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Kursad Turksen *Editor*

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Stem Cell Therapy - Potential and Challenges

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Editor

Cell Biology and Translational Medicine, Volume 11

Stem Cell Therapy - Potential and Challenges

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Preface

This next volume in the Cell Biology and Translational Medicine series addresses the topic of stem cell therapy. Contributors have covered the potential of stem cells for clinical applications and the attendant challenges. Amongst specialized topics, there are chapters on the use of stem cells in SARS-CoV2 infection, mesenchymal stem cells in cardiac repair, the role of exosomes in regenerative medicine and the potential utility of stem cells in diseases including diabetes, arthritis, aging and cancer.

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

I would also like to acknowledge and thank Sara Germans-Huisman and Mariska van der Stigchel, assistant editors, for their outstanding efforts in helping to get this volume to the production stages.

A special thank you goes to Shanthi Ramamoorthy for 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 insights and 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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Induced Pluripotent Stem Cell Derived Human Lung Organoids to Map and Treat the SARS-CoV2 Infections *In Vitro*

Bipasha Bose, Saketh Kapoor, and Muhammad Nihad

Abstract

COVID-19 is the current day pandemic that has claimed around 1,054,604 lives globally till date. Moreover, the number of deaths is going to increase over the next few months until the pandemic comes to an end, and a second wave has also been reported in few countries. Most interestingly, the death rate among certain populations from the same COVID-19 infection is highly variable. For instance, the European populations show a very high death rate, in contrast to the populations from Chinese ethnicities. Amongst all the closed cases with an outcome (total recovered + total died), the death rate in Italy is 13%, Iran is 6%, China is 5%, Brazil is 3%, The United States of America is 2%, India 2%, Israel is 1% as of October 08, 2020. However, the percentage was higher during the early phase of the pandemic. Moreover, the global death rate amongst all the patients with an outcome is 4%. Here we have reviewed virus-transmitted various respiratory tract infections and postulated a better understanding of SARS-CoV2 using lung stem cell organoids *in vitro*. Hence, here we propose the strategies of understanding first the infectivity/severity ratio of COVID-19 infections using various ethnicity originated induced pluripotent stem cell-derived lung stem cell organoids *in vitro*. The greater the infectivity to severity ratio, the better the disease outcome with the value of 1 being the worst disease outcome. This strategy will be useful for understanding the infectivity/severity ratio of virus induced respiratory tract infections for a possible betterment of community-based disease management. Also, such a strategy will be useful for screening the effect of various antiviral drugs/repurposed drugs for their efficacy *in vitro*.

Keywords

Coronavirus · COVID-19 · iPSC · Lung stem cell organoids · Respiratory tract infections · SARS-CoV2

1 Introduction

Virus induced respiratory infections had been known since time immemorial. Categories of such viruses as respiratory syncytial virus (RSV) causing mostly mild upper respiratory tract to

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severe lower respiratory tract infections and pneumonia in children have been extensively studied (Tahamtan et al. 2020). The majority (50–90%) of paediatric hospitalizations caused by RSV are for bronchitis and 5–40% for pneumonia (Hall 2001; Leader and Kohlhase 2002). From the experience of RSV, the severity and fatality mostly depend on the underlying conditions such as birth defects, chronic lung disease, congenital heart disease and immunodeficiencies such as paediatric cancers (Alvarez et al. 2013; Openshaw and Tregoning 2005).

Similar to infants, elderly people with compromised immune system, underlying conditions such as diabetes, hypertension and cardiac conditions, lung disease are most prone to severity of respiratory infections by viruses. Currently, the severe acute respiratory syndrome (SARS), Middle East Respiratory Syndrome (MERS), and now SARS-Corona Virus −2 (SARS-CoV2) infection, also known as Coronavirus disease-2019 (COVID-19) has been infecting people globally at almost an uncontrollable rate.

Of all of these, SARS-CoV2, also known as novel coronavirus, is the most infectious one that has originated in Wuhan, China and has spread worldwide (Wu et al. 2020). SARS-CoV2 is a zoonotic virus that has possibly originated in bats and then possibly transmitted to humans from the wet meat market in China via another intermediate animal host namely the endangered species Pangolin (Zhou et al. 2020). This indicates a cross species transmission of this virus (Guo et al. 2020; Vijaykrishna et al. 2007; Zhang et al. 2020a). This novel SARS-CoV2 has 96% homology with a bat coronavirus (Zhou et al. 2020). Such diverse interspecies transmissions of viruses have been seen earlier in case of SARS, MERS in humans and swine acute diarrhoea in pigs (Yang et al. 2019). Indeed, Wong et al., (2019) had alerted the presence of bats in wet markets in China as the most important cause of such dangerous global outbreaks of coronavirus infections (Wong et al. 2019).

Till October 07, 2020, the total number of people infected globally by novel SARS-CoV2 is 36,391,128 out of which there has been 1,060,443 deaths, and 27,408,566 recoveries (https://www.worldometers.info/coronavirus/). Most important, out of all the mortalities, two-thirds happen to be from Europe. Also, the mortality rates are highest amongst the European and American populations. Hence, there is a strong gap in the knowledge whether ethnicity plays a role in the severity of the novel SARS-CoV2 infection. There are currently no studies available for delineating the ethnicity specific infectivity/severity ratio for this novel SARS-CoV2. Hence, we propose an *in vitro* lung organoid model to delineate the infectivity/severity ratio in various ethnicities in global human populations, better understanding the disease and various therapeutic approaches.

2 History of Virus Transmitted Infections and Respiratory Tract Infections -Early Days Till Date with an Understanding of Global Distribution and Evolution

Various types of viruses infecting the human upper and lower respiratory tracts include influenza virus A and B (Flu-A and B), parainfluenza viruses 1, 2, 3, 4 (PIV-1, 2, 3 and 4); respiratory syncytial virus (RSV) mostly affecting children; human metapneumovirus (hMPV), rhinovirus (RhV) and enterovirus (EnV). Various infections from the above-mentioned respiratory tract attacking viruses are flu, pneumonia, otitis media, parotitis and encephalitis. Moreover, the respiratory tract infections caused by all the viruses present themselves clinically as similar symptoms such as running nose, throat pain, cough and in extreme cases difficulty in breathing that might lead to the depletion of tissue oxygen levels and hence long-term ventilation and often death. Due to similarities in symptoms, it becomes difficult to impossible to distinguish the virus just by clinical diagnosis. However, rapid detection becomes extremely essential to avoid antibiotic therapy, contagion, faster disease management using antiviral therapy, reduced hospital stay and hence, a lesser economic burden.

Moreover, lot of times, due to high mutation rates in viruses, lack of vaccines, severe complications arise. Hence, it is important to understand the origin and outbreak of virus transmitted epidemics and pandemics from a historical perspective.

The virus transmitted infections have been dated back since the Neolithic period around 12,000 years ago. During this time, human habitat changed from hunter-gatherers to more organised and densely populated agricultural communities. Plant viruses such as potyviruses of potato, animal virus- rinderpest of cattle; smallpox and measles for humans are the oldest viruses to have devastated human civilization in the form of endemic or even epidemic. The regions reported to have been infected by viruses are mainly Europe and North Africa before Christ (BC) era (McMichael 2004).

Viruses reportedly got transmitted in the new era during last 1000 years by the Europeans during Spanish conquest times. During this time, the local European people lacked natural resistance to viruses and hence, millions succumbed to the epidemics (McMichael 2004). Regarding respiratory tract infections, the influenza pandemics has been first recorded in 1580 after which they have been occurring with increasing frequency in the recent centuries. The deadliest/devastating flu pandemic was the Spanish flu (H1N1) that affected 500 million people from 1918 to 1920, exactly 100 years ago from today. Although the pandemic was global, the majorly affected people were from the Western countries from the Europe and America. This flu had affected one fourth of the World population then, and killed 40–50 million. Countries such as China were less infected.[13] Hence, ***ethnicity might be playing a role in the infectivity/severity of respiratory tract viral infections***. Similarly, the novel SARS-CoV2 also has the highest infectivity/severity ratio in the European countries and the USA (https://www.worldometers.info/coronavirus/). Moreover, super hygienic circumstances, in the Western World renders less exposure of a child to infections and hence, might contribute to low immunity during the later years of life in Caucasians, as compared to the Asian population.

Finally, viruses became visible to humans with the advent of the electron microscope in 1930s, and virology research expanded. Despite that, various infections caused by viruses continued such as epidemics of poliomyelitis and the most pathogenic virus-human immunodeficiency virus (HIV) causing Acquired Immunodeficiency syndrome (AIDS) etc. Vaccines were also developed for virus transmitted diseases namely rabies, smallpox etc. pioneered by Louis Pasteur and Edward Jenner. Most importantly, from the evolutionary perspective most of the viruses are beneficial to mankind. Viruses reportedly drive evolution by cross-species transmission of genes, thereby playing an important role in the ecosystems and the life in totality (Park 2019). Now, the questions that arise in the current perspective regarding SARS-CoV2 pandemic are:

(a) Are Caucasians more susceptible having a high infectivity/severity ratio?
(b) Is this susceptibility of Caucasian ethnicity due to super hygienic practice during their childhood days resulting in low immunity physiology, or else, their genetic/epigenetic makeup?
(c) Is the cross-species transmission from bats to possibly pangolin and now to humans indicate an evolutionary perspective that is beneficial to mankind?

Finally, the answers to the aforementioned questions from a to c might be able to find a preventive or protective strategy from high infectivity/severity ratio. Hence, in the rest of the sections we have tried to strategize the *in vitro* research technologies that might help us to find the answers to these questions.

3 Lung Stem Cell Organoids Generated from Induced Pluripotent Stem Cells

The severe cases of SARS-CoV2 manifest as lower airways/lower respiratory tract/ lung damage. This lung damage leads to the depletion in the oxygen supply in the entire human body, that cannot be even restored by the artificial process of

ventilation in extreme cases leading to multi-organ failure and deaths. As lung is the organ, that is severely affected by SARS-CoV2, we need to elucidate the effect of this virus on *in-vitro* 3D human lung model systems. Such systems are indeed, better than 2D human lung cell lines. Kaye (2006) had reported the use of multiple cell lines for understanding SARS-CoV (Kaye 2006). However, during that time SARS-CoV2 was not known to the mankind, but it was rather the earlier version of SARS virus that had caused a less severe pandemic in 2002–2003 (de Wit et al. 2016).

Moreover, in this context, animal models will not be the best alternative in this scenario where our question is directed towards checking the infectivity/severity ratio of various human ethnic groups. Regarding the usage of multiple cell lines for understanding SARS coronaviruses, it became evident that SARS coronaviruses could not replicate in various cell lines that are routinely being used for respiratory virus isolation (Kaye 2006). Of all the cell lines tested, interestingly the human lung epithelial carcinoma cell line-A549 did not support the replication of SARS coronavirus (Kaye 2006). On the contrary, human cell lines derived from non-lung tissues such as HEK-293 derived from human fetal kidney, HEPG2 and Huh7-both hepatocellular carcinoma cell lines reportedly supported the replication of SARS coronavirus (Kaye 2006). Common cell line for virus replication studies such as Vero and Vero 6 + both kidney epithelial cell line from African green monkey proved to be conducive of replication of SARS coronavirus (Kaye 2006). Moreover, another coronavirus causing severe respiratory tract infection namely, betacoronavirus 2c EMC/2012, could reportedly be replicated well in A549 cell line (Chan et al. 2013).

In the recent past, human pluripotent stem cells (human embryonic and induced pluripotent) stem cells have been differentiated into this endodermal tissue namely lung in 2D monolayer culture by recapitulating the *invivo* lung development (Kadzik and Morrisey 2012; Longmire et al. 2012; Firth et al. 2014). Such 2D *in vitro* differentiation protocols have been successful to a certain extent and involves modulation of various signaling pathways. Eventually, human lung organoids, 3D *in vitro* counterparts of fetal human lung tissue have been reported by Dye et al *(*Dye et al. 2015*)*. The steps involved firstly, the modulation of developmental signaling pathways for obtaining spheroids from ventral-anterior foregut. Such spheroids upon *in-vitro* expansion resulted in the formation of human lung organoids (HLO). HLOs comprised of multiple cell types and compartments, namely epithelial and mesenchymal compartments of the lung structurally organized resembling a native lung. Moreover, various cell types present in HLOs included human upper airway-like epithelium comprising of basal cells, immature ciliated cells surrounded by smooth muscle and myofibroblasts and most important alveolar like structure with desired cell types (Dye et al. 2015). Hence, such *in vitro* lung organoids derived from human pluripotent stem cells from various ethnic origins are conducive models for testing the infectivity/severity of SARS-CoV2. However, such lung organoids had challenges since they resembled a fetal lung airway, rather than an adult lung airway. Indeed, the SARS-CoV2 had been reported to have a reduced infectivity/severity and asymptomatic infection in pediatric patients, as compared to the elderly (Qiu et al. 2020). Hence, fetal lung 3D organoids derived from hPSCs from various ethnicities may not be the best option for studying the infectivity/severity of SARS-CoV2. So, the ideal lung organoids should be one resembling adult lung airways. Dye et al., have further modified the HLO from the earlier differentiated types by incorporating certain biomaterial such as poly (lactide-co-glycolide) (PLG) scaffolds or polycaprolactone (PCL) to obtain 3D HLO resembling human adult airways (Dye et al. 2015; Dye et al. 2020).

4 Strategies for Using Human Lung Organoids from iPSCs of Various Age Groups and Different Ethnicities for Assessing the Infectivity/ Severity Ratio of SARS-CoV2

As the methods for *in vitro* generation of human lung organoids (HLO) have been reviewed in the previous section, we propose to generate such HLO from the human induced pluripotent stem cells derived from various human ethnicities such as Caucasians, Chinese, Indians, Japanese, Africans, Malaysians. For a decade, recommendations have been made for the generation of such global iPSC library mainly for cell therapy applications, having a stock of iPSCs from all different ethnicities and age groups (Turner et al. 2013). With aggressive efforts, such iPSC bank namely the European bank for the induced pluripotent stem cells (EBiPSC) having a current stock of 894 iPSCs came into existence (De Sousa et al. 2017). The EBiPSC (https://ebisc.org/), opened to the public in 2016 has been generated as a global resource for research that stocks a large collection of quality controlled iPS cells available not only to the European researchers, but also to the worldwide researchers. EBiPSC is a joint initiative of the European Commission, and the European Federation of Pharmaceutical Institutes and Associations (EFPIA), in collaboration with a consortium of international experts from the iPSC community in academia, government and business after facing certain challenges has been operational (George 2018). The EBiPSC mainly operates by receiving the iPS lines from the depositors followed by extensive quality checks and approvals. Under the umbrella of EBiSC, The European Collection of Authenticated Cell Cultures (ECACC) in the UK is involved in distributing the vials to users. Moreover, the Fraunhofer Institute for Biomedical Engineering (IBMT) in Sulzbach, Germany serves as the long-term secure storage facility/ 'mirror bank' of EBiSC. Despite such challenges, we are highly grateful to the research, academia, government and business partners who have successfully created such iPSC repository.

As far as the ethnicity and age of the donor is concerned, all the iPS cell lines of EBiSC are registered with the human Pluripotent Stem Cell Registry (hPSCreg) (https://hpscreg.eu/). This serves as a database maintained and hosted by the Charité Medical University, Berlin, Germany for human pluripotent stem cells (both Human Embryonic and Induced Pluripotent stem cells). The primary role of hPSCreg is collection and public release of the information regarding the various pluripotent stem cell lines and the groups deriving them. For example: The human Pluripotent stem cell line, **UCSFi001-A-1** has been derived by the Allen Institute for Cell Science (AICS), USA from a ***healthy male Asian donor using*** non-integrating episomal vector and is allowed for distribution for research purposes only (https://hpscreg.eu/cell-line/UCSFi001-A-1). Similarly, the iPS cell line named **NIMHi001-A** derived from the peripheral blood mononuclear cells of a Parkinson's disease (PD) **-** ***Indian female of East Indian Ethnicity*** using Sendai virus reprogramming method. The iPS cell line **NIMHi001-A** was derived in the National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore, India is allowed for distribution for research purposes only (https://hpscreg.eu/cell-line/NIMHi001-A) (Datta et al. 2020). In this particular case, although the iPS cell line is from a PD patient, the same can be used for the generation of 3D lung organoids since PD patients do not have any lung defects. Moreover, it is best to use iPSC lines from varying ethnicities derived respectively from healthy donors. Example of another cell line registered in the Human Pluripotent Stem Cell registry from ***Caucasian Ethinicity*** derived from a 40-year-old male is **CSSi001-A (Rosati et al.** 2018a**; Rosati et al.** 2018b). The cell line CSSi001-A (https://hpscreg.eu/cell-line/CSSi001-A) was derived from a healthy individual being a carrier of disease named Joubert syndrome by the Italian institute named Fondazione Casa Sollievo della Sofferenza IRCCS (CSS) from the dermal fibroblast tissue using non-integrative episomal vector method. This

cell line is also available for research purposes. Hence, this iPSC line can be recommended for deriving lung organoids from Caucasian ethnicity as the patients with Joubert syndrome mostly suffer from brain developmental defects and not lung defects (Crawford and Dearmun 2017). Hence, before starting the differentiation of HLO from iPSC from various ethnicities, it is important to have a choice of iPS cell lines from the database. Accordingly, we have tabulated the list of such cell lines from various ethnicities that can be recommended for the derivation of lung organoids for further understanding the infectivity/severity/drug screening for COVID-19. The list of chosen iPS cell lines from different ethnicities is given in Table 1.

More recently, the bronchial transient epithelial cells, a type of progenitor cells has been reported to be more prone to SARS-CoV2 infection, as compared to any other lung cell type (Lukassen et al. 2020). In fact, in addition to HLO comprising of a mixed population of lung cells, the iPSCs from various ethnicities can be differentiated first in 2D into the lung progenitor cell type (bronchial transient epithelial cells) followed by 3D cultures of such cells. This approach will possibly be able to discern the distinct differences in various ethnicities, with respect to, infectivity/severity of SARS-CoV2 infections. Also, such *in vitro* approach for using the differentiated bronchial transient epithelial cells/progenitor cells in 3D will enable us to understand the distribution of Angiotensin-converting enzyme 2(ACE) receptor, the receptor for SARS-CoV2 entry into the cells (Fantini et al. 2020).

As far as infectivity/severity ratio is considered, we postulate using the 3D HLO models, as well as the 2D and 3D cultures of differentiated bronchial transient epithelial cells/progenitor cells. In fact, 2D cultures of related cell types namely (Human primary alveolar epithelial cells; Human bronchial epithelial cells; Human tracheal epithelial cells and human small airway epithelial cells) can also be used that can be procured from commercial vendors such as ScienCell Research Laboratories Carlsbad, California, USA. However, the challenge in commercially available primary cells is that we cannot have the cell types from multiple ethnicities. In this context, the 2D and 3D models can be infected with various doses of viruses, so as to mimic mild, moderate and severe exposure even during the time of initial infection. The postulated experimental designs are represented in Figs. 1 and 2.

Various parameters that need to be fixed for such *in vitro* experiments include, establishment of viral dose of SARS-CoV2 during the initial infection (Tseng et al. 2005; Chen et al. 2018). Secondly, we also can increase the viral dose gradually to obtain mild, moderate and severe infections *in vitro*. Thirdly, we propose to optimize the time with the given viral dose to obtain mild, moderate and severe infection in 2D and 3D human lung/lung cell *in vitro* models. Each of the spatial and temporal effects under 2D and 3D conditions from cells from various ethnicities can be assessed for the severity. The readouts of severity can be final viral load at various time points *in vitro* (Figs. 1 and 2); viral load at one fixed timepoint with mild, moderate and severe infections respectively (Figs. 1 and 2). Moreover, the transcript for the viral spike protein can be assessed at the aforementioned severities and timepoints. Furthermore, in order to understand the difference in severity of infections in cells from various ethnicities, the levels of ACE receptors can also be estimated using a simple western blot.

5 Strategies for Using Human Lung Organoids from iPSCs of Different Ethnicities for Antiviral/Repurposed Drug Screening Against SARS-CoV2

Till now, there is neither any specific drug, nor any vaccine against SARS-CoV2. Hence, the 3D lung organoid *in-vitro* model as proposed and discussed in the previous section can be of immense help in mimicking the *in vivo* conditions of infection. Accordingly, we have tried discussing the various stages at which the SARS-CoV2 can be targeted based on its life

Table 1 List of iPS cell lines from different ethnicities

Sr No	Name of the cell line and URL	Ethnicity/ Gender	Tissue donor-Healthy/Diseased (which disease?)	Reprogramming method used	Reference	Comments
1.	UCSFi001-A https://hpscreg.eu/cell-line/UCSFi001-A	Asian/Male	Healthy	Non-integrating Episomal vector	Mandegar MA et al. CRISPR Interference Efficiently Induces Specific and Reversible Gene Silencing in Human iPSCs. Cell stem cell. 2016;18 (4):541–553.	Can be used for modelling Asian ethnicity HLO
2	NIMHi001-A https://hpscreg.eu/cell-line/NIMHi001-A	Indian/Male	Parkinson's disease	Non-integrating Sendai virus	Datta I et al. generation of induced pluripotent stem cells (NIMHi001-A) from a Parkinson's disease patient of East Indian ethnicity carrying LRRK2 I1371V variant. Stem cell research. 2020 Mar 17;44:101768.	Can be used for modelling Indian ethnicity HLO
3	BIHi001-A https://hpscreg.eu/cell-line/BIHi001-A	Caucasian/ Male	Healthy	Non-integrating Sendai virus	Fenske P et al. Autaptic cultures of human induced neurons as a versatile platform for studying synaptic function and neuronal morphology. Scientific reports. 2019 Mar 20;9 (1):4890.	Can be used for modelling Caucasian ethnicity HLO
4	HELPi001-A https://hpscreg.eu/cell-line/HELPi001-A	Han/Female	Congenital Contractural Arachnodactyly	Integrating Lentivirus	Liu H et al. Establishment of a Beals syndrome patient-derived human induced pluripotent stem cell line HELPi001-A. Stem cell research. 2019 Oct;40:101535.	Can be used for modelling Chinese ethnicity HLO
5	MCRIi001-A https://hpscreg.eu/cell-line/MCRIi001-A	Caucasian/ Male	Healthy	Non-integrating Sendai virus	Nur Patria Y et al. Generation of a SOX9-td Tomato reporter human iPSC line, MCRIi001-A-2, using CRISPR/Cas9 editing. Stem cell research. 2020 Jan;42:101689.	Can be used for modelling Caucasian ethnicity HLO
6	IGIBi001-A https://hpscreg.eu/cell-line/IGIBi001-A	Indian/Male	Sickle cell Anemia	Non-integrating Sendai virus	Bhargava N et al. Generation and characterization of induced pluripotent stem cell line (IGIBi001-A) from a sickle cell anemia patient with homozygous β-globin mutation. Stem cell research. 2019 Aug;39:101484.	Can be used for modelling Indian ethnicity HLO

(continued)

Table 1 (continued)

Sr No	Name of the cell line and URL	Ethnicity/ Gender	Tissue donor-Healthy/Diseased (which disease?)	Reprogramming method used	Reference	Comments
7	ULBi003-A https://hpscreg.eu/cell-line/ULBi003-A	White/Male	Healthy	Non-integrating Sendai virus	Danesi C et al. Increased Calcium Influx through L-type Calcium Channels in Human and Mouse Neural Progenitors Lacking Fragile X Mental Retardation Protein. Stem cell reports. 2018 Dec 11;11(6):1449–1461.	Can be used for modelling Caucasian ethnicity HLO
8	IDVi001-A https://hpscreg.eu/cell-line/IDVi001-A	Caucasian/ Female	Retinitis Pigmentosa	Non-integrating Sendai virus	Terray A et al. Generation of an induced pluripotent stem cell (iPSC) line from a patient with autosomal dominant retinitis pigmentosa due to a mutation in the NR2E3 gene. Stem cell research. 2017 Oct;24:1–4.	Can be used for modelling Caucasian ethnicity HLO
9	IRFMNi001-A https://hpscreg.eu/cell-line/IRFMNi001-A	Caucasian/ M.0ale	Healthy	Integrating Lentivirus	Imberti B et al. renal progenitors derived from human iPSCs engraft and restore function in a mouse model of acute kidney injury. Scientific reports. 2015 Mar 6;5:8826.	Can be used for modelling Caucasian ethnicity HLO
10	IPTi001-A https://hpscreg.eu/cell-line/IPTi001-A	Chinese Han/Male	Alzheimer's disease	Non-integrating Episomal vector	Zhang L et al. Generation of induced pluripotent stem cell line (IPTi001-A) from a 62-year old sporadic Alzheimer's disease patient with APOE3 (ε3/ε3) genotype. Stem cell research. 2019 Dec;41:101589.	Can be used for modelling Chinese ethnicity HLO
11	JTUi001-A https://hpscreg.eu/cell-line/JTUi001-A	Chinese Han/Female	CHARGE syndrome	Non-integrating Episomal vector	He S et al. Establishment of an induced pluripotent stem cell line from a patient with CHARGE syndrome carrying a CHD7 (p. L1151Gfs*17) mutation. Stem cell research. 2020 Mar 20;45:101774.	Can be used for modelling Chinese ethnicity HLO

(continued)

Table 1 (continued)

Sr No	Name of the cell line and URL	Ethnicity/ Gender	Tissue donor-Healthy/Diseased (which disease?)	Reprogramming method used	Reference	Comments
12	JTUi002-A https://hpscreg.eu/cell-line/JTUi002-A	Han/Male	Waardenburg syndrome	Non-integrating Episomal vector	Wang P et al. Establishment of an iPSC line (JTUi002-A) from a patient with Waardenburg syndrome caused by a SOX10 mutation and carrying a GJB2 mutation. Stem cell research. 2020 Mar 7;44:101756.	Can be used for modelling Chinese ethnicity HLO
13	PUMCHi001-A https://hpscreg.eu/cell-line/PUMCHi001-A	Chinese Han/Male	Familial Partial Lipodystrophy Type 2	Non-integrating Episomal vector	Xiao C et al. Generation of an integration-free induced pluripotent stem cell line (PUMCHi001-A) from a patient with familial partial lipodystrophy type 2 (FPLD2) carrying a heterozygous p. R349W (c.1045C > T) mutation in the LMNA gene. Stem cell research. 2020 Jan;42:101651.	Can be used for modelling Chinese ethnicity HLO
14	MUi010-A https://hpscreg.eu/cell-line/MUi010-A	Thai/Male	MYH9-Related Disease	Non-integrating Episomal vector	Tangprasittipap A et al. Generation of a human induced pluripotent stem cell line (MUi010-A) from skin fibroblast of patient carrying a c.2104C > T mutation in MYH9 gene. Stem cell research. 2019 Apr;36:101397.	Can be used for modelling Thai ethnicity HLO
15	IBMSi001-A https://hpscreg.eu/cell-line/IBMSi001-A	East Asian (Taiwanese)/ Male	Autosomal dominant polycystic kidney disease	Non-integrating Sendai virus	Huang CY et al. generation of induced pluripotent stem cells derived from an autosomal dominant polycystic kidney disease patient with a p.Ser1457fs mutation in PKD1. Stem cell research. 2017 Oct;24:139–143.	Can be used for modelling Chinese/ Taiwanese ethnicity HLO

(continued)

Table 1 (continued)

Sr No	Name of the cell line and URL	Ethnicity/ Gender	Tissue donor-Healthy/Diseased (which disease?)	Reprogramming method used	Reference	Comments
16	IBMSi003-A https://hpscreg.eu/cell-line/IBMSi003-A	East Asian (Taiwanese)/ Female	Autosomal Dominant Polycystic Kidney Disease	Non-integrating Sendai virus	Ho MC et al. Generation of an induced pluripotent stem cell line, IBMS-iPSC-014-05, from a female autosomal dominant polycystic kidney disease patient carrying a common mutation of R803X in PKD2. Stem cell research. 2017 Dec;25:38–41.	Can be used for modelling Chinese/ Taiwanese ethnicity HLO
17	MMCi001-A https://hpscreg.eu/cell-line/MMCi001-A	Taiwanese/ Male	Deafness, Autosomal Recessive 1A	Non-integrating Sendai virus	Lu HE et al. Generation of induced pluripotent stem cells MMCi001-A from a Taiwanese hearing loss patient carrying GJB2 pV37I mutation. Stem cell research. 2020 Jan;42:101692.	Can be used for modelling Chinese/ Taiwanese ethnicity HLO
18	WISCi008-A https://hpscreg.eu/cell-line/WISCi008-A	African/ Male	Healthy	Non-integrating Episomal vector	Yin Y et al. Generation of seven induced pluripotent stem cell lines from neonates of different ethnic backgrounds. Stem cell research. 2019 Jan;34:101365.	Can be used for modelling African ethnicity HLO
19	WTSIi001-A https://hpscreg.eu/cell-line/WTSIi001-A	White British/ Female	Healthy	Non-integrating Sendai virus	Kilpinen H et al. Common genetic variation drives molecular heterogeneity in human iPSCs. Nature. 2017 Jun 15;546(7658):370–375.	Can be used for modelling Caucasian/ British ethnicity HLO
20	WTSIi466-A https://hpscreg.eu/cell-line/WTSIi466-A	British Asian – Indian/Male	Hypertrophic Cardiomyopathy	Non-integrating Sendai virus	Nil	Can be used for modelling Indian/ British (mixed?) ethnicity HLO
21	WTSIi479-A https://hpscreg.eu/cell-line/WTSIi479-A	Indian/ Female	Spastic paraplegia	Non-integrating Sendai virus	Nil	Can be used for modelling Indian ethnicity HLO

(continued)

Table 1 (continued)

Sr No	Name of the cell line and URL	Ethnicity/ Gender	Tissue donor-Healthy/Diseased (which disease?)	Reprogramming method used	Reference	Comments
22	WTSIi470-A https://hpscreg.eu/cell-line/WTSIi470-A	African/ female	Rare hereditary Ataxia	Non-integrating Sendai virus	Nil	Can be used for modelling African ethnicity HLO
23	WTSIi483-A https://hpscreg.eu/cell-line/WTSIi483-A	British Asian – Bangladeshi/ Male	Hypertrophic cardiomyopathy	Non-integrating Sendai virus	Nil	Can be used for modelling Asian/ British (mixed) ethnicity HLO
24	WTSIi496-A https://hpscreg.eu/cell-line/WTSIi496-A	Czech/Male	Batten disease	Non-integrating Sendai virus	Nil	Can be used for modelling Caucasian ethnicity HLO
25	WTSIi563-A https://hpscreg.eu/cell-line/WTSIi563-A	Portuguese/ Male	Batten disease	Non-integrating Sendai virus	Nil	Can be used for modelling Caucasian ethnicity HLO
26	PHAi003-B https://hpscreg.eu/cell-line/PHAi003-B	Persian / Female	Primary immunodeficiency	Non-integrating Sendai virus	Arias-Fuenzalida J et al. Generation of a human induced pluripotent stem cell line (PHAi003) from a primary immunodeficient patient with CD70 mutation. Stem cell research. 2019 Dec;41:101612.	Can be used for modelling Eurasian ethnicity HLO
27	GENYOi004-A https://hpscreg.eu/cell-line/GENYOi004-A	Spaniard Caucasian/ Female	ADNP syndrome	Non-integrating Sendai virus	Montes R et al. GENYOi004-A: An induced pluripotent stem cells (iPSCs) line generated from a patient with autism-related ADNP syndrome carrying a pTyr719* mutation. Stem cell research. 2019 May;37:101446. .	Can be used for modelling Caucasian ethnicity HLO

(continued)

Table 1 (continued)

Sr No	Name of the cell line and URL	Ethnicity/ Gender	Tissue donor-Healthy/Diseased (which disease?)	Reprogramming method used	Reference	Comments
28	GENYOi005-A https://hpscreg.eu/cell-line/GENYOi005-A	Spaniard Caucasian/ Female	Familial Platelet Disorder With Associated Myeloid Malignancy	Non-integrating Sendai virus	Lamolda M et al. GENYOi005-A: An induced pluripotent stem cells (iPSCs) line generated from a patient with Familial Platelet Disorder with associated Myeloid Malignancy (FPDMM) carrying a p.Thr196Ala variant. Stem cell research. 2019 Dec;41:101603.	Can be used for modelling Caucasian ethnicity HLO
29	GENYOi006-A https://hpscreg.eu/cell-line/GENYOi006-A	Spaniard Caucasian/ Male	Healthy	Non-integrating Sendai virus	Cabrera S et al. Generation of human iPSC line GRX-MCiPS4F-A2 from adult peripheral blood mononuclear cells (PBMCs) with Spanish genetic background. Stem cell research. 2015 Sep;15 (2):337–40.	Can be used for modelling Caucasian ethnicity HLO
30	UKKi020-A https://hpscreg.eu/cell-line/UKKi020-A	African/ Male	Healthy	Non-integrating Episomal vector	Nil	Can be used for modelling African ethnicity HLO

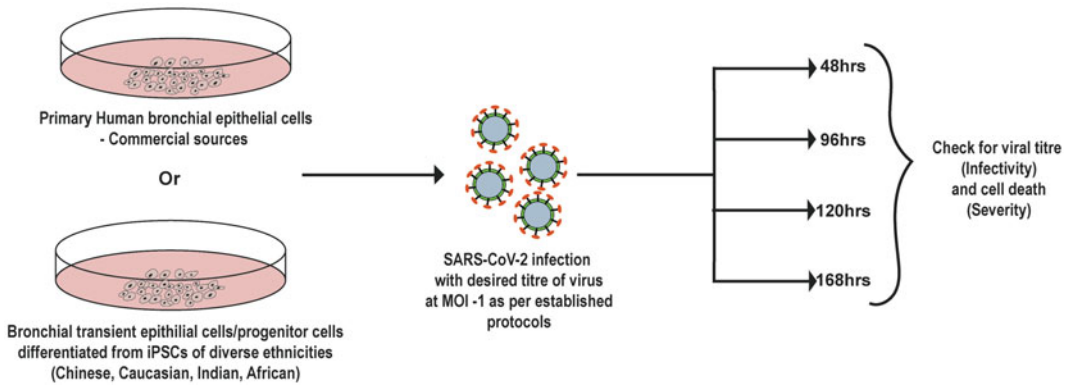

Fig. 1 2D models for *in vitro* assessment of SARS-CoV-2 in various ethnicities

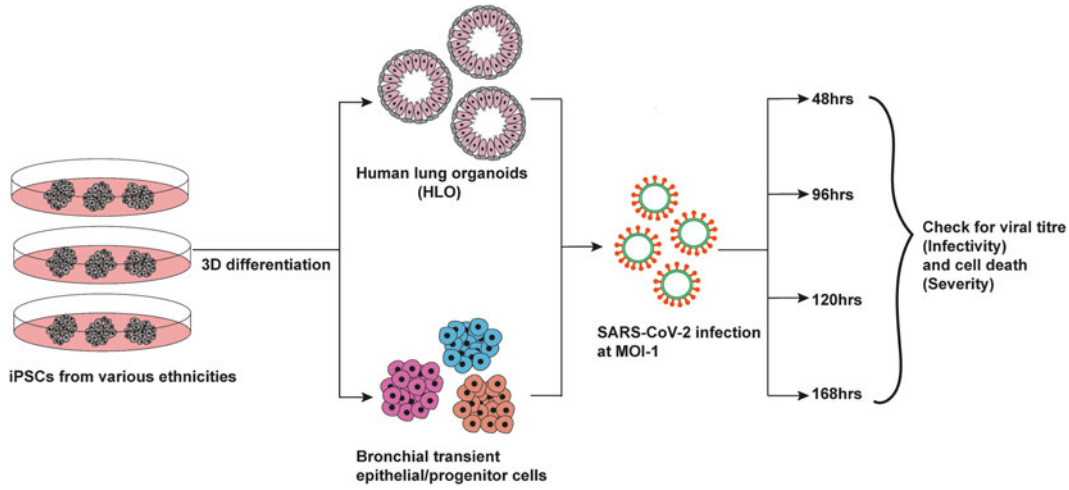

Fig. 2 3D models for *in vitro* assessment of infectivity/severity ratio of SARS CoV2 in various ethnicities

cycle inside the host. The life cycle of the SARS-CoV2, along with possible drugs/groups of drugs to be tested under *in vitro* 3D models is represented in Fig. 3.

First, the group of drugs that can inhibit the entry of virus can be tested on 3D lung organoid models. This can be achieved either by using antibodies against the spike protein of the virus rendering the virus unable to bind to the host cell surface. Monoclonal antibodies against the spike protein, or convalescent plasma from a recovered COVID-19 patient can also be tried (Shanmugaraj et al. 2020; Zhang et al. 2020b). Alternatively, binding of the virus to the host cell surface receptor, ACE, can also be blocked by either blocking the ACE receptor, or else manipulating/inhibiting the other proteins that help in virus binding/stabilizing the ACE receptor. Such proteins are TMPRSS2 (Transmembrane protease serine 2), Furin, sialic acid and Gangliosides present on the cell surface of the host protein (Hoffmann et al. 2020). Importantly, hydroxychloroquine has been reported to bind to sialic acid and gangliosides of the host cells thereby inhibiting the binding of the SARS-CoV2 to the host cells (Fantini et al. 2020). Moreover, recently a clinical trial conducted in France on PCR positive SARS-CoV2 infected patients reported a drastic reduction in viral load by treatment with a combination of hydroxychloroquine and Azithromycin by Day 6 of drug administration (Gautret et al. 2020). The readout of the viral load for the 3D *in vitro* models can be the qRT-PCR of the lysate of the 3D organoids for gene expression for genes corresponding to viral spike protein; human cell surface proteins-ACE receptor, TMPRSS2.

Secondly, after the virus enters the host cells, it releases the viral RNA, the positive strand (+RNA) in the host cytoplasm. Now, the host protein synthesis machinery/ribosome is being used for the synthesis of viral proteins for re-packaging the virus. Hence, the viral protein degradation is another possible target by proteolysis. Antiviral drugs such as Lopinavir-ritonavir which are protease inhibitors that prevent the proteolytic cleavage of new the viral proteins. Such inhibition of synthesis of new proteins hence can prevent viral packaging (Lu 2020). Moreover, a most recent clinical trial reported in the New England Journal of Medicine indicated no improvement in the clinical outcome, in terms of reduction of viral load when the severely ill SARS-CoV2 infected patients/participants have been treated with a combination of Lopinavir-Ritonavir (Cao et al. 2020). However, this drug combination has exhibited a substantial reduction in viral load in another coronavirus namely Middle East Respiratory Syndrome (MERS) (Arabi et al. 2018). Now, connecting the dots to our

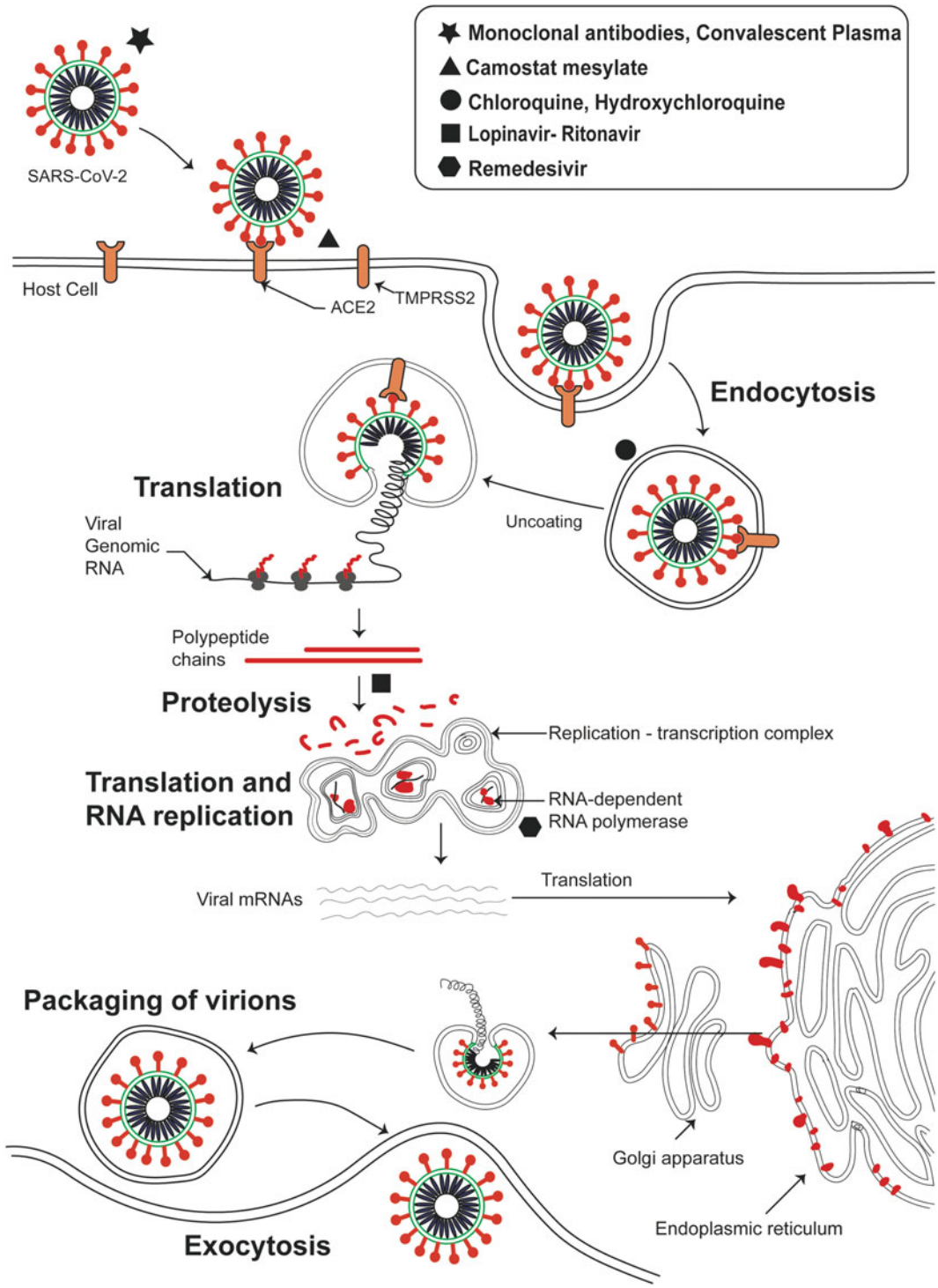

Fig. 3 The life cycle of the SARS-CoV-2 along with possible drugs/groups of drugs to be tested under *in vitro* 3D models

proposed *invitro* 3D lung organoid models infected from different ethnicities infected with SARS-CoV2, the readout of drug response can be assessed by western blot. Western blots for virion protein expression in the whole cell lysates can indicate the presence or reduction in the expression of virion proteins after the drug treatment. Also, the viral titer can also be determined post drug treatment in the lysates of the SARS-CoV2 infected cells.

At the viral RNA replication level, a known antiviral drug called 'Remdesvir', also can be tested in the 3D lung organoid models. Remdesvir is otherwise a nucleoside (adenosine) analog that incorporates into viral RNA leading to its premature termination (Agostini et al. 2018). In the context of SARS-CoV2, a clinical trial with Remdesvir has been reported to reduce the viral load in the infected patients (Grein et al. 2020; Amirian and Levy 2020). Hence, in the SARS-CoV2 infected 3D lung organoid model, the therapeutic effect of remdesvir can be studied. The *in vitro* data readout can be again reduction in viral road from the infected cells or cell culture supernatant.

Having targeted individual viral entry, multiplication and survival machineries, a combinatorial approach can also be tested onto the *invitro* 3D lung organoid models. For example, a combinatorial therapy including the antibodies against the virion proteins, viral entry inhibitors such as hydroxychloroquine, antivirals such as protease inhibitors (Ritonavir-Lopanivir); nucleoside analog (Remdesvir) can be given together (Wang et al. 2020). Again, the dosage of the drug can be determined by extrapolating mg of drug per kg weight of the lung organoid. The best advantage, hence, with the 3D HLO model is that we can specifically check the cell types that are first one to respond to such a drug combination. Secondly, the other proposed 3D lung organoid model using bronchial transient epithelial cells (progenitor cells), would indeed be useful to give a specific readout using single mechanistic drugs, as well as combinatorial drugs.

6 Conclusions

This century has experienced the major outbreaks of coronaviruses such as SARS-CoV, MERS and now SARS-CoV2 (COVID-19). Amongst all the three, the COVID-19 has taken the lives of thousands of people, and affected the lives of millions of others globally. This substantial effect of SARS-CoV2 is mainly due to its highest infectivity. Although the severity of the SARS-CoV2 is lesser as compared to the earlier SARS-CoV and MERS, such high infectivity has taken a substantial toll on the lives of people globally. Hence, the proposition of basic research using 3D *in vitro* modes combined with the reviewed work is likely to give a strong direction to curb the COVID-19. Most importantly, ethnicity and age-specific infectivity/severity ratio, drug responses and combinatorial drug responses can be elucidated using such 3D *invitro* studies.

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Author's Contributions BB conceptualized, designed and wrote the manuscript; designed the figures and approved the manuscript. SK and MN edited the manuscript and made the figures.

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

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Embryonic Stem Cells in Clinical Trials: Current Overview of Developments and Challenges

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Abstract

The first isolation of human embryonic stem cells (hESC) reported in the late 90s opened a new window to promising possibilities in the fields of human developmental biology and regenerative medicine. Subsequently, the differentiation of hESC lines into different precursor cells showed their potential in treating different incurable diseases. However, this promising field has consistently had remarkable ethical and experimental limitations. This paper is a review of clinical trial studies dealing with hESC and their advantages, limitations, and other specific concerns. Some of the hESC limitations have been solved, and several clinical trial studies are ongoing so that recent clinical trials have strived to improve the clinical applications of hESC, especially in macular degeneration and neurodegenerative diseases. However, regarding hESC-based therapy, several important issues need more research and discussion. Despite considerable studies to Date, hESC-based therapy is not available for conventional clinical applications, and more studies and data are needed to overcome current clinical and ethical limitations. When all the limitations of Embryonic stem cells (ESC) are wholly resolved, perhaps hESC can become superior to the existing stem cell sources. This overview will be beneficial for understanding the standard and promising applications of cell and tissue-based therapeutic approaches and for developing novel therapeutic applications of hESC.

Keywords

Cell-based therapy · Clinical trial · Human embryonic stem cells (hESCs) · Macular degeneration · Regenerative medicine

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Abbreviations

ALS	Amyotrophic lateral sclerosis
ART	Assisted reproductive technology
CRISPR	Clustered regularly-interspaced short palindromic repeats
DA	Dopamine
EMA	European Medical Administration
GMP	Good manufacturing practice
hESC	human Embryonic stem cells
hESC-DCs	hESC-derived mature dendritic cells
hiPSC	human-induced Pluripotent stem cells
iPSC	induced Pluripotent stem cells
ISCBI	International Stem Cell Banking Initiative
IVF	in vitro fertilization
LD	Lyme disease
MHC	Major histocompatibility complex
MS	Multiple sclerosis
PD	Parkinson's disease
SCNT	Somatic-cell nuclear transfer
UCBS	human Umbilical cord blood serum
wet-AMD	Wet age-related macular degeneration

1 Introduction

November 2019 was marked the 21th anniversary of the human embryonic stem cells (hESC) report (Thomson 1998). Over the past two decades, the unique characteristics and potency of stem cells have widely attracted the researcher's attention. Regenerative medicine and cell-based therapy have opened a new viewpoint in medicine. However, it has been hampered by the restricted availability of stem cell sources and the potentially hazardous aspects of pluripotent hESCs and induced pluripotent stem cells (iPSC).

Embryonic stem cells (ESC), as the name suggests, are derived from embryos. They were first derived from mice embryos; however, it was the extraction of ESC from the human embryo by James Thomson (1998) in Wisconsin, USA, that drew attention to these cells. Because of their unique properties, ESCs have been long studied and giving hope to many for the treatment of incurable diseases (SEMB 2005). ESC is also known as primary stem cells derived from inner cell mass (ICM) of blastocysts or early-stage pre-implantation embryos that can be used for regenerative medicine and cell therapy purposes. The first clinical study was administered for 3 years (2000–2002), during which time, the efficacy and safety of hESC lines were ascertained in 33 patients suffering from neurological and non-neurological disorders (Shroff 2016a).

Due to their pluripotent feature, ESC has drawn great extensive attention among bio-medical researchers; however, religious and ethical concerns impose limitations on their use (Volarevic et al. 2018). Based on religious and moral ethos, some believe that "human life begins at oocyte and sperm fertilization"; thus, an embryo is a person. According to their belief, an embryo has human rights that must be respected. From this viewpoint, taking a blastocyst and removing the inner cell mass to derive an ESC line is equivalent to killing (Lo and Parham 2009; Monitoring Stem Cell Research: A Report of the President's Council on Bioethics | U.S. Government Bookstore 2004). On the other hand, every year, assisted reproductive technology (ART) clinics create many blastulae. After a couple has completed infertility treatment, they are destroyed because they are made in surplus. Therefore, supporters of hESC research/therapy hold that using ESC from these extra blastulae for research and developing medical treatments, which could help improve and rescue people's lives, is a much better option. Neglect of human rights, fertilization, and abortion only for the sake of deriving ESC, concerns about generating a target population, and seeking mere financial profits are among the ethical concerns (Fan 2007; Saniei and Baharvand 2018; Sivaraman and Noor 2016).

There are two ways to derive hESC, including 1- Oocyte and sperm fertilization and deriving embryo, and 2- Somatic-cell nuclear transfer (SCNT), where the nucleus from a somatic cell is transferred into an oocyte of which the nucleus

is removed. In the first technique, fertilization can be performed in two ways: a- natural fertilization that happens *in vivo*, and b- *in vitro* fertilization (IVF) and culturing fertilized eggs. Deriving ESC from natural fertilization is limited to the cases of compulsory abortion. Under some therapeutic protocols and with the consent of the parents, ESC can be derived for therapeutic services. There are also limitations on culturing *in vitro* fertilized eggs to derive ESC since these cells have limited growth capacity in culture conditions (Golchin and Niknejad 2017). SCNT is carried out to reprogram somatic cells to pluripotent ESC by the somatic cell nuclear transfer (Tachibana et al. 2013). Through this technique, researchers can generate stem cells that are genetically similar to patients' cells, preventing tissue rejection in transplantation. SCNT is used to clone animals like pig, cow, goat, sheep, and recently horse and mule (Galli et al. 2008; Lee and Song 2007).

Depending on the objective, hESC can be used in different injection systems such as intramuscular (IM), intravenous (IV), surgery, tissue engineering protocols and the like (Shroff 2016b). Embryo pluripotent stem cells are considered as candidates of cellular-based therapy for treating different incurable diseases, for instance, degenerative neural diseases like Parkinson's disease (PD) (Lebedeva and Lagarkova 2018), amyotrophic lateral sclerosis (ALS), spinal cord damage, and/or regenerative medicine application for the eye and pancreas tissues (Table 1) (Trounson et al. 2015). Some techniques have been developed to differentiate ESC into germ layers, and each one of these techniques can be a candidate to treat some diseases, and under specific conditions, ESC differentiate into different cell types (Fig. 1).

Table 1 The list of important diseases that clinical usage of hESC for treating of these has been started

Candidate Diseases	
Neural diseases	Spinal cord injury, Amyotrophic Lateral Sclerosis, Parkinson's Disease
Heart diseases	Severe heart failure
Diabetes	Diabetes type I
Eye diseases	Myopic macular degeneration, Wet and dry Age-related macular degeneration, Stargardt's macular dystrophy
Immunotherapy	Immunotherapy vaccine for lung cancer

Recently, several clinical trials reported clinical use of hESC and opened new ideas in hESC-based therapy. Hence, there are many unclear mechanisms of neonatal differentiation that require more molecular studies before the entrance to the clinical application (Roshangar et al. 2010). However, ethical challenges regarding hESC research, such as concerns about the destruction of a human embryo, are the main factors that have limited the development of hESC-based clinical therapies. This study is a review of clinical studies dealing with hESC-based therapy and their advantages, limitations, and other specific concerns. This overview will be beneficial for understanding the standard and promising applications of cell and tissue-based therapeutic approaches and for developing novel therapeutic applications for hESC.

2 hESC for Clinical Trials

Clinical trial studies are carried out to secure clinical licenses for novel medical treatments for which early research (*in vitro* steps) and the preclinical stages have been successfully established. These studies provide satisfactory data as to the safety and efficacy of the new propositions for therapeutic purposes. Every clinical trial involves five phases: Early Phase 1 or Phase 0 (to review how or whether a drug affects the body), Phase 1 (to focus on the safety of a drug), Phase 2 (to investigate whether a drug works in bodies with a specific condition/disease), Phase 3 (to gather more information about a drug's safety and effectiveness by analyzing several populations and distinct dosages and by applying the drug in combination with other medicines) and Phase 4 (to gather additional data about a drug's safety, efficacy, or optimal performance after marketing), each corresponding to the stages of a clinical trial study. There are different databases of privately and publicly-funded

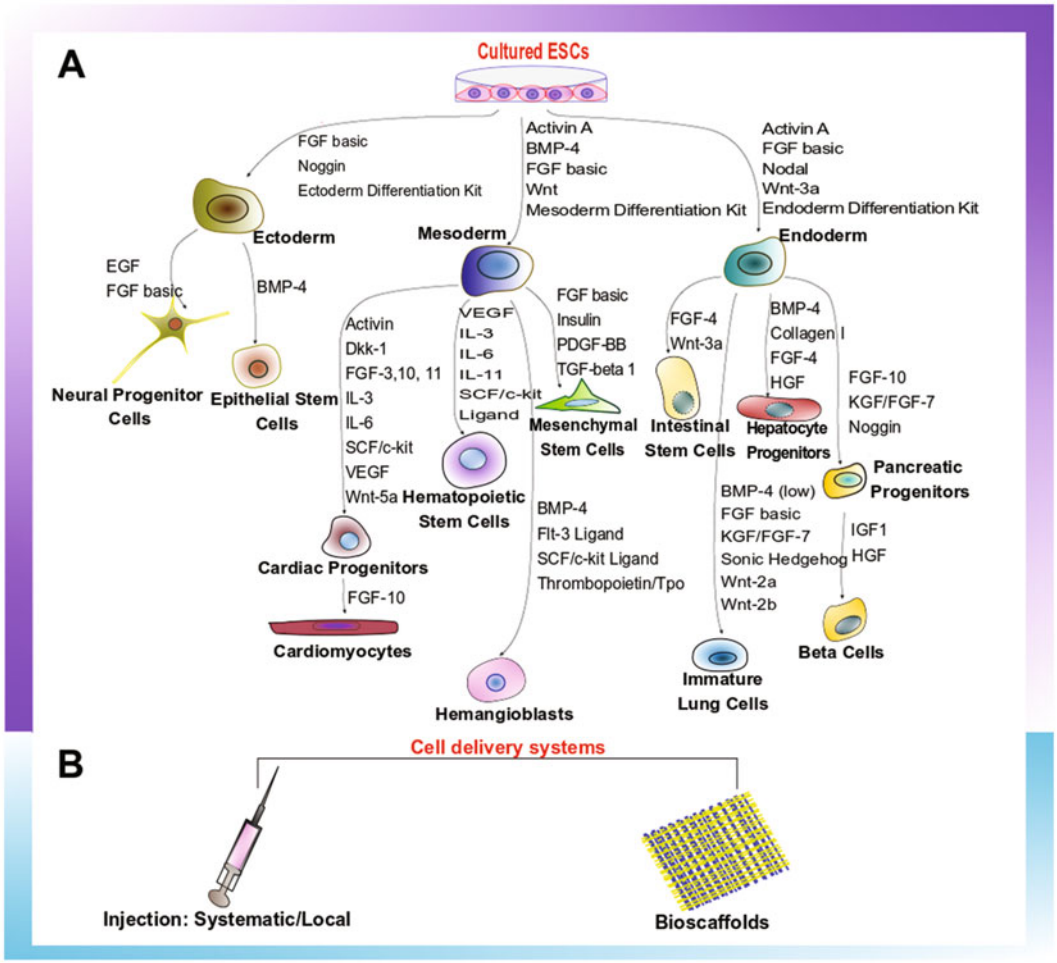

Fig. 1 A schematic representation of ESC differentiation pathways (**a**) and their delivery systems (**b**) for regenerative medicine applications

clinical trial studies conducted at country, region, and international levels. The most important clinical trial database is the U.S. National Library of Medicine (Clinicaltrials.gov), which contains the data of clinical studies from all around the world (Golchin et al. 2018a).

In the following, hESC-based clinical trials are described. Clinical applications of hESC have been summarized in (Fig. 2). Besides, the list of registered hESC-based therapy clinical trials in (https://clinicaltrials.gov/) has been provided in Table 2 that, according to these data, China has the first rank in hESC therapy clinical research, and other countries are in progress (Fig. 3). Since hESC-based therapy approaches are scalable, free of xeno-products, and can be provided expeditiously, they can be used to treat a diversity of incurable and terminal diseases). In the following, hESC-based clinical trials are described.

3 Neurological Disorders

In 2001, a randomized clinical trial by Freed et al. was performed among 40 severe Parkinson's disease patients aged 34–75 years. The patients were

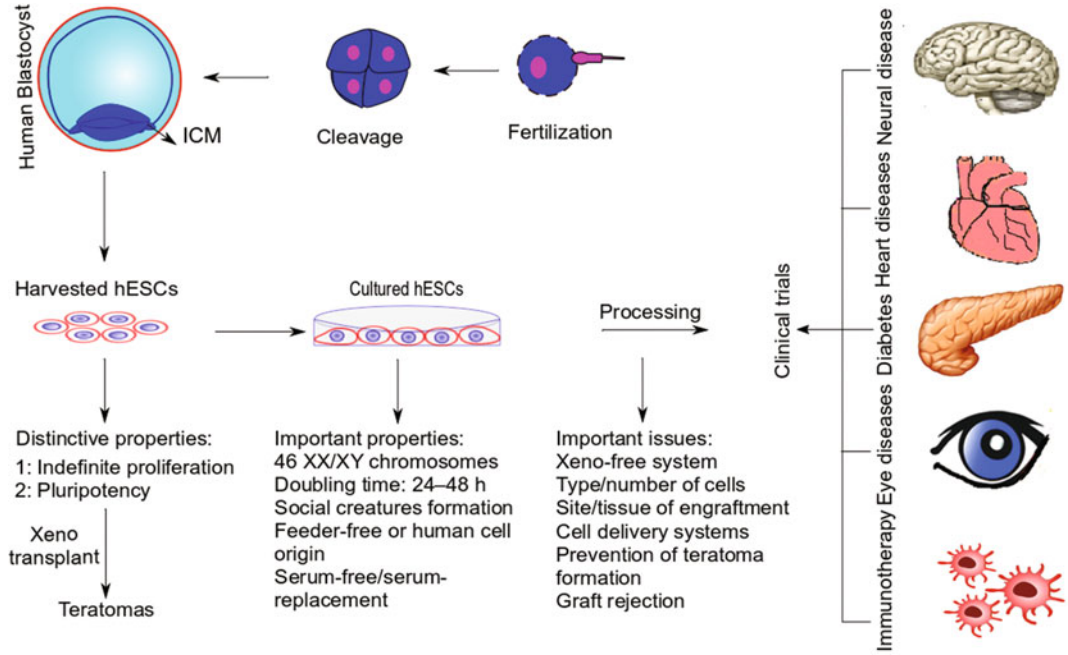

Fig. 2 A schematic representation of ESC culture features and using ESC in clinical trials

Table 2 The list of reported and registered hESC-based clinical trials

	Indication	Phase	Subject	Start/ Finish date	Country	Reference
Neural diseases	Spinal cord injury	Phase1	5	2010–2013	USA	Scott and Magnus (2014)
	Amyotrophic Lateral Sclerosis (ALS)	Phase1 & Phase2	21	2018–2020	Israel	NCT03482050
	Parkinson diseases	Phase1 & Phase2	50	2017–2020	China	NCT03119636
Heart diseases	Severe heart failure	Phase1	10	2013–2018	France	NCT02057900
Diabetes	Diabetes type I	Phase1 & Phase2	69	2014–2021	USA	NCT02239354
Reproductive insufficiency	Primary ovarian insufficiency	Phase 1	28	2019–2021	China	NCT03877471
	Infertility	Not applicable	240	2017–2020	China	NCT02713854
	Infertility	–	40	2002–2025	Israel	NCT00353197
Eye diseases	Retinitis pigmentosa	Phase1	10	2019–2020	China	NCT03944239
	Retinitis Pigmentosa	Phase1 & Phase2	12	2019–2021	France	NCT03963154
	Macular degenerative disease	Phase1 & Phase2	36	2018–2029	UK	NCT03167203
	Dry Age Related Macular Degeneration Disease (Dry AMD)	Phase1 & Phase2	10	2017–2020	China	NCT03046407
	Dry AMD	Phase1 & Phase2	16	2015–2023	USA	NCT02590692

(continued)

Table 2 (continued)

	Indication	Phase	Subject	Start/ Finish date	Country	Reference
	AMD	Phase1 & Phase2	10	2018–2020	China	NCT02755428
	Stargardt's Macular Dystrophy (SMD)	–	12	2013–2019	UK	NCT02941991
	Dry AMD	Phase1	3	2016–2019	South Korea	NCT03305029
	AMD	Phase1&Phase2	12	2012–2020	South Korea	NCT01674829
	Outer retinal degenerations	Phase1 & Phase2	18	2015–2019	Brazil	NCT02903576
	SMD	Phase1 & Phase2	12	2011–2015	UK	NCT01469832
	SMD	Phase1 & Phase2	13	2011–2015	USA	NCT01345006
	Advanced Dry AMD	Phase1 & Phase2	13	2011–2015	USA	NCT01344993
	Age-related macular degeneration	Phase1 & Phase2	24	2015–2024	Israel	NCT02286089
	SMD	Phase 1	3	2012–2015	South Korea	NCT01625559
	SMD patients	–	13	2012–2019	USA	NCT02445612
	AMD	–	11	2012–2019	USA	NCT02463344
	AMD	Phase 1	2	2015–2019	UK	NCT01691261
	Retinal pigment	–	2	2016–2020	UK	NCT03102138
	Macular degeneration diseases	Phase1 & Phase2	15	2015–2019	China	NCT02749734
Immunotherapy	Non-small cell lung cancer	Phase1	48	2018–2022	UK	NCT03371485
Injury	Meniscus injury	Phase1	18	2019–2020	China	NCT03839238

randomized to implant dopamine (DA) neurons from a single embryo and/or sham surgery (Freed et al. 2001). The trial results demonstrated that the transplanted DA neurons were able to survive in patients and provided some clinical benefits in younger individuals as opposed to older patients.

The first clinical trial study on hESC approved by Food and Drug Administration (FDA), USA, was conducted by Geron Corporation in 2009 to transplant differentiated oligodendrocyte cells from hESC for treating patients with spinal cord injury (SCI) (Ilic et al. 2015). Patients with spinal cord injury received injections of oligodendrocyte progenitor cells derived from hESC. However, the company that launched the trial terminated it due to financial restrictions (Safety Study of GRNOPC1 in Spinal Cord Injury – Full Text View – ClinicalTrials.gov n.d.). Achieving the goal of this trial could be a revolution in treating neurological disorders, therefore, in 2011, bio-plasma resumed this trial and after a 3-year follow-up, results of the five patients receiving the transplantation were published in 2014, according to which no patient had suffered from any serious adverse events to Date (Lukovic et al. 2014; Scott and Magnus 2014; Shroff et al. 2017). In 2016 at Keck Medical Center of University of the USA, neuroscientists treated a 21-year-old total quadriplegic patient with the injection of 10×10^6 stem cells (AST-OPC1) into the damaged cervical spine as a part of a multi-center clinical trial. The patient's upper body function had considerably improved 2 weeks after surgery, and 90 days later, he was able to move his hands

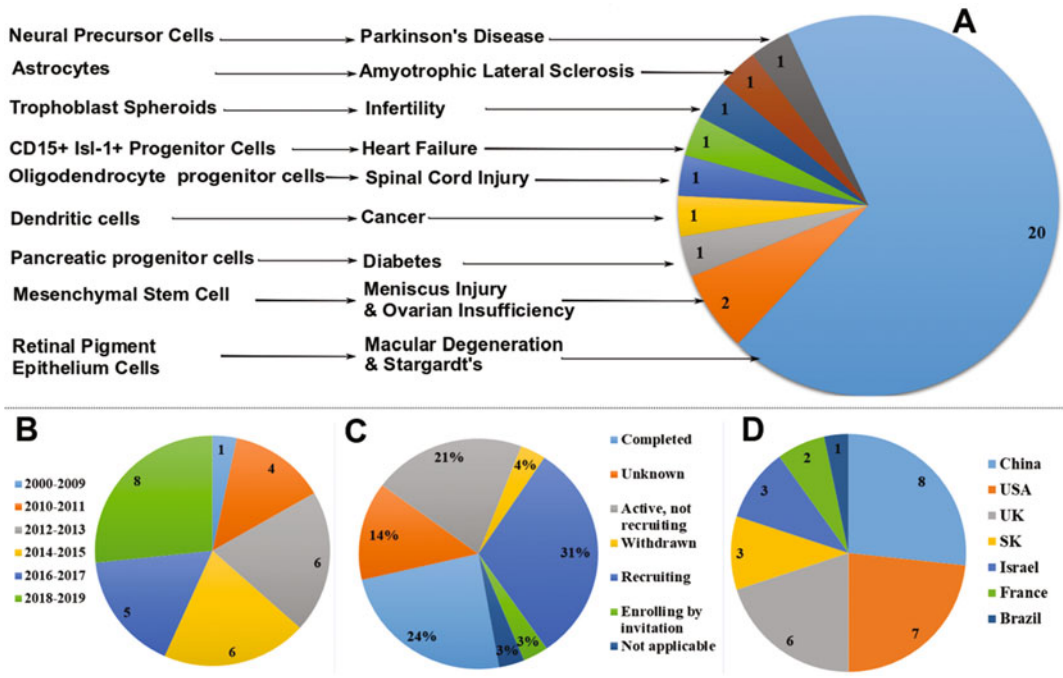

Fig. 3 Review of the clinical trials database (https://clinicaltrials.gov) according to registered studies containing the term "hESC clinical trials", "hESC-derived cell types", plus their "Date", "Status" and "Country". (**a**): Summarize of hESC-based clinical trials and clinical use of differentiated cells from hESC, (**b**): Pie chart of the registered clinical trials according to Date of registration, (**c**): Status of trials in Clinicaltrials.gov, and (**d**): Pie chart of the registered clinical trials according to registrant countries

(First Ever Quadriplegic Treated With Stem Cells Regains Motor Control in His Upper Body – Good News Network 2016; Shroff et al. 2017).

In the area of preclinical studies, Fandel et al. (2016) showed that transplanted hESC-derived cells significantly improved pain and bladder function in rodent SCI models (Blesch 2016; Fandel et al. 2016). A case report was published in 2016 in New Deli, India, on the clinical uses of hESC for one patient with multiple sclerosis (MS) and one with Lyme disease (LD) (Shroff 2016b). The results showed that the clinical use of hESC was a suitable treatment for patients with MS or LD. Also, the patients enjoyed considerable benefits in terms of daily motor skills, physical strength, learning capability, and physical power from cell therapy between 2014 and 2015. MRI-SPECT images and examinations also supported this finding. The two patients under study also received physiotherapy support and antibiotics after cell transplantation (Shroff 2016b).

On the other hand, previous studies on rats have shown that hESC-derived oligodendrocytes had a positive effect on remyelination of nerve fibers and body motor stimulation (Sharp et al. 2009).

A study was initiated in Australia to obtain hESC-derived neural cells to treat PD (Menasché et al. 2015a). One common issue in all such studies is the limitations in separating dopaminergic cells needed for treating PD from other neural cells like serotonergic cells (Schulz et al. 2012). There have been studies on transplanting embryonic abdominal mesencephalon pluripotent cells for treating PD. These cells are abundant in dopaminergic neural cells, which are needed in PD patients. Experiments on animals with the same disease have been promising, such that along with the revival of cells, the reticular formation was

improved (ISCO completes dosing of 2nd cohort in Parkinson's disease clinical trial n.d.; Kushner et al. 2014). Moreover, academic associations have initiated activities to transplant ESC to PD patients, along with observing medical regulations (Canet-Aviles et al. 2014; Lindvall et al. 2004; Takagi et al. 2005). Hence, in future studies, dopaminergic neurons differentiated from pluripotent stem cells, including ESC and iPSC can play a primary role in cellular-based therapy of PD.

4 Cardiovascular Diseases

A large number of clinical trials have shown that stem cell therapy can be a promising novel therapeutic approach for the treatment of cardiovascular diseases. Studies have shown that hESC-derived cardiomyocytes express cardiac transcription factors and display adult cardiomyocyte phenotype and pulsing activity *in vitro* (Cambria et al. 2017; Sanganalmath and Bolli 2013). A study in Paris Public Hospital, France, in 2013 entered trial Phase 1 (NCT02057900) in an attempt to survey the safety of cardiomyocyte progenitor cells (CD15+ Isl-1+) derived from hESC for 10 severe heart failure patients (Menasché et al. 2015b). The study used fibrin gel scaffold as cell-containing implants to provide the condition needed for the growth, proliferation, and differentiation of cardiomyocyte cells. Without developing tumors at the spot or the outer tissues, the cells were capable of generating cardiomyocyte cell lines. The study also surveyed aspects like stimulating the immunity system of the host and electrical stimulation of cardiomyocytes. The results of this clinical study as the first ESC-based clinical trial study were reported in 2015 and 2017. They demonstrated the feasibility of generating a clinical-grade population of hESC-derived cardiac progenitor cells and combining them within a tissue-engineered construct (Menasché et al. 2015b). One year later, the team introduced a protocol that generated a highly purified population of cardiovascular progenitors to be used in their clinical trial study. They demonstrated the technical feasibility of producing clinical-grade hESC-derived cardiovascular progenitor cells. They supported their short- and medium-term safety, thereby setting the grounds for adequately powered efficacy studies (Menasché et al. 2018). Additionally, ESC-derived exosomes displayed enhanced cardiac function and repair in infarcted mice (Khan et al. 2015). Investigations are continued in ESC-based therapy in the treatment of cardiovascular diseases.

5 Diabetes Type I

Literature reviews showed that several preclinical studies support the feasibility of using differentiated hESC for treating diabetes mellitus patients (Han'guk Palsaeng Saengmul Hakhoe, 심중현, 우동훈, & 김종훈 2007; Jacobson and Tzanakakis 2018; Xing et al. 2015). Recently, a clinical study that was initiated in the USA in 2014 and expanded to Canada in 2015 developed a cell culture of insulin-secreting β cells of the pancreas Langerhans Islets derived from hESC and without feeding layers to treat diabetes type I. Totally, 40 patients with type 1 diabetes mellitus participated in the study to place a device containing hESC-derived pancreatic precursor cells under their skin. The study led to exciting and reliable results, and the *in vivo* secretion of insulin in the patients was confirmed at the final stage of cellular differentiation and survey of reactions to glucose (Ilic et al. 2015). This clinical study, registered at www.clinicaltrials.gov (NCT02239354), was supported substantially by the California Institute for Regenerative Medicine (www.cirm.ca.gov) and global type 1 diabetes research organization JDRF (www.jdrf.org).

6 Ophthalmology

Given the regenerative and differentiative capacities of ESC, hESC-based therapy has been tested for retinal disorders, some of which have shown excellent prospects. One of the first hESC-derived cells was transplanted into human patients with retinal disorders. In the first trial,

Schwartz et al. reported that after 4 months, the hESC-derived retinal pigment epithelium (RPE) cells showed no signs of hyperproliferation, tumorigenicity, ectopic tissue formation, or apparent rejection (Schwartz et al. 2012). They extended their study by following up two open-label, Phase 1/2 studies. Their clinical trial results were published in 2015 and supported the primary findings obtained in Ocata Company. The study utilized ECS for the differentiation of RPE to treat macular degeneration (Schwartz et al. 2015). The results showed 99% differentiation into RPE cells. Nine patients with macular degeneration (older than 55 years) and nine patients with Stargardt's disease (older than 18 years) were grouped into cell concentration groups 10×10^4, 15×10^4 and 50×10^4 for each eye. The significant side-effects were rooted in surgery challenges and immunity system suppression. After 22 months, improvement in eyesight of 19 patients was confirmed, seven patients experienced no improvement, and one patient demonstrated symptoms of further loss of eyesight. An unbalanced expansion of the regenerated retinal epithelium was observed in 72% of the patients. The study results also indicated that in 3–12 months, the quality of life index of the patients, in terms of sight, improved from 16 to 25 in macular degeneration patients and from 8 to 20 in patients with Stargardt's disease (Schwartz et al. 2015). However, achieving a more balanced substrate of epithelium cells and better results requires further studies (Liu et al. 2018).

A similar study recently conducted in South Korea employed cell therapy and retinal transplantation with hESC-derived RPE in two patients with age-induced macular degeneration and two patients with Stargardt macular dystrophy (Song et al. 2015). The follow-up term was 1 year and no side effect indicating hyperproliferation at the transplantation site, tumor generation, ectopic tissue, or the like was observed. In addition, visual acuity in three patients improved by 9–19 points and remained unchanged in one patient. Ophthalmologic tests after the surgery indicated no complications (Song et al. 2015).

Recently, Chinese researchers have developed a clinical-grade hESC line under xeno-free conditions that were differentiated into RPE cells for the treatment of wet age-related macular degeneration (wet-AMD) patients (Liu et al. 2018). Other studies in the UK and USA were carried out on treating macular degeneration using RPE cells derived from ESC, where the derived epithelial cells were grown on a very thin scaffold to be injected below the photoreceptor cells. There are also reports by Japanese researchers of using epithelial cells derived from iPSC for retinal transplantation (Cyranoski 2014; Song et al. 2015).

7 Immuno-Therapy

Currently, stem cell studies have provided a new viewpoint for vaccine engineering to employ engineered cells as a vaccine for the prevention and treatment of some incurable diseases. Stem cell vaccines are effective in promoting the pre-existing anti-cancer immune responses. Therefore, Marek's disease (Vautherot et al. 2017), hepatic tumors (Zheng et al. 2017) and lung cancer (Yaddanapudi et al. 2012) are the essential candidate diseases for applying ESC-derived vaccines. GRNVAC2 or AST-VAC2 is an hESC-derived cancer vaccine (allogeneic) designed to stimulate immune systems of patients with non-small-cell lung carcinoma. AST-VAC2 acts against telomerase, the main cancer protein rarely expressed in healthy adult cells. In 2014, a clinical trial of AST-VAC2 as hESC-derived mature dendritic cells (hESC-DCs) was designed to evaluate the safety and toxicity of the vaccine, its feasibility, stimulation of patient immune responses to telomerase and AST-VAC2, and clinical outcomes after

AST-VAC2 administration in lung cancer patients (BioTime Subsidiary, Asterias Biotherapeutics, and Cancer Research UK and Cancer Research Technology Partner for Clinical Trial of Immunotherapy Vaccine for Lung Cancer | Business Wire 2014).

8 Guidelines About the Protocols of Using hESC in Basic Studies and Clinical Trials

Cell therapy techniques based on stem cells are advancing and many of them are in the clinical trial stage (Golchin et al. 2018a). Besides, the International Association of Stem Cells has proposed establishing an international bank of stem cells. The association is a national, and at the same time, an international group and it is aimed at conducting studies on stem cells to develop a set of principles (ethical-scientific) and techniques to store stem cells and to test, examine, and use hESC for therapeutic purposes (Crook et al. 2010). These guidelines are set by FDA, USA, along with recommendations for those in charge of judging proposals of stem cells therapeutic experiments. The good manufacturing practice (GMP) conditions recommended by FDA and European Medical Administration (EMA) are aimed at developing standards of using clinical guidelines and optimizing the safety and quality of stem cells used for cell therapy (Golchin and Farahany 2019).

In Japan, clinical works using hESC were virtually impossible before the adoption of "Guidelines on the Derivation of hESC" in late 2014 (Azuma 2015). All these guidelines and codes are designed to make sure that stem cells are supplied for clinical applications with the highest quality and functionality (Carpenter and Rao 2015; Unger et al. 2008). The notable point is that these guidelines do not guarantee the quality and effectiveness of the cells derived from hESC (Guidance for FDA reviews and sponsors content and review of chemistry, manufacturing, and control (CMC) information for human somatic cell therapy investigational new drug applications (INDS). | Search Results | IUCAT Kokomo n.d.). They are only designed to ensure the replicability of regeneration of stem cells and that it is possible to rely on specific and precise measures to guarantee the health and wellbeing of patients. The following sections discuss the challenges and meaningful solutions in using ESC for clinical trial studies.

9 hESC Culture for Clinical Trial Studies

Among the concerns and limitations of using embryonic pluripotent cells is the potential risk of the development of germ layer tumors, which has been observed in experimental studies on rats and other models (Benninger et al. 2003; Roy et al. 2006). It is essential, therefore, to consider ECS derivatives as candidates for tumor-free cell transplantation and replacement. Another concern is the differentiation of cells derived from embryonic lines into the cells of other tissues. For instance, imperfect transplants of stem cell-derived myocardial cells to damaged myocardium may alter electrical activity (arrhythmic stimulation) (Gepstein et al. 2010). Therefore, there is a need for further optimization and development of differentiation and purification protocols for preclinical tests and clinical treatment to minimize the risk of ectopic cells. Since it is possible to culture and obtain specific types of cells using specific molecules at critical time points, most of these methods yield moderate enrichment that is not adequate or fit for clinical application.

Marker molecules are single-stranded oligonucleotides that generate fluorescent signals when bonding with their target mRNA; therefore, these cells can be recognized based on their fluorescent activities. The notable point is that the marker molecule has a short life term and does not change the genomic structure of hESC. Therefore, this method can be used to enrich cellular populations derived from suitable stem cells or to single out a variety of unwanted cells like undifferentiated ESC that can generate tumors (King et al. 2011).

10 Providing a Cell Culture System Free of any Xenobiotics for hESC

Many of the available ESC lines are obtained through isolation and *in vitro* proliferation in contact with animal products. This creates the risk of contamination with toxic materials, microorganisms, and unknown organisms that might stimulate an immune reaction.

Currently, the ESC line for microbiological tests is recommended by the International Stem Cell Banking Initiative (ISCBI). The FDA requires access to documents, sources, and information of genetic potential of the modified elements and diseases causing factors in each cell derived from hESC for clinical trial purposes. It is essential, therefore, to prevent any exposure to xenobiotics. Recently developed cultures for trial studies are required to ensure that stem cells are kept away from xenobiotics (Catalina et al. 2008; Stasi et al. 2014). Among the measures taken to this end are serum replacement without xenobiotics (e.g., Knockout Serum Replacer [Invitrogen]) and culture medium (e.g., HESGRO [Millipore] or TeSR [STEMCELL])(Hannoun et al. 2010; Ritner et al. 2011; Wong and Bernstein 2010).

There have been advances in the development of culture systems without feeding layers. These cultures minimize the contamination of ESC with xenobiotics compared with the cells cultured on feeding cellular layers (Fukusumi et al. 2013). Cultures without feeding layers and xenobiotics that contain a mixture of recombinant factors with features like controlling differentiation and preserving the pluripotent status of ESC are now commercially available. However, there are reports of high chromosomal instability and high risk of ESC instability from a genetics point of view (Catalina et al. 2008). This is one of the limitations of the clinical use of ESC for clinical trials. Embryonic stem cells fed by layers of human feeder cells like fibroblasts can attenuate these limitations to some extent (Ilic et al. 2009; Ström et al. 2010). Moreover, the human umbilical cord blood serum (UCBS) matrix ensures the highest proliferation speed and preserves karyotype stability without using the feeding layers and serum up to the 10th passage (Ding et al. 2015). However, further progress toward the development of an ESC line suitable for clinical trial studies needs more studies, and the good news is that there are several studies underway in this field.

11 Genetic Anomalies in hESC Lines

Embryonic stem cells derived at the early stage of embryonic life possess the best specifications found in stem cells; however, having the most desired specifications and capacity does not necessarily mean that the cell line suits medical applications. There are reports of chromosome and genome instability in several hESC lines, along with losing heterozygosity or an increase in the diversity of cancer-related genes (Lefort et al. 2008; Närvä et al. 2010). Taking into account that these changes and mutations are less frequent in lower passage cells, it appears that mutations are more likely to appear in long-term cultures. Some of these instabilities and mutations occur through the early proliferation and cellular differentiation stages when cells adapt to the culture condition (Baker et al. 2007). Given the self-renewal and fast proliferation and differentiation of ESC *in vivo* and *in vitro*, the risk of DNA damage or DNA cut and break in double-stranded DNA is higher in the cells' genome. However, studies have recommended that the genome stability of ESC is higher than somatic cells and their genetic response to damage or repair of damage is more robust (Baker et al. 2007). At any rate, these observations prepare the ground for determining the standards of working with hESC lines and their specifications, especially in long-term cultures, which eventually leads to medical applications.

12 Inhibiting Immunity Rejection of Transplantation OF ESC-Derived Cells

The immunity system rejects the transplantation of ESC at the differentiation and proliferation stages as they express both major histocompatibility complex (MHC) I & II, and these tissue adaptation molecules trivially stimulate the immunity system (Bradley et al. 2002). Another factor stimulating the immune system of the receiver is the incompatibility of ABO blood type antigens of the donor and recipient (Lee et al. 2010). These two issues should always be taken into account for tissue and cell transplantation, as they are the causes of stimulation of the immune system and transplant rejection. The best way to avoid transplant rejection by the immune system is to use genetically identical cells from the donor and recipient. Therefore, SCNT is a solution to generate unique ESC lines. Through this method, enucleated egg cells grow to the blastocyst stage and then ESC is derived. The derived stem cells in this method possess an immunological profile compatible with that of the recipient so that they can be used for cell therapeutic purposes. This technique is used for ESC of different species. However, there is no SCNT-derived hESC available yet (Bradley et al. 2002). Another strategy to derive hESC with more compatibility with the recipient is cell engineering to develop a tissue with the lowest tissue incompatibility. For instance, one way is to use cells from a donor with blood type O and suppressed HLA expression (Drukker 2004).

Moreover, there have been proposals to create hESC line banks to supply ABO/HLA combinations that are potentially compatible with the majority of patients. There have been studies supporting such proposals (Nakajima et al. 2007; Taylor et al. 2005). For instance, Taylor et al. concluded that about 150 hESC cell lines express HLA that fit the majority of the USA and UK populations combined (Taylor et al. 2005).

13 Advantages of Using ESC Along with Other Stem Cells

Regardless of all the limitations of using hESC, they are still considered as a critical source of stem cells for clinical trial studies and cell therapy. Currently, there are thousands of hESC lines available for research worldwide; however, only 400 hESC lines are approved for use by the US federal funding, and only five hESC lines were used in preliminary studies (Ludwig et al. 2018). Other sources of stem cells are adult stem cells and iPSC. Despite hESC, iPSC can be derived from patients, which solves the limitation of allogeneic sources.

Moreover, the use of iPSC raises no moral and religious concerns that are the case in using ESC. However, there are many limitations in using adult stem cells and iPSC. Thus, in some cases, hESC are potentially preferred (Drukker 2004).

Adult stem cells are derived from non-embryonic tissues and they are generally positioned in their source tissues. Like hESC, adult stem cells are capable of self-renewal; however, they have a limited potential and can be differentiated into their source tissue cells only once. In general, adult stem cells cannot differentiate into different cells of the source tissue as embryonic stem cells can. Moreover, they cannot preserve their growth capacity in the long run. Another problem lies with using stem cells derived from aged individuals as they have a shallow potential of differentiation and proliferation (Phinney and Prockop 2007; Stenderup et al. 2003).

iPSC is generated by the differentiation of somatic cells that have reached the pluripotent stage through reprogramming. This was first reported in the study by Yamanaka et al. (2006) where cells demonstrated key features of early ESC developed through activating four relatively different factors (i.e., Sox2, Klf4, Oct4, and C-myc) in murine fibroblast cells. The technique was implemented on human cells in 2007 (Ilic

et al. 2015). Different methods have been employed to express these factors and iPSC (Somersall et al. 2013). There are methods available to transfer these factors to somatic cells and some of them, such as retrovirus, lentivirus, and adenovirus, are featured with the risk of hazardous and permanent genome contamination.

Moreover, some iPSC lines are not genetically stable and large-scale genomic rearrangements demonstrate instability and variations in the number of copies, many copies of a gene, and karyotypic anomalies even at the early stages of passage (Gore et al. 2011). There are strategies based on mixing without using plasmid to minimize genome damages and mutations, such as synthetic RNA transfer, viral RNA transfer, or adding purified cell-penetrating recombinant proteins (Kim et al. 2009; Zhou et al. 2009). However, these methods of deriving iPSC are less efficient compared with viral vectors (Golchin et al. 2018b). Also, in some iPSC lines, no positive change in terms of decrease of mutagenicity was observed without a virus vector. Recent studies have also shown that in addition to genomic changes, iPSC includes epigenetic specifications like improper or incomplete reprogramming. Among these are DNA mutations of iPSC, mostly resembling primary somatic cells and indicating that iPSC are not entirely at the pluripotent stage (Lister et al. 2011).

Moreover, there are reports of survival of ESC for 18 years, while there is no similar report for iPSC. It must be considered that iPSC were generated for the first time 13 years ago (Pruksananonda et al. 2012). However, patient-derived iPSC is not suitable for age-related diseases since the somatic cells of the elderly probably have a considerable amount of genomic mutagens and hazardous epigenetic maps. However, the NIH twenty-first Century Cures Act in the USA excludes explicitly funding for hESC-based technologies. At the same time, it supports iPSC, even though an overwhelming amount of associated research, validation, and characterization works have been done on hESC, and the considerable progress in clinical trials has been achieved using hESC (Ludwig et al. 2018).

The most reasonable assessment based on the current knowledge is that hESC-based therapies have no considerable future in terms of clinical application, at least in the next few years. After the introduction of human-induced pluripotent stem cells (hiPSC), a comparison between pluripotency features of iPSC and hESC was made possible. While ethical problems have consistently limited hESC applications, iPSC display differences compared to hESC, including a higher propensity for specification toward particular cell types based on the memory retained from their origin somatic cell source (K. Kim et al. 2010; Noguchi et al. 2018).

The main restrictions regarding the clinical use of hESC are the possible immunogenic responses, a variety of non-differentiated cell types, and teratogens population. However, rapid and reliable methods for isolating hiPSC populations are primarily needed for quality control in cellular-based therapy applications. However, new studies introduced novel methods to distinguish and isolate undifferentiated and teratogenic cells from target cell populations for cell-based therapeutic purposes (Tomuleasa et al. 2014; Wang et al. 2011). Nevertheless, the newest EU innovation program for Horizon 2020 does not restrict researchers in their use of cell type (hESC or hiPSC). In addition to that, currently, iPSC provides a unique opportunity for creating different disease models and has attracted full attention from scientists in the field of regenerative medicine. There are ongoing studies on the safety and efficiency of iPSC. However, studies are continued for improving cellular therapy protocols and products by using different cell sources (Golchin et al. 2019; Golchin 2020).

14 Outlook and Future Directions

Stem cell-based therapy has created much excitement in the regenerative medicine field, as it allows us to the usage of human-derived cells

and tissue in therapeutic targets. The application of hESC as a promising stem cell source for cellular-based therapies is expected to boost progress through to 2025. Specific features of ESC, including self-renewal and pluripotency (Itskovitz-Eldor et al. 2000) enable them to generate three embryonic germ layers (i.e., endoderm, mesoderm and ectoderm), while they can preserve their self-renewal capability. Despite all the difficulties and debates, clinical trial studies on the treatment of several incurable diseases, including spinal cord injury, retinal macular degeneration, type 1 diabetes and heart failure, are already underway. The global hESC market is anticipated to reach US$ 1.06 billion by 2025 (*Human Embryonic Stem Cells (hESC) Market Analysis By Application (Regenerative Medicines, Stem Cell Biology Research, Tissue Engineering, Toxicology Testing), By Country (U.S., UK, Germany, Japan, China), And Segment Forecasts, 2014–2025*, 2017), with Europe accounting for a considerable share of the market following North America (*Human Embryonic Stem Cells (hESC) Market Analysis By Application (Regenerative Medicines, Stem Cell Biology Research, Tissue Engineering, Toxicology Testing), By Country (U.S., UK, Germany, Japan, China), And Segment Forecasts, 2014–2025*, 2017). Hence, hESC technology provides a unique opportunity for regenerative medicine applications in humans.

According to more than two decades of research in the hESC field, the potential applications of hESC in human basic developmental biology and regenerative medicine are vivid. However, several serious problems need to be resolved before the full potential of hESC in this field can be realized. Therefore, further progress in ongoing trials is necessary to develop a more efficient hESC culture system, develop a stable enzymatic passaging technique, improve techniques for genetic manipulation of hESC, enhance ES cell lines capability (SEMB 2005), design and pursue hESC clinical trials, and develop the therapies available in less developed countries. While hESC-based therapy products or approaches have not extensively entered the clinic, the high costs of these therapies have raised some issues about the real cost-benefit analysis of these new therapies. For incurable and rare devastating diseases, these high costs may be supported if the treatment effect is significant, but for more general diseases, cost tags differing from $300,000 to $750,000 are not currently acceptable (Ylä-Herttuala 2019). However, the natural linkage between the destinations of basic and clinical hESC research and the industrial cellular-based therapy sector has continued to permeate the field about prospects for the regenerative medicine and cellular-based therapy fields among external observers, senior academic researchers involved in the field, clinicians, investors, policymakers, and health care manufacturers (Huang and Jong 2019; Klein et al. 2009; Mason and Manzotti 2009).

With recently discovered relatively specific genome editing techniques such as clustered regularly-interspaced short palindromic repeats (CRISPR), disease-specific mutations can be produced in manipulated fine hESC lines that have essential DNA methylation footprint of cells(Ilic and Ogilvie 2017; Jinek et al. 2014). After the progression of hiPSC, because of their free of ethical issues, hESC started to lose their unique appeal(Takahashi et al. 2007; Yu et al. 2007). However, as the critical difference between hESC and iPSC is the potentially modified genomic and epigenetic condition of iPSC, additional standards will be needed in hiPSC-based trials. These additional standards need additional cost and time that may provide some limitations for iPSC-based clinical applications.

On the other hand, hESC derivation provides a different occasion for early human development studies. The discovery of ESC represented a significant advancement in developmental biology, as it allowed the usual manipulation of the cell genome. ESC have been used extensively for generating cell mutants for more than two decades, and their application as a model for developmental biology has been improved.

15 Conclusion

Since the limitations of ESC have been demonstrated by preclinical studies and experiments on animals, the improvement of the culture environment and the derived cells needs further studies. On the other hand, the chance of treating hard-to-cure diseases and helping transplantation candidates using cell therapy is an exciting opportunity that justifies further studies. Contrary to the speed of hESC research from the laboratory to clinical trials, regulation and funding barriers have decelerated the progress. Moreover, ESC lines have been useful in designing therapeutic methods and discovering new drugs and they hold great promises. On the other hand, cell therapy is moving toward using differentiated cells derived from stem cells, among which ESC have attracted a great deal of attention thanks to their unique specifications. There are many scientific, ethical, and legal hurdles to deal with in clinical using ESC. It is good news, however, that clinical studies using hESC for therapeutic purposes have been already initiated and proper steps have been taken to differentiate hESC into specialized cells suitable for cell therapy. Such studies pave the way of using medical advantages of hESC in regenerative medicine, mostly for the age-related diseases that are going to be a severe issue for the health sector soon. Nonetheless, hESC are associated with numerous drawbacks, including the concomitant administration of lifelong immunosuppressive therapy, tumorigenicity, limited effectiveness, and ethical problems. As mentioned before, recent studies support the application of ESC in clinical settings. When all limitations of ESC are entirely overcome, ESC will become superior to the existing stem cell sources.

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Functions of Mesenchymal Stem Cells in Cardiac Repair

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Abstract

Myocardial infarction (MI) and heart failure (HF) are significant contributors of mortality worldwide. Mesenchymal stem cells (MSCs) hold a great potential for cardiac regenerative medicine-based therapies. Their therapeutic potential has been widely investigated in various in-vitro and in-vivo preclinical models. Besides, they have been tested in clinical trials of MI and HF with various outcomes. Differentiation to lineages of cardiac cells, neovascularization, anti-fibrotic, anti-inflammatory, anti-apoptotic and immune modulatory effects are the main drivers of MSC functions during cardiac repair. However, the main mechanisms regulating these functions and cross-talk between cells are not fully known yet. Increasing line of evidence also suggests that secretomes of MSCs and/or their extracellular vesicles play significant roles in a paracrine manner while mediating these functions. This chapter aims to summarize and highlight cardiac repair functions of MSCs during cardiac repair.

Keywords

Anti-fibrotic effect · Cardiac repair · Differentiation · Extracellular vesicles · Heart failure · Immune modulation · Mesenchymal stem cells · Myocardial infarction · Neovascularization · Paracrine function

1 Introduction

Cardiovascular diseases (CVDs) including myocardial infarction (MI) and the resulting heart failure (HF) are important causes of death and disability worldwide. This is because of the inadequate renewal capacity of the heart after a pathologic incident. Approximately one billion cardiomyocytes and two to three billion myocardial cells of the endothelium and the vascular smooth muscle die after an MI and the remaining tissue is replaced by a fibrotic collagenous scar subsequently leading to decline in cardiac performance. This is the main pathophysiological pathway progressing irreversibly to HF and death (Majka

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et al. 2017). Although there have been significant improvements in cardiac therapies that reverse or slow this process partially, none of the available treatments except heart transplantation can completely achieve this goal (Majka et al. 2017; Elnakish et al. 2012).

Stem cell therapies aim to replace damaged heart tissue with fully functional cardiomyocytes that entirely proliferate and couple with the surrounding myocardium electromechanically and promote neovascularization in order to supply blood to the newly existing tissue. There are many types of stem cells that have been demonstrated in-vivo to fit these purposes such as induced pluripotent stem cells (iPSCs), embryonic stem cells (ESCs), hematopoietic stem cells, endothelial progenitor cells and mesenchymal stem cells (MSCs) (Muller et al. 2018). On the other hand, cardiac progenitor cells that locally reside in the heart have been defined and extensively investigated although their existence and regenerative potential have been seriously doubted in recent years (Chien et al. 2019). Among all these cell types, MSCs come to the fore because of their distinct advantages compared to other cell types. These advantages mostly include easy accessibility from several tissues with no ethical issues, expansion in large numbers in a very short time, well-defined characterization and differentiation procedures, multipotent differentiation capacity, low immunogenicity and tumorigenicity, immune modulatory effects, and secretion of therapeutic molecules (Majka et al. 2017; Elnakish et al. 2012; Bagno et al. 2018). Furthermore, their safety profile and beneficial functions have been widely investigated in animal models and patient populations with various CVD phenotypes originating from diverse pathophysiological processes such as acute MI, ischemic cardiomyopathy and non-ischemic HF (White et al. 2016; Chou et al. 2014). This chapter aims to summarize and highlight functions of MSCs during cardiac repair.

2 Mesenchymal Stem Cell Types

MSCs were initially discovered by Alexander Friedenstein and coworkers in the 1970s and described as bone marrow (BM) stromal cells that are able to transdifferentiate into the mesodermal lineage and promote hematopoiesis (Friedenstein et al. 1970; Friedenstein et al. 1974). However, they were defined as "Mesenchymal Stem Cell" by Arnold Caplan in the last decade of twentieth century (Caplan 1986; Caplan 1991). In order to standardize the characterization, the Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy suggested some criteria for characterization of MSCs. These are plastic adherence capacity and differentiation into osteoblast, adipocyte and chondroblast lineages under in-vitro conditions. Furthermore, they should express cluster of differentiation (CD) surface markers including CD73, CD90 and CD105, but not CD11b, CD19, CD31, CD34, and CD45 (Dominici et al. 2006). Besides, MSCs have various terminologies such as mesenchymal stromal cells, multipotent adult progenitor cells, medicinal signaling cells, and mesenchymal progenitor cells, which are used interchangeably (Majka et al. 2017).

MSCs can be induced to transdifferentiate into myocardial cells in-vitro through various methods by culturing them with various chemicals. 5-Azacytidine, bone morphogenetic protein-2, angiotensin-II, dimethyl sulfoxide, fibroblast growth factor-4 are the prevailing chemicals that are used to induce cardiomyocyte differentiation of MSCs. Coculturing MSCs with cardiomyocytes or genetic modification are the featured induction methods for cardiomyocyte differentiation (Chou et al. 2014; Shen et al. 2015). Likewise, there are various protocols in order to induce endothelial differentiation of MSCs including culturing with vascular endothelial growth factor (VEGF), hypoxic conditioning or applying hemodynamic-like forces for mechano-transduction (Afra and Matin 2020; Vittorio et al. 2013). However, it should be noted that all of these differentiation methods are limited for clinical translation yet because of their inability to yield cells at quantitatively, morphologically, genetically and functionally acceptable levels. For example, co-culturing BM-MSCs with rat embryonic cardiomyocytes yielded cardiac specific marker expression while retaining MSC

characteristics of the cells with lack of electrophysiological features of cardiomyocytes such as action potential generation or typical ionic currents (Rose et al. 2008). In a similar manner, 5-azacytidine treated human MSCs obtained from umbilical cord (UC), cord blood and BM did not yield cardiomyocytes at a sufficient level for cardiac repair (Martin-Rendon et al. 2008).

MSCs can be obtained from diverse sources of the body. BM, UC and adipose tissue are the most utilized sources in preclinical and clinical trials (Fig. 1), however MSCs can also be obtained and cultured from amniotic fluid, placenta, skin, dental pulp, endometrium, gingiva, synovium and peripheral blood. However, it should be underlined that MSCs obtained from different sources might differ in terms of cell surface markers, differentiation capacity and paracrine factor secretion (Berebichez-Fridman and Montero-Olvera 2018; Via et al. 2012; Wu et al. 2018). For instance, adipose MSCs can be obtained easily in higher numbers than MSCs obtained from BM and UC (Kern et al. 2006). Moreover, angiogenic activity of adipose MSCs were higher than MSCs obtained from the endometrium and UC both under in-vitro and in-vivo conditions (Lu et al. 2018). On the other hand, comorbid conditions and age of the donor may be a problematic situation during the translational process of autologous MSC applications. Enhanced proliferation rates of adipose MSCs and easy availability of adipose tissue compared to BM harvesting, which is an invasive and painful procedure, highlight the potential of adipose MSCs in autologous applications and emergency situations such as MI and HF (Öztürk et al. 2020).

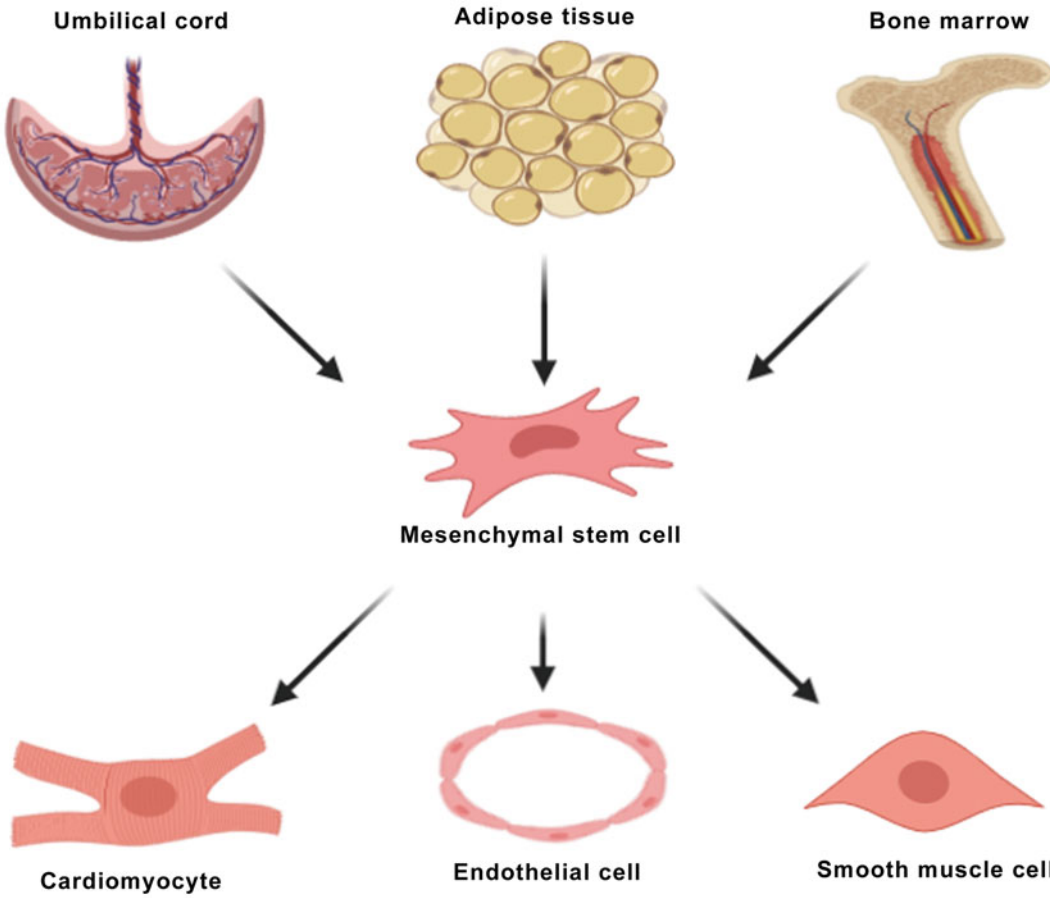

Fig. 1 Major sources of mesenchymal stem cells and cell types of main interest for cardiac repair

MSCs can also be transdifferentiated from pluripotent cells such as iPSCs and ESCs with higher proliferation rates, telomerase activity and lower cell senescence compared to BM-MSCs (Lian et al. 2010; Gao et al. 2017; Sun et al. 2015). Besides, iPSC-derived MSCs possess the basic criteria of MSCs regarding expression of surface markers and differentiation into multilineages with no teratogenic effects observed in animal models (Lian et al. 2010; Gao et al. 2017; Lian et al. 2016).

3 Mesenchymal Stem Cell Functions

3.1 Differentiation

Cardiac tissue mostly originates from the mesodermal layer and cardiomyogenic differentiation is closely related with the signals derived from cells of ectodermal and endodermal origin. Cardiac lineage differentiation, which forms cell types such as cardiomyocytes, smooth muscle cells, endothelial cells and cardiac fibroblasts, is an extremely active process that involves specialization, spatial integration and coordination of diverse cells and signaling pathways and considered as the desired mechanism of action of stem cell-based cardiac regenerative medicine therapies (Leitolis et al. 2019). This emphasis is based on the fact that the heart has almost no regeneration capacity and the healing process occurs with a fibrotic scar after an MI. Theoretically, stem cells engraft to the infarcted cardiac tissue after transplantation, constitute a myocardial mass with a functional vascular network and reverse altered ventricle to normally functioning geometry (Mazo et al. 2012).

MSCs can undergo cardiomyogenic transformation as shown both in in-vitro conditions and in-vivo tracking-based studies (Makino et al. 1999; Pei et al. 2017). Theoretically, there are several molecules and pathways of MSCs such as hepatocyte growth factor (HGF), platelet derived growth factor (PDGF), Wnt and Notch-1 signaling pathways which take significant role in the proliferation and differentiation of MSCs to cardiomyocytes (Farzaneh et al. 2019). Human MSCs tagged with β-galactosidase were shown to engraft in the adult murine myocardium and transdifferentiate into a cardiomyocyte phenotype in a previous study (Toma et al. 2002). Likewise, MSC engraftment and differentiation to cardiomyocyte, smooth muscle and endothelial cells were demonstrated in sex-mismatched experiments of animals with chronically scarred myocardium. Moreover, MSC transplantation contributed significantly to functional improvements and myocardial blood flow while coupling to host myocardium through gap junctions (Quevedo et al. 2009). BM-MSCs were shown to differentiate into smooth muscle and endothelial cells in a canine chronic ischemia model resulting with enhanced neovascularization and cardiac functions (Silva et al. 2005). On the contrary, MSCs did not gain a mature cardiomyocyte phenotype electrophysiologically in a rat MI model but also did not induce ventricular arrhythmias (Wei et al. 2012). Similar discouraging results were also shown in in-vitro studies suggesting a therapeutic effect for MSCs regardless of differentiation (Rose et al. 2008; Martin-Rendon et al. 2008). These conflicting results may originate from the differences in species and culturing conditions of cells that can alter viability or differentiation capacity of MSCs (White et al. 2016). Thus, it might be reasonable to speculate that MSC differentiation to myocardial cell lineage and regeneration is not likely to emerge as the primary therapeutic function of MSCs in cardiac repair.

3.2 Neovascularization

New blood vessel formation from existing endothelial cells, in other words angiogenesis or neovascularization, is an essential task for cardiac tissue repair. This process is coordinated by a complex interaction between extracellular matrix (ECM) components, angiogenic factors and various cells and with the contribution of diverse molecular and biophysical pathways (Elcin 2002; Briquez et al. 2016). There have been intense efforts in the last decades regarding tissue engineering and biomaterial sciences, genetic approaches and clinical trials in order to discover a clinically applicable treatment for therapeutic angiogenesis. However, there is still no clinically

approved pharmacological agent, interventional or surgical procedure yet that enhances the neovascularization and blood supply of infarcted or failing cardiac tissue (Briquez et al. 2016; Elcin et al. 1996; Elcin and Elcin 2006; Koc et al. 2014; Demirdogen et al. 2010).

Stem cells, particularly MSCs, have been extensively studied for therapeutic angiogenesis in diverse animal models and in-vitro assays. MSCs contribute to neovascularization of infarcted cardiac tissues through diverse processes. There is evidence that MSCs are able to transdifferentiate into endothelial cells under in-vitro and in-vivo situations (Quevedo et al. 2009; Silva et al. 2005; Oswald et al. 2004; Li et al. 2007). These MSCs engraft to myocardium, differentiate into endothelial cells and can improve cardiac performance after an MI (Davani et al. 2003). Moreover, MSCs provoke angiogenic processes by secreting various angiogenic agents including VEGF, HGF and angiopoietins (Oskowitz et al. 2011). Among these angiogenic factors, VEGF seems to be the key mediator that supports cardiac protection after an ischemic insult according to a previous rat study demonstrating impaired functions of transplanted MSCs lacking VEGF (Markel et al. 2008). In a similar manner, elimination of vascular cell fate decision but not cardiomyogenic commitment from undifferentiated human BM derived cells was associated with alterations in cardiac functions, decreased capillary and arteriole density as indicated in an in-vivo suicide-gene based study (Yoon et al. 2010). Besides, VEGF gene transfer to MSCs resulted in increased angiogenesis, left ventricular ejection fraction (LVEF) and improved hemodynamic parameters in rats with MI (Gao et al. 2007). Additionally, targeted systemic delivery of VEGF immunoliposomes to ischemic regions of MI rats enhanced engraftment of MSCs, blood vessel density, decreased collagen content in MI tissue and improved left ventricle functions (Tang et al. 2014). Vasculogenic effects of VEGF on MSCs were shown to be mediated through the activation of PDGF receptors acting via the nitric oxide pathway (Gomes et al. 2013). Direct cellular contact of adipose MSCs with early postnatal heart cardiomyocyte fraction is also associated with the formation of capillary tube formation both from the preexisting endothelial cells and stimulation of progenitor cell differentiation to endothelial cells (Rubina et al. 2009). Another proposed mechanism for the angiogenic effects of MSCs is that they functionally and structurally interact with endothelial cells to provide vascular stabilization as resident pericytes (Traktuev et al. 2008).

Although cardiomyogenic differentiation is the desired and suggested therapeutic function of MSCs during cardiac repair, angiogenic contribution of MSCs via diverse mechanisms might be of utmost importance than the other mechanisms.

3.3 Anti-fibrotic Effects

There occurs pathological remodeling of myocardium after an MI that is related with the replacement of myocardial cells by a fibrotic scarred tissue. Cardiac fibroblasts, which are phenotypically transformed to myofibroblasts due to expression of proinflammatory and profibrotic factors after myocardial injury, secrete ECM components including collagen and fibronectin. In fact, this is an adaptive process initially in order to provide the mechanical structure and integrity of the heart but subsequent phases of this process result with impaired cardiac functions, arrhythmias and finally death (Travers et al. 2016; Talman and Ruskoaho 2016).

Despite the tremendous achievements in pharmacological, interventional and surgical management of cardiac diseases, there is still no effective treatment that fully inhibits myocardial scar formation. Stem cell therapies hold promise as antifibrotic agents and alternative to current treatments. Due to their distinctive features in cardio-protection, MSCs have been widely investigated as a possible therapeutic option to inhibit or reverse this myocardial fibrosis process that progresses to HF and death (Elnakish et al. 2013). Increasing lines of data coming from in-vitro and in-vivo models suggest that MSCs reduce cardiac fibrosis and thereby improve cardiac remodeling through the contribution of several pathways and molecules (Elnakish et al. 2012; Elnakish et al. 2013). Additive anti-inflammatory

actions of MSCs also contribute to anti-fibrotic effects after transplantation as demonstrated in a previous rat MI study (Guo et al. 2007). BM-MSC transplantation significantly decreased the synthesis of matrix metalloproteinase-9 (MMP-9), tissue inhibitor of metalloproteinase-1 (TIMP-1) and collagen deposition in injured cardiac tissue after homing as demonstrated in an isoproterenol-induced mice HF model and these anti-fibrotic effects of MSCs were suggested to be mediated through Nuclear factor-ß mediated signaling pathway (Wei et al. 2011). Likewise, MSC transplantation improved cardiac fibrosis in the myocardium by a decrease in collagen volume fraction, collagen type 1 and 3 expression, MMP-2 and MMP-9 in different HF and cardiomyopathy models (Nagaya et al. 2005; Li et al. 2008). MSC therapy was shown to decrease MMP-2, MMP-3, MMP-6, MMP-9, TIMP-1 and TIMP-3 levels in the LV myocardium 28 days after intracoronary transplantation in a pressure overload hypertrophy-induced HF rat model (Molina et al. 2009). Moreover, contribution of antifibrotic and antiapoptotic paracrine factor signaling secreted from MSCs such as HGF, adrenomedullin and insulin-like growth factor-1 was underlined (Li et al. 2008, 2009; Iekushi et al. 2012). In parallel, conditioned medium of MSCs were demonstrated to exert anti-fibrotic effects by modulating proliferation, ~~and~~ collagen secretion and degradation profiles of cardiac fibroblasts (Ohnishi et al. 2007a; Mias et al. 2009). Furthermore, intracardiac injection of MSCs in rats with post-ischemic HF alleviated ventricular fibrosis (Mias et al. 2009). A recent study also highlighted the secretion of prostaglandin-E2 (PGE2) from MSCs that improved cardiac fibrosis and functions in rats with diabetic cardiomyopathy (Jin et al. 2020).

3.4 Immune Modulation

Pathogenesis of MI involves innate and adaptive immune system activation via toll-like receptor-associated pathways with complex interaction of various chemokines, cytokines and inflammatory system cells including neutrophils, mononuclear cells, dendritic cells and lymphocytes. The immune system response plays a decisive function for determining the fate of infarcted cardiac tissue either healing with regeneration or fibrotic scar. Inflammation controls reparative pathways by modulating immune system and inflammatory cells both locally in the infarcted tissue and at a systemic level (Dittrich and Lauridsen 2019; Liu et al. 2016). Moreover, sustained inflammation is associated with progressive impairment in cardiac functions and adverse outcomes in CVD patients (Ruparelia et al. 2017).

The interaction between MSCs and the immune system is highly dynamic and complex. MSCs modulate the properties and functioning of the immune system cells through diverse pathways. For example, MSCs inhibit proliferation of helper and cytotoxic T cells and trigger differentiation to an anti-inflammatory phenotype. MSCs also impair dendritic cell maturation through interleukin-6 (IL-6), which inhibits CD40, CD80 and CD86, and subsequently suppress T-cell activation. Monocytes are induced to differentiate into an anti-inflammatory phenotype by MSCs with the contribution of diverse mechanisms such as transforming growth factor-beta (TGF-ß), IL-10 and PGE2. Moreover, proliferation, cytotoxic activity and cytokine secretion functions of natural killer (NK) cells are decreased, as well as proliferation of B cells is inhibited by MSCs resulting in decreased antibody production (van den Akker et al. 2013).

There is robust evidence that MSCs exert immune modulation and anti-inflammatory effects in preclinical cardiac disease models including MI and HF (Epstein et al. 2017). Intravenous administration of MSCs yielded both local and systemic anti-inflammatory effects such as decrease in NK cells and neutrophils in the heart and NK cells in the spleen in a mice ischemia reperfusion model while improving LVEF and LV end-systolic volume (Luger et al. 2017). Previous studies also underline that intramyocardially-injected MSCs demonstrate anti-inflammatory functions by reducing myocardial tumor necrosis alpha, IL-1 ß, IL-6 levels with a decrease in MMP-1 and TIMP-1 levels that emphasize their anti-fibrotic effects (Guo et al. 2007). Likewise, intravenous MSC injection exerted anti-inflammatory effects

through a decrease in CD68 positive inflammatory cells and monocyte chemoattractant protein-1 expression in myocardial tissue of rats with acute myocarditis (Ohnishi et al. 2007b). Immune modulation capabilities of MSCs were also evident in a phase-2a clinical trial demonstrating decreased number of peripheral NK cells after MSC administration and a significant correlation between the improvement in LVEF and NK cell reduction at 3 months (Butler et al. 2017).

It is known that MSCs have the capacity for immune privilege as a result of human leukocyte antigen (HLA) class I expression and lack of HLA class II expression (Dominici et al. 2006). This feature of MSCs renders remarkable advantage while considering immunologic side effects of allograft applications including graft rejection and graft-versus-host disease (Ringden et al. 2006). For instance, allogeneic BM-MSC transplantation was not associated with significant immunologic reactions in ischemic cardiomyopathy patients at 1 year follow-up as demonstrated in a clinical trial (Hare et al. 2012). MSCs can also have immunosuppressive functions such as directing monocyte differentiation towards an anti-inflammatory phenotype and secreting soluble anti-inflammatory and immunosuppressive mediators including TGF- ß, HGF, PGE2, indoleamine 2,3-dioxygenase, heme oxygenase-1, HLA-G5 and IL-10 (Maggini et al. 2010; Nauta and Fibbe 2007; Uccelli et al. 2008). Despite these striking advantages of MSCs, it should be kept in mind that immune evasion capability of MSCs is altered upon differentiation to cardiac cells that results with major histocompatibility complex antigen expression making them visible to immune system cells (Huang et al. 2010). This alteration occurring through the devastating attack of the immune system against MSCs after differentiation might explain the loss of beneficial effects of MSC transplantation in the long term.

4 Paracrine Functions & Extracellular Vesicles

Engraftment of stem cells into the heart tissue, trans-differentiation into cardiac lineages through coupling electromechanically and functionally with the host tissue is the desired therapeutic function of stem cells in cardiac regenerative medicine. However, emerging data indicate that engraftment and differentiation of stem cells is not at sufficient levels even with intramyocardial and intracoronary applications (Toma et al. 2002; Hong et al. 2014). At this point, it is reasonable to question how MSCs exert their beneficial effects in cardiac disease models and in some clinical trials. It is now evident that beneficial effects of MSCs are mostly mediated through bioactive secretions of MSCs such as growth factors, chemokines, cytokines, cell adhesion molecules, lipid mediators, hormones, gene products and extracellular vesicles (EVs) that act in a paracrine fashion (Fu et al. 2017; Pokrovskaya et al. 2020). Accordingly, the term "paracrine hypothesis" has been suggested in recent years as an alternative mechanism for mechanism of action stem cells (Gnecchi et al. 2016).

Although MSC transplantation was indicated to be relatively safe in previous cardiac studies, there is an undeniable risk for tumorigenesis as well as immune rejection in allogeneic applications. On the other hand, autologous application may be an alternative with its inherent limitations and technical difficulties (Ozturk and Elcin 2018). Therefore, transplantation of MSC secretomes has come to the fore in recent years as therapeutic cell-free agents. For instance, intramyocardial injection of conditioned medium from BM-MSCs that overexpress protein kinase B (Akt) was shown to ameliorate left ventricular functions at the acute phase of MI with no evidence of de novo cardiomyogenesis in rats (Gnecchi et al. 2006). Similar functional effects of the conditioned medium of MSCs were demonstrated in porcine models (Timmers et al. 2011; Timmers et al. 2007). Besides, intravenously-injected human MSCs enhanced cardiac tissue repair in mice with MI without engraftment because a significant proportion of cells were trapped in the lungs (Lee et al. 2009). These secretomes of MSCs are secreted either directly or packaged in a cargo named as EVs and they regulate cardiac lineage differentiation, angiogenic, anti-fibrotic, anti-inflammatory and immune modulation functions of MSCs in the infarcted cardiac tissue and/or failing heart (Gallina et al. 2015).

EVs are nanosized, lipid membrane enclosed complexes that are secreted from all cells in the body. They regulate intercellular communication between cells by transporting bioactive molecules including proteins, lipids and genetic components (Andaloussi et al. 2013). Because they represent the native characteristics of their parent cell both in physiological and pathological conditions, they have been investigated as a potential biomarker in various CVD patient populations. Because they carry cargo between cells, they have been utilized as potential drug delivery systems in CVD (Chong et al. 2019). These unique characteristics of EVs have attracted great interest and they have been proposed as an alternative therapeutic option as cell-free agents instead of stem cells (Chong et al. 2019; Boulanger et al. 2017). For instance, MSC-derived EVs were demonstrated to increase neovascularization under in-vitro and in-vivo conditions. Moreover, these EVs improved cardiac functions in rats with MI (Bian et al. 2014). Likewise, administration of EVs obtained from UC-MSCs significantly ameliorated systolic functions and cardiac fibrosis by protecting myocardial cells from apoptosis and activating angiogenesis in another rat MI model (Zhao et al. 2015).

5 Conclusion

Despite prominent progress in diagnostic and treatment approaches, CVDs will likely contribute to a significant proportion of deaths worldwide. This might be partly due to irreversible loss of heart tissue after a pathological incident. On the other hand, regenerative medicine therapies including stem cell applications continue to improve for years. Due to their distinct advantages compared to other stem cell types, MSCs have attracted great attention. Numerically, MSCs are the most investigated stem cell type in clinical trials and CVDs are at the top of studies in which MSCs are tested (Trounson and McDonald 2015; Kabat et al. 2020). Therefore, understanding the complex mechanism of action of MSCs in cardiac diseases is utmost important (Fig. 2). It should be noted that our limited knowledge about the functions of MSCs mostly comes from in-vitro studies and animal models

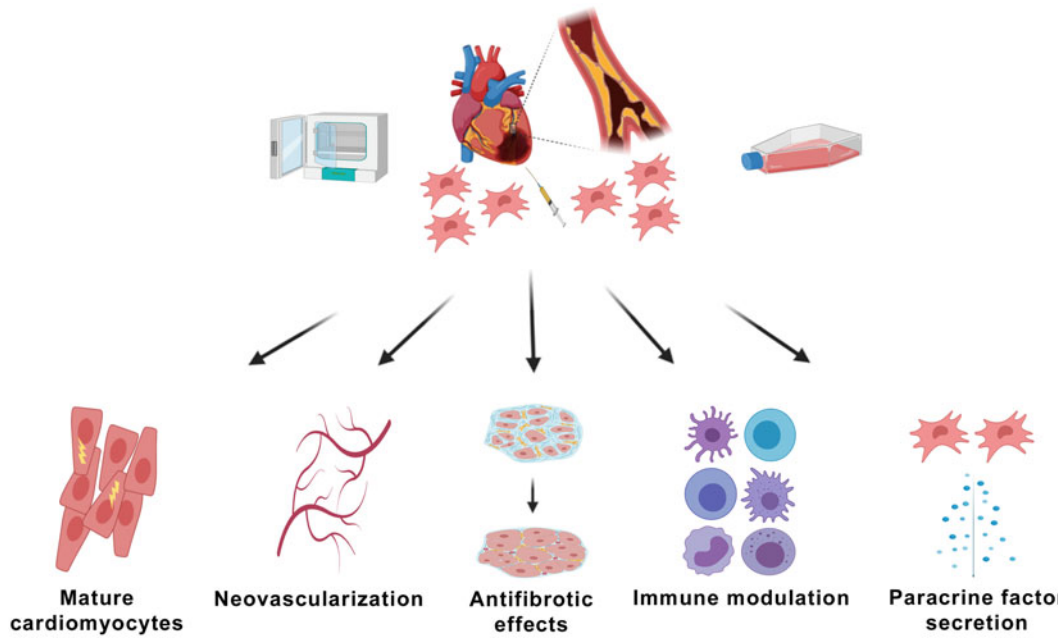

Fig. 2 Prominent functions of mesenchymal stem cells in relation to cardiac repair

emphasizing the significance of preclinical efforts.

There are several challenges of cardiac MSC therapies that need to be clarified such as optimal cell source, donor and number, transplantation route and time, and enhancement strategies before moving to clinical markets. In addition, there is a substantial progress in PSC-based therapies despite tumorigenesis and ethical concerns still exist. Moreover, recent data suggest that most of the beneficial effects of MSCs are regulated through their secretomes and/or EVs emphasizing the role of paracrine functions. Therefore, recent works have focused on the therapeutic effects of these cell-free agents of MSCs instead of cell transplantation. However, these cell-free therapies are in their infancy yet and their functional characteristics have not been fully defined when compared to MSC applications (Öztürk et al. 2020). Thus, MSCs and their functions will likely continue to be at the center of cardiac regenerative medicine research in the near future. Identification of biochemical and molecular regulators of MSC functionality in cardiac diseases will help to enhance safety and functionality of MSC-based cardiac regenerative medicine approaches.

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Cardiac Progenitor Cells

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Abstract

Cardiovascular diseases top the list of fatal illnesses worldwide. Cardiac tissues is known to be one of te least proliferative in the human body, with very limited regenraive capacity. Stem cell therapy has shown great potential for treatment of cardiovascular diseases in the experimental setting, but success in human trials has been limited. Applications of stem cell therapy for cardiovascular regeneration necessitate understamding of the complex and unique structure of the heart unit, and the embryologic development of the heart muscles and vessels. This chapter aims to provide an insight into cardiac progenitor cells and their potential applications in regenerative medicine. It also provides an overview of the embryological development of cardiac tissue, and the major findings on the development of cardiac stem cells, their characterization, and differentiation, and their regenerative potential. It concludes with clinical applications in treating cardiac disease using different approaches, and concludes with areas for future research.

Keywords

Cardiac progenitor cell · Heart embryology · CVD · Stem cell application

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1 Introduction

The heart is the ultimate blood-pumping organ in the body. Anatomically, it consists of four main chambers connected to the rest of the body by a set of vessels, namely, veins and arteries, and wrapped inside the chest by a pericardial sac that provides protection and support (Fig. 1a). The heart consists of two receiving chambers (atria) and two pumping chambers (ventricles). Physiologically, the heart is divided into a right side t and a left side heart. The right heart consists of the right atrium, which receives blood from all

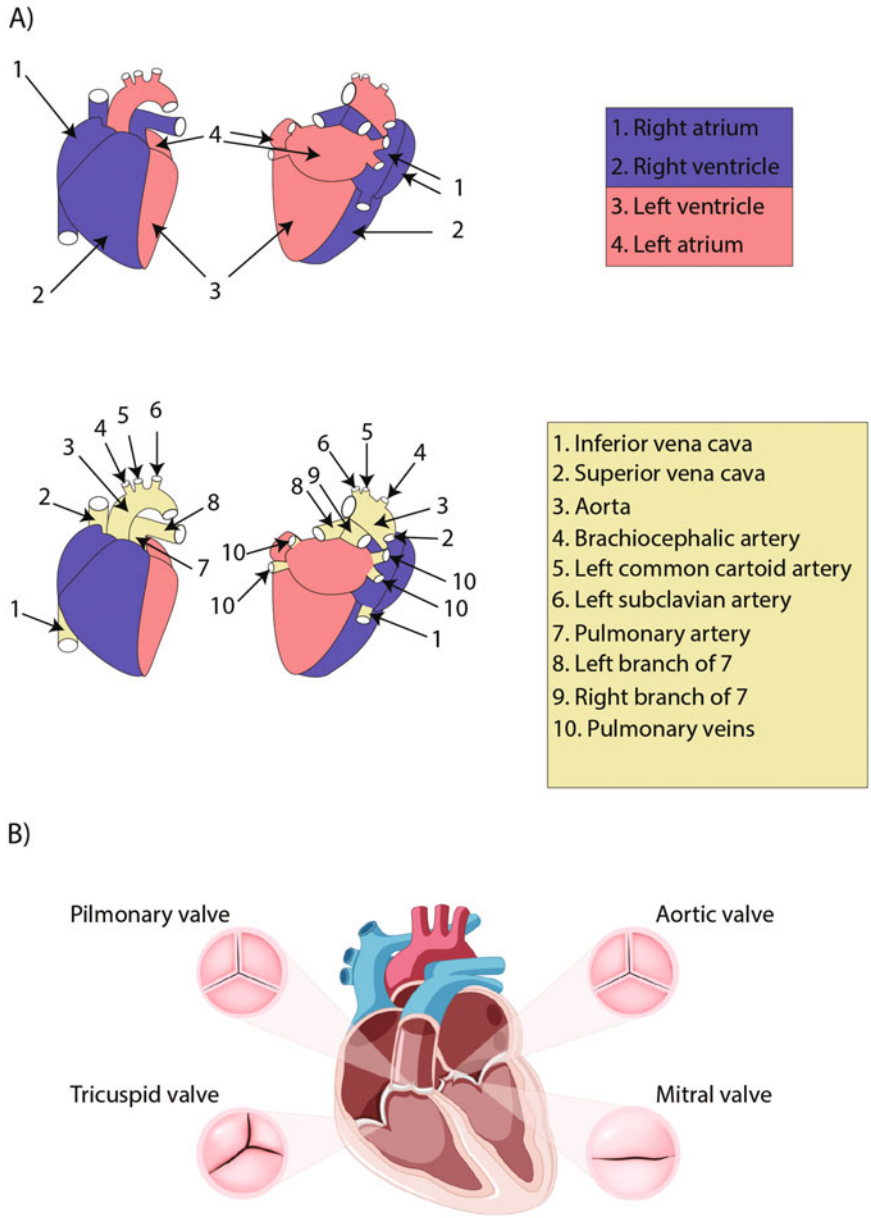


Fig. 1 Heart structure. (**a**) Demonstration of the structure of the heart. (**b**) Heart chambers and inter-chamber valves

over the body via two large veins, the superior vena cava, and the inferior vena cava. Normally, the blood follows a route to the right ventricle where it is pumped to the lungs via the pulmonary artery, where a series of events lead to more oxygenation and less carbonation of the blood. The blood then enters the left heart via four pulmonary veins, where it receives oxygenated blood from the lungs. Oxygenated blood flows to the left ventricle, where it is pumped to all of the organs and tissues of the body via the aorta. This cycle repeats itself with every heartbeat. The cardiovascular circuit is a closed circuit between the two right and left halves of the circulation, which function to perfuse the body with nutrients and carry out metabolic activities in different

organs. Two types of one-way valve between the various chambers maintain the continuity of this circuit and vessels; the atrioventricular (A-V) valves connect the atria with the ventricles, whereas the semilunar valves connect the heart with the aorta and the pulmonary trunk. The mitral valve is bicuspid with two leaflets and connects the left atrium to the left ventricle. The tricuspid valve has three leaflets and connects the right atrium to the right ventricle. The aortic valve connects the left ventricle with the aorta, whereas the pulmonary valve connects the right ventricle with the pulmonary trunk (Fig. 1b). The heart acts as a single functional unit (syncytium), and any defect in this unit will lead to diseases ranging from mild to severe and even fatal conditions. Cardiomyocytes are known to be unable to regenerate after an injury (Laflamme and Murry 2011). To obtain a comprehensive understanding of cardiac regeneration and the role of stem cell therapy in heart repair, it is essential to understand the structure and development of the heart and the role of cardiac progenitors in its regeneration.

2 Development of Cardiac Progenitor Cells

2.1 Embryology of the Heart

Heart development begins soon after gastrulation. The heart develops from the splanchnic mesoderm on both sides of the primitive foregut, forming a heart tube that undergoes a sequence of looping, septation, realignment, and remodeling (Fig. 2). It was recently hypothesized that the heart develops via a sequence of events involving the interaction of different cell groups (Aguilar-Sanchez et al. 2018). In early development, bilateral groups of cells from the splanchnic mesoderm migrate and distribute along the ventral midline in the cardiac crescent (Fig. 2a); this is called the primary or first heart field (Wu et al. 2008). This is followed by another wave of migration from some progenitor cells from the underlying pharyngeal mesoderm, forming the second heart field (Rochais et al. 2009). The cells of the first heart field are positioned more laterally, while those of the second heart field are located more medially and caudally. The cells of the first heart field will form the initial tubular heart. However, the extension of the tubular heart at the venous and arterial pole is assigned to the second heart field. This process gives rise to the right ventricle and outflow tract. The second heart field contributes to the outflow tract, the majority of the right ventricle, and parts of the atria, while the first heart field cells were found to contribute to the entire left ventricle, the majority of both atria, and parts of the right ventricle (Fig. 2b). The merging of the cardiac crescent in the midline forms the primitive heart tube, which is composed of beating cardiomyocytes and is subsequently lined by the endothelium of the endocardium. All parts are surrounded by an extracellular matrix called the cardiac jelly. The endocardial cells appear to be committed even before migration to the heart field.(Ishii et al. 2009) With the first heartbeat, the primitive cardiac tube undergoes canalization and then further elongation, looping, and chambering (Forouhar et al. 2006) (Fig. 2c). This stage is followed by the third wave of cell migration, referred to as the third heart field (Christoffels et al. 2006). However, this time, the neural crest cells come from outside the cardiac mesoderm and are thought to be derived from the dorsal neural tube. They are responsible for completing the separation of the outflow tracts. The third field cells are arranged anteriorly in the early heart tissue forming proepicardium, which is believed to be responsible for forming the membrane covering the entire heart later on, as well as coronary vasculature (Männer et al. 2001). The three waves of cell migration denoted as the first, second, and third heart fields act synchronously to form the ultimate structure of the heart

2.2 Role of Cardiac Progenitor Cells in Heart Development

Cardiac progenitors are precursor multipotent cells that can differentiate into different types of myocytes and non-myocyte cells in the heart (Brade et al. 2013). Identifying the origin of

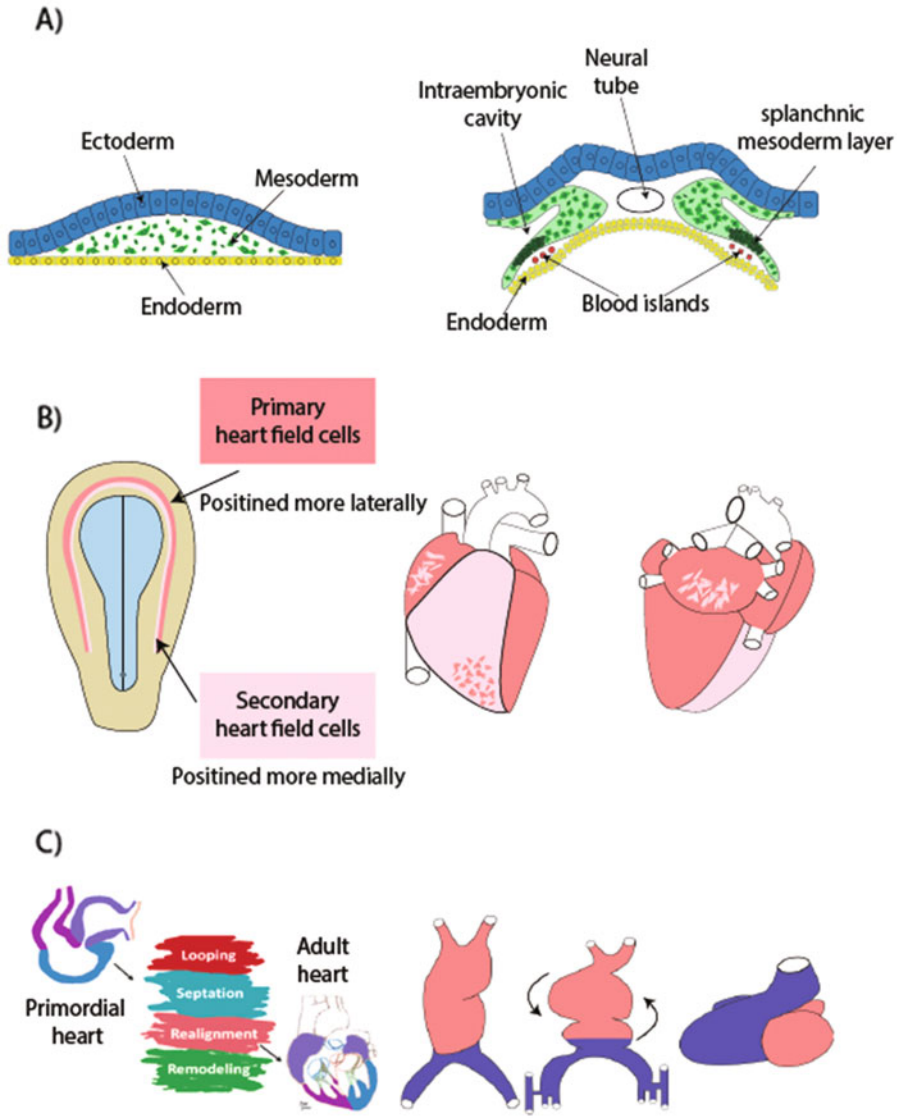


Fig. 2 Heart development. (**a**) Early stage of heart development at embryonic gastrulation**.** (**b**) Development of cardiogenic fields. (**c**) Heart tube formation undergoes a sequence of looping, septation, realignment, and remodeling

these cells and how they differentiate into different cell populations forming a whole functional heart has been the target of extensive research. Determination of the molecular mechanisms that regulate the heart lineage specification provides insight into the potential to reactivate these pathways as a means to treat the loss or damage of adult cardiomyocytes (Xin et al. 2013). During human embryonic development, after the third week of gastrulation, the heart begins to form from the mesodermal germ layer. At this stage, the embryo is converted from being bilaminar (i.e., consisting of two layers of epiblast and hypoblast tissues) into a trilaminar embryonic disk. This process is called gastrulation, where the three germ layers are formed (ectoderm, mesoderm, and endoderm). Initially, a primitive streak (PS) with a node is formed in the bilaminar

embryonic oval disk toward its caudal end. This streak allows the cardiac mesoderm cells (CMCs) to migrate inwardly through PS during gastrulation and to localize in the region anterior of the streak, called the splanchnic mesoderm. Several regulatory pathways from the adjacent endoderm and ectoderm germ layers regulate the induction of the cardiac mesoderm. These include bone morphogenetic protein (BMP), nodal pathway, fibroblast growth factors (FGF), and Wnt signaling pathway, as well as the morphogen gradient (Noseda et al. 2011).

The heart basically develops from migrating CMCs, which transiently express master regulator mesoderm posterior 1 ($Mesp1^+$) and BHLH transcription factor 1, under control of the T-brachyury transcription factor (Bondue et al. 2008). To maintain the cardiac mesoderm lineage, the Mesp1 transcription factor inhibits the Wnt signaling pathway through the activation of Wnt Dickkopf (WNT signaling pathway inhibitor 1) (Bondue and Blanpain 2010). CMCs express the $Mesp1^+$ marker, which is characterized as the earliest sign of heart development (Bondue et al. 2008; Saga et al. 2000). Those uncommitted CMCs proliferate rapidly and migrate craniolaterally to form the cardiac crescent (at week 2 in the human embryo and day E7.5 in the mouse embryo). Sunbsequently, the sequential commitment of $Mesp1^+$ cells is controlled by spatiotemporal signals to give rise to different cardiac progenitor cells (CPCs), expressing specific markers (Saga et al. 2000). Three CPCs have been identified in the embryonic heart: cardiogenic mesoderm cells (CMCs), cardiac neural crest cells (CNCCs), and the proepicardium (PE) (Brade et al. 2013). Cardiogenic mesoderm cells form two fields, the first heart field (FHF) forms the left ventricle and atria, while the second heart field (SHF) forms the right ventricle and outflow tract (OFT) (Buckingham et al. 2005). Cardiac precursors in the FHF and SHF express specific markers such as Gata-4, Nkx2.5, Mef2c, and Islet1 (Laugwitz et al. 2008; Molkentin et al. 1997; Dodou et al. 2004). Cardiac progenitors arising from PE can differentiate into interstitial fibroblasts implanted in the myocardium, smooth muscle, endothelial cells of coronary vessels, and a small number of myocytes located in the atrioventricular A-V septum (Brade et al. 2013). Therefore, the interaction among cardiomyocyte, epicardial, endocardial, and CNCCs results in the formation of the septated four chambers of the fetal heart (Garry and Olson 2006).

3 Types of Cardiac Progenitor Cell

3.1 Cardiac Progenitor Cells in Embryonic Heart

Most of the knowledge obtained about cardiac embryogenesis has been from animal studies, which has resulted in significant gaps of knowledge that limit our understanding of the development of cardiac cells in humans. The anatomical convergence between mice and humans is a key factor in understanding the developmental stages of the heart (Sahara et al. 2019). Animal studies have shown that CPCs play a key role in regulating the sequential assembly of different heart cells during embryogenesis. These progenitors include cardiogenic mesoderm cells (CMCs), CNCCs, and the PE.

3.1.1 Cardiac Mesoderm Cells

In early vertebrate development, CMCs, derived from a common mesodermal lineage, develop into the FHF and SHF. FHF forms on day 7.5 of gestation in mice and from days 16–18 in humans, when the early cardiac progenitor mesoderm forms the cardiac crescent. Markers for FHF cells are the transcription factor NKX2-5 and the cyclic nucleotide-gated ion channel HCN4 (Brade et al. 2013; Wu et al. 2006). In the cardiac crescent stage, FHF progenitor cells are committed to differentiation by the action of BMP (Schultheiss et al. 1997), FGF (Reifers et al. 2000), and Wnt/β-catenin inhibitors (Marvin et al. 2001). In contrast, SHF progenitors remain undifferentiated and in a proliferating state till they enter the heart tube (Brade et al. 2013). SHF is formed from the pharyngeal mesoderm in the medial and anterior regions of the cardiac crescent and is Islet-1

(ISL1)-positive (Cai et al. 2003). The proliferation of uncommitted SHF cells is regulated by FGF, Notch, canonical WNT, and Hedgehog signaling pathways (Dyer and Kirby 2009). By embryonic day 8 in mice, cells from the cardiac crescent migrate to the midline to form a linear heart tube, serving as a scaffold for subsequent heart growth. Further anterior and posterior expansion of the heart results from the migration of cells from the secondary heart field (Garcia-Martinez and Schoenwolf 1993). Many intermediates originate from the first and second heart field-derived CPCs, which subsequently generate all of the major cells in the heart, including cardiomyocytes (CMs), vascular smooth muscle cells (SMCs), arterial and venous endothelial cells (ECs), fibroblasts, and cells of the cardiac conduction system. Moreover, epigenetic regulation mediated by miRNA and lncRNA is also significant for the progression of CPCs to terminally differentiated muscle and non-muscle cardiac lineages (Liu and Olson 2010).

3.1.2 Cardiac Neural Crest Progenitors

The third multipotent distinct embryonic cardiac progenitors are CNCCs, characterized as non-cardiac cell types. CNCCs arise from the ectoderm, from the dorsal neural tube between mid-otic placode and caudal boundary of somit 3 (Achilleos and Trainor 2012). They undergo epithelial–mesenchymal transition (EMT) and migrate toward the heart at pharyngeal arches 3, 4, and 6. CNCCs contribute to the development of the aorticopulmonary septum, conotruncal cushions (i.e., atrioventricular cushions), and smooth muscle and appropriate patterning of the large arteries (Keyte and Hutson 2012; Bergwerff et al. 1998). In addition, the cardiac neural crest generates cardiac parasympathetic innervation and connective tissue insulation of His-Purkinje fibers (Kirby et al. 1983; Gurjarpadhye et al. 2007). Several signaling pathways, transcription factors, and secreted molecules have been shown to interact to instruct CNCCs during their induction, migration, and differentiation. The key players for neural crest induction and specification are BMP/TGF-β growth factors, FGF, the Wnt/β-catenin signaling pathway, as well as retinoic acid (RA) (Aybar and Mayor 2002; Sauka-Spengler and Bronner-Fraser 2008). The migration of CNCCs to a specific site on the heart outflow tract is guided by chemical attractants, such as semaphorin 3C and connexin 43, alongside FGF signaling molecule (Toyofuku et al. 2008; Xu et al. 2006; Sato et al. 2011). The myocardium underlying the outflow tract expresses semaphorin, which binds to its receptor on CNCCs, leading to cytoskeletal rearrangement and cell migration (Toyofuku et al. 2008). The final process of patterning the aortic arch artery is controlled by TGF-β and PDGF signaling pathways. Mutations arising in the genes encoding these signaling pathways or molecules result in various congenital heart diseases (Keyte and Hutson 2012). Unfortunately, no unique molecular marker that allows the identification and tracking of CNCCs is currently available. Instead, molecular lineage labeling and chick–quail chimeras allow the indirect tracking of CNCCs (Phillips et al. 1987). This chick–quail chimera technique allows the transplantation of quail tissues into chick embryo or vice versa, in order to follow the fate of specific regions during embryonic development (Phillips et al. 1987). However, a study reported a multipotent CNCC population in neonatal and adult mouse hearts, precisely within the cardiac side population (Golebiewska et al. 2011). Side population (SP) cells are dormant tissue-resident progenitors that were first identified by their distinctive ability to efflux Hoechst-33342 dyes through ATP-binding cassette (ABC) transporters (Golebiewska et al. 2011). SP cells were isolated and formed a cardiosphere upon culture, similar to the case for neurosphere formation. This cardiosphere was shown to express Nestin and Musashi-1 markers and, upon dissociation, differentiated into neurons, glia, melanocytes, chondrocytes, and myofibroblasts. Once the labeled cardiosphere cells were transplanted into chick embryo, they migrated to the heart, similar to endogenous CNCCs, and contributed to contraction of the cushion and outflow tract (Youn et al. 2003).

3.1.3 Proepicardium (PE)

The outermost layer of the heart enveloping both the endocardium and the myocardium is called the epicardium. PE cells are embryonic progenitor cells that give rise to epicardial cells . During the looping stages of the heart, proepicardial cells migrate to cover the heart surface with an epicardial sheet. Some epicardial cells detach and undergo EMT, invade the myocardial walls, and give rise to the epicardial-derived cells (EPDCs) (Perez-Pomares et al. 2002; Gittenberger-de Groot et al. 1998). Invasive EPDCs differentiate into coronary vascular SMCs, ECs, and subepicardial and intramyocardial fibroblasts (Dettman et al. 1998; Smart et al. 2007). The induction and maintenance of PE are regulated by the opposite interaction between FGF and BMP signaling pathways. FGF signaling induces a proepicardial fate from the splanchnic mesoderm, while BMP signaling induces myocardial differentiation (Kruithof et al. 2006). Important signaling molecules driving EPDC differentiation into primary coronary blood vessels are the TGF-β superfamily, FGFs, retinoic acid, as well as Hedgehog and VEGF (Lavine and Ornitz 2008; Perez-Pomares and de la Pompa 2011).

For a long time, it was thought that the PE is an extracardiac population of cells; however, recent molecular analysis and lineage tracing studies found that CPCs expressed Nkx2-5- and Isl1 markers as SHF progenitors, contributing to the formation of proepicardial cells. In addition, these proepicardial progenitor cells expressed Wt1 and Tbx18 markers and could differentiate into cardiomyocytes, ECs, and SMCs (Zhou et al. 2008a). This supports the assertion that SHF progenitor (Nkx2-5- and Isl1-positive) cells contribute to the formation of PE during cardiac development (Zhou et al. 2008a).

3.2 Cardiac Progenitor Cells in Adult Heart

Cardiac cells were long believed to lack the capacity to self-renew (post-mitotic organ) and thus to have limited potential to regenerate after injury (Laflamme and Murry 2011). The regenerative potential of administering stem cells directly into the heart is still impeded by many challenges, such as limited yield and differentiation potential (Madonna et al. 2016). The benefits of stem cell therapy in cardiac patients have been proposed to be caused by a paracrine action, such as the angiogenesis mediated by the CPC-driven chemokine CXCL6 (Torán et al. 2017; Ptaszek et al. 2012; Mercola et al. 2011; Sebastião et al. 2019). During normal physiological aging, cardiomyocyte genesis is a caused by the slow division of pre-existing cardiomyocytes (Senyo et al. 2013). However, recent studies have shown that the generation of new cardiomyocytes occurs in the adult heart, generating renewed interest in cardiac regeneration (Kuhn and Wu 2010). The discovery of CPCs in embryos encouraged a further search for such progenitors in the adult heart (Kuhn and Wu 2010). An endogenous heterogeneous population of cells that is widely distributed throughout the adult heart, in the atria, ventricles, and other parts, was found to play a role in myocardial regeneration (Anversa and Nadal-Ginard 2002; Bergmann et al. 2009). These CPCs are quiescent cells that make a minimal contribution to repair damage to myocardial cells under normal physiological conditions (Hsieh et al. 2007). The specific biological role of CPCs in maintaining homeostasis or their reparative function in the injured heart is still unclear . Adult CPCs are subclassified into different types, according to their expression of specific cellular markers such as c-kit (CD117), Isl-1 (insulin gene enhancer protein), and Sca-l (stem *cell* antigen-1(Galvez et al. 2008; Oh et al. 2003). However, those markers are not specific and overlap with other tissue markers. The main characteristics of those cells are their self-renewal and clonogenic properties, in addition to their multipotent potential to differentiate into cells of cardiac lineages, such as myocytes, SMCs, and ECs (Beltrami et al. 2003a). The *in vitro* propagation of these cells in culture is characterized by being adherent or spheroid (the adherent cells grow in monolayers, while in the spheroid model the cells grow as 3D aggregates), termed cardiospheres (Messina et al. 2004; Shenje et al. 2008). Spheroids are non-adherent, multicellular

floating clusters of cells that were first defined in neural stem cells (Reynolds and Weiss 1992). Human cardiospheres (CS) were reported from the culture of patients' atrial appendage specimens on non-adhesive substrates supplemented with cardiosphere-forming medium. This medium contains epidermal growth factor, basic fibroblast growth factor, thrombin, cardiotrophin-1, and B27. CS showed heterogeneous populations of both primitive and committed progenitors expressing mesenchymal stem cell markers, such as CD105, CD13, CD73, and CD166, as well as early and late cardiac markers (Nkx2.5, GATA4, and connexin 43) (Barile et al. 2013). CS exhibited the multipotent ability to differentiate into cardiomyocytes, SMCs, and ECs, serving as a promising cell source for treating myocardial infarction in phase 1 clinical trials (Makkar et al. 2012). Additionally, CPCs of proepicardial origin, expressing platelet-derived growth factor receptor-alpha ($PDGFR\alpha^+$) and c-kit, have been found in the adult human heart (Chong et al. 2011). $PDGFR\alpha^+$ cells could differentiate into SMCs and ECs, providing a source of vascular and interstitial tissues of the injured heart . A recent study demonstrated that some types of CPC, such as $Bmi1^+$ cells, contribute to the regeneration of cardiomyocytes after injury, serving as a source of progenitors in cardiac repair (Valiente-Alandi et al. 2016).

3.3 Cardiac Progenitor Cells Derived from Human Pluripotent Stem Cells

Developmental cardiac progenitor cells are the *in vitro* version of CPCs that can be generated from either embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). The main characteristics of these cells are their self-renewal and clonogenic properties, in addition to their multipotent differentiation potential, from which different cardiac lineages such as CMs, SMCs, and ECs arise (Sanganalmath and Bolli 2013; Mauretti et al. 2017; Beltrami et al. 2003b). Generating cardiovascular cells from ESCs has been shown to have many advantages. For example, ESCs are natural pluripotent cells, and can be scaled up and genetically tagged for cell selection or tracing. CPCs were shown to be derived from ESCs by in vitro manipulation of the essential signaling pathways involved in embryonic carcinogenesis, as described by Pucéat's protocol (Jebeniani et al. 1994). ESCs were cultured in serum-free mesogenic induction medium supplemented with small-molecule that inhibit FGF and Wnt signaling pathways in the presence of the cardiogenic morphogen BMP2. CPCs were isolated by the expressions of SEA-1 and MESP1 markers and showed a commitment to three lineages: cardiomyocytes, SMCs, and ECs (Blin et al. 2010a). A phase 1 clinical trial using hESC-derived cardiac progenitors was conducted for patients with severe heart failure (see ClinicalTrials.gov Identifier: NCT02057900).

The discovery of iPSCs and gene editing have promised an attractive approach for generating cardiac cells with specific mutations. This technology allowed the mimicking of inherited cardiac diseases and elucidation of their developmental mechanism. Congenital long QT syndrome is caused by mutation of the KCNH2 gene encoding potassium ion channels that regulate the cardiac action potential (Bohnen et al. 2017). CRISPR/Cas9 editing was thus used to introduce specific mutation into the KCNH2 gene (potassium voltage-gated channel, subfamily H and member 2) of healthy hiPSC lines. These iPSC-derived cardiomyocytes (iPSC-CMs) allowed study of the mechanism underlying inherited cardiac channelopathy (Chai et al. 2018). The third approach for direct cellular reprogramming involves a cocktail of transcription factors (Gata4, Mef2c, and Tbx5) essential for early carcinogenesis being directly injected into the cardiac fibroblasts of elderly mice. The transfected cells were directly reprogrammed into adult cardiomyocyte-like cells. These cells that beat upon cardiac stimulation decreased the infarct size and raised hopes for the achievement of *in vivo* cardiac regeneration (Qian et al. 2012).

4 In Vitro Characterization of Cardiac Progenitor Cells in Embryonic and Adult Heart

Cardiac progenitors express many specific proteins. Their markers have yet to be fully elucidated. Adult CPCs are categorized into seven main types expressing overlapping markers: c-kit, Sca1, islet-, SP cells, epicardium-derived cells, cardiac colony forming unit fibroblasts (c-CFU-Fs), and cardiosphere-derived cells (Le and Chong 2016a). They commonly share the expression of c-kit, but at different levels. Most adult CPC populations express surface markers such as Sca-1, Abcg-2, Flk-1, CD34, CD90, and CD10, and the transcription factors Isl-1, NK2 homeobox 5 (Nkx2.5), GATA binding protein 4 (GATA4), and myocyte enhancer factor 2 (MEF2), which are expressed continuously in both adult and embryonic CPCs. Embryonic CPCs express Oct3/4, Bmi-1, and Nanog, supporting their regenerative potential through enhancing the self-renewal and multiple propagation abilities (Van Berlo et al. 2014; Chong et al. 2013), Surface markers can be identified using flow cytometry or immunohistochemistry (Mishra et al. 2011). Table 1 illustrates the cell surface markers for each population (Takamiya et al. 2011). CPCs can also be characterized by their ability to form cardiospheres (Blue Box 1), SP cells to pump out the DNA binding dye (efflux), and colony formation by c-CFU-F cells (Mishra et al. 2011; Unno et al. 2012; Belostotskaya et al. 2018).

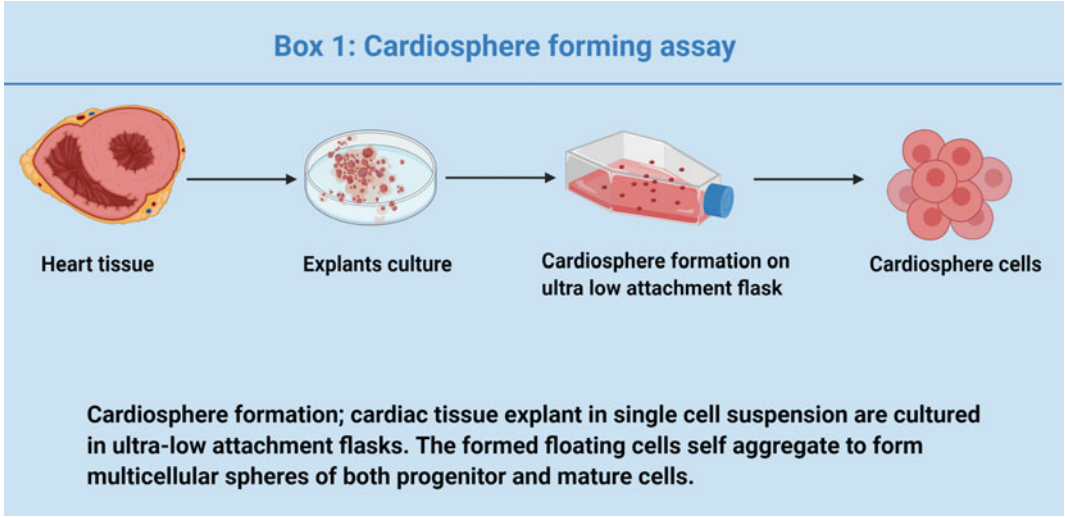
5 Concepts in Cardiac Differentiation

Signaling pathways play an important role in cardiac differentiation (Devalla and Passier 2018). Early-activated signaling pathways are inhibited during the later stages of cardiac differentiation to allow complete differentiation. Wnt proteins encompass a major family of lipid-modified glycoproteins acting as signaling molecules to facilitate cellular communication. They maintain the equilibrium of growth, function, differentiation, and cell death (Willert and Nusse 2012). The activation of Wnt proteins is essential during the generation of the mesoderm, and their inactivation is essential during the differentiation of cardiac progenitors. During early gastrulation, the E-cadherin signaling pathway dominates and the epiblast cells are tightly packed, resulting in an increase in membrane-bound β-catenin and Wnt signaling. The epiblast corresponds to this increase by releasing β-catenin from the membrane into the cytoplasm, leading to its accumulation (Naito et al. 2006; Ueno et al. 2007). Wnt proteins inhibit the phosphorylation of β-catenin in order to prevent its degradation by the proteasome (Pahnke et al. 2016). Subsequently, hypo-phosphorylated β-catenin moves to the nucleus and enhances the transcription of Wnt-induced genes. These genes include Wnt inhibitors that promote cardiac differentiation (Ueno et al. 2007; Lindsley et al. 2008). *In vitro* cardiac differentiation protocols initially used 5-azacitidine, a demethylating agent that alters gene expression and increases Wnt/β signaling, in order to enhance early mesodermal commitment. However, the exact mechanism by which 5-azacitidine acts has not been well characterized. Some protocols combine the usage of 5-azacitidine and TGF-β1 to increase vascularization and certain cardiac markers such as α-smooth muscle actin and vascular endothelial growth factor (VEGF) (Sebastião et al. 2019). Angiotensin II (Ang II) in combination with 5-azacitidine and TGF-β1 at low concentration (Xing et al. 2012) is used to enhance cardiac differentiation. Ang II was shown to stimulate the expression of TGF-β1 in different cell types such as SMCs, cardiac fibroblasts, and myofibroblasts (Williams 2001). To overcome the cytotoxicity of 5-azacytidine, small bioactive lipids were applied to induce Wnt/β-catenin signaling without notable cell damage. Sphingosine-1-phosphate (S-1-P) in combination with lysophosphatidic acid (LPA) was found to activate Wnt/β-catenin signaling, which results in the accumulation of nuclear β-catenin; this in turn facilitates mesodermal induction and subsequent cardiac differentiation. Other differentiation

Table 1 Cardiac progenitor cells types in adult and embryonic heart

Type	Surface marker	Transcription factor	Possible origin	Potency	Differentiates into	Pathway involved in differentiation	Ref.
Cardiac progenitor types (adult)							
Epicardium-derived cells	$CD34^+$	MRTF-A	Proepicardial organ/ epicardium	Multipotent	Smooth muscle myofibroblasts Endothelial cells	TGF-β	Smart et al. (2007), Trembley et al. (2015), Winter et al. (2007), Zhou et al. (2008b)
	c-Kit+	MRTF-B					
	$CD105^+$						
	$CD90^+$						
	$CD44^+$						
	$CD46^+$						
Side population cells	$CD34^+$	Nkx2.5	Bone marrow derived	Multipotent	Cardiomyocytes	c-Jun N -terminal kinase (JNK)	Pfister et al. (2005), Oyama et al. (2007), Liao et al. (2007), Chen et al. (2014)
	$CD45^+$	GATA4	Neural Crest (still heterogenous)				
	c-Kit+						
	Abcg2+						
	Sca-1^+						
Cardio sphere-derived cells	$CD34^+$	MEF2C	Cardiac origin	Multipotent	Cardiomyocytes	Notch 1/J kappa-recombining binding protein (RBPJ) signaling	Walravens et al. (2018), Chimenti et al. (2010), Malliaras et al. (2012), Davis et al. (2009), Chen et al. (2012)
	$CD45^+$	GATA4			Smooth muscle		
	c-$Kit^{(low)}$				Endothelial cells		
	Abcg+						
	$CD31^+$						
	$CD105^+$						
	Sca-1+						
c-Kit+ CPCs	CD34-	Nkx2.5	Cardiogenic mesoderm	Multipotent	Cardiomyocytes	PI3K	Beltrami et al. (2003a), Rota et al. (2007), Fathi et al. (2020)
	CD45-	MEF2C			Smooth muscle	MAPK	
	c-Kit+	GATA4	Bone marrow		Endothelial cells		
	$CD105^+$						
	CD166+						
	Abcg2+						
Cardiac colony forming unit fibroblasts (cCFU-F)	PDGFR	N/A	Proepicardial	Multipotent	Smooth muscle	TGFβ	Chong et al. (2011), Doyle et al. (2015)
	α+				Endothelial cells	BMP	
	CD90+				Cardiomyocytes		
	CD105+						
	CD44+						
	CD29+						

	Sca-1+ CD31- CD45- Flk-1^{-} C-kit$^{(low)}$						
Islet-1+ CPCs	C-kit +/- CD31- Sca-1^{-}	Isl-1+ Nkx2.5+ GATA4+	SHF	Multipotent	Smooth muscle Endothelial cells Cardiomyocytes		Laugwitz et al. (2005), Moretti et al. (2006), Genead et al. (2010)
Sca 1+ CPCs	Sca-1+ C-kit +/- CD105^{+} CD34- CD45-		N/A	Cardiomyocytes Chondrocytes Osteocytes	Cardiomyocytes		Oh et al. (2003), Le and Chong (2016b), Wang et al. (2006), Uchida et al. (2013)
Cardiac progenitor types (embryonic)							
Mesoderm CPCs	Islet-1+ C-kit+ FlK1-	NKX2-5 – Mesp-1+	SHF FHF	Multipotent	Cardiomyocytes smooth muscle cells arterial and venous endothelial cells fibroblasts	Notch, canonical WNT Hedgehog (Bergmann et al. 2009) signaling pathways	Bondue and Blanpain (2010), Wu et al. (2006), Dyer and Kirby (2009)
Neural crest CPCs	CD49+ Integrin-alpha-4 beta 1	Mesp-1- Sox10 MafB Krox20 Nestin	Ectoderm Neural tube	Multipotent	Smooth muscle (in vitro) Aortic arch arteries (in vivo)	TGF-β, and PDGF	Keyte and Hutson (2012), Tani-Matsuhana et al. (2018), Tomita et al. (2005)
Proepicardium	–	Nkx2-5+ Isl1+ Tbx18 WT1(Zhou et al. 2008a)	Extra cardiac mesenchyme and SHF	Multipotent	cardiomyocytes, endothelial cell smooth muscle	TGF-β Hedgehog (Keyte and Hutson 2012; Bergmann et al. 2009)	Keyte and Hutson (2012), Zhou et al. (2008a), Manner et al. (2001)



Blue Box 1 Cardiosphere assay protocol

protocols use glucose synthase kinase 3 inhibitors (GSK3) such as CHIR99021 to increase β-catenin expression, which consequently enhances Wnt signaling, allowing differentiation into cardiomyocytes (Sharma et al. 2018). It was also reported that small molecules such as KY02111 were used to block Wnt/β-catenin signaling in late differentiation in order to efficiently enhance cardiac myocyte differentiation (Minami et al. 2012).

6 Clinical Applications of Cardiac Stem Cells

According to the World Health Organization, the leading cause of mortality globally is cardiovascular diseases (CVDs). In 2016 alone, approximately 31% of deaths worldwide were attributed to these diseases. CVDs include congenital heart diseases, coronary heart diseases, cerebrovascular diseases, peripheral arterial diseases, and rheumatic heart diseases. The use of pharmacological agents and mechanical devices has helped to improve heart function, but most available therapies are symptomatic, do not cure the disease, and require lifelong maintenance. Regenerative medicine could potentially replace damaged heart or vessel cells (Fig. 3).

Stem cells used in cardiac regenerative therapies include:

6.1 Pluripotent Stem Cells

6.1.1 Embryonic Stem Cells

At present, the only performed clinical trial using ESC-derived pluripotent cells (ESCORT) was for the treatment for severe heart failure (ClinicalTrials.gov Identifier: NCT02057900); however, no data from this trial are currently available. Genetic modification of ESCs to promote the expression of the cellular repressor of E1A-stimulated genes (CREG), a glycoprotein that enhances cell survival and differentiation, followed by injection of the CREG-ESC cells into the peri-ischemic region in a myocardial infarction model, showed reductions in infarction, and fibrosis. Moreover, the survival rate of CREG-ESCs was high in the treated mice. Additionally, CREG-ESCs induced reductions in inflammatory cytokines including IL-1β, IL-6, and TNF-α and increases in the pro-inflammatory TGF-β, bFGF, and VEGF165. Enhancing the expression of CREG in CREG-ESCs appeared to prevent teratogenicity as the injection of up to 3.0×10^6 CREG-ESCs did not result in the teratoma formation

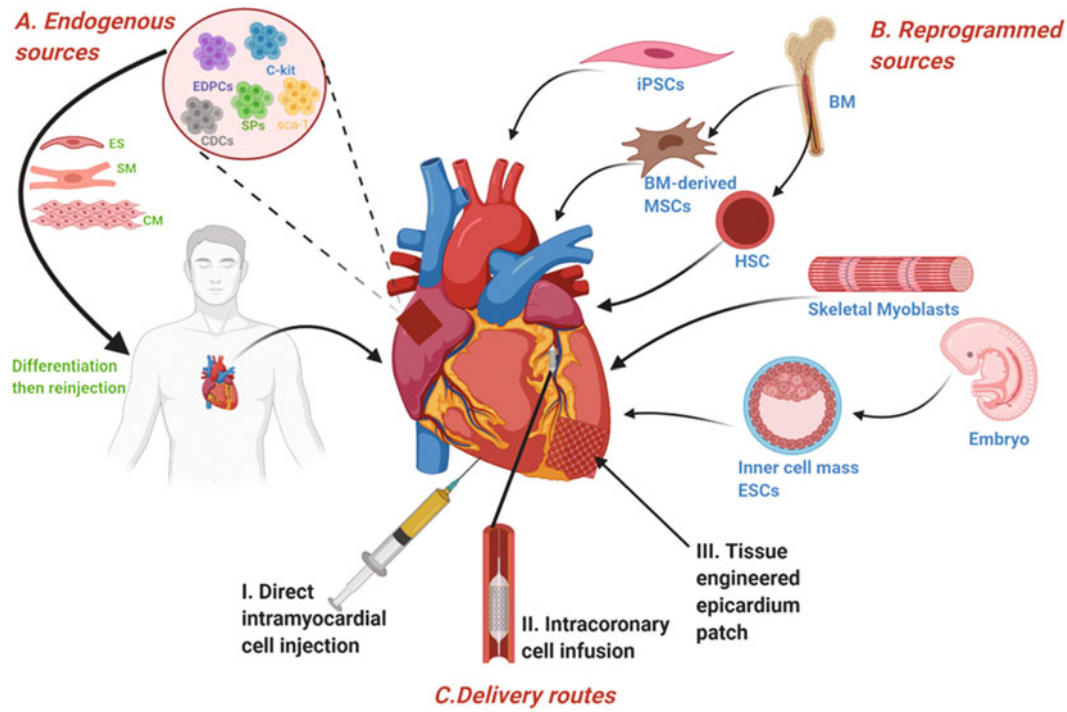


Fig. 3 Summary of different types of stem cell and their roles in heart regeneration

observed with ESCs (Zhang et al. 2018). In addition, Romagnuolo et al. used human ESC-derived cardiomyocytes (hESC-CMs) to treat infarcted heart in a pig model. Although immunosuppressed animals did not reject the grafted cells and no teratoma formation was detected, monomorphic ventricular tachycardia frequently occurred up to 4 weeks after transplantation (Romagnuolo et al. 2019).

6.1.2 Induced Pluripotent Stem Cells (iPSCs)

IPSCs are derived from adult somatic cells (e.g., fibroblasts) that have been reprogrammed into an embryonic-like state, and have the ability to differentiate into different lineages (Takahashi et al. 2007). They display broad differentiation plasticity and are a source of autologous therapy. The in vitro culture of iPSCs treated with BMP2 in the presence of FGF pathway inhibitors was shown to upregulate the expression of connexin-43 and myosin chain complexes in CPCs (Blin et al. 2010b). These progenitors were shown to be multipotent and could generate SMCs, ECs, and cardiomyocytes (Blin et al. 2010b). The cardiomyocyte derivatives of iPSCs (iPSC-CMs) were demonstrated to successfully restore the myocardium after injection into ischemic hearts in animal models (Kawamura et al. 2012; Wang et al. 2013). The administration of CPC-iPSCs was shown to achieve myocardial restoration, increase the formation of new blood vessels, and result in better survival in a hostile ischemic environment compared with terminally committed iPSC-CMs (Mauritz et al. 2011). Carpenter et al. generated CPC-iPSCs that differentiated into smooth muscle and cardiomyocytes and persisted for more than 1 month upon injection into infarcted rat heart (Carpenter et al. 2012). The co-administration of MSC-loaded patch (hMSC-PA) along with hiPSC-CMs in the infarcted heart enhanced their resistance to the hostile ischemic tissue microenvironment and promoted vascular regeneration. This effect was mediated by paracrine factors secreted by hiPSC-CMs. Additionally, the MSC-loaded patch increased the

retention of hiPSC-CMs and prevented their leakage into the epicardial space. Furthermore, the differentiated cells showed striations with Z-bands and more efficient electrical conduction. Dual stem cell therapy led to improved heart function, vascular regeneration, retention, engraftment maturity of hiPSC-CMs, and ameliorated cardiac fibrosis (Park et al. 2019). Ongoing clinical trials [e.g., (HEAL-CHF) ClinicalTrials.gov Identifier: NCT03763136] are limited due to the safety concerns associated with iPSCs.

6.2 Multipotent Stem Cells

6.2.1 Mesenchymal Stem Cells

Human MSCs (hMSCs) are non-hematopoietic, multipotent stem cells capable of differentiating into osteocytes, adipocytes, and chondrocytes, as well as other cell lineages (Ullah et al. 2015). MSCs are extensively used in experimental and clinical studies because of the accessibility of the cell for in vitro modifications, and their immunomodulatory characteristics (Roura et al. 2017; Nauta and Fibbe 2007). HMSCs have been isolated from bone marrow, adipose tissue, umbilical cord, placenta, and amniotic fluid (Nauta and Fibbe 2007). The administration of MSCs into infarcted myocardium resulted in reduction in the size of the infarct and upregulated VEGF secretion, leading to enhanced vascularization and amelioration of the damage to cardiac tissues (Zhao et al. 2014; Rahbarghazi et al. 2014). A paracrine effect mediated by secreted factors and juxtacrine crosstalk between the transplanted MSCs and ECs in the infarcted area were shown to mediate this repair. Soluble factors such as Ang-1, IGF-1, VEGF, SDF-1α, and EGF upregulated the expression of endogenous pro-angiogenic molecules in the infarcted tissue (Rahbarghazi et al. 2014).The proposed mechanisms of repair include the differentiation of administered cells into cardiac cells, release of paracrine signaling factors, and fusion of the administered cells into myocardiac muscle cells (Kajstura et al. 2005; Orlic et al. 2001; Mazhari and Hare 2007). The latter mechanisn was refuted when the injection of Akt-expressing MSCs into infarcted rat heart resulted in transient grafting, infrequent fusion, and very low differentiation (Noiseux et al. 2006). The infarcted microenvironment contributes to the low efficacy of stem cell transplant. Hypoxia and inflammatory cytokines are the main factors that limit the survival of the grafted MSCs in myocardial infarction (Mangi et al. 2003). Transfecting MSCs using genes encoding Akt was shown to enhance their engraftment, differentiation, and ability to repair the damaged heart tissues in a rodent model of MI (Mazhari and Hare 2007; Mangi et al. 2003). In another study, the co-administration of insulin-like growth factor 1 improved the survival of the transplanted MSCs and enhanced their capacity to regenerate the myocardium after MI (Davis et al. 2006). Survival of the MSCs following MI was also enhanced by a hypoxia-regulated heme oxygenase 1-vector modification (Tang et al. 2005). In a phase 1, randomized, double-blind, placebo-controlled clinical trial, allogeneic hMSCs were intravenously injected into patients with a first acute myocardial infarction (Hare et al. 2009). Specific safety monitoring showed that patients who received the cell therapy had better outcomes in terms of cardiac arrhythmias, lung function, left ventricular function, and global symptoms, than those without this therapy (ClinicalTrials.gov Identifier: NCT00114452) (Hare et al. 2009). In addition, autologous BM-MSCs have been injected into 59 patients with ischemic heart failure (ClinicalTrials.gov Identifier: NCT00644410)(Mathiasen et al. 2015). The transplanted patients demonstrated significant improvement in systolic left ventricular (LV) function, left ventricular end-systolic volume, left ventricular ejection fraction (LVEF), systolic volume , and cardiac output, compared with the placebo group. The LV mass and wall thickening were also enhanced(Mathiasen et al. 2015).

Other sources of MSCs include adipose tissues, placenta, cord blood, and Wharton's jelly. Adipose-derived mesenchymal stem cells (AD-MSCs) have also shown promise in MI patients. The cells are usually harvested from subcutaneous lipoaspiration (Davis et al. 2006;

Qayyum et al. 2019). AD-MSCs are easier to obtain and give higher yields of stem cells than bone marrow MSCs. Moreover, a study showed that patients with refractory angina treated with autologous AD-MSCs (ClinicalTrials.gov Identifier: NCT01449032) maintained their exercise abilities, while the exercise performance of patients in a placebo group was significantly decreased. The heart symptoms improved significantly during a 3-year follow-up and the number of weekly angina attacks for patients was significantly reduced (Qayyum et al. 2019)..Umbilical cord MSCs (UC-MSCs), placental MSCs, and those derived from Wharton's jelly present attractive sources for MSCs for cardiac regeneration. UC-MSCs were shown to have a higher capacity for self-renewal than BM-MSCs (Fong et al. 2011). In addition, the intracoronary administration of Wharton's jelly-derived MSCs in patients with ST-elevated MI showed improved myocardial viability and cardiac function, when compared with those transplanted with BMMNC (ClinicalTrials.gov Identifier: NCT01291329) (Gao et al. 2015).

As mentioned above, cardiac repair after MSC therapy is attributed to several mechanisms. The hypoxic conditions of infarcted tissue induce the expression and release of growth factors that promote angiogenesis, the distribution and migration of cardiac progenitors, and the differentiation of MSCs into cardiomyocytes. In addition to the promotion of angiogenesis, factors such as VEGF, hepatocyte increasing factor (HGF), and insulin-like growth factor (IGF) upregulate the expression of cardiac differentiation genes. Additionally, immunomodulatory and trophic factors secreted by MSCs activate resident stem cells to potentiate cardiac repair and enhance vascularization (Madigan and Atoui 2018; Caplan 2017). However, there are still many challenges regarding MSC therapy for cardiac regeneration. Most importantly, poor survival of the transplanted cells after grafting into the host myocardium leads to therapy failure, presumably due to the hostile environment of the ischemic/infarct tissue (Timmers et al. 2011).

6.2.2 Cardiac Progenitor Cells

Upon treatment with 5-azacytidine and TGFβ, CPCs were shown to differentiate into cardiomyocytes that beat spontaneously (Goumans et al. 2008). Additionally, they were differentiated into ECs and SMCs upon treatment with VEGF (Goumans et al. 2008). Upon the transplantation of CPCs into the infarcted zone in mice, cardiac function was improved for 3 months after MI. Over the same period, and despite the remarkable increase in the number of blood vessels, only a small proportion of the infused CPCs survived. The density of the blood vessels increased remarkably when measured only 2 weeks after transplantation, despite of no indication of vascular differentiation. Improved function after MI was not due to their differentiation to replace damaged cardiomyocytes, but due to paracrine mechanisms. The pro-angiogenic potential of extracellular vesicles (EVs) isolated from CPCs was shown to promote revascularization. EVs of CPCs are being introduced as a potential therapy for MI. This is due to the complexity of their content of miRNAs and proteins and their effectiveness as performers of the paracrine therapeutic effect of CPCs (Smits et al. 2009a; Smits et al. 2009b).

Undifferentiated CPCs were found to secrete a higher level of VEGF. In preclinical studies, they were shown to reduce cardiac damage, enhance proliferation in the left ventricle, lead to the promotion of proliferative markers in the border zone, and stimulate the secretion of pro-angiogenic factors such as endoglin (Goumans et al. 2008; Maring et al. 2019). Andrade et al. reported that the subcutaneous injection of 100 $\mu g.kg^{-1}$ of IGF-1 for up to 7 days enhanced the survival and proliferation of CPCs and ameliorated obesity-induced cardiomyopathy in a rat model (Andrade et al. 2020). Moreover, in a preclinical study, Kannappan et al. investigated the effect of enhanced expression of the p53 tumor suppressor gene on CPC function. A high yield of CPCs was isolated from transgenic mice with an extra allele of the p53 gene. Additionally, those cells showed the ability to withstand oxidative stress upon

injection into a rat model of type I diabetes mellitus. High expression of the p53 gene was also shown to play an important role in protecting CPCs and enhancing their ability to replace damaged cardiomyocytes (Kannappan et al. 2017). The intracoronary injection of 0.3 million/kg autologous CPCs to treat single ventricle physiology (TICAP), also known as single ventricular defect (ClinicalTrials.gov Identifier: NCT01273857), showed no adverse cardiac effects in seven study participants (Tarui et al. 2015).

6.2.3 Hematopoietic Stem Cells

Hematopoietic stem cells (HSCs) are blood-forming multipotent cells that are present in the bone marrow at a low level (about 1 in 10,000 cells). Sources of HSCs include the bone marrow, peripheral blood, and umbilical cord (Fong et al. 2011). The injection of lin-c-kit$^+$ HSCs after coronary ligation in mice was shown to repair 68% of the infarcted heart section, leading to a significant improvement in coronary artery disease (Orlic et al. 2001). The COMPARE-AMI clinical trial (phase II, double-blind, placebo-controlled, randomized study) tested the safety and feasibility of administering CD133$^+$hematopoietic progenitor cells by intracoronary injection in acute myocardial infarction (AMI) patients. No serious adverse events such as arrhythmia, angina, stent thrombosis, heart failure, or death were reported during a 1-year follow-up. This study showed that CD133$^+$ injection was safe and feasible, and effectively improved LV perfusion and function for patients with AMI (Mansour et al. 2011). Another trial (the REGENT trial) aimed to compare intracoronary infusion of bone marrow mononuclear cells and hematopoietic cells (CD34$^+$) in patients with AMI. The study showed no significant difference between selected CD34$^+$and unselected bone marrow mononuclear cells. Both groups showed a 3% increase in LVEF from baseline, in contrast to no significant change in the control group (Tendera et al. 2009).

6.2.4 Skeletal Myoblasts (Satellite Cells)

Skeletal myoblasts are multipotent cells, arise from the muscle stem cells (satellite cells), located beneath the basal lamina of muscle fibers (Yin et al. 2013). Myoblasts express a group of markers, including Pax7, CD34, VCAM 1, MRF4, Desmin, CD56, syndecan3, Pax3, M-cadherin, N-CAM, c-met, Leu-19. When activated, especially after injury, myoblasts firstly express MyoD or/and Myf-5, then differentiation markers myogenin and MRF4 (Durrani et al. 2010) (Fig. 4). Satellite cells were among the first stem cells to be tested in for myocardial regeneration in pre-clinical and clinical studies (Tompkins et al. 2018).

When satellite cells isolated from an adult rat were combined with bromodeoxyuridine and transplanted into syngeneic rat heart, they failed to differentiate into cardiomyocytes after 12 weeks follow up (Reinecke et al. 2002). Co-transplantation of skeletal myoblasts and other stem cells showed to be more effective than using the single types of cells. For example, the combination of mononuclear bone marrow stem cells and skeletal myoblasts were more beneficial for myocardial repair than either cell alone (Ott et al. 2004). However, the major limitation of skeletal myoblast transplantation remains due to poor engraftment. Repeated administration of skeletal myoblasts for infarcted tissue was shown to be required for effective treatment (Gavira et al. 2010). Percutaneous injection of three repeated doses of skeletal myblast in infarcted swine heart showed improved efficacy of the aortic valve ejection fraction (AVEF) and improved cardiac function in general (Gavira et al. 2010).

The first skeletal myoblast transplantation was carried out in 2001 in a patient suffering from severe ischemic heart failure (Menasché et al. 2001; Menasche et al. 2001). Clinical applications of this approach however suffered the limitation of absent control groups, and limited number of participants in general. In a 2015 study, seven patients were transplanted with skeletal myoblasts for treatmet of CHF. After 26 weeks, six of the patient showed an improvement in the left ventricular ejection fraction (Sawa et al. 2015). In and study, thirty patients with class II (mild) and class III (moderate) heart failure were treated by using connexin-43 expressing muscle progenitor cells. When followed up for

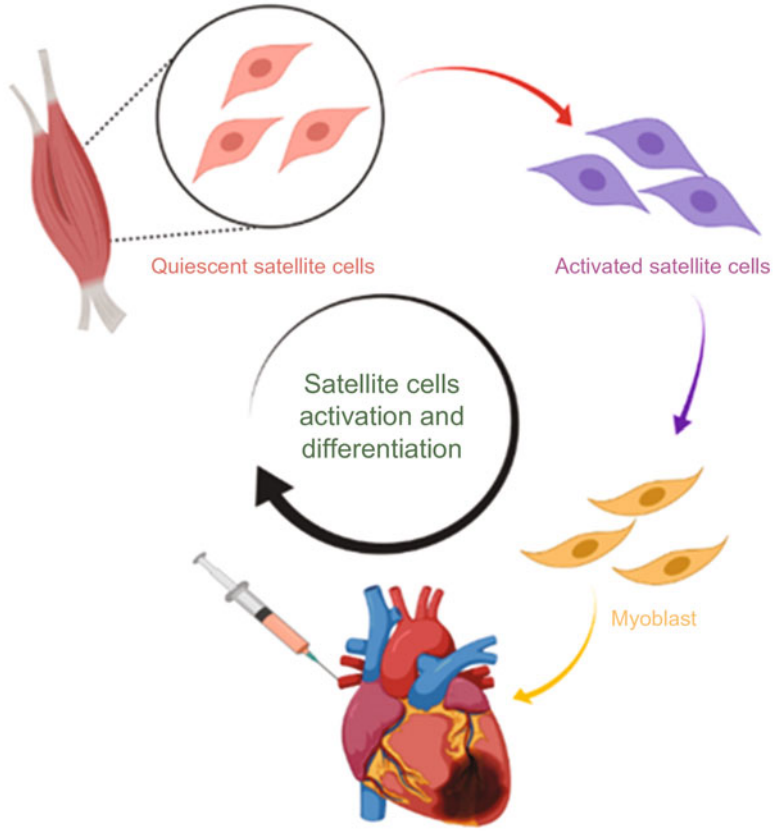


Fig. 4 The pathway cycle for quiescent satellite cell activation and differentiation into myoblasts, followed by transplant into the heart

6 months, the patients showed promising improvement in the targeted tissues especially myocardial viability (Gwizdala et al. 2017). Although, low tumorgenicity encourage the studies at first, but , many side effects were observed ranging from resistant ventricular arrhythmias to ischemic stress (Yin et al. 2013) , in addition to the inability to differentiate into cardiomyocytes in some cases (Cheitlin 2008). Due to these significant risks in patients' lives, the attention on these cells for treatment almost diminished (Müller et al. 2018).

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Cardiac Immunology: A New Era for Immune Cells in the Heart

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Abstract

The immune system is essential for the development and homeostasis of the human body. Our current understanding of the immune system on disease pathogenesis has drastically expanded over the last decade with the definition of additional non-canonical roles in various tissues. Recently, tissue-resident immune cells have become an important research topic for understanding their roles in the prevention, pathogenesis, and recovery from the diseases. Heart resident immune cells, particularly macrophage subtypes, and their characteristic morphology, distribution in the cardiac tissue, and transcriptional profile have been recently reported in the experimental animal models, unrevealing novel and unexpected roles in electrophysiological regulation of the heart both at the steady-state and diseased state. Immunological processes have been widely studied in both sterile cardiac disorders, such as myocardial infarction, autoimmune cardiac diseases, or infectious cardiac diseases, such as myocarditis, endocarditis, and acute rheumatic carditis. Following cardiac injury, innate and adaptive immunity have critical roles in pro- and anti-inflammatory processes. Heart resident immune cells not only provide defense against infectious diseases but also contribute to the homeostasis. In recent years, physiological changes and pathological processes were demonstrated to alter the abundance, distribution, polarization, and diversity of immune cells in the heart. Accumulating evidence indicates that cardiac remodeling is controlled by the complex crosstalk between cardiomyocytes and cardiac immune cells through the gap junctions, providing the ion flow to achieve synchronization and modulation of contractility. This review article aims to review the well-documented roles of both resident and recruited immune cell in the heart, as well as their recently uncovered unconventional roles in both cardiac homeostasis and cardiovascular diseases. We have mostly focused on studies on animal models used in preclinical research, underlying the need for further investigations in humans or *in vitro* human models. It may be foreseen that

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the further comprehensive investigations of cardiac immunology might harbor new therapeutic options for cardiac disorders that have tremendous medical potential.

Keywords

Cardiology · Immunology · Cardiac diseases · Cardiomyocytes · Immunoelectrophysiology · Immunology

Abbreviations

APD	Action potential duration
ATP	Adenosine triphosphate
AVN	Atrioventricular node
CCR	C-C motif chemokine receptors
CD	Cluster of differentiation
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
DAMPs	Damage-associated molecular pattern
DC	Dendritic cell
dsRNA	Double strand RNA
ECM	Extracellular matrix
GM-CSF	Granulocyte-macrophage colony stimulating factor
hsCRP	High sensitivity C-reactive protein
IL	Interleukin
LDL-C	Low-density lipoprotein cholesterol
LPS	Lipopolysaccharide
LQTS	Long QT Syndrome
LV	Left ventricle
MC_T	Tryptase-positive mast cells
MC_{TC}	Tryptase- and chymase-positive mast cells
MHC	Major histocompatibility complex
MPO	Myeloperoxidase
NETs	Neutrophil extracellular traps
NO	Nitric oxide
ROS	Reactive oxygen species
TGF	Transforming growth factor
TNF-α	Tumor necrosis factor-alpha

1 Introduction to Cardiac Immunology

Early observations of infectious diseases lead to the understanding of the immune system's ability to defend the organism against pathogenic agents by discriminating between self and non-self. The inflammation induced by tissue damage of an organ can be sterile or due to the pathogenic infections (Van Linthout and Tschöpe 2017). Although myocardial infarction (MI)-induced trauma or ischemia/reperfusion injury results in sterile tissue damage, viral or bacterial carditis leads to damage activated by infections, both of which induce inflammatory responses. Necrotic cells in damaged tissue release endogenous damage-associated molecular patterns (DAMPs) that constitute of nucleic acids, heat-shock proteins, interleukins, cytoskeletal proteins, and mostly extracellular matrix (ECM) degradation products. Pathogens are recognized by the immune system through pathogen-associated molecular patterns (PAMPs), that include lipopolysaccharide (LPS), flagellin, dsRNA, unmethylated CpG motifs in DNA, and bacterial genomic DNA (Van Linthout and Tschöpe 2017; Cao et al. 2018). Both DAMPs and PAMPs can be recognized by pathogen recognition receptors (PRRs) found in immune and non-immune cells and induce innate immune response by recruiting neutrophils, macrophages, and dendritic cells (DCs) (Epelman et al. 2015). The antigen-presenting cells of innate immune system then stimulate T and B cells to mediate adaptive immune responses (Fairweather et al. 2004; Van Linthout and Tschöpe 2017).

In recent years, immune cells in various tissues have been categorized based on their residency or recruitment to the tissue in response to inflammation. Cardiac resident and recruited immune system elements vary based on specific functions and expression profiles (Epelman et al. 2015; Cao et al. 2018). In steady-state, different immune

cell populations were identified to be localized in various heart regions, such as B cells in the pericardium, macrophages, mast cells and DCs in coronary endothelium, and a specific population of macrophages in valves and AV node (Epelman et al. 2015; Dick and Epelman 2016; Hulsmans et al. 2017). Physiological changes and pathological processes were demonstrated to change the distribution, polarization, and subtypes of immune cells in the heart. As an example, increased hematopoiesis in spleen and bone marrow supplies innate immune cells to the infarct region following MI (Epelman et al. 2015; Dick and Epelman 2016; Swirski and Nahrendorf 2018). Both proliferating resident macrophages and recruited neutrophils or monocytes work in a coordinated way in clearance and repair of the injured heart tissue.

As studies in human heart dependent mostly on small biopsies or cadaveric tissue, and the studies have been extremely limited due to ethical issues. The majority of the physiological, molecular, or functional studies in cardiac immunology were performed in animal models or *in vitro* cell culture models. Interestingly, there are new findings reporting the role of immune dysregulation in rhythm disorders as a result of both pathogenic invasions or sterile infections. In this review article, non-infectious heart diseases were discussed in greater detail than infectious heart diseases, based on the current literature. This review is mostly focused on studies on animal models used in preclinical research, underlying the need for further investigations in human tissues or *in vitro* human models.

2 Immune Cells of the Heart

Both innate and adaptive immune cell subpopulations and their distribution have been described in the cardiac tissue (Swirski and Nahrendorf 2018). In recent years, *resident* cardiac immune cell populations have been identified with distinct transcriptional profile and physiological functions that are dynamically changing in both steady-state and disease-state (Epelman et al. 2015). The aortic valves and coronary arteries contain innate cells, such as macrophages and DCs, arteries also contain mast cells, while AV node has greater amount of cardiac resident macrophages connected to the myocardium. Moreover, pericardial fluid contains macrophages and mast cells as innate immunity, and B cells as adaptive immunity (Swirski and Nahrendorf 2018). Interestingly, the heart of a healthy adult mouse contains 12-fold more CD45+ leukocytes per milligram of tissue than the skeletal muscle (Ramos et al. 2017). This abundance of leukocytes in the heart may be recruited from several local lymph nodes and the lymphatic vessels. Vascular growth factors stimulate lymphangiogenesis following MI by reducing fibrosis, thus this could be a potential regulator for cardiac regeneration (Huang et al. 2017). In this section, the literature investigating resident immune cells of the heart in the steady-state or recruitment following inflammation will be detailed.

2.1 Neutrophils

Neutrophils are present in minute amounts in a healthy heart. Following tissue damage or trauma, neutrophils are recruited to the site of injury that represents the hallmark of acute inflammation. After inflammatory cytokines, chemokines, and cleaved complement proteins are released, neutrophils infiltrate into the damaged cardiac tissue (Epelman et al. 2015). Leukocytosis by the infiltration of circulating leukocytes to the cardiac tissue is crucial for pro- and anti-inflammatory processes (Swirski and Nahrendorf 2018; Puhl and Steffens 2019). Following MI, CXCL2 and CXCL5 chemoattractants produced by cardiac resident macrophages have been shown to specifically contribute to the initial neutrophil extravasation into the ischemic area (Li et al. 2016). Once infiltrated into the infarct zone, neutrophils and Ly6C^{high} monocytes release the granule heme-enzyme myeloperoxidase (MPO) into the ECM, resulting in enhanced oxidative stress, degradation of the ECM, and further leukocyte infiltration (Puhl and Steffens 2019). Neutrophil depletion in experimental MI models

demonstrated impaired inflammatory responses resulting in enhanced fibrotic scar formation with dysfunctional remodeling and decreased cardiac function (Puhl and Steffens 2019). Neutrophils do not persist in the infarcted myocardium for long periods. Neutrophil numbers decrease after 3 days post-MI, and they almost entirely disappear following 7 days of the injury (Swirski and Nahrendorf 2018). Neutrophils activate platelets, for thrombus formation and coagulation, start the reparation process through the release of neutrophil gelatinase-associated lipocalin, mediate endothelial cell activation and cytokine release by forming neutrophil extracellular traps (NETs) (Swirski and Nahrendorf 2018; Puhl and Steffens 2019). Neutrophil degranulation leads to the release of its cytoplasmic and nuclear contents to form NETs for trapping the pathogens and DAMPs, an important initiation step in inflammation and regeneration period. NETs are activated by increased ROS generation in the early stage of MI (Puhl and Steffens 2019). Collectively, neutrophils play critical roles in immunological responses in cardiac tissue through induction of acute inflammation, the formation of NETs, the recruitment of monocytes, and finally stimulating macrophage polarization.

2.2 Mast Cells

Mast cells produce histamines and are associated with the allergic reactions and subsequent vasodilation. In fact, mast cells store and release a variety of biologically active mediators involved in tissue remodeling, including pro-inflammatory cytokines, proteases, growth factors, and fatty acid metabolites (Reber et al. 2015). Based on differences in enzyme secretion and activity, two distinct mast cell phenotypes are classified as the MC_T and MC_{TC}. MC_T is typically found in mucosal tissue, MC_{TC} is found predominantly in connective tissue (Reber et al. 2015). Studies have reported that cardiac mast cells are consistent with the MC_{TC} subtype (Janicki et al. 2015).

The pericardial adipose tissue has been shown to contain a high density of lymphoid clusters and cardiac mast cells (Janicki et al. 2015; Swirski and Nahrendorf 2018). Increased numbers of mast cells have been reported in human hearts explanted from dilated cardiomyopathy patients and in experimental animal models of hypertension and MI (Janicki et al. 2015). Mast cell secretions can activate matrix metalloproteinases (MMPs) that lead to rapid collagen degradation of interstitial collagen matrix after myocardial damage (Janicki et al. 2015). Moreover, gender differences play a role in the incidence of cardiac diseases in humans favors of women, for example, estrogen was shown to keep MMP-2 levels low leading to collagen degradation and ventricular dilatation (Levick et al. 2011; Janicki et al. 2015). As estrogen may have cardioprotective roles via the effect on mast cells, the influence of male and female hormones on cardiac mast cell phenotype, as well as their regulatory pathways, needs to be investigated in detail. Another issue that remains to be demonstrated is understanding how cardiac mast cells interact with other inflammatory cells in the heart during both steady-state and after tissue damage.

2.3 Monocyte/Macrophages

Monocyte/macrophages have diverse functions, such as plasticity, microbicidal activity, antigen-presentation, fibrosis, tissue healing, and immunoregulatory functions (Barin et al. 2012). In the steady-state, phagocytosis by macrophages clears the cell debris as a result of the normal apoptotic process in hemostasis. Macrophages are highly responsive and easily polarized and differentiate from circulating monocytes (Murray et al. 2014). Macrophage polarization and diversity in the tissue depend on the chemokine cues and environmental stimuli varying before and after a disease state (Davis et al. 2013). Macrophages are polarized towards the M1-type in response to pro-inflammatory signals, such as IFN-γ and LPS, leading to the upregulation of lysosomal activation, oxygen radicals, and peroxide production (Barin et al. 2012; Tan et al. 2016). In Th1-associated host defenses, M1 macrophages are considered as a major effector. In the steady-state, IL4/IL13-elicited M2 macrophages play

significant roles in anti-inflammatory and Th2-mediated responses. Importantly, M2 macrophages promote wound healing, angiogenesis, fibrotic, and scavenger processes (Tan et al. 2016).

Cardiac macrophages are present in the murine heart, constituting 6–8% of non-cardiomyocytes within the myocardium (Peet et al. 2020). In a healthy adult mouse heart, macrophages can be distinguished by the expression levels of CCR2 and MHCII (Lavine et al. 2018). Genetic fate-mapping studies reveal that $CCR2^{-}$ cardiac macrophages are established early in development from the primitive yolk sac and self-maintained into adulthood, while $CCR2^{+}$ macrophages are replenished by bone-marrow-derived monocytes after a cardiac damage (Hulsmans et al. 2017; DeBerge et al. 2019). Resident and recruited macrophages have distinct roles during cardiac inflammation (Bajpai et al. 2018). Fate mapping, parabiosis, and single-cell transcriptome analysis revealed that recruited monocyte-derived macrophages modulate cardiac inflammation, but are less efficient in antigen processing and debris clearance (Bajpai et al. 2018; Dick et al. 2019). After a cardiac injury, resident cardiac macrophages produce inflammatory cytokines and chemokines, such as IL-1, IL-6, TNF-α, and CCL2, cardiac fibroblasts release hematopoietic growth factors, such as GM-CSF to activate endothelial cells. Then $Ly6G^{+}$ neutrophils recruit via the help of neutrophil-attracting chemokines such as CCL2 and CCL7 and $Ly6C^{+}$-$CCR2^{+}$ double-positive monocytes mobilize from the blood to the injury site in greater numbers in mice (Murray et al. 2014; Epelman et al. 2015; Swirski and Nahrendorf 2018; Bajpai et al. 2018; Dick et al. 2019). Furthermore, $TIMD4^{+}$ $LYVE1^{+}$ $MHC\text{-}II^{low}$ $CCR2^{-}$ resident murine cardiac macrophages self-renew by regulating G1/S transition, contributing to the regulation of vasculogenesis and wound healing (Bajpai et al. 2018; Dick et al. 2019).

Monocytes/macrophages are also involved in angiogenesis through their secretion of pro- and anti-inflammatory factors. It was shown that macrophages acted as a helper for vascular fusion to create new vessels with the existing network by guiding and physically forming bridges (Fantin et al. 2010). Furthermore, resident mouse $CCR2^{-}$ macrophages were shown to localize into the coronary artery and necessary for remodeling of the primitive coronary plexus through secretion of insulin-like growth factor and other pro-angiogenic signals (Leid et al. 2016).

In addition to the canonical roles of macrophages in inflammation, their unconventional roles have been recently revealed as supportive cells for cardiac conduction, the regulator of membrane action potential, or cardiac cell cycle re-entry (Gomez et al. 2018). In fact, resident cardiac macrophages are shown to abundantly reside at the AV node and express a specific cardiac gap junction protein, allowing electrical coupling with cardiomyocytes and conduction system as depicted in Box 1 (Hulsmans et al. 2017).

Box 1 Immuno-Electrophysiological Roles of Cardiac Macrophages

Pro-inflammatory immune mediators can affect the intracellular Ca^{2+} homeostasis, thereby regulating the electrophysiological properties of the cardiac muscle. It was previously demonstrated that TNF-α, as a pro-inflammatory cytokine, stimulation can decrease the expression of Ca^{2+}-ATPase and Ca^{2+}-regulatory proteins of sarcoplasmic reticulum in adult cardiomyocytes progressively resulting in left ventricular diastolic dysfunction (Tsai et al. 2015; Van Linthout and Tschöpe 2017). Moreover, TNF-α stimulation was found to trigger cardiomyocyte apoptosis, and another pro-inflammatory cytokine IL-1β was shown to mediate cardiomyocyte pyroptosis (Van Linthout and Tschöpe 2017). Furthermore, IL-6 has been demonstrated to increase the cardiac muscle stiffness via reducing the phosphorylation of titin, affecting the contractility (Savvatis et al. 2014). Altogether, those reports underline the importance of the diverse unconventional functions of immune mediators in cardiac electrophysiology.

Apart from their conventional roles in innate immunity, novel modulatory roles of

(continued)

Box 1 (continued)

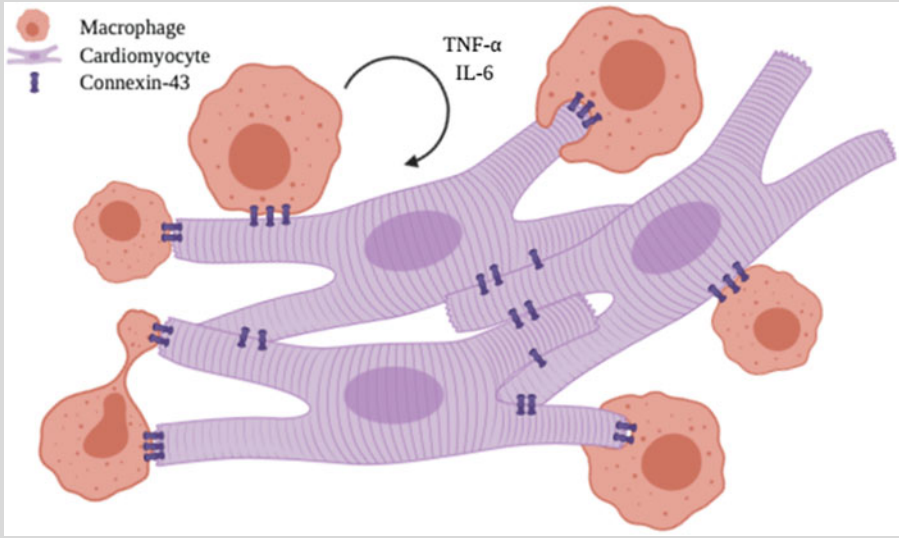

Fig. 1 Cardiomyocytes and cardiac macrophages contact through Cx43 gap junctions. The cartoon depicts the immuno-electrophysiological regulatory role of interaction between cardiomyocytes and cardiac macrophages through Cx43 gap junctions and secreted pro-inflammatory cytokines TNF-α and IL-6

macrophages have been revealed in cardiac electrophysiology. Recently, an unpredicted role of cardiac macrophages in the regulation of the cardiomyocyte membrane potential was demonstrated in mouse models by optogenetic, electrophysiological, and immunohistological studies (Hulsmans et al. 2017). Hulsmans and colleagues reported that tissue-resident macrophages studied in both murine and human heart sections were abundant in the AV node, as well as myocardium (Hulsmans et al. 2017). In this study, cardiac resident macrophages have been shown to directly modulate the electrical activity of cardiomyocytes through gap junctions, particularly Cx43 proteins, as summarised in Fig. 1. Moreover, Cx43 knockout cardiac macrophages were shown to delay or block AV node conduction. Due to this deficiency, slower repolarization occurs in cardiomyocytes connected with Cx43-depleted macrophages resulting in impaired AV conduction (Swirski and Nahrendorf 2018). They further determined that more macrophages physically interacting with a single cardiomyocyte, the higher beating frequency of cardiomyocytes were detected as a result of more cation flux towards the cardiomyocytes from the macrophages. Based on the findings of this remarkable study, heart diseases could be evaluated from a yet largely neglected dimension by considering cardiac macrophage population and the effect of its dysregulation in the progress of cardiac disorders.

3 Inflammatory Heart Diseases

The global prevalence of cardiac infectious diseases is considered rare, but associated with high morbidity and mortality (Murillo et al. 2016). A vast number of studies demonstrate inflammation as a cause or consequence of the majority of heart diseases. Therefore, inflammation through (i) stress factors, such as aging, hypertension, pressure overload, ischemia,

hypercholesterolemia, diabetes, and obesity, and (ii) pathologic factors, such as endothelial dysfunction, atherosclerosis, and acute coronary syndrome, need to be evaluated in detail for cardiac diseases. Without any infectious agents, signaling molecules released by injured or stressed cardiac tissue can induce sterile inflammation, as in the case of post-ischemic or toxic necrosis, massive trauma, or hemorrhage (Van Linthout and Tschöpe 2017). On the other hand, infectious organisms, such as bacteria, viruses, fungi, or parasites, can rapidly initiate the systemic immunological response directly or indirectly affecting the heart. Therefore, inflammatory factors, including high levels of leukocytes, fibrinogen, and CRP levels in the blood, as well as other metabolic or chronic disorders, are routinely assessed by clinical evaluations of the patients and were reported to contribute to the cardiac diseases (Pearson et al. 2003). In fact, certain patients receive anti-inflammatory drugs as a treatment approach for lowering common inflammatory markers, such as CRP as the most relevant (summarised in Box 2).

Box 2 Inflammatory Marker CRP in Cardiac Disorders

C-reactive protein (CRP) is a non-cardiac, specific inflammatory marker, and an acute phase protein with a long plasma half-life. CRP is dominantly synthesized by hepatocytes in response to the pro-inflammatory cytokines and considered as an 'upstream' biomarker for TNF-α or IL-6 (Shrivastava et al. 2015). CRP binds to the phosphorylcholine structures on the membrane of an infectious organism, then macrophages recognize and phagocytose the CRP-bound pathogens through their CRP receptors. CRP also plays a role in atherogenesis, acting on vascular smooth muscle cells and inhibiting NO synthesis (Shrivastava et al. 2015). CRP directly bind to atherogenic oxidized LDL-C and is present within lipid-laden plaques in atherothrombotic and atherosclerotic diseases (Marchio et al. 2019). Moreover, CRP facilitates monocyte adhesion and transmigration into the vessel wall initiating early immunological processes in atherogenesis (Van Linthout and Tschöpe 2017). CRP can be also elevated by a non-infectious situation, as in the case of trauma-mediated alarmin response (Nehring et al. 2020). When it comes to the diagnosis of chronic diseases, high-sensitivity assays are performed, detecting low levels of CRP, named hsCRP. HsCRP levels rise along with various risk factors, such as aging, smoking, and obesity, and hsCRP is widely used to predict the chronic inflammation in cardiac diseases (Yousuf et al. 2013). In SCORE (systematic coronary risk evaluation) study, CRP levels higher than 10 mg/L statistically correlate with a risk higher than 4% to develop a fatal cardiovascular event in the next 10 years (Cozlea et al. 2013). Beyond the direct role of CRP onto the vascular cells of the heart, CRP was reported to direct human macrophage polarization into the M1 phenotype by suppressing the generation of the reparative M2 phenotype (Devaraj and Jialal 2011). Moreover, 5806 and 5382 participants were followed for up to 17 years, who were assessed by high IL-6 and high CRP levels, respectively, and revealing an association of CRP with sudden cardiac mortality (Hussein et al. 2013).

3.1 Endocarditis

Endocarditis is the pathogenic infection of the endocardium or transplanted prosthetic material surfaces, such as valves. Endocarditis prevalence is higher in men than in women (>2:1), and in the clinic, endocarditis is mostly seen in individuals older than 65 years of age, likely associated with aging (Murillo et al. 2016). Diverse clinical outcomes of endocarditis are fever, hypothermia, tachycardia, tachypnea, or abnormal white blood cell count, progressing to septic shock and acute organ failure (Rubio et al. 2019). The most typical

and frequent infectious organism causing endocarditis is *Staphylococcus* species, which attach to the endocardium of the valve or side of the atrium (Murillo et al. 2016). *Staphylococcus* components, lipoteichoic acids, and peptidoglycans, serve as critical pro-inflammatory stimulants. At the first hours of *Staphylococcus* invasion of the endothelial cells, the non-professional phagocytes start to ingest bacterial clusters, causing endothelial cell apoptosis and ultimately endothelial destruction (Rubio et al. 2019). Moreover, macrophages located between endothelial and subendocardial interstitial tissue initiate the acute inflammatory response by releasing CCL5 and other pro-inflammatory cytokines and chemokines. CCL5 was shown to be secreted by endothelial macrophages for recruiting leukocytes to the infected region (Rubio et al. 2019). Higher CD4+ and CD8+ T cells and B lymphocyte cell counts elevated IgM and IgG serum levels, and C4 complement system was found associated with infective endocarditis patients (Forte et al. 2001). Moreover, signaling of endothelin-1, nitric oxide, prostaglandins, prostacyclin, angiotensin I-II, and VEGF between cardiac endothelial cells and cardiomyocytes alter cardiac physiology and electrophysiology. Therefore, deformation of the endothelial layer caused by endocarditis disturbs cardiac physiology, contractile performance, and eventually rhythmicity of the heart (Rubio et al. 2019).

3.2 Myocarditis

Myocarditis, an infectious inflammatory disease of the myocardium, is often caused by viral, bacterial, or protozoa infections, leading to dilated cardiomyopathy, heart failure, or autoimmunity (Swirski and Nahrendorf 2018). Non-infectious, or sterile, myocarditis may also occur in immunological disorders, such as sarcoidosis (Murillo et al. 2016). From the broad infectious agents underlying the basis of myocarditis; coxsackievirus, adenovirus, parvovirus, hepatitis C virus, influenza A and B viruses, and HIV are the most prominent viral factors (Swirski and Nahrendorf 2018). Inflammation due to infectious or sterile myocarditis first activates macrophage and other cardiac resident immune cells through innate immunity. An analysis of autopsy samples isolated from myocarditis patients with sudden death revealed that monocyte and macrophage infiltration to the cardiac tissue were among the most common observations (Barin et al. 2012).

The pathogenic enterovirus, named Coxsackievirus, is one of the most common causes of acute myocarditis (Gaaloul et al. 2014). Coxsackievirus shows a cardiac tropism partly due to the high expression of 'CARs-Coxsackievirus and adenovirus receptors' in cardiomyocytes. Mast cells, which are one of the first cells to respond to myocardial damage after Coxsackievirus infection, degranulate rapidly, and produce inflammatory mediators (Fairweather et al. 2004). In the early asymptomatic days of virus-infected myocarditis, infiltrating killer lymphocytes and NK cells direct the infected cells to apoptosis by also extending tissue damage to the nearby uninfected myocytes. Infiltrating neutrophils and monocytes further contribute to the pro-inflammatory cascades, such as Th1-type immune response mediated by IL-12-induced IFN-γ stimulation. In fact, IL-12 deficient mice showed higher viral replication and limited TNF-α and IFN-γ cytokines (Swirski and Nahrendorf 2018). The subacute phase post-infection is accompanied by a more complex immune response and myocyte cytotoxicity from circulating autoantibodies, cross-reactive epitope mimicry, and finally, autoreactive immune cell recruitment. Beyond 15 days following infection is considered as the chronic phase, in which the viral genome integrated into cardiomyocytes leads to cross-reactivity, accounting for chronic inflammation and dilated cardiomyopathy (Murillo et al. 2016). Against virus-induced myocarditis, T cell responses play a significant role as adaptive immunity through both Th17 cells, which causes dilated cardiomyopathy by affecting cardiac fibroblasts, and Treg cells, which decrease the inflammation and provide protection (Swirski and Nahrendorf 2018).

3.3 Pericarditis

Pericarditis is an inflammatory disease of the fibroserous sac covering the heart, caused by viral, bacterial, or fungal agents (Mayosi et al. 2005; Dababneh and Siddique 2020). After the invasion, the pathogen colonizes in the pericardium stimulating an inflammatory response (Murillo et al. 2016). Elevated hsCRP was identified in approximately three out of four acute pericarditis patients involving viral or idiopathic pericarditis cases (Imazio et al. 2011). Increased anti-myolemmal and anti-fibrillary antibodies in pericardial fluid determined in pericarditis patients indicated high immunological reactivity in chronic pericardial effusion (Karatolios et al. 2016).

Pericardial involvement can also be related to the systemic autoimmune diseases, such as commonly systemic lupus erythematosus, rheumatoid arthritis, and systemic sclerosis. Early-inflammatory events, including complement pathway activation and leukocyte infiltration, were detected in the pericardial fluid in systemic autoimmune disease patients (Ramasamy et al. 2018). Moreover, in the acute phase of MI, pericarditis may occur due to the cardiomyocyte necrosis and vascular injury resulting in pericardial effusion (Ramasamy et al. 2018). On the other hand, the development of pericardial fibrosis at the post-inflammation period was found to be dependent on the secretion of both pro- and anti-inflammatory fibrotic mediators, such as Galectin-3, Ac-SDKP (N-acetyl-seryl-aspartyl-lysyl-proline), and basic fibroblast growth factor (bFGF) (Ramasamy et al. 2018).

3.4 Rheumatic Fever

Rheumatic fever (RF) is mediated by autoimmune reactions triggered by molecular mimicry between *S. pyogenes* and human proteins as a result of post-streptococcal throat infection (Guilherme et al. 2012). Acute RF involves all three layers of the heart, and also displays non-heart symptoms, such as joint swelling, skin rashes, and nodules. Rheumatic heart disease (RHD) is seen after valvular damage triggered by immune responses through the infection in 30–45% of RF patients (Guilherme and Kalil 2015).

M protein and N-acetyl-beta-D-glucosamine, the antigenic structures of the *S. pyogenes,* show structural homology with γ-helical coiled-coil structured human proteins, such as cardiac myosin, tropomyosin, keratin, laminin, vimentin, and several valvular proteins (Guilherme and Kalil 2010). This cross-reaction causes T-cell recognition and T-cell-mediated destruction, and autoimmunity. Autoimmune reactions are mainly mediated by macrophages and heart-infiltrating CD4+ T lymphocytes. A small number of CD8+ T cells also infiltrate into the heart lesions. Th1-type cytokines play a dominant role in lesions of RF. Furthermore, the deficiencies in Th2-type cytokines-producing cells, showed by low levels of IL-4, may contribute to the progression of the lesions in RHD pathogenesis (Guilherme et al. 2006).

The molecular findings show a genetic susceptibility associated with RF pathogenesis. Within the period of an early innate immune response to RF, *MBL2, FCN2, FCγRIIA,* and *TLR2* gene expression have been found to be significantly altered in patients (Guilherme et al. 2012). In addition, variations in both innate and adaptive immunity genes, such as *TNFA, IL1RA, TGFB1*, and *CTLA4,* implied relation to the pathogenesis of RF and RHD (Guilherme et al. 2012). Moreover, immunohistochemical analysis on myocardium and valves from acute and chronic RHD patients showed a large number of mononuclear cells expressing pro-inflammatory cytokines, such as TNF-α, interferons and the regulatory cytokine IL-10 (Guilherme et al. 2012). Therefore, immunological cascades in RHD involve critical roles in the pathogenesis of autoimmunity and chronic inflammation (Guilherme et al. 2012).

3.5 Chagas' Disease

Chagas' disease is caused by a protozoan parasite *T. cruzi* infection in the myocardium that persists throughout a lifetime. Chagas' patients can

remain asymptomatic or turn into a chronic form with cardiac and/or digestive symptoms (Machado et al. 2013). Chagas' infection causes cardiomyocyte necrosis, leading to autoimmunity, thromboembolism, stroke, arrhythmia, and heart failure (Machado et al. 2013). While immunoregulatory cytokines, such as TGF-β and IL-10, increase susceptibility to the acute infection, TNF-α and IFN-γ regulate chemokine production by *T.cruzi*-infected macrophages and cardiomyocytes (Machado et al. 2013). Both human studies and mice models indicated that the production of IL-12, TNF-α, and IFN-γ, a group of Th1 pro-inflammatory cytokines induce macrophages to release NO. Macrophages consequently kill the parasites and contribute to the recruitment and activation of leukocytes via chemokines, such as CLL2 and CCL5 (Machado et al. 2013). Therefore, elevated levels of TNF-α and IFN-γ in the blood and cardiac tissue are shown to be indicators of the severity of cardiac symptoms in chronic Chagas' patients (Machado et al. 2013).

Chagas' disease may also cause initiation of the autoimmunity cascades through the overexpression of CD8+ T cells, especially autoreactive ones. There is an excessive generation of self-antigens in the niche of the damaged region, through the inflammatory mediators, such as cytokines, chemokines, lymphotoxin, NO from cardiac immune cells, and granule components from eosinophils. Therefore, *T. cruzi* infection can activate the subsequent parasite-specific and non-specific immune responses in the heart (Bonney and Engman 2015).

3.6 Lyme Disease

Lyme disease, also named borreliosis, is caused by the bacterium *B. burgdorferi* and is transmitted to humans through the infected tick bites. Lyme disease has multi-systemic effects on various regions of the body, associated with neuroborreliosis, Borrelia arthritis, or carditis (Widhe et al. 2004; Krause and Bockenstedt 2013). *B. burgdorferi* dissemination in the blood could colonize in most parts of the heart, including the conduction system around the AV node, endocardium, myocardium, and coronary circulatory system, or heart valves. Pericarditis and myocarditis may also occur, however, Lyme carditis is typically characterized by AV block and is often treated with antibiotics (Krause and Bockenstedt 2013).

4 Non-infectious Cardiac Diseases and Immunity

4.1 Myocardial Infarct (MI)

MI can occur in the presence of many risk factors, such as hypertension, smoking, dyslipidemia, diabetes, and family history of coronary heart disease (Hajar 2017). The sequence of events in acute MI includes sudden interruption of blood flow by atherosclerotic plaque formation, plaque rupture, coronary artery thrombosis, or coronary occlusion, all of which also trigger the immune response (Weil and Neelamegham 2019). The intramyocardial inflammation proceeding the cardiomyocyte death from ischemia and reperfusion injury in MI, similar to infection or cardiac toxicity, promotes the replacement of myocardium with noncontractile fibrotic scar (Weil and Neelamegham 2019). NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3), IL-1β, and IL-18 mRNA expressions were found to be increased in the left ventricle tissue in the acute phase of MI, indicating the activation of a high inflammatory cascade in the myocardium (Van Linthout and Tschöpe 2017). The inflammation then promotes infiltration of circulating monocytes and neutrophils, as well as the expansion of resident macrophage population at the site of infarction followed by secretion of pro-inflammatory cytokines and chemokines (Peet et al. 2020). In fact, macrophages play an important role in both pro- and anti-inflammatory phases in MI. Cellular fragments released by injured myocardium can trigger resident cardiac macrophage activation and mast cells through endogenous 'alarmins' and DAMPs (Peet et al. 2020). Endogenous DAMPs, such as released mitochondrial or genomic DNA and ROS

released from mitochondria, can also activate the complement system increasing C3 and C5 fragments promoting neutrophil infiltration and leukocyte translocation into the injured myocardium. Cao et al. showed that released genomic DNA fragments from necrotic tissue were recognized by cGAS-STING pathway shifting macrophages towards M1 state (Cao et al. 2018). Thus, to prevent excessive tissue damage through inflammation, the cGAS-STING pathway can be silenced with drugs, polarizing macrophages to the M2 phenotype enabling angiogenesis and ECM remodeling for cardiac repair (Cao et al. 2018).

The generation of a large amount of ROS from mitochondria impairs myocardial function and activates leukocyte migration, reducing the number of functional cardiomyocytes and triggering ECM degradation (Fioranelli et al. 2018). Studies have shown that sustained expression of TNF-α contributes to the development of heart failure; however, TNF-α can also exert cytoprotective effects in a desmin-deficient heart failure model in mice (Papathanasiou et al. 2015). Moreover, mice depleted with IL1R1, the only signaling receptor for IL1, not only attenuated the recruitment of inflammatory monocytes/macrophages, but also diminished the regenerative M2 subtype (Saxena et al. 2014). In other words, excessive M1 macrophage activation may be destructive. In a monocyte-depleted mice model to decrease M1 type macrophage number, the functional outcomes after MI-induced cardiac dysfunction were improved (Hulsmans et al. 2016). Moreover, cardiac-resident macrophages seemed to display M2 phenotype more than infiltrating subtypes. M2 type resident cardiac macrophages provide robust pro-angiogenic and regenerative features and are responsible for the tissue homeostasis (Ma et al. 2018). In order to trigger regenerative phenotype after MI, targeting the up-regulation of canonical M2 genes in myocardial macrophages, using IL-4, IL-10, or $GABA_AR$ (Gamma aminobutyric acid A receptor) agonism, were indicated to protect against degenerative ventricular remodeling (Shiraishi et al. 2016, Peet et al. 2020).

Neutrophils were shown to migrate rapidly to the infarct zone guided by chemoattractants (Hofbauer et al. 2019). NETs formed by neutrophils stimulate cytokine release from macrophages and also activate Th-17 cells, resulting in increased immune cell recruitment to the atherosclerotic plaques (Warnatsch et al. 2015). In ST-elevation following MI, NETs were found to be positively correlated with the infarct size and left ventricular dysfunction through the stimulation of fibrosis (Hofbauer et al. 2019). Over the course of several days of the inflammation, a reparative phase commences dominated by the removal of neutrophils and the recruitment of $Ly6C^{low}$ monocyte derived-macrophages promoting angiogenesis and healing in mice (Swirski and Nahrendorf 2018; Puhl and Steffens 2019). At this post-MI reparative period, hematopoietic progenitor cell proliferation and migration at the infarct side were shown to be regulated by chemokine receptor signaling, such as CXCR4 (Mayorga et al. 2016).

Mast cells are key effectors of the innate immune response during MI. Perivascular localization of mast cells allows storage of inflammatory mediators to be released into the blood following their rapid degranulation in the acute phase of MI (Prabhu and Frangogiannis 2016). In the first 24 h after an ischaemic cardiac event, endogenous DAMPs induce the release of histamine, TNF-α, IL-6, prostaglandins, leukotrienes, tryptase, chymase, and renin from mast cells, leading to an acute immune response, activating resident immune cells (Fioranelli et al. 2018). Moreover, within a few days after MI infiltrating mast cell progenitors into the heart from the white adipose tissue mature, then play a role in the repair and protection against cardiac dysfunction (Ngkelo et al. 2016).

Mast cells are also crucial in cardiac contractility via alteration of PKA-regulated Ca^{2+} signaling in response to MI. Mast cell-dependent mechanism of PKA activity was shown to promote myofilament phosphorylation induced by mast cell-released tryptase (Ngkelo et al. 2016). The main roles of cardiac MC_{TC} after the pro-fibrotic stimuli during MI was depicted in

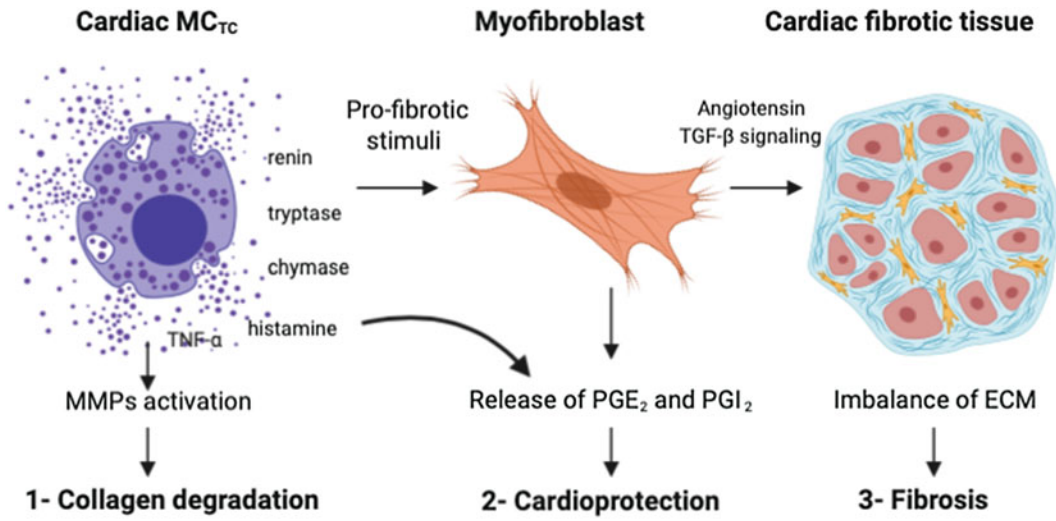

Fig. 2 Cardiac mast cells during and after MI. Cartoon depicting the roles of cardiac mast cells in the case of MI. PGE_2: Prostaglandin E2, PGI_2: Prostaglandin I2

Fig. 2. In the acute phase of MI, cardiac MC_{TC} secretes renin, chymase, tryptase and TNF-α activating MMPs leading to the massive collagen degradation. After the myocardial damage established, released histamine from cardiac mast cells stimulate myofibroblasts to secrete PGE_2 as well as the stable metabolite of the cardioprotective PGI_2 (Levick and Widiapradja 2018). At later stages, the angiotensin and TGF-β signaling cascades initiate irreversible fibrosis. Consequently, after an acute coronary syndrome, repair of damaged tissue can be mediated by collagen formation, myofibroblast proliferation and angiogenesis on the site of infarct with the contribution of the cardiac MC_{TC}.

The subsequent role of the immune system in the acute and reparative periods post-MI are also regulated by the lymphatic system. The cardiac lymphatic vessels may play a role in regeneration by draining excess fluid from the extracellular spaces and transporting the adaptive immune cells between inflammatory tissue and the cardiac lymph nodes (Zhang et al. 2019). Moreover, the impairment of lymphatic drainage was shown to lead to the cardiac lymphedema after MI (Zhang et al. 2019), therefore stimulating lymphangiogenesis through vascular growth factors to improve lymphatic drainage may contribute to the recovery of the infarcted myocardium.

4.2 Atherosclerosis

Atherosclerosis is the leading cause of heart attacks, strokes, and peripheral vascular disease caused by the lipid storage and restriction of blood flow in the arteries. The dysfunctional endothelial cells are the first step towards vascular diseases, affecting endothelium niche of vascular smooth muscle cells and immune cells to promote atherosclerotic lesions accompanying acute coronary events (Fioranelli et al. 2018). Atherosclerotic lesions/plaques develop with the accumulation and trapping of LDL in the intima of the arteries (Linton et al. 2000). Inflammation is involved throughout all atherogenesis steps from foam cell accumulation to fatty streak organization and fibrous plaque formation, until acute plaque fissuring, rupture, and thrombosis occurrence (Fioranelli et al. 2018). In fact, pathological conditions and common cardiovascular risk factors, such as hypertension, hyperlipidemia, hyperglycemia, and smoking, can elicit immune responses that promote the secretion of pro-inflammatory cytokines and chemotactic

factors, inducing monocyte adhesion to the endothelial region and transmigration into the subintimal space (Fioranelli et al. 2018). Later, circulating monocytes are recruited to the atherosclerotic lesions to differentiate into the pro-inflammatory macrophages, namely lipid-laden foam cells, which engulf cholesterol-rich oxidized lipoproteins (Chistiakov et al. 2017; Linton et al. 2000). Vulnerable atherosclerotic plaques caused by an uncontrolled inflammation typically have a necrotic lipid core with a thin, fibrous cap and contain a large number of foam cells. When the plaques rupture, MI, sudden death, or stroke are the possible outcomes (Linton et al. 2000).

From the immunological perspective, the pro-inflammatory stimuli from the atherosclerotic plaques results in recruitment of more macrophages, mast cells, and activated T and B cells that increase vascular lesions. IL-1 and IL-6 act as chemotactic mediators, with IL-1 levels indicating the presence of unstable atheromatous disease (Kouvas et al. 2018). In the presence of CD4+ lymphocytes, TNF-α promotes oxidized LDL uptake by macrophages and facilitates leukocyte migration through VCAM1-expressing endothelial cells, increasing the oxidative stress over the plaque (Kouvas et al. 2018). In fact, through chemokine signaling, a high number of infiltrating immune cells cause an imbalance between the firing of inflammation and modulation of immunity (Linton et al. 2000). In a vulnerable plaque mouse model, the inhibition of antibody-mediated chemokine ligand Cxcl10 resulted in a more stable plaque phenotype (Segers et al. 2011). Targeted depletion or pharmacological inhibition of Cxcr3 decreased plaque formation, recruitment of Th1 inflammatory cells, and increased migration of Treg cells to the lesions in ApoE-knockout mice (Szentes et al. 2018). On the other hand, based on clinical studies, chemokine signaling may be used as a marker for diagnosis. A significant association of increased serum CXCL10 level with the risk of coronary heart disease was reported (Szentes et al. 2018). Moreover, enhanced systemic levels of CXCL9, CXCL10, and CXCR3 were also observed in patients with stable angina pectoris (Szentes et al. 2018).

Macrophage polarization is another key feature of the atherosclerosis progress. Cholesterol crystals, IFNγ, LPS, and oxidized LDL are all known to stimulate M1 macrophage activation. M1-activated cytokines further support the monocyte recruitment and macrophage maintenance in the plaque area (Tan et al. 2016). Due to the involvement in plaque destabilization, the pro-atherogenic M1 macrophage populations predominantly accumulate at the plaque area. On the other hand, M2 macrophages mostly reside at stable cell-rich areas of the plaque and blood vessels tending to prevent foam cell formation and protect against atherosclerosis (Moore et al. 2013; Tan et al. 2016).

4.3 Conduction Disorders

Although cardiac conduction system disorders are focused on pathophysiological studies, the contribution of immune system in disease pathogenesis was largely underestimated. Regarding the role of immune system in conduction diseases, cardiac macrophages became a candidate to be investigated. As cardiac macrophages largely participate in immune surveillance and cardiac development, recent studies suggest an important and specialized cardiac function in regulating the contractility, especially at the AV node (Epelman et al. 2014; Hulsmans et al. 2017). Due to these pioneering studies, new terminology and area of research were introduced as cardiac immunoelectrophysiology, demonstrating the unconventional and essential role of cardiac resident immune cells in the electrical conduction of the heart.

4.3.1 Atrial Fibrillation (AF)

AF is characterized by the rapid, irregular, and dysfunctional beating of the atrial chambers of the heart. The prevalence of AF is 1–2% in the general population, reaching up to 10% in elderly patients (Smorodinova et al. 2017). As an initial inflammation period, higher hsCRP levels were observed to be positively correlated with stroke risk factors, such as diabetes and hypertension, in AF patients (You et al. 2010). Moreover, the high levels of an early inflammation marker IL-6 in the

serum were found to be an associated risk factor for AF in a cohort of subjects with coronary artery disease (Harada et al. 2015). Mostly infiltrating leukocytes and deposition of MPO as the elements of innate immunity were reported to play a role in arrhythmogenesis and AF pathogenesis (Rudolph et al. 2010). Higher plasma levels of MPO have also been observed, suggesting MPO as a profibrotic mediator of AF (Rudolph et al. 2010). In an experimental animal model, increased mainly pro-inflammatory $iNOS^+$, $Arg1^-$ M1 phenotype macrophage population was identified in the arrhythmic atrial tissue samples by protein analysis (Sun et al. 2016). AF induced-pro-inflammatory macrophage polarization was shown to shift towards M1 macrophages exacerbating atrial electrical remodeling by secreting predominantly IL-1β (Sun et al. 2016). In the same study, LPS-stimulated pro-inflammatory macrophages induced atrial electrical remodeling, decreasing the effective atrial refractory period, and L-type calcium currents in both canine and mouse AF models (Sun et al. 2016). In a relevant clinical report, the count of both CD4+ and CD8+ lymphocytes and CD68- and KP1+ DCs were elevated in the left atrial myocardium of patients displaying AF, compared to those with normal sinus rhythm (Smorodinova et al. 2017). Moreover, the number of mast cells and CD20+ B-lymphocytes did not differ between AF and healthy controls. Collectively, in AF cases, the number of cells infiltrating to the heart can be expected to increase, whereas the number of resident cardiac immune cells does not likely to be affected based on the reported literature (Smorodinova et al. 2017). These data also suggested that pro-inflammation may affect electrophysiological properties of myocardial tissue towards AF pathology.

4.3.2 Arrhythmia

Cardiac arrhythmia is caused by genetic or drug-induced abnormalities of the conduction system of the heart displaying irregular beating (Lazzerini et al. 2015). It was newly discovered that immune system modulators are the effectors of the cardiac rhythm. Immune cells and their secreted cytokines and chemokines were found to regulate the rhythmicity of the heart both through systemic and local signaling mechanisms (Lazzerini et al. 2017). Regarding rhythm disorders, it can be inferred that the immune system regulators could contribute to the arrhythmia in several ways; (i) direct interaction between immune cells with cardiac fibroblasts and/or myocytes leading to insulating fibrosis, (ii) direct participation of macrophages in the electrical regulation of conducting myocytes, (iii) direct modulation of ion channel presentation and/or function on the surface of cardiomyocytes promoting cardiac arrhythmia by inflammatory cytokines and autoimmunity (Lazzerini et al. 2017).

Whether inflammatory activation is a cause or consequence of arrhythmia, accumulating data indicate inflammation as a potential contributor of life-threatening forms of congenital arrhythmias. First, severe inflammatory heart diseases, such as myocarditis, can be frequently associated with acquired Long QT Syndrome (LQTS), characterized by a prolonged QT interval on the electrocardiograms (Lazzerini et al. 2015). Other reported causes of acquired LQTS include the adverse effects of drugs, bradyarrhythmias, endocrine disorders, liver diseases, HIV infection, and toxins, all of which also influence immune response through persistent secretion of pro-inflammatory mediators systematically (Lazzerini et al. 2015). The long-term secretion of main pro-inflammatory cytokines from the myocardium in chronic heart failure and ischemic heart disease were shown to recruit the innate immune cells, leading to chronic remodeling and cardiac fibrosis (Kouvas et al. 2018). This persistent pro-inflammatory process in the ventricular tissue may consequently deteriorate the systolic function resulting in ventricular hypertrophy and dilation, inclining the tissue towards arrhythmia (Dick and Epelman 2016).

The cytokines may have a direct effect on membrane ion channel expression and/or function by modulating gap junctions and other cell-cell communication proteins, as summarised in Box 1 (Hulsmans et al. 2017). In other words, the cytokine exposure can alter action potential

duration (APD), clinically affecting QT prolongation and thus the arrhythmia threshold. A pro-inflammatory cytokine TNF-α can upregulate Cx43 expression by improving cell coupling and prevention of arrhythmia in a long term (George et al. 2017). Abnormal Cx43 expression causing uncoupling in the myocardium may increase the risk of arrhythmic events (Fontes et al. 2012). In another study, IL-6 was shown to act on L-type calcium currents and shorten the APD possibly partly due to the cardioprotective effects (Kouvas et al. 2018). Furthermore, TGF-β secreted from the cardiac fibroblasts can also act on the expression of membrane ion channels, modifying the electrical activity of the heart in an *in vivo* rat model (Kaur and Jalife 2017). In addition to its well-documented role as a fibrotic mediator, the study showed that TGF-β was also associated with ventricular arrhythmia (Kaur and Jalife 2017). Collectively, direct immune cell interactions or immune secretions may regulate levels or function of gap junctions affecting APD of cardiomyocytes and rhythmicity of the heart.

Inflammatory mediators can modulate cellular ROS levels. Superoxides, hydroxyl radical, hydrogen peroxide, and other ROS can be formed in splenocytes, blood leukocytes, vascular, and heart tissue through the action of specific oxidases, oxygenases, and peroxidases (Lubos et al. 2008). Cellular ROS can alter ion channel and/or Cx43 expressions leading to changes in sodium and potassium currents in cardiomyocytes, therefore affect the action potential propagation and electrical properties of the heart (Kouvas et al. 2018; Yang et al. 2015). In an LPS-stimulated maternal inflammation model of rats, the direct link between ROS and inflammation-induced heart damage was demonstrated (Zhang et al. 2016). In this study, isoproterenol, a β-adrenoceptor agonist, treatment was shown to increase ROS levels in adult offspring of LPS-treated mothers. The high ROS ratio finally led to left-ventricular systolic dysfunction, cardiac hypertrophy, and fibrosis (Zhang et al. 2016). Consequently, whether the determinant is a cause or consequence of arrhythmia, recent advances in our understanding of immune parameters started to be associated with the pathogenesis of cardiac conduction diseases.

5 Therapeutic Anti-inflammatory Agents for Cardiac Diseases

Many anti-inflammatory agents are used in the clinical management of cardiac disorders. These agents may be classified as synthetic chemicals targeting the inflammatory mediators and anti-cytokine therapies (Ridker and Lüscher 2014; Nguyen et al. 2019). In atherosclerosis, cholesterol crystals induce the generation of NLRP3 inflammasome and IL-1β, and crystals deposit in the arteries (Nguyen et al. 2019). Therefore, developing anti-inflammatory therapies or blocking the inflammasome pathway at the early phase is becoming an important approach to prevent the generation of plaque cholesterol crystals in the vasculature (Duewell et al. 2010; Nguyen et al. 2019). Among the synthetic chemicals, the statins, also known as HMG-CoA reductase inhibitors, are commonly administered to treat atherosclerotic cardiovascular disease by lowering the LDL cholesterol levels in the blood, participating in the immunological switch from a Th1 towards a Th2 cytokine profile (Nguyen et al. 2019). The anti-inflammatory effects of statins were formally tested in a trial named JUPITER (Justification for the Use of Statins in Prevention: An Intervention Trial Evaluating Rosuvastatin) (Mora and Ridker 2006; Nguyen et al. 2019). However, statins and similar molecules were implied to have a limited potential to control plaque inflammation, therefore the alternative anti-cytokine therapies have been widely preferred in both atherosclerosis and other cardiac therapeutic studies.

By using anti-cytokine therapy, blocking the inflammatory signaling pathways has become a novel method for cardioprotection. Among the inflammatory responses activated in cardiovascular diseases, IL-1 and IL-6 signaling pathways and their products have been widely studied in both basic research and clinical trials. At a meta-analysis, polymorphisms in the IL-6 pathway genes were associated with the lower levels of CRP and lower risk of vascular disorders (Sarwar et al. 2012). In atherothrombosis following lipid accumulation and plaque rupture, activated inflammatory pathways degenerate the vessel

wall and neighbouring cardiac tissue. Hence, the inhibition of IL-1, TNF-α, and IL-6 cytokines may serve as a potent approach for atherothrombotic treatment and also prevention (Ridker and Lüscher 2014). In a mice model of Coxsackievirus B3 virus-related myocarditis, IL-6 receptor blockade via anti-IL-6 receptor antibody tocilizumab enhanced systolic and diastolic left ventricle (LV) function by decreasing cardiac inflammation, fibrosis, and titin protein abundance which was responsible for the stiffness of myocardium (Savvatis et al. 2014).

Selective targeting of pro-inflammatory pathways has been an option for improvement of the cardiac function in the post-MI heart. In this manner, CANTOS clinical trial used a specific neutralizing antibody, canakinumab, targeting the inflammatory cytokine IL-1β of IL-1/IL-6 pathway on high-risk atherosclerotic patients with a history of MI (Ridker et al. 2018; DeBerge et al. 2019; Nguyen et al. 2019). The inhibition of IL-1 pathway at the early stages of inflammation has demonstrated the immunomodulatory actions in reducing recurrent cardiovascular events. In a phase III trial, the use of canakinumab has been shown to decrease the incidence of repetitive atherothrombotic event in chronic MI patients by lowering the inflammatory burden as all doses of canakinumab significantly lowered hsCRP in patients (Baylis et al. 2017; Ridker et al. 2017, 2018).

Similarly, in heart failure patients in the post-MI period, elevated pro-inflammatory cytokine levels in the serum and reduced HFrEF (Heart failure reduced ejection fraction) and HFpEF (Heart failure preserved ejection fraction) results indicated that the pro-inflammation may worsen the disease. In HFrEF patients, systemic levels of both IL-1β and TNF-α were detected to increase 2–6 fold compared to control subjects, suggesting persistent inflammation in the body (DeBerge et al. 2019). Therefore, the blockade of IL-1β with the therapeutic monoclonal antibody canakinumab can become a good option for decreasing the rate of recurrent cardiovascular events. Consistent with these, in both human and animal models, administration of the IL-1R antagonist anakinra limited adverse remodeling and preserved LV systolic function after acute MI (DeBerge et al. 2019). Anakinra treatment of HFpEF patients weaken both systemic inflammation and disease symptoms, while a significant reduction of CRP levels in the plasma was detected (DeBerge et al. 2019).

The anti-inflammatory agents are certainly a valid therapeutic approach for the management of cardiovascular diseases. However, the balance between pro- and anti-inflammatory factors are critical for hemostasis. In cardiac diseases, the suppression of inflammation by the administration of synthetic anti-inflammatory drugs, such as NSAIDs (Non-steroidal anti-inflammatory drugs), for the long-term was associated with increased risk of cardiac arrhythmias and AF (Liu et al. 2014). In this context, approaches benefiting from the natural anti-inflammatory response, such as intravenous immunoglobulin and immunoadsorption as immune-modulation therapies, may have better outcomes for managing both local and systemic inflammation in cardiac disease patients (Gilardin et al. 2015). Consequently, targeting the candidate pro-inflammatory pathways in early cardiac events and providing the balance between pro- and anti-inflammatory events may pave the way for developing better therapeutic strategies.

6 Conclusion

The cross-talk and balance between cardiovascular and immune system can be influenced by numerous genetic, physical, and psychological stressors leading to the onset of inflammation and tissue damage. Sterile or infectious diseases in cardiac tissue may result in cardiomyocyte dysfunction and cardiac remodeling associated with systolic and diastolic abnormalities. In response to pathophysiological stress, the cardiac tissue undergoes the remodeling process via immune regulators, that incorporates the elimination of dying resident cells, remodeling of the vascular compartment, and formation of the fibrotic scar. Therefore, the roles of the innate and adaptive immune system in onset, progress, and treatment of cardiovascular disorders have

continuously been investigated through both animal models and clinical trials.

Experimental animal models of cardiovascular disease studies have persistently reported that regardless of infection origin, innate immune system elements drive the initial pro-inflammatory response to overcome the tissue damage and later associated with wound healing and defense against pathogens. Then the adaptive immune system contributes to the immune reaction by generating antibodies or cellular responses. Interestingly, immune system was shown to be also important in regulating the electrical activity of the cardiac conduction system (Hulsmans et al. 2017). The cardiac resident macrophages, together with fibroblasts and endothelial cells, release cytokines that initiate an inflammatory cascade and recruit circulating leukocytes into the injured cardiac tissue. Apart from cardiac macrophages, other cardiac resident and recruited immune cells are involved in inflammation, revascularization, cardiomyocyte dedifferentiation, cardiac fibroblast conversion to myofibroblasts, and fibrotic scar generation. Hence, the immune system modalities have been associated with either as a causative risk factor or as a consequence of cardiac arrhythmia.

The diverse macrophage behaviors, including phenotypic M1-M2 switch, is driven by a complex network of stimuli and have been investigated for understanding their contribution to pathological remodeling following acute cardiac damage. Although a robust inflammatory response is essential to clear the tissue from damaged cells, the prolonged inflammatory response may be associated with severe prognosis. To overcome the excessive damage of pro-inflammatory response, well-designed agents are utilized and being developed in many clinical trials. The therapeutic agents are usually antibodies targeting pro-inflammatory cytokines aiming to neutralize, antagonize, or block the cytokine storm. A shift towards the anti-inflammatory M2 response implicated better regeneration and healing in animal models and clinical trials. The field of macrophage polarization during cardiac disease is moving forward to shift the fate from M1 to M2 phenotypes by chemical or genetic manipulations, however, safer methods using selective drugs may be the best option to overcome heart failures at early stages.

Based on the data summarized in this review article, there are several limitations with the reported literature. First, the reports classifying immune cell populations in the heart are largely dependent on single-cell sequencing analysis (Dick et al. 2019; Martini et al. 2019). Hence, further single-cell sequencing studies should be conducted to have a better and broader representation of the cellular phenotypes and investigate the emerging subpopulation with their physiological roles in disease pathogenesis and normal physiology. Moreover, the majority of the current reports in cardiac immunoelectrophysiology and single-cell studies were dependent on experimental animal models, and thus human relevance of these data await investigation for translating these findings to the clinical practice. Furthermore, disparities regarding gender differences, sex hormones, and immune dimorphism have been raised in cardiac diseases and immune responses (Bhatia et al. 2014), that need to be addressed in the future.

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Conventional and Alternative Mesenchymal Stem Cell Therapies for the Treatment of Diabetes

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Abstract

Diabetes is a public health problem affecting millions of people around the world. Despite the availability of many antidiabetic medications, the adequate level of control of the disease and management of diabetic patients remain a huge challenge. Because of the limitations of current therapies and the tremendous potential of non-conventional treatments such as stem cell therapy, herein, we review the applications of mesenchymal stem cells (MSCs) in treating diabetes. Owing to their unique regenerative and immunomodulatory properties, MSCs have been widely utilized in numerous applications both in animal models and human clinical trials for the treatment of diabetes. This review will summarize the latest experimental and clinical studies that have provided evidence of the beneficial role of MSCs in diabetes treatment.

Keywords

Clinical trials · Diabetes · Mesenchymal stem cell · Pharmacological · Treatment

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Abbreviations

AHEAD	Action for Health in Diabetes
DPP	Diabetes Prevention Program
EMA	European Medicines Agency
GLP-1	Glucagon-Like Peptide 1
GvHD	Graft Versus Host Disease
HbA1c	Hemoglobin A1c
IDF	International Diabetes Federation
IPCs	Insulin-producing cells
MMTT	Mixed-meal tolerance test
MSC	Mesenchymal stem cell
NIH	National Institutes of Health
NOD	Non-obese diabetic
PPARs	Peroxisome proliferator-activated receptors
SGLT2	Sodium glucose co transporter 2
STZ	Streptozotocin
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TZD	Thiazolidinediones
WHO	World Health Organization

1 Introduction

1.1 Diabetes: A Global Epidemic

Diabetes is a serious public health problem that has been recognized by many health organizations including the WHO as a global epidemic (Bassett 2005). It is a chronic, degenerative pancreatic

disease characterized by elevated blood sugar levels and glucose intolerance (Ribeiro et al. 2010). This metabolic disorder results from defects in insulin secretion, or insulin action, or a combination of both (Olokoba et al. 2012). Millions of people around the world are affected by diabetes and are either unaware of the condition or not receiving the appropriate treatment (Boles et al. 2017). The severity of diabetes is determined based on the degree of hyperglycaemia. Chronic hyperglycaemia associated with uncontrolled diabetes may, over time, lead to serious health damage such as heart diseases and long term damage to the eyes (Retinopathy), kidneys (Nephropathy) and nerves (Neuropathy) (Reddy 2017). Feet ulcers, infections, and gangrene are also experienced in diabetic patients (Reddy 2017). In addition, hypertension, hyperlipidemia, negative nitrogen balance and sometimes ketonuria are often associated with diabetes (Reddy 2017). Prevention of this endocrine disorder and its complications is a major challenge to attain health for all (Wareham and Herman 2016).

Multiple forms of diabetes have been recognized including type 1 and type 2 diabetes (Deepthi et al. 2017). Type 1 diabetes (T1D) is generally an autoimmune disorder caused by absolute insulin deficiency where, the pancreas is not able to produce enough insulin or does not produce insulin at all (Deepthi et al. 2017). The virtual absence of insulin requires patients' dependence on insulin injections to regulate glucose levels in the blood and sustain life and that is why it is also known as insulin-dependent diabetes (Deepthi et al. 2017). It can occur at any age but it is most prevalent among children and young adults under 30 years old (previously known as juvenile or childhood-onset) who have genetic predisposition to develop pancreatic β cell failure (Boles et al. 2017). On the other hand, type 2 diabetes (T2D), is more prevalent than other types of diabetes and accounts for 90–95% of diabetes cases (Fox et al. 2015). It is primarily the result of progressive impairment of glucose regulation because the insulin secreted by the pancreas is not utilized properly by the body (Deepthi et al. 2017). Insulin resistance and insulin deficiency are caused by higher-than-normal blood sugar levels (Calonge et al. 2008; Deepthi et al. 2017). T2D can occur at any age if there are risk factors like obesity, family history and physical inactivity; however, it is most commonly diagnosed in people over 40 years old (known as adult onset diabetes) (Boles et al. 2017). The symptoms are often less recognized or absent until complications arise when the disease has been undiagnosed for many years (Deepthi et al. 2017).

1.2 Diabetes Status

Diabetes is a critical health issue whose prevalence is steadily increasing around the globe (Wild et al. 2004). In 2000, there were 151 million adults living with diabetes worldwide. By 2011, the number of adults with diabetes increased by 142% to around 366 million (Fig. 1). According to the International Diabetes Federation (IDF), people between 20–79 years reported to have diabetes have reached a staggering 463 million representing 9.3% of the global adult population, with half of them are unaware that they suffer from the condition and therefore are at a higher risk of developing serious associated complications leading to 4.2 million deaths in 2019 (IDF Diabetes Atlas 9th edition 2019). Thus, in the past 20 years, diabetic cases have more than tripled among adults. As the population is aging and the rates of obesity are increasing, it is projected that by 2030 the number of adults living with diabetes will increase to 578 million (10.2%) and jump to a staggering 700 million (10.9%) by 2045 if no urgent actions are taken (Saeedi et al. 2019).

It is worth mentioning that high-income countries have the highest prevalence of adult diabetes (10.4%), followed by 9.5% in middle-income countries and 4% in low-income countries as classified by the World Bank Income group (Saeedi et al. 2019). Moreover, diabetes prevalence has been reported to be higher in urban areas (10.8%) than in rural areas (7.2%); although, this gap is narrowing due to urbanization of rural areas (IDF Diabetes Atlas 9th edition 2019). All the data indicates that the incidence of the disease will continue to increase due to rapid economic development, urbanization and a very

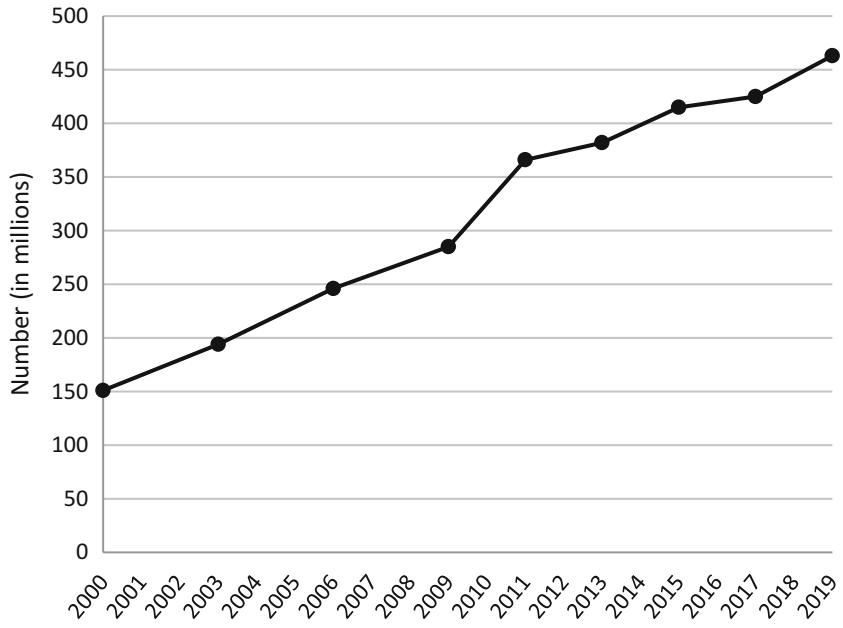

Fig. 1 Total number of adults (in millions) living with diabetes per year from 2000 to 2019

sedentary lifestyle and as long as preventive and control programmes are not effectively followed (King et al. 1998; Nanditha et al. 2016). Thus, all this requires a multisectoral approach to tackle the escalating epidemic (Meng et al. 2019).

2 Non-pharmacological Interventions

Despite the numerous efforts made to control and limit the outspread of diabetes, its high morbidity and mortality rates pose a serious threat which needs to be addressed cautiously (Peng et al. 2018). Diabetes cannot be cured, yet it can be managed with lifestyle modifications at the forefront of the fight against this epidemic especially in T2D.

In the Diabetes Prevention Program (DPP) Randomized Trial that was launched by the National Institutes of Health (NIH) in 1996, the effect of a lifestyle-intervention program through dietary modification and increased physical activity of moderate intensity was investigated in individuals at high risk of developing T2D (DPP Research Group 2002). Results after 2.6 years follow-up showed that the lifestyle modifications were highly effective in delaying or preventing T2D with 58% reduction in its incidence as compared to the placebo group. Astonishingly, these lifestyle changes were significantly more effective than treatment with the anti-hyperglycemic agent, metformin (Knowler et al. 2002). Findings also revealed that after 10 years of follow-up since DPP randomization, diabetes incidence remained lower in the lifestyle group (34%) and in the metformin group (18%) as compared with placebo (DPP Research Group 2009). Another NIH-funded Look AHEAD (Action for Health in Diabetes) trial conducted in T2D patients showed improved hemoglobin A1c (HbA_1c) levels and reduced need for anti-diabetic drugs in the intensive lifestyle intervention group compared with Diabetes Support and Education group (Espeland et al. 2007; Wing et al. 2013).

Yet, sometimes lifestyle modifications are not enough and pharmaceutical interventions are recommended to control hyperglycaemia and prevent disease progression and complications (Solis et al. 2019). The type of diabetes along with its degree of severity affects the type of treatment prescribed (Solis et al. 2019).

3 Pharmacological Diabetes Treatment Options

Several types of oral hypoglycaemic agents have been used in the treatment of T2D. The first line of treatment in patients with T2D is the biguanide, metformin, which was FDA approved in 1994 (Lipska 2017). It improves insulin sensitivity, enhances the ability of peripheral cells to take in glucose, reduces the production of glucose by the liver and aids in weight loss by suppressing appetite (Giannarelli et al. 2003). Some patients may suffer from abdominal bloating, nausea, vomiting and diarrhoea (Siavash et al. 2017). Moreover; patients with kidney problems or heart diseases are advised to be cautious because metformin is thought to increase the risk of developing lactic acidosis (Lipska 2017). However, a review of trials conducted by Salpeter and colleagues showed no evidence of fatal or nonfatal lactic acidosis in subjects on metformin compared to placebo or non-metformin treatment (Salpeter et al. 2010).

The second line of treatment in patients with T2D is sulfonylureas which can be taken as first-line monotherapy if the patient in not overweight or if metformin is intolerable. It can also be given with metformin if glycaemic control is inadequate (Sola et al. 2015). These hypoglycaemic agents are insulin secretagogues that work by stimulating the pancreatic cells to secrete insulin (Sola et al. 2015). They also enhance insulin effectiveness in the body. Sulfonylureas cannot be prescribed to T1D patients who are not able to produce insulin or patients who have had pancreatectomy (Sola et al. 2015). Glinides are another class of oral hypoglycaemic drugs that have similar mechanism of action to sulfonylureas. Both sulfonylureas and glinides have hypoglycaemia as the most common adverse effect (Tran et al. 2015; Keegan 2018).

Thiazolidinediones (TZD) are oral antidiabetic agents that work by increasing insulin sensitivity through its action on peroxisome proliferator-activated receptors (PPARs) (Bailey 2007). Currently, two TZD drugs (Rosiglitazone and pioglitazone) are available in the United States; however, in 2010, rosiglitazone was suspended by the European Medicines Agency (EMA) as the overall risks outweighed its benefits and FDA decided to restrict its use due to increased risk of cardiovascular events (Bourg and Phillips 2012; Pouwels and Van Grootheest 2012). Additionally, in 2011, pioglitazone was suspended by French and Germany Medicines Agencies due to potential increased risk of bladder cancer (Tang et al. 2018).

Alpha-glucosidase inhibitors (acarbose, miglitol and voglibose) are another class of antidiabetic drugs that inhibit the enzymes responsible for breaking down carbohydrates; thus, decreasing their absorption and digestion and subsequently reducing hyperglycaemia (Laar 2008).

Sodium glucose co transporter 2 (SGLT2) inhibitors are also promising oral anti-hyperglycaemic agents that have been developed for the treatment of T2D. They work by reducing blood glucose through inhibition of the glucose reabsorption at the proximal tubule of the kidneys (Simes and Mac Gregor 2019). Examples include canagliflozin and dapagliflozin which have been FDA-approved since 2013. Glycosuria and natriuresis initiated by the inhibition of glucose reabsorption result in modest improvement in weight and blood pressure (Gallo et al. 2015).

Glucagon-Like Peptide 1 (GLP-1) Receptor Agonists, referred to as incretin mimetics, represent another class of pharmacologic treatment for adults with T2D (Hinnen 2017). Incretin hormones, like GLP-1, stimulate insulin secretion and decrease that of glucagon after an oral glucose load. In T2D, this process is reduced and thus insulin release is decreased (Collins and Costello 2020). Therefore, GLP-1 receptor agonists (e.g. exenatide, liraglutide, lixisenatide, dulaglutide) can be prescribed to increase the action of GLP-1 and thereby enhance insulin response (Hinnen 2017). *In vivo*, GLP-1 is inactivated by the hormone dipeptidyl peptidase-4 (DPP-4). So, DPP-4 inhibitors can also be given to increase insulin secretion by inhibiting the enzymatic degradation of the incretin hormone, GLP-1, thereby increasing postprandial GLP-1 activity (Collins and Costello 2020).

In addition to lifestyle modifications and oral hypoglycaemic agents and because of the progressive nature of diabetes, most of the patients with T2D might require insulin replacement therapy to maintain satisfactory blood glucose levels and attain treatment goals (Blonde et al. 2009). However, hypoglycaemia is again a major common side effect of insulin treatment (McCall 2012).

4 Alternative Diabetes Therapies

Although exogenous hypoglycaemic agents and therapeutic insulin provide control over blood glucose and may prevent complications; none of these strategies are able to mimic the natural activity of endogenous insulin especially in T1D patients who are fully insulin-dependent. Moreover, despite the advances made in pharmacology, these antidiabetic drugs are not without significant adverse effects as shown above (Fonseca and Haggar 2014; Peng et al. 2018). Therefore, there is an urgent need for non-conventional therapies that are safe and effective in combatting diabetes. In particular, stem cell-based therapy holds an immense promise as an alternative possible approach to treat diabetes and alleviate its complications.

Mesenchymal stem cells (MSCs) are at the forefront being the most attractive type of adult stem cells under investigation to tackle diabetes. MSCs have been highlighted because of their self-renewal capacity, multipotentiality, low antigenicity, homing ability, reduced toxicity, and ease of culture and expansion *in vitro* (Chen et al. 2007). Moreover, they are abundant and can be easily isolated from different tissues including bone marrow (BM), adipose tissue (AD), umbilical cord (UC), placenta and dental pulp (Orbay et al. 2012).

4.1 Preclinical Applications of Mesenchymal Stem Cells in Diabetes

The ability of MSCs to differentiate into islet-like cells or functional insulin-producing cells (IPCs), to home and induce regeneration of endogenous pancreatic islet beta cells as well as to protect these cells through immunomodulatory properties, have made MSCs a potential novel cell-based treatment for diabetes (Zanini et al. 2011).

Many groups around the world have investigated MSCs transplantation in animal models of diabetes. Madec and his colleagues assessed the effects of a single dose of MSCs infusion in non-obese diabetic (NOD) mice, an animal model for T1D (Madec et al. 2009). MSCs were able to inhibit autoimmune beta cell destruction mediated by progressive islet infiltration of autoreactive (destructive) T cells and macrophages and subsequently prevent diabetes in the NOD mouse model by induction of regulatory (protective) T cells (Madec et al. 2009). These results were in line with other studies reporting that intravenous administration of MSCs into streptozotocin (STZ)-induced T1D mice reverted hyperglycaemia through suppression in autoreactive T cell levels together with increase in pancreatic regulatory T cells (Ezquer et al. 2012). Other preclinical studies have shown that apart from their immunomodulatory properties, undifferentiated MSCs transplantation alleviated hyperglycaemia in NOD mice via differentiation into pancreatic IPCs (Tsai et al. 2015). A more recent study has demonstrated that direct transplantation of MSCs into the impaired pancreas of STZ-treated rats, improved their differentiation into IPCs (Li et al. 2016). Furthermore, intra-pancreatic MSC transplantation stimulated endogenous pancreatic β-cell regeneration resulting in islet neogenesis (Li et al. 2016).

Authors suggested that MSCs might activate endogenous precursor stem cells by providing a supportive pancreatic microenvironment through direct cell-cell contact or the stem cell secretome (Ezquer et al. 2014; Li et al. 2016).

Despite the large number of preclinical studies showing the beneficial effects of MSCs in animal models of diabetes, there are fewer clinical trials utilizing MSCs for treatment of diabetic patients.

4.2 Clinical Applications of Mesenchymal Stem Cells in Diabetes

Twenty five years ago, in 1995, Hillard Lazarus and his colleagues reported the first phase I clinical trial using bone marrow-derived MSCs in human subjects with hematologic malignancies (Lazarus et al. 1995). Since then, clinical trials using MSCs have been rising exponentially to treat a large number of diseases including hematologic, neurodegenerative, autoimmune, liver, lung and kidney diseases. In fact, it was not until 2011 that MSCs (Hearticellgram®-AMI) were approved by Korean Food and Drug Administration for the treatment of acute myocardial infarction. This was followed by Canada and New Zealand granting marrow-derived MSCs (Prochymal®, Mesoblast International Sarl.) conditional approval, in 2012, for the treatment of acute Graft Versus Host Disease (GvHD) in paediatric patients (Waltz 2013). Subsequently, in the last decade, a series of clinical trials using MSCs have been running to assess MSCs safety and efficacy in numerous diseases. Since 2010, more than 1000 MSC-based clinical trials have been listed in the clinical trial registry of the U.S. National Institutes of Health (http://www.clinicaltrial.gov/). According to the data reported by the US NIH, 61 MSC clinical trials have been revealed for diabetes in the last 10 years representing 6% of all trials (Fig. 2). These human trials are roughly evenly divided between T1D and T2D where most of them (51 trials) are still in the early phases (phase I, I/II, or II). There are only few phase III trials either have been completed or ongoing. Most of the MSCs employed in these studies are derived from the umbilical cord (33%), followed by the bone marrow (28%) and adipose tissue (25%) (Fig. 2). Other MSC sources such as wharton's jelly, dental pulp or menstrual blood have also been used. A major issue of note is that more than 90% of these trials are small-sized with less than 100 participants per trial.

Herein, we will review a number of clinical studies, registered at clinicaltrials.gov, that were conducted to evaluate the efficacy and safety of MSCs for treatment of both types of diabetes. In T1D, the important clinical goal is to retain the endogenous secretion of insulin; thereby, attaining long term restoration of glucose metabolism and reducing risk of complications such as hypoglycaemic episodes (Carlsson et al. 2015). Carlson and his colleagues reported the first study on the use of systemic MSC treatment for adult patients newly diagnosed with T1D. The randomized controlled trial under the registration number NCT01068951 aimed to assess the safety and therapeutic effect of autologous BM-MSC treatment in new-onset T1D for a period of one year (Carlsson et al. 2015). All patients tolerated the MSC treatment with no observed adverse effects. Besides, MSC-treated patients preserved pancreatic beta cell function as indicated by preserved or even increased C-peptide response to mixed-meal tolerance test (MMTT) at 1-year follow-up. Despite the promising findings, longer follow-up duration is necessary to validate the results (Carlsson et al. 2015). In 2016, Cai et al. conducted a pilot randomized controlled open-label clinical study (NCT01374854) examining the safety and efficiency of allogeneic Wharton's jelly UC-MSCs plus autologous bone marrow mononuclear cell (aBM-MNC) in patients with established T1D rather than new-onset T1D (Cai et al. 2016). At 12-month follow-up, insulin secretion and C-peptide improved in the treated group compared to the standard care control group. Moreover, HbA_1c, fasting blood glucose and exogenous insulin requirement in the experimental group were lower compared with the control group (Cai et al. 2016). Another interesting phase II pilot study (NCT03920397) evaluated the safety and efficacy of allogenic AD-MSCs

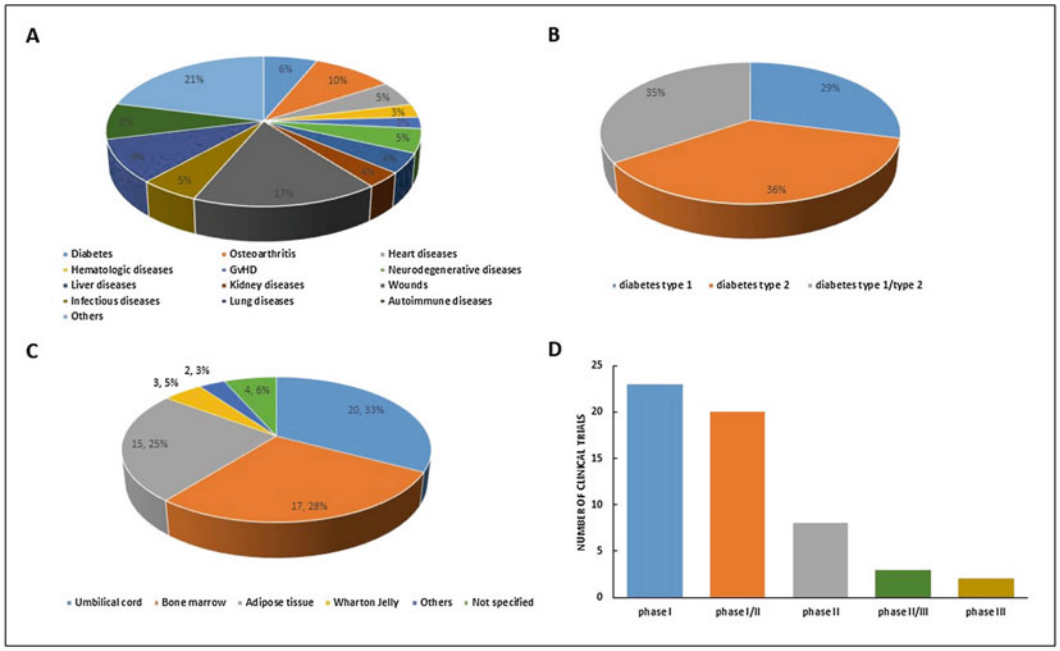

Fig. 2 Mesenchymal Stem Cell (MSC) clinical trials collected from clinicaltrial.gov from 2010 to August 2020 with the term "mesenchymal" listed 1,008 trials. (**A**) Percentages of MSC-based trials by disease classification, including diabetes (6%). (**B**) MSC human trials for diabetes are divided between diabetes type 1 (29%) and type 2 (36%). (**C**) MSCs used for diabetes are isolated from umbilical cord (33%), bone marrow (28%), adipose tissue (25%) and others (14%). (**D**) The majority of these MSC-based diabetes clinical trials are in Phase I (23 trials) and phase I/II (20 trials)

without immunosuppression plus Vitamin D supplementation in patients with early-onset T1D (Araujo et al. 2020). After 3-month follow up, the intervention group showed C-peptide stability, better glycaemic control and lower insulin requirement when compared to the control group with standard insulin therapy (Araujo et al. 2020). However, mild and transient adverse effects were reported in patients 3 months after AD-MSC infusion (Araujo et al. 2020).

Pancreatic beta cell dysfunction is a hallmark of T2D. Despite pharmacological treatment, this process is irreversible. Therefore, there is an utmost need for alternative therapies to restore β-cell function and optimize glycaemia; thus, preventing the occurrence of diabetic complications (Hinnen 2015). Many clinical studies have shown promising results utilizing MSCs for treatment of patients with T2D. Here, we present some of these trials registered in the ClinicalTrials.gov database. In a clinical study (NCT01413035) conducted by Kong et al., 18 patients with T2D were transfused intravenously with UC-MSCs (Kong et al. 2014). Six months later, UC-MSC transfusion effectively ameliorated hyperglycaemia and increased C-peptide levels in patients. Of note, the treatment was well tolerated with only 4 subjects reporting transient slight fever (Kong et al. 2014). A similar study (NCT01759823) was performed to assess the safety and efficacy of autologous BM-MSCs transplantation in 7 T2D patients (Bhansali et al. 2017). At 6 months, 6 out of 7 patients demonstrated reduction in insulin requirement by more than 50% from the baseline, while maintaining HbA1c < 7.0%, accompanied by improvement in beta cell function (Bhansali et al. 2017). Skyler et la also showed that single infusion of allogenic BM-MSCs was safe and feasible for a short period of 3 months in subjects

with T2D (NCT01576328) (Skyler et al. 2015). In another study (NCT01954147), treatment with UC-MSCs was investigated in T2D patients. Chen et al. showed that multiple infusions with UC-MSCs improved glucose metabolism and β cell function in patients diagnosed with T2D for more than 10 years (Chen et al. 2016).

As shown above, most clinical trials have indicated that treatment with MSCs from different sources are relatively safe and effective for both T1D and T2D. However, it is worth mentioning that the above studies have some limitations including the small sample size and short duration of treatment that should be addressed to assess long-term safety and efficacy. Moreover, before the widespread clinical application of MSCs for diabetes treatment, many challenges remain to be overcome such as the most suitable source, dose and route of MSCs for clinical effectiveness. Another critical issue is the cost-effectiveness and scalable generation of MSCs for medical application.

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Mesenchymal Stem Cells: The Past Present and Future

Noha Attia and Mohamed Mashal

Abstract

The biomedical applications of mesenchymal stem cells (MSCs) have gained expanding attention over the past three decades. MSCs are easily obtained from various tissue types (e.g. bone marrow, fat, cord blood, etc.), are capable of self-renewal, and could be induced to differentiate into several cell lineages for countless biomedical applications. In addition, when transplanted, MSCs are not detected by immune surveillance, thus do not lead to graft rejection. Moreover, they can home towards affected tissues and induce their therapeutic effect in a cell-base and/or a cell-free manner. These properties, and many others, have made MSCs appealing therapeutic cell candidates (for cell and/or gene therapy) in myriad clinical conditions. However, similar to any other therapeutic tool, MSCs still have their own limitations and grey areas that entail more research for better understanding and optimization. Herein, we present a brief overview of various pre-clinical/clinical applications of MSCs in regenerative medicine and discuss limitations and future challenges.

Keywords

Drug delivery · Extracellular vesicles · Gene therapy · Mesenchymal stem cells · Secretome

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Abbreviations

AAV	Adeno-Associated Virus
AD-MSCs	Adipose tissue derived Mesenchymal Stem Cells
ALS	Amyotrophic Lateral Sclerosis
BAX	Bcl-2-associated X protein
BDNF	Brain-derived neurotropic factor
BM	Bone Marrow
BM-MSCs	Marrow-derived Mesenchymal Stem Cells
BMP	Bone morphogenetic protein

Caspases	Cysteine-aspartic proteases
CDs	Cluster of Differentiations
CFU-Fs	Colony Forming Unit-Fibroblasts
CM	Condition Media
CXCR2	Chemokine (c-x-c motif) Receptor 2
DCM	Dilated Cardiomyopathy
ECM	Extracellular Matrix
EVs	Extracellular Vesicles
GFs	Growth Factors
GvHD	Graft versus Host Disease
HGF	Hepatocyte growth factor
hHF-MSCs	human hair follicle Mesenchymal Stem Cells
hPMSCs	Human placental Mesenchymal Stem Cells
IGF	Insulin-like Growth Factor
ILK	Integrin-Linked Kinase
ILs	Interleukins
ISCT	International Society for Cellular Therapy
miRNA	MicroRNA
mMSCs	mouse Mesenchymal Stem Cells
MSCs	Mesenchymal Stem Cells
MVs	Microvesicles
NGF	Nerve Growth Factor
NPs	Nanoparticles
PAI-1	Plasminogen activator inhibitor 1
PLGA	Poly (Lactic-co-Glycolic Acid)
PMA	Phorbol 12-myristate 13-acetate
rhBMP	Recombinant human Bone Morphogenetic Protein
RNAi	RNA interference
ROS	Reactive Oxygen Species
SCF	Stem Cell Factor
TGF-β1	Transforming Growth Factor-β1
TNTs	Tunneling nanotubes
tTG	tissue Transglutaminase
UCB-MSCs	Umbilical Cord Blood Mesenchymal Stem Cells
VEGF	Vascular Endothelial Growth Factor
WJSCs	Wharton's Jelly Stem Cells

1 History of MSCs

As early as 1867, the German pathologist, J. Cohnheim was the first to identify non-hematopoietic colony forming unit-fibroblasts (CFU-Fs) in the bone marrow (BM), the cells that will be identified later as mesenchymal stem cells (MSCs) (Anversa et al. 2004). His work discussed the possibility of BM to differentiate into fibroblasts to synthesize collagen fibrils both in normal and wound healing contexts. A century later, an escalating evidence started to accumulate that BM contains cells that are capable of differentiation into other cells of mesenchymal lineage, thanks to the breaking grounds publications by Friedenstein et al. (Afanasyev et al. 2009) Half a century ago, the authors had seeded BM into cell culture vessels and discarded the floating/non-adherent cells after 4 h to eliminate most of the hematopoietic cells. After a lag phase, most of the heterogenous spindle-shaped adherent cells formed foci of cells that are able to multiply excessively. After successive passages, the plastic-adherent cells became capable of self-renewal, acquired a homogeneous fibroblastic appearance, and showed a multi-differentiation potential (Dominici 2006). They could differentiate into several cell types of mesodermal lineage such as, adipocytes, osteoblasts, chondroblasts, myocytes, and tenocytes. Moreover, the plasticity of these adult stem cells can probably generate cells of other lineages, (Gimble et al. 2008) endodermal (e.g. hepatocytes, enterocytes, and islet cells), and ectodermal (e.g. epithelial, glial, and neural cells). Ever since, the term "MSCs" has emerged and the various translational applications of these cells have gained the podium. In addition to BM, MSCs are located in various tissues (Fig. 1), as adipose tissue, amniotic tissue, Wharton's jelly of the umbilical cord, umbilical cord blood, menstrual blood, skeletal muscle, synovium, and pulp of deciduous teeth, just to mention a few (Berebichez-Fridman and Montero-Olvera 2018).

Thanks to their easy acquisition, fast ex vivo proliferation, differentiation plasticity, tropism toward injured tissues, (Rustad and Gurtner 2012) ethical legitimacy, and the feasibility of autologous/allogenic transplantation, MSCs became the stem cells of choice to be applied in the clinical regenerative medicine (Musiał-Wysocka et al. 2019). However, there is still some confusion and lack of uniformity in using the proper nomenclature for MSCs

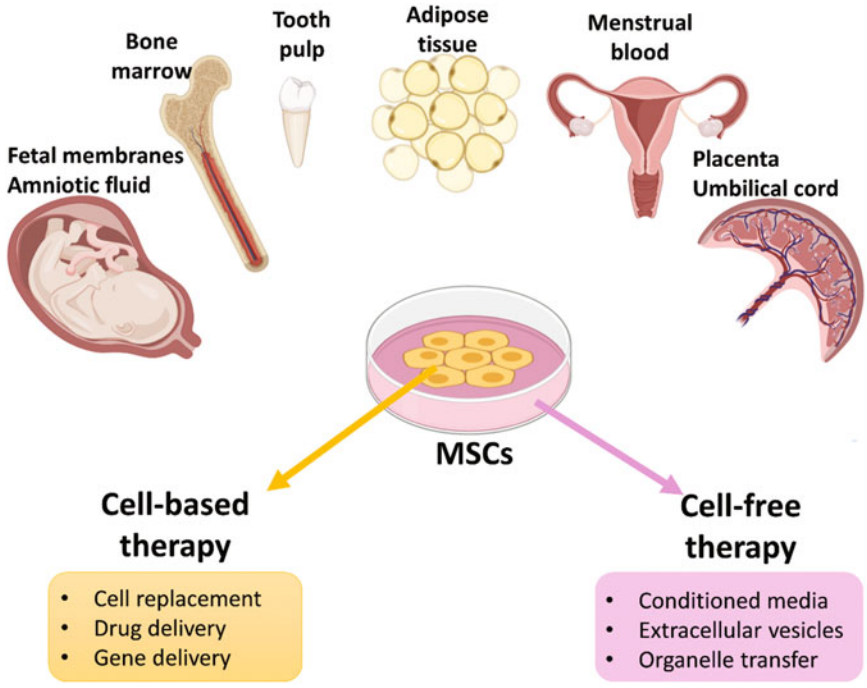

Fig. 1 Various sources of MSCs used in cell-based and cell-free therapeutic approaches

"mesenchymal stem cells" or "mesenchymal stromal cells". Therefore, the International Society for Cellular Therapy (ISCT) (Horwitz et al. 2005) suggests that the fibroblast-like plastic-adherent cells, regardless their tissue of origin, be termed "mesenchymal stromal cells", while the term "mesenchymal stem cells" to be only used to describe cells that meet specified stem cell criteria. Nonetheless, the famous acronym of "MSCs" may be used for both cell populations.

2 Characterization of MSCs

The incredible therapeutic potential of MSCs has fueled a markedly increasing interest in a wide variety of biomedical disciplines. Nevertheless, research studies using MSCs used different methods of isolation and expansion, as well as different approaches for cell characterization. Therefore, it is of utmost importance to standardize the process of MSC characterization in order to be able to compare and contrast the study outcomes, and to ensure progress in the field. The meaningful definition of the word "characterization" is to describe the qualities or peculiarities. In general, characterization of MSCs is crucial to ensure the following:

- Identification/verification of MSCs.
- Classification of MSCs into subclasses according to their tissue of origin where every sub-class depicts peculiar features.
- Isolation/purification of MSCs from other cell populations as an important quality control step that can save time and mitigate experimental variability.
- Tracking of MSCs in vivo.
- Follow up/reassessment of MSC features after application of a new protocol (e.g. expansion, transfection, differentiation, microencapsulation, etc.)
- Targeting of MSCs either in vitro or in vivo.
- Verification of MSC differentiation as some characteristic features may change when they differentiate into other cell types.
- Detection of any aberration from normal as with aging and senescence.

Thereafter, the Mesenchymal and Tissue Stem Cell Committee of the ISCT had proposed a few minimal criteria to define MSCs (Dominici 2006). (a) MSCs must be adherent to plastic when cultured under standard conditions. Isolation of MSCs by plastic adherence is efficient, yet it does not generate a homogeneous population of cells. The isolated cells demonstrate different growth kinetics and differentiation potentials. (b) MSCs must possess a set of positive markers that enabled researchers to recognize them from other cells in their microenvironment such as CDs 29, 44, 73, 90, and 105. At the same time, MSCs should lack another set of markers as the surface antigens expressed by hematopoietic cells, such as CDs 11b, 14, 19, 34, and 79a and HLA-DR. However, the ISCT accepts that these criteria must be met with certain flexibility, particularly when it comes to the lack of expression of markers as HLA Class II that can be conditionally expressed by MSCs when stimulated by certain cytokines. Hence, cells that meet all other criteria, yet are positive for HLA Class II, can be defined as MSCs if they were stimulated. Unfortunately, to date, no marker has been found to be exclusively expressed by MSCs (Chen et al. 2007). Yet, the list of MSC-associated markers is growing over time to enable investigators to simultaneously verify expression of several MSC-associated surface antigens, which increases the confidence in the identification and authentication of the isolated MSCs. (c) MSCs must depict multilineage differentiation potential in vitro. This is considered a function-based method for cell characterization/verification. MSCs can be induced to differentiate into various cell types such as adipocytes, chondroblasts, and osteoblasts, in vitro. This verification tool does not depend on the tissue or species origin of MSCs, while being time consuming. Nonetheless, there are still various challenges facing the process of MSC characterization, such as the redundancy and lack of specificity, experimental variability, and reliance on operator expertise to mention a few (Rohart et al. 2016).

Therefore, numerous research groups have attempted to develop and improve novel molecular markers, such as using the proteomics approach, (Li et al. 2009) epigenetic markers, (Wagner et al. 2016) transcriptome analysis, and the gene signature as 'Rohart MSC in silico test'. (Rohart et al. 2016; Carlini et al. 2019). Although these trials represent an important step towards addressing the thorny question about MSC identity, there is still little consensus among them. May be that is why Caplan (Caplan 2019a) suggested that of characterization of every cell in every MSC population will be of no significance, simply because most cell-preparations have become culture-adapted and can no more display their inherent (in vivo) characteristics, nor their in vivo therapeutic functionality when transplanted to patients.

3 Applications of MSCs

For decades, MSCs have been widely investigated and used in regenerative medicine. In this section, we try to summarize the latest preclinical/clinical applications of various types of MSCs in tissue engineering (Fig. 2). Initially, the vast differentiation potential of MSCs into cardiomyocytes, neurons, blood cells, osteoblasts, hepatocytes, skeletal myocytes, enterocytes, etc., has inspired tremendous cell-based therapeutic purposes for a wide array of disorders.

3.1 Cell Therapy

The enormous therapeutic benefits of MSCs have been well demonstrated in numerous experimental, pre-clinical, and clinical studies. MSCs have been considered as a valuable tool for tissue repair given their ability to suppress the inflammatory response-mediated cell injury, thereby promoting tissue repair. The tissue reparative properties have also been attributed to MSCs' ability to evade cell death by reducing the expression of pro-apoptotic factors (Caspase-3 and Bax), (Li et al. 2013) while increasing the anti-apoptotic activities (Bcl-2), (Wang et al. 2000; Qi and Wu 2013) and restoring the local

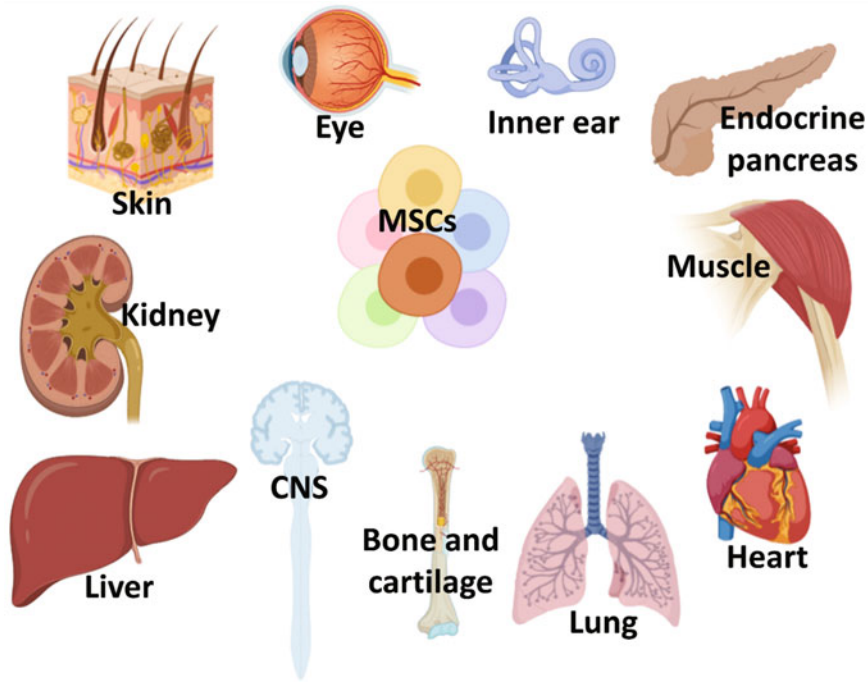

Fig. 2 MSCs are applied in various disorders affecting several organs including the illustrated ones

microenvironment within the damaged tissues. It is also noteworthy that MSCs -transplanted in vivo- could repair various tissues, as muscle, bone, and cartilage via self-renewal and differentiation. However, over the last decade, many of the regenerative abilities of MSCs have been pertaining to their paracrine activity and the secretion of bioactive factors. More recently, MSCs were found to modulate host cell function by transferring their cytoplasm and organelles (e.g. lysosomes and mitochondria) via several mechanisms including so called tunneling nanotubes TNTs, microvesicles and cell fusion (Murray and Krasnodembskaya 2019). Some of the abovementioned mechanisms are illustrated in Fig. 3.

3.1.1 Cell-Based Therapy

MSCs, both autologous and allogeneic, have been used in cell-based therapies to repair damaged tissues, replace lost cells, or to exert immunomodulation. Using MSCs, from various sources, in various cell-based scenarios was strongly supported by the fact that no tumors have been reported in human recipients. Therefore, a great number of clinical trials, for a wide range of clinical conditions, have been documented at the website www.clinicaltrials.gov. Some of the completed studies that have documented results are summarized in Table 1.

Nevertheless, the efficacy of MSC transplantation therapy is currently limited by their low retention and poor survival as demonstrated by several clinical studies. Within a few days post-transplantation, MSCs are challenged with a combination of harsh environmental conditions (Sylakowski et al. 2020) (Oxygen and nutrient deprivation and death signals), and anoikis (due to lack of adhesion to ECM), (Copland and Galipeau 2011) inducing cell death. In addition, in an inflamed tissue, even the recruited inflammatory cells (e.g. neutrophils and macrophages) do generate reactive oxygen species ROS, thereby inducing apoptosis and inactivating the cytoprotective production of nitric oxide. Interestingly, Dong et al. (2019) have reported that transplantation of allogeneic umbilical cord-derived MSCs could play a therapeutic function, despite

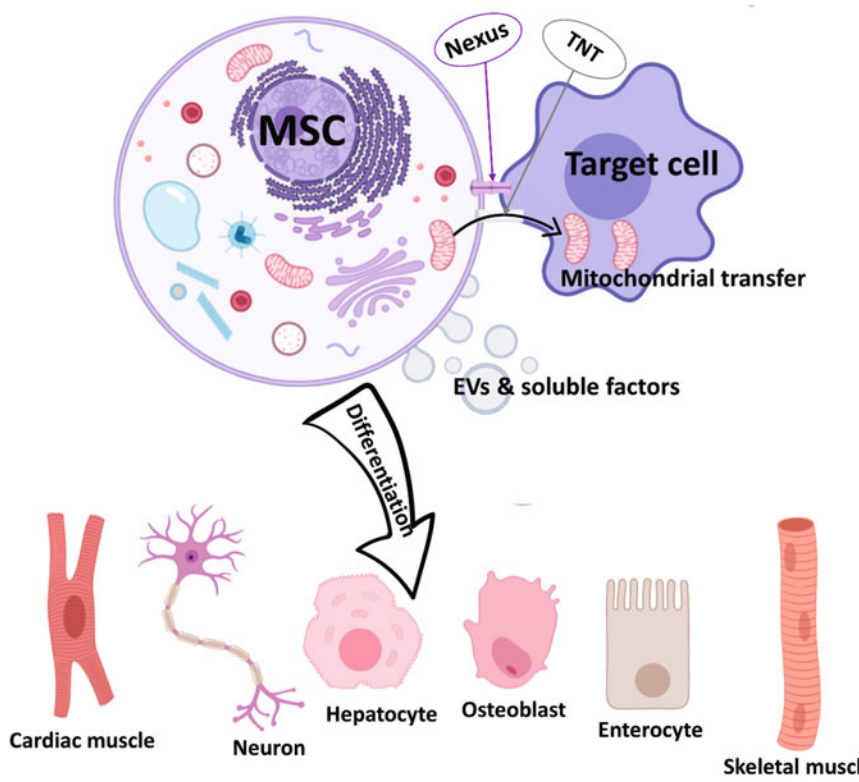

Fig. 3 Some mechanisms of MSCs therapy. (1) Via differentiation into many cell types, including myocytes, neurons, hepatocytes, enterocytes, etc. to replace damaged/abnormal cells (2) Via EVs and secretome. (3) Via organelle (e.g. mitochondria) transfer through gap junctions (nexus) and tunneling nanotubes (TNTs)-like structures

the short life span of transplanted cells in vivo (~ 2 months). The authors argued that cell-based therapy is the main mechanism at the early phase (~ 1 month after cell transplantation), while cell-free therapy takes over at a later stage. In addition, they proposed two different solutions to ensure efficient, practical and feasible application of stem cell-based therapy. First, to improve cell targeting by coating MSCs with antibodies or peptides to make their homing toward the affected tissues more targeted, accurate and efficient. Second, is the use of "cell-free" approach, comprising secretome, exosomes, etc. Likewise, Lee and colleagues (2015) have summarized various other strategies to improve the therapeutic potential of transplanted MSCs in order to surmount the poor cell survival rates. Those approaches comprise a variety of treatments including pre-treatment with growth promoting hormones/drugs (e.g. melatonin and atorvastatin), pre-conditioning with hypoxia, and the use of genetic modification to overexpress anti-apoptosis (e.g. Bcl-2, Survivin, and CCR1/CXCR2) or adhesion (e.g. tTG, ILK and PMA) molecules (Lee et al. 2015).

3.1.2 Cells as a Gene/Drug Delivery Tool

The non-specific targeting of non-viral/viral gene vectors and their systemic distribution throughout the circulation can result in undesired offsite adverse effects. Since the discovery of ability of engineered MSCs to selectively migrate toward the injured/tumor site, the MSC-based gene therapy has experienced a substantial leap ahead. The delivered factors can be receptors, suicide genes, replication inhibitors, apoptosis-inducing genes,

Table 1 Clinical trials (completed) using MSCs in various applications. Data obtained from www.clinicaltrials.gov

Study title	Conditions	Interventions	Completion date	Publications
Human MSCs for acute respiratory distress syndrome	Acute respiratory distress syndrome	Allogeneic human BM-MSCs administered intravenously	February 2015	Wilson et al. (2015)
Effect of MSC transfusion on the diabetic peripheral neuropathy patients	Diabetic peripheral neuropathy	Autogenous BM-MSCs administered intravenously	December 2016	Keilhoff et al. (2006), Morbach et al. (2004), Nakae et al. (2006), Shibata et al. (2008)
Use of MSCs for alveolar bone tissue engineering for cleft lip and palate patients	Cleft lip and palate	Autogenous MSCs derived from dental pulp of deciduous teeth associated with a biomaterial composed of collagen and hydroxyapatite.	December 2015	Hibi et al. (2006), Gimbel et al. (2007)
Intravitreal MSCs transplantation in advanced glaucoma.	Retinal degeneration Primary open-angle glaucoma	Intravitreal transplantation of autogenous BM-MSCs	September 2016	Not provided
Treatment of knee osteoarthritis with allogenic MSCs	Knee osteoarthritis	Intra-articular injection of allogenic BM-MSCs	June 2014	Vega et al. (2015)
Treatment of knee osteoarthritis with autologous MSCs	Knee osteoarthritis	Intra-articular injection of autologous BM-MSCs	September 2014	Orozco et al. (2011), Orozco et al. 2013; Orozco et al. 2014)
Encapsulated MSCs for dental pulp regeneration	Periapical periodontitis	Umbilical cord-derived MSCs encapsulated in a plasma-derived biomaterial	September 2018	Not provided
A study to evaluate the potential role of MSCs in the treatment of idiopathic pulmonary fibrosis	Idiopathic pulmonary fibrosis	Placental MSCs	May 2013	Not provided
The trans endocardial stem cell injection delivery effects on neomyogenesis study (the TRIDENT study)	Chronic ischemic left ventricular dysfunction Myocardial infarction	Allogeneic human MSCs	September 2017	Florea et al. (2017)
The percutaneous stem cell injection delivery effects on neomyogenesis pilot study (the POSEIDON-pilot study)	Ischemic cardiomyopathy	Allogeneic human BM-MSCs administered trans-endocardial during cardiac catheterization	October 2012	Tompkins et al. (2018)
Safety study of local administration of autologous bone marrow stromal cells in chronic paraplegia	Spinal cord injury	Intrathecal injection of autologous BM-MSCs	March 2015	Geffner (2008)
The trans endocardial autologous cells (hMSC or hBMC) in ischemic heart failure trial (TAC-HFT)	Left ventricular dysfunction	Autologous MSCs and BM-MSCs administered transendocardially during cardiac catheterization	September 2013	Heldman et al. (2014), Trachtenberg et al. (2011)

(continued)

Table 1 (continued)

Study title	Conditions	Interventions	Completion date	Publications
A study to assess the effect of intravenous dose of (BM-MSCs) to subjects with non-ischemic heart failure	Non-ischemic heart failure	Allogeneic BM-MSCs	May 2017	Butler et al. (2017)
Subarachnoid administrations of adults autologous MSCs in SCI	Chronic spinal cord injury	Autologous BM-MSCs	May 2016	Vaquero and Zurita (2011), Otero et al. (2011)
Allo-HCT MUD for non-malignant Red Blood Cell (RBC) disorders: Sickle cell, Thal, and DBA: Reduced intensity conditioning, Co-tx MSCs	Sickle cell disease	Parental BM-MSCs	August 2013	Ghavamzadeh et al. (2010)
	Thalassemia			
	Diamond-Blackfan anemia			

regulatory agents [e.g., RNA interference (RNAi), miRNAs], oncolytic viruses, growth factors, and various immunological factors (e.g., cytokines).

Genetic engineering of MSCs was used either to increase their own natural protein production or to empower the expression of new proteins. The tropism capability of MSCs towards many cells and tissues render it an excellent natural vehicle for therapeutic agents. Besides, MSCs show immune suppressive properties and distinct anti-inflammatory and immunomodulatory effects upon transplantation. However, many challenges can significantly decrease their capacity for differentiation and proliferation, such as donor aging and culture senescence, reducing MSCs' culture time and preventing their expandability to the large numbers of cells needed for cell therapy. The adaptable phenotype of MSCs by introduction of exogenous genes is usually the main aim in tissue engineering and regenerative medicine. In comparison to hematopoietic stem cells, MSCs are easier to modify with most viral (biological) vectors, while retaining their in vivo activity (Gonzalez-Fernandez et al. 2017). However, due to the limited safety profile of viral vectors including insertional mutagenicity and the adverse immune responses, non-viral vectors (both chemical and physical) are rising transfection methods. Nonetheless, non-viral carriers showed lower transfection efficiencies when compared to their viral counterparts (Curtin et al. 2012). As a non-viral approach, physical methods provide a simple strategy that facilitates gene transfer using physical forces to overcome the barrier function of cell membranes. They comprise many techniques, like magnetofection, nucleofection, electroporation, mechanical massage, microbubbles, photoporation, sonoporation, hydroporation, jet injection, gene gun, microinjection and needle injection. Although electroporation exhibits a high transfection efficiency in many cell types, most physical methods are still limited by the resulting poor cell viability (Mellott et al. 2013). Nucleofection of MSCs using specific electric field pulse has been proven to increase efficiency of plasmid transfections with respect to the traditional electroporation (Nakashima et al. 2005). In order to increase the transfection efficiency without compromising cell viability, magnetofection is another option that uses magnetic nanoparticles to form complexes with DNA, and then carry DNA into the cells in the presence of a magnetic force. Interestingly, magnetofection could effectively overexpress NANOG in human hair follicle MSCs (hHF-MSCs) (Son et al. 2015). On the other hand, chemical non-viral vectors involve the use of natural or synthetic molecules such as polymers, cationic lipids, peptides, dendrimers and inorganic molecules. These materials can be formulated in diversity of formulations such as nano-emulsions, micelles, liposomes, niosomes, polymersome, polyliposome, solid lipid

nanoparticles and nanostructured lipid carriers. Likewise, the inorganic calcium hydroxyapatite crystals depict strong affinity to DNA binding due to the electrostatic interaction between calcium ions with the negatively charged phosphate groups in DNA. Nano hydroxyapatite is biocompatible, bioresorbable, non-toxic and cost effective to elaborate (Curtin et al. 2012). Meng Yu et al. have designed gold nanoparticles as a highly efficient carrier for miR-5,106-delivery into MSCs to avoid increased cytotoxicity with other conventional inorganic nanocrystals (Yu et al. 2017). Among the ongoing chemical non-viral carriers, cationic liposome is an advantageous and efficient vehicle for transfecting MSCs. However, the transfection efficiency is still markedly lower than with the viral vectors and some other non-viral vectors, namely, electroporation and nucleofection. Interestingly, the clinical safety profile of cationic liposome is advantageous when compared to viral vectors. Generally, lipofection is a good candidate in applications only involving low and transient expression of proteins. Furthermore, MSCs' differentiative power and viability were not compromised (Madeira et al. 2010). Another polymeric vesicle or polymersome has increased applications as a gene delivery tool. Polymersome is self-assembled amphiphilic block or graft co-polymers to form hollow structures, while polymeric nanoparticles are solid colloidal particles. Generally, polymers are the most common molecules used for formulating nanoparticles. Moreover, polymeric micelles are supramolecular assemblies of block co-polymer with enhanced efficacy for gene delivery with minimal side effects (Nishiyama et al. 2005). In addition, micelles, represent an alternative option for the in vivo delivery of siRNA to mMSCs (Raisin et al. 2017).

Niosomes are auto-assembled non-ionic surfactant vesicles. They are consisting of three main parts:(1) a non-ionic surfactant like polysorbates;(2) a neutral support lipid like cholesterol, squalene and lycopene;(3) a cationic lipid. The inclusion of non-ionic molecules in niosomes reduces the toxicity of cationic lipids showing better cellular viability profiles compared to their corresponding anionic or cationic counterparts. Due to their capability to encapsulate both hydrophobic and hydrophilic molecules, niosomes are reported as potential gene carriers (Mashal et al. 2017, 2019; Attia et al. 2018). Regardless all these trials, viral vectors are still considered the most efficient option for cell transduction thanks to their high transduction efficiency (Muhammad et al. 2019). Trials using adenoviral vehicles for gene therapy in humans were the first, followed by a plethora of virus-based gene therapy studies.

Another perspective is the use of MSCs as a drug delivery tool. Thanks to their diversity and ease of preparation of nanomaterials, nanocarriers have become increasingly popular in medical research. Nevertheless, they are facing myriad challenges, as rapid plasma clearance, immunogenicity, uncertain targeting ability and non-optimized pharmacokinetics, to mention a few. Therefore, MSCs started to gain the podium in this relatively new realm due to their innate targeting capability, long circulation time, low immunogenicity, and no tumorigenicity. As natural drug carriers with selective homing abilities, MSCs permit achieving maximal therapeutic efficiency with minimal toxic side effects (Muslimov et al. 2020).

Although many other cell types were initially used in cell-based drug delivery (e.g. leucocytes), their clinical applications were hindered by their limited obtainment in large numbers and insufficient proliferation in vitro, if any. That said, MSCs that are easily obtained and propagated in vitro became an appealing cell candidate for cell-based drug delivery. Moreover, MSCs were found to target immune-privileged tissues as brain tissue where they cross the blood-brain barrier (Shyu et al. 2007; Detante et al. 2012; Lu et al. 2013). Various applications of MSCs as gene and drug delivery vehicle are summarized in Table 2.

3.2 Cell-Free Therapy

Initially, MSC-based therapies were believed to enhance the structure and function of damaged or

Table 2 MSCs as gene/drug delivery tool

Delivery system	Sub-categories	Vector	Disease/model	Cells	Gene/Drug	Ref.
Non-viral	**Physical**	Electroporation	Atopic dermatitis	Human umbilical cord blood-derived MSCs (hUCB-MSCs) have hUCB-MSCs	TGF-β-targeting siRNA	Park et al. (2020)
		Microporation	In vitro/ in vivo (rat brain)	hUCB-MSCs	Brain-derived neurotropic factor (BDNF) plasmid DNA	Lim et al. (2010)
		Nucleofection	Bone senescence	BM-MSCs	miR-196	Candini et al. (2015)
		Nucleofection	Bone formation	BM-MSCs	rhBMP-6	Mizrahi et al. (2013)
		Nucleofection	HGPS	MSCs-iPSCs	miR-9	Nissan et al. (2012)
		Nucleofection	Cardiac repair	hMSCs	mRNA for CXCR4	Wiehe et al. (2013)
		Magnetofection	Myogenic differentiation of human hair follicle	hHF-MSCs	Nanog	Son et al. (2015)
	Chemical	Gold NPs	Bone regeneration	BM-MSCs	miR-5,106	Yu et al. (2017)
		Inorganic NPs	Bone formation	BM-MSCs	BMP2	Curtin et al. (2012)
		Inorganic NPs	Regeneration of articular cartilage and subchondral bone	BM-MSCs	TGF-β1/ BMP-2	Chen et al. (2011)
		Inorganic NPs	Bone tissue engineering	BM-MSCs	pTGF-β3 and pBMP2	Gonzalez-Fernandez et al. (2016)
		Liposome	Spine infusion	C3H10T1/ 2 MSC line	BMP-2	Sheyn et al. (2010)
		Liposome	Islet transplantation	MSC line derived from fetal porcine pancreas	hTERT	Cao et al. (2011)
		Liposome	Myocardial ischemia	BM-MSCs	VEGF	Kim et al. (2011)
		Liposome	Pancreatic cancer	BM-MSCs	IL-25	Piri et al. (2012)
		Niosome	In vitro bone regeneration	D1-MSCs	hBMP-7	Attia et al. (2018)
		Niosome	Theragnostic	hMSCs		Yang et al. (2018)

(continued)

Table 2 (continued)

Delivery system	Sub-categories	Vector	Disease/model	Cells	Gene/Drug	Ref.
					Cy3-labeled siGFP/Anti-miR-138	
		Polymeric NPs	Chondrogenesis of human MSCs	hMSCs	coSOX9-pDNA/ Cbfa-1-siRNA	Jeon et al. (2012)
		Polymeric NPs	Rat myocardial infarction	BM-MSCs	pHI-VEGF	Moon et al. (2014)
		Micelles (polymeric)	Regenerative medicine	BM-MSCs	siRNA targeting mouse Runx2	Raisin et al. (2017)
Viral (biological)		Lentivirus	Ischemic heart disease	BM-MSCs	miR-126	Chen and Zhou (2011)
		Lentivirus	Cancer gene therapy	AD-MSCs	iCasp9	Rossignoli et al. (2019)
		Fiber-modified adenovirus	Matrigel plugs angiogenesis	hPMSCs	kringle1-5/ EGFP	Chu et al. (2014)
		Adenovirus	Intracranial gliomas	BM-MSCs	HSV-TK/GCV	Ryu et al. (2012)
		Gamma-retrovirus	Gastrointestinal adenocarcinoma	Modified autologous MSCs	HSV-TK	Niess et al. (2015)
		Recombinant adenovirus	Cerebral infarction	Modified BM-MSCs	VEGF	Chen et al. (2016)
		Adenovirus	Multiple myeloma	BM-MSCs	IL-2	Trudel et al. (2001)
		Gamma -retrovirus	Colorectal cancer	hBM-MSCs	IL7-IL12	Hombach et al. (2020)
		Retrovirus	Infarcted myocardium	MSCs	AKT	Noiseux et al. (2006)
		Lentivirus	Myocardial infarction	UCB-MSCs	Hepatocyte growth factor (HGF)	Zhao et al. (2016)
		Lentivirus	Traumatic brain injury	BM-MSCs of C57BL/6 mice	FGF21	Shahror et al. (2020)
		Lentivirus	Rat model of stroke	rMSCs	CXCR4	Yu et al. (2012)
		Lentivirus	In vitro cancer cells andhuman vascular endothelial cells	BM-MSCs	PTPN21	Wang et al. (2019a)
		Herpes Simplex Virus (HSV)-1	Rats' ischemic brains	rBM-MSCs	HGF	Zhao et al. (2006)
		Adenovirus	Spinal cord injury	hBM-MSCs	HGF	Jeong et al. (2012)
		Adenovirus	Murine brain tumors	Murine BM-MSCs	EGFR	Sato et al. (2005)

(continued)

Table 2 (continued)

Delivery system	Sub-categories	Vector	Disease/model	Cells	Gene/Drug	Ref.
		Adenovirus	Pancreatic tumors	hBM-MSCs	IFN- β	Kidd et al. (2010)
		AAV	Acute ischemic stroke	hBM-MSCs	IL-10	Nakajima et al. (2017)
Hybrid		Adenovirus/ liposome	Ovarian cancer	hPMSCs	Endostatin	Zheng et al. (2012)
		AAV/ electroporation	Cutaneous wound healing	Human BM-MSCs, AD-MSCs, and UCB-MSCs	CRISPR-Cas9	Srifa et al. (2020)
		Chitosan/ Alginate/ Hydroxyapatite Scaffolds	Bone regeneration	BM-MSCs	BMP-2	He et al. (2014)
		Liposome (DOTAP/ DOPE)/ polymer (PEI)	In vitro	mMSCs	mRNA encoding CXCR4	Rejman et al. (2010)
		Adenovirus/ peptide	Intracranial glioma	UCB-MSCs	stTRAIL	Kim et al. (2008)
		Adenovirus/ peptide	Pulmonary metastasis and solid tumor	BM-MSCs	IL-12 M	Seo et al. (2011)
		Polymersome	Glioblastoma	rMSCs	HSV-TK/ TRAIL	Malik et al. (2018)
		Lipid-polymer	In vitro	hBM-MSCs	Human placental growth factor (PlGF)	Cheung et al. (2018)
Drug delivery		Polymersome	Lung cancer	hPMSCs	Docetaxel	Wang et al. (2019b)
		No-carrier	In vitro tumor growth	BM-MSC line (SR4987)	Paclitaxel	Pascucci et al. (2014)
		Liposomes	Spinal cord injury	GMSCs	Moringin	Mammana et al. (2019)
		Polymer (PLGA) NPs	Lung melanoma	AD-MSCs of C57BL6 mice	Doxorubicin	Zhao et al. (2017)
		Polymeric NPs	Lung tumor	Nano-engineered MSCs	Paclitaxel	Moku et al. (2019)
		Polymeric (PLGA) NPs	Tumor therapy	BM-MSCs	Paclitaxel	Dai et al. (2013)
		Silica Nano rattle	Targeted tumor therapy	BM-MSCs	Doxorubicin	Li et al. (2011)
		Silica NPs (SiO_2 NPs)	Breast Cancer therapy	BM-MSCs	Purpurin-18	Cao et al. (2014)
		Micelles	Neurodegenerative diseases	AD-MSCs	Curcumin	Tripodo et al. (2015)
		Niosome/ tLyp-1 penetrating peptide	Anti-glioma therapy	hAD-MSCs	Doxorubicin and curcumin	Seleci et al. (2017)

diseased tissues via direct cell replacement. Though, it soon became clear that a relatively limited number of MSCs were eventually retained at these sites of injury. Myriad studies on laboratory animals confirmed that intravenously injected MSCs are trapped in the capillaries of the pulmonary circulation or the reticuloendothelial system where most MSCs are generally cleared. However, the heterogenous MSC preparations showed a marked therapeutic functionality when introduced into the body, suggesting that they have a bystander paracrine (i.e. medicinal) capacity. For that reason, A.I. Caplan (2019b) has suggested to change the nomenclature of MSCs to be "medicinal signaling cells".

MSCs are known to secrete a plethora of bio-active factors such as growth factors (GFs), cytokines, chemokines, hormones, interleukins ILs, and extracellular vesicles EVs. Many of which can modulate the immune system, reduce inflammation, and promote healing. As a result, the field adopted a conceptual shift that MSCs enhance tissue repair mainly via their paracrine factors and stimulation of host cells, and it may be via cell-to-cell communication or exosomes and/or some metabolites and cytokines. These paracrine factors have been considered as the invisible heroes that mediated tissue regeneration/repair through their paracrine actions. Therefore, the emphasis has recently shifted toward harnessing the ability of MSCs to secrete cytokines and trophic factors that stimulate innate tissue repair and modulate inflammation and immune responses. In the following Sects. 3.2.1, 3.2.2 and 3.2.3, we highlight the paradigm shift in MSCs' mechanisms of action from the cellular to the paracrine manner, in addition to their relevant pre-clinical/clinical applications.

3.2.1 MSC-Derived Conditioned Media

Following the expansion of MSCs in vitro, they tend to release a set of bioactive factors into the culture medium, now named conditioned medium CM or secretome. The released factors can manipulate the communications between the cells and their microenvironment, facilitating the desired biological function. Therefore, the CM can exert beneficial effects on the recipient that could be considered angiogenic, immunomodulatory, anti-inflammatory, anti-apoptotic, anti-fibrotic or anti-oncogenic. This relatively new strategy has attracted the interest of researchers towards the MSC secretome that can be used as a cell-free therapy for disorders of various vital organs, such as heart, lung, liver, kidney, CNS, etc. Examples of MSC secretome applications are summarized in Table 3.

Although different MSCs are expected to share certain phenotypic features, their tissue of origin seems to affect their secretome, leading to variable effects (Pires et al. 2016; Vizoso et al.

Table 3 MSC secretome/CM in various applications

Disease/model	Cells of origin	Ref.
Severe alveolar bone atrophy	BM-MSCs	Katagiri et al. (2016)
Dilated cardiomyopathy DCM	Human autologous and allogeneic BM-MSCs	Premer et al. (2019)
Hindlimb ischemia	Human amnion and chorion MSCs	Yamahara et al. (2014)
Acute GvHD	Human amnion and chorion MSCs	Yamahara et al. (2014)
Chronic wound	Rat BM-MSCs	Mehanna et al. (2015)
Acute pancreatitis	Human AD-MSCs	Roch et al. (2020)
Anti-GFB glomerulonephritis	BM-MSCs	Iseri et al. (2016)
Liver fibrosis	BM-MSCs	Abdel Aal et al. (2019)
Spinal cord injury	Wharton jelly derived MSCs	Chudickova et al. (2019)
Ischemic stroke	BM-MSCs	Tsai et al. (2014)
Acute lung injury	BM-MSCs	Ionescu et al. (2012)
Corneal wound	BM-MSCs	Fernandes-Cunha et al. (2019)
Acne vulgaris	Human AD-MSCs	Shan et al. (2018)
Systemic sclerosis	BM-MSCs	Dahbour et al. (2017)

2017). For instance, significant differences were reported for the composition of CM of adult bone marrow-derived MSCs (BM-MSCs) and that obtained from adipose tissue AD-MSCs, or Wharton's jelly WJSCs. For BM-MSCs, the CM (Katagiri et al. 2016) held insulin-like growth factor (IGF)-1, (Katagiri et al. 2016) vascular endothelial growth factor (VEGF), (Attia et al. 2014) and transforming growth factor (TGF)-β1. As for AD-MSCs, CM is mainly positive for hepatocyte growth factor (HGF), VEGF, nerve growth factor (NGF), and stem cell factor (SCF), while WJSCs, secrete only NGF and VEGF. Similarly, using a comparative proteomic-based assay, Pires and colleagues (2016) have indicated significant differences in the secretome of MSCs from BM, adipose tissue, and umbilical cord. Nevertheless, crucial elements of success for the CM-based cell-free therapy would be the identification each element of the secretome obtained by different MSC populations, as well as the verification of their exact mechanism(s) of action. Furthermore, the quality control optimization of the process of production of CM from each MSC type (e.g. culture medium, supplements, duration, and other conditions) is key and necessitates more research studies (Sagaradze et al. 2019).

3.2.2 MSC-Derived Extracellular Vesicles

A growing list of attention-grabbing discoveries has demonstrated new ways by which cells communicate with their neighboring cells through the secretion of non-classical secretory vesicles, known as the extracellular vesicles (EVs). At present, and according to the underlying mechanisms responsible for their biogenesis, EVs are basically classified into three major categories. One of which -known as ectosomes, microparticles, and microvesicles (MVs)- has the potential to reach relatively large sizes up to 1 μm in diameter, are. The second category, known as exosomes, are typically way smaller than MVs, with size ranging from 0.04 to 0.1 μm in diameter. The third class of EVs is the apoptotic bodies. They are generally >1,000 nm in size that are released from cells undergoing programmed cell death. They hold numerous cellular components (e.g. cell organelles, DNA fragments, non-coding RNAs, etc.), and are destined for clearance via phagocytosis. The biogenesis of MSC-derived EVs were reported to be regulated by crosstalk of MSCs with their surrounding microenvironment. It is also known that EVs are considered one of the mechanisms by which organelle transfer between cells can take place.

In comparison to MSC-based approach, their EVs have numerous advantages as an alternative cell-free therapeutic too (Rostom et al. 2020). The vesicles are small and circulate easily, whereas MSCs are too large to circulate readily through capillaries, hence most MSCs do not get beyond the first pass capillary bed. Besides, the dose of infused MSCs quickly diminishes quickly, and it may be that the delivery of MSC derived vesicles can achieve a higher "dose" that circulates to a greater extent than their cells of origin. Moreover, EVs lack the capacity to self-replicate providing more control on the dosage and progression. In addition, they do not possess any danger for genetic instability, ectopic differentiation, tumor formation, or immune rejection. On the other side, MSC-EVs exhibit therapeutic effects similar to their parent cells, such as homing to the site of injury and immunomodulatory properties (Rong et al. 2019).

Moreover, MSC-EVs are considered as a drug delivery tool. In addition to their intrinsic tissue regenerative properties, EVs could be further enriched with molecules as microRNAs. In their recent study, Wang and colleagues (2020) have used miR-132-loaded EVs to preserve heart function after direct transplantation into the heart. Therefore, MSC-EVs have gained the podium recently as a novel and attractive approach to be studied in myriad preclinical/clinical studies, including kidney, liver, cardiovascular, immunological, and neurological diseases. Various examples of EVs' applications are summarized in Table 4.

Despite the expanding applications of EVs in various clinical settings, proper testing and dose optimization of EVs are still of paramount importance. Unfortunately, to date, there is no entrusted technique for EVs' enumeration. Therefore, controlling batch-to-batch discrepancies is still a challenging concern. As recently argued by van

Table 4 MSC EVs/exosomes in various applications

Disease/model	EVs/MVs/Exosomes	Cells of origin	Ref.
Myocardial infarction	EVs	BM-MSCs	Bian et al. (2014)
Liver fibrosis	EVs	BM-MSCs	Rostom et al. (2020)
Lethal hepatic failure	EVs	BM-MSCs	Haga et al. (2017)
Acute kidney injury	EVs	Human liver stem cells	Sanchez et al. (2014)
Progressive multiple sclerosis	EVs	Human AD-MSCs	Laso-García et al. (2018)
Spinal cord injury	EVs	BM-MSCs	Ruppert et al. (2018)
Acute graft-versus-host disease	EVs	Human umbilical cord derived MSCs	Wang et al. (2016)
Osteoarthritis	EVs	Human umbilical cord derived MSCs	Vonk et al. (2018)
	Exosomes	Synovial MSCs	Tao et al. (2017)
Acute lung injury	MVs	BM-MSCs	Zhu et al. (2014)
Retinal laser injury	Exosomes	Human UCB-MSCs and AD-MSCs	Yu et al. (2016)
Skin wound	Exosomes	Human AD-MSCs	Hu et al. (2016)
Myocardial infarction	miR-101a-enriched exosomes	Human BM-MSCs	Wang et al. (2020)

Balkom and colleagues, (van Balkom et al. 2019) enormous efforts have to be devoted to developing precise analytical methods for EVs' production, purification, and characterization in terms of molecular composition. Furthermore, the need to enhance EVs' homing and directed targeting to injured tissues (by up-regulating integrin expression as an example). As unmodified exosomes are readily cleared from the circulation, therefore PEG-lipid conjugates inserted into exosome membranes could extend the blood circulation time (Roura and Bayes-Genis 2019).

3.2.3 MSC-Mediated Organelle Transfer

Over the last decade, there has been a substantial interest in different types of cell-to-cell interaction which include the transfer of cytoplasmic material and organelles between cells. Compared to other stem cell types, MSC-mediated organelle transfer has the main focus of researchers to date. MSCs have the capacity to establish sophisticated transport networks that allow effective communication with stressed/injured somatic cells, regardless of their lineage.

In terms of MSCs, intercellular communication is key for all biological processes, including the maintenance of tissue homeostasis, management of the normal cellular functionality and interaction with signals from the external environmental that impact their fate. They can continuously interact with both neighboring as well as distant cells via a wide scale variety of communication networks, such as paracrine signaling, transport through communicating (gap) junctions and electrical coupling, TNTs, and EVs (Murray and Krasnodembskaya 2019). A mounting evidence suggests that MSCs can transfer their cytoplasmic element and organelles (as mitochondria) to various cell types, including neurons, (Babenko et al. 2018) neural stem cells, (Boukelmoune et al. 2018) cardiomyocytes, (Ma et al. 2013) vascular myocytes, (Vallabhaneni et al. 2012) alveolar pneumocytes, (Islam et al. 2012) renal tubular epithelium, (Naoto et al. 2019) corneal epithelium, (Jiang et al. 2016) endothelium, (Liu et al. 2014) and immune cells, (Court et al. 2020) particularly under conditions of cell injury/stress. Interestingly, MSCs' mitochondrial transfer can take place via the secretion of EVs, the formation of connexin 43-containing gap junctions, and TNTs. Two Rho-GTPases, Miro 1 and Miro 2, are shown to link mitochondria to microtubule-associated motor proteins (e.g. kinesin) which facilitate mitochondrial movement along the TNTs connecting two cells. Thus far, most

studies argue that transfer of functional mitochondria results in the improvement of mitochondrial respiration, ATP production and/or alleviation of mitochondrial ROS in the recipient cells, leading to improved cell viability and functionality. Oxidative responses are known to play an instrumental role in the pathogenesis of tissue damage. During oxidative stress, molecular oxygen is inadequately reduced in the mitochondria, resulting in excessive levels of ROS that lead to various forms of injurious effects such as lipid peroxidation, DNA damage, as well as cell death (Hu et al. 2018). In addition, there is evidence indicating the involvement of mitochondrial transfer in the molding of nuclear transcription factors, contributing to cellular reprogramming (Sinclair et al. 2016). In addition to donating functional mitochondria to recipient cells, MSCs could also help to eliminate the defective mitochondria via TNT-mediated lysosomal transfer (Liu et al. 2014). The mechanism of lysosome transfer can involve ATP and motor proteins, as kinesin and dynein, which permit the movement of lysosomes along microtubules. Yet, further investigation is still needed to verify the exact mechanism.

Interestingly, cytoplasmic/organelle transfer does only take place from MSCs to their cell neighbors, it happens the other way around too. In several studies, (Figeac et al. 2014; Mahrouf-Yorgov et al. 2017) it is reported that transport of cytoplasmic contents was mainly directed from differentiated cells toward MSCs. Figeac et al. (2014) argued that TNTs helped bidirectional cytoplasmic transfer between MSCs and stressed cardiomyocytes an experimental model of myocardial infarction. Such bidirectional cytoplasmic exchange was vital for boosting the secretory abilities of MSC for cardioprotective soluble factors, enhancing their therapeutic abilities. Likewise, mitochondrial release from dying cardiomyocytes could be key environmental cues that manipulate the cytoprotective ability of MSCs (Mahrouf-Yorgov et al. 2017). In other context, mitochondrial transfer form vascular smooth muscle cells can regulate MSC proliferation (Vallabhaneni et al. 2012).

4 Limitations of MSC therapeutics

The data available for MSC research to date seems to send two main messages. First, MSCs possess great therapeutic potential, which can be harnessed to treat myriad disorders. Second, clearly not all MSC preparations are equal in potency (Wagner and Ho 2007). It is quite obvious that careful attention to manufacturing protocols is a must for effective reproducibility in MSC-based medicine. Nevertheless, to date, it is still unachievable to pinpoint the pertinent quality attributes, which can be gauged in vitro as predictors of in vivo efficiency, to be used as release criteria and for quality check during the manufacturing processes. Other factors that warrants further optimization could be the timing and dosage of MSC administration, selection of administration routes, detection of injection timepoints, choice of disease models, and the fate of MSCs in vivo (retention, differentiation, etc.).

Another factor that impact MSC efficacy is their source. MSCs obtained from elderly subjects, (Mueller and Glowacki 2001) or from patients developing diabetes, (Cianfarani et al. 2013) or rheumatoid arthritis (Sun et al. 2015) have inferior therapeutic effects compared to those obtained from healthy subjects, resulting in disappointing treatment outcomes. In these conditions, the intrinsic properties of MSCs are altered, thus impairing their protective function. In addition, it is difficult to obtain enough quantities of healthy autologous MSCs with high activity from patients suffering from these medical conditions. Nevertheless, many publications made the opposite argument that compared to cells from healthy donors, MSCs obtained from Amyotrophic Lateral Sclerosis (ALS) patients did not depict any considerable alterations in their functionality, chromosomal alterations or cellular senescence (Ferrero et al. 2008). Anyways, allogeneic MSCs from young healthy donors remains a rational approach to resolve the issue with autologous cells, if any. However, collection of most types of MSCs is still an invasive procedure, highlighting the

importance of perinatal tissues (placenta, umbilical cord blood, and Wharton's jelly) as desirable alternative sources.

MSCs demonstrate specific tumor-oriented migration as well as incorporation capacity in several pre-clinical models, justifying their use as favorable carriers for anticancer drugs/genes. Nonetheless, MSCs show both pro- and anti-cancer features, thus considered as a double-edged sword. MSCs are found to be involved in various stages of tumor progression (Galland and Stamenkovic 2020). They home towards tumor cells attracted by tumor-secreted soluble factors. Once in tumor stroma, MSCs stimulate epithelial to mesenchymal transition (EMT) of cancer cells. Additionally, MSCs enhance metastatic potential of cancer cells (AHN 2020). MSCs also enhance tumor progression by promoting tumor cell stemness and manipulating tumor microenvironment (e.g. by angiogenesis and/or immunomodulation) (Galland and Stamenkovic 2020). On the other side, a variety of pro-tumor trophic factors secreted by tumor cells affect not only the phenotypic features of MSCs, but also their gene expression, illustrating the tumor-MSC bi-directional interactions.

Another dilemma is the ability of MSCs to fuse with host cells forming heterokaryons or entosis-derived hybrids with regenerative potential (Sottile et al. 2016). MSCs are an important adult stem cell resource with great potential for regenerative medicine. However, it is still essential to fully investigate both pros and cons of such hybrids. Future studies are still needed to verify how synkaryons divide and segregate their chromosomes to generate stable or unstable hybrids with regenerative or oncogenic potential, respectively (Berndt et al. 2013). The consequences of cell fusion could be even worse in case MSCs were genetically modified, leading to modification of host cell genome. An appealing approach to guard against any harmful repercussions of cell fusion is the microencapsulation of MSCs within various biomaterials (e.g. hyaluronic acid, alginate, agarose, etc.) that can also provide a physiologic environment that promotes cell survival, (Khatab et al. 2020) functionality, (Attia et al. 2014) and prevent immune response (Hashemi and Kalalinia 2015). Interestingly, microencapsulated MSCs can still provide their paracrine function and influence the site of implantation with their therapeutic protein expression/overexpression. Similarly, MSC microencapsulation might be an effective technique to evade the recently reported pro-oncogenic risk of MSCs mediated by direct cell-to-cell contact with cancer cells (Rodini et al. 2018).

In addition, long-term in vitro cultures/manipulations of MSCs can cause genetic instability and chromosomal abnormalities (Musial-Wysocka et al. 2019). MSCs' expansion in vitro reduces their replicative and differentiation potential, and brings about senescence (Neri 2019). Moreover, it reduces the efficiency of DNA polymerase and repair systems, thus leading to building up of DNA damage, such as mutations (deletions, duplications, rearrangements), as well as epigenetic changes. Therefore, younger passages are more desirable for transplantation in vivo.

5 Future Perspectives

Despite the documented benefits of MSC use in various pre-clinical and clinical applications, the exact mechanisms behind such effects are still under investigation. By time, scientists continue to discover new potential means by which MSCs could help protecting, improving or regenerating tissues. Moreover, long-term research studies will be necessary to investigate any long-term effects of MSCs therapies, including the adverse effects. However, in agreement with A.I. Caplan, (2019a) we believe that if MSCs can halt GvHD, (Elgaz et al. 2019) mitigate the injurious impact of heart attacks on cardiac tissues, (Bagno et al. 2018) and alleviate low-back pain in patients (Mesoblast Phase 2 clinical trial), (Ghosh et al. 2013) scientists should continue to use them. Hopefully, researchers will eventually figure out how MSCs did it, and will try to make them work even better. Therefore, we are still to see better isolation, purification, cultivation, manipulation, and patient presentation protocols. Yet, as any other fascinating therapeutic tool, MSCs are to be used wisely.

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Extracellular Vesicle Therapeutics in Regenerative Medicine

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Abstract

Extracellular vesicles (EVs) are nano-sized, cell-released vesicles which contain lipids, proteins, and nucleic acids derived from the parental cells. EVs play an important role in intercellular communication and influence both physiological and pathological conditions. They are increasingly explored as potential therapeutic agents since they can cross biological barriers, their cargo is protected from degradation and they are involved in the transfer of bioactive components. EVs can promote tissue regeneration and might be alternatives to cell therapy. They can be used both in their native form, and as delivery vehicles for therapeutic agents. However, there are many hurdles to overcome for broad clinical application of EVs as therapeutics. Here, we review recent conditions regarding EVs therapeutics in regenerative medicine.

Keywords

Extracellular vesicle · Graft versus host disease · Induced pluripotent stem cell · Inflammatory diseases · Mesenchymal stromal cell

Abbreviations

EV	Extracellular vesicle
MSC	Mesenchymal stromal cell
CNS	Central nervous system
GvHD	Graft versus host disease
iPSC	Induced pluripotent stem cell
SEC	Size-exclusion chromatography

1 Introduction

Extracellular vesicles (EVs) are nano-sized vesicular particles secreted by cells. Initially, EVs were observed in normal plasma as a platelet-derived particle and were referred to as “platelet dust” in 1967 (Wolf 1989). Intensive research in the past few decades has improved understanding of the origin, content, and function of EVs. It has been proven that EVs play a key role in intercellular communication by transfer of their cargoes, such as lipids, proteins, and nucleic acids. In general, EVs are divided into exosomes, microvesicles and apoptotics bodides according to their biogenesis, surface markers, and size (Théry et al. 2018).

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However, no specific molecular markers can yet confidently divide subtypes of EVs from each other (Théry et al. 2018). EVs exist in all bodily fluids and are likely produced by all types of cells. Numerous studies have shown that they play important roles in both maintaining normal physiological homeostasis and in the development of disease. Based on this, increasing number of researchers are studying the therapeutic applications of EVs.

Conventionally, drug development focused on single small molecular compounds. However, success rate is rather low, especially in diseases of the central nervous system, and other treatment modalities are being investigated, such as recombinant proteins (i.e. growth factors), biologics and nucleic acid medicine. Although some of these strategies have shown efficacy, many researchers started to investigate cell therapy which is a polypharmacological approach and has the potential to target multiple biological pathways. Among them, mesenchymal stromal cells (MSC) is one of the most commonly studied type of cells (Caplan and Correa 2011). Originally, it was expected that transplanted cells would replace damaged tissue through their differentiation capacity. However, many studies showed a low grafting and survival rate of transplanted cells in the target organs (Tögel et al. 2005), indicating that the paracrine effects were primarily responsible for their regenerative potential. This has led to the concept of using the EVs derived from the cells, instead of cells themselves. Furthermore, cell therapy had several disadvantages regarding complex cell handling processes including storing, distributing and using them. Additionally, several adverse effects have been reported, including microbial infection, immune rejection, gene mutation, and tumorigenesis or tumor promotion. In contrast, EV-therapy, which can be called "cell-free polypharmacological approach", can overcome many of these drawbacks, Furthermore, EVs are being explored as vehicles for therapeutic agents, to improve the delivery to the target tissue (Batrakova et al. 2016; Wiklander et al. 2019). Thus, EV-therapy is expected to be the next generation therapy for various diseases and we can expect the number of initiated clinical trials to rise rapidly. Here we review the potential therapeutic applications of EVs in regenerative medicine.

2 Stem Cells-Derived EVs

Stem cell-derived EVs have been used for the treatment of various diseases in preclinical trials. They promote tissue regeneration through numerous mechanisms including induction of regenerative phenotypes, promotion of angiogenesis, immune modulation, inhibiting inflammation, apoptosis, and oxidative stress (Luarte et al. 2016). The field has mainly focused on MSC-derived EVs (MSC-EVs) (Zhang et al. 2020; Álvarez-Viejo 2020). On the other hand, EVs derived from other type of stem cells are also investigated for therapeutic applications (Fig. 1).

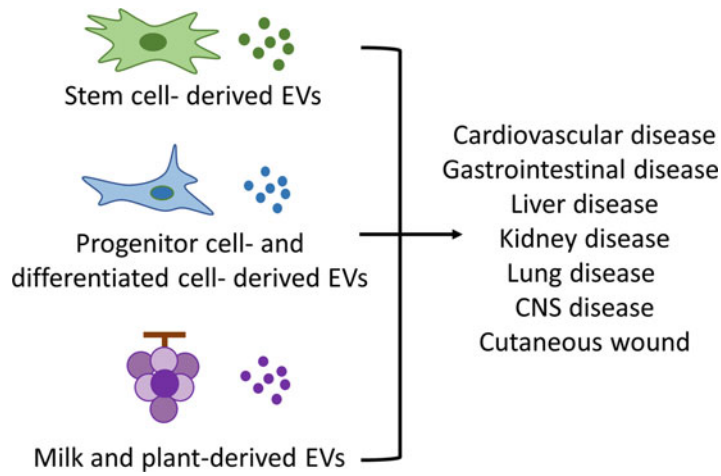

Fig. 1 EVs isolated from several different cell sources and food are increasingly being investigated as treatments for a plethora of diseases in most organ systems

2.1 MSCs-EVs

MSCs are multipotent stem cells which can be derived from several different tissues including bone marrow, adipose tissue, umbilical cord, periodontal ligament, and even solid organs (Caplan and Correa 2011). MSCs have shown therapeutic benefit in a wide variety of diseases in preclinical studies, and have subsequently been applied to several indications in patients (Galipeau and Sensébé 2018; Squillaro et al. 2016). Specifically, allogenic bone marrow MSC therapy has been approved for steroid resistant graft-versus-host disease (GvHD) in some countries including Japan. However, the treatment remains controversial and its efficacy and safety is not entirely clear. For example, a study where MSCs were co-transplanted with hematopoetic stem cells showed a reduced incidence of GvHD, but an increase in tumor relapse (Ning et al. 2008). Currently, many clinical trials are ongoing for inflammatory diseases, central nervous system (CNS) diseases, kidney diseases, liver diseases, and cardiac diseases (Galipeau and Sensébé 2018; Squillaro et al. 2016). However, several concerns regarding efficacy and safety, including pulmonary embolism, uncontrolled differentiation, infection, and tumor formation still exist.

One of the earliest preclinical studies of MSC-EVs showed its efficacy in a mouse model of myocardial ischemia-reperfusion injury in 2010 (Lai et al. 2010). Subsequently, therapeutic effects of MSC-EVs have been reported in various organs including lung, kidney, liver, CNS, cartilage, bone, and heart (Álvarez-Viejo 2020). Direct comparison of the efficacy of MSC therapy with MSC-EV therapy is difficult, due to the potential of MSCs to provide a long-term source of EVs at the injured site. However, several studies showed that MSC-EVs appear to be as effective as their parental MSCs. Furthermore, there are several advantages of using EVs compared with cells. EVs are likely to have less risks of side effects because they are smaller (especially important for embolism), and handling process is less complex enabling development of "off-the-shelf products".

The first case of MSC-EV therapy in human was the administration of allogenic bone marrow MSC-EVs for a patient with therapy-refractory acute GvHD in 2014 (Kordelas et al. 2014). GvHD symptoms recovered within a week after administration of MSC-EV and no significant side effects were observed. To date, several clinical studies of MSC-EVs have been conducted or are ongoing (Zhang et al. 2020). Phase II/III clinical trial of human cord blood–derived EVs for patients with chronic kidney disease showed safety, anti-inflammatory effects and amelioration of kidney dysfunction (Nassar et al. 2016). In addition, clinical trial of MSC-EVs for macular holes (NCT03437759), bronchopulmonary dysplasia (NCT03857841), prevention of fibrosis after cochlear implant surgery, stroke (NCT03384433) and diabetes mellitus type I (NCT02138331) are ongoing. More recently, three clinical trial for MSC-EVs for acute respiratory distress syndrome due to COVID-19 is ongoing in China (NCT04276987, ChiCTR2000030261, ChiCTR2000030484). In summary, the potential of MSC-EVs is widely explored for various diseases.

2.2 Induced Pluripotent- and Embryonic Stem Cell-Derived EVs

The effects of induced pluripotent stem cells-derived EVs (iPSC-EVs) and embryonic stem cells-derived EVs have also been reported in animal models (Zhang et al. 2019; Taheri et al. 2019). Zhang et al. reported the effect of EVs from iPSC-derived MSCs (iPSC-MSC-EVs) in a rat model of cutaneous wound healing in 2015 (Zhang et al. 2015). Subsequently, many researchers reported the effect of iPSC-MSC-EVs or iPSC-derived cardiac progenitor cell-EVs for skin, bone, liver, cardiac, and CNS diseases (Luarte et al. 2016; Rutering et al. 2016). Benefits of these cell types is that they have great proliferation potential and large number of homogenous cells can be acquired. However, the risk of certain adverse effects, especially teratoma-formation (due to cellular

contamination of the EVs), is one of the largest drawbacks (Gutierrez-Aranda et al. 2010).

3 Progenitor Cell- and Differentiated Cell-Derived EVs

Researchers are also trying to apply EVs derived from progenitor cells and differentiated cells as therapeutics. For example, endothelial progenitor cell- derived EVs for myocardial infaction (Xing et al. 2020), cardiac progenitor cell-derived EVs for angiogenesis (Vrijsen et al. 2016), amnion epithelial cell-derived EVs for lung fibrosis (Tan et al. 2018), cardiosphere cells-derived MVs for cardiac diseases (Aminzadeh et al. 2015), umbilical endothelial cells-derived EVs for atherosclerosis (Hergenreider et al. 2012), and oral epithelial cell-derived EVs for skin wounds (Sjöqvist et al. 2019; Sjoqvist et al. 2019).

4 Milk and Plant-Derived EVs

Several research groups have also isolated EVs from milk and different types of fruits or vegetables. Since such material could inflict a foreign body-response in the human body, the immunogenicity is a concern of those EVs, and might be a reason why some researchers opted to use oral administration instead of systemic administration. Oral administration of bovine milk- derived EVs was found to attenuate arthritis (Arntz et al. 2015) and oral administration of grape- derived EVs ameliorated colitis in a mouse model (Ju et al. 2013). Interestingly, these results suggest that EVs can cross intestinal-acid barrier. More recently, a clinical trial to investigate the efficacy of grape- derived EVs to reduce the risk of oral mucositis after radiation- and chemotherapy treatment for head and neck tumors is ongoing (NCT01668849). Furthermore, milk and plant-derived EVs are also used as drug delivery vehicles as described below.

5 Drug-Loaded EVs

Since EVs can transfer bioactive components across biological barriers, EVs are also being investigated as natural delivery vectors for different cargos, including small molecular compounds, proteins, siRNAs and microRNAs (Batrakova et al. 2016). This field especially focuses on CNS diseases, because of the existence of blood-brain-barrier (Fig. 2). For example, EVs loaded with an anti-inflammatory small molecule compound, curcumin, lead to an improved bioavailability and anti-inflammatory effect of this drug in a lipopolysaccharide-induced brain inflammation model (Zhuang et al. 2011). Similarly, administrations of MSC-EVs loaded with phosphatase and tensin homolog siRNA ameliorated spinal cord lesion injury (Guo et al. 2019). EVs released from genetically-modified macrophages that express glial cell-derived neurotrophic factor were suggested as a treatment of Parkinson's disease (Zhao et al. 2014). Milk and plant-derived EVs are increasingly used as drug delivery vehicles,

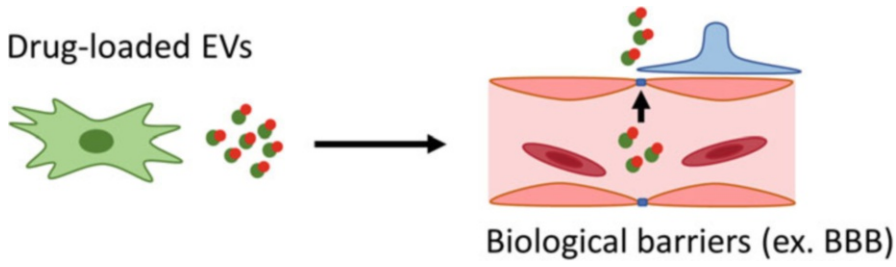

Fig. 2 Evs natural properties to pass through biological barriers, including the blood-brain-barrier, can be exploited to improve the delivery of various compounds to hard-to-reach areas such as the central nervous system

because these EVs may solve one of the main problems in EV-based drug delivery, that is, difficulties to manufacture large amounts of vesicles. A clinical, phase I trial of grape-exosomes loaded with curcumin, given orally to patients with colorectal cancer is currently being conducted (NCT01294702).

6 Hurdles to Consider for Clinical Application of EVs Therapeutics

There are many hurdles to consider for clinical application of EVs therapeutics (Yamashita et al. 2018) (Fig. 3). In particular, establishment of EV-manufacturing is the major barrier due to their heterogeneity and low yield. Development, optimization and standardization of production-methods, including methods for cell culture and isolation of EVs are required for a greater scalability, purity, retained integrity, functionality, sterility, and higher reproducibility. Optimization of storage method of EVs are also required. In addition, appropriate administration route of EVs are also important for therapeutic application.

6.1 Production of Large-Scale EVs

One of the major hurdles for EV-therapeutics is the limited production of EVs from cells. Thus, effective large- scale EV production methods are required. Some researchers reported that yield of EVs can be increased five to tenfold using a hollow fiber bioreactor (Watson et al. 2016). It has been reported that production of EVs was enhanced by applying stress such as hypoxia or low pH (Ban et al. 2015). However, it should be noted that such cellular stress can change the composition and function of EVs. One approach for large scale production of EVs is to immortalize the donor cells. For instance, MSCs transfected by lentivirus carrying MYC gene allows for obtaining of immortalized cells, without any change in fundamental characteristics of MSCs (Chen et al. 2011).

Another approach to increase yield of EVs is using more efficient isolation methods. Currently, ultracentrifugation is the most common method. However, this technique is limited by low EV recovery and laborious and time-consuming process. Other EV-isolation methods, including size exclusion chromatography (SEC), ultrafiltration, polymer-based precipitation, and immunoaffinity chromatography have been developed (Posch 2015). Although these methods can be used for large-scale production of EVs, different isolation methods affect purity and physicochemical properties as described below.

6.2 Isolation of Pure and Uniform EVs

Ultracentrifugation, the most commonly used method for EV isolation, has a risk of contamination of non-vesicular macromolecule and disruption of EV integrity. Polymer-based commercial reagents for EV isolation demonstrate a high recovery of EVs. Unfortunately, the purity is often rather low due to vesicle aggregation and

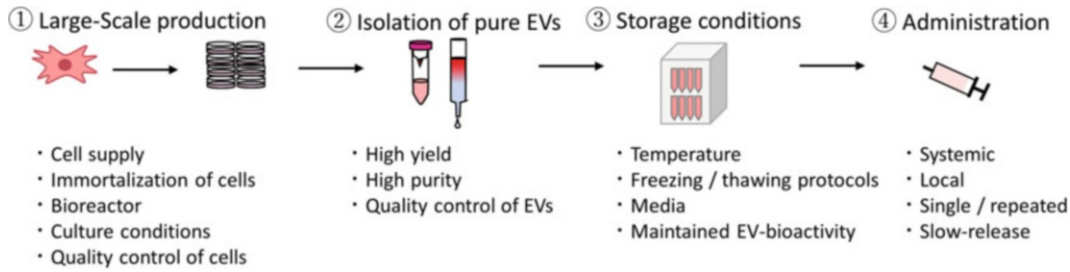

Fig. 3 EV-therapeutics is still a young field with extensive hurdles in several steps, such as production, isolation, storage and administration

contamination. Although this method has been applied in clinical settings for biomarker assessment, it might not always be suitable for therapeutic application. Size-based isolation techniques are being increasingly used for EV isolation. Ultrafiltration devices and tangential flow filtration systems are known to be able to concentrate EVs. Size-exclusion chromatography (SEC) is a method wherein components in a heterogeneous solution are separated based on size. SEC has improved EV integrity, purity, and reproducibility compared to ultracentrifugation. However, there are overlaps in the size of different EVs and SEC is rather time-consuming, which limits its scalability. Immunoaffinity chromatography has been used for EV isolation with high purity. Since this method depends on antibody recognition of EVs proteins, only a subset of EVs, expressing specific antigens, can be captured, resulting in a low yield, but high purity. Lipid-based nanoprobes used in combination with magnetic isolation of EVs, which enables intact, purer EV isolates in a shorter timeframe compared to ultracentrifugation, has been developed.

In conclusion, there are many different methods to isolate EVs, and EVs collected from different isolation methods differ in various properties. Further development of better isolation techniques or combinational approach using the advantage of different isolation techniques will be required. Although little is known about the difference in efficiency of EVs depends on the isolation methods, it is likely that therapeutic potentials vary among the subpopulations. Thus, optimization of the isolation method is important to obtain stable therapeutic effects and to reduce risk of side effects induced by contaminants. Also, virus contamination has to be investigated carefully, since the size can overlap with EVs.

6.3 Optimization of Storage Conditions of EVs

Currently, the International Society of Extracellular Vesicles recommends that EVs are suspended in phosphate buffered saline and stored at −80 °C (Witwer et al. 2013). For therapeutic application, storage at higher temperatures is desirable because it does not require special equipment. Ge et al. have shown that EVs can be stored up to 5 years in a −20 °C without loss of function (Ge et al. 2014). It has been also reported that lyophilization of EVs may improve their stability at higher temperatures. Some researcher showed that EVs can be concentrated, lyophilized, and reconstituted in water solutions (Rutering et al. 2016). However, it is not clear whether lyophilization is applicable to all kinds of EVs. Appropriate storage and stability should be further examined in order to make EVs real "off the shelf" therapies.

6.4 Optimization of Administration of EVs

Biodistribution, pharmacokinetics, bioavailability, and pharmacodynamics of EVs still remain largely unknown. Since the biological effect of EVs is thought to be largely driven by their uptake of target cells, control of EV-biodistribution is required for the therapeutic application of EVs. Wiklander et al. found that the biodistribution of EVs depends on administration routes, using different cell sources (Wiklander 2015). EVs accumulated mainly in liver, spleen, gastrointestinal tract and lungs, but there were differences depending on the cell source. Intraperitoneal injection resulted in higher accumulation of EVs in the gastrointestinal tract and pancreas compared with intravenous injection. In contrast, subcutaneous injection resulted in lower accumulation of EVs in all organs. Several studies showed that systemically injected EVs rapidly disappeared from blood circulation and are taken up by cells (especially macrophages) in the reticuloendothelial system. These findings were consistent irrespective of cell source and route of administration (Takahashi et al. 2013; Imai et al. 2015). This rapid clearance of EVs could be a limit of systemic administration of EVs. To overcome these clearance problems, some researchers have tried local or directed administration of EVs. For example, oral administration of EVs for colitis (Arntz et al. 2015; Ju et al. 2013), intranasal administration of

EVs for CNS diseases (Zhuang et al. 2011), intratracheal administration of EVs for lung diseases (Monsel et al. 2015), and topical administration of EVs for skin diseases (Sjöqvist et al. 2019), etc. In addition to administration route, pharmacokinetic and pharmacodynamic profiles should also be investigated to predict appropriate dose and timing for administration for clinical application.

7 Conclusions

In regenerative medicine field, EVs therapy is gaining momentum and might replace several kinds of cell therapies. Thanks to their advantages regarding safety and easier management process, EVs are expected as next generation "cell free polypharmacological approach". EVs derived from different cell sources will be applied for various indications. EVs are also increasingly explored as delivery vehicles, loaded with bioactive compounds. However, many challenges still exist for broad clinical application of EVs as therapeutics.

Disclosure Statement Aya Imafuku and Sebastian Sjoqvist are founders and owners of ExTherea Inc., Kanagawa, Japan. Sebastian Sjoqvist is employed by Takeda Pharmaceuticals.

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Exosomes as Part of the Human Adipose-Derived Stem Cells Secretome-Opening New Perspectives for Cell-Free Regenerative Applications

Sorina Dinescu, Alexandra Dobranici, Ramona Tecucianu, Aida Selaru, Roxana Balahura, Simona Ignat, and Marieta Costache

Abstract

Human adipose-derived stem cells (hASCs) represent a great resource for regenerative medicine based on their accessibility, self-renewal potential, low immunogenicity, high proliferative rate and potential to differentiate on multiple lineages. Their secretome is rich in chemokines, cytokines and protein growth factors that are actively involved in regeneration processes. In addition, part of this secretome are also the exosomes (hASC-exos), which display high content in proteins, messenger RNAs (mRNAs) and non-coding RNAs (ncRNAs). Due to their content, exosomes promote tissue regeneration by different mechanisms, either by activating or inhibiting several signaling pathways involved in wound healing, extracellular matrix remodeling, immunomodulation, angiogenesis, anti-apoptotic activity and cell migration, proliferation and differentiation. The use of hASC-exos may provide an improved alternative to standard therapies used in regenerative medicine, as a cell-free new approach with multiple possibilities to be modulated according to the patient needs. This review offers an updated overview on the functions and applications of hASC-exos in all areas of tissue regeneration, aiming to highlight to the reader the benefits of using hASCs in modern tissue engineering and regenerative medicine applications.

Keywords

Exosomes · Human adipose-derived stem cells · miRNAs · Signaling pathways in regeneration · Tissue regeneration

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Abbreviations

AFM	atomic force microscopy
AK2	adenylate kinase 2
AKI	acute kidney injury
AKT	Protein kinase B
Alix	ALG2-interacting protein X
Arg-1	arginase-1
AT	adipose tissue
Bad	Bcl associated agonist of cell death
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
Bim	Bcl-2-*like* protein 11
BM-ASCs	bone-marrow derived stem cells
BMEC	brain microvascular endothelial cells
BMP	bone morphogenetic proteins
CD	cluster of differentiation
CM	conditioned medium
Col2A1	type II collagen alpha 1
COX	cyclooxygenase
CSCs	cancer stem cells
CSF-1R	colony-stimulating factor 1 receptor
DDL4	angiogenic inhibitor delta-*like* 4
Dvl	Disheveled
ECM	extracellular matrix
EGF	epidermal growth factor
EPCs	endothelial progenitor cells
ERK	extracellular signal-regulated kinases
ESCRT	endosomal sorting complex required for transport
EVs	Extracellular vesicles
MVBs	multivesicular bodies
FBS	fetal bovine serum
FGF-1	fibroblast growth factor 1
FIZZ	found in inflammatory zone
G-CSF	granulocyte colony stimulating factor
GDNF	glial cell-derived neurotrophic factor
hASCs	human adipose derived stem cells
HDFs	Human dermal fibroblasts
HIPK2	homeodomain interacting protein kinase 2
HR	hypoxia/reoxygenation
HSP	heat shock protein
IDO	3-dioxygenase
IFN-γ	interferon γ
IGF	insulin-*like* growth factor
IL	interleukin
IL-4Rα	IL-4 receptor α
ILVs	intraluminal vesicles
iNOS	inducible nitric oxide synthase
IR	Ischemia/reperfusion
IRAK1	interleukin-1 receptor-associated kinase 1
IWR-1	Wnt-β-catenin signaling inhibitor
JNK	c-Jun N-terminal kinases
LASS2	longevity assurance homologue 2
LECs	lymphatic endothelial cells
lncRNA	long non-coding RNA
MALAT1	metastasis-associated lung adenocarcinoma transcript 1
MAPKs	mitogen-activated protein kinases
MARK1	microtubule affinity regulating kinase 1
Mcl-1	myeloid leukemia cell differentiation protein 1
MCP	monocyte chemoattractant protein
M-CSF	macrophage colony stimulating factor
MHC	major histocompatibility complex
miR	microRNA
MMP	matrix metalloproteinases
mRNAs	messenger RNAs
ncRNAs	non-coding RNAs
NF-kB	nuclear factor-kB
NGF	nerve growth factor
NK	natural killer
NPCs	neural precursor cells
Nrf2	nuclear factor-E2-related factor 2
OA	osteoarthritis
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PDGFA	platelet-derived growth factor subunit A
PDGFR	platelet-derived growth factor receptor
PDK1	3-phosphoi-nositide-dependent protein kinase 1
PI3K	Phosphoinositide 3-kinase

/PIP3	phosphatidyl inositol 3, 4, 5-triphosphat
PKB	Protein Kinase B
PLGA	poly lactic-co-glycolic acid
PPARγ	peroxisome proliferator-activated receptor γ
PREF-1	preadipocyte factor 1
PTEN	phosphatase and tensin homolog
S1P	sphingosine 1 phosphate
S1PR1	sphingosine 1 phosphate receptor 1
SDF-1	stromal cell derived factor
SEM	scanning electron microscopy
Sema	semaphorin
SIRT1	sirtuin 1
SK1	sphingosine kinase 1
STAT3	signal transducer and activator of transcription 3
TCF/LEF	T-cell factor/lymphoid enhancer factor
TEM	transmission electron microscopy
TGF-β	transforming growth factor-β
TIMP	tissue inhibitors of metalloproteinases
TLR4	Toll-*like* receptor 4
TNF-α	tumor necrosis factor-α
TRAF6	NF receptor associated factor 6
TRPM7	Transient receptor potential cation channel subfamily M member 7
TSG101	Tumor susceptibility gene
VEGF	vascular endothelial growth factor
VEGF-R	VEGF receptors
Vsp4	vacuolar sorting protein 4

1 Introduction

In the context of regenerative medicine, mesenchymal stem cells show an immeasurable potential for damaged tissues regeneration (Suman et al. 2019). Mesenchymal stem cells could be obtained from a multiple variety of sources such as bone marrow, adipose tissue, umbilical cord blood, skin dental pulp (Si et al. 2019). Even though, all mesenchymal stem cells are similar in many aspects, human adipose derived stem cells (hASCs) present many advantages. hASCs are multipotent stem cells isolated from a very accessible source, the subcutaneous adipose tissue, a better option compared to the classical use of bone-marrow derived stem cells (BM-ASCs) (Shingyochi et al. 2015; Gentile et al. 2019). They are easily obtained from lipoaspirates after enzymatic digestion and they are highly abundant, as from 300 mL of lipoaspirate, between 1×10^7 and 6×10^8 hASCs could be cultured and expanded in culture (Gimble et al. 2007; Locke et al. 2009; Galateanu et al. 2012; Dinescu et al. 2013). In a comparative study on human adult mesenchymal stem cells derived from bone marrow, adipose and dermal tissue, hASCs proved to secrete the highest levels of paracrine factors involved in tissue regeneration, which recommends their use for regenerative therapies (Hsiao et al. 2012). More advantages of hASCs over other types of stem cells are given by their self-renewal potential, low immunogenicity, high proliferative rate, potential to differentiate on multiple lineages (adipogenic, osteogenic, chondrogenic, myogenic, neurogenic, etc) (Dinescu et al. 2018; Wong et al. 2019; Hong et al. 2019; Shukla et al. 2020). Moreover, through paracrine and immunomodulatory functions, hASCs are involved in directing wound healing and tissue regeneration (Murphy et al. 2013; Dinescu et al. 2018).

Paracrine activity of hASCs and communication with inflammatory microenvironment are important factors for tissue repair mechanisms (Domenis et al. 2018). hASCs display lack of expression of major histocompatibility complex (MHC) class II, MHC class I inferior expression levels and the presence of prostaglandin E2 with the purpose of mediating the immunosuppressive (McIntosh et al. 2006). Also, hASCs intervene in the proliferation of T and B cells, cytotoxicity and activation of natural killer (NK) cells through interleukin (IL)-2 secretion (Domenis et al. 2018). hASCs features influence the production of pro- and anti-inflammatory cytokines and antigen-specific T-reg cells (Gonzalez-Rey et al. 2010). hASCs specific cytokines, highly studied

over the years, are tumor necrosis factor (TNF)-α, interferon (IFN)-γ, 3-dioxygenase (IDO), and IL-17. These cytokines exert effects on immunosuppressive activity of hASCs and mediate the connection between hASCs and T cells. T cells activity is suppressed by hASCs, thus the secretion of TNF-α and IFN-γ is inhibited (Zappia et al. 2005).

hASCs express specific cell surface markers such as cluster of differentiation (CD) 13, CD29, CD34, CD44, CD73, CD90, CD105, CD166, STRO-1, and are negative for CD38, CD45, CD106 (Minteer et al. 2012). Not only that hASCs are able to specifically differentiate in order to ensure cellular restoration where needed, but they also exert paracrine actions on other cells by secreting multiple types of chemokines, cytokines and protein growth factors (Mazini et al. 2019). hASCs secretome has a positive impact on several processes such as immunoregulation, angiogenesis, cell proliferation, wound healing and tissue regeneration. Some of the most important factors produced by hASCs are: epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), insulin-*like* growth factor (IGF) and brain-derived neurotrophic factor (BDNF), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), bone morphogenetic proteins (BMP), interleukins-6,-7,-8,-11,-12, TNF-α (Hsiao et al. 2012; Dinescu et al. 2018; Hong et al. 2019; Shukla et al. 2020). Furthermore, hASCs also act on the surrounding cells and stimulate tissue regeneration by transferring signal molecules through extracellular vesicles (EVs) (Keshtkar et al. 2018).

The aim of this chapter is to provide an updated overview on the content and functions of hASC-exos in all regeneration- related processes, such as wound healing, inflammation, cell proliferation and differentiation, matrix remodeling, etc. and to highlight the ncRNAs that modulate the signaling pathways associated with these regenerative processes.

2 Exosomes and Their Potential for Tissue Regeneration

EVs have been identified as an important hallmark for intercellular communication within a multicellular organism (Raposo and Stoorvogel 2013). They are known to take be part in both normal physiological conditions and during disease development (Kalluri and LeBleu 2020). Originally it has been considered that such structures are just artefacts that can be released only during cell-death and apoptosis (e.g. apoptotic bodies), but the fact that also perfectly healthy cells can secrete vesicles form their plasma membrane has become a matter of interest and has been widely explored over the past two decades (Hristov et al. 2004). EVs can be classified into two major groups, namely endosomes and exosomes (Cocucci and Meldolesi 2015). Whereas endosomes are formed by direct outward budding of plasma membrane generating vesicles with a size of 50 nm to 1 μm in diameter, exosomes have endosomal origin with a size range of 40–160 nm in diameter (Kalluri and LeBleu 2020).

Exosomes are formed during an endosomal process that includes double invagination of the plasma membrane followed by formation of multivesicular bodies (MVBs), which carry intraluminal vesicles (ILVs) (Zhang et al. 2019). During these events, a series of proteins, messenger RNAs (mRNAs) and non-coding RNAs (ncRNAs) are encapsulated within the ILVs (Anand et al. 2019). Further, ILVs are secreted as exosomes via MVBs fusion to the plasma membrane and exocytosis, process that ends with the release of exosomes in the extracellular environment (Zhang et al. 2019) or trafficked to lysosomes for degradation. Recently, it has been described that MVBs formation, followed by ILVs generation are dependent on the endosomal sorting complex required for transport (ESCRT) machinery (Raiborg and Stenmark 2009; Schmidt and Teis 2012).

ESCRT is a complex composed of four proteins 0, I, II and III ESCRTs that are actively involved in MVBs formation and protein cargo

sorting (Henne et al. 2011; Hurley 2015). Upon recognition of ubiquinated proteins of the endosomal membrane the ESCRT complex is activated, thus generating the promotion of the budding process. After cleavage and ILVs formation, the complex separates from MVBs with energy provided by vacuolar sorting protein 4 (Vsp4) (Henne et al. 2011). As ESCRT complex takes part in exosome formation, it is expected that its ***accessory proteins*** will be identified within exosomes regardless of their cell origin: ALG2-interacting protein X (Alix), Tumor susceptibility gene (TSG101), heat shock proteins (HSP60, 70 and 90) and tetraspanins CD63, CD81, CD82, CD9. This specific set of proteins is known as the exosomal marker proteins and is widely used in studies for exosome identification and characterization (Hong et al. 2019). Even though CD63, CD9 and CD81 are commonly found in exosomes, they are not specific markers as they can be also identified in MVBs and apoptotic bodies (Doyle and Wang 2019).

Since the discovery of exosomes, lots of isolation methods have been developed. Novel techniques are constantly evolving, among them ultracentrifugation, precipitation, size-based methods or immuno-affinity capture-based isolation. As for exosome characterization, lots of analysis can be used- such as atomic force microscopy (AFM), scanning electron microscopy (SEM) but the golden standard for exosome morphology validation is transmission electron microscopy (TEM) (reviewed by (Zhou et al. 2020)).

Besides being secreted by a large range of mammalian cells, such as mesenchymal stem cells (Han et al. 2016), neurons (Janas et al. 2016), cancer cells (Bae et al. 2018), exosomes can also be found in body fluids such as plasma (Whiteside 2018), saliva (Zlotogorski-Hurvitz et al. 2015) and the lymph (Park et al. 2018). Exosomal cargo consists in a large pool of various molecules, including transcription factors, receptors, extracellular matrix proteins, lipids and nucleic acids (mRNAs and ncRNAs). As previously mentioned, these molecules are recruited during ILVs formation (Anand et al. 2019), but the exact mechanisms have not been explored. All these components are transported from the donor cell to the receiving one via exosomes, thus generating various signals that active or influence major biological processes such as cancer, inflammation, tissue degeneration and repair. MicroRNA (miR)-21 found in tumor derived-exosomes has been demonstrated to be present in significant amounts in case of ovarian (Taylor and Gercel-Taylor 2008), lung (Fortunato et al. 2019) and breast cancer (Shi 2016). Whereas, exosomal miR-9, miR-128 and miR-107 have been identified in regulating neuronal differentiation and their downregulation has been correlated with Alzheimer's disease (Van Giau and An 2016), miR-155, miR-146a and miR-125b were found to regulate inflammation in mouse models of alcoholic liver disease (Bala et al. 2012).

The fact that exosomal cargo has a beneficial effect upon tissue repair and regeneration has been widely demonstrated over the past few years. It has been reported that exosomes derived from mouse embryotic stem cells could promote cardiac function via delivery of miR-294 to cardiac progenitor cells (Khan et al. 2015). Choi et al. (2016) proved that differentiating human skeletal myoblasts-derived exosomes could promote muscle regeneration by collagen deposition reduction in a laceration mouse model. Also, in case of cutaneous damage it was found that human umbilical cord mesenchymal stem cells-derived exosomes were administered and were able to inhibit the inflammatory reaction caused by burn injury. More specific, it was found that miR-181c downregulated Toll-*like* receptor 4 (TLR4) signaling pathway, therefore attenuated inflammation and enhanced tissue regeneration (Li et al. 2016). The same exosomes were shown in another study by Zhou et al. (Zhou et al. 2013) to ease acute kidney injuries in cisplatin treated rats. In this case it was found that exosomes reduced renal oxidative stress and prevented apoptosis. The beneficial effect of

exosomes has been also demonstrated for bone regeneration, when exosomes could efficiently generate proliferation and osteogenic differentiation in bone marrow-derived mesenchymal stem cells (Qi et al. 2016). Moreover, it was found the hepatocyte-derived exosomes promoted cell proliferation paired with liver regeneration in mice suffering from ischemia/reperfusion injuries or partial hepatectomy (Nojima et al. 2016a). Curiously, the regenerative effects of exosomes have also been investigated in nervous tissue regeneration. Oligodendrocyte-derived exosomes were found to have multiple effects upon neuronal physiology such as, ensuring neuronal survival when neurons were deprived from oxygen and glucose in a model of cerebral ischemia (Fröhlich et al. 2014).

Taking into consideration both hASC unique properties and exosomes significant contribution in the field of regenerative medicine, hASC-derived exosomes present promising tools for stimulating tissue regeneration via signal transduction. The signaling cues encapsulated within hASC-exos have been found to promote damaged tissue repair targeting a large pale of proteins and transcription factors actively involved in key signaling pathways. hASC-exos have been found to contribute to repair in acute kidney injuries (Zhu et al. 2017), to boost neurite outgrowth (Chen et al. 2019b) and promote neuronal survival (El Bassit et al. 2017), but also be involved in wound healing (Li and Guo 2018) and bone regeneration (Tan et al. 2020). Based on all this evidence it can be stated that hASC-exos are responsible for the transport of key signals which can change the behavior and physiology of target cells. The pro-regenerative signals of hASC-exos act as activators or inhibitors of key signaling pathways involved in the tissue repair and homeostasis maintenance. Thus, hASC-exos could present a powerful tool for regenerative medicine purposes. Further, we will describe in depth events triggered by the bioactive molecules carried within hASC-exos upon important signaling pathways active during tissue regeneration (Fig. 1).

3 hASC-Exos Interaction with the Signaling Pathways Involved in Tissue Regeneration

Exosomes promote tissue regeneration by different mechanisms, among them by activating or inhibiting several signaling pathways involved in processes such as cell survival, proliferation and differentiation. These pathways will be further discussed in relationship with regenerative properties of hASC-exos: immunomodulation, angiogenesis, wound healing and anti-apoptotic effects.

3.1 hASC-Exos Interaction with Wnt-β Catenin Pathway

The Wnt-β-catenin is one of the highly conserved signaling pathways which participates in processes such as cell migration and proliferation, has anti-apoptotic effects and regulates stem cell pluripotency. It also plays major roles in embryonic development and development decision (MacDonald et al. 2009; Majidinia et al. 2018). Activation of Wnt-β-catenin pathway was reported in wound (Carre et al. 2018) and fracture (Sun et al. 2019) healing, osteogenic differentiation (Gong et al. 2018) and liver regeneration (Zhao et al. 2019) The Wnt-β-catenin signaling pathway is early activated in regenerative processes, mainly by post-transcriptional changes, highlighting its importance and relevance (Shiah et al. 2016; Majidinia et al. 2018). Wnt ligands are glycoproteins rich in Cys that bind to Frizzled or LRP5/6 receptors (LDL receptor-related proteins 5 and 6). This represents the external stimulus necessary to activate other two proteins, Dishevleled (Dvl) and β-catenin. The latter is then translocated in the nucleus where it binds to T-cell factor/lymphoid enhancer factor (TCF/LEF) family of transcription factors, regulating gene expression (MacDonald et al. 2009).

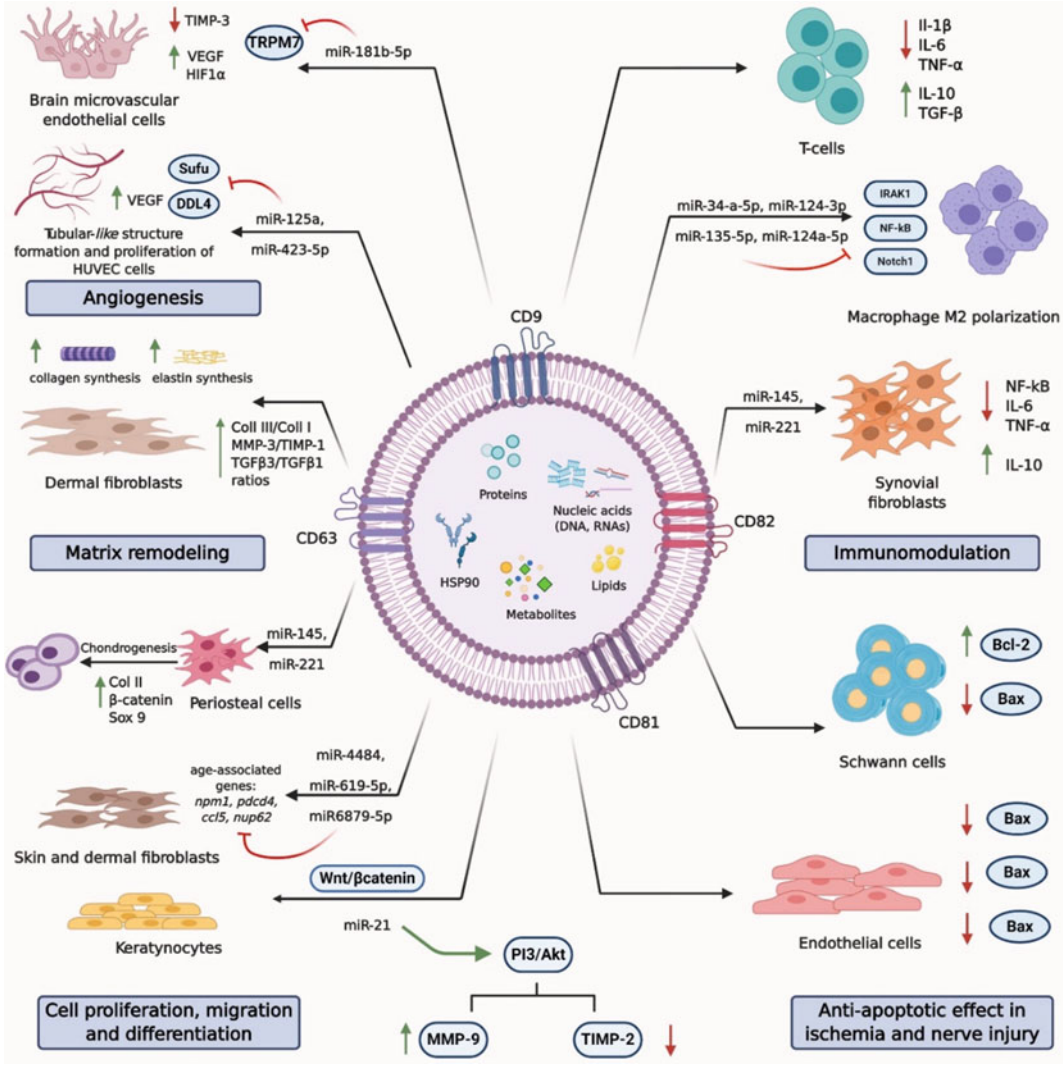

Fig. 1 Human adipose-derived stem cells exosomes composition and role in molecular events associated to tissue regeneration- cell proliferation, migration, differentiation, immunomodulation, angiogenesis, matrix remodeling and anti-apoptotic effect

hASC-exos have the ability to stimulate or enhance tissue regeneration via Wnt-β-catenin activation. In a study conducted by Cui et al. (2017), cardioprotective role of hASC-exos against ischemia/reperfusion injury was strongly correlated with Wnt-β-catenin activation. Ischemia/reperfusion (IR) *in vivo,* and hypoxia/reoxygenation (HR) *in vitro*, determined a decrease in Wnt3, p-GSK-3β(Ser9) and β-catenin expression levels, these proteins being essential in signal transduction. hASC-exos significantly counteracted IR and HR effects, by increasing expression levels of the aforementioned proteins. Overall, hASC-exos determined an increase in cell viability along with a decrease in cell apoptosis. An inhibitor of Wnt-β-catenin pathway, XAV939, reversed hASC-exos' cardioprotective effect *in vitro*. Further investigations are needed to provide insight into the mechanisms underlying Wnt-β-catenin activation by hASC-exos. Inhibition of cell apoptosis and stimulated proliferation was also observed in case of cutaneous

wound healing, where activation of Wnt–βcatenin pathway occurred after treatment with hASC-exos (Ma et al. 2019). A possible mechanism of Wnt-β-catenin activation by hASC-exos was offered by He et al. (2020) in a recent study regarding wound healing. hASC-exos containing metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) are responsible of silencing another ncRNA, miR-124 (He et al. 2020). It was observed that Wnt-β-catenin pathway was activated in MALAT1 presence and inactivated in condition of MALAT1 knockdown. Furthermore, miR-124 silencing determined regaining the promotion of Wnt-β-catenin signaling.

hASC-exos were used in diabetic cutaneous wound healing as a nanodelivery vehicle for exogenous miR-21-5p to promote regeneration *in vivo* and *in vitro* (Lv et al. 2020). miR-21-5p along with hASC-exos were expected to have higher regenerative properties than each component alone, the two of them acting in a synergic manner. Activation of Wnt-β-catenin pathway *in vitro* was the result miR-21-5p being efficiently delivered to HaCaT cells by hASC-exos, determining the promotion of keratinocyte migration. *In vivo,* Wnt-β-catenin signaling promoting keratinocyte migration played a major role in wound regeneration by re-epithelization (Lv et al. 2020).

Wnt-β-catenin signaling pathway was also reported to be active in case of osteogenic differentiation. Lu et al. (2017) used TNF-α as a pre-conditioning agent for hASCs to stimulate exosomes secretion, highlighting its role in acute inflammation, a process necessary for regeneration. Exosomes belonging to pre-conditioned hASCs had a potentiated effect towards human primary osteoblastic cells' migration and differentiation. These exosomes had higher expression levels of Wnt-3a protein, while β-catenin levels in correspondent osteoblast cells were increased. In the presence of Wnt-β-catenin signaling inhibitor (IWR-1), osteogenic markers, such as Runx2, bone sialoprotein and osteopontin were decreased. Wnt pathway activation by hASC-exos was also reported in chondrogenic differentiation of periosteal cells. Incubation of these precursor cells with hASC-exos led to elevated levels of β-catenin mRNA and consequently this proved Wnt-β-catenin signaling activation. Simultaneously, other mRNAs of chondrogenic markers (Sox9 and Col II) were also increased along with a change in chondrocyte morphology. Similarly, treatment with an antagonist, ICG-001, prevented the change in morphology and decreased chondrogenic markers (β- catenin, Sox9 and Col II) (Zhao et al. 2020).

3.2 hASC-Exos Interaction with PI3K-Akt Pathway

Phosphoinositide 3-kinase (PI3K)/Akt is a complex, survival pathway, with a variety of upstream stimulators that consist of growth hormones, cytokines and foreign molecules (Chen et al. 2013). Its activation can occur through tyrosine kinase receptors, cytokine receptors, integrins, B and T cell receptors or G protein coupled receptors (Shi et al. 2019). The first event implies activation of PI3K enzyme that produces phosphatidylinositol3,4,5-triphosphat (PIP3). PIP3 recruits 3-phosphoi-nositide-dependent protein kinase 1 (PDK1) and Akt, which is phosphorylated by PDK1. Akt, known as Protein Kinase B (PKB), meets three different izoformes, Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ), participating in cell growth and survival, angiogenesis or regulation of glucose homeostasis. These processes are regulated through other proteins, such as mTOR for proliferation, VEGF for angiogenesis B-cell lymphoma 2 (Bcl2) and Rac-1 for survival and migration respectively (Hers et al. 2011; Shi et al. 2019).

For tissue regeneration purposes, it was observed that different PI3K/Akt pathways were activated in cases of bone (Zhang et al. 2016), liver (Zhong et al. 2019) and nervous tissue regeneration (Dong et al. 2019). hASC-exos were used to stimulate processes necessary for tissue regeneration, by activating PI3K/Akt signaling cascade, especially for wound healing results. Paracrine function of hASCs was emphasized by Zhang et al. (2018c) who used a cell-free strategy with exosomes to characterize underlying mechanisms in cutaneous wound

healing. Treatment with hASC-exos resulted in human dermal fibroblast proliferation *in vitro* and collagen secretion, mediated by PI3K/Akt signaling pathway. As expected, signaling inhibition resulted in blocked proliferation and decreased expression of Col I. Col III, Akt and phosphorylated Akt. More insight into the wound healing properties of hASCs secretome via PI3K/Akt pathway was provided by Ren et al. (2019), who assessed its role on proliferation, migration and angiogenesis. Time-depended phosphorylation of Akt protein was observed in HUVEC, HaCaT cells and fibroblasts after exposure to hASCs secretome. This observation was correlated with enhanced migration and proliferation of all three types of cells, as well as enhanced angiogenesis *in vitro*. Furthermore, keratinocyte migration was demonstrated to be the result of Akt/HIF-1α activation. HIF-1α actions downstream PI3K/Akt pathway and is activated by phosphorylated Akt. HIF-1α levels decreased after inhibiting Akt phosphorylation, along with proliferative activity of keratinocytes (Zhang et al. 2020).

However, none of these studies offered explanation of the exact mechanism by which PI3K/Akt signaling pathway is activated in response to hASCs paracrine function. Recently, An et al. (2019), as well as Yang et al. (2020) discovered a small non-coding RNA, miR-21 possibly involved in PI3K/Akt signaling activation, having proliferative and angiogenic effects in wound healing. miR-21 was found to be one of the important factors that promote tumor angiogenesis and it was overexpressed in hASC-exos. In response to this type of hASC-exos, HUVEC cells were capable of vascularization, having higher protein expressions for HIF1-α, VEGF and stromal cell derived factor (SDF-1). hASCs that overexpressed miR-21 presented increased levels of phosphorylated Akt and decreased levels of phosphatase and tensin homolog (PTEN), which is a tumor suppressor protein (An et al. 2019). Yang et al. (2020) observed positive effects of miR-21 from hASC-exos on HaCaT cells migration that was attributed to PI3K/Akt signaling pathway activation. This led to changes in expressions levels of matrix metalloproteinases 9, 2 (MMP-9 and MMP-2) and in tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1 and TIMP-2). Additionally, mir-21 does not act alone in modulating cell signaling, but it cooperates with TGF-βI protein, in a negative feedback loop, both inhibiting each other.

hASC-exos exercised therapeutic effects in urinary stress incontinence, having high expression levels of proteins involved in various signaling pathways, PI3K/Akt included. Therapeutic potential comprised of enhancing the growth of skeletal muscle and Schwann cell lines. Thus, overexpressed proteins were associated not only with PI3K/Akt, but also with Jak- signal transducer and activator of transcription (STAT) and Wnt pathways that participate in nerve and skeletal muscle regeneration (Ni et al. 2018).

3.3 Other Signaling Pathways Involved in Regenerative Properties of hASC-Exos

Mitogen-activated protein kinases/Extracellular signal-regulated kinases (MAPK/ERK) signaling pathway is activated by growth factors and mitogens through various types of receptors, in order to regulate gene expression and consequently promote cell survival. Genes that are targeted by it, code for proteins involved in apoptosis control, such as Bcl associated agonist of cell death (Bad), Bcl-2-*like* protein 11 (Bim), induced myeloid leukemia cell differentiation protein 1 (Mcl-1) or caspase 9. MAPK/ERK signaling pathways comprises a series of kinases that activate each other by downstream phosphorylation. It interacts with PI3K/Akt pathway in cell growth and tumorigenesis (McCubrey et al. 2007). The studies conducted by Ren et al. (2019) and An et al. (2019) also discovered ERK pathway to be activated along with PI3K/Akt in wound healing promoting cell migration and angiogenesis. Additionally, activation of ERK/MAPK signaling by hASC-exos contribute extracellular matrix (ECM) remodeling by increasing expression of MMP3 (Wang et al. 2017). On the other hand, hASC-exos with immunoregulating effect suppressed MAPK and

nuclear factor-kB (NF-kB) pathways in neural tissue regeneration. This led to inhibition of microglia activation, which take part in neuroinflammation (Feng et al. 2019).

PKA pathway is another cell signaling cascade activated by hASC-exos that was found to be involved in regenerative processes such as angiogenesis. More precisely, hASC-exos from cells that were kept in hypoxic condition stimulated angiogenesis of HUVEC cells by activating PKA pathway. Its inhibition suppressed angiogenic effects, confirming PKA activation as an underlying mechanism to promote vascularization (Xue et al. 2018). Other signaling pathways regulated by hASC-exos are sphingosine 1 phosphate(S1P)/sphingosine kinase 1(SK1)/sphingosine 1 phosphate receptor 1 (S1PR1) which have immunomodulating effects in cardiac damage (Deng et al. 2019), or sirtuin 1 (SIRT1) with immunomodulating effects in acute kidney injury (Gao et al. 2020) (Fig. 2).

4 Biological Functions of hASC-Exos and Contribution to Tissue Regeneration

The biological processes in which exosomes are involved are numerous. Due to their varied content of proteins and nucleic acids, they can be found to take part in multiple crucial cellular events. In depth molecular analysis revealed 148 miRNA molecules in hASC-exos and proteomic investigations detected the presence of over 1400 exosomal proteins which play different roles in biological processes (Hong et al.

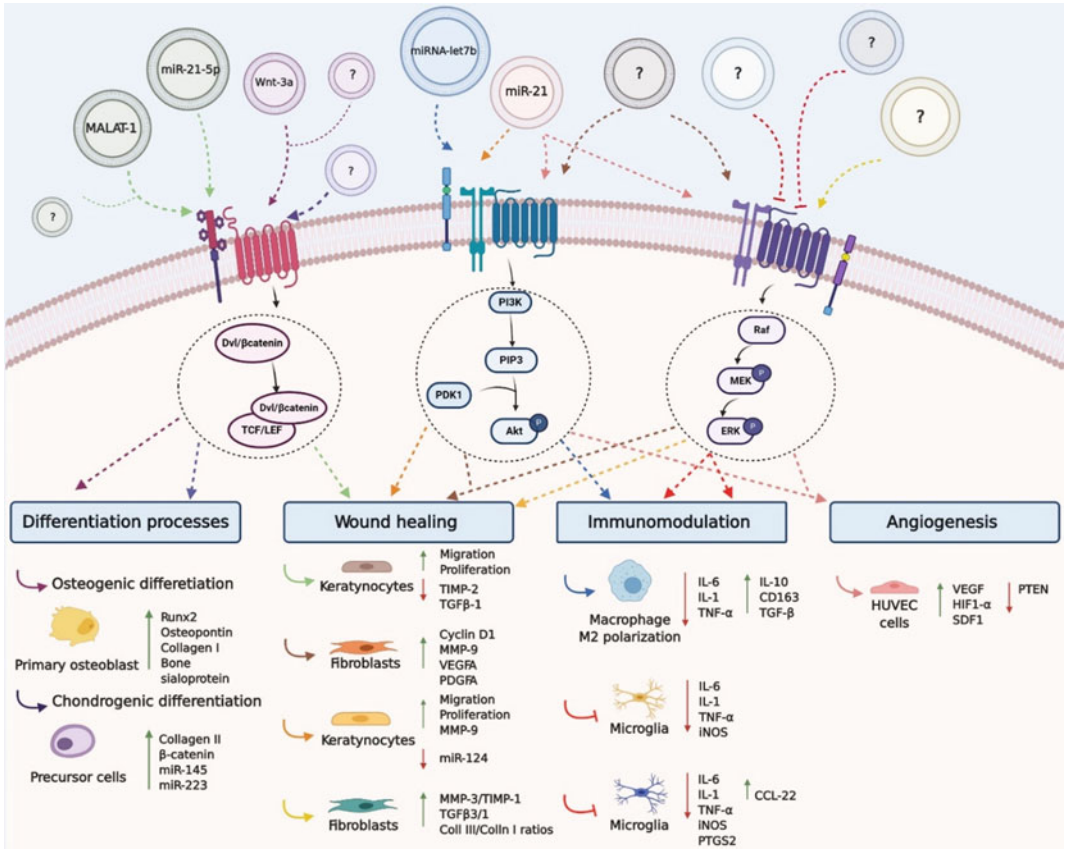

Fig. 2 The major tissue regeneration-related signaling pathways modulated by hASC-exos cargo in ncRNAs and the downstream effects associated with wound healing, immunomodulation, angiogenesis and differentiation processes during regeneration

2019) such as, wound healing, extracellular matrix remodeling, immunomodulation, angiogenesis, anti-apoptotic activity and cell migration, proliferation and differentiation.

Wound healing represents a complex process that involves multiple mechanisms and many cell types in order to regenerate the damaged tissue. This process has three stages: inflammation, proliferation and remodeling, each represented by a main cellular event (cell migration, cell proliferation and ECM deposition) (Broughton et al. 2006; Shingyochi et al. 2015; Hassanshahi et al. 2019). Upon injury, the inflammatory stage is initiated and involves the formation of a provisional wound matrix and initiation of clotting cascade. The clot serves as scaffolding for the inflammatory cells that are attracted to the injury site and further contribute to clearing the invading bacteria and cellular debris (Broughton et al. 2006; Reinke and Sorg 2012). The next phase is the proliferative stage that represents the response of progenitor cells to the chemoattractants secreted by the inflammatory cells. Epithelial progenitor cells, fibroblasts and keratinocytes migrate to the wound site, proliferate, some of them differentiate, and then start producing collagen and other extracellular matrix proteins. Moreover, angiogenesis is induced, aiming to restore the vascular system. During the last stage, as the wound matures by replacing collagen III in stronger collagen I, the cells that produced the granulation tissue undergo apoptosis (Reinke and Sorg 2012). Prolonged healing may lead to excessive scar formation that is usually associated with defective healing (Hong et al. 2019). The correlation between hASC-exos activity and these biological functions will be further discussed in detail in the following section.

4.1 hASC-Exos Function in Immunomodulation and Anti-Inflammatory Activity

In some tissue regeneration processes, inflammation is an important first step. Inflammation is a response of the body to damaging stimuli and which presents as a goal homeostasis restoration in organisms. A regulated inflammatory response is normal and favorable for tissue regeneration (Wu et al. 2018).

The immunomodulatory characteristics of hASCs are associated with cell-to-cell connection and production of immunosuppressive mediators (Blazquez et al. 2014). hASCs are involved in regulation of immune system through cell-to-cell intercommunication and secretion of different factors, such as EVs, growth factors, soluble mediators (Fang et al. 2019). Due to hASCs interaction with immune cells in the adipose tissue, they can direct the T and B cells and macrophages activity. Through cell-to-cell contact, hASCs are able to decrease the proliferation of allogenic lymphocytes (Yañez et al. 2006). Also, hASCs can be used for allogenic and xenogenic transplantation, without immunosuppression (Lin et al. 2012).

hASCs support the activation of T-reg cells, macrophages interaction, IL-10 secretion and TH1 cells proliferation downregulation (Al-Ghadban and Bunnell 2020). hASCs secretion of soluble mediators, EVs and growth factors is useful for regenerative medicine. In addition, immune diseases could be alleviated using cell-free therapy represented by hASC-exos.

hASC-exos have the property to decrease the intensity of the inflammatory responses. This is possible due to the pro-inflammatory cytokines TNF-α, IL-1β, inducible nitric oxide synthase (iNOS), monocyte chemoattractant protein (MCP)-1and cyclooxygenase (COX)-2 down-regulation, and the anti-inflammatory cytokines IL-10 up-regulation (Yu et al. 2016). hASC-exos include in their composition different cytokines, metabolites or ncRNAs which have anti-inflammatory and regenerative properties (Seo et al. 2019). Due to the already proven role of hASCs in immunosuppression, hASC-exos started to be used for similar applications (Al-Ghadban and Bunnell 2020). The therapeutic effects of hASC-exos are performed through a paracrine mechanism of content release into target cells cytosol.

The immunomodulatory characteristics of hASC-exos are represented by peripheral tolerance (Mokarizadeh et al. 2012), regulation of

immune response (Zhang et al. 2014). Like hASCs, hASC-exos influence the activity of B and T lymphocytes. Immunosuppressive activity of hASC-exos are represented by the conversion of T lymphocytes into the T-regulatory phenotype (Wu et al. 2018).

hASC-exos influence the secretion of IL-10 and TGF-β, reduce the secretion of IL-1β, IL-6 or TNF-α, and decrease the activation and differentiation capacity of T cells (Blazquez et al. 2014).

In 2017, Jaimes et al. reported that in microglia cells, the control of inflammatory responses is determined by p38, MAPKs, c-Jun N-terminal kinases (JNK) and ERK 1/2 suppression. The reduced inflammatory responses and microglia cells activity can support the regeneration of neural tissue (Jaimes et al. 2017).

A study employed by Domenis et al., showed that the presence of inflammatory cytokines influences the hASC-exos immunosuppressive and anti-inflammatory reactions and M2 phenotype polarization (Domenis et al. 2018). It is known that M2 phenotype polarization is supported by hASC-exos. Balaphas et al., studied the role of EVs in steatohepatitis. One of the observations was that hASC-exos determine the polarization of M2 macrophages, contributing to decrease of steatosis and promoting liver regeneration (Balaphas et al. 2019).

Another study conducted by Sanchez-Margallo et al. proved the inhibitory effect of hASC-exos on the propagation and differentiation of CD4 and CD8 T cells and IFN-γ production. In the end of the study, they suggested that hASC-exos represent an alternative for regeneration of damaged tissue and local immunosuppression, because IFN-γ is involved in immune and anti-inflammatory responses (Blazquez et al. 2014).

In 2017, Zhao et al. investigated the effect of hASC-exos treatment on mice with obesity-associated inflammation. The treatment influenced M2 macrophages polarization, arginase-1 (Arg-1) activation and reduce the inflammatory responses. M2 macrophages stimulation determines STAT3 activation, Arg-1 and IL-10 expression and inhibition of inflammatory responses after lipopolysaccharides and IFN-γ recognition. The results indicated the interplay between hASC-exos and macrophages, control over the progression of obesity, hepatic steatosis mitigation, regulation of homeostasis and increased insulin sensitivity (Zhao et al. 2018).

Different studies were employed in order to evaluate the immunomodulatory characteristics of hASC-exos. Among the immunomodulatory effects are the increased CD163, CD206, Arg-1, STAT6, MAF BZIP transcription factor B (MafB) expression, characteristics to M2 macrophages activation (Heo et al. 2019), increased neovascularization and reduced inflammation (Bai et al. 2018), decreased infiltration of mast cells and inflammatory dendritic cells (Shin et al. 2020) and low levels of TNF-α, IL-1β, IFN-γ expression (Patel et al. 2018).

On the other hand, Nojima et al., indicated that hASC-exos increased the proliferation of hepatocytes and supported the regeneration of the liver (Nojima et al. 2016a). The same group showed that EVs production is dependent on two chemokine receptors CXCR1 and CXCR2 (Nojima et al. 2016b).

4.1.1 Anti-inflammatory miRNAs Specific to hASCs-Exos and Their Effects

miRNAs contribute to inflammatory responses regulation and manage the immune cell phenotype (Ti et al. 2016). Ti et al. reported that regulation of macrophage polarization and the decrease of the inflammatory response are performed by miRNA-let7b through inhibition of TLR4/NF-kB pathway and STAT3/AKT signaling pathways activation (Ti et al. 2015).

Hutchison et al., studied the role of miR-181 in inflammation and tissue regeneration. Therefore they used an experimental neuroinflammatory model treated with LPS and concluded that miR-181 is important for astrocytes activation, anabolic metabolism in immune cells and to reduce inflammation during regeneration (Hutchison et al. 2013).

Zhao et al. researched the activity of miR-145 and miR-221 from hASC-exos used as treatment for bone regeneration. They observed that

miR-145 is involved in TNF-α mediated inflammation suppression and miR-221 is involved in proliferation and differentiation of periosteal cells, contributing to tissue regeneration (Zhao et al. 2020).

Withal, miR-126 was studied in myocardial cells. miR-126 secreted by hASC-exos has the ability to prevent inflammation, tissue damage and apoptosis and to contribute to decrease of myocardial ischemic injury and regeneration initiation (Luo et al. 2017).

Wang et al. indicated that miR-233 has suppressing influence over TNF-α, IL-1β, and IL-6 secretion. miR-223 intervenes at the level of the STAT3 signaling pathway, interacting with semaphorin (Sema) 3A and STAT3 in macrophages. Sema3A is involved in axon branching and synapse formation, but also in bone and cardiac development and regeneration processes and immune disorders (Wang et al. 2015).

M2 macrophages polarization is supported by miR-34a-5p, miR124-3p, miR135b-5p and miR146a-5p activity. An interesting observation was that M1 and M2 polarization depend on the miR-21-5p activity. Diversely, miR-34 is involved in pro-inflammatory cytokine inhibition by targeting Notch1 signaling pathway, miR-146 support the expression of the genes associated with M2 phenotype and targets interleukin-1 receptor-associated kinase 1 (IRAK1) and TNF receptor associated factor 6 (TRAF6) mediators of NF-kB signaling pathway (Domenis et al. 2018). Common to both inflammation and tissue damage is the production of miR-21 by exosomes. Caescu et al. noticed that M2 phenotype of macrophages is supported by colony-stimulating factor 1 receptor (CSF-1R) downregulation provoked by miR-21. They demonstrated that the knockdown of miR-21 in mice models, reduced the Arg-1, mannose receptor 1, IL-4 receptor α (IL-4Rα) and found in inflammatory zone (FIZZ) protein expression levels. MiR-21 supported the M2 macrophages responses through suppression of a MEK/ERK1/2 pathway activator, named SIRPb1 (Caescu et al. 2015).

4.2 hASC-Exos Effect on Cell Growth, Proliferation and Differentiation

Adult stem cells are quiescent cells that once prompted, they proliferate with the potential to differentiate into multiple cell types providing novel tools for cure and rehabilitation in regenerative medicine. Thus, the key to developing novel and effective therapies lies with the understanding of cell growth, proliferation and differentiation (Lambrou and Remboutsika 2014).

Many studies have described the involvement of hASC-exos in proliferation and migration processes through several mechanisms in different cell types, such as vascular endothelial cells, epithelial cells, fibroblasts, and cancer cells (Hong et al. 2019). Thus, it has been reported that the expression of some growth factors and receptors, such as platelet-derived growth factor subunit A (PDGFA), platelet-derived growth factor receptor (PDGFR), VEGFA, VEGF receptor 2 (VEGFR2), fibroblast growth factor 2 (FGF2), and hypoxia inducible factor 1 subunit alpha (HIF-1A) was increased by hASC-exos in endothelial cells. Moreover, hASC-exos upregulated PDGFA, VEGFA, VEGFR2, epidermal growth factor (EGF), and FGF2 in HaCAT cells and fibroblasts, thus contributing to the neovascularization of endothelial cells in wounds (Ren et al. 2019).

Several studies have shown that hASC-exos play an important role in wound healing through the Wnt/β-catenin (Ma et al. 2019) and PI3K/AKT signaling pathways (Yang et al. 2020). For example, it has been reported that hASC-exos stimulated proliferation and migration of HaCaT cells after their exposure to H_2O_2, by activating the Wnt/β-catenin pathway (Ma et al. 2019), and via exosomal miR-21, which increases MMP-9 and decreases TIMP-2 expression by regulating the PI3K/AKT pathway (Yang et al. 2020). Furthermore, it has been demonstrated that hASC-exos participate in the proliferation and tube formation of endothelial cells through the activation of the ERK and AKT signaling pathways, by increasing the phosphorylation of p-ERK1/2 and p-AKT (Hong et al. 2019).

Many studies have demonstrated that hASC-exos significantly promoted the proliferation of dermal fibroblasts during wound healing, by activating the PI3K/Akt signalling pathway, which intervenes in cell cycle progression and in the expression of growth factors, including TGF-β1 and bFGF (Zhang et al. 2018c). Human dermal fibroblasts (HDFs) could incorporate hASC-exos, which stimulated HDFs' migration and expression of long non-coding RNA MALAT1, which plays an important role in the regulation of different molecular signaling pathways and participates in cell cycle, proliferation, angiogenesis, migration, and tumorigenicity (Hong et al. 2019). Choi et al. (2018) reported that hASC-exos can promote proliferation of dermal fibroblasts and regeneration of skin fibroblasts through a series of miRNAs (miR-4,484, miR-619-5p, miR-6,879-5p), which can inhibit some aging-associated genes, including *npm1*, *pdcd4*, *ccl5* and *nup62*. Thus, hASC-exos secrete a number of growth factors and cytokines, which potentially affect cellular growth and proliferation and play important roles in the wound healing process (Zhang et al. 2018b).

The promotion of epithelial cells, endothelial cells, and fibroblasts proliferation by hASC-exos has been supported by upregulation of *cyclin A1*, *cyclin A2*, *cyclin D1*, *cyclin D2*, and partially supported by overexpression of *c-myc* gene, which are involved in cell cycle progression, being considered proliferative markers (Ren et al. 2019).

Zhang et al. (2018b) demonstrated that hASC-exos have the potential to increase the number of chondrocytes in the cartilage repair process, through a synergistic combination of increased proliferation, reduced apoptosis and enhanced recruitment while promoting matrix synthesis. They also observed exosome-mediated increases in the expression of genes associated with proliferation (proliferating cell nuclear antigen (*PCNA)* and *FGF-2*) and anti-apoptosis (*Survivin* and *Bcl-2*). hASCs derived exosomal lncRNA KLF3-AS1 transplantation could contribute to chondrocyte proliferation and inhibition of apoptosis via miR-206/GIT1 axis, thus facilitating cartilage repair. Therefore, cellular delivery of exosomal lncRNA KLF3-AS1 could be a possible mechanism for osteoarthritis (OA) therapy. hASC-exos treatment led to an increase in type II collagen alpha 1 (Col2A1) and aggrecan, which are essential for normal cartilage function, and a decrease in MMP-13 and runt-related transcription factor 2 (Runx2), that are involved in chondrocyte differentiation and cartilage degradation, thus indicating the potential role of hASC-exos in inhibiting chondrocytes degradation (Liu et al. 2018).

Li et al. examined the effects of hASC-exos on bone growth and they observed that hASC-exos combined with a poly lactic-co-glycolic acid (PLGA) scaffold led to rapid repair of critical-sized mouse skull defects. Besides, they reported that hASC-exos increased the proliferation and osteogenic differentiation of human BM-MSCs (Li et al. 2018a). hASC-exos also have an important role in treating many bone diseases such as osteoporosis, osteoarthritis etc. In this regard, it has been demonstrated that hASC-exos, under the influence of TNF-α, could induce the proliferation and differentiation of osteoblastic cells by stopping the Wnt signaling pathway (Maqsood et al. 2020).

Studies have shown that hASC-exos could increase neurite lengths via neural growth factors (nerve growth factor (NGF), glial cell-derived neurotrophic factor (GDNF), brain-derived neurotrophic factor (BDNF), fibroblast growth factor 1 (FGF-1), and IGF-1), and by overexpressing cyclin Ki67 in Schwann cell nuclei, thereby stimulating neurite outgrowth (Ching et al. 2018; Bucan et al. 2019; Hong et al. 2019). Moreover, it has been demonstrated that miR-222 from hASC-exos enhance Schwann cell proliferation and migration by targeting longevity assurance homologue 2 (LASS2) which suppresses cell growth, promotes neurite outgrowth with increased expression of PTEN, a known inhibitor of nerve regeneration. hASC-exos were shown to incorporate miR-222 and miR-21, with an increased expression noted upon differentiation (Ching et al. 2018). It has been reported that hASC-exos enhanced secretion of BDNF and NGF by Schwann cells, which led

to increased proliferation, myelination, and migration of cells in a dose-dependent manner. Also, it was reported that treatment with the hASC-exos improved muscle atrophy by promoting axonal regeneration and myelination, but exosomal components that exerted these effects remained unidentified (Chen et al. 2019b).

Studies have demonstrated that mice treated with hASC-exos exhibited decreased renal injury and increased proliferation of renal tubular epithelial cells, thus attenuating acute kidney injury (AKI). Also, treatment with hASC-exos led to upregulation of tubular *SOX9* gene expression, a key gene involved in renal repair and renal tubule epithelial cell regeneration, and inhibited TGF-β1-induced renal fibrosis (Zhu et al. 2017). In addition, Jin et al. introduced hASC-exos derived from rat into the iliac veins of rats in an acute liver disease model, thereby enhancing their survival rate at 72 h by over 70% compared to controls (Wong et al. 2019).

hASC-exos could increase the proliferation of the ovarian granule cells and inhibit their apoptosis through the regulation of SMAD signaling pathway, thus managing to restore ovarian function in premature ovarian failure (Huang et al. 2018).

Regarding the role of hASC-exos in cancer, it appears that hASC-exos have dual (or contradictory) functions in regulating tumorigenesis, both by promoting and inhibiting the growth of cancer cells (Shukla et al. 2020). For example, it has been shown that hASC-exos stimulated the proliferation and migration of the breast cancer cell line MCF7, through the activation of Wnt pathway and accumulation of Wnt target genes, including *Axin2* and *Dkk1* (Lin et al. 2013). In contrast, miR-122-transfected hASCs can generate miR-122 encapsulated exosomes to deliver miR-122 into hepatocellular tumor cells, which elevated tumor cell sensitivity to chemotherapeutic agents via gene expression alternations and tumor proliferation *in vitro* and *in vivo* (Vakhshiteh et al. 2019). Studies have shown that internalized hASCs-CM (conditioned medium)-derived exosomes inhibited the growth and proliferation of A2780 and SKOV-3 ovarian cancer cells, thus suggesting that the relationship between hASCs and cancer could be partially explained by the mechanisms involved in exosome activity, given that hASC-exos contain miRNAs, which could be involved in cancer suppression or progression (Reza et al. 2016). Besides, it has been demonstrated that hASC-exos could inhibit prostate cancer via delivery of miR-145 by reducing the activity of Bcl-xL and promoting apoptosis through the caspase-3/7 pathway (Vakhshiteh et al. 2019).

A study has reported that miR-503-3pf, released by adipocyte secreted-exos, may inhibit tumor growth regulating cancer stem cells (CSCs) proliferation and self-renewal, reducing the expression of pluripotency genes. Also, xenograft tumor growth is inhibited by the administration of miR-503-3p, supporting this miR as a stemness-attenuating factor via cell-to-cell communication (Gentile and Garcovich 2019).

Many miRNAs from hASC-exos are very important in proliferation and differentiation processes. For example, they are involved in cell cycle and proliferation (miR-191, miR-222, miR-21, let-7a), and in endothelial cells differentiation (miR-6087) (Gurunathan et al. 2019). Wang et al. have shown that exosomal miR-132 derived from hASCs/VEGF-C can be transferred directly to lymphatic endothelial cells (LECs), thus promoting LECs proliferation and migration, also lymphangiogenic response through the modulation of TGF-β/Smad signaling (Wang et al. 2018). Moreover, it has been demonstrated that in nasopharyngeal carcinoma, miR24-3p, miR-891a, miR106a-5p, miR-20a-5p, and miR-1908 from exosomes participate in cell proliferation and differentiation through down-regulation of the microtubule affinity regulating kinase 1 (MARK1) signaling pathway, while in lung cancer, exosomal miR-302b inhibits cell proliferation through the TGFβRII/ERK pathway (Gurunathan et al. 2019).

As a novel type of cell communication, exosomes have drawn much attention in cell differentiation. Several studies have demonstrated that exosomal miRNAs participated in multiple differentiation processes, such as osteogenesis, neurogenesis, myogenesis and angiogenesis, thereby indicating that miRNAs may be the

main mechanism for exosomes-mediated differentiation (Zhang et al. 2017). From another point of view, exosomes are mediators of communication between stem cells and mature tissue cells. Once the stem cells differentiate into mature cells, they in turn also induce stem cells towards differentiation through exosomes, thus a positive-feedback loop mechanism is formed during differentiation (Zhang et al. 2017).

It has been shown that hASC-exos are involved in cutaneous wound regeneration through extracellular matrix reconstruction. This process is accomplished by modulating the levels of collagen type III: type I, MMP3: TIMP1, TGF-β3: TGF-β1, and stopping the differentiation of fibroblasts into myofibroblasts to reduce scar formation (Wang et al. 2017).

Shen et al. have reported that hASC-exos-miR-19a could be used in the treatment of corneal fibrosis, because miR-19a suppressed fetal bovine serum (FBS)-induced differentiation of rabbit corneal keratocytes into myofibroblasts through the inhibition of homeodomain interacting protein kinase 2 (HIPK2) expression, which participate in the enhancement of p53 and Smad3 pathways in FBS-induced keratocytes (Shen et al. 2020).

Li et al. (2019) have demonstrated that the adipogenic differentiation process, which is involved in adipose tissue regeneration and certain problems related to obesity, can be regulated by an axis consisting of lncRNA H19, miR-30a, and C8orf4. It has been reported that adipogenesis could only be induced by adipose tissue (AT)-Exos, while hASC-exos had small effect (Hong et al. 2019). Thus, 14 miRNAs enriched in AT-Exos were previously reported to promote adipogenic differentiation of MSCs. For example, miR-450a-5p is a regulator of hASCs differentiation into adipocytes by downregulating WISP2 in hASCs. Moreover, a number of adipogenesis-related proteins were detected in hASC-exos including preadipocyte factor 1 (PREF-1), adenylate kinase 2 (AK2), peroxisome proliferator-activated receptor γ (PPARγ), and MMP-2, MMP-9 (Zhang et al. 2017).

Furthermore, hASC-exos promoted chondrogenesis in periosteal cells and increased chondrogenic markers, including collagen type II and β-catenin. Periosteal cells treated with exosomes exhibited higher levels of miR-145 and miR-221, which were associated with the enhanced proliferation of periosteal cells and chondrogenic potential (Zhao et al. 2020).

hASC-exos can regulate the Wnt signalling pathway and endocytosis, thus contributing to the modification of miRNAs profiles and promotion of osteogenic differentiation, according to the phase of differentiation (Gurunathan et al. 2019). Studies have indicated that miRNAs such as let-7a and miR-218 were up- or downregulated at different time points and the alteration was helpful to the osteogenic differentiation of hASCs (Li et al. 2018a). Moreover, it has been demonstrated that exosomes, from TNF-α-preconditioned hASCs (which is supposed to mimic the acute inflammatory phase following a bone injury), stimulated the osteogenic gene expression through the Wnt signaling pathway, which is a fundamental pathway in osteogenic differentiation. Also, the mir-130a-3p/SIRT7/Wnt axis could be a molecular mechanism underlying the effectiveness of exosomes in the modulation of hASCs osteogenic differentiation (Storti et al. 2019).

Studies have demonstrated that neurons-conditioned medium-derived exosomes contributed to differentiation of hASCs into neural cells (Zhang et al. 2017). Moreover, hASC-exos assist in neural differentiation by miR-124 delivering to neural precursor cells (NPCs). miR-124 suppresses *Sox9* expression, involved in NPC multipotent capacity and maintenance, hence the effect of miR-124 on *Sox9* promotes NPC differentiation (Reza-Zaldivar et al. 2018).

It has been shown that hASC-exos have cardioprotective roles through their paracrine effects (reduction in the levels of apoptotic proteins detected (e.g. Bcl-2-associated X protein (Bax)), increase in the levels of proteins Bcl-2 and Cyclin D1, activation of Wnt/β-catenin pathway) rather than the direct differentiation into cardiomyocytes (Shukla et al. 2020).

hASC-exos participate in the modulation of immune response through their paracrine activity. For example, hASC-exos inhibit the differentiation of CD4+ and CD8+ T cells into effector or

memory cells *in vitro* via anti-CD3/CD2/CD28 stimuli (Hong et al. 2019).

4.3 hASC-Exos Involvement in Angiogenesis

Angiogenesis implies the formation of new blood vessel such as capillaries starting from a preexisting vascular network. This could take place either by the phenomenon of endothelial cells sprouting from old blood vessels or by inserting tissue pillars within already existing vessels in order to split them. In regenerative medicine, angiogenesis is very important for generating functional tissues (Rouwkema and Khademhosseini 2016). It is essential mainly in cases of ischemic diseases when damaged tissues need supply with oxygen and nutrients. Since sometimes stem cell therapy could pose ethical impediments or biological limitation (immune rejection/tumorigenicity), stem cell EVs have emerged as intensively exploited alternative to promote angiogenesis (Bian et al. 2019).

hASC-exos, as being carriers for different types of molecules, especially ncRNA species, can work as stimuli to promote angiogenesis of endothelial cells. Human umbilical vein endothelial cells (HUVEC) are widely used to study angiogenic properties of hASC secretome (Liang et al. 2016; Han et al. 2019; An et al. 2019). hASC-exos can be absorbed by HUVECs, determining higher proliferation rates and tubular-*like* structures formation, both *in vitro* and *in vivo* in BALB/c male nude mice, along with augmented expression of VEGF (Zhang et al. 2018a). Two models, HUVECs and a nude mouse model of subcutaneous fat grafting, were used by Han et al. (2019) to identify the molecular mechanism underlying angiogenic properties of exosomes from hypoxia-treated hASCs. These hASC-exos had elevated levels of angiogenic proteins, like VEGF, VEGF-R, FGF, EGF, MCP-2 and MCP-4. *In vivo,* neovascularization is most probably mediated by VEGF/VEGF-R signaling, as their expression is also increased in grafted tissues. Xu et al. (2019) identified miR-423-5p as a mediator delivered by hASC-exos to HUVEC to promote angiogenesis. Sufu protein is targeted by miR-423-5p which downregulates its expression in HUVEC cells with increased proliferation and tube forming capabilities. miR-125a is another angiogenesis' regulatory molecule delivered by hASC-exos to HUVECs. Its target is represented by the angiogenic inhibitor delta-*like* 4 (DDL4), whose protein expression levels are decreased after hASC-exos internalization (Liang et al. 2016).

In ulcerative injuries, hASC-exos overexpressing nuclear factor-E2-related factor 2 (Nrf2) transcription factor increased cutaneous wound healing by promoting vascularization. hASC-exos were capable to reduce glucose-induced senescence of endothelial progenitor cells (EPCs). Nrf2 inhibit ROS formation and cytokine expression in EPCs, while treatment with EPC and hASC-exos resulted in improved vessel density in a diabetic rat model (Li et al. 2018b). *In vitro*, EPCs' migration was also improved by exosomes derived from hASC overexpressing miR-126. Treatment with these exosomes promoted angiogenesis *in vivo*, showing protective effects in acute myocardial ischemic injury in a rat model (Luo et al. 2017). hASC-exos could improve vascular remodeling processes after stroke in rats. Positive effects were reported towards brain microvascular endothelial cells' (BMEC) migration and angiogenesis, through miR-181b-5p/TRPM7 axis, after oxygen-glucose deprivation. Transient receptor potential cation channel subfamily M member 7 (TRPM7) is directly targeted by miR-181b-5p, decreasing its expression in BMEC. Furthermore, hASC-exos expressing miR-181b-5p regulated other protein expressions, upregulating VEGF, hypoxia- inducible factor 1α and downregulating TIMP-3 (Yang et al. 2018). In mice frat graft retention, hASC-exo manifested increased angiogenesis similar to their cell source, meaning they could be effectively used in cell-free therapies in regenerative medicine (Chen et al. 2019a). Another strategy is proposed by Du et al. (2018), who transfected hASC with a miR-199-3p mimic to assess exosomes effects on angiogenesis. Thus, exosomes derived from those modified hASCs promoted migration and

proliferation of human peripheral blood mononuclear cells (endothelial tip cells). miR-199-3p acted by downregulating expression of Sema3 protein. Additionally, there was also increased expression of MMP9, PCNA and a decreased expression of TIMP3.

4.4 hASC-Exos Influence on Matrix Remodeling

As part of the wound healing process, matrix remodeling represents the last stage and it is typically associated with the extent of scar formation. This mainly depends on ECM production and reorganization, processes that are targeted as a way to reduce excessive scarring (Hong et al. 2019; Qiu et al. 2020). Even though hASC-exos main impact on wound healing is focused on the first two stages, inflammation and proliferation, there are some studies that investigate the positive effect of hASC-exos on matrix remodeling.

Hu et al. (2016) stimulated dermal fibroblasts with hASC-exos and observed that collagen and elastin synthesis is promoted during the first steps, but then it is inhibited in the late stages to reduce scar formation. A more complex study was conducted by Wang et al. (2017) on mice with full-thickness dorsal wounds, that received hASC-exos intravenously and were investigated over 21 days. hASC-exos induced a reticular pattern of collagen that resembled the surrounding unwounded skin and the ratio of collagen III to collagen I was increased compared to control group. In addition, after 14 days of hASC-exos treatment, fibroblasts were inhibited to differentiate to myofibroblasts, which are responsible for granulation tissue formation. Furthermore, the study comprised *in vitro* results as well, on dermal fibroblasts treated with hASC-exos. Remodeling of ECM was regulated by hASC-exos by activating ERK/MAPK pathway and increased the ratio of MMP3 to TIMP1. TGF-β isoforms play an important role in scar formation, and more importantly TGF-β1/3. TGF-β1 promotes fibroblasts to myofibroblasts differentiation and granulation tissue formation, while TGF-β3 is involved in inhibiting myofibrobast differentiation and reduces scar formation. In this study, the collagen III to collagen I and TGF-β3 to TGF-β1 ratios were also increased that helped to reduce excessive scarring. Another study by Zhang et al. (2018c), showed that human dermal fibroblasts stimulated with hASC-exos, promoted collagen synthesis by activating the PI3K/Akt signaling pathway. Collagen III to collagen I ratio was also increased by hASC-exos, by approximatively 50%, positively influencing the extent of scar tissue.

In a recent study, Yang et al. (2020) correlated the highly expressed miR-21 in hASC-exos with increased MMP-9 expression and reduced TIMP2 expression through PI3K-AKT pathway. Moreover, mirR-21 from hASC-exos specifically targeted TGF-β1 in HaCaT cells, reducing its expression and therefore having a positive impact on scar formation.

4.5 Anti-apoptotic Signals Mediated by hASC-Exos

Among other biological behaviors, it was found that hASC-exos contain signals that exhibit anti-apoptotic effects in pathological conditions. For example, during ischemic reperfusion injuries, oxidative stress and inflammation lead to apoptosis of myocardial cells which will further decrease cardiac function. Liu et al. (2020) found that treatment with hASC-exos reduced oxidative stress-induced apoptosis in myocardial cells *in vitro*, thus demonstrating their exosomes protective effect against cardiac affections. Evidence indicate that hASC-exos inhibit the apoptotic process, by transfer of several proteins and miRNAs (Hong et al. 2019). Also, in case of HaCaT cells exposed to H_2O_2 it was demonstrated that hASC-exos managed to enhance cell proliferation and stopped apoptosis via increasing the expression of anti-apoptotic protein Bcl-2 and inhibiting the expression of pro-apoptotic protein Bax (Ma et al. 2019). Furthermore, the same outcomes were confirmed in ischemia-reperfusion injuries where treatments with hASC-exos decreased apoptotic biomarkers such as Bax, caspase 3 and poly ADP-ribose polymerase (Lin et al. 2016; Bai et al.

2018). Additionally, the effect of hASC-exos has been also studied in case of peripheral nerve injuries models. Liu et al. (2019) elaborated a study where they induced sciatic nerve injuries in rats. They harvested and purified Schwann cells and analyzed proliferation and apoptosis of these cells post-injury. They treated Schwann cells cultures with various hASC-exos concentrations and the results indicated that the proliferation rate of cells increased significantly in a concentration-dependent manner. Flow cytometry analysis and immunofluorescence staining also indicated a significant decrease in apoptotic cells for the Schwann cells treated with hASC-exos. Last, qPCR results demonstrated that exosomes upregulated Bcl-2 anti-apoptotic expression and downregulated the pro-apoptotic factor, Bax. To sum up, there is clear evidence that hASC-exos have an impressive control over the apoptotic process and present tremendous potential for therapeutic use against serious affections.

5 Conclusions

Human adipose derived stem cells show great potential for regenerative medicine applications, mediated by the molecules produced in their rich secretome. While chemokines, cytokines and protein growth factors in hASCs secretome have been widely studied for their properties, the EVs secreted by hASCs and the effect of their content on the regenerative process and its phases is just starting to be explored. There is clear evidence that hASC-exos can be used as a cell-free therapy in patients in order to promote regenerative processes such as wound healing, extracellular matrix remodeling, immunomodulation, angiogenesis, anti-apoptotic activity and cell migration, proliferation and differentiation. hASC-exos are able to modulate signaling pathways such as Wnt-β-catenin, PI3K-Akt, MAPK/ERK and others involved in tissue regeneration. Therefore, one of the future targets in tissue engineering and regenerative medicine would be to explore the properties of hASC-exos in order to activate the first steps of tissue regeneration more efficiently than the currently used methods and to provide a personalized alternative for regenerative applications.

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A Museum of Stem Cells Points to Muse Cells as Robust Transplantable Cells for Stroke: Review

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Abstract

Stem cell-based therapy stands as a robust experimental treatment for ischemic stroke. Stem cells derived from fetal, embryonic, and adult tissues serve as potential sources for transplantable cells in the setting of ischemic stroke. However, the search continues for finding an optimal cell line for clinical use. Muse cells, a distinct subset of mesenchymal stem cells found sporadically in the connective tissue of nearly every organ, may be a suitable candidate due to its safety and accessibility. These cells have been investigated for therapeutic usage in chronic kidney disease, liver disease, acute myocardial infarction, and stroke. Muse cells display the ability to engraft and differentiate into the host neural network unlike many other cell lines which only display bystander immunomodulating effects. Taking advantage of this unique engraftment and differentiation mechanism behind Muse cells' therapeutic effects on the central nervous system, as well as other organ systems, will undoubtedly advance the cells' utility for cell-based regenerative medicine in stroke.

Keywords

Stem cells · Stroke · Transplantation · Regenerative medicine · Brain repair

1 Introduction

Stroke is currently the fifth leading cause of death in the United States and can cause disabling neurological deficits including cognitive impairment, hemiparesis, sensory disturbance, and aphasia (Ovbiagele et al. 2013). Ischemic stroke comprises 87% of all stroke cases and is characterized by inadequate perfusion to vital organs like the brain, leading to oxygen and nutrient deprivation and eventually cell death (Benjamin et al. 2019; Sacco et al. 2013). The ischemic cascade following stroke is divided into three phases. The acute phase occurs within the first few hours after the ischemic event. Blood flow, ATP, and energy stores in the affected brain tissue plummet, causing ionic disruption and metabolic failure. The ensuing ionic imbalance and neurotransmitter release (glutamate excitotoxicity) promotes an excess influx of sodium and calcium into the cell. Increased intracellular calcium activates downstream phospholipases and proteases that degrade

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integral membrane components and proteins, while the surplus of sodium leads to cellular swelling (Lo et al. 2003). In addition, the production of oxygen free radicals and other reactive oxygen species during the acute phase causes further damage and cell death (Hao et al. 2014; Lakhan et al. 2009). The subacute phase occurs after the acute phase and lasts for the first few days after the ischemic event. During this phase, injured cerebral tissue releases cytokines, chemokines, cellular adhesion molecules (CAMs), and matrix metalloproteases (MMPs) (Lo et al. 2003; Stonesifer et al. 2017). The release of MMPs and immune cell modulators increases the permeability of the blood-brain barrier (BBB), allowing peripheral leukocytes to infiltrate and upregulate the inflammatory process (Hao et al. 2014; Stonesifer et al. 2017). However, neuroinflammation is a self-regulated process and eventually subsides to prepare for structural and functional reorganization (Iadecola and Anrather 2011). Thus, in the transition from the subacute to the chronic phase, inflammation resolves and tissue repair begins, but such endogenous regenerative process is not sufficient to confer functional recovery in stroke patients. Although the mechanism behind the reestablishment of homeostasis is still poorly understood, evidence suggests that it is orchestrated by mediators that suppress the inflammatory response. Major steps include the removal of dead cells and the introduction of exogenous treatments designed to deliver anti-inflammatory and pro-survival factors that promote tissue reconstruction and repair (Iadecola and Anrather 2011; Nathan and Ding 2010).

The complex regulation of the ischemic cascade changes the neural, vascular, and connective tissues in the affected areas of the brain (Krause et al. 2019). These changes and subsequent neurological deficits can persist long after the stroke itself and prevent patients from fully reintegrating into society. With the country's aging population, the number of yearly cases is expected to increase. Projects indicate that 3.88% of the US population over the age of 18 will have a stroke by 2030 and the total annual stroke-related costs are expected to reach $240.67 billion (Ovbiagele et al. 2013). There are only two approved acute treatment options currently available – tissue plasminogen activator (tPA) and endovascular thrombectomy – despite the American Heath Association and American Stroke Associations' emphasis on implementing effective acute and chronic stroke care. Unfortunately, their use is limited by short therapeutic time windows and risks of additional damage. Although rehabilitation is an option for chronic stroke care, functional recovery remains modest. With the central nervous system's (CNS) limited capability to recover after injury, treatments to regenerate neural cells is an unmet need.

In 1988, Sharp et al. described the first successful cell transplant in animal models of ischemic brain injury using rat fetal neocortical cells (Mampalam et al. 1988). Studies that followed illustrated the ability of the grafted cells to integrate with the injured host brain and receive afferent fibers and vascularization (Grabowski et al. 1992a, b). Since these discoveries, the fields of stem-cell therapy and regenerative medicine have amounted impressive preclinical evidence of stem cell transplantation's restorative effects on disorders of the CNS including ischemic stroke (Lindvall and Kokaia 2006; Song et al. 2018). However, evidence for the donor cells' survival, differentiation, and functional integration in the host brain have repeatedly failed to translate in human clinical trials (Kondziolka et al. 2000, 2005; Savitz et al. 2005). As the search continues for cell source targeted for ischemic brain injury, it is important to keep in mind that the degree of repair depends primarily on the selection of appropriate cell types for transplantation. Embryonic stem cells (ES) and adult tissue-derived stem cells have unique characteristics that determine their specific responses to stroke. The following sections provide a concise overview of different stem cell types and their potential value in targeted stroke therapy.

2 Identifying the Optimal Cell Type for Stem Cell Transplantation

2.1 Embryonic Stem Cells

ES cells are derived from the embryonic inner cell mass (ICM) prior to the 5th day of development post-fertilization. These pluripotent cells can

replicate indefinitely and differentiate into any cell type in the body. ES are isolated from the surrounding embryo by fine-needle aspiration, laser dissection, or by growing the ICM on the surface of feeder cells (Lee and Lee 2011). After purifying the cell-isolate, ES cells can be grown and maintained *in vitro* until they are ready for transplantation. In the context of targeted stroke therapy, ES develop into neuronal progenitor cells to assist in repairing damaged neurons and brain tissues. In addition, they promote angiogenesis, release neurotrophic factors such as erythropoietin, and upregulate neuroprotective factors such as BcL-2 (Liu et al. 2014). However, ethical concerns surrounding the destruction of embryos and high risk of tumorigenicity severely limit the use of ES cells in clinical applications (Stonesifer et al. 2017).

After implantation of the embryo on the 5th day of development, the ES cells of the ICM begin to permanently differentiate into more specialized cells and are no longer pluripotent. These new, more differentiated stem cells are multipotent and still have a strong capacity to self-renew but can only give rise to cells of one lineage. The ES cells eventually disappear completely, and the 'adult' multipotent stem cells are responsible for maintaining adult tissues. There are several key adult-tissue derived stem cells that may be beneficial in the post-stroke care of patients. Neural stem cells (NSCs) directly differentiate into the various neuronal cell types to expedite recovery (Zhao and Moore 2018). Induced pluripotent stem cells (iPSCs) are a recently discovered source of autologous ES-like cells. Extraembryonic, adipose, and dental-derived stem cells also improve stroke outcomes. Next, we will discuss bone-marrow derived stem cells, in particular mesenchymal stem cells (MSCs). Finally, we focus on Multilineage-differentiating stress enduring (Muse) cells which are primarily derived from bone marrow, but subsequently harvested in other tissues, such as adipose and umbilical cord.

2.2 Neural Stem Cells

NSCs form the entire central nervous system (CNS) by differentiating into neurons, astrocytes, and oligodendrocytes (Okano and Temple 2009). However, many NSCs terminally differentiate once neural development is complete, leaving only a small population in the subventricular zone (SVZ) and subgranular zone (SGZ) (Kempermann et al. 2015). The markedly reduced quantity of these stem cells limits the brain's ability to renew itself after injury. Harvesting techniques using needle-aspiration or biopsy are dangerous, and while newer techniques such as magnetic isolation may be safer, they are still constrained by the scarcity of NSCs. Nevertheless, NSCs remain a prime candidate for stroke-therapy because hypoxia and injury stimulate these cells to migrate from the SVZ and SGZ to damaged tissue where they promote angiogenesis, neurogenesis, and secretion of various neuroprotective factors (Santilli et al. 2010; Zhang et al. 2014). These effects are most pronounced if NSC transplantation is autologous and administered within 72 h of insult (Chen et al. 2016). However, maintaining a premade store of autologous NSCs is impractical given the difficulty of obtaining these cells.

2.3 Induced Pluripotent Stem Cells

Unlike the other stem cell types, iPSCs are not normally present throughout development. Cellular differentiation is naturally a unidirectional process; however, scientific advancements have allowed researchers to reverse cell development such that stem cells can be artificially generated from terminally differentiated somatic cells like fibroblasts and blood cells. Exposing the cell to specialized genes and signals reprograms adult cells to become embryonic-like iPSCs capable of asymmetric division. The major advantage to using iPSCs is that adult cells can be easily harvested from any tissue source, converted into stem cells, then induced to become nearly any other type of cell, including T-regulatory cells, microglia, and other neural cell types. Like traditional stem cells, iPSCs reduce infarct size and modulate the immune system to create more suitable environments for recovery (Zents and Copray 2016). Despite the numerous benefits of iPSCs, their value is offset by two major flaws. First, although autologous in nature, they may

still be rejected by the host (Zhao et al. 2011). Second, iPSCs have the highest tumorigenicity of any of the studied stem cell types (Liang et al. 2013).

2.4 Other Sources of Adult Stem Cells

Bone marrow, adipose and extraembryonic tissues (e.g., umbilical cord, placenta) are two sources of mesenchymal stem cells (MSCs). Adipose tissue is a type of loose connective tissue composed primarily of adipocytes. Adipose tissue-derived mesenchymal stem cells (AD-MSCs) are acquired by enzymatically digesting fat samples obtained from fat suctioning or excision. Transplantation with AD-MSCs improves neurological recovery, decreases the size of the infarct, and reduces inflammation (Gutiérrez-Fernández et al. 2013). Furthermore, treatment is very accessible due to the high prevalence of adipose tissue and the ability to administer treatment intravascularly with encouraging results (Gutiérrez-Fernández et al. 2013). However, treatment with AD-MSCs is diminished by its' propensity to cause cancer cells to rapidly proliferate (Eterno et al. 2014).

While adipose tissue is plentiful, extraembryonic tissues like the umbilical cord and placenta are not. The umbilical cord arises from the placenta during gestation and together these organs connect the circulatory systems of the mother and fetus. As they are both shed after delivery, stem cells can be easily harvested from them (Gutiérrez-Fernández et al. 2013; Shinozuka et al. 2013). Tissue injury induces extraembryonic tissue-derived MSCs to inhibit immune cell migration, increase angiogenesis and neurogenesis, and potentially preserve neuroplasticity (Shinozuka et al. 2013). Despite the promising potential for neurological repair, extraembryonic stem cell use is constrained by the availability of placentas or umbilical cords to harvest cells from (Stonesifer et al. 2017).

Other sources of adult-tissue derived stem cells include breastmilk, menstrual blood, and dental tissue. Preliminary stroke models have shown that breast milk and menstrual blood-derived stem cells may also have beneficial effects, but few studies have investigated this thoroughly enough to warrant their consideration as a transplantation source (Stonesifer et al. 2017). Similarly, dental tissue-derived stem cells have been shown to preserve neurological function post-stroke, but their utility is minimized by the higher availability of other tissue sources with comparable outcomes (Stonesifer et al. 2017).

2.5 Bone Marrow-Derived Stem Cells

Because of its long track record of safety as graft source for hematologic diseases, the bone marrow has been extensively studied for stem cell therapy in stroke. Bone-marrow is a highly active spongy tissue that produces billions of new cells each day (Higgins 2015). There are four key multipotent cells that accomplish this extraordinary feat: hematopoietic stem cells (HSCs), endothelial stem cells (ESCs), very small embryonic-like stem cells (VSELs), and MSCs. This diversity in cell type makes bone-marrow an attractive target for stem cell harvesting. Typically, marrow is extracted from the iliac crest of anesthetized patients using needle-aspiration and is cryopreserved until it is ready for purification (Gorin 2019). These cells can then be isolated and transplanted into stroke patients, with each stem cell type having different effects. We will briefly consider HSCs, ESCs, and VSELs before discussing MSCs in detail.

HSCs develop into all the different types of blood cells in the body. In response to stroke and hypoxia, they preferentially differentiate through the myeloid lineage, which may be important in resolving the hypoxic environment (Felfly et al. 2010). The beneficial effects of HSC are limited by its tendency to promote inflammation, thereby delaying and possibly diminishing recovery (Kasahara et al. 2016).

EPCs are a potentially valuable transplant source due to their ability to repair the blood-brain barrier and brain vasculature, which are often compromised during or prior to the onset

of stroke (Stonesifer et al. 2017). Damage to the blood-brain barrier allows inflammatory cells from the systemic circulation to migrate into the site of injury, leading to inflammation and even more damage. The strong angiogenic properties not only result in increased vessel density and reduced quantity of apoptotic cells, but also provide mild anti-inflammatory effects by limiting inappropriate immune cell access to the brain (Chen et al. 2008). Nevertheless, the usefulness of EPCs is limited by the difficulty in producing purified cell cultures.

VSELs are present in both the brain tissue and the blood in low quantities. They have excellent potential for stroke treatment due to their ability to differentiate into neurons, microglia, and oligodendrocytes (Hsiao et al. 2014). However, like EPCs, they are very difficult to harvest in clinically relevant numbers.

2.6 Mesenchymal Stem Cells

MSCs were originally isolated from bone marrow but have been harvested from multiple tissues including the umbilical cord, amniotic fluid, placenta, and adipose tissue (Friedenstein et al. 1966; McElreavey et al. 1991; Zuk et al. 2002). MSCs have been found to have a high potential for regeneration while maintaining multipotency. These cells exhibit plastic adherence, have the ability to self-renew, and exhibit a specific set of cell surface markers, such as cluster of differentiation (CD)73, CD90, and CD105, while lacking expression of CD14, CD34, CD45, and human leukocyte antigen-DR (HLA-DR) (Mushahary et al. 2018). MSCs have the ability to differentiate into mesodermal cells such as adipocytes, chondrocytes, myocytes, and osteocytes (Ullah et al. 2015). MSCs express various growth factors that are proven to facilitate tissue repair and maintain homeostasis within the immune system (Ma et al. 2014). MSCs therapeutic potential allows for the treatment of chronic diseases including Parkinson's disease, Alzheimer disease, and Type 1 diabetes because of their ability to secrete anti-inflammatory molecules and immunoregulatory effects (Ullah et al. 2015). MSCs can interact with cells of the innate and adaptive immune system to control effector functions (Li and Hua 2017). The mechanism of MSCs involves the migration to injured tissues through specific target pathways where they inhibit the release of pro-inflammatory cytokines and help promote the survival and growth of the damaged cells.

MSCs have been used *in vitro* to expand the cells and differentiate into specific cell lineages. Cultured MSCs have been shown to modulate immune responses and reroute the progression of inflammatory diseases (Ma et al. 2014). As tissue injuries correspond with inflammation, MSCs can effectively mobilize to damaged tissue sites. Their mechanism of action involves modulating inflammatory processes and releasing growth factors to facilitate tissue repair (Ma et al. 2014). MSCs contain immunomodulatory features and secrete cytokines and immune receptors to maintain homeostasis and regulate the environment in the host tissue. Their multilineage potential and secretion of anti-inflammatory molecules make MSCs an effective treatment for chronic diseases (Ullah et al. 2015). When MSCs migrated to the site of damaged tissue, cytokines, toxins of infectious agents, and hypoxia allow for the release of growth factors that promote the development of fibroblasts, endothelial cells, and tissue progenitor cells which carry out tissue regeneration and repair (Ma et al. 2014). MSCs are useful for treatment of chronic diseases due to their functions in inflammatory niches but also immunomodulatory properties. The immunosuppressive functions of MSCs are triggered by the environment of the cells and allows for the release of inflammatory factors.

The therapeutic effects of MSCs allow for the cells to work in action with immune cells, stromal cells, and endothelial cells to promote tissue repair. In vitro, MSCs have the ability to differentiate into all the three lineages: ectoderm, mesoderm, and endoderm and act as a potential source for stem cell therapy for ischemic stroke (Ullah et al. 2015) (Kondziolka et al. 2005). The ability of MSCs to differentiate into several different types of tissues and expansive properties allows

them to be used in stem cell-based therapies. (Mushahary et al. 2018)The use of MSCs to protect against ischemia/reperfusion, however, is influenced by the culture conditions that influence function and depends on how the MSCs are administered and expanded *in vitro* (Ma et al. 2014). In studies of ischemic stroke, MSCs are able to modulate an immune response and act neuroprotective, through stimulation of neurogenesis, oligodendrogenesis, astrogenesis, and angiogenesis (Dabrowska et al. 2019). MSCs derived from bone marrow are commonly used due to the secretion of neurotropic factors which help to stimulate cerebral repair processes. The use of MSCs demonstrates the ability to promote cell survival and modulate the immune response, however, *in vivo* studies indicate that MSCs do not functionally replace the injured cells and do not serve as a promising stem cell therapy to regenerate the injured neurons after an ischemic stroke.

2.7 Multilineage-Differentiating Stress Enduring (Muse) Cells

Reported in 2010 by Kuroda et al., Multilineage-differentiating stress enduring (Muse) cells are a subset of endogenous regenerative MSCs that reside in the peripheral blood and connective tissue of nearly all organs (Wakao et al. 2018). They are also found in mesenchymal tissues but are hypothesized to originate in the bone marrow where they make up ~0.03% of the mononucleated cell fraction (Tanaka et al. 2018). These cells possess the ability to self-renew, exhibit triploblastic differentiation, and regenerate a plethora of tissues when administered topically or intravenously. A small concentration is also present in peripheral blood, 0.01–0.2% of the mononucleated cell fraction, however this number may increase during injury or disease due to activation via stress (Tanaka et al. 2018; Wakao et al. 2014). Muse cells may be isolated and distinguished using the marker SSEA-3 (Wakao et al. 2011). Muse cells also reside in extraembryonic tissues such as the umbilical cord making them distinct from other somatic cells (Leng et al. 2019). Muse cells' unique regenerative capacities could provide a feasible treatment for many diseases.

When compared to MSC's, Muse cells have demonstrated the potential to fully engraft into the site of injury and replenish dead or ischemic tissue *in vivo* (Kuroda et al. 2018; Hu and Longaker 2017; Minatoguchi et al. 2018; Nishina et al. 2018; Uchida et al. 2018). In terms of ischemic stroke, MSC's have shown the ability to regenerate damaged tissue *in vitro*, however *in vivo* models have not indicated a full incorporation into area of infarct (Ikegame et al. 2011). Although attenuation of post-stroke inflammation was visible *in vivo,* it is plausible that this is due to stimulation of MSC secretome inducing endogenous paracrine-mediated brain regeneration pathways (Dabrowska et al. 2019; Leong et al. 2012; Ishizaka et al. 2013; Doeppner et al. 2015). Muse cells have demonstrated paracrine characteristics alike those of MSC's as well as the ability to travel and reside at injured sites (Tanaka et al. 2018). The regenerative capacities of Muse cells may provide a more beneficial cell-based therapy to treat ischemic stroke and other diseases when compared to MSC's, however further investigation is necessary.

The pluripotency exhibited by Muse cells allows for Muse-cell based therapy to treat a wide range of diseases such as myocardial infarct (MI), stroke, chronic kidney disease, liver disease, chronic skin wounds, and soft tissue defects (Kuroda et al. 2018; Hu and Longaker 2017; Minatoguchi et al. 2018; Nishina et al. 2018; Uchida et al. 2018). These cells are non-tumorigenic and exhibit low telomerase activity making them a great candidate for cell-based therapy (Tanaka et al. 2018). Many of these diseases do not have a standard treatment besides end-stage transplantation, and a regenerative cell-based therapy may be a possible avenue to treat these diseases.

Endogenous Muse cells have shown to play an important role in the acute phase of MI. Acute Myocardial Infarct (AMI) patients with a higher concentration of endogenous Muse cells in the peripheral blood have shown greater progress in cardiac remodeling, cardiomyocyte regeneration, and cardiac function during chronic phase. Upon

intravenous administration of exogenous allogenic Muse cells, AMI rabbit model exhibited a significant decrease in myocardial infarct size, a 6-month cardiac remodeling phase, and improved cardiac function over a long period of time without immunosuppressant treatment (Uchida et al. 2017). Muse-cell based therapy could also potentially be beneficial in treating patients suffering from an acute myocardial infarct (Minatoguchi et al. 2018). In a recent murine stroke model, cultured human bone marrow-derived Muse cells were administered to 2 weeks post-lacunar infarction. Transplantation during the subacute phase resulted in differentiation into neurons and oligodendrocytes, promoted neuronal reconstruction and improved overall brain function (Minatoguchi et al. 2018). Muse cells have demonstrated positive results both *in vitro* and *in vivo* to treat chronic liver disease (Ogura et al. 2014). Cell-based therapies using bone-marrow derived stem cells and peripheral blood-derived stem cells did not display efficacy in clinical trials when treating chronic liver disease (Ogura et al. 2014). Muse cells derived from human bone marrow were intravenously administered to immunodeficient mice with liver fibrosis. Spontaneous differentiation of the Muse cells into tissue compatible cells was exhibited as well as homing at the site of injury in the liver (Ogura et al. 2014). Chronic kidney disease may also utilize a Muse cell-based therapy (Uchida et al. 2018). There are many underlying causes of renal dysfunction and a cell-based therapy is needed as alternative to dialysis and transplantation. A rodent model of chronic kidney disease indicated differentiation at the site of injury of intravenously administered Muse cells into glomerular cells as well as improvement in renal function (Uchida et al. 2018). Chronic wounds and soft tissue defects have shown favorable improvement when treated with exogenous Muse cells. Research has shown that through differentiation of Muse cells into fibroblasts, keratinocytes and melanocytes, Muse cells may be an avenue of therapy for skin reconstruction (Hu and Longaker 2017). Muse cells exhibit regenerative characteristics that make them a potential candidate as a cell-based therapy for many diseases. The non-tumorigenic, pluripotent and regenerative abilities of these cells warrant their potential as therapy.

The ability of Muse cells to regenerate tissues have been exhibited in the brain, kidneys, liver, heart, and skin (Kuroda et al. 2018; Hu and Longaker 2017; Minatoguchi et al. 2018; Nishina et al. 2018; Uchida et al. 2018). Exogenous and endogenous Muse cells migrate to the injury through the peripheral blood, and home to the damaged host tissue site through the sphingosine-1-phosphate (S1P)-S1P receptor 2 (S1PR2) system (Yamada et al. 2018). Muse cells are able to survive the harsh environment at the target site due to their high stress tolerance (Alessio et al. 2018). Muse cells also possess immunomodulatory abilities allowing them to evade host immune cells at the injury site. Muse cells integrate into the site of injury and spontaneously differentiate into tissue compatible cells. In addition to differentiation at the site of injury, Muse cells were also found to exhibit paracrine characteristics through secretion of therapeutic factors such as hepatocyte growth factor, stem cell-derived factor 1, and epidermal growth factor that promoted functional recovery in tissue injuries (Tanaka et al. 2018). In the case of an ischemic stroke rodent model, 2–-3 months after Muse cell transplantation the Muse cells had formed synapses with host neurons as well as integrated axons into the pyramidal tract (Uchida et al. 2016). Both of these findings resulted in improved motor function and somatosensory evoked potential. Findings indicate that Muse cells could potentially be used as a regenerative stem cell therapy for many diseases however further elucidation is necessary.

3 Preclinical Studies on Muse Cells

There have been several recent *in vitro* and *in vivo* studies exploring the efficacy of Muse cells in treating ischemic injury (Leng et al. 2019; Uchida et al. 2017; Yamada et al. 2018; Alessio et al. 2018). Preclinical studies showed that Muse cells migrate to the site of injury, incorporate

into peri-infarct tissue, and differentiate spontaneously into cells that are congruous with injured tissue (Leng et al. 2019). In addition, Muse cells derived from adipose tissue have been shown to have anti-inflammatory activities, decreasing the secretion of cytokines, such as interferon-γ, which indicates their potential efficacy in ameliorating post-ischemic neuroinflammation (Uchida et al. 2016). When comparing Muse cells to non-Muse cells (cells other than Muse cells in MSCs), Muse cells differentiate into neurons and oligodendrocytes, remain integrated in the peri-infarct while non-Muse cells release therapeutic factors but do not replace ischemic cells (Dezawa et al. 2019). However, before the clinical application of Muse cells can be considered, the optimal timing, dosage, and means of delivery need to be further investigated.

In order to establish the optimal timing for Muse cell stroke therapy, preclinical trials investigating the differential effects of acute, subacute, and chronic delivery need to be examined. In a recent study, human fibroblast-derived Muse cells were transplanted stereotaxically into three regions near the ischemic cortex 2 days after the middle cerebral artery occlusion (MCAO). The Muse cells remained in the rat's brain for 84 days. Substantial amelioration in neurological and motor performance was observed after more than 84 days (Alessio et al. 2018). Another study found that immunodeficient MCAO rat models showed recovery 35 days after acute transplantation of Muse cells (Dezawa et al. 2019). In an immunodeficient lacunar infarction mouse model, human bone marrow-derived Muse cells were transplanted into the site of the peri-lesion at the subacute stage of lacunar infarction (Uchida et al. 2017). At 56 days post transplantation, the Muse cells differentiated into NeuN, MAP 2 expressing neurons and GST-pi expressing oligodendrocytes, and the mice with the Muse cell transplantation showed substantial improvement in neurological function (Uchida et al. 2017). The cylinder test in a different study fetal porcine cells found that neurological and motor recovery were not significantly different between immunodeficient lacunar mice given either subacute treatment or chronic treatment (Abe et al. 2020). Moreover, the recovery time for ischemic animal models varies between acute and subacute treatment but may not differ between subacute and chronic delivery (Abe et al. 2020). While delivering Muse cells in the acute phase is ideal, delivery during subacute or chronic phases may still confer benefits.

Additionally, preclinical studies have investigated the efficacy of various does for Muse cell ischemic stroke treatment. Multilineage-differentiating stress-enduring cell-based product (CL2020) was injected through the cervical vein in an immunodeficient mouse lacunar model. CL2020 was administered in three different doses: high dose (5×10^4 cells/body), medium dose (1×10^4 cells/body) low dose (5×10^3 cells/body) at both the subacute phase and chronic phase. As seen in the cylinder test, the mice that were given the high dose demonstrated the greatest neurological and motor function improvement in both the subacute and chronic group when compared to the vehicle. For the mice which were given the high dose, their rehabilitation lasted up to 22 weeks (Abe et al. 2020). In another study, comparing the effects of Muse cells and non-Muse cells, a dosage 2.5×10^4 cells/body was used. The results indicated that the Muse cells improved neurological function of MCAO mice, as observed 35 days after transplantation (Dezawa et al. 2019). Furthermore, preclinical studies suggest that higher doses of Muse cell treatment are more effective in alleviating stroke-induced injury, bringing these cells closer to clinical application.

Before moving to clinical trials, the least invasive mode of delivery for Muse cell treatment must be established. Preclinical trials have examined intravenous injection as possible means of delivery for Muse cells (Dabrowska et al. 2019). The reparative properties of Muse cells through intravenous injection can be observed in a variety of tissues, such as the brain, liver, and skin (Leng et al. 2019). In the study that intravenously administered CL2020 to lacunar mice through the cervical vein, neurological and motor recovery was observed. When the human cells were depleted by the intraperitoneal injection of diphtheria toxin, the recovery was abolished, indicating that intravenous administration may be a viable, non-invasive method to deliver the cells (Abe et al. 2020).

Although the preclinical trials involving Muse cells are promising, there are some limitations. Allogeneic Muse cells can stay in the host brain as differentiated neuronal tissue for longer than 6 months (Dezawa et al. 2019). However, before moving to clinical trials, a long-term engraftment system, where donor cells continue to integrate themselves into the host's nervous tissue must be engendered. Preclinical studies have demonstrated encouraging results regarding the efficacy of Muse cells in treating ischemic stroke models.

4 Clinical Studies on Muse Cells

Treatment of neuronal cells through stem cell transplantation evidently enhance the motor and cognitive recovery in rodent stroke models (Kondziolka et al. 2000). However, the same significant improvement was not seen in clinical trials (Kondziolka et al. 2000, 2005; Savitz et al. 2005). Savitz et al. (2005) conducted a clinical trial to observe the effects of fetal porcine neural cell transplantation. A burr hole was created during the surgical process to implant the fetal cells at the infarct site in the basal ganglia (Savitz et al. 2005). In preclinial studies, the specific cell transplantation was deemed safe in animal models for basal ganglia infarcts (Savitz et al. 2005). In the clinical trial, some patients did not display adverse effects while some improved in speech and motor functions over long periods of time (Savitz et al. 2005). Other patients, however, temporarily experienced motor deficits weeks after transplantation (Savitz et al. 2005). One patient specifically developed seizure a week after treatment, and the study was terminated by the FDA (Savitz et al. 2005). Some studies have suggested that stem cells are doing more harm than good. Amariglio et al. (2009) first reports a human brain tumor after the 13-year-old patient with Ataxia Telangiectasia (AT) underwent fetal neural stem cell therapy. Although the patient was healthy after the treatment, a small tumor was discovered adjacent to the site 4 years later (Amariglio et al. 2009). Through cytogenetic and molecular examinations, the tumor was concluded to have originated outside the body, suggesting that the tumor was derived from the transplantation (Amariglio et al. 2009). Incidents mentioned above raises concern for the safety of patients receiving cell therapy. With inconsistent results in clinical trials, a new method of approach should be developed for cell therapy to improve the safety and efficacy of the treatment.

Compared to other stem cells, MSC is the most suitable type of stem cell for neural cell therapy for ischemic stroke patients. One study examined the safety and efficiency of MSC treatment in nonacute ischemic strokes (Valeria Battistella et al. 2011). In the study, patients received the maximal amount of MSC (5×10^8 cells) during the clinical trial, but no adverse events were reported for 180 days after transplantation (Valeria Battistella et al. 2011). Even with large amounts of stem cells were introduced to stroke patients, the intervention did not cause detrimental effects to their health. This finding ensures the safety of nonacute stroke patients when undergoing this method of treatment. Additionally, long-term studies have demonstrated no significant side effects in patients treated with MSC (Jin Soo Lee et al. 2010). Patients treated with MSC did not develop malignant tumors and no significant structural change was observed after a year of treatment (Jin Soo Lee et al. 2010). Studies investigating cell therapy with MSC have consistently provided no evidence regarding health concerns or consequences. Due to its safety and accessibility, MSCs are a favorable source for cell therapy. However, some MSC studies are concerned about the lack of evidence of the stem cell's efficiency.

Recent clinical trials have been conducted using Muse cells (CL2020) in Japan, further highlighting its safety and efficiency in ischemic strokes. Japanese studies consist of treatments for neonatal hypoxic ischemic encephalopathy, myocardial infarction, ischemic stroke, spinal cord injury, and epidermolysis bullosa (JapicCTI-183834, J.I. 2020; JapicCTI-184103, J.I. 2018; JapicCTI-184563, J.I. 2018; JapicCTI-194841, J.I. 2019; JapicCTI-195067, J.I. 2019). All clinical trials administered patients with allogenic CL2020 through intravenous infusion. Immunosuppression was not necessary during

the clinical trials because of the HLA-G expression in human Muse cells. This molecule allows the stem cells to function in the target site without the living body and its immune system reacting (Shohei Wakao et al. 2014). Muse cell's unique ability to develop into various types of cells and tissues allows it to target and repair damaged sites in vivo (Shohei Wakao et al. 2014). During clinical treatments for spinal cord, epidermolysis bullosa, and ischemic stroke, no significant evidence suggested that the intervention was detrimental or ineffective for patients with the mentioned conditions. Clinical studies for the three health conditions deemed CL2020 to be safe and efficient, further supporting the notion to utilize MSC and Muse cells for stem cell therapy (JapicCTI-184103, J.I. 2018; JapicCTI-184563, J.I. 2018; JapicCTI-194841, J.I. 2019). Muse cell therapy has also been explored for neonatal hypoxic ischemic encephalopathy patients (jRCT2043190112, J.I. 2020).

Muse cells can be directly administered to patients because of their unique anti-inflammatory and anti-immune mechanisms (Dezawa 2018; Young 2018). This mechanism prevents the body from rejecting the stem cell, avoiding the need to genetically manipulate the Muse cells for acceptance (Dezawa 2018). Additionally, unlike embryonic stem cells (ES) and induced pluripotent stem cells (iPS), Muse cells do not need to be administered to the target site directly (Dezawa 2018). Instead, Muse cells can be administered through intravenous injections, removing the need for surgical operations (Dezawa 2018). Due to these advantages, Muse cells bring new light to stem cell therapy. Further research and clinical studies should be conducted to investigate the efficiency and reliability of Muse cells in ischemic stroke.

5 Summary

Ischemic stroke, caused by areas of the brain being deprived of oxygen and nutrients, lead to neurological damages and cognitive impairments (Benjamin et al. 2019; Sacco et al. 2013). The affected areas acutely experience ionic disruptions and metabolic failures due to lack of sufficient blood flow, ATP, and energy. Chronically, the release of free radical oxygen species and inflammation cause additional damage and cell death within the affected regions of the brain (Hao et al. 2014; Lakhan et al. 2009). Unfortunately, tPA and endovascular thrombectomy are the only approved treatments for acute stroke and their use is limited by the narrow effective time window and risk for additional damage. Rehabilitation helps chronic management and stroke care but is does treat the loss of function. An intervention that could regenerate neural cells and restore lost function of the brain would be a valuable addition in our toolkit to treat stroke.

Stem cell therapy offers a potential solution, and preclinical studies have highlighted the regenerative abilities of donor stem cells to neural tissue (Lindvall and Kokaia 2006; Song et al. 2018; Kondziolka et al. 2000). However, ethical and logistical concerns limit the use of stem cells in regenerative medicine. For example, the extraction of ES involves the destruction of human embryos (Bernard and Parham 2009). iPSC, despite its impressive ability to become any cell type, is infamous for having the highest tumorigenicity, where cultured cells give rise to tumors over time (Liang et al. 2013). These issues bring controversy to stem cell research, hindering the advancement of stem cell therapy. Compared to previously mentioned stem cells, adult stem cells, such as MSCs, are more suitable for stem cell therapy. No ethical issues regarding their procurement is present because MSCs are harvested from the placenta, bone marrow, and umbilical cord (Shinozuka et al. 2013). Additionally, MSCs evidently possess the same proliferative ability as iPSC while also being safe to use (Valeria Battistella et al. 2011). However, MSCs possess their own limitations. Studies have shown the variability and heterogeneity of MSC when the stem cell is extracted from different donors and tissue source, making it difficult for different research groups to compare methods and results (Mohamed-Ahmed et al. 2018). To circumvent this and previous limitations, Muse cells, a distinct subset of MSCs, are favored in cell therapy for ischemic stroke. Preclinical

studies have investigated the efficiency of Muse cells. Studies have highlighted the mobility and anti-inflammatory activities of Muse cells (Leng et al. 2019; Uchida et al. 2016). Unlike non-Muse MSCs, Muse cells are able to differentiate into neurons, replace ischemic cells, and remain integrated (Dezawa et al. 2019). Clinical trials have commenced to investigate the safety and efficiency of Muse cells. Muse cells were administered through intravenous injections, removing the need for surgical operations (Dezawa 2018). This was possible due to Muse cell's ability to identify and repair damaged neural sites. Additionally, Muse cells were not genetically modified and no signs of rejection were observed in ischemic stroke patients because of the stem cells' anti-inflammatory mechanism (JapicCTI-184103, J.I. 2018). The evidence generated from preclinical and clinical studies makes Muse cells the most suitable candidate for cell therapy in ischemic stroke due to its safety, accessibility, and efficiency. Further clinical studies should be conducted to determine the consistency of Muse cells.

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