Adenocarcinomas accounting for 95% of the colorectal tumors usually begin as benign polyps also known as adenomas that have the potential to develop into cancerous outgrowths as a result of accumulated mutations having a profound impact on the signalling pathways involved in maintenance of cell proliferation and tumor suppression (Colangelo et al. 2017). It is the third most common form of cancer worldwide leading to escalating mortality rates. The aberrated cell signalling pathways controlling the cell proliferation, stem cell maintenance and tumor suppression ultimately lead to the invasive forms of CC that metastasizes to distant organs wherein the tumor cells detach from the primary tumors, intravasate the vascular networks and reach the neighbouring tissues leading to rapid deaths. The late diagnosis of this form of cancer often leads to reduced treatment options as the tumor cells tend to metastasize at faster rates. The predominant cause for the progression of CC is the development of therapy refractory metastatic disease. Complex surgical resections of the tumors mostly do not completely cure the patient of cancer as there usually occurs a relapse of CC in a more invasive metastatic form as most of the tumor cells tend to metastasize even prior to the surgical removal of the tumor. Prolonged chemotherapy has also been observed to have evident effects on the patients however it did not prove to be of great use in the metastatic disease and rather lead to predominant drug resistance and tumor development (Tauriello and Batlle 2016). Several upcoming treatment options involve chemotherapy in combination with targeted therapy that targets not only the tumor cells but also its unique tumor *niche* that can lead to a more effective treatment of the metastatic forms of CC. Multifold studies have now highlighted that the escalation and recurrence of tumors are governed by the genetic alterations in the tumor microenvironment factors as well apart from the aberrated cancer cells (Quail and Joyce 2013). Tumor microenvironment (TME) plays a dominant role in influencing the tumor cells for their development and progression of CC. A coordinated network of interface cell types mainly include pericytes, adipocytes, immune cells, endothelial cells, fibroblasts, and mesenchymal stem cells through the extracellular matrix and soluble factors such as cytokines, chemokines, growth factors and various metabolites enhancing the tumorigenesis. Tumor assorted macrophage and myeloid suppressive cells characterize tumor-promoting immune cells enclave with their derived cytokines such as interleukin (IL-6, IL-1β, IL-23) and tumor necrosis factor (TNF- α). VEGF stimulates vascular endothelial cells for

the formation of blood capillaries and is enhanced in the perivascular environment of tumor blood vessels exhibiting an abnormal physiology, due to deviant pericytes and permeable endothelial layers leads to the development of hypoxia and metastasis. It has been reported that the existence of MSCs can enhance the metastatic ability of various cancer including colon cancer.

Cancer-associated fibroblasts (CAFs) in the TME from the produce an excess of growth factors, cytokines, chemokines, structural protein components, and metabolites which are resultant of diverse precursors like mesenchymal stem cells (MSC) or endothelial cells so as to further endorse oncogenesis. An abnormal tumor-associated MSC can gain distinct functions such as secretion of TGF-ß to be a factor of epithelial-to-mesenchymal transition (EMT) and immune-suppressive activities following their interactions with tumor cells. Further, deviant MSC are known to secrete CXCL12 (SDF-1) and also liberate VEGF to sustain tumor cell growth and survival. MSC have the potential to either impede or elevate tumor progression within the TME by their distinctive kind of cellular interactions. MSCs can be recognized as RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) which are expelled by the CC-chemokines ligand 5 (CCL-5) and further act together with suitable cytokine receptors like CCR1, CCR3 and CCR5. The adaptation of tumor growth and propagation is influenced by the extracellular matrix (ECM).

The precise contribution of the TME in promoting cancer in highlighted when only a confined success rate in met when the cancer cells are only targeted. The tumor microenvironment has therefore been considered to play an additional critical role in cancer progression as targeting only the cancer cells has led limited success rates in most of the CC patients (Ribatti et al. 2006). This review will briefly summarize the current understanding of the role of various cellular compartments of the tumor microenvironment in which CCs proliferate and metastasize and thereafter focus the discussion on the therapeutic aspect of targeting the major paracrine factors that govern the fate of colon cancer progression and metastasis.

2 Tumor Microenvironment (TME)

The escalating significance towards ecological therapy for various forms of cancer has given rise to extensive analysis of the cellular and non-cellular compartments of tumors which are commonly referred to as the tumor microenvironment (Wang et al. 2017). These solid tumors are predominantly a mass of several cell types providing a tumor specific *niche* having a profound effect on the immune status, neovascularization and establishment of an ECM that promotes the interactions among the various cells present in the TME. Tumor *niche* has been found to play a crucial role in the tumor growth and metastasis that was well explained by the "seed and soil" hypothesis put forward by Stephen Paget years back in 1889 (Ribatti et al. 2006). According to him the cancer cells that are represented by the "seed" can be maintained well only in its specific environment that was represented by the "soil" thereby, highlighting the importance of the TME in the maintenance and progression of cancer (O'Malley et al. 2016). The tumor microenvironment or *niche* is mainly composed of the tumor associated cells, extracellular matrix, inflammatory cytokines and matrix associated molecules that play a crucial role in the tumor cells maintenance, progression and metastasis. The tumor associated cells mainly include, immune cells such as T-cells, tumor associated macrophages, monocytes, neutrophils, natural killer cells, dendritic cells along with endothelial cells, platelets, cancer-associated fibroblasts and mesenchymal stromal cells (Peddareddigari et al. 2010). Due to excessive infiltration of the TME by the immune cells a chronic inflammatory response is elicited that plays a critical role in the neoplastic process. The essential role of inflammation in cancer progression was first described by Rudolf Virchow in 1863 (David 1988). The neoplastic process has several signaling pathways similar to those observed during an inflammatory response such as apoptosis, angiogenesis and escalated proliferation rates. The inflammatory process involved in normal tissue repair is resolved post tissue regeneration and pathogen elimination

and homeostasis is maintained. However, chronic inflammation of the tissues in some cases has been observed to give rise to malignant transformation of the normal stromal cells and inhibit the restoration of normal homeostasis thereby leading to carcinogenesis (Landskron et al. 2014). The TME enables the malignant cells to evade clearance by the immune system and promote vascularization of the tumor by the release of various cytokines and matrix molecules which ablate the efficacy of several therapeutic regimens (O'Malley et al. 2016). The chronic inflammation and release of chemokines by the tumor cells as well as the stromal cells of TME are the key factors governing the tumor niche (Melzer et al. 2016). The complex mechanisms of tumor development and progression can therefore be analysed clearly by observing the interactions between the several factors and cells residing in the tumor specific *niche* (Melzer et al. 2016) (Fig. 1).

2.1 Structural Scaffold for the Tumor Stroma

Fibrous protein in the tumor stroma includes elastin, collagen, fibronectin, proteoglycans like chondroitin sulphate and hyaluronic acid which are subsisted in the ECM and is also mainly enriched with collagen. Soluble factors such as growth factors, angiogenic factors, cytokines and chemokines are abundantly present in tumor stroma. Collagen involves the deposition and tight organization of matrix proteins like elastin, laminins and also the altering enzymes such as lysyl oxidase, leads to a more inflexible phenotype of complete tumor. Fibronectin is associated in tumor invasion and metastasis (Chen and Huang 2014).

2.2 Cellular Components of the TME in Colon Cancer

Tumor niche consists of a class of non-malignant cell types such as immune cells, endothelial cells, fibroblast and MSC which expand the tumor-assisted functions along with the soluble factors and ECM components interconnects with the cancer cells to disseminate tumorigenesis. The tumor microenvironment in CC is comprised of multiple components such as vasculature, tumor-infiltrating cells, extracellular matrix (ECM), and other matrix-associated molecules. A detailed characterisation of the cellular landscape and their role in cancer progression has been explained in the below sections.

2.2.1 Immune Cells

Immune cells play a prominent role as gatekeepers and protectors of the body from various infections and cancers. Cancer immune-surveillance involves the role of both the innate and the adaptive immune system to eradicate the tumor from the body. However, the immune-surveillance tends to be affected due to the interactions of the immune cells with other cellular components, cytokines and ECM molecules present in the TME. Due to varied response of the immune system during cancer progression the concept of immune-editing has been introduced which includes three stages namely, elimination, equilibrium and escape. These cancer cells are either eliminated by the immune cells else they acquire adaptations making them resistant to the immune clearance and thereby maintain their pool resulting in cancer progression (Colangelo et al. 2017). The first stage mainly involves the complete clearance of the tumor cells effectively by the immune cells and various other critical signaling molecules involved in the immune clearance process. Post tumor clearance these immune cells and active molecules result in immune-editing wherein the immune cells in the equilibrium state are unable to completely eliminate the tumor from the body as the cancer cells have adapted to the immune-surveillance by establishing phenotypic alterations such as epithelial to mesenchymal transition. However, at this stage even though the immune cells are unable to completely eliminate the tumor cells they are capable to limit the tumor growth to a certain extent. Therefore, the immune microenvironment have been found to play a role in the retaining a pool of dominant cancer cells that can repopulate the tumor and maintain tumor growth.

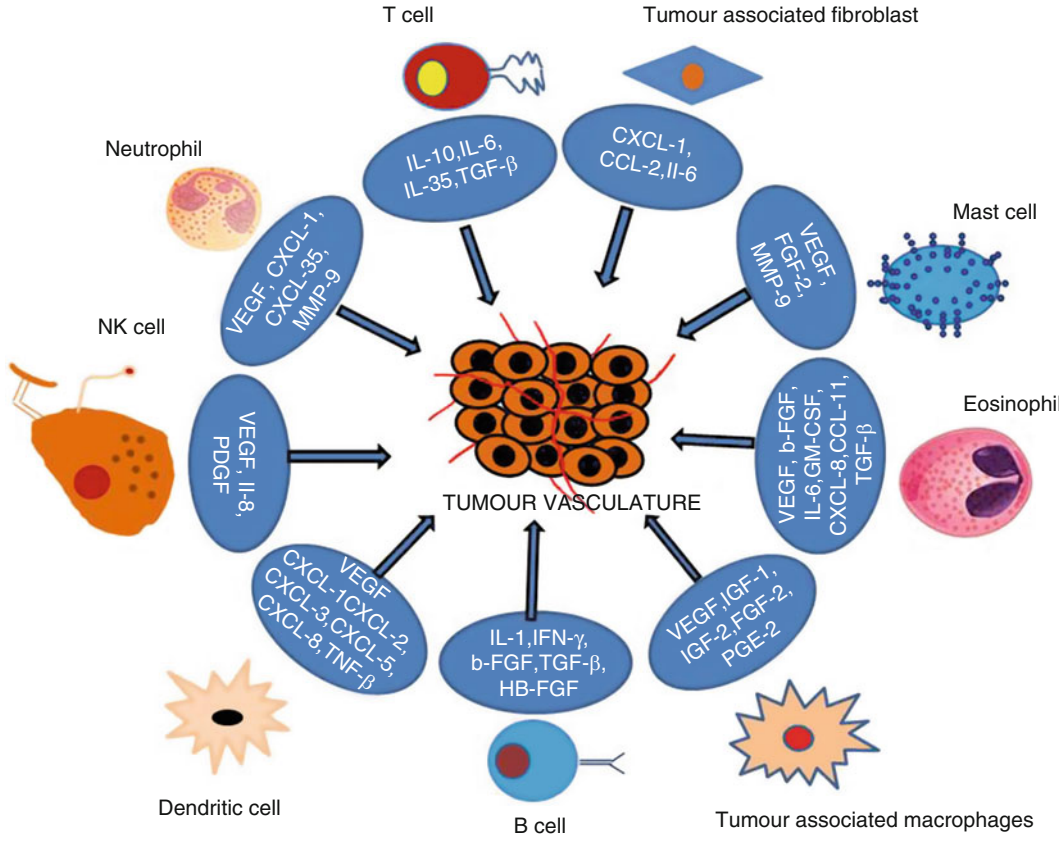

Fig. 1 The cytokines/paracrine factors secreted by cellular components of tumor microenvironment

In the escape stage, the immune cells assist the tumor cells in effectively escaping from the body's immune clearance and promote cancer invasion by releasing anti-cancer proteins and cytokines. Therefore targeting these interactions of the cellular components of the tumor *niche* will be proving to be a more potent cancer therapeutic regimen (David 1988).

2.2.2 Tumor Associated Macrophages (TAM's)

Macrophage plasticity has been observed to massively impact the tumor growth and invasion potential. TAM's play a critical role in the regulation of the tumor niche in colon cancer. Macrophages have always been recognized as critical effector cells for the clearance of the tumors. However, recent studies have highlighted the role of macrophages as TAM's in assisting the cancer cells in altering their phenotypes as well as promoting tumor invasion. TAM's have been observed to crowd at the tumor edges and induce apoptosis of the cancer cells however; those populated near the tumor invasion sites have been found to possess reduced potential in tumor ablation (Wang et al. 2017). TAM's are main contributors in promoting tumor angiogenesis (Balkwill et al. 2012). This dual role of the macrophages can be explained by their ability to alter their phenotypes and their potent plasticity that can be attained by altering their polarization according to the current conditions. Macrophages are mainly classified into two distinct types based on their polarization states such as M1 and M2 macrophages. The M1 macrophages are the normally activated macrophages that release type I pro-inflammatory cytokines and elicit an anti-tumorigenic response. However, the M2

macrophages that are alternatively activated release type II cytokines that elicit an anti-inflammatory response and thereby promote tumor growth and invasion. These TAM's have been observed to enhance tumor invasiveness by intricate paracrine interactions between the cancer and the macrophages mainly including factors such as tumor-derived CSF-1 and macrophage-derived EGF. Targeting this switch of macrophage toward tumor enhancing phenotype and characteristics can be useful to block tumor increase (Quail and Joyce 2013).

2.2.3 Mesenchymal Stem Cells (MSCs)

MSCs are distinctive multipotent cells which possess distinct characteristics such as self-renewal, anchorage- dependent and their differentiation potential that exist in perivascular environment of the human tissues and organs such as bone marrow, adipose tissue and fetal tissues comprising of placenta, amniotic membranes and umbilical cord. MSCs have an excellent potential to drift to the site of inflammation and to sustain tissue repair, angiogenesis, stem cell homeostasis, immune inflection and thus elevate tumor progression by liberating various endocrine and paracrine signals. Exosomes are extracellular vesicles which aid in the intercellular contact with the neighbouring tumor cells and mesenchymal stroma cells. Movement of MSCs to the site of inflammation or tumor microenvironment is facilitated by the exosomes to secrete chemokines such as CXCL1, CCL2, IL-6 and growth factors like TGF-β1,VEGF and PDGF-BB (Rhee et al. 2015). Thus, the focal point of the extracellular vesicles to the tumor cells helps in their migration and paves the way to tumor malignancy. Exosomes arising from MSCs receive their stimulation from matrix metalloproteinase-2 (MMP-2) and ecto-5′-nucleotidase activity along with miRNA facilitates cancer progression.

Migration of MSC's to the TME and Their Potential Interface

The MSCs can be attracted and recruited into damaged tissues by releasing enormous amounts of inflammatory cytokines and chemokines. The malignant tissues are well known for the release of several chemokines during the tumor development. The chemokines such as CCL2, CCL15, CCL20, CCL25, CXCL1 and CXCL8 have been identified to greatly impact the homing of the MSC's to the tumor *niche*. The possible paracrine interactions between MSCs and CC cells in the TME during CC progression have been summarized in Fig. 2. These active factors can be secreted to implement profound effects on the cells via paracrine signaling or by releasing exosomes that are extracellular vesicles containing substantial amounts of the secreted factors. The recruitment of MSC's therefore activates these stem cells and results in spontaneous release of a set of other inflammatory cytokines that promote tumor development and maintenance of tumor *niche* (Ali et al. 2015a). These MSC's upon activation in the TME transform into malignant cells commonly known as the cancer associated fibroblasts (CAFs). These CAF's promote tumor progression and metastasis. These CAF's are mainly characterized by expression of α-smooth muscle actin, PDGF receptor-β. These malignant fibroblast have also been observed to induce epithelial to mesenchymal transitions in the cancer cells by the expression of proteins such as MMP, TWIST, WnT5A and TGF-β type-I receptors that in turn give rise to aggressive forms of tumors (Peddareddigari et al. 2010). Based on recent research for the underlying mechanism of interaction between MSCs and CC cells in tumor niche, some significant factors has emerged which upon targeting might lead to suppression of cancer progression (Fig. 3).

2.2.4 Cancer Associated Fibroblasts (CAF's)

The stroma is the most essential component that maintains the tissue architecture that provides a base for the residing cells. Fibroblasts are the most common cells present in almost all the tissues of the body. These spindle-shaped cells predominantly found the colon in the normal colonic mucosa are situated adjacent to the colon mucosal epithelium. They are known to play a role in the synthesis, deposition and turnover of the basement membrane components. The

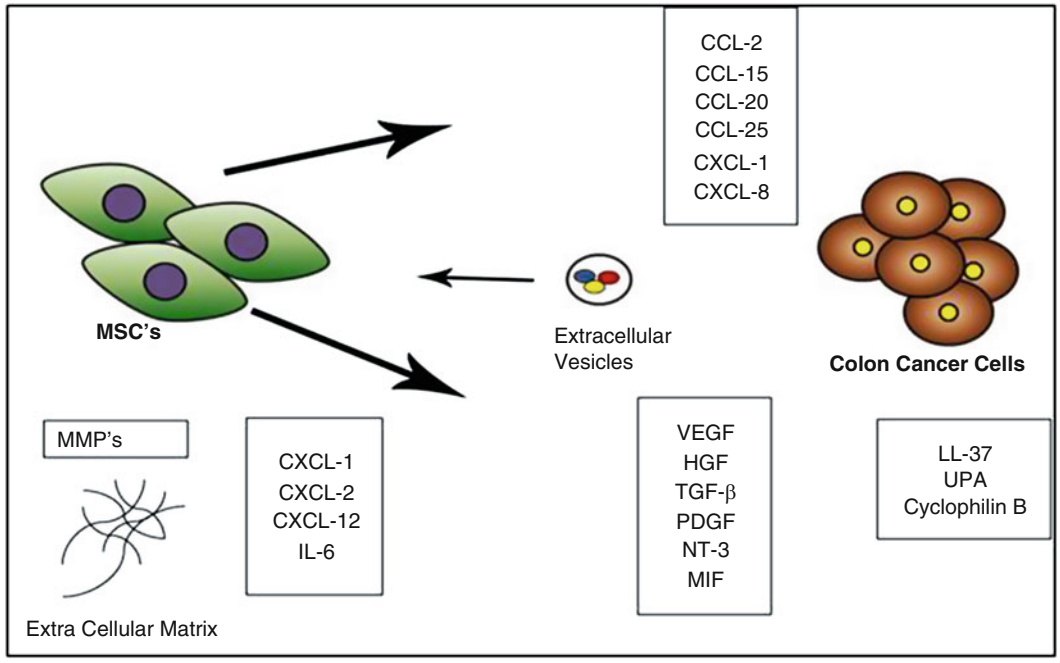

Fig. 2 Paracrine interactions between the colon cancer cells and the MSCs in the tumor microenvironment

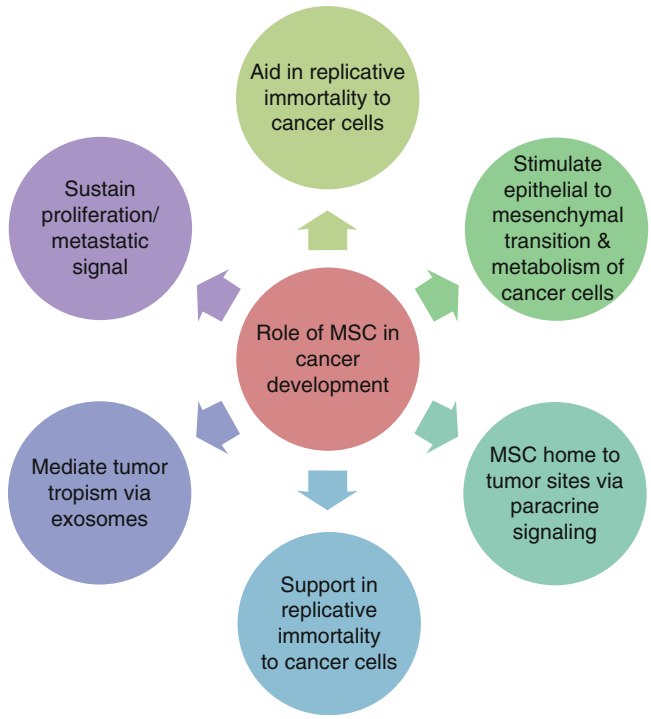

Fig. 3 Factors with therapeutic potential for targeting the cancer progression emerging from the interaction of MSCs and CC cells in TME

crosstalk between these fibroblasts and the epithelium via paracrine signaling results in the maintenance of tissue integrity (Wang et al. 2017). Therefore these fibroblasts are also found to infiltrate the tumors and play a major role in maintaining the tumor growth and TME. These fibroblasts get activated in the TME and give rise to cancer associated fibroblasts (CAF). The CAF's have been found to promote cancer cell proliferation and metastatic potential. These cells are mainly characterized by the altered expression of α-SMA, vimentin, fibroblast activating protein (FAP), platelet-derived growth factor receptors α and β, fibroblast specific protein-1 (FSP1), the chondroitin sulphate proteoglycan neuron-glial antigen-2 (NG2), and prolyl-4-hydroxylase (Colangelo et al. 2017). These cells secrete considerable amounts of stromal-cell derived factor 1 that specifically binds to the CXCR-4 expressed by the tumor cells thereby promoting tumor invasion and angiogenesis (Chen and Huang 2014). The CAF's formed from the modified fibroblasts present in the tumor *niche* have been observed to secret multiple factors which influences the invasive potential of the tumor, its angiogenesis as well as tumor progression. These factors mainly include miRNAs 200b and 155, angiogenesis factor VEGF, chemokines such as SDF1 also known as CXCL-12, hepatocyte growth factor (HGF), epidermal growth factor (EGF), macrophage migration inhibitory factor (MIF), and various other interleukins as well (Wang et al. 2017; Bahrami et al. 2018). TGF-β released by the fibroblasts has been known to induce epithelial to mesenchymal transition of the cancer cells thereby contributing to the immune-suppressive tumor *niche*. Studies have indicated that elimination of these FAP- positive cells resulted in spontaneous tumor necrosis mediated by the factors IFN-γ and TNF-α (Quail and Joyce 2013).

2.2.5 Vascular Endothelial Cells and Pericytes

Tumor cells are well known to promote the infiltration of endothelial cells into the tumor compartment in order to promote neovascularization. Several soluble factors such as VEGF, FGF, PDGF and various other chemokines play important roles in promoting tumor growth by promoting tumor vasculature. When a blood vessel ruptures in the tumor vicinity the angiogenic signals are released from the malignant cells stimulating formation of new blood vessels. The formation of these new aberrant and uneven blood vessels results in the leakiness which is one of the major reasons of excessive metastasis of the malignant cells (Chen and Huang 2014). Pericytes are cells of mesenchymal origin that form a support system for blood vessel formation and function. These mainly provide structural support and therefore depletion in their pool results in the formation of excessive aberrant and leaky blood vessels which results in a higher rate of tumor metastasis (Wang et al. 2017).

2.2.6 Myeloid Derived Suppressor Cells (MDSC)

MDSC or immature myeloid precursor cells are of immense interest as they are implicated in cancer immune suppression as well as immune suppressive states of colon cancer. These cells are broadly characterized as CD11b+, CD33+ and LIN−, HLA-DR−, cells in humans. MDSC consists of a heterogeneous set of precursor myeloid cells capable of suppressing the adaptive immune response. MDSCs proliferation is influenced by pro-myelopoetic factors produced by colon cancer cells which include M-CSF, IL-6, PGs, GM-CSF, VEGF and stem cell factor (SCF). As the MDSC proliferate, they induce a negative feedback inhibition on the T cell and NK cells activity thereby arresting the immune response against the colon cancer progression (Wang et al. 2017).

3 Non-cellular Components of the TME of Colon Cancer

The colon cancer TME is not only composed of several cell populations but also includes multifold active molecules and ECM components that equally contribute in maintaining the malignant microenvironment as well as promote the tumor growth and metastasis.

3.1 miRNA in Colon TME

These small non-coding RNA's have been known to play a crucial role in several physiological processes and also have conspicuous role in various pathological conditions including cancers. Interestingly this microRNA's have been observed to not only regulate the cancer cells but also have an impact on the tumor stroma which in turn gives rise to drastic progression of the cancer (Jiang et al. 2017). The alteration in the function and the stages of different tumors has been found to be associated with altered miRNA expression. The miRNA expression in colorectal cancer has been well elucidated. The miRNA's with escalated expression levels during CRC include miR-21, miR17, miR-155, miR-146, miR-221, miR-31, miR-25 and miR-196 (Strubberg and Madison 2017). Around 35 microRNAs have been observed to be either escalated or abated in CC. A detailed chart of the highly up and down regulated miRNAs, their target genes and possible signaling pathways involved are depicted in Table 1 (Inoue et al. 2012; Sansom et al. 2010; Ibrahim et al. 2011; Wang et al. 2015; Zhang et al. 2010, 2018; Xu et al. 2014; Schee et al. 2010; Mohammadi et al. 2016). These variations in the miRNA expression are found to be result of repeated chromosomal aberrations. Both normal as well as malignant cells have been found to release miRNA's into the peripheral blood that are protected from RNase degradation by protective vesicles known as exosomes. The crosstalk between the other cells of the tumor *niche* and the cancer cells via autocrine or paracrine signalling is crucial for the progression of cancer. These interactions are brought about by these exosomal miRNA that are released by the cancer cells in order to promote the neighbouring cells to maintain the required tumor *niche* for enhanced cancer progression and metastasis. The released exosomal miRNA's have also been observed to provide a premetastatic environment that enhances permeability and vascularization of the tumor that in turn promotes the metastasis of the malignant cells to the neighbouring organs (Strubberg and Madison 2017). This exosomal microRNA's can be easily detected in the plasma levels thereby coming forward as an effective target for diagnosis as well as CC treatment. The microRNA's miR-17-3p and miR-92a have been observed to be drastically abated in CC patient's post-surgery thereby indicating their excessive expression during cancer progression. MiRNA-29a has been observed to help differentiate between advanced stages of CC from other bowel disorders. Colon cancer progression can be effectively regulated by directly targeting these miRNA expressions by directly blocking/inhibiting these miRNA's or by downregulating the expression of these miRNA's by antisense oligonucleotides or promoter methylation (Inoue et al. 2012). Multifold tumor suppressor miRNA's have also contributed widely to the regulation of CC progression. These beneficial miRNA's are mostly transferred by viral vectors that release these RNA's at the tumor site to achieve maximal tumor abatement. Several tumor suppressive miRNA's are now being delivered not only to the malignant cells but also the tumor stromal cells present in the immediate tumor microenvironment. The delivery of these miRNA's have been found to modulate the tumor associated dendritic cells from an immunosuppressive to an immune-stimulatory state resulting in enhanced reduction of the tumor as they are subjected to the body's immune system.

3.2 miRNA from TAMs in Colon Cancer TME

Recent studies have highlighted the role of the immune cells to also play a vital role in the initiation, progression and metastasis of various cancers. The macrophages in the tumor *niche* have been observed to be polarized from an anti-tumorigenic (M1) to a pro-tumorigenic (M2) state by alterations in their metabolic pathways. This has been analysed due to the predominant dysregulation of the miRNA expression profiles of the normal macrophages after the onset of cancer. Therefore, several

Table 1 A detailed table of the highly up and down regulated miRNAs, their target genes and possible signaling pathways involved in colon cancer

S. No	Name	Target genes	Role in colon cancer	Signalling pathway	Regulation up/down	References
1	miR- 107	*HIF-1β, VEGF*	Inhibit angiogenesis	HIF-1β Hypoxic signalling	↓	Inoue et al. (2012)
2	miR 802	*AG-II,HATIR*	Mediate angiogenesis	Angiotensin II, ERK pathway	↑	Sansom et al. (2010)
3	miR 145	*HIF-1,VEGF*	Inhibit angiogenesis	IRS I	↓	Ibrahim et al. (2011)
4	miR 194	*THBS1,*	Promote angiogenesis	MAP4K4/c-Jun/MDM2	↑	Wang et al. (2015)
5	miR 27a	*GT-094*	Promote angiogenesis	PINK1 regulation in mitophagy	↓	Zhang et al. (2010)
6	miR-7	*XRCC2, PAX6, YY1*	Tumor suppressor	CDK6 signalling	↑	Xu et al. (2014)
7	miR-21	*PTEN, PDCD4,SPRY2*	Tumor progression	Akt signalling pathway	↑	Schee et al. (2010)
8	miR-22	*HIF-1α*	Tumor suppressor	HIF-1α Hypoxic signalling	↓	
9	miR-31	*SATB2*	Oncogenesis	Autophagy	↑	
10	miR-92a	*KLF4*	Proliferation and migration	MMP2 and E-Cadherin	↑	
11	miR-101	*COX2*	Tumor suppressor	Oxidative stress pathway	↓	
12	miR-155	*MSH2, MSH6 AND MLH1*	Tumor supressor	Cell cycle	↓	
13	let-7c	*AURORA KINASE A AND B*	Tumor supressor	Cell cycle arrest	↓	
14	miR-126	*CXCR4*	Tumor suppressor	NF -κB pathway	↓	
15	miR-130b	*INTEGRIN B1*	Tumor suppressor	Not reported	↑	
16	miR-132	*ZEB2*	Tumor suppressor	EMT	↓	
17	miR-139-3p	*INSULIN-LIKE GROWTH FACTOR -1 RECEPTOR*	Tumor suppressor	Not reported	↓	
18	miR-15	*BCL*	Tumor suppressor	NF-κB	↓	
19	miR-16	*KRAS*	Tumor suppressor	p53	↓	
20	miR- 81b	*PDCD4*	Tumor progression	STAT3	↑	
21	miR-183	*ABCA1*	Tumor progression	Cholesterol pathway	↑	
22	miR-195	*BCOX1, BCL-2, AND CARMA3*	Suppress tumor cell proliferation and metastasis	RAS	↓	
23	miR-196a	*HOXD8*	Tumor suppressor	PI3K-AKT-mTor	↑	
24	miR-196b	*GATA6*	Tumor suppressor	Wnt/β-catenin	↑	
25	miR-203	*CDK6*	Tumor progression	Not reported	↑	

(continued)

Table 1 (continued)

S. No	Name	Target genes	Role in colon cancer	Signalling pathway	Regulation up/down	References
26	miR-215	*THYMIDYLATE SYNTHASE (TS)*	Tumor suppressor	Not reported	↑	
27	miR-224	*KRAS,*	Tumor suppressor	ERK, Akt pathway	↑	
28	miR-340	*PKM*	Tumor suppressor	Glycolysis	↓	
29	miR-35p	*NOTCH-1*	Tumor suppressor	Notch signalling	↓	
30	miR-320e	*SOX4*	Tumor suppressor	Wnt/β-catenin	↑	
31	miR-17-5p	*E2F1*	Tumor progession	Not reported	↑	
32	miR-106a	*RUNX3, PRB*	Tumor suppressor	pRB pathway	↓	
33	miR-144	*NRF2*	Tumor suppressor	mTor	↓	
34	miR-145	*KRAS, C-MYC*	Tumor suppressor	Not reported	↓	
35	miR-494	*APC*	Tumor progression	Wnt/β-catenin signalling	↑	Zhang et al. (2018)

approaches have been made to target these dysregulated miRNA profiles to revere the polarization of the TAM's to normal macrophages and bring about effective anti-tumorigenic effects. The miR-511-3p that encodes for the macrophage-mannose receptor has been found to possess altered expression in the $MRC1^{+}$ TAM's by escalating the expression of this miRNA in the TAM's resulted in enhanced suppression of the pro-tumoral genes thereby inhibiting tumor growth and alterations in the blood supply to the tumors. The escalated release of cytokines from this TAM's resulted in aberrated cancer progression. Excessive expression of miRNA-155 has been found to attenuate the release of multifold cytokines such as IL-6, IL-10 and TNF-alpha that play a vital role in promoting malignancies. The escalated expression of miRNA-155 resulted in evident reversal of the TAM's to normal anti-tumorigenic macrophages (Sansom et al. 2010).

3.3 Extracellular Matrix (ECM)

Almost all the mammalian cells remain in close contact with their surrounding stromal matrix whose components and composition varies in different organs, cell types and medical conditions such as cancer. The extra cellular matrix is mainly composed of 5 essential components namely collagens, laminins, fibronectins, proteoglycans and hyaluronans. These ECM components bind to the integrins present on the cell surface and provide the required mechanical and physicochemical support to the cells. The ECM is also critically involved in the cell migration and bears several growth factors that play a major role in maintaining the cell pool. The stromal cells and epithelial cells in contact with each other coordinate and produce the basement membrane (BM) that has a significant role in cancer progression. The loss of the BM components or their inappropriate synthesis alters the cell physiology thereby being a key player in the onset of the disease. For instance the loss of the ECM component laminin-5 is major contributor in colon cancer progression. An altered ECM composition has been found to give rise to a switch in the role of the integrin α6β4 that is also known as the tumor antigen. Enhanced expression of this integrin has been found to give rise to escalated aggressiveness and poor prognosis of the developing tumors. In colon carcinoma this integrin promotes cell migration on laminins-1 thereby contributing to the metastatic potential of the malignant cells. The metabolism of the ECM

molecules is an essential aspect involved during tissue homeostasis and the response of cells towards acute and chronic stresses. Several types of proteinases participate in ECM turnover, however matrix metalloproteinases (MMPs) are known to be the major ECM degrading enzymes along with urokinase-type plasminogen activator (Rhee et al. 2015). The ECM degradation being a common phenomenon during cancer development, these enzymes involve the interactions of the stromal and the cancer cells upon ECM loss thereby inducing a profound effect on the stromal cells via direct contact or paracrine signaling. These interactions therefore promote the metastasis of the malignant cells (Ali et al. 2015a).

3.4 Matrix Metalloproteinases

The MMP's are a group of zinc-dependant endopeptidases that play a crucial role in the hematogenous metastasis process by carrying out the degradation of the ECM. Many matrix metalloproteinases have been linked to the colon cancer progression and metastasis (Ibrahim et al. 2011) MMP-9 is a common 92-kDa proenzyme which has the potential to degrade type IV collagen post activation. MMP-2 another important matrix metalloproteinase plays a crucial role in CC progression and invasion. Elevated expression of MMP-2 has been observed in bladder cancer, lung carcinoma, colorectal cancer gastric and breast cancer. Studies working on the silencing of MMP-2 in colon cancer cell lines have indicated significant ablation of the cell proliferation, invasion and colony forming capacity (Wang et al. 2015; Zhang et al. 2010). Particularly, MMP-7 overexpression has been observed to occur during the intiation of the carcinogenic cascade post transformation of the normal mucosa into carcinogenic adenomas. Data obtained from *in vitro* studies have shown that MMP-7 expression is related to the invasiveness of the primary tumor cells (Xu et al. 2014) in colon cancer and overexpression of MMP-7 has also been reported in pancreatic (Schee et al. 2010) and breast (Zhang et al. 2018) carcinomas, respectively. Recent studies reported that MMP-7 appears to be an early event in the adenoma-to-carcinoma pathway (Mohammadi et al. 2016), hence multiple experiments are being carried out by utilizing MMP inhibitors for the prevention or treatment of colorectal cancer.

3.5 Cytokines

The colon cancer onset is mainly observed in patients initially afflicted by inflammatory bowel disease that occurs due to a noticeable loss of balance between the pro-inflammatory and regulatory cytokines. The malignant cells possess the ability to modulate the stroma and the immediate microenvironment by secreting multifold factors that recruit inflammatory cells and activate stroma cells in the TME. These cells in turn release several factors such as cytokines, chemokines, growth factors and proteases that promote tumor progression and metastasis. Along with these factors these cells also release several relative oxygen and nitrogen species which induce genetic alterations thereby promoting cancer. Chemokines can be directly secreted into to the extracellular space or via vesicles. Multifold cytokines such as TNFα, IL-8, IL-6 and VEGF, have been noticed to be elevated in colorectal carcinoma (CRC) patients (Kuninty et al. 2016). Escalated levels of TNF-α and IL-6 have been observed to result in the activation of NF-κB and STAT3 pathways. Interleukin 1β a potent pro-inflammatory cytokine secreted in voluminous amounts by the macrophages in the tumor *niche* is known to promote the secretion of other inflammatory cytokines and chemokines such as TNFα, IL-6, IL8, IL-17, COX-2 and PGE2 that promote the colon cancer cell growth and progression. The colon cancer cells stimulate the macrophages present in the TME to secrete multifold amounts of IL-6 that is known to activate STAT3 in the malignant cells. This cytokine plays a crucial role in maintaining the growth of the tumor cells as the inhibition of this chemokine resulted in evident decrease in the growth potential of the malignant cells. This cytokine is also secreted in substantial quantities by various other stimulated cells residing in the tumor vicinity mainly including fibroblasts, monocytes,

endothelial cells and immune cells namely the B and T-lymphocytes. Another critical cytokine in the TME is IL-10, this cytokine has predominant inhibitory effects on the pro-inflammatory cytokines such as IL-1β, TNFα and IL-6and is mainly secreted by Th2 cells, B cells, tumor cells and macrophages. Abated levels of this cytokine result in drastic progression of the tumors. Several chemokines released by the cancer cells such as CC15, CCL-2, CCL-20, CXCL-8 that promote the attraction of the MSC's towards them. The tropism of the MSC's is also affected by the release of certain active factors such as VEGF, HGF and TGFβ. The MSC's upon activation by the colon cancer cells secrete detectable amounts of CXCL-1, CXCL-2, CXCL-12 and IL-6 (Zucker and Vacirca 2004). These pro-inflammatory cytokines and its associated pathways have emerged as potential targets for effective and alternative cancer therapy.

4 Future Perspectives and Therapy

4.1 Effective Molecular Interaction of MSCs with Tumor Cells

Cellular and molecular mechanisms involved when mesenchymal stem cells are co-cultured along with distinctive cancer cell types such as colon cancer, lung cancer, breast cancer, ovarian cancer cell lines exemplify an intercellular contact with tumor microenvironment evolving the expression of MSC surface markers such as CD90, CD105, and CD73 on the cell surface both *in vitro* by appropriate differentiating conditions (Melzer et al. 2016). For instance, when co-culturing adipose derived MSCs with colon cancer cell lines such as HCT116 cells, LoVo cells, SW480, LS174T, and CCD-18 Co, it has been recognized to explicit epithelial- mesenchymal transition (EMT) correlated genes like ZEB1, ZEB2, Slug, Snail, Twist and also stemness genes such as Oct4, Sox2, Nanog, Bmi1 which are known to be intensified in co-cultured system along with other associated cancer genes namely MMP1, IL10, TGF-(α, β), COL1A1, IFN-γ, VEGFA etc. (Dong et al. 2011). Thus, it is proven that the MSCs enhance the tumor propagation and malignancies in conjunction with knocking down or up-regulating certain genes along with other growth factors and signaling pathways in their tumor niche (Melzer et al. 2016). In tumor malignancy, it has been identified as tumor cells along with MSCs imply certain gene transcripts and other growth factors trigger various signaling pathways. They are instigated that according to their diverse cancer cell types in the tumor niche, it is comprised of notch, Hedgehog, Wnt, PI3K, NF-κB, and STAT pathways which are specifically activated or inhibited depending on the paracrine signals in their tumor stroma. Therefore, paracrine signals are proven to enhance the MSCs residing in the tumor niche of colon cancer more precisely (Illemann et al. 2006; Gout and Huot 2008). In contrast to the tumor-enhancing ability of MSCs, different studies have shown that MSCs inhibit tumor progression and metastasis by inhibiting angiogenesis, suppressing immune responses, suppressing Wnt and Akt signaling, and inducing apoptosis or cell cycle arrest in the G0-G1 phase of the cancer (Lazennec and Lam 2016). Due to their tropism to the tumor niche, mesenchymal stem cells are considered to be a promising vector for the delivery of antitumor agents.

4.2 Targeting of TME by miRNAs

A number of malignancies have been associated with characteristic miRNA signatures (Chen et al. 2015; Lu et al. 2005; Bullock et al. 2013). Compared to normal tissues, miRNAs have been found to be dysregulated in cancers. Recent studies have highlighted the inevitable role of miRNAs in the progression of CRC. The miRNAs of the cancer cells via the non-cell autonomous mechanism have been found to elicit an impact on the TME by altering the miRNA profiles of the non aberrated neighbouring cells and resulting into their conversion into cells possessing cancer promotive effects. Many studies have highlighted the role of CAF in the initiation and progression of cancer. Changing of phenotype to CAFs is responsible for trans-

differentiation properties as well as CAF associated tumorigenic actions of stromal cells in TME (Sansom et al. 2010). One of the most common miRNAs that has been reported to be predominantly present in the tumor cells as well as the CAFs of pancreatic and colorectal tumors is miRNA-21 (Lu et al. 2005; Bullock et al. 2013; Ali et al. 2015b). Hence studies that utilized antagomiR for the inhibition of miR-21 resulted in decreased migration/invasion potential of the CAFs (Chen et al. 2015; Lu et al. 2005). The CAF phenotype can be induced by both altered escalated as well as abated expression of certain miRNAs (Bullock et al. 2013; Ali et al. 2015b). Abrupt expansion of the roles of miRNAs in tumor microenvironments has come forward. For the use of miRNAs in therapeutics, it is vital to deliver miRNA mimics cells or antagomiRs directly into the target cells. However, the polyanionic nature, hydrophilicity, and high molecular weight of naked miRNAs make it impossible of them to pass through cell membranes. Rapid removal by urine has been observed when the chemically modified anti-miR oligonucleotides are administered in the absence of a carrier as they exhibit limited tissue distribution. Viral and non-viral encapsulation strategies and nanoencapsulation of miRNAs to protect miRNAs from degradation by nucleases, along with improved circulating half-life had been reported earlier (Sansom et al. 2010).

5 Concluding Remarks

The progression and metastatic potential of the colon cancer cells is predominantly supported by the immediate tumor microenvironment that consist of several cellular and non-cellular components that promote the sustenance and spread of these malignant cells to other organs of the afflicted patient. The TME mainly comprising of the immune cells such as T-cells, B-cells, Tumor associated Macrophages, fibroblasts, CAF's, and acellular components mainly comprising of the ECM, cytokines and chemokines that have predominant effects on the signalling pathways involved in colon cancer progression and metastasis. Therapeutic agents that can alter the colon cancer ecosystem may be effective in preventing or treating the metastatic diseases. Colon cancer cells rely on stromal factors to proliferate and migrate (Rhee et al. 2015; Heslin et al. 2001). Therapeutic targeting of stromal cellular components, including inflammatory cells such as CAFs, TAMs, immune cells, endothelial cells and the vasculature, ECM, and matrix-associated molecules, must therefore be considered eventually. Therapies targeting the tumor *niche* mainly the small molecule inhibitors, antibodies blocking the interactions between the tumor cells and the other cells of the TME will give rise to a more effective alternative therapy as the cancer progression is blocked at the molecular level by targeting the cytokines and the exosomal interactions and thereby abating the activation of signalling pathways involved in the progression and metastasis of colon cancer cells. In order to develop multifold strategies for the *in-vivo* delivery of miRNA the mechanisms of miRNAs have been extensively analysed. However, there are a few setbacks that are being worked on. One of the major issues is the poor cancer tissue permeability in miRNA based therapy. Heterogeneous tumor perfusion and interstitial fibrosis has resulted in the inefficiency of the penetrating miRNA-containing delivery vesicles with or without targeting moieties in the tumor microenvironment. In order to promote alternative and more effective cancer therapies the complicated nature of the cancer cell–host cell interactions and cell–ECM interactions in the tumor are to be understood.

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