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Contents

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Contents

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OPINION REVIEW

Role of induced pluripotent stem cells in diagnostic cardiology

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Abstract

Ethical concerns about stem cell-based research have delayed important advances in many areas of medicine, including cardiology. The introduction of induced pluripotent stem cells (iPSCs) has supplanted the need to use human stem cells for most purposes, thus eliminating all ethical controversies. Since then, many new avenues have been opened in cardiology research, not only in approaches to tissue replacement but also in the design and testing of antiarrhythmic drugs. This methodology has advanced to the point where induced human cardiomyocyte cell lines can now also be obtained from commercial sources or tissue banks. Initial studies with readily available iPSCs have generally confirmed that their behavioral characteristics accurately predict the behavior of beating cardiomyocytes *in vivo*. As a result, iPSCs can provide new ways to study arrhythmias and heart disease in general, accelerating the development of new, more effective antiarrhythmic drugs, clinical diagnoses, and personalized medical care. The focus on producing cardiomyocytes that can be used to replace damaged heart tissue has somewhat diverted interest in a host of other applications. This manuscript is intended to provide non-specialists with a brief introduction and overview of the research carried out in the field of heart rhythm disorders.

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Core Tip: The introduction of induced pluripotent stem cells (iPSCs) has supplanted the need for human stem cells, thus eliminating most ethical controversies. This methodology has advanced to the point where induced human cardiomyocyte cell lines can also be obtained from commercial sources or tissue banks. iPSCs can predict the behavior of cardiomyocytes *in vivo*, so that new ways are paved in cardiology research to study arrhythmias and heart disease in general, accelerating the development of new, more effective antiarrhythmic drugs, clinical diagnoses, and personalized medical care.

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INTRODUCTION

Human induced pluripotent stem cells (iPSCs) are produced by reprogramming adult mesenchymal cells, most often fibroblasts. The process is complicated, requiring the introduction and activation of four gene regulatory networks, each comprised of molecular regulators that interact with each other and with other substances in the cell to control gene expression of mRNA and protein-specific genes. Acting together these four transcription factors can produce mature cells that behave in a completely different manner to the original fibroblasts used to form them, leading to the formation of multiple cell types[1-3].

Myocytes created from fibroblasts are basically identical to native cardiomyocytes. There are three different types of native cardiomyocytes, and iPSC production yields all three types in variable and unpredictable proportions, presenting difficulties for researchers. The most obvious and most publicized cardiological application of iPSCs is the production of new cardiac tissue to replace tissues destroyed by infarction or other diseases[4-9], but this goal has yet to be successfully realized in humans. Initially, this was partly because the subtype of iPSCs could not be assured. Obviously, atrial cardiomyocytes would not be a suitable substitute for damaged ventricular cardiomyocytes and, regardless, there is always the danger that introducing a mixture of cells might lead to teratoma formation[10]. Nonetheless, substantial advances have already been made, and success seems to be mainly a matter of time. Once these problems have been fully resolved, iPSCs, in various configurations, could be used to repair damaged hearts. They could also be used to predict interactions between drugs and the cardiac conduction system.

The occurrence of any specific conduction abnormality - including QT prolongation, altered action potential duration, triggered activity, the blockade of human-ether-a-go-go-related channel (hERG) and other ion conduction channels-and the occurrence of lethal arrhythmias-such as Torsades de Pointes (TdP)-cannot reliably be predicted with currently available screening methods (Langendorf preparations, patch-clamp, or even arterially perfused isolated rabbit left ventricular wedge)[11]. Animal models are problematic predictors of arrhythmia occurrence because of anatomic varia-tions[12-16]. Such an issue poses a huge difficulty for drug makers trying to produce effective antiarrhythmic drugs; animal-to-human extrapolation is an uncertain process, which can pose a danger to patients if unrecognized differences emerge between animal and human models[17].

The problems associated with the use of amiodarone and sotalol illustrate the difficulties of drug development[18,19]. Both drugs are used to treat atrial and ventricular tachyarrhythmias and can be life-saving, but can also produce lethal side effects. Unfortunately, predicting side effects is, at this moment, impossible. Amiodarone's most feared side effect is fatal pulmonary interstitial fibrosis, but

hepatitis, hypothyroidism (probably irreversible) and mixed sensorimotor polyneuropathy have all been reported with some regularity. Amiodarone's most important complications are QT prolongation and TdP[20]. If new and safer replacement drugs are ever to be developed and approved by the United States Food and Drug Administration and the European Medicines Agency, developers will first have to establish that new drugs do not produce predictable untoward side effects or exacerbate the conditions they were designed to treat.

The availability of iPSCs allows researchers to make reasonably accurate predictions about what effect any new drug will have on the heart and its electrical system. Cultured iPSCs, can be used to construct *in vitro* models of the human cardiac conduction system. The effects observed *in vitro* can then be used to predict how, and/or whether, a drug will alter electrical conduction, or produce structural alterations in humans. The process is not as simple as it sounds and some knowledge of the subject is crucial to clinicians for the safe use of new drugs.

UNDERLYING PHYSIOLOGY AND CLINICAL MANIFESTATIONS

Cures for cardiac conduction diseases will only be found when their root causes are fully elucidated. Even physicians who have nothing to do with arrhythmia research should retain some knowledge of the molecular biology that underlies cardiac conduction.

The cardiomyocyte repolarization/depolarization cycle begins with a current generated by the outward flow of potassium ions through specific pores or channels. Potassium pores exist in all life forms and many different types have been identified (more than 20). Two types of potassium channels are absolutely critical to the process of cardiac repolarization: The rapid delayed rectifier current (identified as IKr) and the slow delayed rectifier current (identified as IKs). If a drug or a mutation disrupts either of these two currents, the action potential of the cell is prolonged with an increase in the time required for electrical depolarization and repolarization of the ventricles[21]. Prolonged repolarization leads to the occurrence of early after depolarization (EAD) currents. EADs are dangerous because they favor the occurrence of triggered activity (defined as the occurrence of spontaneous action potentials occurring during phase 2 or phase 3 of repolarization, leading to the production of inappropriate action potentials and arrhythmia)[12]. Blockade of the IKr also causes the QT interval to be prolonged, leading to the triggered activity *via* a slightly different mechanism[22]. Such a situation is likely to occur when a drug molecule interferes with potassium channels as in the case of type III antiarrhythmic drugs. Slowing of the potassium current is associated with a repolarization dispersion, where one area of the myocardium recovers from depolarization faster than an adjoining region, which also makes TdP more likely to occur[23]. Repolarization dispersion is thought to be the reason that myocardial hypertrophy is associated with arrhythmias[24]. The farther the depolarization front has to travel, the greater the interval between depolarization and repolarization. Dispersion is especially likely to occur if the area of abnormal delay and dispersion is located within the Purkinje system or, alternatively, if the area is located in the mid-wall of the left ventricle where the "M cells" are located. These cells have prolonged action potentials that act to further increase the dispersion of repolarization, making the occurrence of TdP ever more likely[25]. For a new antiarrhythmic drug to be introduced, it must first be proven that it exerts none of the effects enumerated above.

Sudden cardiac death (SCD) due to ventricular tachycardia (VT) or ventricular fibrillation (VF) accounts for approximately half of all deaths in patients with heart failure (HF) and may be considered a heritable trait[26-32]. Current guidelines[33] recommend an implantable cardioverter-defibrillator (ICD) in patients with symptomatic and severe left ventricular dysfunction of any origin. However, SCD may occur in asymptomatic patients with only mild HF. On the contrary, as many as two-thirds of patients with severe HF implanted with an ICD do not experience device interventions over 3 to 5 years follow-up[34]. A similar clinical scenario leaves unanswered the question of whether selected gene variants may affect the risk of SCD in HF patients. Genomic science provides us with new approaches to identify gene variants or mutations that predispose patients with inherited electrical diseases to SCD. However, a growing body of evidence suggests that DNA changes in the same genes that convey risk in primary electrical diseases may enhance susceptibility to VT/VF even in a polygenic condition such as HF. Sustained VT and VF often occur as a consequence of delayed after-depolarizations triggered by diastolic sarcoplasmic

reticulum (SR) calcium leak[35]. Genes encoding calcium handling proteins involved in electrical homeostasis of the failing heart may represent suitable candidates for defining individual susceptibility to life-threatening arrhythmia[26,27]. However, only very few genes belonging to the major candidate systems have been characterized and screened for possible association with SCD in HF. The cardiac ryanodine receptor 2 (RyR2), a calcium-releasing channel located in the SR membrane, plays a key role in the electrical homeostasis of cardiomyocytes. RyR2 dysfunction has been described in both HF patients and animal models and is critical to many of the aspects of the disease, including life-threatening arrhythmia[36]. In a large cohort of HF patients, Ran *et al*[37] found that the A allele of RYR2 c.5656G>A was associated with an increased risk of SCD. Arvanitis *et al*[38] reported that the Ser96Ala variant in histidine-rich calcium-binding protein was associated with ventricular arrhythmia in idiopathic dilated cardiomyopathy. It is known that a serine residue replacing glycine at position 1886 (G1886S or rs3766871) in the *RyR2* gene prompts a significant increase in intracellular calcium oscillation and creates a site of phosphorylation for protein kinase C (PKC) entailing PKC-mediated calcium diastolic leak from the SR[39,40]. While the RYR2 rs3766871 variant has been previously described only in the setting of arrhythmogenic right ventricular cardiomyopathy, a role of RyR2 rs3766871 minor allele for increased susceptibility to VT/VF has been recently reported also in patients with HF[41]. The SERCA calcium ATPase (ATP2A2) belongs to a large family of P-type cation pumps that couple adenosine triphosphate (ATP) hydrolysis with cation transport across membranes[42]. Alternative splicing of the *ATP2A2* gene produces two isoforms, SERCA2a (primarily located in the heart and slow-twitch skeletal muscle) and SERCA2b (present in smooth muscle and non-muscle tissues). Mutations in the *ATP2A2* gene affect the expression level, ATP affinity, calcium affinity, and phosphorylation of ATP. In an attempt to investigate whether variants of the genes encoding major calcium handling proteins affect the occurrence of VT/VF in HF patients, it was found that the ATP2A2 c.2741+54G>A gene variant was associated with decreased susceptibility to life-threatening arrhythmia. Indeed, patients carrying the ATP2A2 c.2741+54A allele variant had an approximately 70% reduction in the relative risk of VT/VF during follow-up[43]. Defective calcium handling in failing cardiomyocytes has long been recognized as a cause of ventricular arrhythmia, and recent evidence suggests that selected calcium gene variants may modify the risk of SCD even in a complex and polygenic disease such as HF. While statistically associated with a modified risk of SCD, the biological role of many of these gene variants is presently unknown. The recent breakthrough discovery of iPSCs could enable the investigation of mutated cardiomyocytes generated from patient's somatic cells, allowing functional characterization of iPSC-derived mutated cardiomyocytes. A similar approach represents an interesting and promising solution for the biological relevance of genetic substrates in secondary arrhythmogenic conditions.

Microelectrode array

Microelectrode arrays are used in many fields of study, although the basics of the system are the same no matter what kind of test is being performed; improvements and refinements in this methodology are being reported almost continuously. These tests are performed in wells that look just like those in any clinical laboratory test plate used to observe chemical reactions, however, they differ in one important respect; electrodes are located at the bottom of each well.

When the electrodes and iPSCs are joined together they form the backbone of the system. The idea was derived from earlier networking studies, designed to test neural interactions. Networking electrodes were originally made of titanium salts and gold conductors[44], but other materials have been used. The system is now so advanced that these wells, indeed, the entire networking system, including software, are all available off the shelf.

IPSCs, can either be studied singly or as part of an integrated network. For most intents and purposes these cells have all the same capabilities as embryonic stem cells that have been allowed to mature. In 2012, Shinya Yamanaka outlined a method to induce pluripotency by inserting genes that acted as reprogramming factors, also called transduction factors, by attaching them to carrier viruses and inserting the virus into the cells, which eventually causes the cells to express the exogenous genes. The cells are then cultured and finally harvested[45]. Since the technique was first introduced, many other iPSCs, and related transcription factors, have been identified and used, including, miRNAs (a type of non-coding RNA that inhibits translation in many species). Whatever the precise role of these diverse factors, other epigenetic processes are critical for the process of converting maturing stem cells back to inducible pluripotent cells[46].

Once the multiple electrode arrays have been constructed, beating cardiomyocytes, derived from pluripotent stem cells are plated over each well, without the electrodes ever actually penetrating the cells. Such a methodology essentially recreates many aspects of a working myocardium, including the generation of waveforms not very different from those seen on clinical electrocardiograms. Introducing an experimental drug into the system, the probable effect on a beating human heart can be confirmed with a high degree of accuracy.

For example, experimental drugs have been tested in networked iPSCs that alter the duration and shape of the QT interval in almost exactly the same pattern as seen in humans. Not only do drugs produce the same electrocardiographic changes, but physiological stressors also produce changes similar to those that occur *in vivo* with the same rate and QT interval alterations seen in humans[47,48]. If animal studies suggest that a drug can cause dangerous QT prolongation, it is simple enough to test the drug on networked beating human cardiomyocytes. Another obvious application of this technology is the measurement of calcium transients by using fluorescence microscopy. Calcium indicators are introduced into the cells and the resulting fluorescence can be quantitated noninvasively and used to measure calcium ion flux, which controls inotropy. In the past, such experiments required the use of isolated small animal muscle[49].

The same type of cellular network can be used to study the effect of genetic mutations known to cause cardiac arrhythmias, including channelopathies such as hERG; more than 90 long QT syndrome (LQTS) mutations have been mapped to date. It is possible to measure the effect of mutations on IKr and IKs, although debate still exists over the exact mechanism by which some mutations alter potassium flow, answers to at least some of these questions should soon be forthcoming[50]. With the availability of high-throughput networked cardiomyocytes, it is now possible to evaluate a drug's effects on potassium flow before it is ever given to an animal, let alone evaluated in human clinical trials.

IPSCs from a patient with a novel *KCNQ* mutation were used by Egashira *et al*[51] to identify the mutation. The patient had survived VF, thanks to the nearby presence of an automated external defibrillator. Using a slight variation multi-electrode array system (where the electrical activity of clumps of cells, rather than sheets of cells was measured), abnormal repolarization, as manifested by electrical field potential duration, was observed in the spontaneously beating iPSC cardiomyocytes. Egashira *et al*[51] then added an assortment of potassium ingress and egress blockers to prove that the repolarization abnormality lay within the slow inward potassium channel[51]. At present, the technology is too cumbersome for routine clinical use. In the future, however, it should be possible to use this approach when exome screening fails to identify one of the usual culprits.

The recent discovery of the *TECRL* gene, an arrhythmia-inducing gene that produces features of catecholaminergic VT (CPVT) and LQTS, was accomplished using much the same technology[52]. Three patients were studied; two with a history of cardiac arrest and one with an episode of recorded CPVT. Once iPSCs had been produced and the mutation identified and sequenced, electrophysiological studies were then performed. These demonstrated exactly the same features (catecholamine sensitivity, triggered activity, delayed afterdepolarizations as had been seen in the patients. The abnormalities were all reversed by the addition of flecainide, a class 1c antiarrhythmic drug. Had iPSCs not been available, finding a remedy would have been purely by empiric trial and error. However, the real significance of the study is that there is now a reliable methodology with which to screen drugs for effectiveness.

Even without going to the effort of creating an entire iPSCs network, it is still possible to clinically diagnose some disorders from the electrical behavior of a single iPSC. A very recent report describes two patients with known Brugada Syndrome. When compared to the findings in two healthy controls, it was observed that each of the Brugada Syndrome patients carried one of two different sodium voltage-gated channel alpha and subunit 5 variants. The electrical characteristics of iPSCs produced from the patient's own skin fibroblasts were studied. The studies showed reductions in inward sodium current density and reduced maximal upstroke velocity of action potential when compared with healthy controls. Furthermore, iPSC cardiomyocytes from the Brugada Syndrome patients demonstrated increased triggered activity, abnormal calcium (Ca2fl) transients, and beating interval variation, the very same abnormalities previously reported in other studies, using different methodologies[53]. Late in 2016, a study using individual iPSCs was used to confirm results observed in a previous knock out mouse study. The studies had suggested the existence of a new cardiac regulatory mechanism that appeared to play a key role in the association between arrhythmias and myocardial hypertrophy. When the mouse studies were

repeated in human iPSCs, it was possible to confirm that the same stress-activated kinase was operative in human cells[54].

Heart disease screening

Another obvious application for iPSCs is screening for suspected heart disease, and for determining the significance of a mutation once it has been identified. Hypertrophic cardiomyopathy (HCM) is a very good example. The clinical diagnosis can be difficult to make (left ventricular hypertrophy with wall thickness > 15 mm, in the absence of ventricular dilation or any apparent disease that could cause hypertrophy)[55]. Unfortunately, it is not uncommon for there to be a complete disconnect between phenotype and genotype: Abnormal genes may be present but symptoms and signs absent.

Both sarcomeric mutations and non-sarcomeric mutations in HCM can be identified by whole-exome sequencing, and these studies demonstrate that the same genotype may be responsible for sudden death in one individual, but remain asymptomatic in another[56]. Multiple mutations have been detected in patients with HCM: Nine sarcomeric genes are known to carry most HCM-related mutations and encode sarcomeric mutations, while an additional nine mutations code for sarcomeric Z-disc proteins such as muscle LIM protein, α-actinin, or telethonin[57,58].

Since iPSCs cardiomyocytes became available, the pathogenic effects of some mutations (MYH7 and MYBPC3) associated with HCM have already been identified [59], and calcium blockade has been found to be an effective treatment for another HCM mutation (MYH7-R663H)[60]. Whole-exome sequencing almost never yields the identity of a single culprit gene, but rather detects multiple mutations, some of which may be relevant and some not. If one single mutation is responsible for the obvious phenotype of HCM, it has yet to be identified. It hardly needs saying, but exactly the same methodology used to identify culprit genes could be applied to genomic studies of countless other disorders, just by inducing the required cell type from transformed fibroblasts.

DISCUSSION

Overcoming the ethical problems related to the use of stem cells through the introduction of iPSCs opens up an interesting scenario on the study of the cellular basis of diseases[61,62]. The use of pluripotent cells makes it possible to reproduce models for the study of cardiological pathologies which frequently cause SCD and are often diagnosed post-mortem such as structural cardiomyopathies and channelopathies[63-66].

Furthermore, iPSCs can be exploited in the personalization of therapies in relation to the possibility of carrying out pharmacological tests on cells derived from the patient[67-70].

Although the principles are easy to understand, at present there are some important caveats. One is that fibroblast generated iPSCs demonstrate an immature phenotype so that they more closely resemble mid-gestation human fetal hearts[71-73]. These differences may well alter final experimental and clinical results, depending on the stage of development of the iPSCs being used. When used in other fields, the same caveat applies. Now that this difference has been recognized, finding ways to make sure the cells are organized and function as adult cells is the object of intense research, which has already begun to generate results. Recent reports indicate that iPSCs can be stimulated and made to mature by a combination of pacing and increasing mechanical stress[74-77].

Another issue that had been delaying progress is that protocols used to produce iPSCs do not produce just one kind of cell, but rather yield a mixed population of cardiomyocyte subtypes including ventricular-, atrial- and pacemaker-like cells[78-81]. Birket and colleagues[82] made the early observation that even though the iPSCs can behave like normal human cardiomyocytes, the production process leads to unequal numbers of each of the subtypes. Obviously, different results will be generated depending on which type of cell predominates. Many laboratories are working on effective cell separation methods and standardized methods should soon be available.

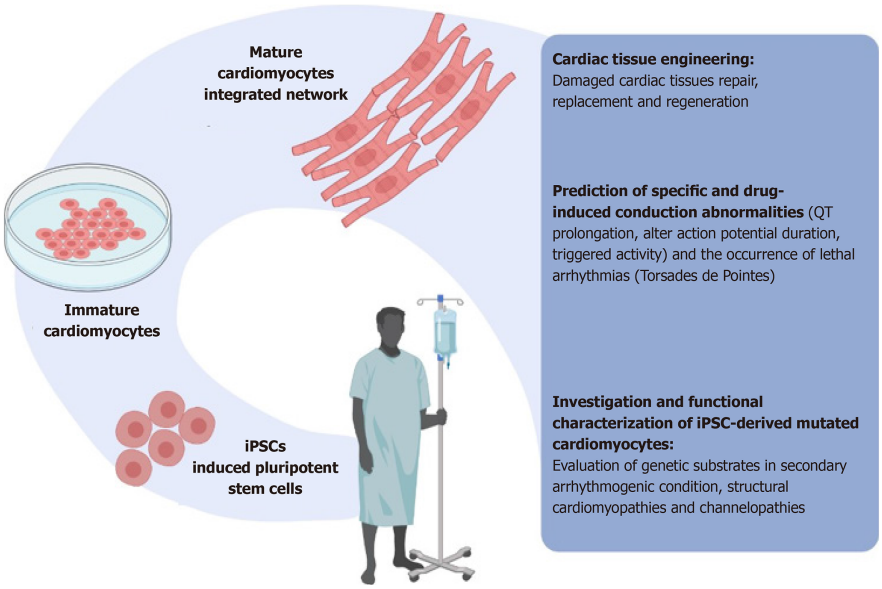

Figure 1 Induced pluripotent stem cells can provide new ways to study arrhythmias and heart disease in general, accelerating the development of new, more effective antiarrhythmic drugs, clinical diagnoses, and personalized medical care. iPSCs: Induced pluripotent stem cells. Figure created with BioRender (https://biorender.com).

CONCLUSION

In summary, the main applications of stem cells include disease modeling, cell diagnostics, and therapy personalization (Figure 1). Such tasks involve molecular profiling, the identification of biomarkers of the expression of the pathological phenotype, as well as the identification and testing of targeted therapies. The availability of pluripotent cardiac stem cells, especially networked beating cardiomyocytes, is likely to revolutionize our understanding of many cardiac rhythm disorders and diseases, provide a rational testing method for the development of drugs, permit clinicians to assess effectiveness before drug administration and, most importantly, save lives.

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REVIEW

Multidifferentiation potential of dental-derived stem cells

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Abstract

Tooth-related diseases and tooth loss are widespread and are a major public health issue. The loss of teeth can affect chewing, speech, appearance and even psychology. Therefore, the science of tooth regeneration has emerged, and attention has focused on tooth regeneration based on the principles of tooth development and stem cells combined with tissue engineering technology. As undifferentiated stem cells in normal tooth tissues, dental mesenchymal stem cells (DMSCs), which are a desirable source of autologous stem cells, play a significant role in tooth regeneration. Researchers hope to reconstruct the complete tooth tissues with normal functions and vascularization by utilizing the odontogenic differentiation potential of DMSCs. Moreover, DMSCs also have the ability to differentiate towards cells of other tissue types due to their multipotency. This review focuses on the multipotential capacity of DMSCs to differentiate into various tissues, such as bone, cartilage, tendon, vessels, neural tissues, muscle-like tissues, hepatic-like tissues, eye tissues and glands and the influence of various regulatory factors, such as non-coding RNAs, signaling pathways, inflammation, aging and exosomes, on the odontogenic/osteogenic differentiation of DMSCs in tooth regeneration. The application of DMSCs in regenerative medicine and tissue engineering will be improved if the differentiation characteristics of DMSCs can be fully utilized, and the factors that regulate their differentiation can be well controlled.

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Core Tip: Dental mesenchymal stem cells have been widely used in tissue engineering and regenerative medicine due to their multipotential differentiation ability. We herein discuss the multipotency of dental mesenchymal stem cells and some related factors influencing the odontogenic/osteogenic differentiation, which provide guidance for fully utilizing the multipotency of dental mesenchymal stem cells.

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INTRODUCTION

Over the past three decades, in the search for treatments for a variety of degenerative diseases and irreversible forms of tissue and organ damage, the emerging field of tissue engineering and regenerative medicine (TERM) has attracted a lot of interest, and great efforts have been made to realize the regeneration of different types of tissues and organs to restore normal physiology and body function. As one of the important aspects of regenerative medicine, tissue engineering mainly takes advantages of the following three methods: (1) Cell/biomaterial complex systems with cell-seeded biomaterials implanted into the body to restore and regenerate tissues/organs; (2) Cell systems, such as stem cell transplantation; and (3) Biomaterial systems implanted into the body and integrated into tissues[1]. As a vital part of TERM, a suitable source of stem cells is a significant initial requirement. Since the 1990s, the field of stem cell biology has gradually developed and rapidly become a main research trend in regenerative medicine. Induced pluripotent stem cells, progenitor cells from various tissues, human embryonic stem cells and adult stem cells are all potential seed cells for TERM[2]. Cells derived from induced pluripotent stem cells or differentiated from human embryonic stem cells can be used to build related tissue cell models. Progenitor cells and adult stem cells from various tissues can differentiate into mature tissues.

As adult stem cells, dental mesenchymal stem cells (DMSCs), including dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), stem cells from apical papilla (SCAPs), gingival mesenchymal stem cells (GMSCs), stem cells from human exfoliated deciduous teeth (SHED) and dental follicle stem cells (DFSCs) have been widely studied because of their ready availability, easy accessibility and lack of complex ethical issues. DMSCs have multiple differentiation potential and can differentiate into a variety of tissue-like cells under specific induction conditions, providing potential seed cells for TERM. For example, SHED are capable of inhibiting bone loss, decreasing neuronal apoptosis and forming pancreatic islet-like clusters[3-5]. DPSCs can differentiate into myogenic lineage and corneal stromal-like constructs[6,7] and can also reduce bone loss in an osteoporosis mouse model, prevent retinal ganglion cell loss and repair spinal cord injury[8-10].

DMSCs, in particular, have great potential for application in engineering regeneration of dental tissues. In 2006, Sonoyama *et al*[11] transplanted a hydroxyapatite/SCAP-Gelfoam/PDLSC structure into a swine alveolar socket, which regenerated mineralized root-like tissue and formed periodontal ligament space[11]. In 2012, Guo *et al*[12] identified a method of combining DFSCs with treated dentin matrix scaffolds in the alveolar fossa that proved to be a promising strategy for tooth root regeneration[12]. In 2013, Iohara *et al*[13] transplanted autologous DPSCs with granulocyte-colony stimulating factor into a dog pulpectomized tooth and found that newly formed pulp tissue, including innervation and vasculature, fully filled in the root canal[13].

Efforts have been made to promote tooth regeneration by DMSCs, but many factors affect this complex regeneration process, such as correlative non-coding RNAs, signaling pathways, inflammation, aging and exosomes. In the process of induced differentiation of DMSCs, many non-coding RNAs, including microRNAs and long noncoding RNAs (lncRNAs) and related signaling pathways are involved to regulate the expression of odontogenic/osteogenic differentiation genes. In addition, donor

age, cell senescence and the complex oral inflammatory microenvironment also pose great challenges to tooth regeneration by DMSCs. Moreover, the hot topic of research in recent years, exosomes, which carry a variety of contents, have also captured the attention of researchers in inducing the differentiation of DMSCs. If we can regulate these factors well, it will enable a big step forward in the application of DMSCs in the field of tooth regeneration. This review focuses on the multidirectional differentiation potential of DMSCs and the effect of the above-mentioned factors on the odontogenic/osteogenic differentiation of DMSCs in the field of tooth regeneration, hoping to provide a reference for the efficient use of DMSCs.

DIVERSE DIFFERENTIATION OF DMSCS

In addition to the odontogenic differentiation ability of DMSCs, in recent years the research on the differentiation of DMSCs into other tissue-like cells, such as osteogenesis, chondrogenesis, angiogenesis, neurogenesis and differentiation potential toward tendon-like cells, insulin-producing cells, hepatic-like cells, corneal stromal-like cells, *etc*. has become popular (Figure 1). To explore the diverse differentiation ability of DMSCs is an issue worth exploring.

DPSCs

In 2000, Gronthos *et al*[14] identified that DPSCs can form alizarin red-positive condensed nodules with high levels of calcium cultivated by L-ascorbate-2-phosphate, glucocorticoid, dexamethasone and inorganic phosphate[14]. As a seed cell for bone regeneration, DPSCs usually attached to some materials for bone defect models. For example, Wongsupa *et al*[15] fabricated a scaffold combination of poly-ε-caprolactone–biphasic calcium phosphate with the modified melt stretching and multilayer deposition technique seeded with human DPSCs (hDPSCs), which increased the newly formed bone in calvarial defects rabbit models[15]. However, Jin *et al*[16] showed that adipose tissue-derived stem cells exhibited greater osteogenic differentiation potential compared to DPSCs[16].

In vitro, DPSCs can differentiate into chondroblasts, which suggests that it can be useful for cartilage injuries[17]. CD146 marked DPSCs can express the chondrogenic inducing factor transforming growth factor (TGF)-β3 and form three-dimensional cartilage constructs when seeded on poly-L-lactic acid/polyethylene glycol electrospun fiber scaffolds[18]. Costal chondrocytes are able to supply a chondro-inductive niche that promote the DPSCs to undergo chondrogenic differentiation and enhance the formation of cartilage[19]. Xenotransplantation of DPSCs in platelet-rich plasma and 3% alginate hydrogels significantly regenerated cartilage in rabbit models of cartilage damage[20,21].

In 2016, Chen *et al*[22] first identified expression of tendon-related markers such as scleraxis, tenascin-C, tenomodulin, eye absent homologue 2, collagen I and collagen VI in dental pulp tissues. Also, DPSCs seeded in aligned polyglycolic acid fiber scaffolds can promote the expression of tendon-related markers under mechanical stimulation and form mature tendon-like tissue in a mouse model[22]. As neural crest-derived cells, DPSCs can be induced to differentiate into neuron-like cells with the use of growth factors, including basic fibroblast growth factor and epidermal growth factor, which are preferable to the chemical-induction method[23-25]. DPSCs transplanted into a rat model of middle cerebral artery occlusion, peripheral nerve injuries and retinal injury expressed related neuronal markers[26-28].

Three-dimensional culture promoted the differentiation of hDPSCs into insulin-producing cells[29], and pancreatic islets were also generated from DPSCs[30]. The potential toward insulin-producing cells of hDPSCs was superior to human PDLSCs (hPDLSCs)[31]. DPSCs also exhibited angiogenic potential when implanted into mouse brain and into a rat model of acute myocardial infarction by promoting neovasculogenesis[32,33]. Furthermore, DPSCs differentiated into bladder smooth muscle cells in a particular culture medium[34], while the Wnt-GSK3β/β-catenin pathway played an important role in this process[35]. DPSCs had the potential to form a high-purity hepatic lineage when cultured in serum-free medium[36], and DPSCs derived from cryopreserved dental pulp tissue of vital extracted diseased teeth also showed the potential to differentiate into hepatic-like cells[37]. Additionally, DPSCs had the capacity to differentiate into melanocyte-like cells when cultured in a specific melanocyte differentiating medium[38].

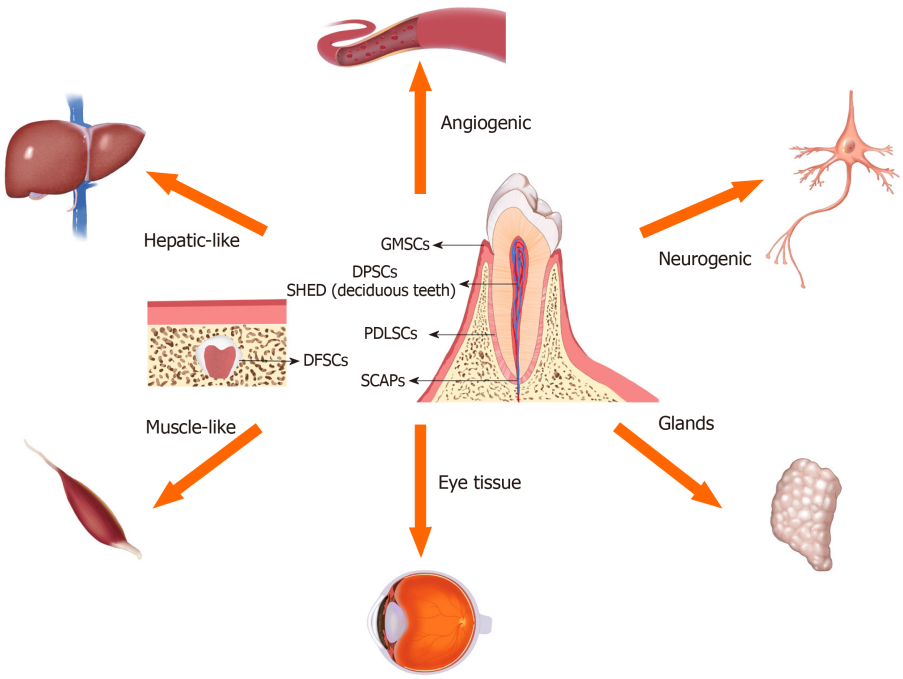

Figure 1 Location of dental mesenchymal stem cells and their diverse differentiation potential. Dental mesenchymal stem cells can be isolated from different tissues of the teeth. Dental mesenchymal stem cells have multidifferentiation ability and can differentiate into many tissue-like cells. DPSCs: Dental pulp stem cells; PDLSCs: Periodontal ligament stem cells; SCAPs: Stem cells from apical papilla; GMSCs: Gingival mesenchymal stem cells; SHED: Stem cells from human exfoliated deciduous teeth; DFSCs: Dental follicle stem cells.

PDLSCs and GMSCs

PDLSCs have great osteogenic differentiation potential. Kato *et al*[39] observed that PDLSCs have the highest levels of some bone differentiation markers without osteogenic differentiation among mesenchymal stromal cells derived from bone marrow and adipose-derived mesenchymal stem cells[39]. Seeded on nanohydroxyapatite-coated genipin-chitosan conjunction scaffold, PDLSCs exhibited significantly greater viability and alkaline phosphatase activity and promoted calvarial bone repair [40]. Moshaverinia *et al*[41,42] reported that PDLSCs and GMSCs capsulated in an injectable arginine-glycine-aspartic acid tripeptide-coupled alginate microsphere delivery system promoted bone regeneration and chondrogenesis, respectively, for a calvarial defect animal and subcutaneous implantation of nude mice, and PDLSCs showed significantly higher osteogenic and chondrogenic differentiation capability compared with GMSCs.

In 2021, Shen *et al*[43] showed that 6-bromoindir-ubin-3′-oxime promoted mineralized nodule formation in PDLSCs[43]. PDLSCs from beagle dogs and humans can both be induced to differentiate into neural-like cells by various protocols[44,45], and the Wnt/β-catenin signaling pathway has been implicated in this process[46]. Bueno *et al*[47] found that the nuclear shape of hPDLSC-derived neural-like cells was similar to cells in neurogenic niches from adult mouse brain, and no cell proliferation occurred in the course of neurogenesis. The potential for neurogenesis is improved by the addition of specific short peptides or phytocompounds[48-50]. As another stem cell type derived from periodontal tissue, GMSCs also have neurogenic differentiation potential and displayed action potential capacity when tested by a neurosphere-mediated induction method[51], while hypoxia preconditioning activated more genes associated with neuronal development[52]. In addition, over prolonged passages, human GMSCs have been found to spontaneously differentiate into neural precursor cells[53].

Encapsulated PDLSCs and GMSCs in an alginate/hyaluronic acid three-dimensional scaffold promoted the regeneration of neurogenic tissue[54]. Besides, PDLSCs had the ability to differentiate into corneal stromal keratocyte-like cells[55] and constructed a multilamellar human corneal stromal-like tissue *in vitro* when seeded onto orthogonally aligned, multilayered silk membranes and supplemented with the neuropeptide substance P[56]. PDLSCs also could be directed to develop into retinal progenitors and islet-like cell clusters with competence for photoreceptor differentiation and secretion of insulin[57,58]. Moreover, both PDLSCs and GMSCs differen-

tiated into tendon-like cells using an injectable and biodegradable arginine-glycine-aspartic acid tripeptide-coupled alginate hydrogel scaffold[59]. The GMSCs could also be induced to differentiate into functional keratinocytes when treated with *Acalypha indica* in a three-dimensional microenvironment[60].

DFSCs

Human DFSCs can differentiate to osteogenic lineage cells in osteogenic induction medium without dexamethasone, and BMP6 is a key gene in the osteogenic differentiation[61]. Plasma rich in growth factors and soluble silica can promote osteogenic differentiation of DFSCs[62,63]. Lucaciu *et al*[64] indicated that DFSCs could be used for promoting bone regeneration on titanium implant surfaces[64]. DFSCs were loaded into poly-ε-caprolactone scaffold and implanted into skulls defects of Sprague Dawley rats, and bone regeneration was observed[65]. Undifferentiated DFSCs expressed some neural markers, such as nestin, β-III-tubulin and S100β and exhibited a spindle-like morphology[66]. Using a two-step strategy for neuronal differentiation, DFSCs could be differentiated into neurosphere-like cell clusters, and finally developed a cellular morphology with small bodies and long cellular extrusions while exhibiting increased expression of neural cell markers[67].

It has been suggested that human DFSCs may have the potential to differentiation toward the glial lineage rather than the neuronal lineage[66]. Induced cardiomyocytes derived from DFSCs, which were cultured in medium with suberoylanilide hydroxamic acid, could be intraperitoneally injected into experimental mice and exhibited homing capacity into the heart muscle[68]. Comparing the differentiation potential toward pancreatic β cell-like cells among the stem cells from dental pulp, papilla and follicle, the DFSCs demonstrated higher potency and secreted more insulin upon glucose challenge[69]. Furthermore, epithelial stem-like cells from the human dental follicle were able to differentiate into salivary gland acinar and duct cells[70].

SHED

SHED represent a promising cell source for bone regeneration, which are usually combined with many biomaterials. Combined hydroxyapatite scaffold and SHED can promote alveolar bone regeneration, and interleukin-17A can enhance osteogenic differentiation of SHED, both due to increasing osteoprotegerin/receptor activator of nuclear factor κB ligand ratio[71,72]. FGF-2 pretreated SHED represent a faster formation of intramembranous bone after implanted in craniofacial bone defects than hypoxia pretreated[73]. A carbon nanomaterial named graphene oxide quantum dots promotes osteogenic differentiation of SHED *via* the Wnt/β-catenin signaling pathway [74]. In addition, SHED have the chondrogenic differentiation ability. After transplantation into the subcutaneous space on the back of nude mice, SHED recombined with β-TCP scaffolds were able to produce new cartilage-like tissues[75].

In 2011, SHED were successfully induced to differentiate into neural-like cells by a simple short-term growth factor-mediated induction protocol[76], and then in 2013, a novel three-stage method was established[77]. Yang *et al*[78] found that Noggin overexpression combined with the Rho kinase inhibitor Y-27632 exhibited a synergistic effect in promoting differentiation of SHED into neuron-like cells[78]. The lncRNA C21orf121 promotes SHED differentiation into neuronal cells by upregulating the expression of BMP2, acting as a competing endogenous RNA to compete with BMP2 binding to miR-140-5p[79]. SHED in polyglycolic acid tubes combined with autografting can regenerate the mandibular branch of the rat facial nerve[80]. Also, SHED have been used to repair a Parkinsonian rat model, an acute contused spinal cord injury model and a model of diabetic peripheral neuropathy[81-83].

In addition, SHED can differentiate into angiogenic endothelial cells, and when cultured with decellularized extracellular matrix of human umbilical vein endothelial cells can improve endothelial differentiation[84,85]. Using shear stress *via* the downstream pathway of vascular endothelial-derived growth factor-Notch signaling or by inhibiting TGF-β signaling in SHED can enhance endothelial differen-tiation[86, 87]. SHED transplanted into immunodeficient mice using Matrigel with human umbilical vein endothelial cells form extensive vessel-like structures[88].

SHED also have the potential for hepatic differentiation, which can be improved by using liquorice or angelica extracts in the culture medium[89]. $CD117^+$ SHED hepatically differentiated *in vitro* were used to repair either acute liver injury or induced secondary biliary cirrhosis in a rat model[90]. Meanwhile SHED or SHED-converted hepatocyte-like cell-based spheroids transplanted into a CCl_4-induced chronic liver fibrosis mouse model improved hepatic dysfunction[91,92].

Furthermore, SHED can differentiate into epidermal cells and accelerate wound repair when seeded onto polyvinyl alcohol/silk fibroin nanofiber dressings[93]. CD117$^+$ SHED also have the potential to differentiate toward all functional endocrine and exocrine subsets of pancreatic cells in serum-free conditions[94]. When cocultured with immortal corneal epithelium cells *in vitro*, SHED display the potential for transdifferentiation to corneal epithelium-like cells[95]. Li *et al*[96] indicated that SHED can transdifferentiate into retinal photoreceptor-like cells *in vitro* and retain good viability *in vivo* after transplantation into mice with a normal immune system[96]. Moreover, functional smooth muscle cells can be differentiated from SHED by TGF-β1 induction, while the ALK5 signaling pathway may regulate this process[97].

SCAPs

In 2020, Deng *et al*[98] reported that platelet derived growth factor BB promoted SCAPs osteogenic differentiation and enhanced bone formation in calvarial defects combined with a thermosensitive hydrogel[98]. Both conditioned culture medium containing traditional Chinese herbal remedy, Yunnan Baiyao, and high glucose α-Minimal Essential Medium can promote the odonto/osteogenic differentiation of SCAPs through the nuclear factor κB signaling pathway[99,100]. Depletion of lysine-specific demethylase 2A enhanced the adipogenic and chondrogenic differentiation potentials of SCAPs[101]. In 2020, Yang *et al*[102] reported that DLX5 and HOXC8 enhanced the expression of chondrogenic markers including type II collagen, type V collagen and sex-determining region Y box protein 9[102].

In 2017, Kim *et al*[103] first formed a three-dimensional cell-based nerve-like tissue with axons and myelin structures using SCAPs through a three-dimensional organotypic culture method[103]. The secreted frizzled-related protein 2, a Wnt signaling modulator, and insulin-like growth factor (IGF)-2 improved the neurogenic differentiation potential of SCAPs[104,105]. Adding graphene dispersion and water-soluble single-walled carbon nanotubes to the neuroinductive medium enhanced the neural differentiation of SCAPs[106].

SCAPs show angiogenic potential, and SCAPs and/or DPSCs transplanted in three-dimensional-printed hydroxyapatite scaffolds can form vascularized dentin/pulp-like tissue[107]. Coculture of human umbilical vein endothelial cells and SCAPs under hypoxic conditions promotes the construction of vessel-like structures *in vitro*, and ephrinB2 may play an important role in stabilizing the vascular-like struc-tures[108,109]. Furthermore, erythropoietin enhances the endothelial differentiation of SCAPs [110]. In addition, SCAPs also have hepatogenic potential[111], and mesenchymal stem cells derived from dental papilla can also be differentiated into pancreatic β cell-like cells[69].

MULTIPLE FACTORS INFLUENCING THE ODONTOGENIC/OSTEOGENIC DIFFERENTIATION OF DMSCS

MicroRNAs

MicroRNAs (miRNAs) play important roles in regulating the tooth regeneration process (Table 1). Downregulation of miR-143-5p and miR-143-3p promotes the odontoblastic differentiation of DPSCs through the osteoprotegerin/receptor activator of nuclear factor κB ligand signaling pathway[112,113]. Acting *via* the p38 mitogen-activated protein kinases (MAPK) signaling pathway, downregulated miR-143-5p and miR-488 are capable of inducing DPSCs to differentiate into odontoblast-like cells by targeting MAPK14 and MAPK1, respectively[114,115]. Wang *et al*[116] found that miR-125a-3p regulates odontoblastic differentiation of DPSCs in an inflammation model by targeting Fyn, a member of the protein tyrosine kinase Src family[116].

Meanwhile miR-let-7c-5p can restore the osteogenic differentiation of inflamed DPSCs by suppressing the lipopolysaccharide (LPS)-induced inflammatory phenomena[117]. In inflamed pulp tissues, miR-223-3p is remarkably upregulated, and overexpression of miR-223-3p in DPSCs can increase the protein levels of dentine sialophosphoprotein (DSPP) and dentine matrix protein 1[118]. Sun *et al*[119] found that during LPS-mediated odontoblastic differentiation of DPSCs, the expression of miR-140-5p is markedly decreased, while when miR-140-5p is expressed in DPSCs after LPS treatment, the odontoblastic differentiation ability is inhibited[119].

Additionally, during odontogenesis of hDPSCs, the expression of miR-508-5p decreases gradually, while significant inhibition of odontogenesis is observed after overexpression of miR-508-5p, which targets glycoprotein nonmetastatic melanomal

Table 1 Summary of the microRNAs influencing the odontogenic/osteogenic differentiation of dental mesenchymal stem cells

Ref.	MicroRNA	Cell type	Signaling pathway or targets	Outcome
Zhan *et al*[112], 2018	miR-143-5p	DPSCs	OPG/RANKL	Downregulation promoted odontoblastic differentiation
Yang *et al*[113], 2020	miR-143-3p	DPSCs	OPG/RANKL	Downregulation promoted odontogenic differentiation
Wang *et al*[114], 2019	miR-143-5p	DPSCs	MAPK14	Downregulation promoted odontoblastic differentiation
Yu *et al*[115], 2019	miR-488	DPSCs	MAPK1	Downregulation enhanced odontoblastic differentiation
Wang *et al*[116], 2020	miR-125a-3p	DPSCs	Fyn	Regulated odontoblastic differentiation in an inflammation model
Yuan *et al*[117], 2019	miR-let-7c-5p	Inflamed human DPSCs	-	Restored the osteogenic differentiation
Huang *et al*[118], 2019	miR-223-3p	Inflamed human DPSCs	-	Increased the proteins levels of DSPP and DMP-1
Sun *et al*[119], 2017	miR-140-5p	DPSCs	-	Inhibited odontoblastic differentiation after LPS treated
Liu *et al*[120], 2019	miR-508-5p	DPSCs	GPNMB	Inhibited odontogenic differentiation
Xu *et al*[121], 2018	miR-21	DPSCs	STAT3	Downregulation caused the decreasing expression of DMP-1 and DSPP
Qiu *et al*[122], 2019	miR-146a-5p	STRO-1 + human DPSCs	-	Promoted osteo/odontogenic differentiation
Zhang *et al*[123], 2018	miR-143	DPSCs	TNF-α/NF-κB	Suppressed the osteogenic differentiation
Yao *et al*[124], 2019	miR-215, miR-219a-1-3p	DPSCs	HspB8	Inhibited the osteogenic differentiation
Wei *et al*[125], 2017	miR-21	PDLSCs	Smad5	Inhibited osteogenesis
Li *et al*[126], 2019	miR-24-3p	PDLSCs	Smad5	Inhibited osteogenic differentiation
Wei *et al*[127], 2015	miR-21	PDLSCs	ACVR2B	Performed a positive function in mediating the stretch-induced osteogenic differentiation
Yao *et al*[128], 2017; Cao *et al*[129], 2017	miR-214	PDLSCs	ATF4, Wnt/β-catenin	Downregulation decreased the osteogenic differentiation
Bao *et al*[130], 2019	miR-148a	PDLSCs	-	Downregulation rescued the inhibition of osteogenesis triggered by LPS stimulation
Yan *et al*[131], 2017	miR-22	PDLSCs	HDAC6	Promoted osteogenesis
Li *et al*[132], 2018	miR-17	PDLSCs	HDAC9	Promoted osteogenesis in an inflammation condition
Xu *et al*[133], 2019	miR-132	PDLSCs	GDF5, NF-κB	Inhibited the osteogenesis
Zhen *et al*[134], 2017	miR-31	PDLSCs	Satb2	Took part in the high glucose-suppressed osteogenic differentiation
Wan *et al*[135], 2012	miR-34a	Human dental papilla cells	-	Increased the expression of DSPP and decreased the expression of ALP
Sun *et al*[136], 2014	miR-34a	SCAPs	-	Upregulated odonto/osteogenic markers
Wang *et al*[137], 2018	miR hsa-let-7b	SCAPs	MMP1	Suppressed the odonto/osteogenic differentiation
Dernowsek *et al*[138], 2017	miR-450a-5p,miR-28-5p	SHED	-	Supported the osteogenesis
Klingelhöffer *et al*[139], 2016	miR-101	DFSCs	-	Enhanced the osteogenic differentiation
Han *et al*[140], 2019	miR-3940-5p	GMSCs	-	Promoted the osteo/dentinogenic differentiation

DPSCs: Dental pulp stem cells; PDLSCs: Periodontal ligament stem cells; SCAPs: Stem cells from apical papilla; MAPK: Mitogen-activated protein kinases; OPG/RANKL: Osteoprotegerin/receptor activator of nuclear factor κB ligand; GPNMB: Glycoprotein nonmetastatic melanomal protein B; TNF-α: Tumor necrosis factor-α; NF-κB: Nuclear factor κB; ATF4: Activating transcription factor 4; LPS: Lipopolysaccharide; DSPP: Dentine sialophosphoprotein; ALP: Alkaline phosphatase; SHED: Stem cells from human exfoliated deciduous; GMSCs: Gingival mesenchymal stem cells; DFSCs: Dental follicle stem cells; HspB8: Heat shock protein B8; ACVR2B: Activin receptor type IIB; GDF5: Growth differentiation factor 5; MMP1: Matrix metalloproteinase 1; miR: MicroRNA; DMP-1: Dentine matrix protein 1; Smad5: SMAD family member 5.

protein B[120]. Xu *et al*[121] reported that during odontoblast differentiation of DPSCs, the expression of miR-21 can be regulated by treating with TNF-α, while downregulation of miR-21 causes a decrease in the expression of dentine matrix protein 1 and DSPP by interacting with STAT3[121]. Moreover, miR-146a-5p promotes odontogenic/osteogenic differentiation of STRO-1$^+$ DPSCs[122]. miR-143 suppresses the osteogenic differentiation of DPSCs by regulating the TNF-α/nuclear factor κB pathway[123], while miR-215 and miR-219a-1-3p inhibit the osteogenic differentiation capability of DPSCs by downregulation of heat shock protein B8[124].

During osteogenic differentiation of PDLSCs, the expression of miR-21 and miR-24-3p decrease, and their downregulation markedly inhibits osteogenesis of hPDLSCs by targeting SMAD family member 5 (Smad5)[125,126]. miR-21 also performs a positive function in mediating the stretch-induced osteogenic differentiation of hPDLSCs by regulating the expression of activin receptor type IIB[127]. Inhibition of miR-214 in PDLSCs can decrease osteogenic differentiation by targeting activating transcription factor 4 and regulating the Wnt/β-catenin signaling pathway[128,129]. Downregulation of miR-148a in PDLSCs rescues the inhibition of osteogenesis triggered by LPS stimulation[130]. miR-22 and miR-17 promote osteogenesis of PDLSCs by inhibiting HDAC6 and HDAC9 expression, respectively, the latter under inflammatory conditions[131,132]. In addition, in osteogenic differentiation of PDLSCs, miR-132 decreases, and overexpression of miR-132 inhibits osteogenesis by targeting growth differentiation factor 5 and activating the nuclear factor κB signaling pathway[133]. Meanwhile miR-31 plays a role in the high glucose-suppressed osteogenic differentiation of PDLSCs by targeting Satb2[134].

Upregulation of miR-34a in human fetal dental papilla cells increases the expression of DSPP and decreases the expression of alkaline phosphatase (ALP)[135]. In addition, miR-34a mimic transfection in SCAPs significantly upregulates odontogenic/osteogenic markers[136]. miR-hsa-let-7b suppresses the odontogenic/ osteogenic differentiation of SCAPs partly by targeting matrix metalloproteinase 1[137]. Moreover, overexpression of miR-450a-5p or miR-28-5p in SHED supports osteogenesis[138]. miR-101 enhances osteogenic differentiation in human DFSCs[139], and miR-3940-5p promotes the osteo/dentinogenic differentiation of GMSCs[140].

LncRNAs

LncRNAs significantly regulate the multiple differentiations of mesenchymal stem cells, and there are several reports of the regulatory effect of lncRNAs in regenerative engineering of dental-tissue-derived stem cells (Table 2). In 2020, Liu *et al*[141] identified a total of 89 lncRNAs differentially expressed after osteo/odontogenic induction of hDPSCs, and downregulation of lncRNA SNHG7 was found to inhibit the differentiation of DPSCs, upregulating the expression of miR-1226-3p and miR-210-5p at the same time[141]. In 2020, Chen *et al*[142] reported that 132 lncRNAs were differentially expressed between the odontoblastic-differentiated and undifferentiated hDPSCs and that lncRNA-G043225 exerted a positive regulatory effect through miR-588 and fibrillin 1[142]. Additionally, 47 lncRNAs were differentially expressed in hDPSCs between normoxic and hypoxic induction conditions, and 561 lncRNAs were differentially expressed between young and old donors in hDPSCs after osteoinduction[143,144]. Overexpression of lncRNAs CCAT1 and lncRNA H19 promotes odontogenic differentiation of hDPSCs by inhibiting expression of miR-218 and regulating expression of the *DLX3* gene, respectively[145,146]. Knockdown of lncRNA STL and lncRNA X-inactive specific transcript inhibits the osteogenic potential of DPSCs, and the latter is essential for efficient osteogenic differentiation induced by TNF-α[143,147].

In 2016, Qu *et al*[148] demonstrated that 2171 lncRNAs were differentially expressed between osteogenic-differentiated and undifferentiated PDLSCs, and 393 lncRNAs were strongly associated with osteogenesis-related mRNAs[148]. Zheng *et al*[149] indicated that downregulation of lncRNA maternally-expressed 8 and lncRNA MIR22HG markedly suppressed the osteogenic differentiation of PDLSCs[149]. Knockdown of lncRNA maternally-expressed 3 inhibits the osteogenesis of PDLSCs in

Table 2 Summary of the long noncoding RNAs influencing the odontogenic/osteogenic differentiation of dental mesenchymal stem cells

Ref.	LncRNA	Cell type	Signaling pathway or targets	Outcome
Liu *et al*[141], 2020	lncRNA SNHG7	DPSCs	miR-1226-3p, miR-210-5p	Downregulation inhibited osteo/odontogenic differentiation
Chen *et al*[142], 2020	lncRNA-G043225	DPSCs	miR-588, FBN1	Positively regulated odontoblastic differentiation
Zhong *et al*[145], 2019	lncRNA CCAT1	DPSCs	miR-218	Overexpression promoted odontogenic differentiation
Zeng *et al*[146], 2018	lncRNA H19	DPSCs	DLX3	Upregulation enhanced odontogenic differentiation
Shi *et al*[143], 2019	lncRNA STL	DPSCs	-	Knockdown inhibited osteogenesis
Tao *et al*[147], 2019	lncRNA XIST	DPSCs	-	Downregulation inhibited osteogenic differentiation
Zheng *et al*[149], 2018	lncRNA MEG8, lncRNA MIR22HG	PDLSCs	-	Downregulation suppressed osteogenic differentiation
Liu *et al*[150], 2019; Deng *et al*[151], 2018	lncRNA MEG3	PDLSCs	miR-27a-3p/IGF1 axis, Wnt/β-catenin	Knockdown inhibited osteogenesis
Wang *et al*[152], 2016	lncRNA-POIR	PDLSCs	miR-182	Enhanced osteogenic differentiation
Xu *et al*[153], 2019	lncRNA-TWIST1	PDLSCs	TWIST1	Improved osteogenic differentiation
Jia *et al*[154], 2019	lncPCAT1	PDLSCs	-	Reversed the suppression effect of osteogenesis caused by miR-106a-5p overexpression
Huang *et al*[155], 2020	lncRNA FER1L4	PDLSCs	miR-874-3p	Promoted osteogenic differentiation
Feng *et al*[156], 2020	lncRNA XIST	PDLSCs	miR-214-3p	Enhanced osteogenic differentiation
He *et al*[160], 2018	lncRNA TUG1	PDLSCs	lin-28 homolog A	Improved osteogenic differentiation
Wang *et al*[161], 2020	lncRNA DANCR	PDLSCs	-	Positively regulated osteogenic differentiation
Li *et al*[162], 2019	lncRNA H19	SCAPs	lncRNA-H19/miR-141/SPAG9/MAPK	Promoted the osteo/odontogenesis
Jia *et al*[157], 2016; Jia *et al*[158], 2015; Peng *et al*[159], 2018	lncRNA ANCR	DPSCs, PDLSCs, SCAPs	Wnt, lncRNA-ANCR/miR-758/Notch2 (PDLSCs)	Downregulation facilitated osteogenic differentiation

DPSCs: Dental pulp stem cells; FBN1: Fibrillin 1; lncRNAs: Long noncoding RNAs; MAPK: Mitogen-activated protein kinases; MEG3/8: Maternally-expressed 3/8; miR: MicroRNA; IGF1: Insulin-like growth factor 1; PDLSCs: Periodontal ligament stem cells; SCAPs: Stem cells from apical papilla; XIST: X-inactive specific transcript.

periodontitis *via* the miR-27a-3p/IGF1 axis, while it plays a positive role in human DFSCs by activating the Wnt/β-catenin signaling pathway[150,151]. In 2016, Wang *et al*[152] identified a novel lncRNA named lncRNA-POIR, while Xu *et al*[153] first named lncRNA-TWIST1 in 2019; both are osteogenesis impairment-related lncRNAs of PDLSCs from periodontitis patients and can enhance the osteogenic differentiation of PDLSCs from healthy individuals and periodontitis patients by interacting with miR-182 and inhibiting TWIST1 expression, respectively[152,153]. Prostate cancer-associated ncRNA transcript-1 upregulation reverses the suppression effect of osteogenic differentiation in PDLSCs caused by miR-106a-5p overexpression[154].

LncRNA FER1L4 and lncRNA X-inactive specific transcript can promote the osteogenesis of PDLSCs by sponging miR-874-3p and miR-214-3p, respec-tively[155, 156]. In addition, downregulation of antidifferentiation noncoding RNA can facilitate the osteogenic differentiation of DPSCs, PDLSCs and SCAPs[157], while this regulatory effect on PDLSCs is related to the canonical Wnt signaling pathway[158]. The antidifferentiation noncoding RNA/miR-758/Notch2 axis may also participate [159]. Furthermore, lncRNA TUG1 improves osteogenic differentiation of PDLSCs by regulating the expression of lin-28 homolog A[160]. Knockdown of lncRNA differentiation antagonizing nonprotein coding RNA positively regulates the osteogenic differentiation of PDLSCs[161]. Moreover, lncRNA H19 overexpression promotes the osteo/odontogenesis of SCAPs *via* the lncRNA-H19/miR-141/SPAG9/MAPK positive

feedback loop[162].

Signaling pathways

Wnt signaling pathway: The Wnt/β-catenin signaling pathway plays an important role in regulating DMSC differentiation, which is a key signaling pathway. For odontoblastic differentiation, activating the Wnt/β-catenin signaling pathway partially reverses the vacuolar protein sorting 4B knockdown-driven suppression of odontoblastic differentiation of hDPSCs[163] and rescues the osteoblastic/odontoblastic differentiation of stathmin-deletion hDPSCs[164]. These studies revealed that activation of the Wnt signaling pathway promotes osteogenic/odontoblastic differentiation of DPSCs. However, Scheller *et al*[165] first reported that Wnt/β-catenin inhibits odontoblastic differentiation of DPSCs in 2008[165]. The reason for the conflicting effects of Wnt signaling on odontoblastic differentiation in these studies is undefined and needs to be further explored. For osteoblastic differentiation, Rolph *et al*[166] confirmed that ferutinin promoted osteoblastic differentiation of DPSCs by modulating the Wnt/β-catenin signaling pathway[166] when Wnt5a was reported to inhibit osteoblastic differentiation of human periodontal ligament stem cell-like cells[167].

MAPK signaling pathway: The MAPK signaling pathway includes the ERK signaling pathway and the p38/MAPK signaling pathway[168]. In odontoblastic differentiation, one study showed that a combination of mineral trioxide aggregate and propolis significantly promoted the expression of DSPP and Dentine matrix protein 1 as well as mineralized nodule formation through activating the ERK signaling pathway in hDPSCs[169]. Kong *et al*[170] confirmed that a magnesium-enriched microenvironment enhanced the odontoblastic differentiation of hDPSCs by activating the ERK/BMP2/Smad signaling pathway[170]. In osteoblastic differentiation, berberine was reported to bind to epidermal growth factor receptor in hPDLSCs to activate the ERK signaling pathway and upregulate the nuclear-related gene FOS, thus promoting osteoblastic differentiation of PDLSCs[171]. In addition, mineral trioxide aggregate was confirmed to promote osteo/odontoblastic differentiation of SCAP through activation of the p38 and ERK signaling pathway. Another study showed that parathyroid hormone promoted the osteo/odontoblastic differentiation of DPSCs by activating the ERK and p38 signaling pathway[172].

Mechanistic target of rapamycin signaling pathway: Mechanistic target of rapamycin (mTOR), a highly conserved serine/threonine protein kinase, is involved in regulating interactions between proteins[173]. The mTOR signaling pathway has been confirmed to play a significant role in the osteo/odontoblastic differentiation of DMSCs. Tanaka *et al*[174] confirmed that inhibiting mTOR signaling promoted osteo/odontoblastic differentiation of SCAPs[174]. However, activation of the mTOR signaling pathway promoted osteogenic differentiation of hDPSCs in the process regulated by IGF-1 in which rapamycin blocked osteogenic differentiation induced by IGF-1[175] while inhibiting mTORC1 limited mineralized nodule formation by SHED[176]. Taken together, these data suggest that the mTOR signaling pathway plays different roles in different cell types of DMSCs.

AKT signaling pathway: The AKT signaling pathway is critical for cell proliferation, growth, metabolism and differentiation, especially in differentiation of DMSCs. Recent studies have shown that metformin and miR-let-7c-5p enhance the osteogenic differentiation of PDLSCs by activation of the AKT signaling pathway[117,177]. Another study reported that activation of the AKT signaling pathway could enhance the osteogenic differentiation of DPSCs in LPS-induced inflammation. In short, the AKT signaling pathway may play a positive role in odontogenic/osteogenic differentiation of DMSCs.

Notch and shh signaling pathway: The Notch signaling pathway is critical for development and cell differentiation. Notch signaling has been confirmed to inhibit odontoblastic differentiation of hDPSCs[178]. Interestingly, another study showed that overexpression of CCN3 activated the Notch signaling pathway to promote odontoblastic differentiation of DPSCs, which suggested that Notch signaling pathway activation promotes odontoblastic differentiation of DPSCs[179]. The reasons for these contradictory effects in odontoblastic differentiation of DPSCs remain undefined and need to be explored.

It is worth noting that the Shh signaling pathway is also involved in odontogenic/osteogenic differentiation of DMSCs. A recent study has shown that stathmin regulates odontogenic/osteogenic differentiation of DPSCs *via* the Shh signaling

pathway[180].

Inflammation

In an inflammatory microenvironment, DMSCs from inflamed tissue contact and interact closely with extrinsic irritants, local cells or their components, immune cells and multiple soluble regulatory molecules[181]. For example, dental caries are one such gram-negative microbial infection that is primarily responsible for pulpal inflammation. LPS was used to create *in vitro* inflammatory conditions that initiate infection-stem cell interaction, which has been used widely to induce an inflammatory microenvironment[182].

Immunophenotyping of cell surface antigens by flow cytometry showed that DMSCs and inflamed DMSCs have similar expression patterns of surface markers[181,183]. The cells are positive for STRO-1, CD105, CD73, CD90, CD29 and CD44[184] and negative for CD45, CD34, CD14 and HLA-DR, indicating a mesenchymal stem cell phenotype[183,185-187]. In addition, inflamed DMSCs have the potential to differentiate into multiple lineages. Mesenchymal stem cells isolated from inflamed pulp possess stemness and multidifferentiation potential similar to DPSCs from healthy pulp[185]. Like DPSCs, inflamed DPSCs are capable of adipogenic and osteo/dentinogenic differentiation under the corresponding *in vitro* induction conditions. However, chronic inflammation impairs differentiation of DPSCs[188]. On the other hand, inflamed DPSCs show increased ALP and osteocalcin. In the inflammatory microenvironment, PDLSCs from inflamed periodontal tissue show higher proliferation rates but express lower levels of osteogenic differentiation markers[189-191]. Both inflamed hPDLSCs and hPDLSCs have been successfully differentiated under osteogenic and adipogenic conditions[192]. Because of evident similarities in their immunomodulatory properties, inflamed PDLSCs can provide a promising alternative to PDLSCs[193]. Cells isolated from human periapical cysts demonstrate a strong osteogenic but weak adipogenic capacity[184,194]. Osteogenic differentiation of inflamed DFSCs results in decreased ALP activity and alizarin red S staining compared to normal DFSCs[195]. Similarly, the osteogenic differentiation of LPS-treated DFSCs is suppressed, and the cells display low levels of TGF-β1 and high levels of TGF-β2.

Aging

Aging is an intricate degenerative process during which the regenerative capacity of MSCs progressively declines[196]. Unavoidably, DMSCs also undergo physiological age-related changes with declines in proliferation and osteo/odontogenic differentiation potentials with increased age[197,198]. Improving the performance of aging DMSCs is important for tissue regeneration engineering. Yi *et al*[144] demonstrated that the osteogenic potential of DPSCs from young donors was superior to that of those from old donors, and 304 mRNAs and 561 LncRNAs were differentially expressed between age-groups[144]. Wang *et al*[199] found that miR-433 may be one of the important senescence-related miRNAs of human dental pulp cells, which inhibits mineralization of human dental pulp cells by negatively regulating GRB2 and the RAS–MAPK signaling pathway[199]. SHED and DPSCs undergo senescence, including declines in the proliferation rate and osteogenic differentiation capability, following serial expansion from P4 to P20. SHED exhibit a better performance than DPSCs, which indicates that mineralization capacity is related to replicative senescence *in vitro* and to donor age[200].

As a significant factor regulating the function of differentiated odontoblasts[201], sclerostin advances the aging process of human dental pulp cells through the Wnt/β-catenin pathway and reduces the proliferation and odontoblastic differentiation capability of senescent human dental pulp cells[202]. The Wnt/β-catenin signaling pathway is one of the important pathways that regulates cell differentiation, increasing the osteogenic/dentinogenic differentiation potential of DPSCs[203]. It has been reported that the rate of dentin deposition and neurogenic differentiation potential declines with advanced age, which may be related to a decrease in endogenous Wnt/β-catenin signaling[204,205].

In 2014, Feng *et al*[206] compared the characteristics of DPSCs from five different age groups (5–12 years, 12–20 years, 20–35 years, 35–50 years and > 50 years) and found that the expression of $p16^{INK4A}$ markedly increased with age and inhibited osteogenic/odontogenic differentiation when upregulated[206]. Then in 2017, Mas-Bargues *et al*[207] indicated that $p16^{INK4A}$ also played a part in oxidative stress-related premature senescence of DPSCs caused by long-term culture in 21% ambient oxygen tension compared with 3%-6% physiological oxygen tension[207]. Replicative senescence of DPSCs resulted in decreases of B-lymphoma Mo-MLV insertion region

1, organic carbon, DSP and bone sialoprotein compared with rapidly proliferating cells and increases of $p16^{INK4A}$, while B-lymphoma Mo-MLV insertion region 1 transduction promoted the expression of organic carbon and DSP, ALP activity and mineralized nodule formation. Therefore, this may indicate that the odontogenic differentiation potential of DPSCs weakens during senescence, partly due to decreased B-lymphoma Mo-MLV insertion region 1 expression[208].

In contrast, Ma *et al*[209] reported that adult DPSCs cultured in juvenile dental pulp cell-conditioned medium demonstrated decreased osteogenic differentiation capability, whereas juvenile DPSCs induced by adult dental pulp cell-conditioned medium showed improved osteogenic differentiation capability, indicating that the activity of DPSCs can be modulated by the extrinsic microenvironment[209]. A certain degree of inflammatory stimulation promoted the proliferation and mineralization of both adult and juvenile rat DPSCs, but this effect declined with age[210]. Furthermore, Horibe *et al*[211] isolated a type of mobilized dental pulp stem cells induced by granulocyte colony-stimulating factor from young and old donors, which showed minimal characteristic changes with aging, suggesting that mobilized dental pulp stem cells act as an advantaged source in dental pulp regeneration[211].

Exosomes

Exosomes are vesicles secreted by different cells with a diameter of 30–100 nm. They can function as carriers for different components to impact intercellular communication, including various miRNAs, lncRNAs and proteins. Exosomes play an important role in mediating some signaling pathways to influence the physiological function of cells. In recent years, increasing research into the effect of exosomes on the odontoblastic/osteogenic differentiation of DMSCs has been proposed (Figure 2).

In 2016, Huang *et al*[212] indicated that the exosomes derived from hDPSCs cultured with growth (DPSC-Exo) or odontogenic differentiation media (DPSC-OD-Exo) enhanced the odontogenic differentiation of DPSCs *in vitro*, and DPSC-OD-Exo showed stronger induction differentiation-inducing ability than exosomes derived from hDPSCs cultured with growth media in a three-dimensional environment consisting of type I collagen hydrogels and a tooth root-slice regeneration model[212]. In 2019, Hu *et al*[213] further identified the miRNA profile of human exosomes derived from hDPSCs cultured with growth media and DPSC-OD-Exo by miRNA sequencing, and the results indicated that miR-27a-5p was highly expressed in DPSC-OD-Exo, promoting odontogenic differentiation of DPSCs through the TGF-β1/Smad signaling pathway[213].

In 2019, Chew *et al*[214] reported that human MSC exosome-loaded collagen sponge used in an immunocompetent rat model with periodontal intrabony defects significantly repaired the defects by regenerating newly formed bone and periodontal ligament as a result of periodontal ligament cell migration and proliferation[214]. Meanwhile in 2020, Wang *et al*[215] reported that conditioned SHED-Exos derived from a 3 d osteogenic supernatant improved the osteogenic ability of PDLSCs by activating the BMP/Smad and Wnt/β-catenin signaling pathways and that BMP2 and Wnt3a carried by SHED-Exos played a pivotal part in this process[215].

Moreover, extracellular vesicles (EVs) are a type of mixed vesicles, consisting of endosome-derived exosomes and cell membrane-derived ectosomes. In 2017, Li *et al*[216] demonstrated that the EVs derived from Schwann cells promoted the osteogenic differentiation of hDPSCs[216]. In 2019, Čebatariūnienė *et al*[217] indicated that hPDLSC EVs did not influence osteogenic mineralization of PDLSCs but reversed the inhibitory effect on PDLSC osteogenic differentiation of an anti-TLR4 blocking Ab. They also revealed that the EVs may have a potential regulatory ability of genes related to osteogenesis and interfere with TLR4 signaling[217]. Additionally, Pizzicannella *et al*[218] reported that EVs derived from human GMSCs combined with a three-dimensional polylactide biomaterial enhanced the osteogenic differentiation of human GMSCs *in vitro*[218].

CONCLUSION

At present, most studies of the multidirectional differentiation of DMSCs focus on the following areas: the regeneration of teeth, bone, cartilage, tendon and blood vessels; the repair of nerve injury; the formation of retina and cornea; and the secretion of insulin. Different types of DMSCs have different abilities towards differentiation into diverse lineages. It is significant to explore the potential of DMSCs to differentiate into various tissues. In addition to the application of oral tissue regeneration, these studies

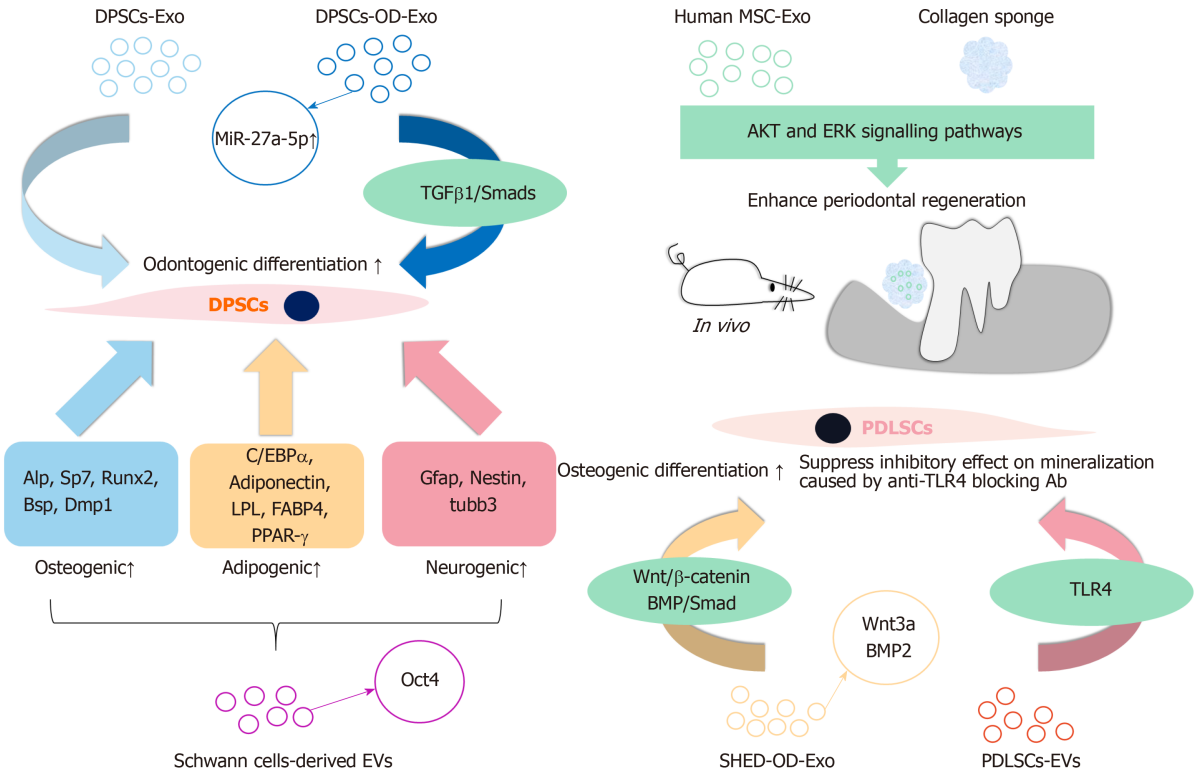

Figure 2 Reported extracellular vesicles that mainly contributed to the odontogenic/osteogenic differentiation process of dental mesenchymal stem cells. Extracellular vesicles (EVs) from a variety of cell sources can influence the osteogenic, adipogenic and neurogenic differentiation process of dental mesenchymal stem cells. Exo: Exosomes; DFSCs: Dental follicle stem cells; LPL: Lipoprotein lipase; MSC: Mesenchymal stem cells; PDLSCs: Periodontal ligament stem cells; PPAR-γ: Peroxisome proliferator-activated receptor-γ; SHED: Stem cells from human exfoliated deciduous teeth; TGFβ1: Transforming growth factor β1.

are helpful to the future application of DMSCs in neurovascular injury-related diseases, retinal and corneal injury-related diseases and endocrine diseases such as diabetes. The induction of DMSCs to differentiate insulin-producing cells and neuron-like cells *in vitro* requires the conditioned-culture medium with a variety of auxiliary inducing factors, like some growth factors and peptides, and sometimes it needs to be induced in several steps, which takes a long time and is relatively complex. The cells induced by the conditioned culture medium express the specific molecules of related tissue-like cells. Researchers detect the specific expression molecules to determine whether the cells differentiate into specific tissue-like cells. Such *in vitro* differentiation is often limited and may not represent the true differentiation of the cell itself. It is of great significance to improve the induction mode and shorten the induction time for the application of DMSCs in the future. In addition, combining DMSCs with materials possessing good biological compatibility may provide a better approach to tissue regeneration.

Making full use of the odontogenic/osteogenic differentiation ability of DMSCs is of great significance to the application of DMSCs in dental tissue regeneration engineering. In this review, some factors related to the regulation of DMSCs in odontogenic/osteogenic differentiation are reviewed. The regulation process of DMSC odontogenic/osteogenic differentiation is complex. A variety of non-coding RNAs and multiple signaling pathways participate in the differentiation process of DMSCs. The application of DMSCs should consider the donor age and cell aging. With increasing donor age and number of cell passages, differentiation ability may decrease accordingly. At the same time, the future clinical application of DMSCs should account for the impact of the inflammatory microenvironment. How to increase the anti-inflammatory ability of DMSCs is a difficult problem for clinical application of DMSCs in the future. In addition, exosomes, as a crucial medium for communication and transmission of information between cells, have become a hotspot in recent years. In the process of normal tooth development, exosomes also seem to play an important role in regulating gene expression of target cells through their rich and varied contents. Utilizing the characteristics of exosomes endocytosed by cells, discovering other exosomes or transforming contents to promote DMSC odontogenic/osteogenic

differentiation will be a future research direction. If we can positively regulate the related factors that advance the odontogenic/osteogenic differentiation of DMSCs and make full use of their differentiation potential, there will be great progress in the application of DMSCs in dental tissue regeneration engineering. Future research should emphasize effectively combining the various types of DMSCs with odontogenic/osteogenic, neurogenic, vascularization and other multipotencies to provide a potential scheme for dental tissue regeneration with normal functions.

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REVIEW

Stem cell therapy in ocular pathologies in the past 20 years

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Abstract

Stem cell therapies are successfully used in various fields of medicine. This new approach of research is also expanding in ophthalmology. Huge investments, resources and important clinical trials have been performed in stem cell research and in potential therapies. In recent years, great strides have been made in genetic research, which permitted and enhanced the differentiation of stem cells. Moreover, the possibility of exploiting stem cells from other districts (such as adipose, dental pulp, bone marrow stem cells, *etc.*) for the treatment of ophthalmic diseases, renders this topic fascinating. Furthermore, great strides have been made in biomedical engineering, which have proposed new materials and three-dimensional structures useful for cell therapy of the eye. The encouraging results obtained on clinical trials conducted on animals have given a significant boost in the creation of study protocols also in humans. Results are limited to date, but clinical trials continue to evolve. Our attention is centered on the literature reported over the past 20 years, considering animal (the most represented in literature) and human clinical trials, which are limiting. The aim of our review is to present a brief overview of the main types of treatments based on stem cells in the field of ophthalmic pathologies.

Key Words: Stem cells; Multipotent mesenchymal cells; Adipose stem cells; Novel therapies; Eye pathology; Cornea; Cell therapies; New materials

Core Tip: Stem cell therapies have shown great potential in ophthalmopathies. Interesting results have arisen in the treatment of ocular surface diseases and for their neuroprotective effect. Stem cells can be transplanted, injected or topically applied.

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The main goals of treatments include preventing vision loss, restoring sight, recreating the connections with the central nervous system, and regenerating eye tissues. Recent discoveries have permitted the use of stem cells taken from other districts. From literature analysis, it is clear that stem cells can provide a potentially successful solution; however, ethical issues, costs and possible long term side effects limit the current use.

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INTRODUCTION

Numerous studies in scientific medical literature continually show that cell therapy tends to be an effective alternative and innovative method to regenerate damaged tissue[1]. In the last decades, studies have shown that stem cell therapies can bring substantial benefits to patients suffering from a wide range of diseases and injuries[2]. For this reason, huge investments, resources and important clinical trials have been performed in stem cell research and potential therapies based on stem cells. In these past years, there has been a continuing expansion in the number and types of stem cells assessed for potential treatment use. Stem cells are now being used in various fields of medicine, ranging from hematology to cardiology, in addition to neurology, plastic surgery, dentistry, *etc.*[3-5]. Although literature has reported limited success to date, clinical trials continue to evolve as our understanding enhances regarding the physiology and mechanisms underlying potential healing benefits behind stem cells. Recent advancements in regenerative medicine have also considered the use of stem cells for cellular repair and regeneration[6].

Stem cell therapies have shown great potential in numerous studies reported in literature concerning injuries and diseases of the eye[3]. This progress is a result of several factors, including the relatively small numbers of cells required, easy accessibility for surgery and straightforward assessment and visualization of grafts. Numerous types of cells have been used in clinical trials for the eye[2]. Research on stem cell therapies has been applied to almost all parts of the eye.

Based on our preliminary animal experiments regarding stem cells therapies in corneal healing[3,7], and the growing number of studies that show great clinical potentials of stem cells for this therapeutic approach, we decided to assess the literature reported in the last 20 years. Considering that the number of studies reported in literature since 2000 is immense, and our experience in this field is limiting, we do not intend to provide an exhaustive meta-analysis, but a quick overview of the use of stem cells for ophthalmology treatment; thus, we apologize in advance if opinion leaders and experts in this field of study have not been cited in our paper. The aim of our review is to present a brief overview of the main types of treatments based on stem cells in the field of ophthalmic pathologies, by briefly addressing the what, why, which, how, when and where of this issue. We have tried to concentrate or review on pertinent human studies; however, considering most of the literature to date tends to be based on animal experimentation, mention has also been made regarding this vast body of literature.

MATERIALS AND METHODS

We conducted a search of the literature published between January 1, 2000, to December 20, 2020, using MEDLINE (PubMed). The database was first searched using the key words "stem, cell and eye", in which 7486 studies were found. We considered only studies in English and those referring to humans, thus reducing the count to 3941. The reference lists of all retrieved articles were scanned to identify additional relevant studies. We then considered only articles based on "stem cells therapy" (2194 papers), and we excluded "case reports", "case series", "conference papers", "letters" and "*in vitro*" (1179 articles). Results were then divided and sorted by when/where stem cells

were used, which included: Ocular surface; corneal epithelium, stroma, endothelium, limbus; trabecular meshwork; lens; optic nerve; retina. The details regarding the selection of papers considered in the manuscript are listed in Figure 1.

Only articles with abstracts were considered (1148 articles). After a selection by title and abstract, 185 articles were analyzed. A quality score was calculated for each article using a checklist from the American Society of Plastic Surgeons guidelines for therapeutic studies[7]. Each study was appraised by at least two reviewers (GM and MZ), and rating decisions were based on the consensus of the reviewing authors. A summary of the most significant studies and conclusions is reported in Tables 1 and 2.

What are stem cells

In brief, stem cells are undifferentiated cells that are present in the embryonic, fetal and adult stages of life and give rise to differentiated cells, which are the building blocks of tissue and organs. In the post-natal and adult stages of life, tissue-specific stem cells are found in differentiated organs and are instrumental in repair following injury to the organ. The main characteristics of stem cells are self-renewal (the ability to proliferate extensively), clonality (usually arising from a single cell) and potency (the ability to differentiate into different cell types)[8].

Stem cells can be classified as totipotent (zygote), pluripotent [embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)], multipotent [mesenchymal stem cells (MSCs)] and oligopotent. Totipotent cells form embryonic and extra-embryonic tissues. Pluripotent cells form all three germ layers, while multipotent cells generate cells limited to one germ layer.

The human body develops from the zygote and blastocyst from which ESCs are derived into germ layers endoderm, mesoderm and ectoderm. Specific organs arise from germ layers. Some of the progenitor cells that have contributed to organ formation do not terminally differentiate but are retained as tissue stem cells and can be found in bone marrow, bone, blood, muscle, liver, brain, adipose tissue, skin and gastrointestinal tract[9]. The tissue stem cells may be called progenitor cells since they give rise to terminally differentiated and specialized cells of the tissue or organ. These cells may be dormant within tissues; however, have the ability to proliferate under circumstances of injury and repair[10]. The dynamics of tissue stem cells or progenitor cells varies from tissue to tissue. In bone marrow, liver, lung and gut, for example, stem cells regularly proliferate to supplement cells during normal turnover of cells or tissue injury. In other organs, however, like pancreas, heart or nervous system, stem cells proliferate to replace damaged cells following injury[11,12].

Why use stem cells in ocular diseases

The human eye is a remarkable structure produced from the coordinated development of multiple tissues. It is the result of a combination of tissues deriving from neuroectodermal (*e.g.*, retina), ectodermal (*e.g.*, lens and cornea) and mesodermal lines. Diseases and injuries that compromise the function of any of these major ocular tissues can lead to blindness[13,14]. Considering the fundamental importance and influence on quality of life of sight, research has invested enormous resources and studies towards the search for new therapies to prevent and treat ocular disorders. There is a notable history of trail-blazing work in ocular medicine, exemplified by tissue transplantation, the use of laser therapy, the recent gene-therapy and cell therapy[14]. Due to burden of eye disease, and its relative ease of accessibility, the eye is a prime target for stem cell transplantation therapies, in which complications tend to be rare (*e.g.*, overgrowth and tumor formation)[15]. In addition, advanced methods currently exist to examine easily and thoroughly assess the clinical results of eye transplant therapies. Modern technology is readily available to provide a non-invasive quantifiable visualization of most structures of the eye, and visual functions can routinely be assessed rapidly, quantitatively and accurately.

Which stem cells can be considered for treatment in ophthalmology

Human pluripotent stem cells: ESCs and iPSCs: Human pluripotent stem cells (hPSCs) have been considered promising sources for regenerating damaged tissues and organs because of their ability to differentiate into cells from three embryonic germ layers[16]. These cells can also be maintained in an undifferentiated state for a prolonged period in culture[8]. There are numerous scientific studies regarding therapy with hPSCs, specifically, in the form of iPSCs. Several studies have reported the use of iPSCs in retinal degenerative pathologies[2,17,18]. hPSCs have the greatest potential for cell replacement and can be successfully pre-differentiated prior to transplantation in the eye. iPSCs are pluripotent stem cells generated from somatic

Table 1 Clinical trials in ocular diseases

Ophthalmic disease	Ref.	Stem cells used	In animals or humans	Conclusions
Ocular surface, cornea and limbus	Kenyon and Tseng[52], 1989	Limbal tissue autograft transplantation (LSCs)	Humans	Patients have consistently shown improved visual acuity, rapid surface healing, stable epithelial adhesion without recurrent erosion, arrest or regression of corneal neovascularization
	Lindberg *et al*[53], 1993	LSCs	Humans	Corneal epithelial stem cells are located in the limbus and indicate that cultured autologous limbal cells may function as grafts to permanently restore the corneal epithelium after severe ocular surface injury
	Ma *et al*[54], 2006	MSCs like LSCs on amniotic membrane	Animals	Therapeutic effect due to the inhibition of inflammation and neovascularization
	Kwitko *et al*[57], 1995	Conjunctival autograft transplantation	Humans	Conjunctival transplantation proved to be an adequate method of treating severe bilateral surface disorders, with minimal complications
	Croasdale *et al*[58], 1999	Keratolimbal allograft	Humans	KLAL transplantation for patients with severe ocular-surface disease is an important management option
	Tsai *et al*[59], 2000	Limbal epithelial cells	Humans	By 1 mo, the ocular surface was covered with corneal epithelium, and the clarity of the cornea was improved in 83% of patients
	Sangwan *et al*[60], 2012	Limbal tissue	Humans	After surgery, a completely epithelialized, avascular and stable corneal surface was seen in all recipient eyes by 6 wk
	Kushnerev *et al*[61], 2016	Dental pulp stem cells	Animals	Dental pulp stem cells were successfully isolated, labeled, and delivered to the corneal surface
	Chan *et al*[66], 2013	iPSCs	Animals	hES cells can be induced to differentiate into keratocytes *in vitro*. Pluripotent stem cells may provide a renewable source of material for development of treatment of corneal stromal opacities
	Susaimanickam *et al*[67], 2017	iPSCs	Animals	PSC-derived corneal epithelial cells offer an alternative tissue source for regenerating different layers of the cornea and eliminate the need for complicated cell enrichment procedures
	Zeppier *et al*[3], 2017	MSCs	Animals	Better corneal repair in epithelium and stromal layers in stem cell treated eyes
	Reinshagen *et al*[68], 2011	BM-MSCs	Animals	BM-MSCs could differentiate into cornel epithelial like cells *in vivo* in rat damaged corneas
	Gu *et al*[70], 2009	BM-MSCs	Animals	BM-MSCs could differentiate into cornel epithelial like cells
	Beyazyildiz *et al*[46], 2014	MSCs	Animals	Topical application of MSCs could be a safe and effective method for the treatment of DES
	Reza *et al*[73], 2011	LSCs	Animals	Transplantation of a bioengineered CLEC-muc sheet in limbal stem cell-deficient rabbit eyes resulted in regeneration of a smooth, clear corneal surface
	Nishida *et al*[76], 2004	Oral mucosal stem cells	Humans	Complete reepithelialization of the corneal surfaces occurred within 1 wk in all four treated eyes. Corneal transparency was restored and postoperative visual acuity improved
Corneal stroma and endothelium	Sepsakos *et al*[79], 2017	Ocular surface SCs	Humans	Without addressing the underlying stem cell deficiency, keratoplasty in patients with total limbal stem cell deficiency will ultimately fail in all cases
	Naylor *et al*[81], 2016	iPSCs	Humans	The hiPSC-derived NCCs acquired a keratocyte-like morphology and an expression profile similar to corneal keratocytes *in vivo*
	Alió Del Barrio *et al*[84], 2017	ASCs	Humans	Safety of corneal stromal transplantation of autologous ASCs in humans, showing cell

				survival in vivo and the ability of these cells to produce a low amount of new collagen in patients with advanced keratoconus
	Coulson-Thomas *et al* [85], 2013	UC-MSCs	Animals	UC-MSCs transplantation may be a feasible alternative to keratoplasty in treating congenital disorders of the cornea secondary to keratocyte dysfunction
	Yam *et al*[88], 2018	Perodontal ligament SCs	Animals	Potential translation of PDL cells for regenerative corneal cell therapy for corneal opacities
	Joyce *et al*[89], 2012	UC-MSCs	Animals	UCB MSCs are able to "home" to areas of injured corneal endothelium and that the phenotype of UCB MSCs can be altered toward that of HCEC-like cells
Trabecular meshwork	Abu-Hassan *et al*[93], 2015	iPSCs	Animals	TM-like iPSCs became similar to TM cells in both morphology and expression patterns. When transplanted, they were able to fully restore intraocular pressure homeostatic function
	Manuguerra-Gagné*et al* [95], 2013	MSCs	Animals	MSC and their secreted factors induced reactivation of a progenitor cell pool found in the ciliary body and increased cellular proliferation
Lens	Lin *et al*[96], 2016	LECs	Both	Surgical method of cataract removal that preserves endogenous LECs and achieves functional lens regeneration in rabbits and macaques, as well as in human infants with cataracts
	Murphy *et al*[98], 2018	PSCs	Animals	We demonstrate large-scale production of light-focusing human micro-lenses from spheroidal masses of human lens epithelial cells purified from differentiating pluripotent stem cells
ON	Kuwahara *et al*[102], 2015	hESCs	Animals	Multipotent stem cells within the CM contribute to *de novo* retinal tissue growth
	Mesentier-Louro *et al* [105], 2019	iPSCs	Animals	Between 1% and 7% of iPSCs-derived RGCs integrated into the ganglion cell layer after intravitreal injection, and about 20% after combined injection of RGCs and iPSCs
	Zhang *et al*[107], 2015	UC-MSCs	Animals	Human umbilical cord blood stem cells and brain-derived neurotrophic factor effectively repair the injured optical nerve, improve biomechanical properties, and contribute to the recovery after injury

ASC: Adipose-derived stem cell; BM-MSC: Bone marrow-mesenchymal stem cell; CLEs: Cutaneous lupus erythematosus; CM: Ciliary margin; DES: Discrete event simulation; HCEC: Human corneal endothelial cell; hESCs: Human embryonic stem cells; hiPSC: Human-induced pluripotent stem cells; iPSC: induced pluripotent stem cell; KLAL: Keratolimbal allograft; LEC: Lens epithelial stem/progenitor cell; LSC: Limbal stem cell; MSC: Mesenchymal stem cell; NCC: Neurocysticercosis; ON: Optic nerve; PDL: Periodontal ligament; PSC: Pluripotent stem cell; RGC: Retinal ganglion cell; TM: Trabecular meshwork; UC-MSCs: Umbilical cord-mesenchymal stem cell; UCB: Umbilical cord blood.

cells by cellular genetic reprogramming using defined transcription factors[8]. First described in 2007 by Takahashi *et al*[19], iPSCs used in the experiments were derived from skin fibroblasts produced using retroviral technology[19]. iPSCs provide a unique *in vitro* model that allows the generation of retinal progenitor cells. An advantage of iPSCs made from a patient's own cells could reduce the need for immunoprotective regimens post-transplantation[15]. It has been demonstrated that these iPSCs have similar characteristics features of PSCs, conserving the possibility to generate tissues from each of the three germ layers[20]. The greatest problem reported regarding iPSCs was genomic instability, which may induce teratoma. The aim of research in recent years has been to create safety protocols capable of guaranteeing genomic stability[21]. The greatest success of hPSCs has been in retinal pigment epithelial cell (RPE)/photoreceptor replacement for aged-related macular degeneration (AMD), but studies have also reported the potential successful use in almost all structures of the eye[22,23].

Adult stem cells: The use of adult stem cells represents an easier route to regenerative-cell therapies. The ability of some adult tissues (*i.e.* skin, haemopoietic system, bone, liver, *etc.*) to repair or renew, indicates the presence of stem or progenitor cells[9]. Numerous clinical trials based on the use of adult stem cells have also been developed

Table 2 Retinal diseases and therapies

Ophthalmic disease	Ref.	Stem cells used	In animals or in humans	Conclusions
Retina	Van Meurs *et al* [109], 2004	RPE cells	Humans	A pigmented area was seen in the extraction bed of the neovascular membrane in only one patient
	Tucker *et al* [110], 2011	iPSCs	Animals	Adult fibroblast-derived iPSCs provide a viable source for the production of retinal precursors to be used for transplantation and treatment of retinal degenerative disease
	Lamba *et al* [111], 2006	hESCs	Animals	hES cell derived retinal progenitors integrate with the degenerated mouse retina and increase in their expression of photoreceptor-specific markers
	Nakano *et al* [48], 2012	IPSCs/hESCs	Humans	We demonstrate that an optic cup structure can form by self-organization in hESC culture
	Gonzalez-Cordero *et al* [113], 2013	ESCs	Animals	We show that rod precursors integrate within degenerate retinas of adult mice and mature into outer segment-bearing photoreceptors
	Schwartz *et al* [40], 2015	hESCs	Humans	First evidence of the medium-term to long-term safety, graft survival, and possible biological activity of pluripotent stem cell progeny in individuals with macular diseases
	Schwartz *et al* [114], 2012	hESCs	Humans	The hESC-derived RPE cells showed no signs of hyperproliferation, tumorigenicity, ectopic tissue formation, or apparent rejection after 4 mo
	Gaddam *et al* [120], 2019	Adult stem cells	Humans	The paracrine nature of adipose stem cells, in particular, has been highlighted as a potential solution to the lack of a homing and conducive environment that poses a challenge to the implantation of exogenous stem cells in the target tissue

hESCs: Human embryonic stem cells; iPSC: induced pluripotent stem cell; RPE: Retinal pigment epithelium.

in the field of ophthalmology.

Considering that the component of the eye, mainly the retina, is directly derived from the central nervous system (CNS), studies regarding cell therapy have shown to be difficult, limiting, yet potentially fascinating. The nervous system was thought to be rigidly constructed, with no capacity for physical repair. However, studies of nerve development have led to isolation of cells from the developing and mature mammalian CNS that shows numerous properties of stem cells [neural stem cells (NSCs)][9]. NSCs have been studied with success in spinal cord injury and traumatic brain injury[24]. NSCs secrete trophic factors, and therapies involving these cells may have potential for the neuroprotection of photoreceptors rather than replacement of retinal neurons, including retinal ganglion cells (RGC)[22,25].

Bone marrow stem cells (BMSCs) are progenitors of bone, cartilage and skeletal tissues, in addition to the hematopoiesis-supporting stroma and adipocyte cells. These cells can differentiate into mesenchymal cells, visceral mesoderm, neuroectoderm and endoderm characteristics *in vitro*[26]. In ophthalmology, BMSCs can be used to prevent graft *vs* host disease in corneal transplantation. These cells have also been studied in retinal diseases. The rationale for exploring the use of BMSCs as a potential therapy is a paracrine trophic effect on degenerating ischemic retina[22,27].

The previous view of adult stem cells has been that the differentiation potential was strictly limited to cell lineages found within the tissue of origin studied in tissues such as skin and bone marrow. During the past few years, this view has changed. Several studies have shown apparent plasticity of adult stem cells, like the ability to differentiate to cell types other than the tissue of origin[9]. This remarkable finding challenged the long-held assumption that truly multipotent or pluripotent stem cells did not persist beyond early stages in embryogenesis.

Multipotent stem cells can be inserted in this context because they express markers of pluripotency previously seen only in ESCs or pregastrulation embryos[9]. The most commonly studied multipotent SCs in ophthalmopathies are MSCs. MSCs are classically the "post-natal, self-renewing and multipotent stem cells giving rise to all skeletal tissues"[2]. They have been found in different fetal tissues, in extraembryonic tissues [placenta, umbilical cord (UC) and amniotic fluid] and in adult tissues (bone marrow, peripheral blood, adipose tissue, dermis, synovium, periosteoum, cartilage, skeletal muscle, fallopian tubes, menstrual blood, gingiva and dental tissue and eye) [28]. Placental and adipose-derived MSCs are, by far, considered in the largest number

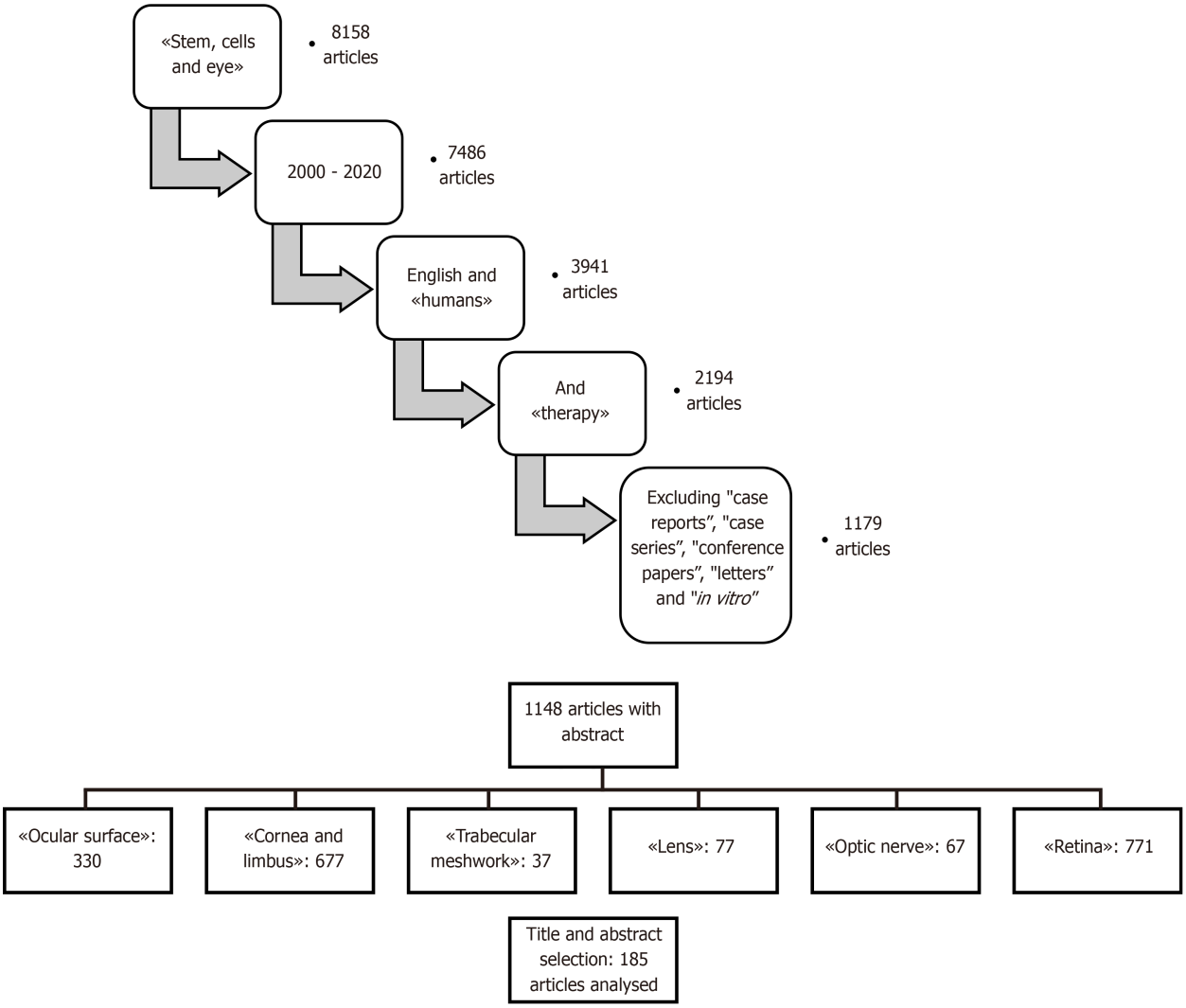

Figure 1 The selection of PubMed literature published from 2000 to 2020, which was considered in the manuscript.

of clinical trials.

Fetal MSCs are scarcely considered in reported studies probably due to the difficulties in achieving sufficient cell numbers after expansion[29].

UC-MSCs have excellent proliferation and differentiation properties. These cells are readily available in large quantities and cultured rapidly. Moreover, UC incorporates both mesenchymal and epithelial stem cells with anti-inflammatory and immune-privilege properties that can be differentiated into corneal epithelial, stromal and endothelial cells[29].

Human adipose-derived stem cells (ASCs) are an easily accessible autologous stem cell source. They have been shown to support the growth of many types of stem cells including human embryonic stem cells (hESCs), iPSCs and limbal stem cells (LSCs) [30]. ASCs resemble BMSCs in terms of morphology, proliferation and multipotency [31]. These cells have been considered for various ocular disorders, from retinal degeneration (neuroprotection and neurogenesis)[22,31] to corneal dise-ases[1,3,32].

Dental pulp stem cells (DPSCs) were the first human dental stem cells isolated from the dental pulp of permanent teeth[33]. These cells show the unique characteristic of the potential to differentiate into not only typical mesodermal cell lineages but also ectodermal and endodermal cell lineages. DPSCs have exhibited the potential to differentiate into active neurons, cardiomyocytes, myocytes, melanocytes and hepatocyte-like cells. These cells have shown higher angiogenic, neurogenic and regenerative potential when compared to BMSCs. In the field of ophthalmology, these cells have been studied for corneal repair[34], treatment of glaucoma and retinal diseases[35].

Going even more specifically, let us analyze the eye structure. Within the healthy eye, different types of adult stem cells can be found, which can be considered as eye stem cells. Retinal pigment epithelium stem cells (RPESCs) represent one of the latest

discoveries in terms of stem cells of the eye. Retinal epithelium arises from the CNS neuro-epithelium. Interestingly, studies have shown how these cells can proliferate in some animal species and in certain conditions (*e.g.*, injury). Salero *et al*[36] identified a subpopulation of RPE cells that can be activated to self-renew and that exhibit multipotentially, producing either stable RPE progeny or neural, osteo, chondro or adipo-lineage mesenchymal progeny[36]. Studies have demonstrated how RPESCs, to date unknown, can proliferate pathologically in specific conditions, giving rise to retinal disorders. RPESCs are considered as a new type of SC capable of producing both CNS and mesoderm-associated lineages[36]. Autologous RPE cell replacement has provided proof-of-concept evidence that cell therapy may have promising applications for retinal degenerative diseases. RPE cell source, however, is restricted for applications in RPE cell replacement due to the limited number of donor eyes and ethical issues concerning this type of treatment[37].

Hallmark features of the corneal epithelium include the high regenerative potential and the capacity for rapid ocular surface repair through proliferation and centripetal migration of progenitor cell populations residing at the border of the cornea and the sclera in a location called limbus. LSCs represent a quiescent cell population with high proliferative potential, which enables efficient corneal regeneration and repair[38]. LSCs are fundamental for corneal homeostasis. A loss or deficiency [LSC deficiency (LSCD)] of these cells causes the disruption of homeostasis[39]. LSCs can be collected using biopsy and expanded in culture, and then they can be transplanted in the diseased eye[1].

Criteria for selecting stem cells type: As explained above, stem cells for the treatment of ocular disorders can be obtained from the same eye (or fellow eye), from other tissues of the patient, or from a donor. As with all treatments, clinicians seek safe, widely available and economically viable therapies. Obtaining SCs from the patient's own tissues have changed the therapeutic perspectives. This option has the advantage of not requiring immunosuppressive therapies and guarantees no immune reactions [40]. Another element that guides the choice of SCs is the availability of cells. Alternatives to pluripotent stem and eye tissue cells have been considered in literature, however, these options tend to be limiting due to ethical constraints, possible unknown adverse effects, little availability and unsustainable costs[21,37]. Autologous sources, such as MSCs (UC and adipose stem cells in particular), have been widely studied in recent years because of the large availability and the confirmed safety in other medical uses[3,4], and seem at the moment to preferential options for the future.

How can stem cells be used in clinical research

Multiple techniques to approach stem cell therapy for ophthalmic disorders have been described and studied over time.

Transplantation is the most used technique for stem cell therapy in the eye. SCs can be transplanted either as a cell suspension[40] or on different substrates, like autologous sheets of cells, biomaterial-based patches or three-dimensional (3D) structures[41,42]. SC transplantation is a technique substantially used in all eye structures. The key sites currently targeted include: The cornea, mainly the clear tissue covering the front of the eye that helps focus the incoming light; the neural retina, which contains the photoreceptors; and the RPE, a single layer of pigmented cells that plays a key role in maintaining the photoreceptor cells and the blood-retina barrier[15, 41,42].

Stem cells injection (pluripotent, MSCs or hematopoietic stem cells)[27,40] is a procedure still under study today, considered especially for the treatment of AMD. To date, these therapies are not yet proven to be safe and effective in ophthalmic use, however, have shown to be widely validated in other body districts[43]. Several clinical experiments have proposed the injection of SCs into the intravitreal space (exploiting the paracrine effect of SCs) or in the subretinal space; however, results were not as expected, and several collateral effects have been reported in literature[44] that have partially "extinguished" the enthusiasm for results obtained in animal clinical trials.

Topical stem cells therapies (eye drops) have been extensively studied to treat ocular surface diseases. Hematopoietic stem cells from UC blood serum[45] have been proposed for topical treatment of ocular surface diseases. The rationale for applying serum to the ocular surface is that, compared to conventional lubricant treatments, it more closely resembles natural tears due to several of its biochemical constituents. It has produced satisfactory results in terms of efficacy and safety. Moreover, MSCs (in particular BMSCs[46] and ASCs[3]) have been used topically for dry eye syndrome or other corneal injuries or diseases with promising results. The rationale for MSCs eye

drops is the wide-ranging differentiation potential, anti-inflammatory and immunomodulatory effects of MSCs[46-49].

In what ocular disorders can stem cells be used

Ocular surface, cornea and limbus: The ocular surface is the interface between the functioning eye and the environment. This surface provides anatomic, physiologic and immunologic protection and comprises the palpebral and bulbar conjunctival epithelium, the corneoscleral limbus, the corneal epithelium and the tear film[50]. The functions of ocular surface include maintaining optical clarity of the cornea and protection of the structures of the eye from microbes, trauma and toxins. Disorders of the ocular surface include a variety of conditions. The most common are dry-eye disease, blepharitis, ocular allergies and pterygia. Less common diseases are LSCD and ocular surface diseases due to systemic diseases. The narrow zone between cornea and bulbar conjunctiva is defined as limbus, which contains layers of cells populated by Langerhans cells, melanocytes and LSCs (the stem cells that generate corneal epithelium). If a patient has an extensive destruction of the limbus, a functional corneal epithelium can no longer be formed, and the cornea reacquires an epithelium by invasion of bulbar conjunctival cells. The only way to prevent the corneal conjunctivation is indeed to restore the limbus. Keratoplasty has been considered the conventional gold standard treatment before the introducing of regenerative medicine [51].

The first attempt to restore limbus, using free limbal tissue grafts from the uninjured eye, was published by Kenyon and Tseng in 1989[52]. The potential of limbus grafts promoted additional studies in this field, which brought the discovery of LSCs[38,53]. In 2006, Ma *et al*[54] showed how transplantation of human MSCs on amniotic membrane, like LSCs on amniotic membrane, could reconstruct severely damaged rat corneal surface. They demonstrated how the therapeutic effect did not come from epithelial differentiation of MSCs but was probably due to the inhibition of inflammation and neovascularization.

Since then, cultures of LSCs have been used to treat corneal diseases and injuries, initially engrafting the injured eye from the uninjured one[55], then treating bilateral pathologies with expanded cultures of LSCs[56]. The fragility and limited number of active SCs of the corneal epithelium in culture, in addition to the induction of iatrogenic damage to the healthy fellow eye, however, tend to slow-down and limit the clinical application of LSCs. For these reasons, several materials have been proposed to enhance results, which include fibrin glue, amniotic membrane, polymers, collagen sponges or strips and devitalized membrane or polymers[57].

LSCD is the most challenging disease of the ocular surface for ophthalmologists. Corneal LSC grafting procedures have been utilized. This procedure involves either direct transplantation of limbal tissue or transplantation of *in vitro* expanded cells on a variety of biological or synthetic carrier materials[29]. These techniques include conjunctival limbal autograft, living-related conjunctival allograft[57], keratolimbal allograft[58], autologous *ex vivo* cultivated limbal epithelial transplantation[59], simple limbal epithelial transplantation[60], cultivated oral mucosal epithelial transplantation[61] and transplan-tation of peripheral corneal cells[62]. Clinical studies using conjunctival limbal autografts have shown excellent short-term and good long-term results, however, with high risks of complications, which mainly include induced damage to the donor eye[63]. For these reason, other types of stem cells have been proposed to produce LSCs and corneal epithelium cells.

In 2012, an efficient protocol, with positive preliminary results, was proposed to produce corneal epithelial cells from human-iPSCs (hiPSCs)\ESCs obtained from hair follicles or dermal fibroblasts[64]. Results were also then confirmed in successive studies[65,66]. The evolution of this approach led to the creation of corneal organoids from iPSCs[23], initially in two-dimensional, and then in 3D culture systems, with promising results as alternative for the treatment of bilateral LSCD[67].

MSCs have also been considered for ocular surface therapy[3,55]. Preliminary results in animal experiments are encouraging, and the potential is great. Studies have shown how bone marrow-MSCs (BM-MSCs) are safe and can restore corneal epithelia from LSCD with the same results of cultivated limbal epithelial transplantation[68]. BM-MSC associated with amniotic membranes have successfully been evaluated in LSD[69]. Gu *et al*[70] demonstrated how BM-MSCs could differentiate into cornel epithelial like cells *in vivo* in rat damaged corneas[70]. MSCs have been proposed also to promote graft survival[71,72]. This can be explained by the potent immunomodulatory properties of SC, suppressing maturation and activation of atrial premature complexes and dendritic cells and cytotoxicity of natural killer cells. Several studies have attempted to exploit these features of MSCs and have shown interesting results

by using topical therapies with eye drops containing MSCs (mainly BM-MSC and ASCs)[45,46]. Topical application of MSCs can lead to an increase in aqueous tear volume and improvement in ocular surface evaluation tests. Moreover, it is a safe and easy applicable method.

Cells derived from epithelial UC-MSCs are capable of forming a stratified epithelial layer when seeded on artificial matrices. Reza *et al*[73] first described the properties of UC-MSCs, finding how they have similar characteristics of LSCs[73]. Garzón *et al*[74] utilized an epidermal growth factor enriched medium to induce UC-MSCs to transdifferentiate into corneal epithelial cells on a 3D human artificial anterior cornea model [74]. The aim of the use of UC-MSCs derived epithelial cells is to circumvent the problem of the limited amount of limbal tissue available for transplantation, especially in cases of bilateral LSCD[29].

With regards to MSCs, human immature dental pulp and oral mucosa stem cells have been reported to be a promising cell source to correct LSCD, as described by Hassan and AbdelAziz[75] and Nishida *et al*[76]. Studies about ocular surface diseases and their treatment are reported in Table 1.

Research is currently searching for new ways of applications of stem cells therapy. As described above, MSCs derived exosomes is one of these[28]. The other way recently described to facilitate and carry cells to the ocular surface is the use of contact lenses[77].

Corneal stroma and endothelium: The corneal stroma is the thickest corneal layer (approximately 90% of corneal thickness) and is composed of specialized extracellular matrix components and collagen fibrils. Corneal stromal keratinocytes (CSKs) are the major cell type in corneal stroma and are located between the collagenous lamellae, which are generally quiescent[29]. Corneal insults cause death of CSKs at the injured site. Surviving CSKs near the injured site are activated to proliferate, while some fibroblasts are induced to transform in highly contractile myofibroblasts. When fibroblasts action prevails, scaring and consequent opacity of cornea can occur. The presence of dense opacities and persisting scars can interfere with the passage of light and cause an important loss of visual acuity if the opacity is large and localized in the center of the visual axis of the cornea, thus have an important burden on quality of life of patients[78].

Keratoplasty is a primary treatment option and current gold standard to treat numerous corneal conditions, including corneal injury, corneal dystrophy, keratoconus and corneal infractions. The ever-increasing number of patients needing keratoplasty has led to the shortfall of viable donor corneas. Moreover, it is important to note that post-surgical complications include important and sight threatening conditions, in addition to the risks of transplant rejections and elevated permanently induced astigmatism[23]. Sepsakos *et al*[79] demonstrated that patients who underwent an ocular surface SCs transplantation (*e.g.*, with LSCs) before keratoplasty have better results than patients that did not do that[79].

Various trials (Table 1) trying to obtain keratinocytes derived from hiPSCs have been proposed in the past several years[80,81]; however, the genetic reprogramming of iPSCs tends to be quite intricate. The differentiation of iPSCs to keratinocytes involves two steps[81]. The possibility of obtaining CSKs from iPSCs is very interesting in animal models; to date, clinical studies involving injection in humans are lacking in literature[82].

MSCs express high levels of hepatocyte growth factor (HGF) in an inflamed environment, as is the case in an injured cornea. HGF inhibits the generation of opacity-inducing myofibroblasts and, alone, can restore corneal transparency in an *in vivo* model of eye injury[83]. This is the rationale that has been proposed for the use of these cells. All MSCs seem to have similar behavior *in vivo*, being able to achieve keratocyte differentiation and modulate the corneal stroma. ASCs have been demonstrated to be an ideal source of autologous stem cells, while BM-MSCs have the same profile, but the extraction by bone marrow puncture is more complicated, not free of risks and painful. A pilot study by del Barrio *et al*[84] demonstrated the apparent safety of corneal stromal transplantation of autologous ASCs in humans, showing cell survival *in vivo* and the ability of these cells to produce a low amount of new collagen in patients with advanced keratoconus. Several studies on animals have suggested that UC-MSC transplantation may be a feasible alternative to keratoplasty in treating congenital disorders of the cornea secondary to keratocyte dysfunction such as mucopolysaccharidoses, giving rise to potential future possibilities[85,86]. UC-MSCs could represent an attractive alternative, but the autologous use of these cells tends to be expensive and currently almost impossible.

An alternative stem cell population currently reported in clinical trials for the therapy of corneal stroma disorders include corneal stromal stem cells (CSSCs). These cells show similar properties of MSCs and can be obtained by specific cultures of LSCs. Experimental studies have shown that CSSCs can differentiate in CSKs after injection in the stroma[87].

Periodontal ligament stem cells and DPSCs could differentiate in CSKs and prove to be of potential clinical use[28,88].

The corneal endothelium is the posterior corneal surface. It is formed by a monolayer of endothelial cells (CECs) that regulate the stromal hydration, preventing edema and maintaining corneal deturgescence, which are all fundamental for normal vision. These cells are non-mitotic and have limited regenerative capacity. Diseases that affect the corneal endothelium cause stromal edema, central opacity and vision loss. Full thickness or partial corneal transplantation has been the gold standard for endothelial diseases to date; however, preliminary studies have shown that stem cells therapies could be of potential clinical use[28,29]. MSCs could serve as a potential source to generate corneal endothelial cells for the treatment of corneal endothelial diseases, like Fuchs' endothelial dystrophy and aphakic/pseudophakic bullous keratopathy. Joyce *et al*[89] demonstrated the potential of UC-MSCs to differentiate in CECs when treated in particular cultures[89]. At this moment, however, there are no concrete data in current literature on the possible application of SCs in human endothelial disorders, which remains to be the primary cause of corneal transplantation[29].

Trabecular meshwork: Glaucoma is a degenerative optic neuropathy affecting approximately 70 million people worldwide. The most frequent cause of glaucoma is the increase of intraocular pressure (IOP) due to an alteration of aqueous outflow. Humor aqueous is continuously produced, and its outflow is controlled by the trabecular meshwork (TM), an avascular tissue located within the uvea and posterior to the corneal margin[90]. TM is populated by specialized cells that decline in number with age, and these cells are particularly low in individuals with primary open glaucoma. It is probable that TM cells become dysfunctional prior to death and increasingly fail to carry out the physiological roles, leading to enhanced aqueous humor outflow resistance and the development of pathologically elevated IOP[91]. Despite the importance of controlling IOP, the ultimate cause of glaucoma-associated vision loss includes axonal damage and the progressive loss of RGCs, the axons of retinal neurons that make up the optic nerve and transmit visual information from the eye to the brain[91]. The presence of MSCs in TM have been suspected for years and then confirmed and propagated *in vitro*[92].

TM cells are difficult to obtain from a living eye. Studies have faced this problem by using iPSCs generated *via* genetic reprogramming of adult somatic cells[19]. To date, the use of TM-like cells derived from iPSCs from a patient's own dermal fibroblasts may offer the best solution to the challenge of TM cell replacement therapy[92]. Abu-Hassan *et al*[93] evaluated the results of iPSCs derived TM cell transplantation. The study showed that iPSCs differentiate to resemble TM cells in several keyways and have the potential to restore the primary TM cell function, thus maintaining IOP homeostasis[93].

Recently, other stem cells have been proposed to find the possibility of obtaining TM cells (Table 1). MSCs have been used in attempt to repair TM tissue predominantly in animals[94]. Kumar *et al*[31] described the differentiation of ASCs in TM cells[31], and Manuguerra-Gagné *et al*[95] studied the regeneration of TM from BM-MSCs with encouraging results[95]. Data in stem cell models are of utmost importance and the basis when designing future studies with potential clinical benefits.

Lens: Cataracts are the leading cause of blindness in the world. The current standard of care in congenital cataracts involves surgical removal of the cataractous lens and implantation of an artificial intraocular lens. Surgery is not free of complications, especially in patients with complicated cataracts and with other underlying ophthalmic conditions.

Human lens regeneration has not been demonstrated yet, however, lens epithelial stem\progenitor cells (LECs) have been isolated[96]. The most significant functions of LECs are sustained self-renewal and protective capacities against external injuries[97,98]. Li *et al*[99] have recently reported clinical trials on animals and humans (Table 1). They showed that with a new minimally invasive surgical procedure, with preservation of LECs, lens regeneration can be achieved with increased visual axis transparency and decreased rate of complications. The same conclusion has also been reported by Li *et al*[99]. A further possibility of obtaining lenses through cell differen-

tiation has been proposed by Murphy *et al*[98], who showed the possibility of creating micro-lenses starting from iPSC differentiation.

Optic nerve

RGCs are a population of CNS neurons located in the innermost layer of the retina and convey visual signals from the retina along their axons to the brain. Axonal injury leads to the functional loss of RGCs and subsequently induces death of neurons. Axon growth is essential for the restoration of neuronal connectivity and reestablishment of a functional visual system after optic nerve injury[100]. Some optic neuropathies are very common, like glaucoma that is the first cause of irreversible blindness; while other conditions tend to be rare. Leber's hereditary optic neuropathy is just one example of this heterogeneous category of ophthalmic pathologies. There is currently no treatment available for inherited optic neuropathies such as Leber's hereditary optic neuropathy or dominant optic atrophy. With regards to glaucoma, current treatments aim at lowering the IOP. It is well known how the regenerative potential of the mammalian CNS is very limited. Research regarding the regeneration of elements of the nervous system is thus fascinating and usually limited to animal models.

There are two main objectives of cell therapy, including the delivery of a trophic and neuroprotective support[22,27] and (more ambitious) replacement of lost cells and functional restoration[101]. The first step in developing cell replacement-based strategies for optic nerve regeneration requires a reliable, high-volume source of healthy RGCs[102].

iPSCs\ESCs represent the first source studied for the optical nerve regeneration. Takahashi *et al*[19] in 2007 reported a remarkable break-through technology whereby adult fibroblasts could be reprogrammed into iPSCs. These transdifferentiated cells exhibit similar characteristics to hESCs, including the ability to propagate indefinitely and the ability to differentiate into many different cell types, including RGCs. As explained by Gokoffski *et al*[100], there are two different methods for generating RGSs from pluripotent stem cells: Organoid differentiation and planar differentiation. Organoids are self-organized 3D miniature organs developed *in vitro* from PSCs. Kuwahara *et al*[102] first developed 3D retinal organoids from hESCs[102]. Planar-derived RGCs are transdifferentiated from traditional two-dimensional cell culture techniques[103,104]. One of the limitations of this technique is the ability of iPSCs\ESCs to integrate into the ganglion cell layer. Preliminary results by Mesentier-Louro *et al*[105] reported that between 1% to 7% of iPSCs-derived RGCs integrated into the ganglion cell layer after intravitreal injection and about 20% after combined injection of RGCs and iPSCs[105] (Table 1).

The other limitation regarding the use of iPSCs currently being studied involves the possibility of formation of synaptic connections. Axonogenesis of derived RGCs is another crucial point: Neurotrophic factors, which theoretically could promote it, keep RGCs alive longer. The ideal local cellular environment is essential to permit and enhance RGCs in creating new axons; however, neuronal survival does not equate to new axon formation[106]. An additional unresolved question involves the possibility of new axons in establishing functional synapse.

To overcome the difficulties in integrating RGCs, gene therapy has developed possible solutions, which are currently under investigation. Zhang *et al*[107] demonstrated the utility of pigment epithelium-derived factor[107]. It is constitutively expressed by various ocular tissues, but its exogenous delivery is therapeutically advantageous to promote RGCs survival and axon regeneration. Zhang *et al*[107] used NSCs as cellular vectors to deliver continuously pigment epithelium-derived factor to adult retinas after optic nerve injury. These studies showed the utility of genetically modified NSCs to protect RGCs and promote axonal regeneration.

The effects of MSCs on RGCs have also been studied (mainly in animals). MSCs provide neuroprotection against RGC death; however, studies showed that these SCs did not tend to integrate[14]. Preliminary studies by Zhang *et al*[107] have described the possible usefulness of UC-MSCs, which have shown a partial recovery of injured optic nerve.

Retina: Visual signalling starts in photoreceptor cells. In the human retina, rods and cones are responsible for dim light vision and daylight vision, respectively. Rods are located mainly in the peripheral retina, while cones are concentrated in a small portion of the retina, called macula. Bipolar cells receive visual signals from photoreceptors and transmit it to RGCs. RPE cells form a monolayer underneath the outer segment of the photoreceptors, constituting the outer blood-retinal barrier. In the visual system, RPE cells play diverse roles, which include absorption of scattered light, regulation of nutrients, ions and solutes, secretion of growth factors, regulation of the retinal cycle

and phagocytosis of photoreceptor outer segment.

Retinal degenerative diseases are characterized by retinal cell loss, such as RPE and/or photoreceptor cell loss in AMD and retinitis pigmentosa, and RGC death in glaucoma, as seen previously. Studies that report retinal diseases and treatment are summarized in Table 2.

Binder *et al*[108] and van Meurs *et al*[109] described the therapeutic potential of autologous RPE cells taken from the peripheral region of the retina in AMD. These promising results have prompted research towards stem cell therapies to extend it to degenerative retinal diseases, where cell replacement is needed[37]. Retinal degenerative disorders requiring cell replacement include AMD, retinitis pigmentosa, Stargardt's disease and glaucoma, which represent current challenges in cell therapy. RPE cell source is restricted for applications in RPE cell replacement, thus stem cells have been studied to solve this limitation.

The greatest potential for cell replacement has been seen with ESC/iPSCs[19], which can be successfully pre-differentiated prior to transplantation in the eye, with the greatest success shown in RPE/photoreceptor replacement for AMD[22]. Studies involving cell reprogramming by Tucker *et al*[110] and Lamba *et al*[111] have shown the possibility of creating ESC/iPSC-derived retinal progenitors capable of maturing in RPE or photoreceptors and capable of integrating in the retina[112]. The same results, but with hESCs and hiPSCs, were confirmed by Nakano *et al*[48] and Gonzalez-Cordero *et al*[113]. The first clinical trials in humans have been done by Schwartz *et al*[40,114] in 2012 and 2015[40,114]: They successfully used hESCs-derived RPE cells in Stargardt's disease without collateral effects (also noted by Mandai *et al*[17]). Cell reprogramming allows the use of autologous PSCs, which guarantee no immune reactions with respect to allogenic cells. One of the observed collateral effects is the long-term hyperpigmentation of the macula[114]. Potential future alternative approaches include the implant of stem cell derived RPE growing on a bioengineered scaffold, which can improve stability and maintain cell polarization[14].

Considering the functions of RPE on the overlying retina, it is fundamental to know that subretinal RPE transplantation aims at replacing these functions. Transplantation of non-RPE cells has been pursued with the rationale that they may counter disease through trophic factor secretion. For this reason, clinical trials with subretinal UC-MSCs, NSCs and retinal progenitor cells are based on trophic support mechanism of action. These procedures, however, need further studies considering the potential increase of complications[115].

The future of retinal therapies could involve bioengineering. Stern *et al*[14] have reported that incorporating bioengineering approaches may better preserve retinal layering and integration *in vivo* models. The possibilities of this approach have been successfully seen in animal clinical trials[116]. Nakano *et al*[48] has described the formation of optic cup and stratified retina from hPSCs. Several successive studies have confirmed the possibility of creating retinal organoids and layers of differentiated photoreceptors, which can develop outer segment structures[117]. Organoids may prove valuable in producing specific retinal cell types or 3D retinal structures for transplantation. This is particularly important for conditions such as geographic atrophy[14].

An alternative approach to retinal degenerative pathologies with SCs is the possibility of exploiting the paracrine capacities of some type of cells. This stem cell therapy is not disease specific and can have broad clinical applications, as seen previously. This is the potential of BM-MSCs and places them as a candidate cellular therapy to combat ocular neurodegeneration, even if these cells are less ideal for replacement cell therapy[22,27]. Transplantation of BM-MSCs in experimentally induced glaucoma and optic nerve transection show no evidence of the differentiation into mature retinal cells (RPE or photoreceptors), despite some integration into the retina; but, improvement of retinal function by preserving photoreceptors and RPE cell viability have described[118].

ASCs have also been studied for this clinical use, which could prove to be beneficial in eye therapies for the paracrine effects, as seen for disorders of the SNC. Several studies suggest that ASCs have therapeutic potential for neurodegenerative conditions through neurotrophic factor production, with several of the active factors being different from those produced by BM-MSCs and DPSCs[27,119]. ASCs and BM-MSCs have recently been evaluated with promising results in diabetic retinopathy. ASCs have shown the potential to be therapeutically effective in early-stage of diabetic retinopathy through paracrine factors and physical contact with endothelial cells[120].

Limits and why not

Stem cell therapy in ocular diseases certainly represents the future in the treatment of numerous serious eye diseases, especially if organ transplantation can be avoided. One of the main problems of these therapies, mainly the immunological tolerance to transplanted cells, has been solved thanks to the use of autologous cells[17]. Graft *vs* host disease has been limited in this way. Despite all the advantages and promising results, what we know about stem cells therapies is still incomplete, especially concerning the possible side effects. The tumorigenicity of transplanted stem cells, to date, remains to be the most concerning issue for cell transplantation. Advances in genetic research and reprogramming have made it possible to reduce the incidence of this event[20]. Other side effects may be caused by tissue sampling, particularly if performed in the contralateral, healthy eye. This can be avoided using SCs derived from other tissues, like ASCs[32].

Stem cell transplantation has the huge limit of the survival and engraftment of cells. To improve outcomes, methods considered include pre-treating the host tissue or the SCs culture with growth factors or cytokines, embedding SCs in biomaterials or expanding SCs in culture. Obtaining an adequate number of SCs is fundamental. For example, the expansion of ASCs in culture has been considered, but extensive cultures may change the properties of SCs *in vivo*, rendering them unfit for restoring injured or diseased tissues. Genetic research is thus of fundamental importance in this field. A further problem related to SCs treatment is the cost of this type of therapy, which is still very high today due to limited cost efficient technology to render this therapy available on a large scale[37]. Conserving samples of one's own tissue (*i.e.* adipose stem cells, which are the most represented) taken during other operations, similar to the methods used during lipofilling for aesthetic or reconstructive aims, could be a possible solution. Moreover, the ethical problem of the use and collection of certain cell types, such as ESCs, UC-MSCs and NSCs, remains considerable, which further limits SCs use in clinical trials.

Future prospectives

The vast literature and resources used to date underline the importance of stem cell therapies, especially for the future. To date, the results are still primordial, but the conditions seem leading to an important turning point. Further studies are surely needed to provide additional knowledge behind the mysterious physiological and potentially curative properties of stem cells, which can pave the way to clinical applications in ophthalmic care. Numerous current trials have been proposing different perspectives for the future of cell therapy in ophthalmic diseases. The therapeutic effects of MSCs in regenerative medicine, for example, can also be attributed to exosomes, which are secreted soluble factors[45]. Exosomes are extracellular vesicles that are produced in the endosomal compartment of most eukaryotic cells and contain proteins that regulate tissues biogenesis. Several studies have considered the injection of MSCs exosomes in ocular tissues, with interesting results[28]. Research should not only be limited at stem cell studies alone but also need to include a whole series of innovations brought on by bioengineering and nanotechnology[14,48,118]. Modern studies seem to be aimed at the 3D development of fundamental parts of the eye, the use of specific vectors that can guarantee the replacing of tissues, and organoids[67]. Novel studies are geared at the possibility of reproducing eye tissues in a normal 3D configuration. Transplantation of sophisticated multicellular 3D tissues can be an exciting opportunity. Nakano *et al*[48] have shown that hPSCs can generate differentiated 3D structures similar to the embryonic eye cup, containing RPE and neural retina. Garzón *et al*[74] described the generation of the anterior cornea by tissue engineering. In addition, bioengineers have proposed to build ocular structures by incorporating biocompatible materials with stem cells products, for example to create TM or RPE monolayer[47]. Bioengineering is fundamental for the development of optimal cell culture biomaterials for the expansion of SCs and the differentiation into ocular cells[16]. Modified cultures (*e.g.*, using growth factors, biomaterials, injectable hydrogels, scaffolds or membranes containing cells) allow the maturation of cells and dedifferentiation, recreating the micro-environment of the host tissue. Consequently, there is an easier integration and a better survival of these SCs in the recipient site[42]. The advantage of these approaches is to guarantee a reduction of the culture time to obtain a viable ocular tissue (such as a cornea[75]), thus avoiding massive tissue harvesting (preserving LSCs, for example[75]).

CONCLUSION

The potential therapeutic goals include being able to restore the sense of sight in patients who have lost it, by replacing or recreating the damaged eye structures, including restoration of physiological and functional connections with the CNS. Stem cell therapies seem to be interesting and promising, even concerning the more external structures, including the ocular surface and cornea. Finally, the possibility of using these cells topically, without having to subject patients to surgery, organ transplantation or hospitalization, is an element of great importance and hope.

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REVIEW

Programmed cell death in stem cell-based therapy: Mechanisms and clinical applications

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Abstract

Stem cell-based therapy raises hopes for a better approach to promoting tissue repair and functional recovery. However, transplanted stem cells show a high death percentage, creating challenges to successful transplantation and prognosis. Thus, it is necessary to investigate the mechanisms underlying stem cell death, such as apoptotic cascade activation, excessive autophagy, inflammatory response, reactive oxygen species, excitotoxicity, and ischemia/hypoxia. Targeting the molecular pathways involved may be an efficient strategy to enhance stem cell viability and maximize transplantation success. Notably, a more complex network of cell death receives more attention than one crucial pathway in determining stem cell fate, highlighting the challenges in exploring mechanisms and therapeutic targets. In this review, we focus on programmed cell death in transplanted stem cells. We also discuss some promising strategies and challenges in promoting survival for further study.

Key Words: Programmed cell death; Apoptosis; Autophagy; Stem cell; Therapeutic strategies

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Core Tip: The point of interest of this work is the complex mechanisms of the programmed cell death in stem cells (SCs), which suggests a series of targets as an efficient, reliable, and potential strategy to promote the SC-based therapy.

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INTRODUCTION

Cell-based therapies have raised tremendous expectations and presented favorable curative effects in repairing damaged tissue and enhancing functional repair[1-3]. Stem cells (SCs) could serve as a cellular reservoir to maintain, produce, repair, and even regenerate multiple tissues with the characteristic properties of self-renewal and differentiation. Thus, SCs are developed as the preferred sources for cell-based therapies due to their ability to differentiate into a wide range of cell types and their capacity of secretion regulated by the microenvironment, also termed the "niche"[4]. Based on the stage of development, SCs can be divided into three types: Embryonic SCs (ESCs), induced pluripotent SCs (IPSCs), and adult SCs (ASCs)[5]. ESCs are derived from the inner cell mass of a blastocyst[6]. There are ethical limitations to the use of ESCs in therapy[7]. Compared with ESCs, IPSCs derived from mature body cells could be regulated to dedifferentiate into pluripotent SCs as a renewable source of alternative cells and tissues[8]. ASCs or somatic SCs (SSCs) can be found in various adult tissues, including neural SCs (NSCs), hematopoietic SCs (HSCs), mesenchymal SCs (MSCs), and epidermal SCs. Many trials have shown that ASCs can be used to treat diseases[9,10]. For example, bone marrow mononuclear cells[11], NSCs[12], and MSCs[13] are usually used to treat stroke.

SCs-based therapies are widely used in the treatment of various diseases[14-18]. Limbal stem cell therapy is used for treating burn-related corneal destruction[19], NSCs in gastrointestinal tract disorders[20], bone marrow-derived mesenchymal SCs (BM-MSCs) in diabetic cardiomyopathy[21], and MSCs in multiple sclerosis[22] and several clinical conditions. However, SC-based therapies also have limitations. Impaired cell homing regulated *via* various factors (such as chemokines) causes *in situ* tissue regeneration failure[23]. Also, a high death rate of transplanted SCs limits the therapies[24,25]. After MSC injection, over 99% of injected cells die in the left ventricular myocardium within 4 d[26].

Accumulated evidence shows a close tie between multiple types of programmed cell death (PCD) and SCs, including apoptosis, autophagy, ferroptosis, pyroptosis, and necroptosis. Studies demonstrate that p53 induces apoptosis of human ESCs (hESCs) through a mitochondrial pathway shown to be extremely sensitive to FasL-induced cell death in MSCs[27,28]. Ohgushi *et al*[29] observed that Rho-associated coiled-coil-containing protein kinase (ROCK)-dependent hyperactivation of myosin directly caused dissociation-induced apoptosis in hESCs and immediate activation of the Rho/ROCK/MLC2 signaling cascade. In 2010, the María group found that inhibitors of apoptosis proteins (IAPs) could promote the numbers of hematopoietic stem and progenitor cells and improve resistance to cell death[30]. Moreover, reports suggest that high levels of pro-apoptotic B-cell lymphoma 2 (Bcl-2) family members were overexpressed in hESCs[31]. Autophagy in SCs traces its history to 1980 where marrow cells revealed several abnormalities within an intrinsic myeloid precursor cell defect[32]. Lately, the role of autophagy in SC fate and aging is drawing attention due to the ability of the autophagy activator rapamycin to restore the biological properties of aged SCs by increasing their differentiation and proliferation capacity and decreasing adipogenic differentiation capacity, including the molecular mechanisms targeting 5′ AMP-activated protein kinase (AMPK) and rapamycin (mTOR)[33,34]. Research on necroptosis in SCs started relatively late but progressed rapidly to show that tumor necrosis factor α (TNF-α) could act on HSCs and progenitors for facilitating hematopoietic clearance and promoting regeneration. Furthermore, pharmaceutical inhibition of receptor-interacting protein kinase-3 (RIP3) showed a curative effect in

promoting SCs, such as targeting necroptosis of intestinal SCs[35]. Some other cell death-related molecules have been increasingly recognized in SCs, such as the PI3K/AKT signaling pathway[36], MAP kinases ERK[37], JNK, and p38[38].

Some methods have been used to control programmed cell death in SCs. The concept of preconditioning was proposed by Charles E. Murry in 1986[39]. Presently, several strategies, such as using heat shock, free radical scavengers, over-expressing anti-apoptotic proteins, anti-inflammatory therapy, and co-delivery of extracellular matrix molecules, have been introduced[40-45]. Besides genetic strategies, three-dimensional culture technology and co-transplantation are novel ideas to enhance SC-based therapies.

Exploring cell death mechanisms in SCs and targeting these potential therapeutic molecules are vital to successful SC-based therapies (shown in Table 1[19-21,46-92]). In this review, we highlight the conditions or reasons leading to cell death in SC-based therapeutic approaches. Also, we demonstrate the cell death mechanism in SCs, which may provide a novel, efficient, reliable, and potential strategy in promoting SC-based therapy.

A QUICK LOOK AT PCD

According to the death inducers, cell morphologic changes, and molecular mechanisms, cell death can be divided into two types: Non-programmed cell death caused by an external injury leading to instantaneous and irreversible cell damage[93,94], and PCD (*e.g.*, apoptosis, autophagy, necroptosis, and pyroptosis), a common occurrence in the development of organisms without strong immune responses[95].

PCD occurs extensively during the development of pathology in various tissues. It is closely related to the therapeutic efficacy and prognosis of SC-based treatment. Robey *et al*[25] indicated that most cell death occurs in the first week post-transplantation. In NSC transplantation for neurological disorders in the brain, less than 4%-10% of primary NSCs survived within the first few days[96]. Similarly, Yasuda and Hayashi's groups showed that 15% of transplanted cells survived at 1 wk and 9% at 4 wk in a rat infarction model[97]. A significantly high death rate occurred, and over 99% of MSCs died within 4 d after transplantation into the left ventricular myocardium of mice[26]. Thus, cell death may be a significant concern that needs attention.

Apoptosis

Apoptosis is the classic form of PCD without spillage of contents into the surrounding environment[98]. Apoptosis plays an important role in the orderly and efficient removal of damaged SCs to prevent cancer through two classical apoptotic pathways: The intrinsic pathway and the extrinsic pathway[99]. The intrinsic pathway, also called the mitochondrial pathway, shows a close relation with SCs[100,101]. It is closely regulated by a group of cytokines, especially the Bcl-2 family[102,103]. The extrinsic pathway is triggered by ligand-receptor binding. TNF-family receptors and cysteine-aspartic proteases, known as caspases, play a vital role in the extrinsic pathway[104].

Autophagy

Autophagy is a eukaryotic cell recycling process involving the degradation of cytoplasmic organelles, proteins, and macromolecules with the recycling of decomposition products *via* the mTOR/Ras-cAMP-PKA axis to maintain cellular homeostasis and enhance stem cell survival[105]. Autophagy is divided into three major types: Microautophagy, macroautophagy, and chaperone-mediated autophagy (CMA)[106]. During microautophagy, cargos are captured by lysosomal membrane invaginations or protrusions[107]. In macroautophagy, autophagosomes are regarded as typical signatures[108]. CMA focuses on molecular chaperones to identify cargo proteins containing specific pentapeptide sequences without using membrane structures to isolate cargo[109].

Necroptosis

Necroptosis is a pro-inflammatory lytic form of PCD. Necroptosis could be induced through several innate immune signaling pathways triggered by stimulating RIG-I-like receptors, TLRs, and death receptors[110,111]. Receptor-interacting serine-threonine kinases 1 and 3 (RIPK1 and 3) are phosphorylated and activated through these signaling pathways[112]. Subsequently, mixed lineage kinase domain-like (MLKL) could be activated[113].

Table 1 Summary of programmed cell deaths in stem cell-based therapy

Disease	SCs	Therapy models	Therapeutic effects	PCDs in SCs	Ref.
Myocardial infarction	MSCs	Canine; porcine; mice; human	Inducing cardiac regeneration; increasing angiogenesis; repair by differentiating into cardiomyocytes	Apoptosis, autophagy, pyroptosis	[46-48]
	iPSCs	Porcine; murine; rats; mice; non-human primates	Showing heart regeneration potential; regenerating the injured tissues; promoting a cardiomyogenic and angiogenic response	Apoptosis, autophagy, ferroptosis	[48,49]
	ESCs	Non-human primates	Showing heart regeneration potential; increasing angiogenic differentiation	Apoptosis, autophagy, pyroptosis	[48,50]
Intracerebral hemorrhage	MSCs	Rats; primates; human	Repairing *via* differentiating into neurons or neuron-like cells; promoting axonal regeneration, neurogenesis, and angiogenesis	Apoptosis, autophagy, pyroptosis	[51-54]
	NSCs	Mice, rats	Differentiating into neurons or glial cells; promoting neurogenesis and angiogenesis; promoting regeneration	Apoptosis, autophagy	[51,55-57]
	ESCs	Rats	Differentiating into neurons or glial cells; promoting neurogenesis and angiogenesis	Apoptosis, autophagy, pyroptosis	[51,58,59]
	iPSCs	Rats	Differentiating into neuroepithelium-like/neuroepithelioid SCs and neural cells; promoting neurogenesis and angiogenesis	Apoptosis, autophagy, ferroptosis	[51,60-62]
Corneal reconstruction	LSCs	Human	Regenerating the corneal epithelium; differentiating into cells of the corneal epithelium	Apoptosis.	[19]
	MSCs	Mice; rats; rabbits; human	Regenerating the corneal epithelium and corneal stroma; angiogenesis	Apoptosis, autophagy, pyroptosis	[63]
Neurodegenerative disorders of the gastrointestinal tract	ESCs	Mice	Differentiating into enteric neuronal and glial cells	Apoptosis, autophagy, pyroptosis	[20,64]
	iPSCs	Rats, mice	Differentiating into neural and glial cells	Apoptosis, autophagy, ferroptosis	[20,65]
	CNS-NSCs	Mice	Differentiating into neurons; regenerating and repairing ENS	Apoptosis, autophagy	[20,66,67]
	ENSCs	Mice; rats	Stimulating a local regenerative response; regenerating and repairing ENS; differentiating into new neurons	Apoptosis, autophagy	[20,68,69]
Diabetic cardiomyopathy	MSCs	Mice; rats	Promoting angiogenesis; regenerating tissues; differentiating into cardiomyocytes and vasculature cells	Apoptosis, autophagy, pyroptosis	[21,70]
	EPCs	Rats	Differentiating into endothelial cells to form new blood vessels and promoting neovascularization	Apoptosis	[70,71]
	CSCs/CPCs	Rats	Differentiating into newborn cardiomyocyte; promoting heart regeneration	Apoptosis	[70,72]
	iPSCs	Rats; mice	Attenuating oxidative stress and fibrosis; diminishing pro-oxidant expression and enhancing antioxidant (catalase and MnSOD) concentration; promoting heart regeneration	Apoptosis, autophagy, ferroptosis	[70,73]
Diabetic retinopathy	ASCs	Rats; mice	Promoting angiogenesis; improving ischemia; offering protection against nerve damage; differentiating into photoreceptor and glial-like cells in the retina	Apoptosis	[74-77]
	HSCs	Murine; rats	Promoting angiogenesis	Apoptosis, autophagy	[74,78]
	BM-MSCs	Murine; rats; mice	Differentiating into retinal glial cells; stimulating angiogenesis; promoting resident neural progenitors to regenerate neuro-retinal tissue	Apoptosis, autophagy, pyroptosis	[74,79,80]
	iPSCs	Rats; mice	Differentiating into cells expressing features of retinal pigment epithelial cells, retinal progenitor	Anti-apoptosis, autophagy,	[75,81]

			cells, and retinal ganglion cells, and slowing down retinal degeneration	ferroptosis	
Neurological disorders	NSCs	Mice, rats, monkeys, pigs, human	Differentiating into neurons and supporting glial cells; releasing angiogenic factors to promote local tissue regeneration	Apoptosis, autophagy	[82-85]
	HSCs	Human	Promoting cell survival; stimulating proliferation and migration of NSCs; inducing regeneration of damaged brain cells; promoting angiogenesis	Apoptosis, autophagy	[82,86]
	MSCs	Human	Promoting neuronal regeneration; promoting angiogenesis	Apoptosis, autophagy, pyroptosis	[82,86]
Diabetes	ESCs	Mice, rats	Differentiating into cluster of insulin producing beta cells	Apoptosis, autophagy, pyroptosis	[87-89]
	Hepatic and intestinal stem cells	Mice	Differentiating into beta cells in response to high glucose concentration	Apoptosis	[87,90]
	Spleen stem cells	Mice	Differentiating into insulin secreting beta cells; regenerating islet	Apoptosis	[87,91]
	HSCs	Mice	Differentiating into beta cells and vascular endothelial cells of the pancreas; inducing beta cell regeneration from the host cells residing in pancreas	Apoptosis, autophagy	[87,92]

SC: Stem cell; MSCs: Mesenchymal stem cells; NSCs: Neural stem cells; ESCs: Embryonic stem cells; iPSCs: Induced pluripotent stem cells; LSCs: Limbal stem cells; CNS-NSCs: CNS-derived NSCs; ENSCs: Enteric neural stem cells; CSCs/CPC: Cardiac stem/progenitor cells; ASCs: Adipose stem cells; HSCs: Hematopoietic stem cells; BM-MSCs: Bone marrow derived mesenchymal stem cells; ENS: Enteric nervous system; EPCs: Endothelial progenitor cells; PCD: Programmed cell death.

Others

Pyroptosis, dependent on multiple molecules, such as caspase-1 and caspase-11, is widely believed to play an important role in resisting the invasion of pathogens[114]. Ferroptosis, an iron-dependent form of regulated cell death (RCD), is induced through an excessive accumulation (*e.g.*, ROS and lipid peroxidation products) characterized by mitochondria shrinkage or dysmorphic small mitochondria[115,116]. Moreover, other types of cell death are also crucial during a series of events, such as failures in SC-based therapies. The biological correlations between the different PCD pathways are complex, where it is especially significant as a network among these pathways regarding PCD of transplanted SCs[117,118].

PCD AND ITS KEY MOLECULES IN STEM CELLS FOR TRANSPLANTATION THERAPY

PCD of SCs is usually caused by a hostile pathological environment created due to multiple conditions, including apoptotic cascade activation, excessive autophagy, inflammatory response, ROS, excitotoxicity, and ischemia/hypoxia[39]. This section systematically reviews the molecular mechanisms involved in cell death pathways and we also summarize these key molecules in Table 2[35,38,119-134].

Apoptosis

Recently, an emerging body of evidence has highlighted a vital role of the apoptosis effect on several cell types, including SCs[135]. Hence, it is crucial to investigate and understand the mechanisms underlying apoptosis for analysis of SC transplantation and the development of drugs targeting specific apoptotic molecules. According to the inducing signaling, apoptosis could be divided into two types: Intrinsic pathway initiated by intracellular stresses (shown in Figure 1), and extrinsic pathway responding to extracellular cues (shown in Figure 2).

The intrinsic pathway of apoptosis: In the intrinsic pathway, the initiators (*e.g.*, ROS and radiation induced DNA damage) cause various cascade reactions resulting in the release of cytochrome C (cyt C), p53, and mitochondrial outer membrane permeabilization (MOMP). For example, hematopoietic stem and progenitor cells (HSPCs) are

Table 2 Molecular mechanisms and therapeutic targets of programmed cell deaths in stem cells

PCDs	SCs	Molecular pathways of PCDs	Therapeutic target(s)	Therapeutic effects	Ref.
Apoptosis	hESCs	Mitochondrial priming and p53 signaling pathway	Bcl-2	Preventing damaged cells from compromising the genomic integrity of the population	[119]
	HSCs	ASPP1 stimulated p53 signaling pathway	ASPP1, RUNX1	Preventing hematological malignancies	[120]
	ISCs	ARTS/XIAP/caspase 9 axis	XIAP	Controlling ISC numbers and preventing the propagation of abnormal progeny	[121]
	MSCs	p38 MAPK regulated early apoptosis while JNK regulated late apoptosis	p38	Protecting MSCs from oxidative stress damage	[38]
	NSCs	p38 MAPK signaling	TNF-α, p38	Impairing cell viability, decreasing therapeutic effects	[122]
Autophagy	iPSCs	AMPK/mTOR/ULK1 complex/PI3K complex/conjugation cascade complexes with LC3 and Atg9 during macroautophagy;KFERQ domain/Hsc 70/LAMP2A during CMA	LC3	Removing unnecessary or dysfunctional components	[123]
	HSCs	type III PI3K mammalian Atg6/PIP3/(Atg12-Atg5-Atg16) or (Atg4/LC3-I/Atg7/Atg3/LC3-II/PE) axis	LC3-II	Recycling cytoplasmic constituents and restoring metabolic homeostasis, and maintaining cells survival under harsh conditions	[124]
	NSCs	PI3K-AKT-mTOR/ULK1/the class III PI3-kinase-Beclin1 complex/PI3/PI3P/ the complex of Atg12–Atg5–Atg16L1/LC3-I/LC3-II axis	mTOR	Being involved in modulation of the embryonic neurogenesis as well as the injury repair of adult brain	[125]
	MSCs	PI3K/AKT/mTOR/ULK1/the class III PI3-kinase-Beclin1 complex/PI3/PI3P/the complex of Atg12–Atg5–Atg16L1/LC3-I/LC3-II axis	AKT, mTOR	Eliminating damaged organelles and biomacromolecules to maintain cellular homeostasis	[126,127]
	ESCs	AMPK/ mTORC1/ULK1 axis	Atg5, Atg12	Maintaining the undifferentiated state of ESCs *in vitro*	[128]
Necroptosis	ISCs	ZBP1/RIP3/MLKL axis	ZBP1	Disrupting homeostasis of the epithelial barrier and promoting bowel inflammation	[35,129]
	SSCs	RIP1 signaling pathway	RIP1	Using Nec-1 to target RIP1 for reducing both necroptosis and apoptosis, which benefits for recovery rate and proliferation potential	[130]
	NPSCs	RIPK1/RIPK3/MLKL axis	HSP90	Protecting SCs from PCD *via* alleviating mitochondrial dysfunction (mitochondrial membrane potential loss and ATP depletion) and oxidative stress (production of mitochondrial ROS), cellular total ROS and MDA, and downregulation of superoxide dismutase	[131]
Pyroptosis	MSCs	Exosome/circHIPK3/ FOXO3a axis	circHIPK3	Preventing pyroptosis and repairing ischemic muscle injury through a novel exosome	[132]
	ESCs	Caspase-1 signaling pathway	N/A	Embryonic stem cell-derived exosomes inhibit doxorubicin-induced pyroptosis	[133]
Ferroptosis	NPCs and IPSCs	Ferritin/ROS/lipid peroxidation axis	NCOA4, p53	Decreasing stem cells and triggering neuronal death	[134]

ISCs: Intestinal stem cells; iPCs: Induced pluripotent stem cells; HSCs: Hematopoietic stem cells; ESCs: Embryonic stem cells; NSCs: Neural stem cells; MSCs: Mesenchymal stem cells; EPCs: Endothelial progenitor cells; CPCs: Cardiac progenitor cells; IPSC: Pluripotent stem cells; ZBP1: Z-DNA-binding

protein 1; RIP3: Receptor-interacting serine/threonine kinase 3; MLKL: Mixed lineage kinase domain like protein; PUMA: p53 upregulated modulator of apoptosis; NOXA: Known as PMAIP1, phorbol-12-myristate-13-acetate-induced protein 1; Bax: Bcl-2 associated X protein; Bak: Bcl-2 antagonist/killer 1 protein; cyt c: Cytochrome C; Apaf-1: Apoptosis protease activating factor-1; casp: Caspase; FADD: Fas-associated death domain; Bcl-2: B-cell lymphoma 2; AMPK: AMP-activated protein kinase; mTOR: Mammalian target of rapamycin; ULK1: Unc-51-like kinase complex; ROS: Reactive oxygen species; MDA: Malondialdehyde; GPX4: Glutathione peroxidase 4; circHIPK3: One of the most abundant circRNA in muscle; FOXO3a: A transcription factor of the O subclass of the forkhead family; LncRNA: Long non-coding RNAs; KLF3-AS1: Localize at chromosome 4p14 according to the exocarta database; mTOR: Mammalian target of rapamycin; ULK1: Atg1/unc-51-like kinase; LC3: Light chain 3; PI3K: Beclin-1/class III phosphatidylinositol 3-kinase; CMA: Chaperone-mediated autophagy; Hsc 70: Heat shock cognate71 kDa protein; LAMP2A: Lysosomal-associated membrane protein type 2; Atg: Autophagyassociated gene; Atg6: Vps34/Beclin-1; PIP3: Phosphatidylinositol (3,4,5) P3; PE: Phosphatidyl ethanolamine; SSCs: Spermatogonial stem cells; Nec-1: Necrostatin-1, a necroptosis inhibitor; NPSCs: Nucleus pulposus-derived stem/progenitor cells; HSP90: Heat shock protein 90; ROS: Reactive oxygen species; PCD: Programmed cell death.

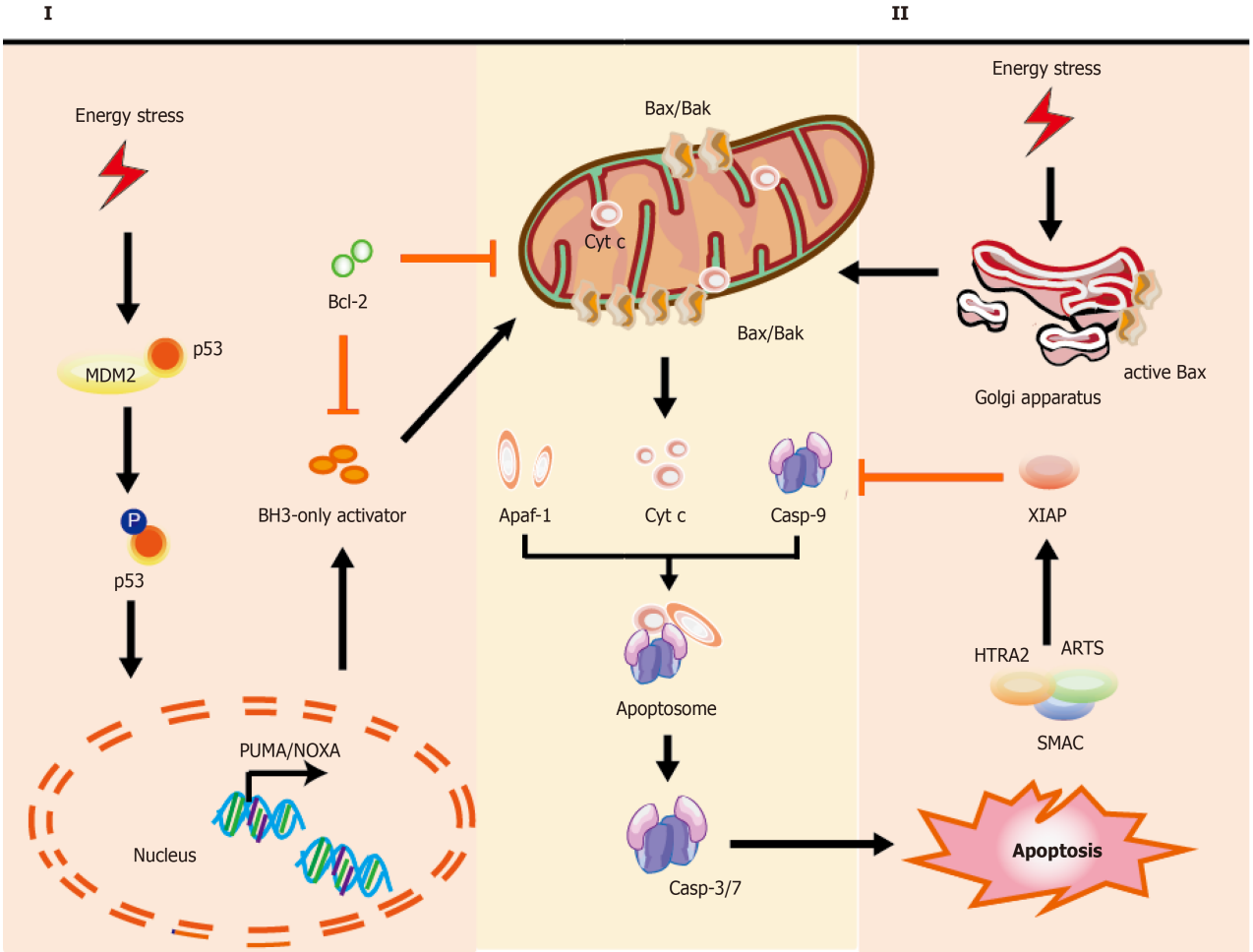

Figure 1 Mechanisms of intrinsic apoptotic pathways in stem cells. Cell stress from various damage causes a rapid response leading to apoptosis *via* BH3-only activator (Way I) or active Bax directly from the Golgi (Way II) to the mitochondria, which subsequently induces a co-pathway [MOMP, cytochrome C (cyt C) releasing, *etc.*]. I: Stress inducers, such as DNA damage could stabilize and activate p53, which leads to p53 nuclear translocation. Subsequently, p53 exerts an impact on transcription of apoptotic genes *via* DNA-binding activity and its transcriptional activity (*e.g.*, PUMA, NOXA, and Bax); II: Bax, which is monomeric in the cytoplasm, could be activated *via* stabilized p53 and active-Bax translocates from the Golgi to the mitochondrion. Once instigated with the apoptotic signals, active-Bax could lead to the alteration of MOMP, which undergoes dimerization and transfers to the OMM, so that relevant proteins (such as cyt C) are released into the cytosol usually confined in the intermembrane space. The released cyt C is involved in apoptosome formation *via* binding to the cytosolic Apaf-1. This complex recruits and activates initiator pro-casp-9, and then act-casp-9 activates downstream executor casp-3/-6/-7, leading to apoptotic cell death. In the cytoplasm, IAP antagonists (*e.g.*, SMAC, ARTS, and HTRA2) could bind and suppress XIAP, causing the activation of casp-9 for the apoptotic pathway. The T-shaped lines indicate inhibitory interactions involved in this pathway, while the solid arrows indicate activating interactions. Bax: Apoptosis regulator Bcl-2 associated X protein; OMM: Outer membrane permeabilization; MOMP: Mitochondrial outer membrane permeabilization, cyt C: Cytochrome C; PUMA: p53 upregulated modulator of apoptosis, NOXA: Pro-apoptotic BH3-only protein, also known as PMAIP1, phorbol-12-myristate-13-acetate-induced protein 1; Apaf-1: Apoptosis protease activating factor-1; IAP: Inhibitor of apoptosis; SMAC: Second mitochondria-derived activator of caspase; ARTS: Apoptosis-related protein in the transforming growth factor-β signaling pathway; HTRA2: High-temperature-required protein A2.

used for treating acquired and primary immunodeficiencies, thalassemia, and sickle cell disease. However, the presence of intrinsic apoptosis is shown in HSPC-based

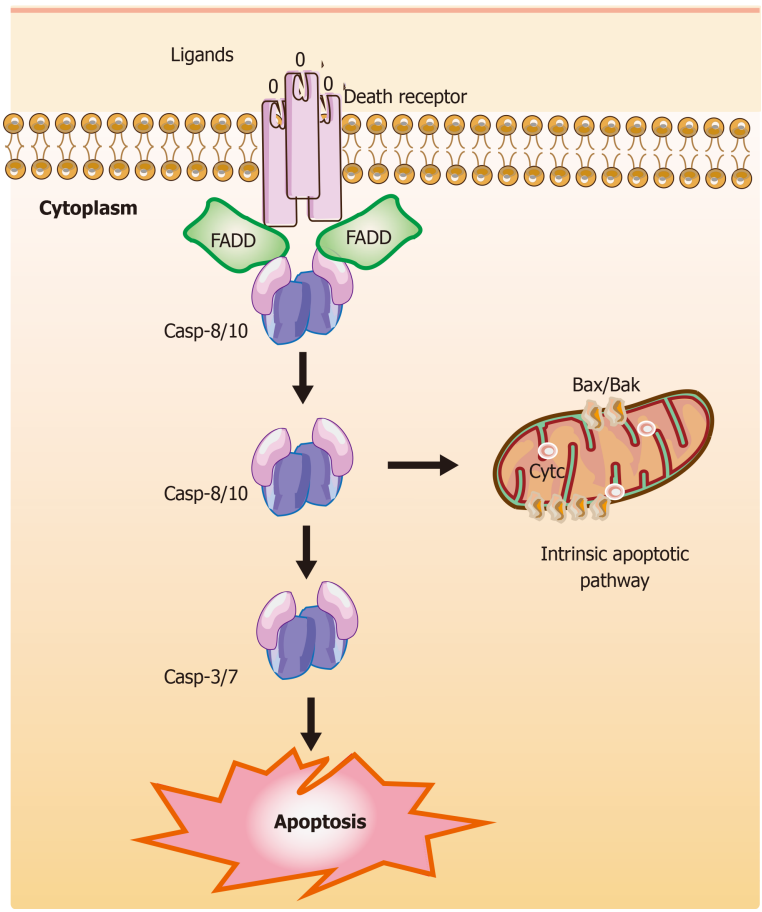

Figure 2 Mechanisms of extrinsic apoptotic pathways in stem cells. The extrinsic apoptotic pathway (also known as the death receptor-dependent pathway) is induced by the connection between death receptors exposed on the cell surface [tumor necrosis factor (TNF) receptor] and the specific TNF family ligands. Subsequently, this signaling causes a conformational change leading to the recruitment of Fas-associated death domain (FADD) and allows interactions between FADD and casp-8 and/or the casp-10, resulting in the cleavage and activation of casp-3 and casp-7 through their death domain. Finally, the active and cleaved casp-3 induces changes in phosphatidylserine exposure, DNA fragmentation, and the formation of apoptotic bodies. Also, casp-8 can target the BH3-only protein Bid and cleave Bid to a truncated fragment t-Bid, which could connect to the extrinsic apoptotic pathways. The T-shaped lines indicate inhibitory interactions involved in this pathway, while the solid arrows indicate activating interactions. FADD: Fas-associated death domain.

therapy in which excess DNA damage can trigger cumulative p53 pathway, constraining proliferation, yield, and engraftment of HSPCs, while moderate damage can lead to reversible function impairment by transient p53 inhibition[136]. According to the downstream activators of p53, two main pathways could be described: BH3-only activator (Way I shown in the left part of Figure 1) and active BAX from the Golgi (Way II shown in the right part of Figure 1) to the mitochondria.

Part I during the intrinsic pathway: During the intrinsic pathways, DNA damage, as a significant inducer, can stabilize and activate p53 by phosphorylation (for example, the phosphorylation of p53 at Ser46 can induce the p53-dependent apoptotic pathway caused by DNA damage[137]), leading to p53 nuclear translocation[119]. Subsequently, p53 exerts an impact on transcription of apoptotic proteins (namely, the related proteins) *via* DNA-binding activity and its transcriptional activity, such as the pro-apoptotic proteins p53 upregulated modulator of apoptosis (PUMA), NOXA (the pro-apoptotic BH3-only proteins, also known as PMAIP1 [phorbol-12-myristate-13-acetate-induced protein 1]), and apoptosis regulator Bcl-2 associated X protein (Bax) [138,139].

PUMA and NOXA can bind and activate Bax and Bcl-2 antagonist/killer-1 protein (Bak) in the cytoplasm, resulting in MOMP and release of cyt C[140]. Further, p53 can directly interact with Bax and Bak to modulate MOMP[141,142]. Of note, in the absence of cellular stress, p53 could rapidly produce and degrade in human pluripotent SCs (hPSCs), and the stabilization of p53 occurred upon DNA damage or *via* inhibition of MDM2 (the E3 ubiquitin ligase mouse double minute 2 homolog, which maintains low p53 levels through triggering p53 degradation)[143,144]. Interestingly, the activation of p53 is also involved in other types of cell death, such as

ferroptosis[134].

Part II during the intrinsic pathway: Typically, Bax is monomeric in the cytoplasm. Studies show that active Bax localized to the Golgi held away from the mitochondrion in some hPSC lines, whereas active BAX could transform the mitochondria after cell stress as DNA damage *via* a rapid p53-dependent pathway during apoptosis[145]. Once instigated with the apoptotic signals, Bax could undergo dimerization and transfer to the outer membrane of mitochondria, leading to the alteration of MOMP [146], so that relevant proteins (such as cyt C) were released into the cytosol usually confined in the intermembrane space[147]. The released cyt C is involved in apoptosome formation *via* binding to the cytosolic apoptosis protease activating factor-1 (Apaf-1)[148]. This complex recruits and activates initiator pro-caspase-9, and then act-caspase-9 activates downstream executor caspases-3/-6/-7, leading to apoptotic cell death[148,149]. In the cytoplasm, the inhibitor of apoptosis (IAP) antagonists could bind and suppress XIAP (X-linked inhibitor of apoptosis, E3 ubiquitin-protein ligase), causing the activation of caspase-9 for the apoptotic pathway[121]. These IAP antagonists include second mitochondria-derived activator of caspase (SMAC), apoptosis-related protein in the transforming growth factor-β signaling pathway (ARTS), and mitochondrial serine protease high-temperature-required protein A2 (HTRA2)[121,148]. Koren *et al*[121] found highly expressed ARTS in cells comprising the intestinal SC niche, which protects Paneth cells from undergoing apoptosis.

The extrinsic pathway of apoptosis: The extrinsic apoptotic pathway is also known as the death receptor-dependent pathway induced *via* the connection between death receptors exposed on the cell surface (one of the numbers in the tumor necrosis factor receptor (TNFR) family) and the specific TNF family ligands mentioned above[150]. Previous research reported the effect of TNFα on the development of human hematopoietic progenitors *in vitro* within the role of inhibition[151] or promotion[152]. These TNFα-driven mechanisms play a vital role in HSC response to inflammatory stress for removing damaged cells and activating SCs[153]. Recently, HSC transplantation for malignancy has shown anti-tumor activity *via* TNFα-driven pathways[153,154]. Death receptors and their ligands cause a conformational change, which leads to the recruitment of Fas-associated death domain (FADD)[155] and allows interactions between FADD and caspase-8 and/or the caspase-10, resulting in the cleavage and activation of caspase-3 and caspase-7 through interactions between their death domain (DD)[156]. Finally, the active and cleaved caspase-3 induces changes in phosphatidylserine exposure, DNA fragmentation, and the formation of apoptotic bodies. However, reports suggest that caspase-3 activity could be elevated in nonapoptotic pathways in neural SCs[157].

Remarkably, caspase-8 can target the BH3-only protein Bid (BH3-interacting domain death agonist) and cleave Bid to a truncated fragment t-Bid[158]. Capper *et al* [159] and Jia *et al*[160] showed that decreased Bid could inhibit apoptosis, promote proliferation, and delay senescence in human periodontal ligament SCs (h-PDLSCs) *via* activated Yes-associated protein, and low levels of caspase-8 were detected in stem cell features through hypermethylation. Subsequently, t-Bid could directly translocate to the outer mitochondrial membrane after activating apoptotic regulator Bax and inhibiting Bcl-2, leading to co-engages between the intrinsic apoptotic pathway and the extrinsic apoptotic pathway[158]. Some evidence shows that activation of the extrinsic pathway and inhibition of caspase-8 can induce necroptosis[161,162].

Emerging findings indicate that Bcl-2 family proteins play a vital role in SCs (*e.g.*, overexpression of Bcl-2 in MSCs[163], ESCs[164], and neuroepithelial SCs (NESCs) [165] improved their survival). The three functional groups Bak and Bax, BH3-only proteins, and Bcl-2 maintain a balance between SC survival and death. For example, high levels of Bcl-2 were measured in HFSCs for antiapoptosis in contrast to differentiated cells[166,167]. In the SCs, Bax performs as an activated conformation sequestered in the Golgi apparatus held away from the mitochondrion. Following stresses such as DNA damage, active Bax translocates to the mitochondrial outer membrane to initiate MOMP and the apoptotic cascade, which bypasses the conventional intrinsic and extrinsic apoptotic pathways[168,169]. However, the mechanism underlying the localization of active Bax at the Golgi and active Bax-induced pore formation in the Golgi stacks is unclear.

Autophagy

As a self-protective catabolic mechanism within the cells, autophagy exerts a key influence in sustaining SC homeostasis by maintaining stemness, upregulating quiescence, managing differentiation *via* remodeling, and self-renewal *via* metabolic

reprogramming[170-173]. Autophagy contributes to metabolic regulation through increased glycolysis to generate ATP in the hypoxic milieu for balancing SC fate[174, 175]. For example, autophagy plays a vital role in maintaining the quiescence of SCs (*e.g.*, HSCs and muscle SCs (MuSCs)) *via* rejuvenating aged quiescent SCs controlled by various autophagy pathways such as the p38/mitogen-activated protein kinase (MAPK) signaling pathway[176,177]. Uncovering the autophagy mechanisms underlying SC quiescence presents novel therapeutic strategies to release the cells out of the quiescent state, promoting their proliferation and differentiation (such as induced activation of quiescent NSCs for neuron injury), or re-establishing quiescence to prevent aberrant proliferation and differentiation or premature senescence (such as anti-cancer therapeutics), which carry the risk of cancer SCs (CSCs)[178,179]. These stressors (*e.g.*, starvation, oxidative stress, infection, and hypoxia) stimulate the cascade of autophagy as follows (shown in Figure 3)[180].

During autophagy, the formation of multi-protein complexes is associated with morphologic changes (shown in Figure 3). Initiation of autophagy is controlled by nutrient sensors, namely, mTOR and AMPK[173,181]. Typically, the mTORC1 complex functions as an inhibitor for autophagy. Under environmental stresses and physiological stressors, AMPK is activated to inhibit the activity of mTORC1, leading to a release of the ULK1 (Unc-51-like kinase complex, also known as ATG1) complex to induce autophagy, which is usually inhibited by mTORC1[182]. This initiation process is known as the phagophore assembly site (PAS) formation, which is regarded as indispensable for nucleation in the next stage. Compared with somatic mouse embryonic fibroblasts, whole-cell extracts of iPSCs and ESCs express high levels of AMPK and phosphorylated AMPK[183]. Interestingly, AMPK inhibition in mouse bone marrow-derived MSCs can upregulate both autophagy and apoptosis in hypoxia and serum deprivation conditions, suggesting crosstalk between autophagy and apoptosis through AMPK-ULK1 pathways[184,185]. Mutations in mTOR lead to smaller brains in mouse cortical development, and fewer proliferating neural progenitors result from disruption of NSC self-renewal[181].

Next, PI3 is phosphorylated to PI3P *via* the class III PI3-kinase-Beclin1 complex formed by core subunits of Beclin1 (Atg6), Atg14 L, and Vps34-Vps15, resulting in autophagosome formation[186,187]. The Atg12-Atg5-Atg16L1 complex acts as a regulator for enveloping and translocating the cytoplasmic cargo to the lysosome within misfolded-protein degradation[188]. Atg4 can cleave LC3 (Atg8) to generate cytosolic LC3-I. Atg3 (E2 enzymes) and Atg7 (E1-like enzymes) can lead the conjugation of PE to LC3-I to form lipidated LC3-II, which is combined with the autophagosome membrane to complete and elongate autophagosome formation[189]. Finally, the autophagosome contents undergo degradation due to low lysosomal pH. Some evidence demonstrates that autophagy plays an important role in reprogramming to form iPSCs, while iPSCs colony formation shows reprogramming failure due to the lack of Atg3, Atg5, or Atg7[190,191]. Autophagy is necessary for SC survival and sustenance. It is critical for SC differentiation in which co-localized dots of Tuj1-positive and GFP-LC3-positive cells are monitored and progress increasingly during NSC differentiation[192].

In microautophagy, misfolded or/and toxic proteins can be directly engulfed by the lysosomal membrane and degraded in the lysosome[193]. During chaperone-mediated autophagy, the heat shock cognate 70 kDa protein (HSC70) chaperones attach to the pentapeptide motif KFERQ (namely Lys-Phe-Glu-Arg-Gln) for delivery to lysosomes *via* a specific receptor LAMP2A. Reports suggest that targeting peptide HSC70 during autophagy can dramatically decrease amyloid-β (Aβ) oligomers in iPSCs with superior neuroprotective activity[194]. However, the molecular mechanism between autophagy and SCs is still unclear and remains to be further explored.

Apart from these vital targets, key transcription factors are closely linked to the stem cell state and the occurrence of autophagy (shown in Figure 3). For example, FOXO3A can enhance autophagosome formation *via* autophagy gene expression in hematopoietic SCs and breast cancer stem-like cells, which is needed to mitigate an energy crisis and allow cell survival[182,195]. Moreover, an elevated level of SOX2 is detected in NSCs, which is important for self-renewal; downregulation of SOX2 is observed in differentiated neurons and glia[196]. Besides SOX2, other transcription factors such as STAT3, OCT4, KLF4, and c-Myc are also vital for reprogramming in the initial creation of iPSCs at the genetic level[197].

Necroptosis

The occurrence of necroptosis in SCs has recently been reported. Wang *et al*[35] found that gut stem cell necroptosis resulting from genome instability triggered bowel inflammation. Moreover, TNF-α could promote the survival and myeloid differen-

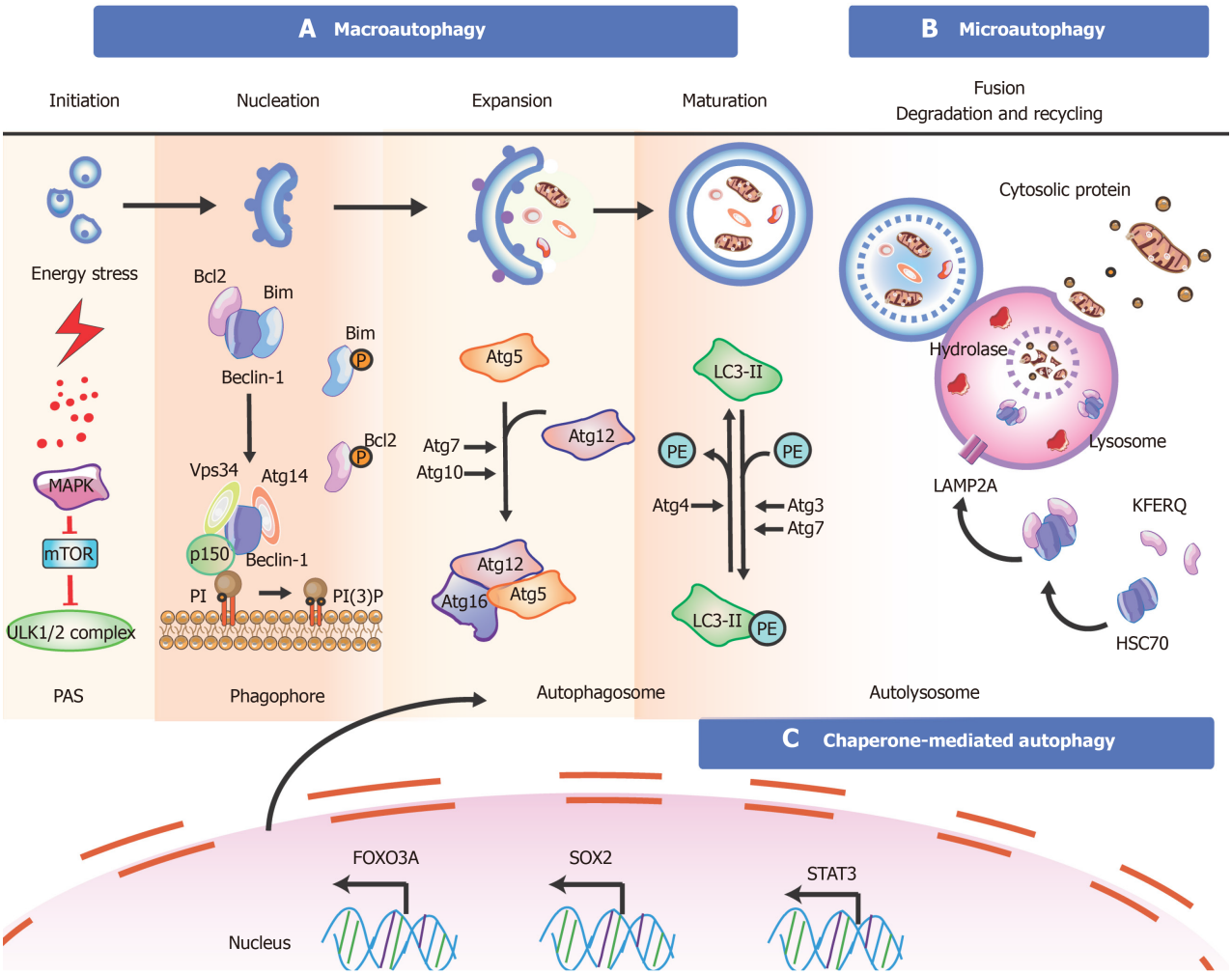

Figure 3 Overview of the mechanisms during autophagy in stem cells. There are three types of autophagy [macroautophagy (section a), microautophagy (section b), and chaperone-mediated autophagy (section c)] based on different pathways; however, they produce the same results. Besides these proteins, key transcription factors closely related to autophagy are shown. The T-shaped lines indicate inhibitory interactions involved in this pathway, while the solid arrows indicate activating interactions. A: Typically, the mTORC1 complex functions as an inhibitor to control the initiation of autophagy. Under environmental stresses and physiological stressors, AMPK is activated to inhibit the activity of mTORC1, leading to a release of the ULK1 (Unc-51-like kinase complex, also known as ATG1) complex to induce autophagy. This initiation process is known as the phagophore assembly site (PAS) formation. Next, PI3 is phosphorylated to PI3P *via* the class III PI3-kinase-Beclin1 complex formed by core subunits of Beclin1 (Atg6), Atg14 L, and Vps34-Vps15, resulting in autophagosome formation. The Atg12-Atg5-Atg16L1 complex acts as a regulator for enveloping and translocating the cytoplasmic cargo to the lysosome in misfolded-protein degradation. Atg4 can cleave LC3 (Atg8) to generate cytosolic LC3-I. Atg3 (E2 enzymes) and Atg7 (E1-like enzymes) can lead the conjugation of PE to LC3-I to form lipidated LC3-II, which is combined with the autophagosome membrane to complete and elongate autophagosome formation. Finally, the autophagosome contents undergo degradation due to low lysosomal pH; B: In microautophagy, misfolded or/and toxic proteins can be directly engulfed by the lysosomal membrane and degraded in the lysosome; C: During chaperone-mediated autophagy, the heat shock cognate 70 kDa protein (HSC70) chaperones attach to the pentapeptide motif KFERQ (namely Lys-Phe-Glu-Arg-Gln) for delivery to lysosomes *via* a specific receptor LAMP2A. Also, some of the key transcription factors are closely linked to the stem cell state and the occurrence of autophagy (bottom). FOXO3A can enhance autophagosome formation *via* autophagy gene expression in hematopoietic stem cells and breast cancer stem-like cells, which is needed to mitigate an energy crisis and allow cell survival. Besides FOXO3A, other transcription factors such as SOX2, STAT3, OCT4, KLF4, and c-Myc are also vital for reprogramming in the initial creation of stem cells at the genetic level during autophagy.

tiation of HSC *via* activating a strong and specific p65-nuclear factor κB (NF-κB)-dependent gene program that prevents necroptosis rather than apoptosis to poise HSCs for myeloid cell production[153].

Others

In addition to apoptosis and autophagy (mentioned above), reports on other cell death types have led to studies exploring cell death mechanisms, such as ferroptosis and pyroptosis[35,132,198-203]. Notably, different cell death mechanisms can simultaneously occur in disease (termed as 'PANoptosis'), suggesting a complex but practical integrated network between various cell death mechanisms in SCs[204,205].

Ferroptosis had been observed in SCs with an imbalance of iron homeostasis, a significant upregulation of cytosolic free iron content, and DNA/protein/lipid

oxidative damage, leading to an obvious senescence phenotype and spontaneous death in iPSC-derived neuronal precursor cells (NPCs)[134,206]. iPSCs and gene-correction are used for treating Pelizaeus-Merzbacher disease (PMD) but subsequently undergo cell death after the pre-myelinating stage with evidence for caspase-3-dependent apoptosis in approximately 40% of cells and ferroptosis[205]. Thus, iron chelators and lipophilic antioxidants can lead to downregulation of apoptosis and ferroptosis[205]. Further, transfusional iron overload (IOL) may have clinical importance as a character close to transplant-related mortality in hematopoietic stem cell transplantation (SCT) for hematologic malignancies (HM)[198].

For pyroptosis (TLR4-NLRP3-mediated cell death pathway), a large body of evidence shows that stem cell transplantation can function as an inhibitor for pyroptosis, suggesting a novel approach called stem cell-derived exosome treatment [207,208], and numerous molecular pathways, such as exosome/LncRNA KLF3-AS1/miR-138-5p/Sirt1 axis and exosome/circHIPK3/FOXO3a axis, are presented[132, 133,209].

All kinds of RCDs contribute to making a constant effort to maintain a homoeostatic balance, in which it is especially significant for the therapeutic effects of SC-based therapy. As for apoptosis in SCs, the intrinsic and extrinsic pathways play a synergistic role in ensuring the multi-cellular organisms to keep normal cells, and remove abnormally proliferating cells or other defective cells. Failure to regulate apoptosis would lead to the uncontrolled growth and division of cells during pathological process. In this regard, whether the SCs that we utilized in transplantation would be uncontrolled someday is also a potential challenge. Compared with apoptosis, autophagy could be regarded as a source of energy through digestion of cellular structures and/or organelles against multiple stresses such as nutrient deprivation (caloric restriction). These two main RCD pathways are widely studied and also some novel ways such as active-Bax in Golgi to inducing apoptosis will be further dug out. Remarkably, Bcl-2 as a co-regulator during these two pathways might be a potential target not only for apoptosis but also for autophagy. Others RCDs such as neroptosis, pyroptosis, and ferroptosis are also found in transplanted SCs, but their detail signaling and application need to keep digging. All in all, various cell death mechanisms are under investigation (apart from the cell death types described). Notably, it is necessary to focus on the overall network between different molecular cell death pathways.

STRATEGIES TO PROMOTE STEM CELL SURVIVAL FOR TRANSPLANTATION THERAPY

As mentioned above, the microenvironment exerts a vital role in the survival of SCs. Many studies have contributed to providing a wide range of strategies to enhance stem cell transplantation therapy *via* improving the microenvironment, including preconditioning strategy (*e.g.*, exposure to oxidative stress, heat shock, and ischemic/hypoxic injury), pretreatment (*e.g.*, drug treatment, cytokines, antioxidants, nitric oxide, glucose deprivation, growth factors, miRNAs, and exosomes), genetic modification, and co-transplantation of different cell types (shown in Figure 4 and Table 3[210-228).

Preconditioning strategy

Preconditioning strategies mainly help to promote tolerance of SCs and progenitor cells derived from SCs. These triggers aim to alter cell signaling and metabolism for adaptation to appropriate and mild stress conditions and sublethal insults [*e.g.*, ischemic preconditioning (IPC), hypoxia, anoxia, hydrogen sulfide (H_2S), hydrogen dioxide (H_2O_2), and carbon monoxide (CO)].

In detail, IPC of SCs is considered an efficient method to promote cell survival. After a repeated short cycle of ischemic/reperfusion (I/R), some of the chemical signals (*e.g.*, ROS, NO, and adenosine) can release and trigger cell protection *via* a cascade of survival factors such as the activation of protein kinase C (PKC), protective protein kinase B (PKB or Akt), nuclear factor κB (NF-κB), and Src protein tyrosine kinases, and subsequent upregulation of cyclooxygenase-2 (COX-2), inducible NO synthase (iNOS), heme oxygenase-1 [HO-1], Mn superoxide dismutase, aldose reductase, and anti-apoptotic genes (*Bcl-xL, Mcl-1, c-FLIPS,* and *c-FLIPL*)[210]. During ischemia/hypoxia or heat shock preconditioning, the level of Hsp70 and Hsp90 is upregulated. Reports suggest that Hsp70/90 can inhibit SMAC in the myocardium to prevent activation of caspase-3/9 (pathway described above)[211,212].

Table 3 Strategies to enhance stem cell transplantation therapy

Strategy	Method	Target	Effects	Molecular mechanisms	Ref.
Preconditioning	Short repeated ischemia/reperfusion	ESCs	Enhancing the tolerance of subsequent prolonged lethal ischemia	Promoting the expression of trophic factors, inducing the release and activation of PKC, PKB, or Akt, NF-κB and Src protein tyrosine kinases, and subsequently upregulating COX-2, iNOS, HO-1, Mn superoxide dismutase, aldose reductase, and antiapoptotic genes	[210-212]
	Hypoxia	MSCs	Promoting mesenchymal stem cell migration and survival	Increasing the expression of lncRNA-p21, HIF-1α, and CXCR4/7(both were chemokine SDF-1 receptors)	[213]
		CSCs	Promoting survival and cardiogenic differentiation	Inducing the activation of the HIF-1α/apelin/APJ axis	[214]
		NSCs	Promoting survival and neuroprotective properties, and facilitating functional recovery *in vivo*	Upregulating HIF1-α and HIF target genes such as *EPO* and *VEGF* and neurotrophic, and growth factors	[215]
	Hydrogen peroxide preconditioning	BMSCs	Improving the therapeutic potential for wound healing	Upregulating cyclin D1, SDF-1, and its receptors CXCR4/7 expression, and activating the PI3K/Akt/mTOR pathway, but inhibiting the expression of p16 and GSK-3β	[216]
	Nitric oxide donor preconditioning	hCSCs	Enhancing survival	Upregulating phosphorylation of NRF2, NFκB, STAT3, ERK, and AKT, as well as increasing the protein expression of HO-1 and COX2	[217]
	Heat shocking	MSCs	Promoting migration	Triggering the activation of ERK and PI3K/Akt signaling pathways *via* HSP90	[218]
Pretreatment	Oxytocin	MSCs	Antiapoptosis and cell protection	Increasing the expression of Akt and phospho-ERK1/2 proteins, rapid calcium mobilization, and upregulation of antiapoptotic and angiogenic genes including *HSP27/32/70, TIMP-1/2/3, VEGF*, thrombospondin, and matrix metalloproteinase-2	[219]
	Minocycline	NSCs	Increasing the capacity of migration, proliferation, and differentiation to improve neurological recovery	Increasing the expression of Nrf2	[220,221]
	Melatonin	MSCs	Inducing fewer fibrotic damage	Downregulating the levels of TNF-α, TGF-β, and α-SMA, and upregulating the expression of E-cadherin	[222]
	Extremely low-level lasers	MSCs	Enhancing the migration of MSCs; promoting the proliferation rate of SCs	Allowing the FAK and ERK1/2 pathways and increasing PDGF and HGF; inducing the up-regulation of mitochondrial ROS and NO	[223,224]
Genetic strategies	Overexpressing pro-survival factors	hNSCs	Improving short- and long-term survival	Overexpression of Bcl-2, Bcl-xl, Hif1a, or/and Akt1	[225]
	Genetic modification	MSCs	Potentiating MSC survival	Overexpression of ERBB4 and ILK	[226]
3D technology	Hydrogels mimicking	MSCs, ESCs, EPCs	Role in stem cell differentiation, changing matrix stiffness, mechanical stress and strain, nonlinear elastic, microenvironments and viscoelastic microenvironments	N/A	[227]
Co-transplantation	Co-transplantation of MSCs and HSCs	MSCs HSCs	Enhancing therapeutic effects	N/A	[228]

ESCs: Embryonic stem cells; NSCs: Neural stem cells; MSCs: Mesenchymal stem cells; HSCs: Hematopoietic stem cells; EPCs: Endothelial progenitor cells; hNSCs: Human neural stem cells; SCs: Stem cells; Hsp70/90: Heat shock protein

70/90; ERK: Extracellular regulated protein kinases; Nrf2: Nuclear factor erythroid 2; TNF: Tumor necrosis factor; TGF: Tumor growth factor; SMA: Smooth muscle actin; HGF: Hepatocyte growth factor; ROS: Reactive oxygen species; Bcl-2: B-cell lymphoma 2; ERBB4: Erb-b2 receptor tyrosine kinase 4; ILK: Integrin-linked kinase; SDF-1: Stromal-derived factor-1; EPO: Erythropoietin; VEGF: Vascular endothelial growth factor; TIMP: Tissue inhibitor of metalloproteinase; PDGF: Platelet-derived growth factor.

Similarly, hypoxia-inducible factor (HIF-1) is upregulated during hypoxia preconditioning to inhibit tumor suppressor p53, reduce oxidative phosphorylation, upregulate VEGF receptor levels, and promote the activation of Akt to target caspases and Bcl-2 for anti-apoptosis[229,230]. Recent findings reveal that OM-MSC (olfactory mucosa mesenchymal SC) with hypoxic preconditioning functions as an inhibitor for apoptosis and pyroptosis in microglial cells through activation of HIF-1α *in vitro*[231]. Hypoxia-preconditioned SCs can also upregulate paracrine activity, and their exosomes are also considered a novel transplantation therapy. For example, MSC-derived exosomes with hypoxia preconditioning show promising potential as an effective means for optimized bone fracture healing *via* exosomal miR-126 and the SPRED1/Ras/Erk signaling pathway[232].

Besides preconditioning with ischemia and hypoxia, oxidative stress and heat shocking are also the most common preconditions for SCs within a similar rationale. Chronic exposure to oxidative stress (*e.g.*, H_2O_2, H_2S, and CO) produces protective effects by activating mitochondrial ROS production, resulting in ERK activation and anti-apoptotic protein expression for cell proliferation, migration, anoikis, autophagy, and survival[216,233,234]. Moreover, heat shocking precondition of mesenchymal SCs can induce HSPs to activate ERK and PI3K/Akt signaling pathways, resulting in increased expression of trophic factors, proteins, and genes for cell protection[218].

Pretreatment strategy

Pretreatment is a strategy for successfully protecting transplantable SCs, using various factors before implantation, whereas preconditioning refers to providing a specific environment within sublethal insults. These factors include antioxidants, cytokines, growth factors, and drug therapy (phosphodiesterase inhibitors, glucose deprivation, pro-survival protein expression, and anti-apoptotic proteins).

To date, various drugs have been developed for the pretreatment of SCs. Pretreatment with pharmacological inhibitors can result in increased expression of survival signaling and a high Bcl-2/Bax ratio in the early phase (2 h), and activation of the JAK/STAT signaling pathway in the late phase (24 h) for cardioprotection[210]. Also, Ji group has reported the protective effect of histochrome pretreatment against oxidative stress in cardiac progenitor cells (CPCs) *via* upregulating Bcl-2 and Bcl-xL and downregulating Bax and H_2O_2-induced cleaved caspase-3[235]. Moreover, short-term incubation either with an antioxidant N-acetyl-L-cysteine (NAC) or a specific inhibitor of TNFR 1 signaling can prevent TNF-α-mediated ROS accumulation in HSCs[154]. MSC pretreatment with oxytocin (OT) [10(-10) to 10(-6) M] in response to signaling events can induce Akt and phospho-Ras-dependent extracellular signal-regulated kinase (ERK)1/2, rapid calcium mobilization, and upregulation of anti-

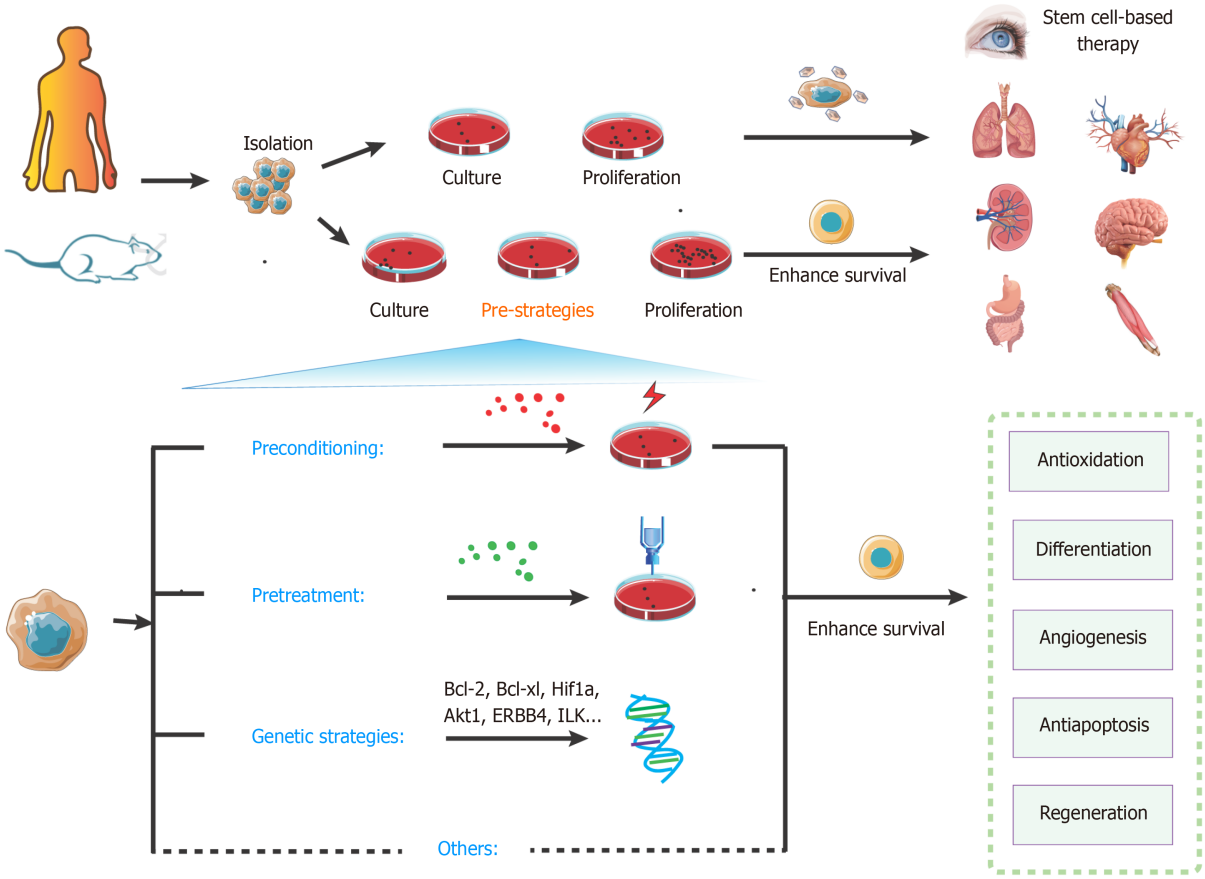

Figure 4 Overview of key strategies to enhance stem cell transplantation therapy. The steps of stem cell-based transplantation therapy include drawing the materials, isolation, culture, proliferation, and transplantation. Compared with the classic approaches, pre-strategies could enhance survival of stem cells. These pre-strategies mainly include preconditioning, pretreatment, genetic strategies, and other methods. They can effectively activate various signaling pathways for protecting cells from injury and promoting survival.

apoptotic and angiogenic genes, including *HSP27/32/70*, tissue inhibitor of metalloproteinase (TIMP)-1/2/3, vascular endothelial growth factor, thrombospondin, and matrix metalloproteinase-2[219]. Minocycline preconditioning increases Nrf2 expression and neuroprotective paracrine secretion. It promotes migration, proliferation, and differentiation of NSCs to improve neurological recovery after NSC transplantation[220,221]. The molecular mechanism involves upregulation of antioxidant genes and reduced oxidative stress grafted cell death following transplantation, resulting in low-rate cell death[221]. Some studies have shown the benefits of melatonin pretreatment on MSC-based therapy with a reduction in the levels of TNF-α, TGF-β, and α-SMA, and upregulation of E-cadherin expression that induces less fibrotic damage[222].

Trophic factors and cytokines are also considered effective pretreatment approaches for regulating MSC fate. For example, SC pretreatment with IL-1β can promote migration and survival of MSCs and improve function in type 2 diabetes, acute myocardial infarction, and neural disorders *via* upregulating the expression of various cytokines, chemokines, and adhesion molecules [*e.g.*, IL-6/8/23A, TNF-α, CCL5/20, CXCL1/3/5/6/10/11, VCA-1 (vascular cell adhesion molecule 1), and ICAM-1/4 (intercellular adhesion molecule 1 and 4)]. IL-1β can induce phosphorylation of NF-κB, but not PI3K/AKT and ERK1/2 pathways[236]. In the NSC pretreatment strategy, a series of experiments using IL-6 show that it can reprogram NSCs to tolerate hostile environments *via* activating STAT3 to increase the levels of superoxide dismutase 2 (SOD2) for anti-apoptosis against inflammatory cytokines and oxidative stress *via* mitochondrial-dependent apoptotic pathways[237,238]. Some other molecular targets, including Rho-associated kinase inhibition, TGF-β2 treatment, SDF-1 signaling of PI3K/Akt, and p38 MAPK inhibition *via* anti-apoptotic pathways, also enhanced SC survival during treatment[239].

Compared with chemical pretreatment methods discussed above, physical factors such as extremely low-level lasers, pulsed electromagnetic fields (PEMF), mechanical stretch, and nanochelating-based nanocomplexes (*e.g.*, GFc7) are also used as pretreatment methods to enhance SC-based therapy[240-243]. For example, pretreatment with extremely low-level lasers improves the migration ability of MSCs *via* activation of FAK and ERK1/2 pathways and increased expression of platelet-derived growth factor (PDGF) and HGF. Furthermore, it also promotes the proliferation rate of SCs by inducing the upregulation of mitochondrial ROS and NO and enhancing the expression of the S-phase proportion in MSCs[223,224].

Genetic strategy

Genetic strategies have raised hopes for better SCs-based therapy since they were introduced more than a decade ago[244,245]. The core idea of this technology is to target key genes and the expression of factors related to the fate of SCs. Under different death stimuli, overexpression of various factors such as TNFR, Akt1, stromal cell-derived factor-1 (SDF-1), and hepatocyte growth factor (HGF) is beneficial for the repopulation of SCs[246]. Studies on modified transplanted hNSCs show improved short- and long-term survival of transplanted hNSCs *via* overexpression of these pro-survival factors, including Bcl-2, Bcl-xl, Hif1a, or/and Akt1[225]. Genetic modification for ERBB4 (erb-b2 receptor tyrosine kinase 4) and ILK overexpression could potentiate MSC survival[226]. In recent years, the CRISPR/Cas9 system has been widely used for genome editing applied in genetic modification of SCs for *in vivo* applications such as neural regeneration, bone regeneration, treatment of blood disorders, and cartilage tissue engineering[247]. Although gene modification promises to enhance tolerance to damage "at the root," there are still formidable predictability challenges and potential long-term side effects.

Others

Recently, three-dimensional culture technologies (*e.g.*, MSC encapsulation technique) mimicking the physical environment to sustain the viability of SCs to induce multi-lineage differentiation are used to protect SCs from PCD as an innate immune system and provide favorable mediators such as cytokines and growth factors[227,248]. However, the time, cost, and labor efficiency of three-dimensional technologies for SCs may be non-negligible challenges, and a combination of biocompatible materials based on simple and easy methods is needed for SC-based therapy. Moreover, co-transplantation of different cell types offers an alternative strategy to improve outcomes of SC-based treatment. Studies show promising results with co-transplantation of human fetal mesenchymal and hematopoietic SCs in type 1 diabetes, epidermal neural crest SCs (EPI-NCSC), and olfactory ensheathing cells (OEC)[228,249]. However, the significance of co-transplantation for SC-based therapy is still unclear[250,251].

As described above, these pre-strategies could provide transplanted stem cell with a certain microenvironment to improve the survival. The core ideas of these methods are to upregulate the survival factors (*e.g.*, Bcl-2, Akt, SMAC, mTOR, SOD2, STAT3, HSC 70, ERK, and Nrf2) and downregulate the death catalyzers (*e.g.*, caspase, p53, TNFa, Bax, cyt C, XIAP, MAPK, and Atg) (shown in Figure 5). Bcl-2 might be regarded as a key molecule that raised tremendous expectations, which plays a vital role in both apoptotic and autophagy pathways. Given the fact that gene strategies seem to be hardly accepted in clinical trials to improve effectiveness of SC-based transplantation, preconditioning and pretreatment may provide a cost-effective and handy option. Remarkably, distinct types of transplanted cells or distinct aiming organs show noticeable differences not only in their signaling but also their response to the local area, so studies need to find a right composition as well as an effective target of any applied transplanted SC system.

CONCLUSION

The SC pool plays a driving role in tissue homeostasis and harm repair. Lately, SC-based therapies may be regarded as a potential strategy that raised tremendous expectations and presented favorable curative effects in enhancing functional repair and repairing damaged tissue. Given the fact that a considerable number of studies on SC-based therapy verify that RCDs occur extensively during the development of the transplanted SCs, RCDs show a crucial role in the therapeutic efficacy and progression of this treatment. Also, RCD interventions may offer opportunities for a better clinical

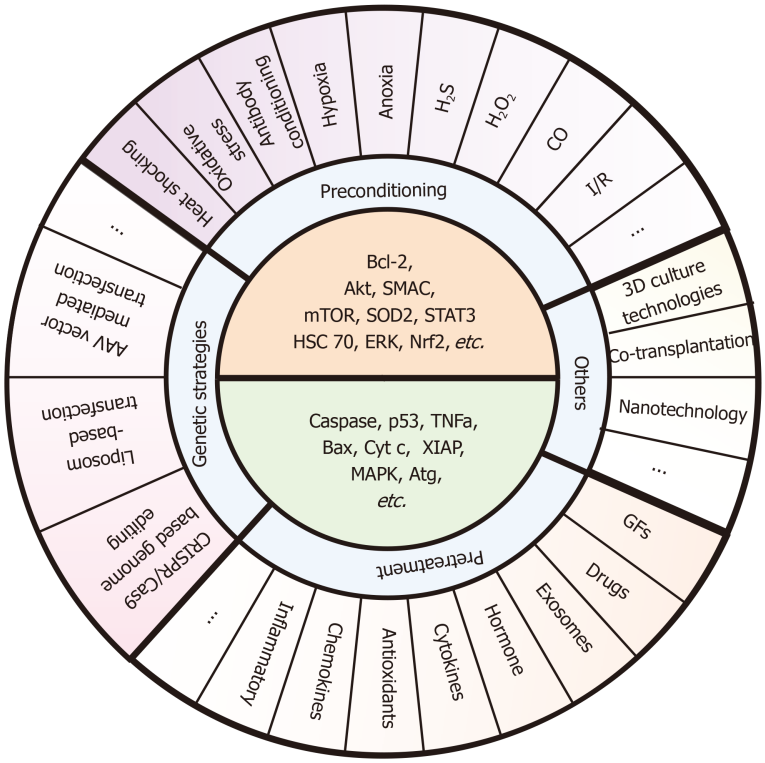

Figure 5 Specific pre-strategies and their key molecule targets for enhancing stem cell transplantation therapy. These pre-strategies mainly include preconditioning (*e.g.*, exposure to oxidative stress, heat shock, and ischemic/hypoxic injury), pretreatment (*e.g.*, drug treatment, cytokines, antioxidants, nitric oxide, glucose deprivation, growth factors, miRNAs, and exosomes), genetic strategies (*e.g.*, AAV vector mediated transfection, Liposome-based transfection, and CRISPR/Cas9-based genome editing), and other methods (*e.g.*, 3D culture technologies, co-transplantation, and nanotechnology). The core ideas of these pre-strategies are to upregulate the survival factors (*e.g.*, Bcl-2, Akt, SMAC, mTOR, SOD2, STAT3, HSC 70, ERK, and Nrf2) and downregulate the death catalyzers (*e.g.*, caspase, p53, TNFa, Bax, Cyt c, XIAP, MAPK, and Atg). However, there are few methods targeting all of these molecules at the same time during the co-network. Also, studies pay more attention to certain signaling such as Bcl-2 and mTOR, and other signals such as Atg or XIAP still need further mining.

application.

Recently, there have been tremendous strides in understanding the fate of SCs post-transplantation related to self-condition and microenvironment. Along this line, targeting multiple signal transduction pathways in PCDs and survival processes would provide novel approaches for enhancing SC-based therapies. However, the interactions are complex and involve multiple networks rather than one crucial pathway (as the recent term 'PANoptosis'), thus necessitating further research. Moreover, various factors involved in specific pathways may change during stem cell differentiation or show microenvironmental divergence in different cell types, stages of development, and stimuli.

Several approaches can prevent the loss of a vast majority of transplanted SCs, such as preconditioning, pretreatment, and genetic strategies. Important insights into the molecular pathways that control PCD of SCs may unlock novel and potential avenues for regenerative drugs and more efficient therapy. These pre-strategies provide SCs with harsh or nutrient-rich environment to improve the SCs *via* upregulating the survival factors and downregulating the death catalyzers. A summary diagram is shown in Figure 6. Recently, some of the novel technologies such as 3D culture technologies, co-transplantation, and nanotechnology also show promising prospects. Furthermore, safer use, better results, and highly feasible and beneficial methods are required for clinical applications.

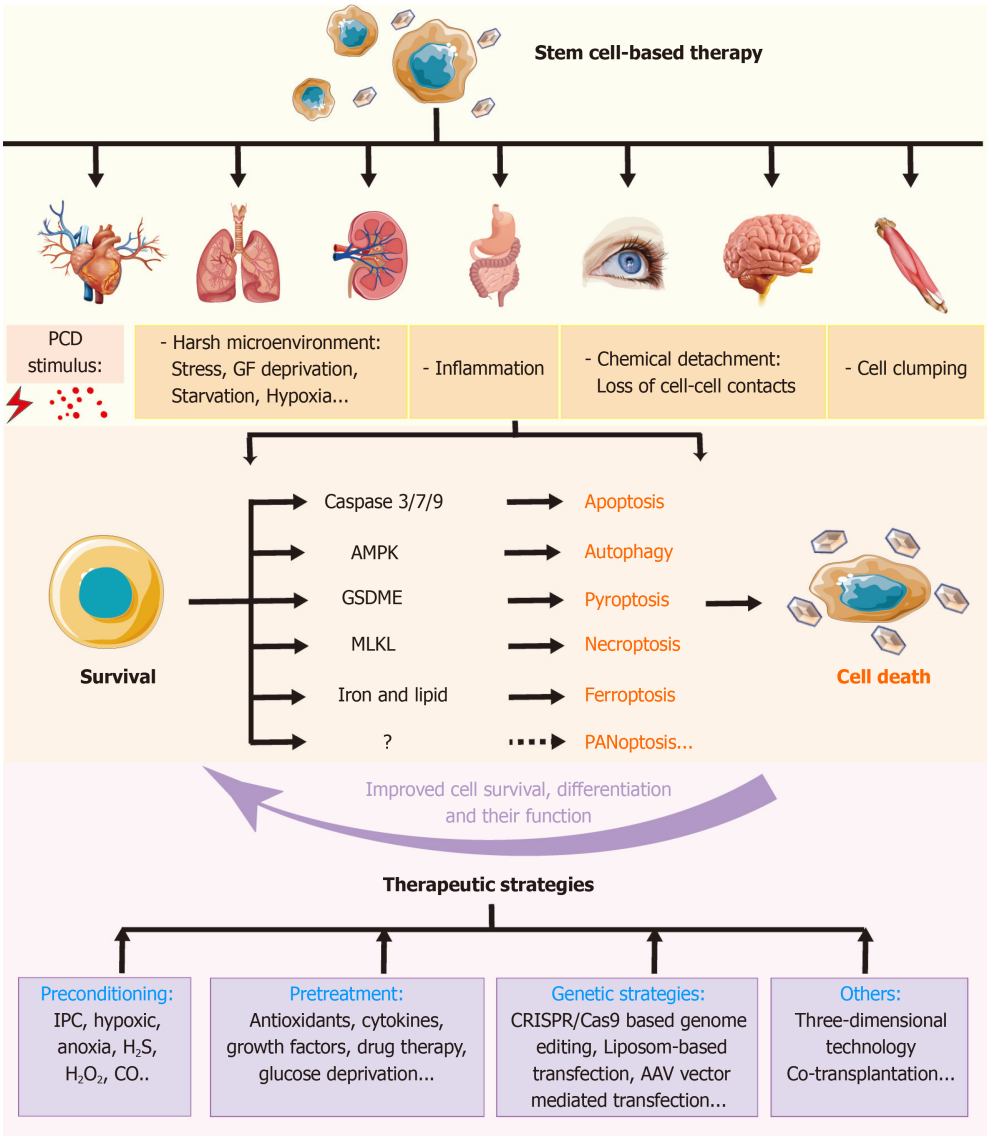

Figure 6 Role of regulated cell deaths in stem cell-based transplantation and therapeutic pre-strategies to improve the therapy. Stem cell-based therapy has been used in various diseases. A number of stimuli may induce regulated cell deaths (RCDs) in transplanted stem cells (SCs), which results in poorer outcomes. Different signals involved in distinct types of RCDs may provide some targets to improve SC-based transplantation. These therapeutic strategies include preconditioning, pretreatment, gene strategies, and so on. IPC: Ischemic preconditioning; PCD: Programmed cell death; MLKL: Mixed lineage kinase domain like protein; GSDME: Gasdermin E.

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REVIEW

Low complexity domains, condensates, and stem cell pluripotency

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Abstract

Biological reactions require self-assembly of factors in the complex cellular milieu. Recent evidence indicates that intrinsically disordered, low-complexity sequence domains (LCDs) found in regulatory factors mediate diverse cellular processes from gene expression to DNA repair to signal transduction, by enriching specific biomolecules in membraneless compartments or hubs that may undergo liquid-liquid phase separation (LLPS). In this review, we discuss how embryonic stem cells take advantage of LCD-driven interactions to promote cell-specific transcription, DNA damage response, and DNA repair. We propose that LCD-mediated interactions play key roles in stem cell maintenance and safeguarding genome integrity.

Key Words: Liquid-liquid phase separation; Embryonic stem cell; Pluripotency; Low complexity domain; Transcription; DNA damage response

Core Tip: This review article discusses recent findings regarding the role of low-complexity domain proteins and phase separation in regulating key cellular processes in embryonic stem cells, including transcriptional activation and repression, cellular signaling integration, DNA damage response, and DNA repair.

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INTRODUCTION

Embryonic stem cells (ESCs) are derived from pluripotent cells in the inner cell mass of the blastocyst[1,2]. ESCs are highly proliferative cells that can self-renew indefinitely *in vitro*. In addition to replication stress due to an abbreviated cell cycle[3], it has been shown that ESCs are transcriptionally hyperactive[4]. The increased replication and transcriptional burdens in ESCs promote genome instability[5-8]. Therefore, ESCs are under increased pressure to conduct transcription and DNA repair efficiently to maintain stem cell identity and genome integrity. Deciphering the mechanisms by which ESCs safeguard transcriptional and genomic fidelity is important for understanding pluripotency, and for translating stem cell-based therapies.

In response to developmental signals, ESCs exit from self-renewal and undergo differentiation to generate every cell type in the body. This highly dynamic process requires coordinated changes in gene expression patterns. Genes required for stem cell self-renewal are silenced, while genes encoding developmental regulators that are normally repressed are reactivated to direct the differentiation of ESCs into cell types representing the three embryonic germ layers[9]. Wholesale changes in gene expression are accompanied by reconfiguration of chromatin structure in differentiating ESCs, whereby previously euchromatic regions associated with pluripotency genes are packaged into repressive heterochromatin[10,11]. Conversely, genomic loci associated with lineage-specific genes become euchromatic, thus permissive to transcriptional activation[12].

A fundamental problem in stem cell biology (and cell biology in general) is how complex biochemical reactions (*e.g.*, transcription, DNA replication, repair, chromatin remodeling, and signal transduction) are organized and regulated inside a densely packed cellular space. While specific cellular reactions can be compartmentalized within classic membrane-enclosed organelles such as endoplasmic reticulum and Golgi apparatus, those that occur inside the nucleus present a unique challenge because the nucleus lacks such organelles to spatially and temporally control biological reactions, where inadvertent "mixing" of these reactions could prove fatal to a cell. Indeed, it has been shown that proteins in the nucleus are often enriched in discrete membraneless compartments. For example, factors involved in mRNA splicing are concentrated in the Cajal bodies to facilitate assembly of spliceosomal machinery[13]. Nucleoli are sites of ribosome biogenesis enriched in factors required for ribosomal RNA transcription and processing[14], and were recently identified as a protein quality control compartment[15]. Under specific conditions such as biomolecular concentration, temperature, pH, and salt concentration, biomolecules can coalesce and separate from bulk solution in cells, as condensates reminiscent of oil droplets in water[16-18]. This process, termed liquid-liquid phase separation (LLPS), underlies the formation of membraneless compartments such as the nucleolus and Cajal body[19,20]. Recent work also implicates biomolecular condensates in a wide range of cellular processes, enriching specific macromolecules within distinct compartments and increasing local concentration to overcome activation barriers[13,14,21-23].

In this review, we examine the emerging roles of protein hub formation and condensation in compartmentalizing and coordinating biochemical reactions in the complex nuclear environment. We discuss how protein condensates enhance cellular reactions critical for stem cell function, facilitate crosstalk between cellular processes to generate complex responses to changing cellular environment, and how these responses collectively safeguard stem cell fidelity.

PHASE SEPARATION OF PROTEINS CONTAINING INTRINSICALLY DISORDERED REGIONS

Intrinsically disordered regions (IDRs) are prevalent in eukaryotic proteome, particularly among regulatory proteins such as transcription factors[24]. These unstructured regions are often composed of low-complexity sequences limited in amino acid diversity. Low complexity domains (LCDs) are enriched in glycine, and polar residues such as serine, asparagine, glutamine, and tyrosine. Other IDRs are characterized by clusters of positively and negative charged amino acid (*e.g.* lysine, glutamic acid) interspersed with hydrophobic residues such as phenylalanine[25]. These unique amino acid compositions found in LCDs have been shown to promote LLPS by polar or charge-charge intermolecular interactions in a concentration dependent manner[25,26]. In addition, the flexible nature of LCDs is thought to facilitate their interaction

with multiple protein partners, by rapidly adopting an ensemble of conformations[27, 28] Indeed, LCD's ability to bind multiple proteins, also known as multivalency, is a major driving force of LLPS by lowering threshold concentration[29]. It is worth emphasizing that while LCDs are unstructured sequences, they do not always bind promiscuously to any proteins; instead, they can be selective for binding partners[30-32]. More importantly, because these selective multivalent interactions are usually weak and transient, as opposed to the high affinity (but low valency) "lock-and-key" interactions found in ligand-receptor complexes, they allow dynamic regulation of LLPS properties, condensate composition, and biochemical reactions that take place inside these bodies. In the following sections, we discuss examples wherein LCD-driven interactions play a critical role in regulating cellular processes relevant to ESC biology.

LCDS IN TRANSCRIPTIONAL ACTIVATION AND REPRESSION IN ESCS

Transcriptional activation

During early embryonic development, pluripotent cells in the inner cell mass of the blastocyst rapidly expand through self-renewal[33]. Buttressing this critical developmental period is a robust gene regulatory network that functions to maintain pluripotency in these cells[34-36]. High transcriptional activity in ESCs has been shown to skew towards genes that encode transcription factors and chromatin remodeling machinery[4], likely as an adaptive measure to meet the increased transcriptional demand. How expression of these factors stabilizes the pluripotent state in ESCs has become apparent through a number of seminal studies. Transcription factors octamer-binding transcription factor 4 (OCT4) and sex-determining region Y-box 2 (SOX2) play a pivotal role in activating stem cell pluripotency[37-42]. Cooperative binding of OCT4 and SOX2 along with a wide array of transcription factors and transcriptional coactivators at gene enhancers lead to the formation of "super enhancers." Super enhancers differ from typical enhancers by their unusually high density of transcription factors spread over a relatively large genomic region measured in kilobases[43-45]. These transcription factor-rich domains are thought to fuel higher transcriptional output by the RNA polymerase II (Pol II) machinery. The cooperative nature of transcription factor assembly at super enhancers is thought to allow the formation or collapse of super enhancers over a relatively small concentration range of transcription factors[44], and is therefore proposed to play an important role in dynamic gene expression during ESC self-renewal and differentiation. Recent studies on LCDs, which are highly enriched in transcription factors, provide important insights into how these high-density transcription factor hubs are formed to drive cell-specific transcription in ESCs (Table 1) [46-54].

Mediator

The ubiquitous transcriptional coactivator Mediator is a large, multisubunit complex that is required for transcription of most Pol II genes, by virtue of its ability to interact with a wide array of transcription factors and Pol II[55,56]. Mediator stimulates transcription by functionally and physically connecting transcription factors at enhancers to the Pol II machinery at promoters[57], where distal enhancers are brought to proximity to their target promoters through DNA looping by cohesion-CTCF (CCCTC-binding factor)[58,59] (Figure 1A). Small hairpin RNA-mediated screens indicated that downregulation of subunits of the Mediator complex compromises expression of OCT4/SOX2-dependent genes in mouse ESCs[60]. Consistent with its role as a coactivator for OCT4/SOX2, Mediator colocalizes extensively with OCT4 and SOX2 across the ESC genome[61].

The mediator complex subunit 1 (MED1) of the Mediator complex contains an LCD at the C-terminus that is rich in serine residues[62]. Studies have shown that MED1 LCD and Mediator holocomplex undergo LLPS *in vitro*. Substitution of serine residues in MED1 with alanine abolishes phase separation, indicating the importance of serine-mediated polar intermolecular interactions in LLPS. To examine the mechanism by which Mediator interacts with OCT4 and SOX2, *in vitro* droplet assays showed that MED1 LLPS droplets readily incorporate OCT4 and SOX2[46]. Furthermore, mutations of acidic amino acids in the activation domain of OCT4, which abrogate transactivation activity, also compromise its ability to phase separate with MED1. These observations indicate a functional correlation between MED1-OCT4 LLPS and transcriptional activation, and suggest LCD-dependent phase separation as a potential mechanism by which activator-coactivator complexes are assembled at gene enhancers

Table 1 Low complexity domain-containing proteins in transcriptional activation and repression in embryonic stem cells

Ref.	LCD-Factors	LCD-domain	Nature of LCD	Experimentally confirmed		Modifications
				In vitro	*In vivo*	
Boija *et al*[46]	MED1	C-terminus	Polar	Yes	Yes[1]	NA
Boija *et al*[46] Brehm *et al* [47]	OCT4	N- and C-terminal domains	Acidic amino acids (electrostatic)	Yes	Yes[1]	Phosphorylation
Boija *et al*[46] Xue *et al*[48]	SOX2	N-terminus	NA	Yes	NA	NA
Boija *et al*[46] Metallo *et al* [49]	c-MYC	Entire polypeptide	NA	Yes	NA	NA
Boija *et al*[46] Jenkins *et al* [50]; Oldfield *et al*[51]	p53	N- and C-terminal domains	Acidic amino acids (N-terminus)	Yes	NA	Phosphorylation
Boija *et al*[46] Xue *et al*[48]	NANOG	N- and C-terminal domains	NA	Yes	NA	NA
Boija *et al*[46]	ER	NA	NA	Yes	NA	NA
Choi *et al*[30]	ABCF1	N-terminus	NA	Yes	Yes[2]	NA
Boehning *et al*[52]	RNA Pol II	C-terminus	Hydrophobic and electrostatic	Yes	Yes[1]	Phosphorylation
Lau *et al*[53]	HP1α	N-terminus	Acidic amino acids	Yes	Yes[1]	Phosphorylation
Plys *et al*[54]	CBX2	Entire polypeptide	Positively charged	Yes	Yes[2]	Phosphorylation

[1]Endogenously-tagged fluorescent protein-fusion.
[2]Overexpression of protein fused with fluorescent tag. LCD: Low complexity domain; MED1: Mediator complex subunit 1; OCT4: Octamer-binding transcription factor 4; SOX2: Sex-determining region Y-box 2; ABCF1: ATP-binding cassette subfamily F member 1; CBX2: Chromobox 2; ER: Estrogen receptor; NA: Not available.

(Figure 1B). It is worth noting that diverse transcription factors (*e.g.* p53, myelocytomatosis viral oncogene homolog, NANOG, estrogen receptor) can also phase separate with MED1 *in vitro*[46]. These results demonstrate that the LCD of MED1 is rather promiscuous in binding, consistent with Mediator acting as a ubiquitous coactivator.

RNA Pol II: Carboxy-terminal domain

Biochemical studies demonstrated that Mediator interacts with RNA Pol II through the carboxy-terminal domain (CTD) of the largest subunit of Pol II complex[63,64]. Mammalian CTD contains 52 heptad repeats of the consensus sequence $Y_1S_2P_3T_4S_5P_6S_7$. This LCD plays important roles at all steps of transcription from initiation to elongation to termination[65]. Initiation requires the assembly of the preinitiation complex (PIC), composed of general transcription factors (GTFs), Mediator, and Pol II with unphosphorylated CTD, at gene promoters[66,67]. As Pol II leaves the promoter and initiates transcription, the CTD becomes phosphorylated on serine 5 (Ser5) by GTF TFIIH-associated kinase, cyclin dependent kinase 7 (CDK7)[68-71]. It is known that Mediator interacts preferentially with the unphosphorylated CTD[72,73] (Figure 1A). This suggests that phosphorylation of Pol II CTD may disrupt its interaction with Mediator, thus providing a mechanism by which Pol II can dissociate from PIC to initiate transcription. Two recent studies support this notion and implicated LLPS in regulating Mediator-Pol II interaction[32,52]. They demonstrated that the ability of the CTD to undergo LLPS by itself, or with MED1, is disrupted by phosphorylation of Ser5 by CDK7. Therefore, Mediator-Pol II interaction and promoter-enhancer communication can be modulated by phosphorylation status of the CTD during the transcription cycle.

Positive transcription elongation factor b

After Pol II escapes the promoter, the CTD becomes hyperphosphorylated at Ser2 by CDK9 of the positive transcription elongation factor b (P-TEFb), while Ser5 previously phosphorylated by TFIIH is gradually removed by phosphatases[74-76]. This switch in Ser phosphorylation pattern is thought to promote elongation by aiding the recruitment of elongation and chromatin-modifying factors to the transcribing Pol II [75] (Figure 1A). A recent study indicated that the histidine-rich LCD of the cyclin T1 subunit of P-TEFb (a heterodimer of CDK9 and cyclin T1) stabilizes the binding of P-

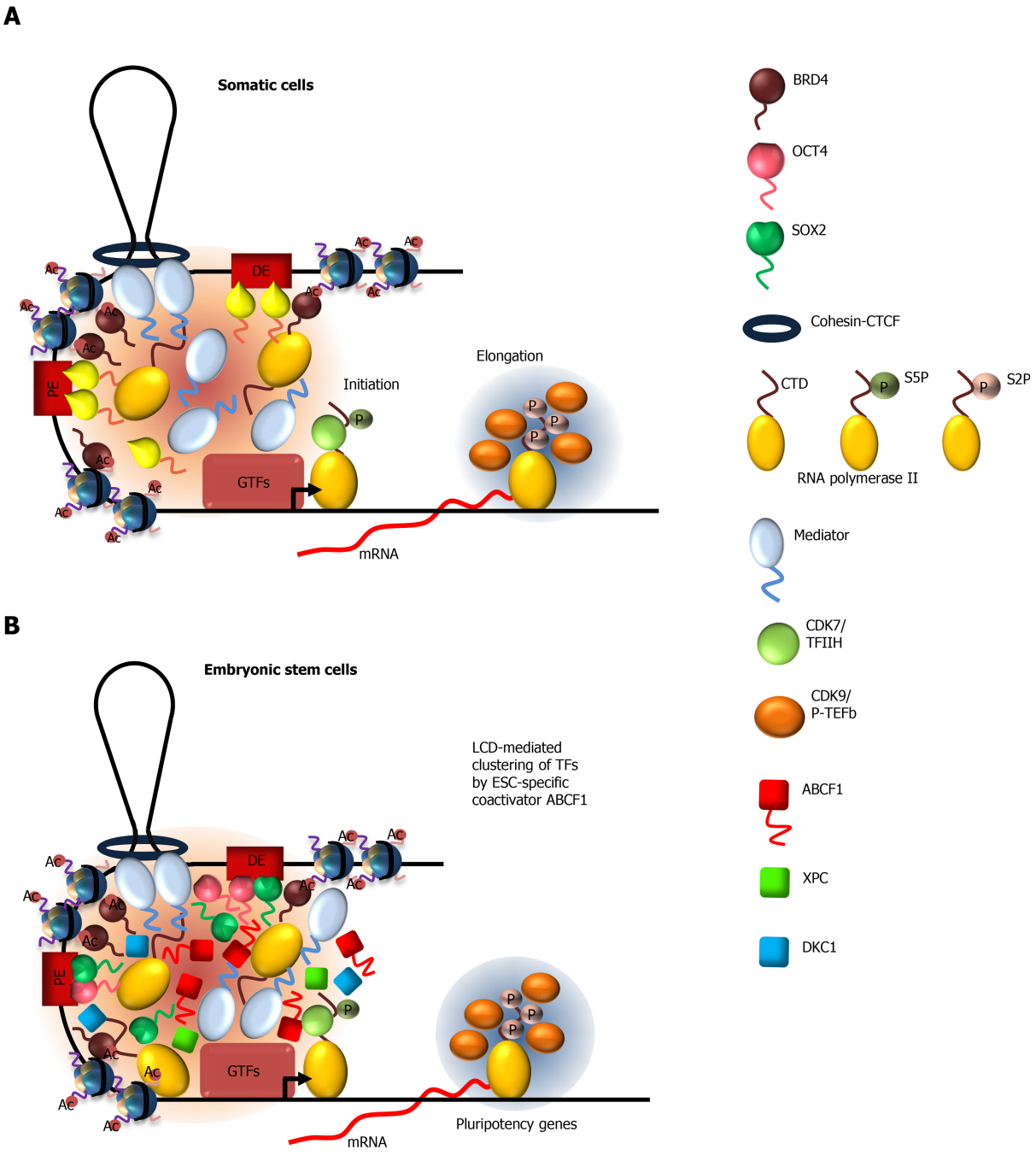

Figure 1 Models depicting the mechanisms by which low-complexity sequence domain-driven interactions between transcription factors and coactivators at gene enhancers contribute to transcriptional activation. A: Mechanism of transcriptional activation in somatic cells. Low complexity domain (LCD)-mediated interactions between Mediator and TFs, Mediator-bromodomain-containing protein 4 (BRD4), and Mediator-RNA polymerase II (Pol II), as well as binding of BRD4 to acetylated histones, facilitate the formation of a transcription factor-rich compartment at proximal enhancer, and at distal enhancers brought into close proximity through DNA looping by cohesion-CTCF. Increasing local concentration of these factors promotes the formation of the pre-initiation complex composed of general transcription factors and Pol II. Phosphorylation of the C-terminal domain (CTD) at serine 5 (S5P) of Pol II by cyclin-dependent kinase 7/TFIIH disrupts Mediator-Pol II condensates, allowing transcriptional initiation and promoter escape by Pol II. During the early elongation phase of transcription, positive transcription elongation factor b preferentially forms condensates with S5P CTD of Pol II. This results in efficient hyperphosphorylation of the CTD at serine 2 and productive transcription elongation by Pol II; B: Optimal activation of pluripotency genes by stem cell-specific transcription factors octamer-binding transcription factor 4 (OCT4) and SOX2 in ESCs requires cell-specific coactivators ATP-binding cassette subfamily F member 1 (ABCF1), xeroderma pigmentosum, complementation group C (XPC), and dyskerin (DKC1). The LCD of ABCF1 forms selective multivalent interactions with SOX2, XPC, DKC1 and Pol II to promote the assembly of Pol II transcription machinery at pluripotency genes. Activation of OCT4/SOX2-target genes *in vivo* likely requires both promiscuous LCD-mediated interactions by Mediator and selective LCD-dependent interactions by ABCF1. LCDs are represented by wavy lines. ABCF1: ATP-binding cassette subfamily F member 1; Ac: Acetylated; BRD4: Bromodomain-containing protein 4; CDK: Cyclin-dependent kinase; DE: Distal enhancer; DKC1: Dyskerin; GTFs: General transcription factors; ESC: Embryonic stem cell; General transcription factors; LCD: Low complexity domain; OCT4: Octamer-binding transcription factor 4; P-TEFb: Positive transcription elongation factor b; PE: Proximal enhancer; S2P: Phosphorylated serine 2; S5P: Phosphorylated serine 5; SOX2: SRY-box 2; TF: Transcription factor; XPC: Xeroderma pigmentosum, complementation group C.

TEFb to active genes and to the Pol II CTD to catalyze Ser2 hyperphosphorylation[77]. They showed that cyclin T1 forms liquid-like puncta in the nucleus in an LCD-dependent manner. Formation of these nuclear condensates and the ability of P-TEFb to hyperphosphorylate the CTD are sensitive to 1,6-hexanediol that disrupts LLPS. Consistent with these observations, the LCD is also required for phase separation of cyclin T1 with CTD *in vitro*. Interestingly, pre-phosphorylation of the CTD by CDK7/TFIIH significantly enhances cyclin T1-CTD LLPS, suggesting that a potential function of Ser5 phosphorylation by TFIIH after promoter escape is to prime LLPS of P-TEFb with the CTD, thereby increasing the efficiency of Ser2 phosphorylation. Taken together, these observations underscore the role of LCD-mediated interactions in regulating transition from transcriptional initiation to elongation.

Chromatin readers

Bromodomain-containing protein 4 (BRD4) is a critical transcriptional and epigenetic regulator in ESCs[78-80]. It contains two bromodomains that recognize acetylated lysines on histone H3 and H4 that are associated with active gene promoters[81]. BRD4 also acts as a scaffold for recruiting P-TEFb and chromatin remodeling proteins to facilitate transcription by Pol II[78,82,83]. BRD4 has been shown to colocalize with Mediator at super enhancers that control genes important for stem cell identity[62] (Figure 1B). BRD4 contains an LCD at its C-terminus with high proline and glutamine content. Studies showed that BRD4 LCD by itself can form LLPS droplets *in vitro* and can be incorporated into MED1 condensates. These results suggest that LLPS between Mediator and BRD4 represents a mechanism by which they are concentrated at super enhancers[62,84]. This is supported by the observation that treatment of cells with 1,6-hexanediol reduced their occupancy at enhancers. It would be interesting to examine whether binding of BRD4 to acetylated nucleosomal DNA promotes its LLPS with Mediator, due to increased valency (*i.e.*, cooperativity) in interactions by BRD4[85]. While the mechanism by which BRD4 recruits P-TEFb to gene promoters is unknown, it is tempting to speculate that their interaction could be promoted by their respective LCD.

Stem cell-specific coactivators

Most if not all of the regulatory factors described thus far are utilized by many transcription factors to activate their target genes in both ESCs and somatic cells. Our work and others indicated that robust transcriptional activation by OCT4 and SOX2 in ESCs requires additional coactivators that are distinct from Mediator[30,86-88]. Using a fully reconstituted *in vitro* transcription assay, we detected multiple novel coactivators that work in concert with OCT4 and SOX2 to activate pluripotency gene transcription. Biochemical purification of these coactivators led to the discovery of three stem cell-specific coactivators - the nucleotide excision repair protein xeroderma pigmentosum, complementation group C (XPC)[87-90], dyskerin (DKC1) ribonucleoprotein complex[86], and the ATP-binding cassette subfamily F member 1 (ABCF1)[30] (Figure 1B). We found that the ability of XPC and DKC1 to stimulate OCT4/SOX2-activated transcription is strongly dependent on ABCF1, indicating a pivotal role of ABCF1 in mediating stem cell-specific transcription.

ABCF1 contains an LCD at the N-terminus that is unusually rich in charged amino acids, of which about 40% are divided between lysine and glutamic acid residues. These clusters of positively and negatively charged amino acid, interspersed with hydrophobic residues such as phenylalanine, are known to promote LLPS[91,92]. Indeed, we showed that ABCF1 undergoes LLPS in an LCD-dependent manner. More importantly, the LCD is also required for transcriptional activity *in vitro* and in ESCs, due to its ability to selectively interact with SOX2 (but not OCT4), its co-dependent coactivators XPC and DKC1 as well as Pol II. These LCD-driven interactions are also detected at OCT4/SOX2-target gene enhancers and are sensitive to disruption by 1,6-hexanediol treatment. It is worth noting that the conformationally flexible XPC protein also contains several highly disordered regions that we found, however, to be dispensable for transcriptional activation[87-90]. These observations revealed the unique ability of ABCF1 LCD to integrate multiple lines of information encoded by SOX2, XPC, DKC1, and the Pol II machinery, likely by forming a hub of these factors at target gene promoters through selective multivalent interactions (Figure 1B). In summary, cell type-specific transcriptional activation in ESCs requires an interconnected network of LCD-driven interactions by both general and cell-specific coactivators for optimal and gene-specific transcriptional activation.

Transcriptional repression

During stem cell self-renewal, developmental genes must be properly silenced. Failure to repress these genomic regions compromises stem cell identity and pluripotency of ESCs[93-96]. Studies have shown that heterochromatin is essential for silencing the autosomal imprinted genomic loci, *HOX* gene clusters and other differentiation-associated genes[97,98]. Heterochromatic regions are characterized by hypoacetylated histones and repressive modifications such as trimethylated histone H3 Lysine 9 (H3K9me3), trimethylated histone H3 Lysine 27 (H3K27me3), and mono-ubiquitination of histone H2A lysine 119 (H2AK119ub)[99-103]. These modifications not only control nucleosomal interactions but also regulate the association of non-histone chromosomal proteins that together influence nucleosomal packaging and gene repression. For example, heterochromatic regions are established and protected by chromatin components and trans-acting factors such as heterochromatin protein 1 (HP1) and Polycomb repressive complexes 1 and 2 (PRC1, PRC2)[104] (Table 1). Understanding how histone binding proteins and histone modifying enzymes are assembled at heterochromatin will elucidate the mechanisms by which a repressed chromatin state is initiated and maintained to silence developmental genes during stem cell self-renewal, and how these heterochromatic regions are decondensed to facilitate their reactivation when ESCs undergo differentiation (Figure 2A). The highly compact heterochromatin structure has led to a number of studies that evoke LLPS for heterochromatin domain formation.

HP1

Compaction of chromatin is a key process in maintaining the repressed state of heterochromatin. HP1 recognizes H3K9me3 modifications through its chromo shadow domain and nucleates chromatin condensation[105,106]. Underscoring a direct role of HP1 in chromatin condensation, artificial targeting of HP1 to a genomic locus is sufficient to cause local condensation and formation of high-order chromatin structure [107]. In mammals, HP1 exists in three isoforms: HP1α, β and γ. HP1α is commonly associated with silenced heterochromatic regions, while the other two isoforms appear to have both gene silencing and activating functions[108-111]. These HP1 proteins possess three LCDs (LCD1, 2, and 3). Interaction between LCD1 and LCD2 has been shown to contribute to multivalent interactions with nucleo-somes[112-114]. HP1α LCD1 in N-terminal extension (NTE) region has also been shown to bind DNA, which in turn induces DNA compaction and phase separation *in vitro* and in cells (Figure 2B). Phosphorylation of NTE of HP1α was shown to disrupt the cooperative binding between HP1α and DNA, resulting in reduced DNA compaction with less defined compaction domains and slower compaction rate[115,116]. These observations are consistent with another study demonstrating that specific loss of HP1α leads to dysregulation in establishing heterochromatin domains[117]. Interestingly, these phosphorylation sites are absent in HP1β and HP1γ, making regulation of HP1 LLPS and chromatin compaction by phosphorylation a unique property of the α isoform [118]. However, a recent study challenges the role of phase separation of HP1 in heterochromatin formation[119]. They demonstrated that HP1 proteins do not form stable LLPS droplets in mouse cells and do not regulate the size, accessibility, and chromatin compaction. Chromatin compaction tolerates loss of HP1 and H3K9me3. Relaxation of heterochromatin upon transcriptional reactivation occurs independent of HP1/H3K9me3. Future studies will be required to resolve the apparent discrepancy.

PRCs

The recruitment of PRC1 complexes to chromatin drives nucleosome compaction and transcriptional silencing[10,103,120,121]. This is mediated by the chromobox 2 (CBX2) subunit of PRC1, which recognizes H3K27me3 that is deposited by histone methyltransferase Enhancer of zeste homolog 2 subunit of the PRC2 complex[122,123]. Once PRC1 is recruited to H3K27me3, it monoubiquitinates H2A at lysine 119 (H2AK119ub), which is essential for maintaining gene repression in ESCs[124]. It has long been observed that PRC1 complexes form concentrated nuclear compartments known as Polycomb bodies[54,125]. Recent studies indicated that CBX2 is responsible for PRC1 LLPS and chromatin compaction[54] (Figure 2C). CBX2 is a low-complexity disordered protein containing a serine-rich patch and positively charged amino acid rich region. It has been shown that phosphorylation of serine residues by casein kinase 2 enhances CBX2 LLPS *in vitro*, likely by facilitating electrostatic intermolecular interactions between phosphorylated serines and positively charged lysines. Consistent with this hypothesis, mutation of 23 Lysine and arginine residues to alanine abolishes CBX2 LLPS *in vitro*. Importantly, lysine to alanine substitutions in CBX2

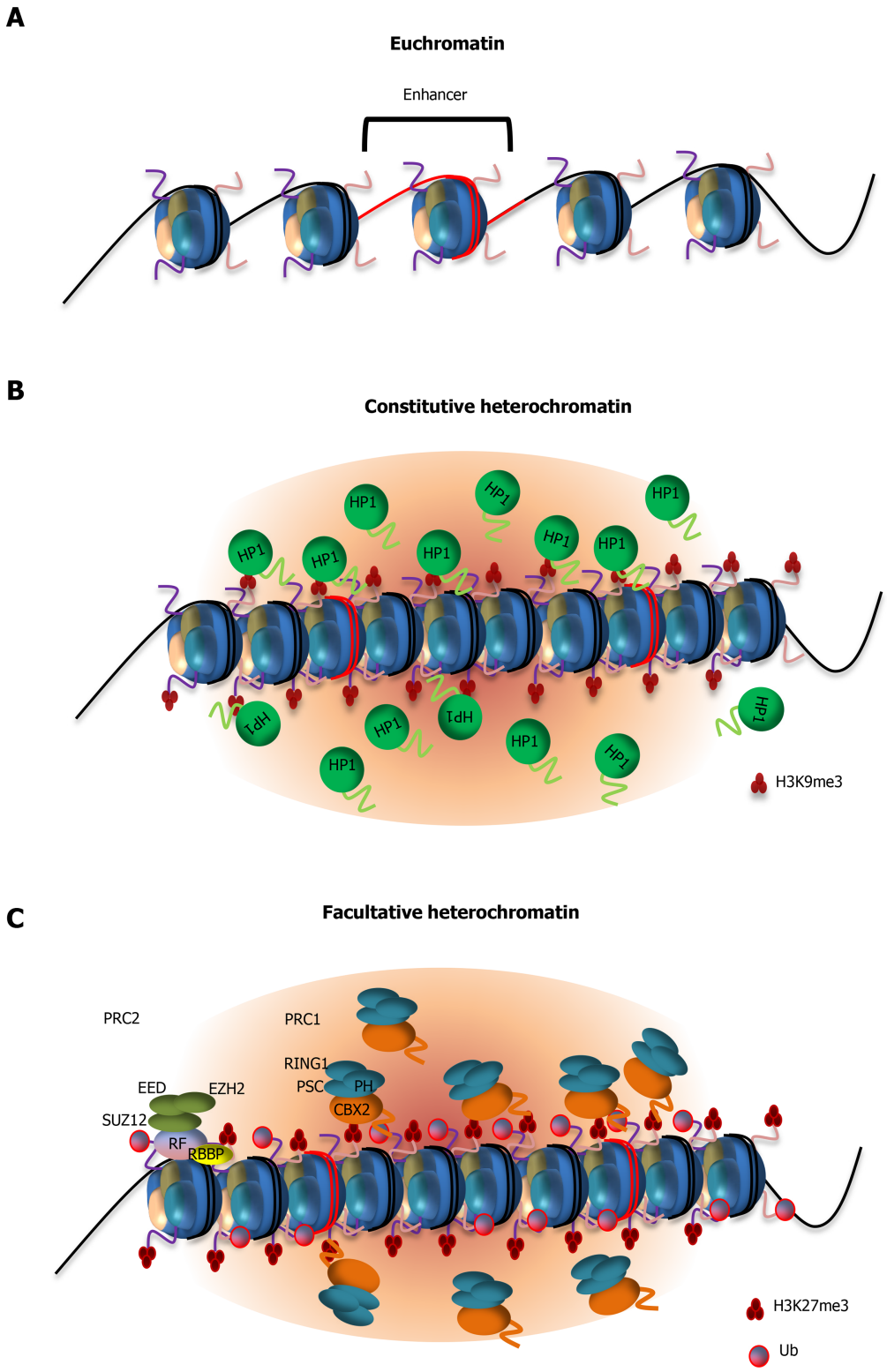

Figure 2 Models showing the role of low complexity domain-mediated protein condensation in gene silencing by heterochromatin formation. A: Euchromatic regions showing nucleosome-depleted regions containing transcription factor binding sites such as enhancers. Nucleosome-free regions are accessible by transcription factors and thus permissive to gene activation; B: Formation of constitutive heterochromatin requires interactions between heterochromatin protein 1 (HP1) and histone H3 Lysine 9 trimethylation and low complexity domain (LCD)-driven condensation of HP1 with DNA; C: Establishment of facultative heterochromatin is initiated by deposition of histone 3 Lysine 27 trimethylation (H3K27me3) by the histone methyltransferase subunit of polycomb repressive group 2 (PRC2), enhancer of zeste homolog 2. Binding of PRC2 to chromatin is regulated by non-core subunits (*e.g.*, Jumonji and AT-rich interaction domain containing 2, polycomb-likes) which act as recruitment factors[178]. Chromatin compaction is then mediated by the recruitment of PRC1, through recognition of H3K27me3 by its subunit chromobox 2 (CBX2). CBX2 contains an LCD that drives phase separation of PRC1 and is required for proper heterochromatin formation. Subsequent mono-ubiquitination of histone 2A lysine 119 by the ring finger protein 1 subunit of PRC1 is essential for gene silencing. CBX2: Chromobox 2; EZH2: Enhancer of zeste homolog 2; H3K9me3: Histone 3 Lysine 9 trimethylation; H3K27me3: Histone 3 Lysine 27 trimethylation; HP1: Heterochromatin protein 1; JARID2: Jumonji and AT-rich interaction domain containing 2; PCL: Polycomb-like; PRC: Polycomb repressive group; RING1: Ring finger protein 1.

result in axial patterning defects in mice, indicating altered *Hox* gene expression patterns during development[53]. Thus, these results support a functional link between CBX2 LLPS and gene silencing. Taken together, these studies suggest a role of LLPS in gene repression through LCD-driven chromatin condensation, and in the proper reactivation of developmental genes in a spatially and temporally regulated manner. It appears that LLPS may play a role in concentrating factors that are critical for chromatin compaction and maintenance of the repressed chromatin state, and in excluding factors that would otherwise gain access to these repressed domains and interfere with gene silencing[115].

INTEGRATION OF SIGNALING PATHWAYS AND TRANSCRIPTION BY LCDS

In mouse ESCs, Hippo/Yes-associated protein (YAP)/transcriptional coactivator with PDZ-binding motif (TAZ), Janus kinase (JAK)/signal transducer and activator of transcription (STAT), Wingless-related integration site (Wnt)/β-catenin, and transforming growth factor beta (TGF-β) pathways play important roles in supporting stem cell self-renewal and pluripotency[126-130]. How ESCs integrate and interpret these signals and generate an appropriate transcriptional response to these cues are key to understanding fundamental mechanism governing self-renewal *vs* differentiation cell fate decision.

Hippo

The Hippo pathway controls cell proliferation and survival by regulating the activity of YAP, a transcriptional coactivator for transcriptional enhancer factors (TEFs)[131-133]. The Hippo pathway regulates YAP activity primarily by controlling its nucleocytoplasmic shuttling through phosphorylation. Activation of the Hippo pathway by signals derived from cell-cell contact, mechanosensing (*i.e.*, substrate stiffness), and cellular stress inhibits YAP by phosphorylation at serine 127, leading to its sequestration in the cytoplasm. When Hippo signaling is inactivated, YAP translocates to the nucleus and stimulates TEF-activated transcription by forming complexes with Mediator and BRD4/P-TEFb[134,135]. Other studies added complexity to this model, by showing that hyperosmotic stress also activates nemo-like kinase, which leads to YAP phosphorylation at serine 128 and, unexpectedly, translocation to the nucleus and activation of YAP-dependent genes, despite simultaneous phosphorylation at serine 127 by the Hippo pathway[136,137].

YAP is enriched in pluripotent ESCs but its level significantly decreases upon differentiation and is further inactivated by phosphorylation at serine 127[127]. YAP supports stem cell maintenance by binding to key pluripotency-associated genes such as *Nanog*, *Oct4*, and *Sox2* and regulate their expression. How YAP stimulates the transcription of these genes was unknown but recent studies implicated phase separation of YAP and its paralogue TAZ as a key mechanism. In one study, YAP was shown to form liquid-like condensates with TAZ and TEF in the nucleus upon hyperosmotic stress[138]. In another study, TAZ but not YAP was shown to undergo LLPS when the Hippo pathway is inhibited, even though YAP and TAZ show extensive sequence similarities[139]. Formation of TAZ condensates in cells is regulated by Hippo pathway, where signals that promote nuclear retention of TAZ induce the formation of nuclear puncta that colocalize with Pol II, BRD4, MED1 and CDK9/P-TEFb, indicating that these condensates likely represent transcriptionally active compartments. Protein domain swapping experiments demonstrated that the ww and coiled-coil (cc) domains of TAZ (but not YAP) contribute to LLPS. This result is in contrast to studies by Cai *et al*[138] showing that YAP can in fact phase separate *in vitro*. Differences in protein preparation, concentration, and *in vitro* droplet formation assay condition may explain the apparent discrepancy. Nevertheless, both studies demonstrated that the ability of YAP or TAZ to activate its target genes requires their LCDs, suggesting that transcriptional activation by TEF is facilitated by LCD-mediated interaction with YAP/TAZ.

Wnt, TGF-β, JAK/STAT pathways

Master transcription factors such as OCT4 and SOX2 define ESC identity in part by integrating extracellular signals at gene enhancers to drive cell-specific transcription. It has been shown that terminal effectors of the Wnt, TGF-β, and JAK/STAT signaling pathways, β-catenin, small mothers against decapentaplegics (SMADs), and STAT3,

respectively, converge onto cell-specific super enhancers[140]. How these enhancers "hijack" signal-regulated transcription factors are not well-understood. A recent study showed that these signaling effectors synergize with OCT4, SOX2, and Mediator by forming transcription condensates at super enhancers[141]. Upon activation of the signaling pathways, β-catenin, SMADs, and STAT3 translocate to the nucleus and form condensates at super enhancer at the *Nanog* locus in mouse ESCs (Figure 3). By contrast, activation of Wnt signaling was not sufficient to target β-catenin to the transcriptionally silenced *Nanog* locus in the muscle cell line C2C12. These results indicate that recruitment of β-catenin to *Nanog* enhancer likely requires open chromatin, active transcription, and presence of other transcription factors bound at active enhancers. Perhaps the high density of transcription factors and abundance of LCD-mediated multivalent interactions at super enhancers promote efficient concentration of signal-dependent transcription factors. Indeed, β-catenin, SMADs, and STAT3 were shown to form condensates with Mediator *in vitro* through their LCDs. Mutations that disrupt β-catenin LLPS also compromise recruitment to its target gene enhancers and transcriptional activation, supporting a functional correlation between LLPS propensity and transcription factor recruitment and gene activation. Compartmentalization of these signaling effectors not only concentrates these factors at the appropriate enhancers but may also insulate these factors from activating the wrong targets. These LCD-dependent multivalent interactions at enhancers likely permit dynamic regulation of transcription - a key feature of regulated gene expression in response to extracellular signaling.

LCDS IN DNA REPAIR AND DNA DAMAGE RESPONSE

Unlike terminally differentiated somatic cells, the fast replication rate of ESCs makes them prone to replication stress-induced DNA damage such as double strand breaks (DSBs)[142-144]. At the same time, high proliferation rate poses significant challenge to DNA repair because DNA lesions that are left unrepaired prior to cell division will be inherited by daughter stem cells and then propagated to their progenitors, likely leading to deleterious effect in development[145]. Therefore, ESCs are under increased pressure to efficiently and accurately repair DNA damages. Indeed, it has been shown that ESCs express higher levels of DNA repair factors and favor high fidelity DSB repair by homologous recombination (HR)[28]. It has also been shown that ESCs are hypersensitive to DNA damage and readily undergo spontaneous differentiation and apoptosis[145,146]. This is likely a fail-safe mechanism by eliminating compromised ESCs from the self-renewing population. In the following sections, we will examine the role of LCD and LLPS in DNA repair and DNA damage response (DDR) and discuss how they safeguard stem cell genome integrity.

HP1 and F-actin

The abundance of repetitive sequences in heterochromatin poses unique challenges to DNA repair due to increased risks of aberrant recombination induced by DSBs, which can lead to deletion, duplication, and translocation[147]. Cells have developed elaborate mechanisms to promote efficient and error-free DNA repair by taking advantage of LLPS. Upon DSB, it has been shown that phosphorylation of threonine 51 in HP1 Leads to dissociation of HP1 from heterochromatin, as evidenced by loss of binding to H3K9me3 and dispersal of HP1 nuclear puncta[115]. Dissociation of HP1 Likely alters LLPS status at DSBs, which in turn facilitates chromatin relaxation and engagement of downstream effectors to initiate DNA repair[148]. In another study using *Drosophila* as a model system, phase separated heterochromatic domain at DSBs appears to be able to exclude repair factors such as Ku80 that are involved in error-prone non-homologous end joining (NHEJ), and enrich factors required for the initial steps of HR repair[149]. It was proposed that such exclusion mechanism favors repair by error-free HR, assuming that HR repair factors can still efficiently assess the damaged site. It will be interesting to test whether this exclusion mechanism is mediated by selective interaction between HP1 condensates and repair factors in HR but not NHEJ pathway.

Expansion of the HP1-organized heterochromatin domain is also thought to facilitate the physical relocation of the DSB DNAs to the nuclear periphery in *Drosophila* or to the heterochromatin domain periphery in mouse cells, locations that are believed to be more conducive to repair by HR[150,151]. Studies demonstrated that physical movement of heterochromatic DSBs depends on polymerization of F-actin and mobilization of DSB DNAs by tethering the DNA and 'walking' along the F-actin

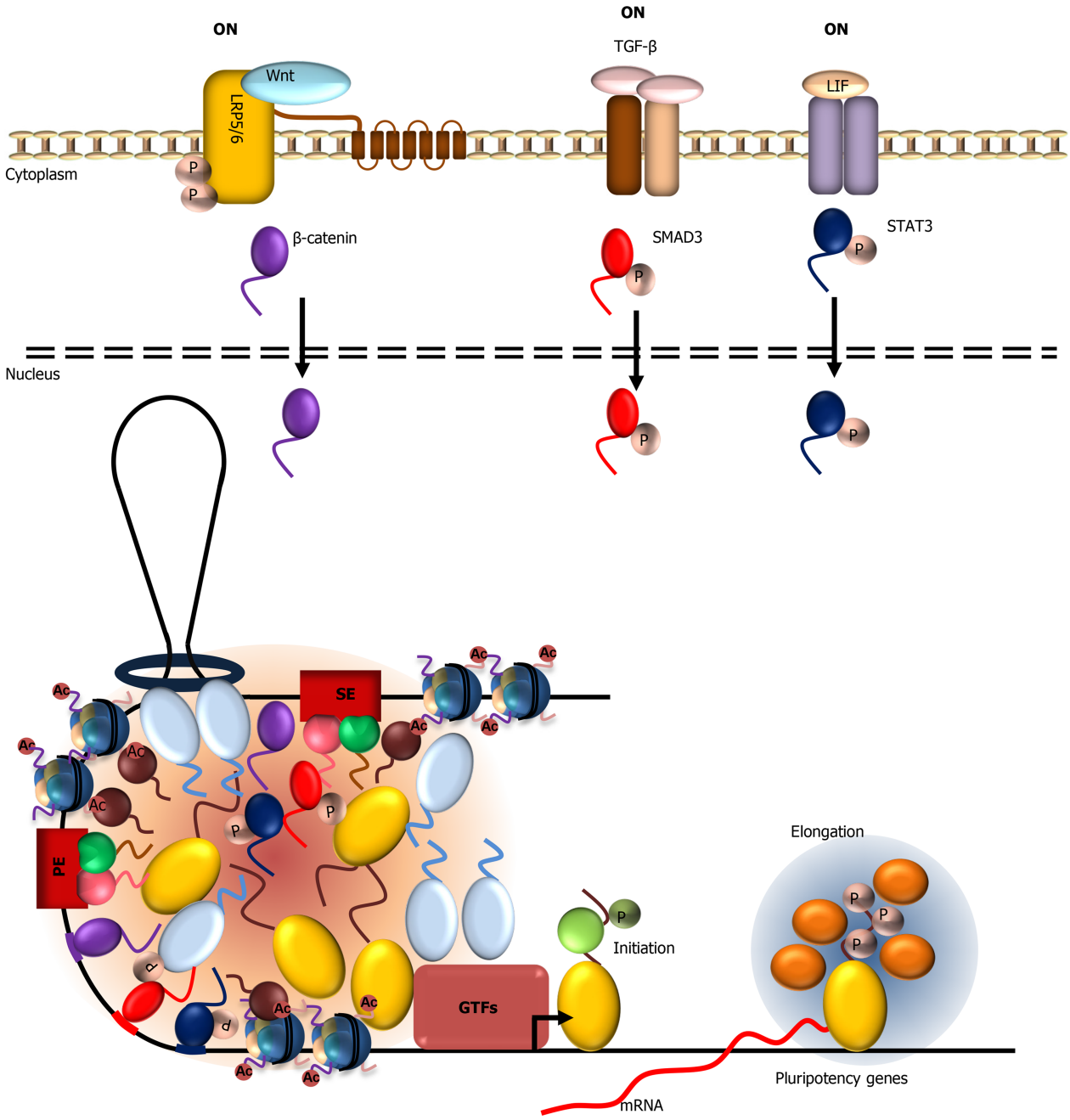

Figure 3 Activation of the wingless-related integration site, transforming growth factor beta, and Janus kinase/signal transducers and activators of transcription signaling pathways leads to nuclear translocation of their respective terminal signaling effectors: β-catenin, SMAD family member 3, and signal transducers and activators of transcription 3. These low complexity domain (LCD)-containing transcription factors bind their respective signal-responsive elements in pluripotency gene promoters and form LCD-mediated condensates with Mediator to modulate ESC-specific transcriptional activation in a signal-dependent manner. JAK: Janus kinase; STAT: Signal transducers and activators of transcription; SMAD3: SMAD family member 3; TGF-β: Transforming growth factor beta; Wnt: Wingless-related integration site.

filaments by myosins[150]. It has been shown that F-actin crosslinked by filamin spontaneously assembled into phase-separated F-actin filament bundles that can extend and contract[152]. We speculate that changes in actin filament dynamics driven by phase separation could facilitate the relocation of heterochromatic DSBs to appropriate subcellular compartments as DSB repair progresses.

DDR factors and post-translational modifications

Fused in sarcoma, phosphorylation, and poly-adenosine diphosphate (ADP)-ribosylation: Fused in sarcoma (FUS, also known as translocated in liposarcoma, TLS) is one of the most studied proteins known to undergo phase separation. Its unstructured N-terminal prion-like domain is required for phase separation[153-155].

In addition to its role in RNA metabolism, recent studies highlighted a role of FUS in DDR. Upon DNA damage, FUS is rapidly recruited to DSB sites[156,157]. It has been shown that poly-ADP-ribosylation (PARylation) at DSBs by poly-ADP-ribose polymerase enzymes triggers the translocation of cytoplasmic FUS to the nucleus and formation of large phase separated FUS-containing compartments at DSB sites[158-160] (Figure 4A). These FUS compartments are thought to contribute to DNA repair by facilitating the recruitment of downstream effectors of DNA repair such as p53-binding protein 1 (53BP1)[161] (Figure 4B). The C-terminal arginine-glycine-glycine repeat (RGG) domain of FUS likely plays a role in LLPS by directly binding PAR[159]. Therefore, the high propensity of FUS to generate large phase separated domains in cells could be due to increased valency in interaction using both the N-terminal prion-like LCD as well as C-terminal RGG domain when PAR accumulates at DSBs. It is worth to stress that these phase separated compartments are not static structures. Indeed, it has been shown that multivalent interactions by FUS can be destabilized by phosphorylation of the prion-like LCD[162] and PAR hydrolysis by PARG[158]. The reversible nature of FUS LLPS compartments is likely a necessary feature of DNA repair where recruitment of repair factors to and exclusion from damaged sites must be dynamically regulated.

53BP1: In addition to PARylation at DSBs, phosphorylation of histone variant 2AX is another early event in DDR and is required for the recruitment of 53BP1 to DSB sites [163] (Figure 4B). 53BP1 has been shown to generate sizeable chromatin domains in the nucleus that persist throughout the repair process[164] and is thought to recruit downstream effectors to regulate DDR and repair (Figure 4C)[163]. Recent studies demonstrated that these 53BP1 domains display liquid-like properties[164,165]. In one study, it showed that 53BP1 can concentrate p53 into 53BP1 condensates and activates p53-target gene expression, thereby inducing a cell cycle checkpoint DDR[164] (Figure 4D). They showed that conditions that perturb 53BP1 condensate formation also compromise p53 signaling, indicating that the recruitment of p53 to 53BP1 condensates is likely important for proper activation of a p53 response in damaged cells. It will be interesting to examine whether 53BP1-dependent activation of p53 contributes to repression of pluripotency genes and activation of differentiation-associated genes observed in damaged ESCs[166]. Surprisingly, while 53BP1 contains a largely unstructured N-terminal domain, it is dispensable for LLPS *in vitro*[164]. Rather, the structured Tudor domain is required for phase separation by 53BP1. It is speculated that multivalent interactions between tyrosines (Y) and arginines (R) in Tudor domain promote LLPS, similar to what was observed regarding the role of Y/R in phase separation of the FET (FUS, Ewing sarcoma breakpoint region 1, TATA-box binding protein associated factor 15) protein family[167]. Future mutagenesis studies should help clarify the LLPS mechanism employed by 53BP1. The ability of 53BP1 Tudor domain to undergo LLPS demonstrated that structured domains can also contribute to phase separation. Another study highlighted the involvement of damaged-induced long non-coding RNA (dilncRNA) at DSBs in organizing 53BP1 condensates[165]. They showed that PIC assembly at DSBs containing Pol II, Mediator, and P-TEFb, and transcription of dilncRNAs facilitate molecular crowding and phase separation of DDR factors including 53BP1 (Figure 4E). Supporting this notion, inhibition of dilncRNA transcription reduces the size 53BP1 condensates and repair efficiency. Given that FUS binds RNA[168] and phase separates with Pol II CTD[155], it is tempting to speculate that transcription of dilncRNAs by Pol II at DSBs may also facilitate the incorporation of FUS into repair condensates.

ABCF1 and intracellular DNA sensing: ABCF1 was previously identified as a sensor for intracellular DNAs that arise from infection or DNA damage[169]. Binding of these DNAs by ABCF1 triggers an innate immune response in somatic cells. However, because ESCs lack a canonical innate immune response to DNAs[170-172], the functional consequence of DNA sensing by ABCF1 in ESCs is unknown. Our identification of ABCF1 as a critical stem cell coactivator prompted us to examine whether ABCF1 can couple DNA sensing with stem cell transcription in response to DNA damage[30] (Figure 5A). We found that ABCF1 specifically binds double-stranded (ds) but not single-stranded (ss) DNAs in an LCD-dependent manner. Remarkably, binding of ABCF1 to dsDNAs dramatically stimulates LLPS *in vitro*. These results suggest that upon DNA damage, ABCF1 may preferentially form condensates with dsDNAs in damaged ESCs instead of binding SOX2 and Pol II. Consistent with this model, we found that ABCF1's interaction with SOX2 and assembly of Pol II transcription machinery at pluripotency gene promoters are disrupted upon DNA damage, resulting in downregulation of pluripotency genes critical for stem cell

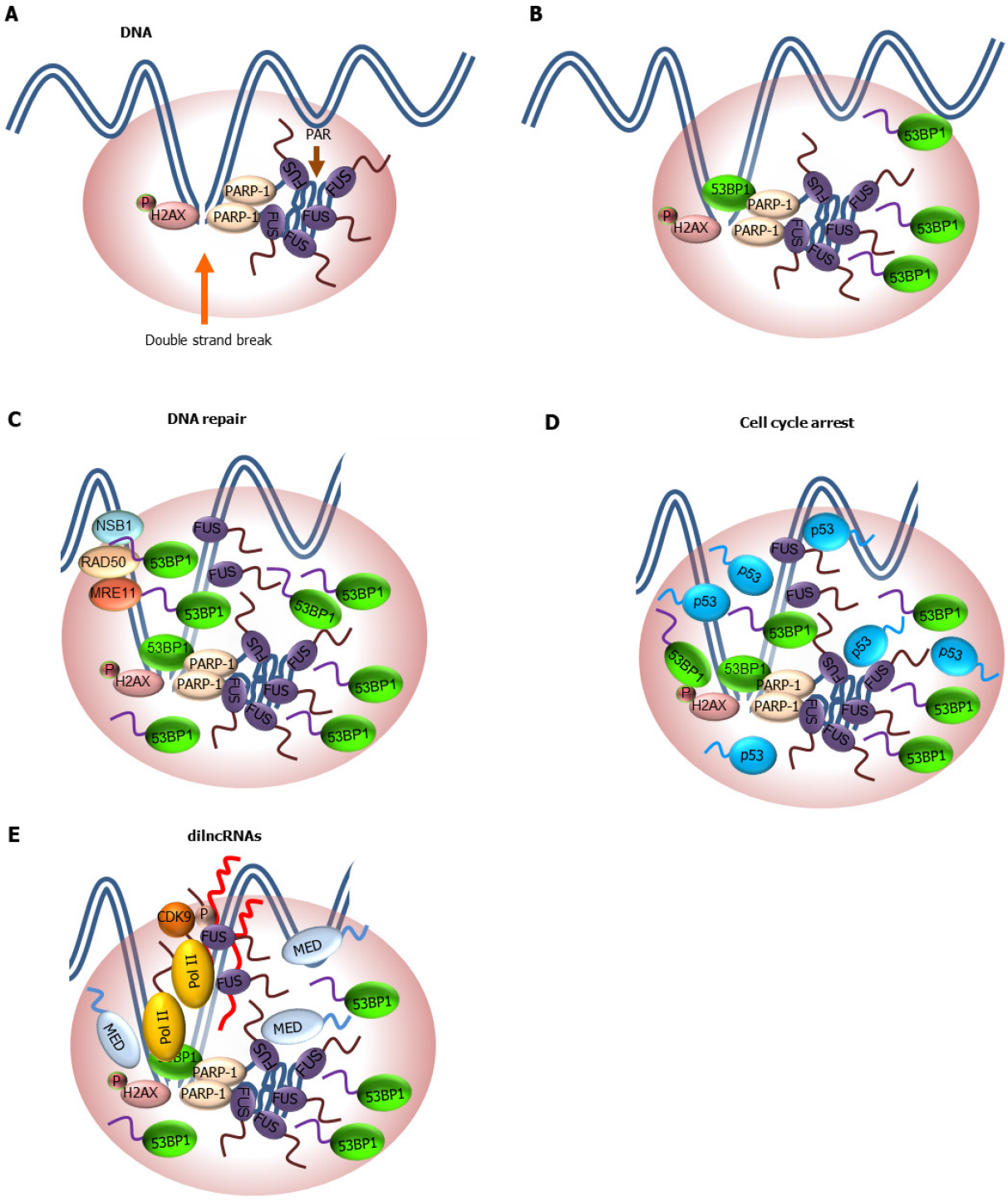

Figure 4 Role of low complexity domain-driven condensate formation in DNA damage response and DNA repair. A: Double strand break (DSB) triggers phosphorylation of histone variant H2AX (γ-H2AX) and poly-ADP-ribosylation (PARylation) by PARP-1. PARylation facilitates the recruitment of fused in sarcoma (FUS) to DSB site through its RGG domain and formation of FUS condensates driven by its prion-like low complexity domain (LCD); B: γ-H2AX and FUS condensates at DSBs promote the incorporation of a critical downstream effector of DSB repair factor, p53-binding protein 1 (53BP1). However, the Tudor domain but not its disordered region of 53BP1 is required for 53BP1 phase separation; C: Formation of 53BP1/FUS condensates facilitate the recruitment of downstream repair machinery such as meiotic recombination 11 homolog. RAD50 homolog, double strand break repair protein, and Nijmegen breakage syndrome 1 involved in homologous recombination repair of DSBs; D: p53 is incorporated into 53BP1 condensates and activate a p53-dependenent gene expression that results in cell cycle arrest. Disruption of 53BP1 condensates blunts p53-dependent response to DNA damage; E: Assembly of Pol II, mediator and cyclin dependent kinase 9/positive transcription elongation factor b at DSBs leads to transcription of dilncRNAs. dilncRNAs facilitates molecular crowding and phase separation of 53BP1 and other repair factors. It is likely that a network of LCD-mediated protein-protein and protein-nucleic acid interactions drives the formation of repair condensates at DSBs. 53BP1: p53-binding protein 1; dilncRNA: Damage-induced long non-coding RNA; DSB: Double strand break; FUS: Fused in sarcoma; H2AX: Histone variant 2AX; MRE11: Meiotic recombination 11 homolog; NBS1: Nijmegen breakage syndrome 1; PARP-1: Poly-ADP-ribose polymerase-1; RAD50: RAD50 homolog, double strand break repair protein.

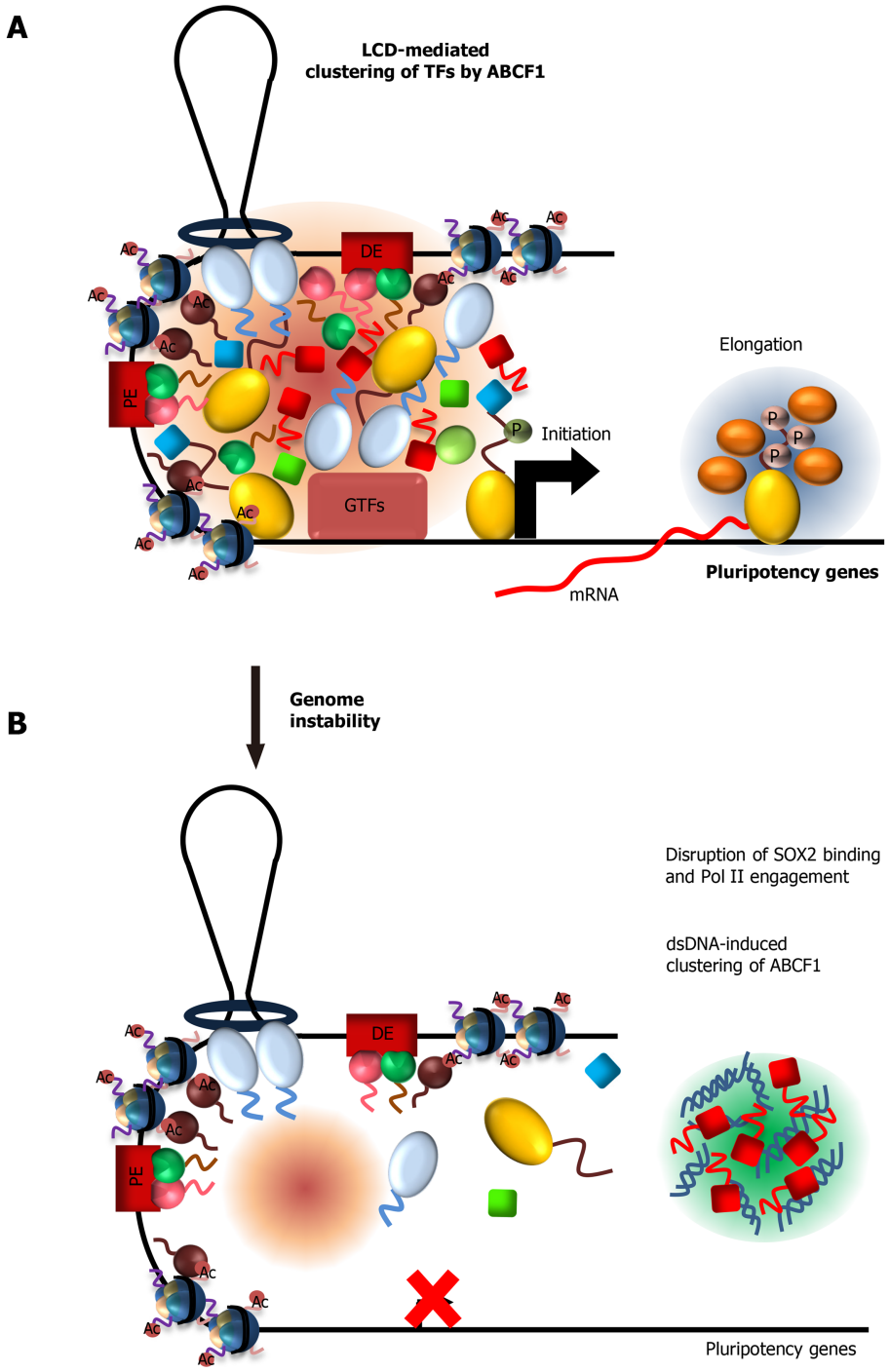

Figure 5 ATP-binding cassette subfamily F member 1 couples stem cell-specific transcription with DNA sensing in Embryonic stem cells. A: ATP-binding cassette subfamily F member 1 (ABCF1) low complexity domain promotes specific clustering and formation of a hub comprising of sex-determining region Y-box 2 (SOX2), xeroderma pigmentosum, complementation group C (XPC), dyskerin (DKC1), and RNA polymerase II (Pol II) molecules at target gene promoter to stimulate transcription, presumably by increasing local concentration of these factors; B: ABCF1 proteins available for transcription are diverted to bind intracellular double-stranded DNA (dsDNAs) generated from genome instability, due to increased propensity of ABCF1 to form condensates with dsDNAs. Decrease in ABCF1 at gene promoters destabilizes the multivalent interactions between SOX2, XPC, DKC1, and Pol II. This leads to disruption of the protein hub and decrease in gene transcription by Pol II. Downregulation of pluripotency-associated genes promotes differentiation of compromised ESCs and their elimination from the self-renewing population, thereby preserving genome fidelity in ESCs. LCD: Low complexity domain; TF: Transcription factor; dsDNA: Double-stranded DNA; Pol II: RNA Polymerase II; ABCF1: ATP-binding cassette subfamily F member 1; SOX2: Sex-determining region Y-box 2.

maintenance (Figure 5B). We propose that ESCs may leverage ABCF1's ability to switch between transcription factor and dsDNA condensates to modulate pluripotency gene transcription. Direct coupling of DNA sensing and stem cell-specific transcription *via* ABCF1 may represent an effective strategy to safeguard genome integrity by eliminating compromised ESCs from the self-renewing population through enforced differentiation.

CONCLUSION

A growing number of factors have been shown to form condensates with the MED1 subunit of the Mediator complex. Less clear are the mechanisms by which MED1 forms these numerous, functionally distinct condensates. Changes in their composition upon signaling pathway activation, and at different stages of gene transcription where "cargoes" are handed off from one condensate (*e.g.* initiation) to another (*e.g.* elongation) must be tightly regulated. A key challenge is how to avoid accidental mixing of these MED1 condensates. Post-translational modifications of the CTD of Pol II provide one such strategy wherein different phosphorylated forms of the CTD (Ser5 *vs* Ser2) condense preferentially with regulatory factors in initiation or elongation. In addition, we propose that the requirement of coactivators such as ABCF1 to form stem cell-specific multivalent interactions adds another layer of specificity for gene regulation in ESCs.

Evidence of LLPS in cells, particularly with respect to transcription factors, relies in part on observations of their phase separation behaviors *in vitro*, that they are spherical in shape, can fuse and fission, and allow exchange of biomolecules. However, these properties are not unique to LLPS. Indeed, a recent study on Pol II compartment formation during herpes simplex virus type 1 infection highlighted that, despite sharing several properties that are consistent with phase separated condensates, these Pol II compartments are formed by non-specific interactions with viral genomic DNA, distinct from behaviors typically attributed to Pol II condensates[173]. In another study, it was shown that at physiological concentration, TFs activate Pol II transcription at endogenous genomic loci by forming dynamic LCD-driven hubs in the absence of LLPS[31]. Therefore, there are likely multiple pathways with which clustering of biomolecules in cells can be achieved without undergoing LLPS. In fact, a recent study provides evidence that formation of transcription factor droplets can actually be counterproductive to gene activation[174], suggesting that the topology and binding dynamics of multivalent interactions are critical for protein function in transcription and likely other cellular processes. For discussion on the role of phase separation in biological reactions, we recommend several excellent reviews on evidence for and against LLPS in cells[23,175-177].

Whether or not these LCD-driven domains in cells meet the criteria of LLPS, it is evident that an intricate network of multivalent interactions controls various steps in transcription, their integration with signaling pathways, and in DNA repair and DDR - processes essential for maintenance of stem cell pluripotency and genome integrity. Transient and weak protein-protein and protein-nucleic acid interactions mediated by LCDs in regulatory factors enhance efficiency of biological reactions by enriching relevant factors in distinct hubs or compartments, specificity by combinatorial assembly, and dynamic regulation in response to changing cellular environment by modulating LCD-LCD interaction affinity and specificity.

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MINIREVIEWS

Different kinds of stem cells in the development of SARS-CoV-2 treatments

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Abstract

On February 11, 2020, the World Health Organization officially announced the coronavirus disease 2019 (COVID-19) caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), as an emerging recent pandemic illness, which currently has approximately taken the life of two million persons in more than 200 countries. Medical, clinical, and scientific efforts have focused on searching for new prevention and treatment strategies. Regenerative medicine and tissue engineering focused on using stem cells (SCs) have become a promising tool, and the regenerative and immunoregulatory capabilities of mesenchymal SCs (MSCs) and their exosomes have been demonstrated. Moreover, it has been essential to establishing models to reproduce the viral life cycle and mimic the pathology of COVID-19 to understand the virus's behavior. The fields of pluripotent SCs (PSCs), induced PSCs (iPSCs), and artificial iPSCs have been used for this purpose in the development of infection models or organoids. Nevertheless, some inconveniences have been declared in SC use; for example, it has been reported that SARS-CoV-2 enters human cells through the angiotensin-converting enzyme 2 receptor, which is highly expressed in MSCs, so it is important to continue investigating the employment of SCs in COVID-19, taking into consideration their advantages and disadvantages. In this review, we expose the use of different kinds of SCs and their derivatives for studying the SARS-CoV-2 behavior and develop treatments to counter COVID-19.

Key Words: COVID-19; SARS-CoV-2; Stem cells; Exosomes; Pluripotent stem cells; Mesenchymal stem cells; Artificial stem cells

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Core Tip: The use of stem cells (SCs) to address the coronavirus disease 2019 (COVID-19) pandemic has been widely studied in various fields; for example, human embryonic SCs and human induced pluripotent SCs have been used to generate functional human cells, tissues, and organoids that are used for modeling COVID-19 and discovering drugs. Mesenchymal SCs and their exosomes have been used in clinical trials to control the severe acute respiratory syndrome coronavirus 2 immune response, showing absorption of pulmonary lesions and clinical improvement.

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INTRODUCTION

In the last two decades, humanity has experienced outbreaks of Ebola, Severe acute respiratory syndrome (SARS), H1N1, and now severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). On February 11, 2020, the World Health Organization (WHO) and the International Committee on Taxonomy of Viruses officially announced coronavirus disease 2019 (COVID-19) and designated the virus SARS-CoV-2[1,2]. Although the majority of patients evolve clinically well and recover quickly, showing mild symptoms, a significant portion of the affected patients develop acute lung injury (ALI), devastating pulmonary edema and atelectasis caused by capillary membrane injury, which can subsequently trigger a cascade of serious complications, such as severe pneumonia with acute respiratory distress syndrome (ARDS), resulting in multiorgan failure and death[3-6].

Cases of this pandemic illness have been reported in more than 200 countries, taking the lives of more than two million persons, which is the reason that medical, clinical, and scientific efforts are needed[4,6]. Currently, basic research and clinical investigation are urgently required. For basic research, it is essential to establish models to reproduce the viral life cycle and mimic the pathology of COVID-19[7] and to develop new prevention or treatment strategies. In this sense, regenerative medicine and tissue engineering focusing on the use of stem cells (SCs) have become promising tools. In these approaches, cells are utilized to replace or rebuild damaged organs and tissues. There are currently 1135 clinical trials related to COVID-19 registered in the International Clinical Trials Registry Platform (ICTRP), which is an initiative of the WHO, and 16 of these 1135 trials involve the use of SCs[2]. On the other hand, in a recent review in ClinicalTrials.gov (February 2021), a resource provided by the United States. National Library of Medicine, 4793 COVID-19 studies were reported; 88 of them used different types of SCs or their derivatives. However, it is essential to mention that the use of SCs in this pandemic illness is focused not only on treatment options but also on organoids' development to study the virus's behavior.

In this review, we expose the use of different kinds of SCs and their derivatives for studying the SARS-CoV-2 behavior and develop treatments to counter COVID-19 (Figure 1).

SCs GENERALITIES

SCs are unspecialized cells with the potential to differentiate into any organism's cell and have the capability of self-renewal. According to their origin, SCs can divide into embryonic and adult cells. Embryonic SCs (ESCs) can be obtained from the zygote, morula, or the blastocyst's inner cell mass and possess high potentiality (range of differentiation potential). Contrary, adult SCs can be isolated from neonatal and adult tissues such as the umbilical cord, placenta, bone marrow, adipose tissue, dental pulp, and peripheral blood; adult SCs show restricted potentiality. Regarding their potentiality, they are divided into totipotent, pluripotent, multipotent, and oligopotent

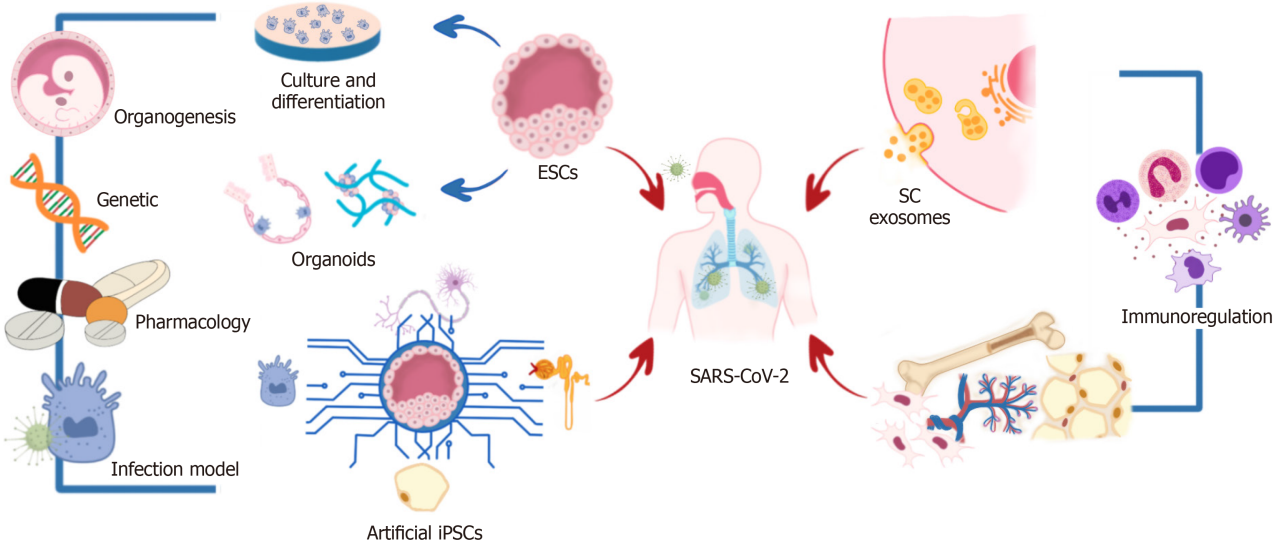

Figure 1 Stem cells in the development of severe acute respiratory syndrome coronavirus 2 treatments. Different kinds of stem cells (SCs), such as embryonic, mesenchymal, or artificial induced pluripotent SCs (iPSCs) or their products (SC exosomes), have been used in clinical trials and basic research to understand severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) behavior in humans. For example, Embryonic SCs (which can be differentiated in lung airway, lung alveolar, and intestinal epithelial cells or used for organoid development) and artificial iPSCs have been used in the progress of organogenesis knowledge, the comprehension of genetic alterations, pharmacological treatments or interactions, and the combination of SARS-CoV-2 in different kinds of cells (infection model). Mesenchymal SCs and SC exosomes have mainly been used in clinical trials as immunoregulators, and some authors have also stated their regeneration capability. SCs: Stem cells; iPSCs: Induced pluripotent SCs; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ESCs: Embryonic stem cells; MSCs: Mesenchymal SCs.

SCs (Figure 2)[4,8].

Totipotent

SCs have the highest differentiation potential; there are two definitions for totipotency. One declares that a totipotent cell is a single cell that can give rise to a new organism given appropriate maternal support, and the other states that a totipotent cell can give rise to all the extraembryonic tissues plus all the tissues of the body, differentiating into any of the three germ layers. One example of a totipotent cell is the zygote[8,9].

Pluripotent SCs

Pluripotent SCs (PSCs) are defined by their capability of differentiating into cell types derived from the three embryonic germ layers but not extraembryonic structures. PSCs were initially established in culture as ESCs and obtained from the morula or the blastocyst's inner cell mass (4-14 d after oocyte fertilization). Induced pluripotent SCs (iPSCs) are also a type of PSC derived from adult somatic cells that have been genetically reprogrammed into PSCs. The advantage of reprogramming iPSCs has created new opportunities for understanding human diseases and physiopathology, including a growing number of viral infections[6].

Multipotent SCs

Multipotent SCs (MSCs) have a narrower spectrum of differentiation than PSCs. However, they can differentiate into all cell types of one particular lineage; one example is a hematopoietic stem cell, which can differentiate into several blood cells.

Oligopotent SCs

Oligopotent SCs are characterized by the narrowest differentiation capabilities and have the competency to differentiate into only one lineage[8,10,11]. Considering the characteristics mentioned above, we will discuss some clinical trials and basic science assays focused on using SCs and their derivatives in COVID-19.

ESCs AND COVID-19 INFECTION MODEL

Human pluripotent SCs (hPSCs), human embryonic SCs (hESCs), and human induced

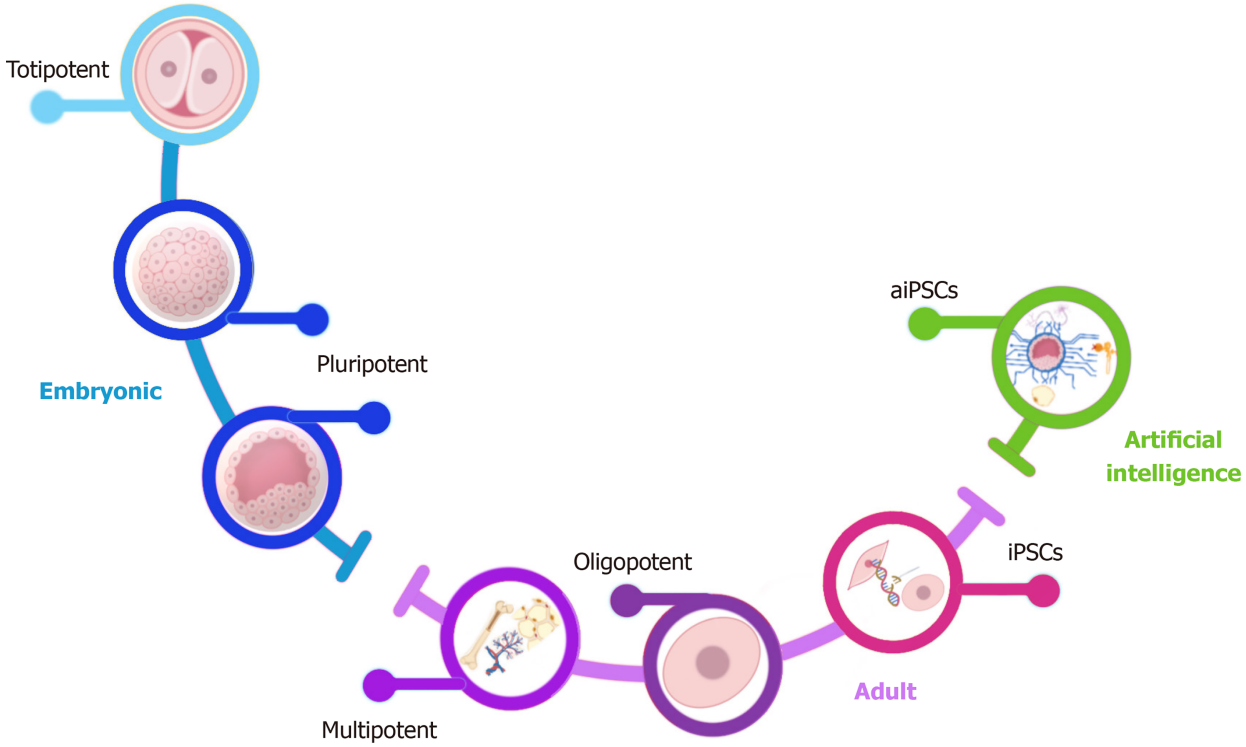

Figure 2 Stem cells classification. According to their origin, stem cells (SCs) are divided into embryonic and adult cells. Embryonic SCs can be obtained from the zygote, morula, or the blastocyst's inner cell mass and possess high potentiality. Adult SCs can be isolated from neonatal and adult tissues (umbilical cord, placenta, bone marrow, adipose tissue, among others). Likewise, the induced pluripotent SCs are considered another kind of adult SCs once they are derived from adult somatic cells but have been genetically reprogrammed. Concerning their potentiality, SCs are divided into totipotent, pluripotent, multipotent, and oligopotent SCs. Thanks to artificial intelligence, the generation of artificial induced pluripotent SCs was reported. iPSCs: Induced pluripotent stem cells.

pluripotent SCs (hiPSCs) are being used to generate functional human cells, tissues, and organoids that are used for modeling human disease and drug discovery, including modeling infectious disease. hPSC derived neuronal progenitor cells (hNPCs), and brain organoids were used to study the Zika virus's impact on human brain development[12]. Likewise, another research group demonstrated the infection capacity of the protozoan *Trypanosoma cruzi* in hiPSC-derived cardiomyocytes, demonstrating the potential of these cells as a human model for studying cardiomyopathy in Chagas disease and for the development of new therapies against the parasite. In the same way, these cells have been used to study hepatitis B[11].

Considering those mentioned above, currently, the use of these cells has been proposed in differentiation protocols for generating lung airway, lung alveolar, and intestinal epithelial cells. Abo *et al*[13] declared that iPSC-derived lung and intestinal epithelial cells derived in their protocols could be banked and used to generate multiple organ lineages from a single individual cell. Likewise, these cells can recapitulate appropriate cell-intrinsic phenotypes of a genetic disease and respond to immune stimuli. Moreover, it is essential to mention that they established a novel iPSC-derived alveolar epithelial type 2 cell air-liquid interface culture system to enable modeling of environmental exposures of the human alveolar epithelium, including viral infection, observing the expression of angiotensin-converting enzyme 2 (*ACE2*) and *TMPRSS2*, two genes encoding host cell proteins essential for SARS-CoV-2 cell entry in the iPSC-derived airway, alveolar, and intestinal epithelial cells.

PSCs AND ORGANOIDS FOR SARS-CoV-2

In biological research history, one of the significant challenges to understanding human biology and disease and the clinical development of novel drugs includes a limited number of suitable animal models, which try to recapitulate human physiology *in vivo*. However, there are significant differences in metabolism between humans and laboratory models, and common drugs for humans, such as ibuprofen and warfarin, are toxic to rats, highlighting that humans are not inbred in contrast to

all animal models[5,14].

The most used models for SARS-CoV-2 studies have been African green monkey-derived Vero cells or human cancer cell lines, which show limitations for modeling complex human organ systems. Therefore, relevant human models to study SARS-CoV-2 infection are critically important[15,16]. Recently, with the discovery of PSCs, knowledge about human development and morphogenesis in healthy and disease contexts has significantly improved. With the recapitulation of human organogenesis *in vitro*, the concept of an "organoid" emerged. Currently, the term organoid refers to three-dimensional systems formed by self-assembly of SCs *in vitro* that allow the recreation of the architecture and physiology of human organs in detail; as the functions are similar to natural organs in the body, organoids have provided opportunities for studying human diseases, infectious diseases, genetic disorders, and cancers[14,17,18].

The use of these organoids in SARS-CoV-2 research is not an exception. Pei *et al*[18] developed an optimized method to differentiate human airway organoids and alveolar organoids from hESCs, carrying out differentiation through six stages (ESCs, definitive endoderm, anterior foregut endoderm, ventralized anterior foregut endoderm, lung progenitors, and human airway or alveolar organoids), demonstrating that SARS-CoV-2 infects and extensively replicates in these organoids.

In the same way, Han *et al*[16] developed a lung organoid model and colonic organoids using hPSCs, reporting a robust induction of chemokines following SARS-CoV-2 infection in the lung organoid, similar to what is seen in patients with COVID-19, and that multiple colonic cell types, especially enterocytes, express ACE2 and are permissive to SARS-CoV-2 infection.

Therefore, organoids recapitulate many biological parameters, including the spatial organization of heterogeneous tissue-specific cells, cell-cell interactions, cell-matrix interactions, and certain physiological functions generated by tissue-specific cells within the organoid. Moreover, organoids provide a stable system amenable to extended cultivation and manipulation while being more representative of *in vivo* physiology[19,20] and thereby have significant advantages in studying this pandemic illness. Table 1 resumes the organoid infection models developed for the SARS-CoV-2 study.

Although significant advances have been reported in the use of organoids in SARS-CoV-2, one possible concern is whether hPSC-derived cells can recapitulate the biology of SARS-CoV-2 infection in adults since the vertical infection of the fetus is not entirely clear[15]. Moreover, Tindle *et al*[21] have declared that existing lung organoid models available for modeling COVID-19 do not recapitulate all the heterogeneous epithelial cellularity of both proximal and distal airways, lack propagability, and/or cannot be reproducibly generated for biobanking. Similarly, Monteil *et al*[22] have stated that their studies' design focuses on the early stages of infection, limiting predictions concerning the effect in later phases. So, organoids remain rudimentary compared to the adult human organ[21].

ARTIFICIALLY IPSCs IN COVID-19

Despite significant advances in iPSCs, cellular reprogramming remains a challenge due to the high costs, time-consuming, and the tendency of iPSCs to revert to their original somatic genotypes over time, adding ethical limitations; reason by which new technologies have been used to avoid these limitations. Artificial intelligence (AI) has demonstrated that it can shorten the process and increase efficiency.

AI is the way to model human intelligence to accomplish specific tasks without much intervention of human beings. It is defined as "the science and engineering of making intelligent machines." The term was first used in 1956 with The Logic Theorist program, designed to simulate human beings' problem-solving ability. Since then, a significant subset of AI called machine learning (ML) has emerged at the forefront of AI research. An ML is conceptualized as "a field of study that gives the computer the ability to self-learn without being explicitly programmed." The impact of AI has been transferred to the field of healthcare with its use in pharmaceutical and biomedical studies[23,24], is useful in a wide range of applications across public health, disease prediction, and drug development, including the analysis of real-time data for disease detection, and ML-based disease risk models. Moreover, AI has also helped model human behavior[25], and currently, SCs have also been evolved in this field.

Esmail and Danter[6] created the DeepNEU platform, a validated hybrid deep-machine learning system[6]. This platform enables the generation of artificial iPSCs

Table 1 Resume of organoid infection models developed for severe acute respiratory syndrome coronavirus 2 study

Ref.	Organoid infection model	Reported advantages
Yang *et al* [15]	hPSC-derived cells/organoids, including pancreatic endocrine cells, liver organoids, endothelial cells, cardiomyocytes, macrophages, microglia, cortical neurons, and dopaminergic neurons	Permissiveness to SARS-CoV-2 infection; ACE2 expression was detected; Chemokine induction
Han *et al* [16]	hPSC-LOs	hPSC-LOs (particularly alveolar type-II-like cells) are permissive to SARS-CoV-2 infection; Robust chemokine induction; Discovery and test therapeutic drugs
	hPSC-COs	Permissiveness to SARS-CoV-2 infection hPSC-Cos especially enterocytes, express ACE2; Discovery and test therapeutic drugs
Pei *et al* [18]	hAWOs and hALOs from hESCs	Permissiveness to SARS-CoV-2 infection and replication; Infected cells express ACE2 but not all ACE2 expressing cells were infected; Chemokine induction; Discovery and test therapeutic drugs
Yiangou *et al*[20]	hPSC-derived cardiac models	Permissiveness to SARS-CoV-2 infection; Activation of the innate inflammatory response; Show contractility, electrophysiology, and sarcomeric fragmentation
Monteil *et al*[22]	Human capillary organoids from iPSCs; Kidney organoids from hESCs	Permissiveness to SARS-CoV-2 infection and significantly inhibited by human recombinant soluble ACE2. ACE2 expression

hPSC: Human pluripotent stem cells; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2; ACE2: Angiotensin-converting enzyme 2; hPSC-LOs: Lung organoid model using human pluripotent stem cells; hPSC-Cos: hPSC-derived colonic organoids; hAWOs: Human airway organoids; hALOs: Alveolar organoids; iPSCs: Induced pluripotent stem cells; hESCs: Human embryonic stem cells.

(aiPSCs) (Figure 2). In the same way, they also reported the generation of artificially induced neural SCs and artificially induced cardiomyocytes from aiPSCs[23]. The authors have also used DeepNEU v5.0 for creating computer simulations of artificially induced type 1 (AT1) and type 2 (AT2) alveolar lung cells (aiLUNGs). Moreover, they exposed aiLUNGs to the simulated SARS-CoV-2, reproducing the genotypic and phenotypic profiles associated with the infection. Furthermore, these infected cells were treated with drug repurposing of a small group of approved drugs with well-known action mechanisms. This study also demonstrated that aiLUNG-COVID-19 simulations could be used to rapidly repurpose novel and known drug combinations with anti-SARS-CoV-2 therapeutic potential for animal and human trial validation[6].

Nonetheless, this technology's employment must consider ethical and societal implications, in addition to the requirement of systematic examination (*e.g.*, issues around security, privacy, and confidentiality). Even though significant advances have been developed, it is mandatory to recognize that AI is still at the early stages of its development for its application across the healthcare industry[25].

MSCs AND THEIR IMMUNOMODULATORY RESPONSE IN SARS-CoV-2

As previously mentioned, MSCs have high proliferative potential and limited differentiation capacity; nevertheless, one of their most promising characteristics is their immunomodulatory properties because they secrete many types of cytokines by paracrine secretion or make direct interactions with immune cells, leading to immunomodulation. In this sense, these cells help modulate the proliferation, activation, and function of various immune cells, altering innate and adaptive immune responses. These immunomodulatory effects are triggered by the activation of TLRs in MSCs stimulated by pathogen-associated molecules such as lipopolysaccharides[4,26].

It has been reported that MSCs, as well as human bone marrow SCs (BMSCs), possess anti-inflammatory properties, which have also been demonstrated in virus-induced lung injury models. Intravenous injection of mouse BMSCs into H9N2 virus-infected mice reduced inflammatory cell recruitment into the lungs and provoked a reduction in chemokine and proinflammatory cytokine levels. Similarly, human umbilical cord-derived MSCs showed a similar effect on the inflammatory response, secreting growth factors in an *in vitro* lung injury model induced by the H5N1 virus[5].

MSC therapeutic effectiveness is probably limited to a niche of immunological disorders and immune-mediated illnesses such as graft-*vs*-host-disease, for which MSCs have demonstrated varying levels of efficacy in phase 1/2 clinical studies[27].

In SARS-CoV-2 research, it has been reported that inflammation is the driving force behind coronavirus infections. The majority of deaths caused by COVID-19 are the result of ALI and ARDS due to rapid virus replication, massive inflammatory cell infiltration, and elevated proinflammatory cytokine/chemokine responses (cytokine storm), which are events that are associated with a dysregulation of the immune response and are crucial to controlling inflammation as early as possible[4,28]. ALI and ARDS are severe clinical manifestations of COVID-19, and while administration of MSCs to subjects with ARDS was well tolerated, efficacy data at the clinical level is not as compelling[27].

Due to no pharmacological therapies halt the disease and progress very fast, immunomodulation using SCs seems to be an effective therapy. In this sense, MSCs represent one of the most promising candidates since their safety and efficacy have been shown in pre-clinical models of ARDS[1].

For the reasons mentioned above, MSCs have been widely used in cell-based therapy, from basic research to clinical trials, highlighting that most of the trials that use MSCs are focused on the immune response; for example, the clinical trial reported by Leng *et al*[26] showed that the inflammatory and immune functions were corrected, based on measurement of cytokine levels [*i.e.*, tumor necrosis factor (TNF)-α, IL-10] and a subset of immune cells (D4, CD8, NK, DC cells) without adverse effects.

SCs and exosomes

All cells in the organism release exosomes, described as extracellular vesicles enclosed by a membrane, transporting biologically active molecules, such as lipids, chemokines, growth factors, nucleic acids, metabolites, and proteins. The molecular contents of exosomes differ depending on their cellular origin, environment, developmental phase, and epigenetic modification, among other factors. Active molecules are taken up by surrounding cells or circulate in the blood and eventually are taken up by distant cells, mediating autocrine, paracrine, and endocrine effects that can be exploited therapeutically[29-31].

The terms "exosomes," "microvesicles," "microparticles" are used interchangeably, including all extracellular vesicles produced by SCs. These were first described in the 1970s. Exosomes' therapeutic potential was first described in MSC exosomes when it was observed that they were cardioprotective in a murine model of acute myocardial ischemia/reperfusion injury[32,33].

In this sense, many research types have focused on SC exosomes, emerging the exciting prospect of "cell therapy without the cells"[29]. In addition to the great regenerative potential, the content of SC exosomes has anti-inflammatory and immunomodulatory properties. Moreover, unlike SCs, exosomes do not raise immunological reactions, survive in an inflamed medium, do not develop teratomas, and protect their content against degrading enzymes[34].

It is also important to mention that exosomes play a significant role in intercellular communication and trigger physiological responses, and also have the ability to transfer horizontal micro ribonucleic acid. All these processes are facilitated through the exosome's cargo function[33]; they deliver their cargo to the cell of interest, enter the cell, interact with cellular organelles, and contribute to chemical reactions at the cellular level with their enzymes, respectively. For this reason, the employment of exosomes to living tissues is more feasible and less threatening in comparison to SCs [34].

As of June 13, 2020, 174 clinical trials have been registered to utilize exosomes (Clinicaltrials.gov). However, there are no Food and Drug Administration-approved exosome products available on the market[28].

Moreover, preclinical studies have shown encouraging effects of exosomes in animal models of acute respiratory distress syndrome and other respiratory diseases, showing reduced alveolar inflammation and restoration of leaky epithelial membranes [4]. Besides, it has been reported that the MSC secretome can be administered through inhalation, which is a beneficial characteristic of respiratory diseases. Preliminary studies suggest that MSC exosomes might also be efficient for the treatment of COVID-19[35].

Nevertheless, it is essential to mention that some other authors have declared the role of exosomes in COVID-19 reinfection or reactivation[36], supporting this declaration of basic virus biology and physiopathology. Viruses enter the cells using the endocytic pathway and exit the host cell by direct budding through the membrane. During viral infections, exosomes incorporate pathogen-derived nucleic acids, proteins, and lipids, becoming a vector of viral materials for the "Trojan exosome hypothesis," promoting the viral spread and evading the immune response, which discourages the use of exosomes[35].

As a perspective, these exosomes could also be considered for the design of SARS-CoV-2 vaccine trials, since a research group in Italy at the Istituto Superiore di Sanità in Rome declared that they might have a platform for vaccines for the emerging diseases based on exosomes, using deoxyribonucleic acid vectors where the antigen is fused to an exosome-anchoring protein, which was demonstrated for the Ebola and influenza virus, as well as Crimean-Congo hemorrhagic fever, West Nile virus, and hepatitis C virus[37].

SARS-CoV-2 CLINICAL TRIALS EMPLOYING SCs OR THEIR DERIVATIVES

Many countries, including the United States, Italy, the United Kingdom, France, Germany, Brazil, and Jordon, have proposed SCs as a COVID-19 treatment; as expected, China hosts almost 50% of these trials. As of June 12, 2020, over 2100 clinical trials were officially registered for COVID-19 treatment (ClinicalTrials.gov). These clinical trials range from the application of antiviral drugs to novel therapies such as cell therapies. At the same time, 169 "cell therapy" trials were registered on ClinicalTrials.gov. As of August 2020, 38 registered clinical trials were using MSCs, of which seven used exosomes for the treatment of COVID-19 (https://clinicaltrials.gov/)[11,35]; only 16 trials were registered in the ICTRP[2,4,29].

In a recent review in ClinicalTrials.gov (February 2021), we found 4793 COVID-19 studies, 88 of them used different types of SCs or their derivatives (one employing ESCs, two PSCs, three SC exosomes, and 82 MSCs), highlighting that nine of these studies are reported as completed, which are summarized in Table 2.

One of these trials was conducted in Beijing's YouAn Hospital in China, from January 23, 2020, to February 16, 2020, and seven confirmed COVID-19 patients were included in the study and received an intravenous transplant of 1×10^6 MSCs per kilogram body weight. The patients had a clinical follow-up of 14 d in which no adverse effects were observed, reporting a decreased ratio of serum proinflammatory cytokine TNF-α and a subset of immune cells; in the same way, the symptoms of these seven patients improved two days after MSC transplantation[1,27].

In another case report, Liang *et al*[38] administered three doses of UCMSCs to a 65-year-old woman who had tested positive for SARS-CoV-2 and had clinical signs of ALI and severe organ injury caused by an inflammatory response; no side effects were reported, and most of the laboratory indexes and computed tomography (CT) images showed remission of the inflammation symptoms. The patient was subsequently transferred out of the intensive care unit (ICU), and the throat swab test was negative four days later. Sánchez-Guijo *et al*[39] described an additional case report in which 13 adult COVID-19 patients under invasive mechanical ventilation were included. All the patients previously received antiviral and/or anti-inflammatory treatments, including steroids. Ten out of thirteen patients received two doses of allogenic adipose tissue-derived MSCs (AT-MSCs), two patients received one dose, and one patient received three doses. Each dose contained 0.98×10^6 AT-MSCs/kg of body weight. As a result, no adverse effects were reported, and 70% of the patients exhibited clinical improvement and were discharged from the ICU; however, four patients remained intubated, and two patients died.

As previously mentioned, MSC exosomes have also been used in clinical trials. Chu *et al*[40], from February 26, 2020, to April 30, 2020, recruited six patients diagnosed with COVID-19 pneumonia (two patients with severe symptoms and four patients with minor symptoms) who received nebulization of MSC-derived exosomes in different modalities at the end of the beginning of antiviral treatment for a while. No acute allergic reactions or secondary allergic reactions were observed. Their results indicated that MSC-derived exosome nebulization is a safe and feasible therapeutic approach for treating patients with COVID-19 pneumonia, showing clinical benefits. Moreover, chest CT revealed absorption of pulmonary lesions. Although the research has shortcomings, it is considered an essential antecedent for further research.

Another clinical trial that evaluates the use of exosomes is being developed in Ruijin Hospital Shanghai Jiao Tong University School of Medicine in Shanghai, China, by Qu *et al*[41], who evaluate aerosol's safety and efficiency inhalation of allogenic AT-MSC-derived exosomes in the treatment of severe COVID-19 pneumonia patients. In this research, participants will receive conventional treatment plus one dose of aerosol inhalation of MSC-derived exosomes a day at 2×10^8 nanovesicles/3 mL for five consecutive days.

Table 2 Clinical trials completed using different types of stem cells or their derivatives

No.	Title	Conditions	Interventions	Locations
1	Study Evaluating the Safety and Efficacy of Autologous Non-Hematopoietic Peripheral Blood SCs in COVID-19	COVID-19	Biological: Autologous NHPBSC; Drug: COVID-19 standard care	Abu Dhabi SCs Center, Abu Dhabi, United Arab Emirates
2	Mesenchymal SCs Therapy in Patients With COVID-19 Pneumonia	COVID-19, Pneumonia	Other: Mesenchymal SCs	University of Health Sciences, Istanbul, Turkey
3	Treatment with Human Umbilical Cord-derived Mesenchymal SCs for Severe Corona Virus Disease 2019 (COVID-19)	COVID-19	Biological: UC-MSCs; Biological: Saline containing 1% Human serum albumin solution without UC-MSCs	General Hospital of Central Theater Command, Wuhan, Hubei, China
4	Mesenchymal SCs for the Treatment of COVID-19	COVID-19, Prophylaxis	Biological: PrimePro (UC-MSCs); Other: Placebo	Southern California Hospital at Culver City/Southern California Hospital at Hollywood, Culver City, California, United States
5	Use of UC-MSCs for COVID-19 Patients	COVID-19, ARDS	Biological: Umbilical Cord Mesenchymal; SCs + Heparin along with best supportive care. Other: Vehicle + Heparin along with best supportive care	Diabetes Research Institute, University of Miami Miller School of Medicine, Miami, Florida, United States
6	Therapeutic Study to Evaluate the Safety and Efficacy of DWMSC in COVID-19 Patients	COVID-19, SAR	Drug: allogeneic mesenchymal stem cell; Other: Placebo	Site 550: University of Hassanudin/Dr. Wahidin Sudirohusodo Hospital, Makassar, Indonesia
7	Investigational Treatments for COVID-19 in Tertiary Care Hospital of Pakistan	COVID-19, Cytokine Release Syndrome, Critical Illness, ARDS	Procedure: Therapeutic Plasma exchange; Biological: Convalescent Plasma; Drug: Tocilizumab; Drug: Remdesivir; Biological: Mesenchymal stem cell therapy	Pak Emirates Military Hospital, Rawalpindi, Punjab, Pakistan
8	Evaluation of Safety and Efficiency of Method of Exosome Inhalation in SARS-CoV-2 Associated Pneumonia.	COVID-19, Pneumonia	Drug: EXO 1 inhalation; Drug: EXO 2 inhalation; Drug: Placebo inhalation	Medical Centre Dinasty, Samara, Russian Federation
9	A Pilot Clinical Study on Inhalation of Mesenchymal SCs Exosomes Treating Severe Novel Coronavirus Pneumonia	COVID-19	Biological: MSCs-derived exosomes	Ruijin Hospital Shanghai Jiao Tong University School of Medicine, Shanghai, Shanghai, China

United States National Library of Medicine, ClinicalTrials.gov, 02/21/2021. COVID-19: Coronavirus disease 2019; NHPBSC: Non hematopoietic peripheral blood SCs; SCs: Stem cells; MSCs: Mesenchymal stem cells; UC-MSCs: Umbilical cord-derived MSCs; ARDS: Acute respiratory distress syndrome; SARS-CoV-2: Severe acute respiratory syndrome coronavirus 2.

One published study used BMSC-derived exosomes in COVID-19-infected patients and evaluated the treatment's safety and effectiveness, reporting an overall improvement in clinical symptoms and laboratory tests within 3-4 d after treatment without adverse effects[36].

However, it is crucial to recognize that all these clinical trials have shown advantages and disadvantages as any other treatment in development, which are resumed in Table 3.

THE INCONVENIENCE OF USING MSCs IN COVID-19

As previously mentioned, the use of MSCs as an immunomodulatory and regenerative treatment has been suggested to treat COVID-19. Nevertheless, there are many challenges associated with MSC therapy, including low *in vivo* survival rates, dosing, cell isolation and growth strategies, and donor variability issues[27]. Moreover, it is essential to mention the intervention of the ACE2 receptor in COVID-19, as Desterke *et al*[42] reported that ACE2 is highly expressed in MSCs from adult bone marrow, adipose tissue, or umbilical cord[42].

ACE2 is a type I transmembrane metallocarboxypeptidase that is homologous to ACE, an enzyme that plays a vital role in the renin-angiotensin system. It is mainly expressed in alveolar type I and II cells, fibroblasts, endothelial cells, and macrophages. Currently, ACE2 protein expression has also been reported in the kidney and gastrointestinal tract, tissues that have been shown to harbor SARS-CoV-2,

Table 3 Advantages and disadvantages of stem cells in clinical trials

	Advantages	Disadvantages
ESCs		
	High differentiation capability which can recapitulate appropriate cell-intrinsic phenotypes	Ethical dilemma
	Grow up in the laboratory from a single cell for later transplantation	Tumorigenesis risk
MSCs		
	Immunomodulatory properties	A narrower spectrum of differentiation
	Widespread availability and accessibility	High expression of ACE2 receptor
	Limited ethical concerns	Expression of MHC I and II

ESCs: Embryonic stem cells; MSCs: Mesenchymal stem cells; ACE2: Angiotensin-converting enzyme 2; MHC: Major histocompatibility complex.

highlighting treatments with ACE inhibitors or the receptor antagonist angiotensin II notably increase the expression of ACE2. The reason is that patients with these pathologies who are treated with these medicines have an increased risk of developing COVID-19[42].

Some studies have demonstrated that SARS-CoV-2 enters the human cell through the receptor ACE2, acting as a receptor-binding domain for the virus spike complex, allowing viral attachment, fusion, intracellular entry, and COVID-19 infection[43,44]. Once the virus reaches the circulatory system, after replicating in type II pneumocytes, it infects other organs that express ACE2, which can generate multiple organ failure [45].

Considering the abovementioned findings, the expression level of ACE2 in MSCs could make MSCs not beneficial in COVID-19 patients if its expression is high. Currently, there are no data concerning ACE2 expression levels in MSCs of different origins used for therapy. Moreover, Desterke *et al*[42] reported that ACE2 expression was significantly higher in MSCs derived from adipose tissue and adult bone marrow, and lower expression levels were found in placenta-derived MSCs but only in early passages of cultures. However, it is imperative to highlight that Desterke *et al*[42] have also declared that hESCs and hiPSCs express deficient levels of ACE2. Therefore, it is crucial to determine if the SC population that would be transfused to patients could also be a target for SARS-CoV-2 entry into the human body.

ETHICAL RISK OF THE SCs USE IN CLINIC

As previously mentioned, many clinical trials employ SCs in the COVID-19 illness. Nevertheless, there has always been insecurity in the use of this type of cells. The ESCs show the disadvantage of tumorigenesis risk, widely discussed in the literature[46]. However, a fact of great weight that limits its use is the ethical dilemma. Since the beginning of the ESCs use in the research field, the thought of human embryo destruction has existed. Being the fundamental question: whether it is morally acceptable to pursue novel therapies for curing illnesses at the expense of destroying an early human embryo? Reason by which many countries such as Italy and United States have forbidden the research using hESCs. Since then, new strategies to avoid this problem have been proposed; in this sense, iPSCs technology has provided new opportunities[47].

Moreover, MSCs have arisen as a leading contender for cell sources due to their limited ethical concerns and low risk of tumor formation. Additionally, MSCs show widespread availability and accessibility[48]. However, no matter what type of SCs are used as novel treatments, there is a concern in the unproven commercial practices marketing that involve SC treatments, reason by which the International Society for Stem Cell Research in 2007 established a task force of scientific, medical, and bioethical experts to develop guidelines for the clinical translation of SC research. These guidelines address any attempt to develop novel clinical applications of SCs and their direct derivatives[49]. It is essential to mention that there are two central aspects to the definition of innovative therapies: the departure from standard medical therapy and that the employed therapy has not been validated or there is not enough available evidence to support the safety and efficiency of the therapy. Moreover, the only adult

stem cell therapy currently accepted for therapeutic use as standard best practice are hematopoietic SCs[50].

CONCLUSION

With all the above, pre-clinical and clinical trials in early-stage have demonstrated the efficiency and safety of SC treatment in COVID-19 patients; however, it is important to continue investigating different types of SCs and their derivatives in large-scale researches to confirm and validate the safety and efficacy profile of these therapies with reliable evidence. Likewise, considering their advantages and disadvantages, it is essential to change the paradigm using some types of SCs that could help obtain better results and are not used by the persistence of some taboos.

Finally, it is imperative to recognize that we are not prepared to face outbreaks of this magnitude. Worldwide medicine must be prepared for future pandemics since it needs to face globalization and the factors it carries, such as international travel, global economic exchange, and social behavior. We must advance in regenerative medicine and SCs therapies to improve the immune response, regenerate damaged tissues or systems, and understand virus behaviors in cultured cells and organoids.

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ORIGINAL ARTICLE

Basic Study

Disease modifying treatment of spinal cord injury with directly reprogrammed neural precursor cells in non-human primates

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Author contributions: Baklaushev VP and Ahlfors JE designed and coordinated the study and wrote the manuscript; Durov OV and Kim SV conducted the surgery; Kalsin VA, Revkova VA, and Samoilova EM propagated and characterized stem cells; Gulaev EV registered and interpreted evoked potentials; Gubskiy IL conducted magnetic resonance diffusion tensor imaging; Melnikov PA conducted confocal microscopy; Karal-Ogly DD and Orlov SV coordinated animal care; Troitskiy AV, Chekhonin VP, and Averyanov AV coordinated data

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Abstract

BACKGROUND

The development of regenerative therapy for human spinal cord injury (SCI) is dramatically restricted by two main challenges: the need for a safe source of functionally active and reproducible neural stem cells and the need of adequate animal models for preclinical testing. Direct reprogramming of somatic cells into neuronal and glial precursors might be a promising solution to the first challenge. The use of non-human primates for preclinical studies exploring new treatment paradigms in SCI results in data with more translational relevance to human SCI.

AIM

To investigate the safety and efficacy of intraspinal transplantation of directly reprogrammed neural precursor cells (drNPCs).

METHODS

Seven non-human primates with verified complete thoracic SCI were divided into two groups: drNPC group (n = 4) was subjected to intraspinal transplantation of 5 million drNPCs rostral and caudal to the lesion site 2 wk post injury, and lesion control (n = 3) was injected identically with the equivalent volume of vehicle.

RESULTS

Follow-up for 12 wk revealed that animals in the drNPC group demonstrated a significant recovery of the paralyzed hindlimb as well as recovery of somatosensory evoked potential and motor evoked potential of injured pathways. Magnetic resonance diffusion tensor imaging data confirmed the intraspinal transplantation of drNPCs did not adversely affect the morphology of the central nervous system or cerebrospinal fluid circulation. Subsequent immunohistochemical analysis showed that drNPCs maintained SOX2 expression characteristic of multipotency in the transplanted spinal cord for at least 12 wk, migrating to areas of axon growth cones.

CONCLUSION

Our data demonstrated that drNPC transplantation was safe and contributed to improvement of spinal cord function after acute SCI, based on neurological status assessment and neurophysiological recovery within 12 wk after transplantation. The functional improvement described was not associated with neuronal differentiation of the allogeneic drNPCs. Instead, directed drNPCs migration to the areas of active growth cone formation may provide exosome and paracrine trophic support, thereby further supporting the regeneration processes.

Key Words: Direct cell reprogramming; Neural precursor cells; Directly reprogrammed neural precursor cells; Spinal cord injury; Nonhuman primates; Regenerative therapy, Evoked potentials

Core Tip: Here, we describe a novel regenerative therapy of spinal cord injury by means of intraspinal transplantation of directly reprogrammed neural precursor cells (drNPCs). We showed that after transplantation of drNPC non-human primates demonstrated a significant recovery of the paralyzed hindlimb and recovery of somatosensory and motor evoked potential of injured pathways. Immunohistochemical analysis showed that drNPCs maintained multipotency in the transplanted spinal cord for at least 12 wk, migrating to areas of axon growth cones. Our data demonstrated that drNPCs transplantation was safe and contributed to improvement of spinal cord function after acute complete spinal cord injury in non-human primates.

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INTRODUCTION

Traumatic spinal cord injury (SCI) is a severe and often incurable disease of the central nervous system, with an annual age-standardized incidence rate of 13 (11-16) per 100000[1]. Every year nearly 500000 or even more new people suffer an SCI[1,2]. Due to the extremely high degree of disability it causes, SCI is considered to be the highest priority candidate for the development of regenerative approaches for clinically unmet needs[3]. All current approaches to medical rehabilitation (physical neurorehabilitation, neurostimulation, kinesiotherapy, *etc.*) after severe complete SCI (severe ASIA A and B) have poor efficacy, providing no noticeable restoration of lost function[4].

Neural stem cell (NSC)/neural progenitor cell (NPC) transplantation is generally considered one of the most promising future therapies for SCI[5-10]. Development of methods for generating autologous human NSCs/NPCs by direct reprogramming of somatic cells[11-15] has offered fundamentally new possibilities for this approach. Several studies on animal models of SCI (including primates) have established proof of efficacy for allogeneic or even xenogeneic NSC/NPC transplantation[9,12,16,17]. However, there are still very few clinical trials using these cells, and therefore the possibility of functional restoration after spinal cord injury remains an open question [7,10].

Autologous NSCs obtained by directly reprogramming cells from various starting cell types are the most promising for clinical use because of better genome stability and lower risk of tumor transformation compared to induced pluripotent stem derived NPCs. Recently it has been shown that directly reprogrammed neural precursor cells (drNPCs) obtained through transient transfection of bone marrow mononuclear cells with non-integrating synthetic plasmids expressing musashi-1, neurogenin-2, and methyl-CpG binding domain protein 2 demonstrated normal karyotype and all fundamental features of neural stem cells[11]. Transplantation studies in small animal models have provided very promising preliminary results for the use of human drNPCs in the treatment of experimental SCI and stroke[12,13] prompting us to initiate a study in non-human primates (NHP).

Previously we described the novel model of controlled complete SCI on NHP[18]. The goal of the current study was to investigate the safety and efficacy of intraspinal transplantation of allogeneic drNPCs in this NHP (*Macaca mulatta*) model of complete subacute SCI.

MATERIALS AND METHODS

Directly reprogrammed neural precursor cells

Allogeneic drNPCs were created from the bone marrow mononuclear cells of one female *Macaca mulatta* according to methods previously described[11]. The bone marrow (5 mL) was collected from the head of the humerus under ketamine anesthesia (10 mg/kg). Briefly, direct reprogramming was made by means of transient transfection of a cocktail of three transcription factors: musashi-1, neurogenin-2, and methyl-CpG binding domain protein 2.

Two weeks before transplantation, the cryopreserved drNPC cells were thawed and seeded onto laminin-coated plates and expanded in neuro Cult-XF basal medium (Stem Cell Technologies) with 1% Pen-Strep (Gibco), 1 × B-27 Supplement (50 ×) (Gibco), 20 ng/mL of bFGF (Peprotech), and 20 ng/mL of epidermal growth factor (Peprotech) (complete growth medium). The cells were incubated at 37 °C in 5% CO_2 and 5% O_2. Shortly before intraspinal injection, the cells were detached from the plates with Stem Pro Accutase (Thermo Fisher Scientific). A sample was taken for flow cytometry analysis, and the rest were divided into two vials. Cells from one vial were seeded onto laminin-coated cover glass Petri dishes for immunocytochemistry (ICC) analysis, as described below. The cells from the other vial were tested for viability using a Luna 2 cell counter and injected into animals. Viability was no less than 98% in all cell preparations, and lapsed time between formulation and injection did not exceed 20 min.

Immunophenotyping of drNPCs

Cells were cultured for 14 d in complete growth medium followed by fixation with ice-cold buffered 4% paraformaldehyde for 30 min. For flow cytometry cells were detached with Stem Pro Accutase followed by fixation with ice-cold buffered 4% paraformaldehyde for 10 min. The following antibodies were used: nestin (R and D and Abcam), SOX2 (BD Biosciences), βIII-tubulin (R&D), microtubule associated protein 2 (MAP2) (Sigma-Aldrich), glial fibrillary acidic protein (GFAP) (DAKO), NF-200 (Sigma-Aldrich), macro H2A.1 (Abcam), human leukocyte antigen (HLA)-ABC (BD Pharmingen), and HLA-DR (Miltenyi Biotec). For flow cytometry, directly labeled primary antibodies were used at a concentration of 10 μg/mL. For ICC, all primary antibodies were diluted in PBS-TT (PBS with 0.2% Tween 20, 0.3% Triton X-100, and 1.0% normal goat serum) at a concentration of 1-5 μg/mL. Goat anti-mouse IgG (H + L) labeled with Alexa Fluor 488 and goat anti-rabbit IgG (H + L) labeled with Alexa Fluor 633 (Life Tech, United States), all diluted at 1:400 in PBS-TT, served as secondary antibodies. Cell nuclei were counterstained with Hoechst (1 μg/mL; Invitrogen, United States). A Nikon A1 scanning laser confocal microscope (Nikon Company, Japan) was used to evaluate all ICC, while an S3e cytometer (Bio-Rad) was used for flow cytometry.

Animals

Seven mature *Macaca mulatta* male NHPs were enrolled in this study. Animals were kept under natural light conditions with free access to water and fed two times a day. All procedures were performed in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (European Treaty Series No. 123, Strasbourg, March 18, 1986; Directive 2010/63/EU of the European Parliament and Council of 22 September 2010 On the Protection of Animals Used for Scientific Purposes). The protocol of the study was approved by the Bioethics Committee of Research Institute of Medical Primatology (Statement from July 13, 2016), and the Local Ethical Committee of the FRCC of FMBA of Russia (Statement No. 10b of September 12, 2016).

Prior to surgery, NHPs were housed in an open-air cage (20 m^2 enclosure with enriched environment such as climbing/hanging gear and toys providing an opportunity for games and socialization) at the Sochi Institute of Medical Primatology. After surgery, NHPs were housed in large individual cages with toys and climbing/hanging gear, adopted for the animals with distal paralysis in one limb. The cages were near each other, which allowed physical contacts between NHPs and their socialization. The length of the study was minimized. Subjects were kept under natural light conditions with free access to water and fed two times a day. To avoid distress, NHPs were contacted only with the staff they knew well, whom they allowed to do intramuscular injections. All manipulations out of the cage [preparation for the surgery, detection of the evoked potentials, neurological examination of the paralyzed left limb, magnetic resonance diffusion tensor imaging (MRI), *etc*.] were conducted under sedation by ketamine (10 mg/kg, intramuscular). Health and well-being of the animals were monitored daily by vet staff using exterior, activity, and appetite as criteria and were followed by medical examination, if necessary.

The animals were randomly assigned to two groups: the lesion control group [(LC), n = 3] and drNPC transplantation group (NPC, n = 4) (Table 1).

SCI induction

SCI induction was performed as described before[18]. Briefly, animals were anesthetized by endotracheal inhalation with isoflurane (1.2–2.0 vol%). After skin incision and paraspinal muscle separation, Th 8 interlaminectomy was performed. The dura mater was dissected, and the spinal cord exposed. Guided by intraoperative recording of somatosensory evoked potentials (SSEPs) and motor evoked potentials (MEPs), 25% of the spinal cord cross-section in the projection of the left dorsal funiculus and left lateral corticospinal tract was excised with a length of about 5 mm. Further excision was continued until the SSEPs and MEPs from the corresponding segments of the left hindlimb disappeared. Before closing the wound, duraplasty was performed using the autofascia, followed by sealing with neurosurgical fibrin glue. In the postoperative period, all animals received antibiotic therapy (ceftriaxone, 50 mg/kg, intramuscular, once a day). Pain was managed by the administration of ketonal (15 mg/kg).

Implantation of drNPCs

All NHPs underwent a second surgery 2 wk after the SCI induction. In the experi-

Table 1 Characteristics of experimental groups

Group	No/Age[1]/Weight[2]	First surgery (Week 0)	Second surgery (2 wk after the injury)	Hindlimb score	MRI	SSEPs, MEPs	Histology and IHC
Lesion control	LC1/4.3/5.2 LC2/4.2/5.3 LC3/4.1/5.1	Resection of 25% area of spinal cord in the projections of lateral pyramidal tracts and the dorsal column at the level Th 7-8 (as described)	Sham surgery + vehicle	Before transplant and 2, 4, 6, 8, and 12 wk post transplant	Before transplant and 2, 4, 8, and 12 wk post transplant	Before lesion, (intraoperatively, D0), 2 wk after lesion (D14), 8 and 12 wk post transplant	Nissl, van Gieso; IHC: GFAP, NF200, BDNF
NPC	NPC1/3.3/4.1 NPC2/4.3/4.6 NPC3/3.8/4.6 NPC4/3.6/4.1		Implantation of 5×10^6 drNPCs				

[1]Age in years.

[2]Weight in kilograms at the beginning of the experiment. BDNF: Brain derived neurotrophic factor; drNPCs: Directly reprogrammed neural precursor cells; GFAP: Glial fibrillary acidic protein; IHC: Immunohistochemistry; MEP: Motor evoked potential; MRI: Magnetic resonance imaging; NPC: Neural progenitor cell group; SSEP: Somatosensory evoked potential.

mental (drNPC) group, the spinal cord around the injury was exposed again, and a drNPC suspension was injected into the perifocal zone in the projection of the dorsal funiculus and lateral corticospinal tract above and below the lesion (Supplementary Figure 1). A dose of 5×10^6 cells, resuspended in a total volume of 100 mL in Hanks' solution, was injected at four sites at a rate of 5 mL/min (25 mL per injection) by means of a sterile system consisting of a silicone tube and a 28G needle attached to a Hamilton 500 microsyringe, which was connected to a nanoinjector (Leica Microsystems). After each injection, the needle was left in the spinal cord tissue for 3 min and then slowly withdrawn. To prevent spinal cord compression by the scar tissue of the dura mater, duraplasty with the autofascia was repeated. In the control (LC) group, the same surgery was performed, with the equivalent number of injections and volume of Hanks' solution. No immunosuppression therapy was administered for either group.

Neurophysiological and imaging assessment

Hindlimb function: The degree of neurological deficit was determined using the NHP hindlimb score system suggested with our modifications[18] to assess the severity of lower monoplegia. The scores were assessed for the ipsilateral (left) limb. The score included an assessment of active flexion in large joints, reliance on the limb, tendon and periosteal reflexes, muscle tones, toe gripping, activity, and movement coordination.

MRI morphometry: MRI morphometry was conducted as described previously[18]. Briefly, T2-weighted images were obtained in two orthogonal planes at the thoracic and cervical level as well as at the level of the head. The structures were measured using the RadiAnt software (Medixant). The area was calculated using the ImageJ freeware package (National Institutes of Health, Bethesda, MD, United States).

Neurophysiological examination: Intraoperative monitoring of transcranial myogenic electrical potentials, MEPs and SSEPs, was performed during all the surgical interventions using the Neuro-IOM system (Neurosoft, Russia), as described previously[18]. Briefly, the registration of the latency and amplitude of the muscle response for abductor hallucis (AH), musculus tibialis anterior (TA), and musculus quadriceps femoris (QF) was performed with the active electrode placed in the region of the motor point. The amplitude and latency parameters of the cortical SSEP response of the hindlimbs in the form of the first positive (P1) and negative (N1) peaks were evaluated by sequential stimulation of nervus tibialis. The absolute values of the SSEP and MEP parameters varied in different animals and different muscle groups; therefore, we used a scoring system from the study[19] modified by us[18] from 0 (no evoked potentials) to 5 [the amplitude is 50%–100% of the baseline (before the injury) and the latency is no higher than 110% of the baseline] for SSEP-nervus tibialis as well as MEP-AH, MEP-TA, and MEP-QF.

Animal termination

Twelve weeks post transplantation, all animals were deeply anesthetized with an

intravenous administration of ketamine (20 mg/kg) followed by infusion of a single extra-high dose of propofol (5 mL of a 1% solution). Transcardial perfusion was performed with a buffered, cooled 10% formalin solution as described previously[18]. The vertebral columns were post-fixed for 24 h in the same solution at 4 °C. The spinals cords were isolated from the fixed preparations and sagittal sections (vibratome, 100 μm and paraffin 5–7 μm) were prepared from the region of injury and transplantation.

Histological and immunohistochemical analysis

Morphological studies were carried out on cresyl violet (Nissl), and hematoxylin and eosin stained paraffin sections, as described previously[18]. Immunohistochemical staining was performed on 3–5 μm paraffin sections as well as on 50–100 μm vibratome sections, both using fluorescence detection. Sections of the spinal cord were stained with antibodies to β-tubulin III (2 μg/mL), nestin (2 μg/mL; R&D), SOX2 (5 μg/mL; BD Biosciences), MAP2 (5 μg/mL; Sigma-Aldrich), NF200 (5 μg/mL; Sigma-Aldrich), GFAP (2 μg/mL; DAKO), brain derived neurotrophic factor (5 μg/mL; Abcam), and macro H2A.1 (Abcam). Secondary antibodies used were Alexa Fluor 488 goat anti-mouse IgG (H + L) and Alexa Fluor 633 goat anti-rabbit IgG (H + L) (highly cross-absorbed, all dilutions 1:400; Invitrogen, United States); counterstaining was done with Hoechst. Fluorescence was detected by a confocal microscope Nikon A1 (Nikon, Japan). For the quantification of positive cells, we used NIS Elements software (Nikon).

Statistical analysis

Statistical analysis of the hindlimb score, SSEP, MEP, and MRI was carried out on the three NHPs of the LC group and the four NHPs of the NPC group at each time point. The data were summarized as the median and the first and third quartiles or as the mean ± SD.

To compare baseline data in the groups, the nonparametric Mann–Whitney test for quantitative data and the χ^2 or Fisher exact test for qualitative data were used. The correlation between quantitative variables was estimated by Spearman's method. The hindlimb score and MEP/SSEP score data were analyzed by calculating Pearson's linear correlation coefficient.

For the main analyses (hindlimb score, MEPs, SSEPs), we used a mixed linear model with time points as nested data, the group and timeline being fixed factors; their interaction was also estimated. A two-sided probability threshold of 0.05 was used to determine statistical significance. The analyses were performed using IBM® SPSS® Statistics Version 23.0.

RESULTS

Immunophenotyping of drNPCs

Flow cytometry and immunocytochemical characterization of cultured allogeneic *Macaca mulatta* drNPCs was performed prior to transplantation. Expression of the immature neural stem/progenitor markers SOX2 and nestin was detected in 85.6% and 90.5% of the cells, respectively, as determined by flow cytometry, while GFAP expression was ubiquitous with up to 99.4% of the cells positive for this marker. The neuronal markers βIII-tubulin and MAP2 were detected in 85.8% and 58.6% of the cells, respectively (Figure 1A-C). Human leukocyte antigen DR expression was detected in a very small subpopulation, not more than 4.4% (Figure 1A), while HLA-ABC was not detected in a noninflammatory environment at all (Supplementary Figure 2). When drNPC were cultured on laminin coated plastic in complete growth media most cells coexpressed nestin and GFAP (Figure 1B). A smaller GFAP positive population appeared to have decreased nestin expression, indicating glial fate differentiation. drNPC cultures exhibited spontaneous early differentiation with formation of βIII-tubulin and MAP2 positive networks (Figure 1C-E) with most cells maintaining SOX2 expression, confirming their immature status. Taken together, flow cytometry and ICC demonstrated that drNPC cells are a relatively homogeneous neural stem/progenitor population that can initiate neuronal and glial differentiation in culture.

Time course of neurological deficit

Analysis of hindlimb score changes with time showed no noticeable improvement of

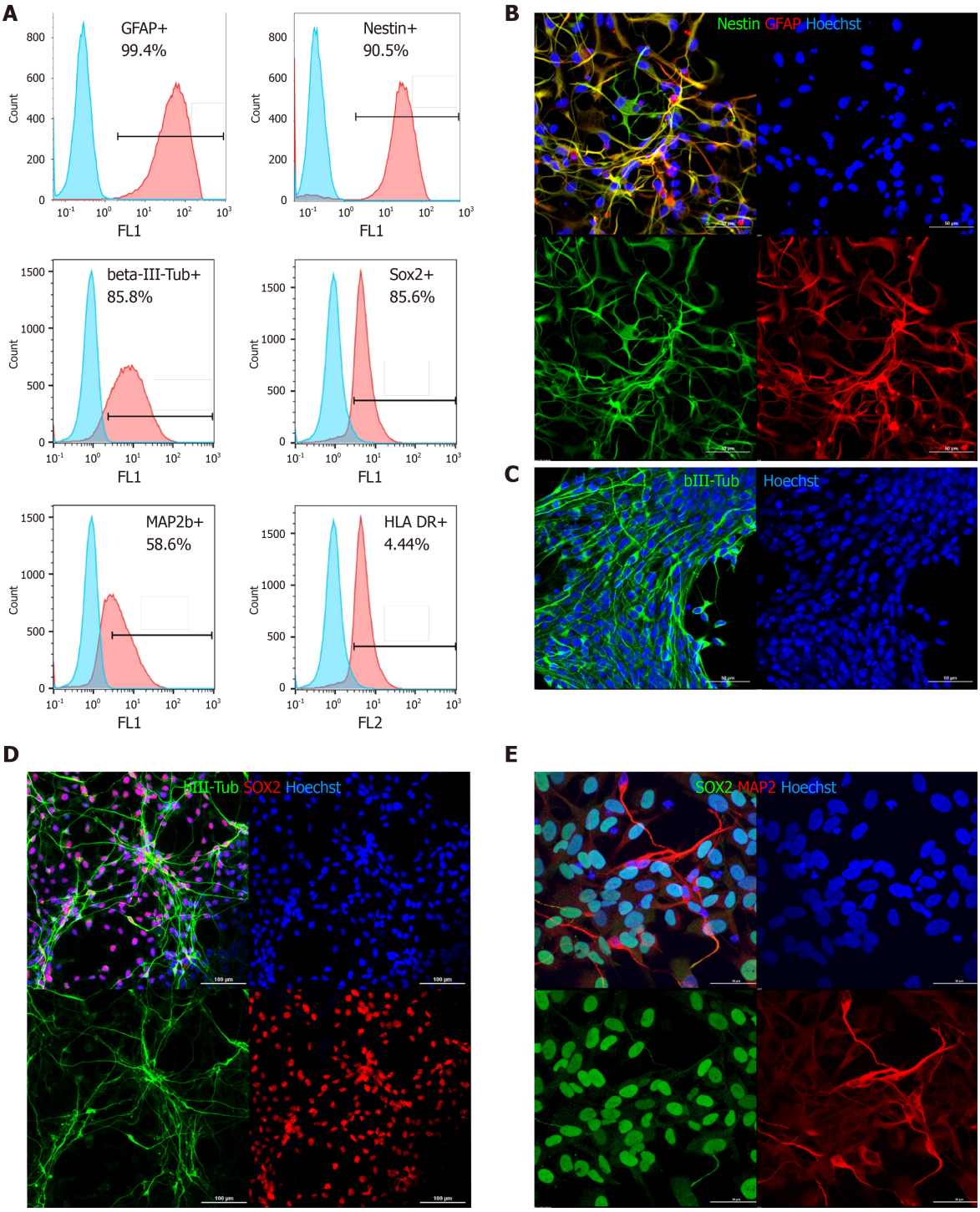

Figure 1 Phenotypic characterization of directly reprogrammed neural precursor cells by flow cytometry and immunocytochemistry. A: Flow cytometry: blue peaks-negative control (isotype immunoglobulins); from left to right, top to bottom: Glial fibrillary acidic protein, nestin, βIII-tubulin SRY-box transcription factor 2 (SOX2), microtubule associated protein 2 (MAP2), human leukocyte antigen (HLA)-DR; B: Nestin and glial fibrillary acidic protein staining (most cells are double positive); C: βIII-tubulin (green); D: βIII-tubulin (green) and SOX2 (red); E: MAP2 (red) and SOX2 (green) (D and E: Partial spontaneous differentiation of directly reprogrammed neural precursor cells on laminin/poly-L-lysine coated plastic). In all panels, nuclei are counterstained with Hoechst (blue). Scale bar, 50 μm (B, C, and E) and 100 μm (D).

the neurological state of control animals (LC group) that underwent SCI induction surgery and were administered vehicle. In contrast, the animals with implanted drNPCs (NPC group) exhibited recovery of motor function beginning on the fourth week after transplantation. Statistical analysis showed that the time course of the hindlimb score recovery was significantly different in the NPC group ($P < 0.01$, as estimated using the mixed linear model) as compared to the LC group (Figure 2).

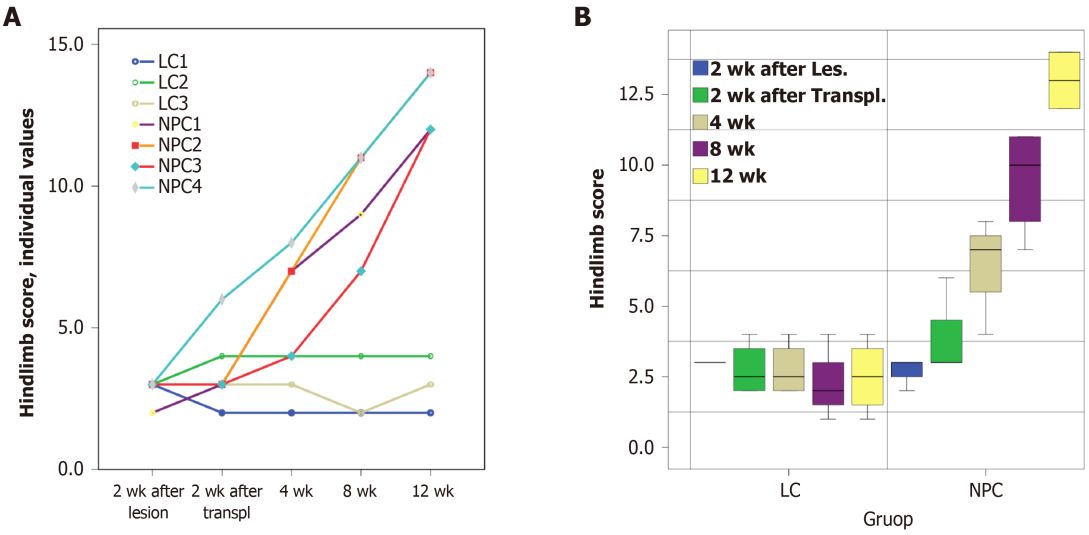

Figure 2 Changes in the hindlimb score in the experimental groups up to 12 wk after directly reprogrammed neural precursor cell transplantation or vehicle injection. A: Hindlimb scores of individual animals; B: Box plots of the parameters in the lesion control (LC) group and neural progenitor cell (NPC) groups (the median, the minimum, and maximum values and the first and third quartiles). The hindlimb scores of the LC group did not change over time. However, the hindlimb scores in the NPC group significantly increased over time and were statistically significant at 4, 8, and 12 wk post transplantation when compared to the pretransplantation time point ($P < 0.01$). Differences between the LC and NPC groups are significant from the 4th wk ($P < 0.01$, as estimated using the mixed linear model).

Analysis of individual parameters of the hindlimb score in drNPC treated animals showed that the muscle tone and the tendon and periosteal reflexes were normalized by 12 wk after transplantation (Supplementary Table 1). By that time, all NHPs were able to flex the hindlimb at large joints, use the hindlimb when walking, and use its digits for grip when climbing. Two of the four animals (NPC1 and NPC4) exhibited the most significant functional recovery as they were able to jump using the ipsilateral hindlimb for support and actively use this limb when climbing. In general, their behavior differed little, if at all, from that of healthy NHPs, indicating near complete recovery of function.

Analysis of SSEPs and MEPs

Estimation of MEPs and SSEPs both immediately after the injury and 2 wk post injury showed that the SSEPs from nervus tibialis of the left hindlimb and MEPs from the left AH and TA were either absent (NPC3, NPC4) or had amplitudes decreased by more than 80% (LC1-3, NPC1, NPC2) in all of the seven animals shortly before the second surgery and implantation, indicating a complete SCI (Supplementary Table 2 and Figure 3A). The absolute values of amplitude and latency varied substantially in different animals and different groups of muscles (Supplementary Tables 2-4); therefore, when performing comparative analysis, we took the values of these parameters in each animal before the injury to be 100%. We also used the point scale for estimation of SSEPs and MEPs suggested by Ye *et al*[19], which we adapted for our model[18] (Supplementary Table 5). In both groups the amplitude of SSEP, MEP-AH, and MEP-TA at day 14 (before the second surgery) varied from 0 points (absence of EP) to 1 point in the EP score (the maximal value was 17.4% in NHP LC2) (Supplementary Table 2). Due to this heterogeneity, we decided to define, as criteria for recovery at week 12, an increase in amplitude of 1 point or more. In the LC group, neither MEP nor SSEP parameters changed considerably at 12 wk after the vehicle injection. Even though NHPs in the LC group had residual MEP-AH (from 10% to 17%, 1 point) at day 14, we did not find MEP-AH at week 12 in two of the three NHPs (LC1, LC3), indicating deterioration over time. In LC2, which had 17.4% of residual MEP-AH amplitude at day 14, we detected MEPs from the left AH; however, their amplitude was as low as 4% of the baseline value, *i.e.* lower than the residual MEP-AH (Figure 3A and Supplementary Table 2). In contrast, the amplitude of the MEPs of the ipsilateral AH recovered over 50% of the baseline value in two of the four NHPs with implanted drNPCs (NPC1, NPC2) and to 14% and 17%, respectively, in the other two NHPs (NPC3, NPC4) (Figure 3A and Supplementary Table 2). Thus, all NHPs in the NPC group demonstrated recovery of MEP-AH by our criteria: from 1 to 3 points for NPC1 and NPC2 [NPC1: 9% (day 14)–56% (week 12); NPC2: 11% (day 14)–55% (week

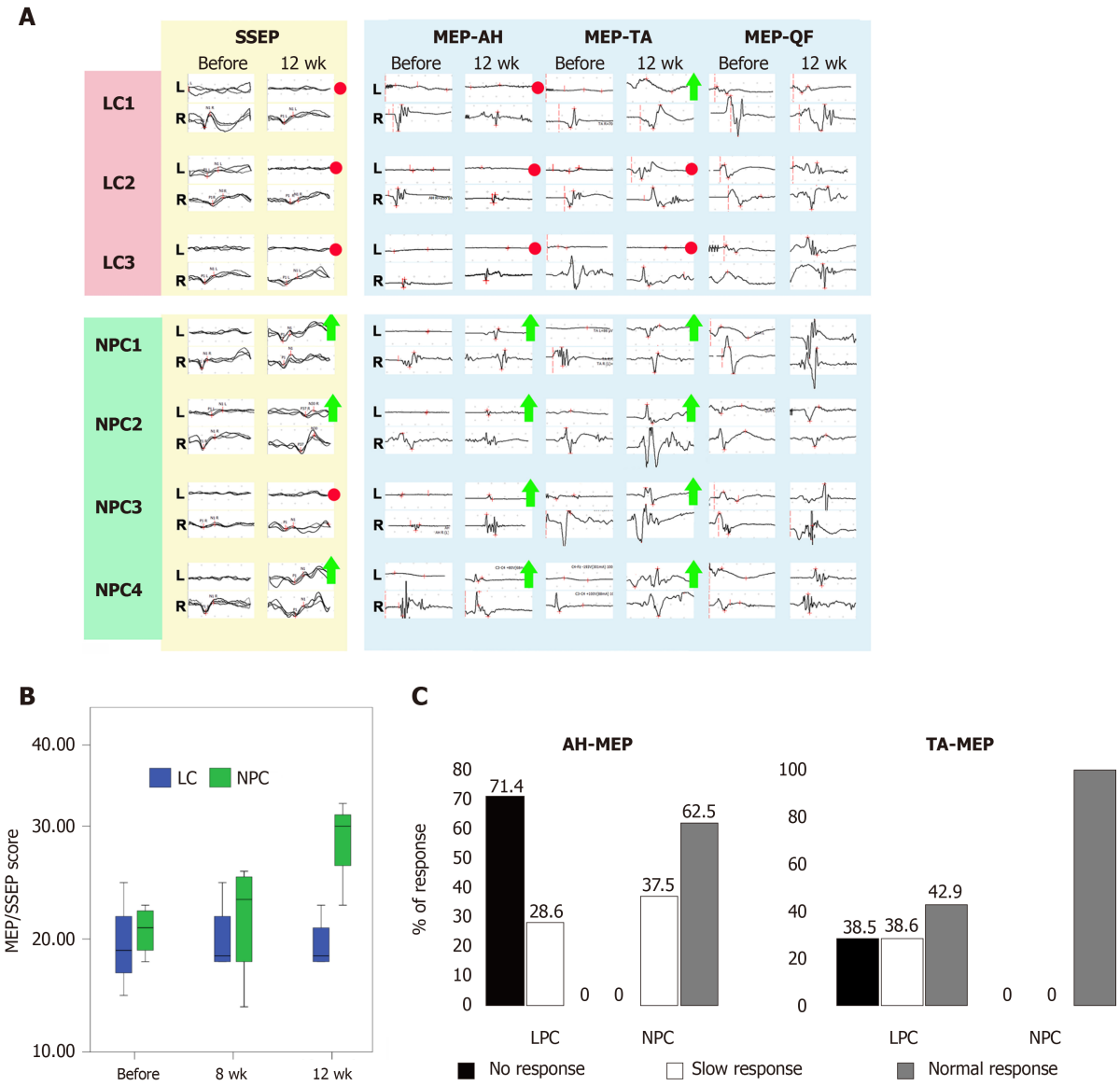

Figure 3 Functional assessment. A: Examples of somatosensory evoked potential (SSEP) and motor evoked potential (MEP) from all animals in the lesion control (LC) group and neural progenitor cell (NPC) group. Red circles indicate lack of recovery. Green arrows indicate recovery by 1 point of the EP scale. The MEP-musculus quadriceps femoris (QF) served as an internal control as the neighboring quadriceps femoris pathways were not specifically dissected and thus minimally affected. The MEP-quadriceps femoris showed no deterioration over time indicating the safety of the transplant procedure and the directly reprogrammed NPCs; B: Box plots of evoked potential scores in the LC and NPC groups (median, minimum, and maximum values and first and third quartiles). Differences between the groups 12 wk after the transplantation were significant ($P < 0.01$, as estimated using the mixed linear model); C: Semiquantitative analysis of the MEP-abductor hallucis and MEP-tibialis anterior latencies on the ipsilateral side 12 wk after directly reprogrammed NPC transplantation or vehicle injection. AH: Musculus abductor hallucis; TA: Musculus tibialis anterior.

12)]; and from 0 to 1 point for NPC3 and NPC4 (from 0 at day 14 to 14% and 17% at week 12 for NPC3 and NPC4, respectively) (Supplementary Table 5).

In the LC group only one NHP (LC1) demonstrated some spontaneous recovery of MEP-TA from 0 at day 14 to 1 point (6%) at week 12 (Figure 3A and Supplementary Table 3). MEP-TA amplitude in the other two NHPs did not change from day 14 to week 12. In contrast, all NHPs of the NPC group demonstrated recovery from 1 point at day 14 to 3 points at week 12 (Supplementary Table 3).

The differences between the NPC and LC groups in the degree of recovery of the MEPs from AH became significant at 12 wk ($P < 0.05$, as estimated using the mixed linear model, Figure 3B). There were no significant differences between groups in the MEPs from the ipsilateral TA. In both groups the MEPs from musculus quadriceps spontaneously recovered up to 3–5 points at day 14 indicating that the intersection of the medial corticospinal tract fibers was not complete (Figure 3A). We decided to analyze MEP-QF at all timepoints as an internal control. The amplitude and the latency of MEP-QF did not differ significantly between timepoints or treatment

groups, demonstrating safety of the drNPC injection.

None of the animals in the LC group exhibited SSEPs at 12 wk after vehicle injection; SSEPs were absent even in the animal LC2 that retained residual (approximately 10%) evoked potentials 2 wk after the injury. In three of the NHPs with implanted drNPCs (NPC1, NPC2, NPC4), the SSEP amplitude recovered to 63.1%, 27.7%, and 21.8% of the baseline level, respectively. NPC3 did not show any recovery of SSEP.

Statistical analysis showed significant differences between the LC and NPC groups in the ipsilateral nervus tibialis SSEP amplitude 12 wk post drNPC transplantation ($P < 0.05$, as estimated using the mixed linear model). Qualitative comparison of the MEP-AH and MEP-TA latencies at 4 and 12 wk post transplantation showed that the groups significantly differed in these parameters as well ($P < 0.01$ and $P < 0.05$ for the MEP-AH and MEP-TA latencies, respectively, as estimated by the χ^2 test) (Figure 3C). Statistical analysis of the SSEP/MEP scale showed significant differences between the treatment groups in the total score 12 wk post transplantation ($P < 0.01$, as estimated using the mixed linear model) (Figure 3B). Taken together, our data showed that 12 wk after drNPCs intraspinal transplantation of three of the four animals exhibited SSEP recovery, and all animals had recovery of MEP-AH and MEP-TA by at least 1 point. We detected no impairment of residual EP from TA or QF or any other negative adverse effects to innervation both in the ipsilateral and contralateral sides of all animals in the study.

MRI morphometry

To examine the potential tumorigenic effect of drNPCs and any impact on cerebral liquid flow after intraspinal drNPCs transplantation, the brain and spinal cord were evaluated by MRI followed by morphometry of the cerebral ventricles and aqueduct, the anterior and posterior subarachnoid spaces, and central canal at 2 wk after the SCI induction and 4, 8, and 12 wk post intervention (drNPC transplantation or vehicle injection) (Figure 4A). Additionally, we used postmortem high-resolution MRI for morphological investigation of SCI volume after drNPC transplantation in comparison with the vehicle control group (Figure 4B). None of the animals displayed signs of hydrocephalus syndrome or morphological signs of syringomyelia; there were no noted alterations in the cerebral ventricles or subarachnoid spaces at 12 wk in all four animals (Figure 4A, 4C and 4D). Dilation of the central canal was observed in one NHP in the vehicle control group (Supplementary Figure 3) and appeared dependent on the degree of postoperative epidural fibrosis.

To investigate possible structural alterations of the spinal cord after intraspinal drNPC transplantation, we determined the spinal cord area at the site of the injury as well as below and above it before the transplantation and for the 12 wk post intervention (Figure 4F). Statistical analysis of these data did not show significant differences in morphometric parameters between the LC and NPC groups, indicating that all animals tolerated the injection procedure and the drNPC transplantation.

At week 12 post intervention, the volume of injury (the sum of all areas with pathological hyperintense signals in T2 weighted coronal sections, Figure 4B) was in the range of 28.0-36.5 mL and did not differ significantly between groups (Figure 4G). This suggests that drNPCs transplantation did not add measurable volume to the spinal cord nor contribute significantly to a gliomesodermal scar at the macroscopic level.

Taken together, our MRI data confirmed the relative consistency of the SCI lesion in the LC and NPC groups and demonstrated that the intraspinal transplantation of drNPCs did not adversely affect the morphology of the central nervous system or cerebrospinal fluid circulation.

Immunohistochemical analysis

We next sought to detect transplanted cells and to investigate their fate post transplantation using IHC. The allogeneic drNPCs were derived from the bone marrow of a donor female, whereas the recipient NHPs were males. By using an antibody against macro-H2A.1, a histone overexpressed in the inactivated X chromosome of female cells[20], we were able to distinguish donor cells from the recipient. We determined that macro-H2A.1 expression levels reliably differentiated between the transplanted female cells and the host male cells (Figure 5A, 5B, and 5D). Macro-H2A.1-positive cells were found both in the white and grey matter of the injured spinal cord in all animals of the NPC group. Double staining with macro-H2A.1 and SOX2 antibodies revealed that the majority of the cells were positive for both markers (Figure 5B), as confirmed using the colocalization feature of the NIS elements application (Pearson correlation = 0.78). The donor cells were predominantly

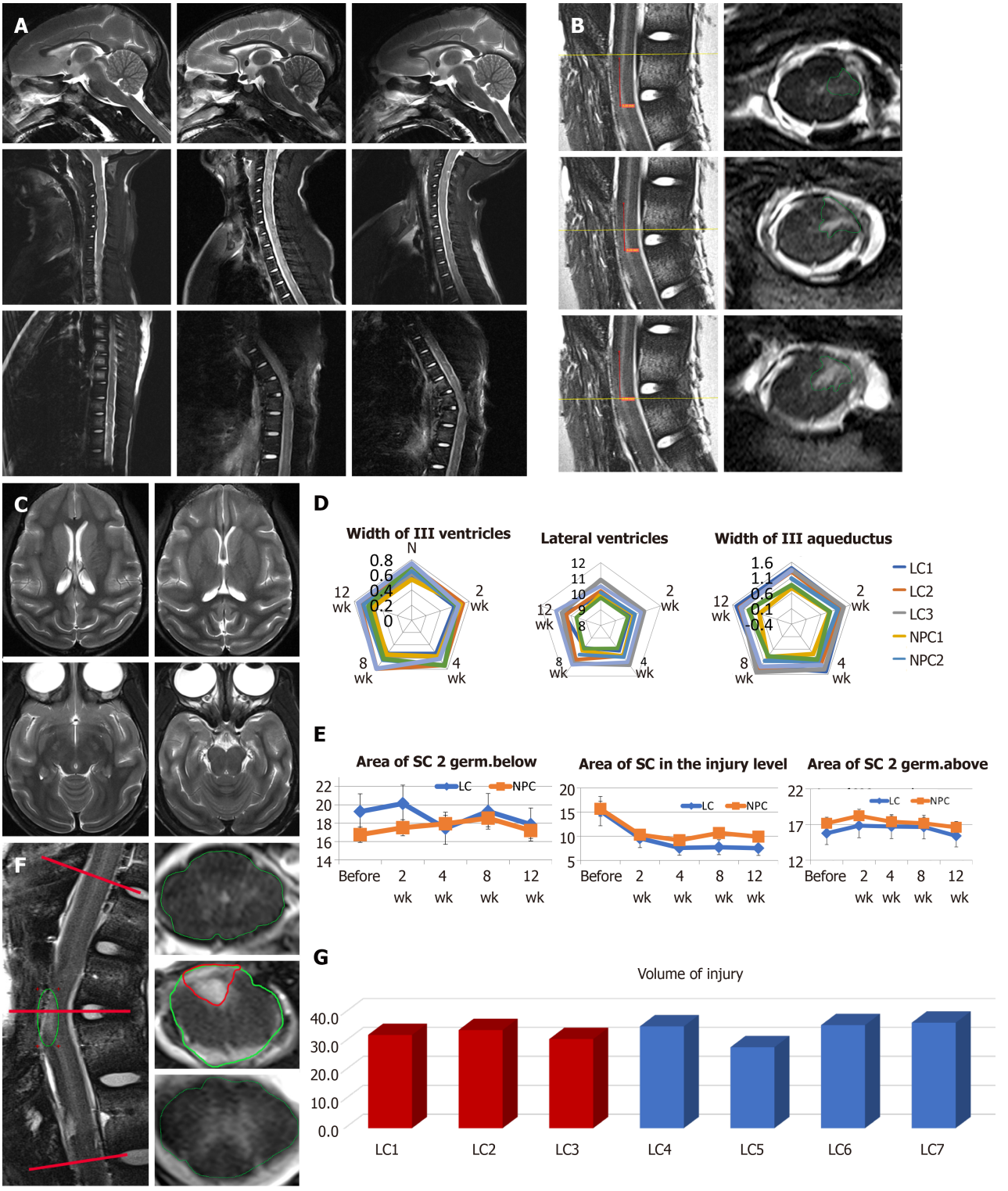

Figure 4 T2 weighted magnetic resonance imaging and the results of the brain and spinal cord morphometry. A: Sagittal sections of the brain and the cervical and thoracic spinal cord of animal NPC1 before the lesion (left column), 2 wk after the lesion, just before the transplantation (middle column) and 12 wk after the transplantation (right column). The site of the injury is shown by white arrows; B: Postmortem high-resolution magnetic resonance diffusion tensor imaging of the spinal cord of animal NPC1. Left column: sagittal sections, right column: coronal sections from the upper part, middle part, and lower part of the injury (from top to bottom). The projection planes of coronal sections shown by yellow lines on corresponding sagittal sections. The middle part of the injury was shown by green arrow; C: Coronal sections of the brain, animal NPC2; D: Morphometry of the brain ventricles monitored 12 wk after the transplantation in animals of the lesion control (LC) group and neural progenitor cell (NPC) group; E: Morphometry of the spinal cord area in the lesion site, and two segments above and below; F: The example of sagittal (left column) and coronal (right column) sections with highlighted spinal cord and injury areas. The projection planes of coronal sections shown by red lines of the sagittal one; G: The results of spinal cord lesion volume calculation at week 12.

localized around endogenous NF200-positive growth cones (Figure 5C and 5D) as well as around reactive astrocytes (Figure 5B and 5F). In the LC group, neither expression of macro-H2A.1 nor SOX2 was detected (Supplementary Figures 4-6). Quantification

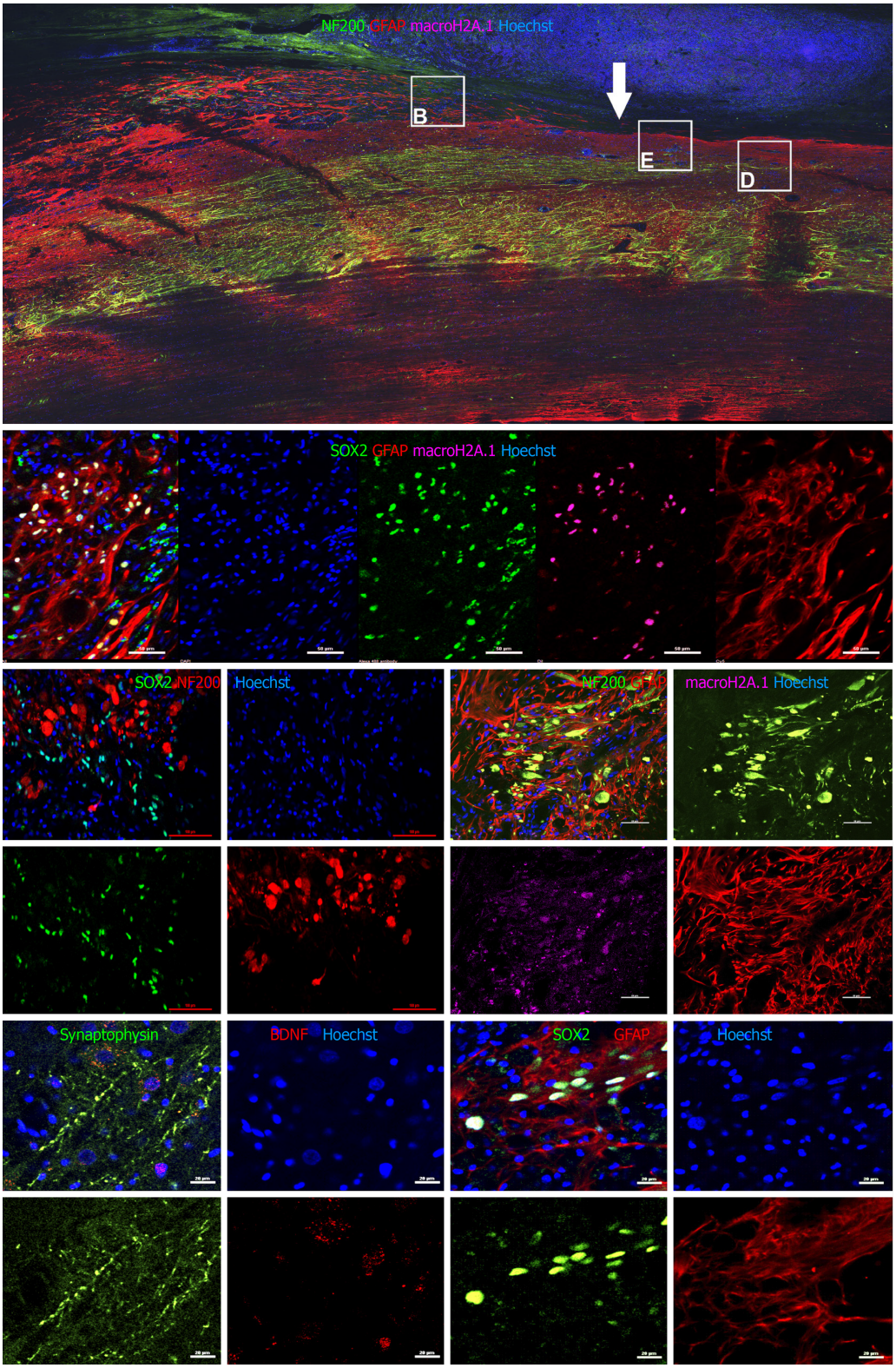

Figure 5 Immunohistochemical analysis on spinal cord sagittal sections 12 wk after transplantation. A: Large image showing the center of injury (white arrow) of animal NPC1 stained with antibodies to NF200 (green) glial fibrillary acidic protein (GFAP) (red) and macro H2A.1 (magenta; staining is visible only in enlarged panels). Squares show approximate localization of enlarged panels B, D, and E (B and E stained with different antibody cocktails); B: Enlarged fragment of a sagittal section of animal NPC 1 stained for SRY-box transcription factor 2 (SOX2) (green), GFAP (red) and macro-H2A.1 (magenta). SOX2 and macro H2A.1 colocalized in the same cells (Pearson correlation = 0.78); C: Enlarged fragment of sagittal section of animal NPC4 stained for NF200 (red) and SOX2 (green). NF200 and SOX2 did not colocalize (Pearson correlation = 0.20); D: Enlarged fragment of sagittal section stained for NF200 (green, growth cones are shown), GFAP (red, astrogliosis area), and macro H2A.1 (magenta), animal NPC1. NF200 and macro H2A.1 did not colocalize (Pearson correlation = 0.20); E: Enlarged fragment of sagittal section stained for synaptophysin (green) and brain derived neurotrophic factor (red), animal NPC1; F: A sagittal section of animal NPC4 spinal cord near the injury epicenter stained for SOX2 (green) and GFAP (red). Size bar 50 μm (A-D), and 2 μm (E-F).

of the number of drNPC-derived cells in confocal images revealed that the density of macro-H2A.1 and SOX2-positive cells was almost 2-fold higher around damaged axons, which formed growth cones and presented high intensity NF200 staining *vs* areas of low NF200 staining intensity (Figure 6). The estimated total number of transplanted cells in a tissue slice was less than 1000. Taking into consideration a slice thickness of 100 μm and that the area of transplantation did not exceed 2 mm, it appears that the total number of surviving cells was less than 20×10^3 cells per NHP, indicating an estimated survival rate of less than 1% at 12 wk.

Despite the abundance of macro-H2A.1+ cells in areas positive for NF200, there was no colocalization of the markers (Pearson correlation = 0.20), indicating that none of the surviving drNPC derived cells were terminally differentiated neurons. Interestingly, we found high expression of synaptophysin and brain derived neurotrophic factor in the axonal growth cone-rich areas where macro H2A.1/SOX2-positive cells were located (Figure 5E). In astrogliosis foci, the SOX2-positive cells were located along GFAP-positive processes of reactive astrocytes and appeared GFAP-positive (Figure 7A). However, three dimensional reconstruction of confocal z-stacks showed that some SOX2/macro H2A.1-positive cells were GFAP negative (Figure 7B-D) and oftentimes were surrounded by GFAP-positive processes (Figure 7D). Taken together, our careful single cell analysis indicated that surviving SOX2-positive cells had not differentiated into astrocytes.

DISCUSSION

Research focused on regenerative approaches for central nervous system diseases in general and SCI in particular indicates upon close examination of the published literature on this topic that one of the most important aspects driving clinical translation of promising preclinical data is the choice of the *in vivo* models. The data obtained in small rodents has not always been successfully reproduced in large animals, and therefore its extrapolation to humans is still challenging[21,22]. Because of this, research conducted on anthropoid primates has experienced renewed interest from the scientific community based on the rationale that the phylogenetic proximity of NHPs to humans may provide a basis for more successful clinical trans-lation[19,21-23]. Based on these considerations, we had previously developed a bioethically acceptable NHP model of regionally complete SCI with the use of intraoperative EP detection, resulting in a regionally complete and irreversible model of SCI[18]. This was evidenced in the current study by the absence of EP from nervus tibialis, AH, and TA both intraoperatively, *i.e.* immediately after excision of a fragment of the spinal cord, as well as 2 wk after surgery, except for some residual potentials.

In the current study we used allogeneic rather than autologous drNPC cells because that allowed us to carefully characterize donor cell behavior postmortem without relying on permanent genetic modification of the donor cells or pretransplant physical labeling that may lend itself to errors in the case of cell fusion or endocytosis of the label by host cells. This likely resulted in a lower number of surviving cells at 12 wk post transplantation and likely did not allow for the survival of any differentiated cells. The surviving $SOX2^+$ donor cells nevertheless allowed for significant functional recovery by supporting neurogenesis and synaptogenesis of the host neurons despite the absence of immunosuppression.

Previous studies in NHPs addressing the safety and efficacy of cell-based therapies in SCI have showed that xenogeneic (human) neural stem cells might improve spinal cord regeneration[9], but for clinical purposes, we argue that the most desirable cell type is autologous[7]. The potential challenges of using nonautologous cells in the clinic was demonstrated in a recent study whereby pharmacological immunosuppression did not provide adequate long-term survival of transplanted cells and failed to improve functional recovery after SCI[24], indicating that both longer term and more extensive immunosuppression may be required than previously thought for nonautologous cell transplants for SCI. This may not be feasible for SCI patients who are already more prone to suffer from infections. To provide a solution to the challenge of generating autologous neural progenitor cells for large scale clinical use in SCI repair, a realistic source of such autologous cells are the patient's own somatic cells that are obtained, *e.g.*, from the bone marrow and directly reprogrammed into neural precursor cells[11-13]. Direct reprogramming skips the pluripotent state and therefore rapidly generates cells with considerable safety advantages over pluripotent derived cells[14].

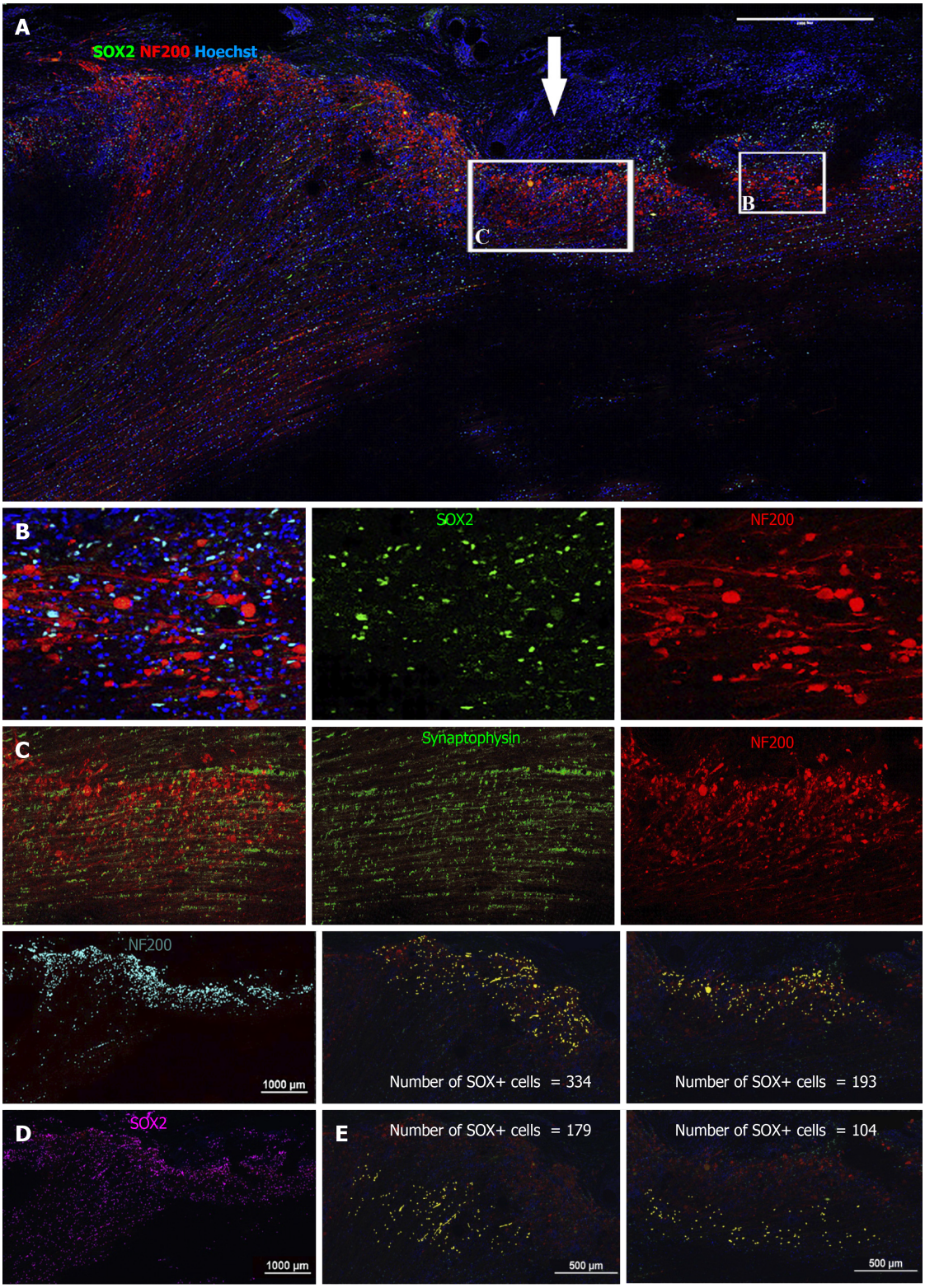

Figure 6 Immunohistochemical analysis of NF200 and SRY-box transcription factor 2 on spinal cord sagittal sections 12 wk after transplantation (animal NPC4). A: Large image showing the center of injury (arrow). Squares show approximate localization of enlarged panels B and C; B: Enlarged image of SRY-box transcription factor 2 (SOX2) (green) and NF200 (red) staining; C: Enlarged image of synaptophysin (green) and NF200 (red) staining; D: The results of quantification of NF200 (blue) and SOX2 (magenta); binary selection; and E: Upper images: yellow dots-number of SOX2-positive cells in the high intensity zone of NF200 fluorescence (area of growth cones); Lower images: yellow dots-number of SOX2-positive cells in the low intensity zone of NF200 fluorescence. Bar size 500–1000 μm.

Based on the hindlimb score[22], modified for our model, we observed restoration of function in the paralyzed limb for all animals in the drNPC transplanted group (NPC group), while the vehicle control group showed no recovery. The best recovery was observed in subject NPC1, which correlated with the restoration of MEP to over

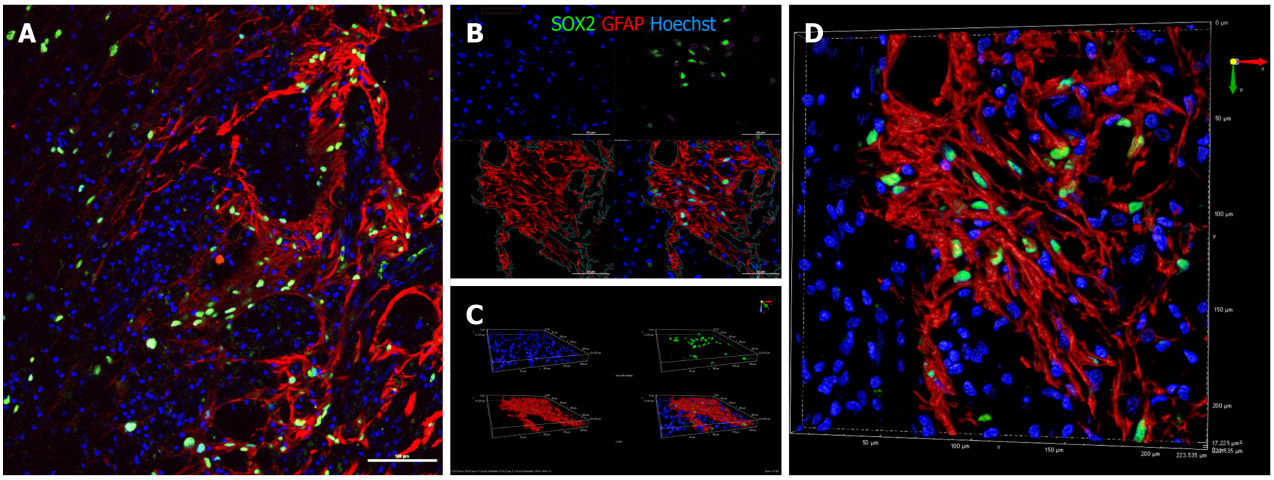

Figure 7 Immunohistochemical analysis of SRY-box transcription factor 2 (green) and glial fibrillary acidic protein (red) on sagittal sections of animal NPC4 spinal cord. A: Large image showing that in the site of astrogliosis SRY-box transcription factor 2 (SOX2)-positive cells predominantly localized along glial fibrillary acidic protein (GFAP)-positive processes; B and C: Three dimensional reconstruction revealed that SOX2 was not expressed by GFAP positive cells; and D: High resolution three dimensional reconstruction. Arrows show the GFAP-positive processes, surrounding SOX2-positive cells. Bar size 100 μm.

50% of baseline (preinjury) levels. Importantly, none of the three control animals showed any recovery of SSEP and MEP up to 12 wk post vehicle injection. Therefore, the MEP restoration observed in all four animals and the SSEP restoration observed in three animals in the drNPC transplanted group suggests preliminary efficacy of drNPCs transplantation in SCI.

Because we transplanted female drNPCs into males, we used antibodies against macro H2A.1, a histone marker overexpressed in the inactivated X chromosome of female cells[20] to identify the donor cells. Transplanted cells could be identified by the high fluorescence intensity of macro H2A.1; it was further confirmed that the bright macro H2A.1-positive cells were indeed female drNPCs because they were also labelled with SOX2, a specific marker of neural multipotency that is not expressed in the naïve adult spinal cord[25]. This was further confirmed by the absence of any $SOX2^+$ cells in the vehicle transplanted LC NHPs (Supplementary Figures 4-6). Using this approach, we were able to detect double positive macro H2A.1 and $SOX2^+$ cells (drNPCs) that mainly accumulated in the formation zone of axonal growth cones. This biased distribution of grafted drNPCs suggests active migration towards the spinal cord regeneration zone. Interestingly, our histology data estimated that less than 1% of the transplanted drNPC cells were detected at 12 wk post transplantation. Although the drNPC retained neural multipotency (SOX2 expression) and low human leukocyte antigen DR expression, the use of allogeneic rather than autologous drNPCs in the absence of immunosuppressive therapy (a major limitation of this study) may help explain the low survival rate of the graft. The development and testing of autologous NHP drNPC in SCI is the subject of ongoing studies.

Despite the regenerating axonal environment into which donor cells integrated, we were unable to detect macro H2A.1-positive cells that expressed neuronal markers. We cannot rule out the possibility that neuronal differentiation did occur before early immune detection and elimination by the host immune system. Nevertheless, our study suggests that drNPC-based efficacy does not depend on substantial survival of the grafted cells nor neuronal differentiation. While unravelling the mechanism of drNPC based efficacy was beyond the scope of our study, it is anticipated that it involves various factors, of which neurite outgrowth and synaptogenesis appear significant, as previously discovered in a recent study employing the cells in an unrelated rodent model of stroke[13]. The mechanism may also have similarities to spinal cord regeneration mechanisms seen in more primitive vertebrates[26]. The hypothesis according to which transplanted NPCs must undergo neuronal differentiation, integrate into functioning neural networks (*i.e.* be directly involved in conducting an electrical impulse[9,17]) to provide meaningful clinical benefit is the focus of intense debate and investigation. Our study with drNPCs suggests that significant functional recovery of anatomically disturbed pathways can potentially be accomplished through a regeneration mechanism that may be unique to drNPC-like cells.

CONCLUSION

There was no evidence of safety concerns regarding drNPC transplantation into the spinal cord for at least 12 wk post transplantation, as evidenced by the absence of pathological changes in the spinal cord and cerebrospinal fluid as assessed by MRI and histological analysis. There were also no observed ectopic cell colonies.

drNPCs injection contributed to significant improvement of spinal cord function after subacute complete SCI, based on neurological status assessments and neurophysiological recovery during 12 wk post transplantation. Functional improvement was not associated with the neuronal or glial differentiation of drNPCs but rather by the presence of multipotent $SOX2^+$ drNPCs. Directed drNPC migration to the areas of active host growth cone formation, including in the areas of corticospinal axons, may provide some paracrine trophic support that activate the regeneration processes.

ARTICLE HIGHLIGHTS

Research background

The research is based on two points: the discovery of direct reprogrammed neural progenitor cells (drNPCs) and the development of an evoked potential-driven model of spinal cord injury in non-human primates.

Research motivation

The key problem to be solved is the restoration of brain-spinal cord connection and functions after the complete spinal cord injury.

Research objectives

The main objective of the study was to investigate the safety and efficacy of intraspinal transplantation of drNPCs in the treatment of complete spinal cord injury on non-human primates.

Research methods

Experiments were conducted on non-human primates with behavioral, neurophysiological, histological, and immunohistochemical assessment.

Research results

Injections of drNPCs were accompanied by restoration of anatomically resected afferent and efferent neuronal pathways. No evidence was found that drNPCs were directly involved in the restoration of neuronal pathways.

Research conclusions

Using a primate evoked potential guided spinal cord injury model we demonstrated safety and efficacy of intraspinal injections of allogeneic drNPCs.

Research perspectives

At the next stages of research, it is necessary to increase the survival rate of transplanted cells, such as by transplanting predifferentiated tissue-engineered constructs.

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ORIGINAL ARTICLE

Retrospective Study

Feasibility of allogeneic mesenchymal stem cells in pediatric hypoxic-ischemic encephalopathy: Phase I study

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Author contributions: Kabataş S, Karaöz E, and Akkoç T performed concept and design of the study; Kabataş S, Civelek E, Karaöz E, and Akkoç T performed the supervision; Kabataş S, Civelek E, Kaplan N, Savrunlu EC, Can H, Genç A, Boyalı O, Diren F performed the analysis and/or interpretation; Kabataş S, Civelek E, Can H, Genç A, Boyalı O, Diren F, Karaöz E performed the literature search; Kabataş S, Kaplan N, Savrunlu EC, Karaöz E, and Akkoç T was responsible for writing; Kabatas S, Civelek E, Savrunlu EC, Karaöz E, and Akkoç T conducted critical reviews.

Institutional review board statement: Ethical approval to report this case was obtained from the IRB of Turkish Ministry of Health, Department of Organ, Tissue Transplant and Dialysis Services' Scientific Committee,

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Abstract

BACKGROUND

Ankara, Turkey, No. 56733164-203-E. 2351.

Informed consent statement: There is human subject in this article and written informed consents were obtained from the patient for their anonymized information to be published in this article and before the stem cell therapies.

Conflict-of-interest statement: The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Data sharing statement: No additional data are available.

Manuscript source: Invited manuscript

Specialty type: Cell and tissue engineering

Country/Territory of origin: Turkey

Peer-review report's scientific quality classification

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Grade B (Very good): B, B
Grade C (Good): 0
Grade D (Fair): 0
Grade E (Poor): 0

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Hypoxic-ischemic encephalopathy (HIE) is one of the leading causes of death and long-term neurological impairment in the pediatric population. Despite a limited number of treatments to cure HIE, stem cell therapies appear to be a potential treatment option for brain injury resulting from HIE.

AIM

To investigate the efficacy and safety of stem cell-based therapies in pediatric patients with HIE.

METHODS

The study inclusion criteria were determined as the presence of substantial deficit and disability caused by HIE. Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) were intrathecally (IT), intramuscularly (IM), and intravenously administered to participants at a dose of 1×10^6/kg for each administration route twice monthly for 2 mo. In different follow-up durations, the effect of WJ-MSCs administration on HIE, the quality of life, prognosis of patients, and side effects were investigated, and patients were evaluated for neurological, cognitive functions, and spasticity using the Wee Functional Independence Measure (Wee FIM) Scale and Modified Ashworth (MA) Scale.

RESULTS

For all participants (n = 6), the mean duration of exposure to hypoxia was 39.17 + 18.82 min, the mean time interval after HIE was 21.83 ± 26.60 mo, the mean baseline Wee FIM scale score was 13.5 ± 0.55, and the mean baseline MA scale score was 35 ± 9.08. Three patients developed only early complications such as low-grade fever, mild headache associated with IT injection, and muscle pain associated with IM injection, all of which were transient and disappeared within 24 h. The treatment was evaluated to be safe and effective as demonstrated by magnetic resonance imaging examinations, electroencephalographies, laboratory tests, and neurological and functional scores of patients. Patients exhibited significant improvements in all neurological functions through a 12-mo follow-up. The mean Wee FIM scale score of participants increased from 13.5 ± 0.55 to 15.17 ± 1.6 points (mean ± SD) at 1 mo (z = - 1.826, P = 0.068) and to 23.5 ± 3.39 points at 12 mo (z = -2.207, P = 0.027) post-treatment. The percentage of patients who achieved an excellent functional improvement (Wee FIM scale total score = 126) increased from 10.71% (at baseline) to 12.03% at 1 mo and to 18.65% at 12 mo post-treatment.

CONCLUSION

Both the triple-route and multiple WJ-MSC implantations were safe and effective in pediatric patients with HIE with significant neurological and functional improvements. The results of this study support conducting further randomized, placebo-controlled studies on this treatment in the pediatric population.

Key Words: Hypoxic-ischemic encephalopathy; Pediatric; Stem cell; Wharton jelly

Core Tip: Hypoxic-ischemic encephalopathy (HIE) emerges as one of the leading causes of morbidity and mortality in children. There are a limited number of options for treating HIE. Recently, stem cell and cellular therapies appear to be a potential treatment option for ischemic brain injury caused by HIE. The aim of this phase I open-label clinical study is to investigate the efficacy and safety of one of these stem cell-based therapies in a group of pediatric patients with HIE. Both the triple-route and multiple Wharton`s jelly-derived mesenchymal stem cells administrations were safe and effective in pediatric patients with HIE with significant neurological and functional improvements.

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INTRODUCTION

Hypoxic-ischemic encephalopathy (HIE) is a type of ischemic brain injury especially in pediatric population. It is caused by a lack of oxygen supply to the brain, resulting in oxygen deprivation. HIE has high morbidity and mortality rates[1]. Today, there is a limited number of treatment options for HIE *e.g.*, cooling[2]. Different therapeutic approaches have been used to treat and improve functional and neurological outcomes of HIE patients. Among these approaches, stem cell therapies combined with new protocols are an adopted method to prevent ischemic brain injury caused by HIE[1,2]. Bone marrow (BM) is used as the most common source to derive mesenchymal stem cells (MSCs). Yet, taking MSCs from BM requires a highly invasive procedure, and the age of the donor is an effective factor for the maximal life span of obtained cells. Nowadays, Wharton's jelly (WJ), an umbilical cord (UC) tissue, comes to the fore as a potential source of stem cells since this tissue is discarded at birth, providing an opportunity for the isolation of MSCs. With their immune-privileged status, high proliferation capacity, and absence of ethical issues, UC-MSCs appear to be an optimal therapeutic tool[3].

The issue of selecting the most appropriate route for MSC implantation is of critical importance and needs to be discussed to successfully treat HIE. Each strategy has its advantages and disadvantages, for example, the intravenous (IV) route of transplanting MSC might provide diffuse implementation while avoiding adverse reactions associated with invasive approaches. Notwithstanding, when systemically transplanted, MSCs are able to cross the blood-brain barrier; however, they can also reach to other organs such as the liver, lungs, kidneys, and spleen and be retained by them[4]. For this reason, carrying out transplantation through multiple routes can be more effective than the use of a single route. Patients tolerated the IV and intrathecally (IT) administrations well with no adverse reactions or side effects in 24 wk after treatment[5]. In our previous study, we evaluated the safety, efficacy, and practicability of both the triple-route and multiple administrations of WJ-MSCs with this treatment approach in a patient with HIE, traumatic brain injury, and cerebral palsy[6-8]. As further studies have been conducted on this subject, it is now possible to use WJ-MSCs for the clinical treatment of HIE.

The present study was designed as a phase I clinical trial to investigate the effects of both triple-route and multiple administrations of WJ-MSC. The study population was selected as pediatric HIE patients with significant functional impairments who have a limited number of treatment options. The primary outcome of the study was to investigate the safety of this treatment with magnetic resonance imaging examinations, electroencephalographies, laboratory tests, and neurological and functional scores of patients. The efficacy of this treatment was also studied.

MATERIALS AND METHODS

Study design

The present study was designed as a phase I open-label, multi-center study. The aim of the study was to assess the safety and efficacy of both triple-route and multiple administrations of WJ-MSCs. The study inclusion criteria are given in Table 1. Pediatric patients with radiologically confirmed HIE and significant functional and cognitive impairments were included in the study. Participants were followed up for a period of 1 year following the administration of WJ-MSCs. Participants were not restricted in terms of receiving any kind of medical therapy or treatment (occupational, physical, or speech therapy) during their follow-ups. The legal representatives of participants were informed about the procedure and gave written informed consent in accordance with the principles of the Helsinki Declaration. The study protocol was approved by the Turkish Ministry of Health, General Directorate of Health Services, Department of Tissue, Organ Transplantation and Dialysis Services, Scientific Committee with the protocol number of 56733164- 203-E.2351. The data of patients are given in detail in Table 2.

Table 1 Enrollment criteria

No.	Inclusion criteria
1	Age < 18
2	HIE radiologically confirmed at initial diagnosis and at study enrollment
3	The patients who does not have any chronic illness (cancer, kidney, heart/hepatic failure etc.) other than HIE. Adequate systemic organ function confirmed by normal ranged laboratory values
4	Life expectancy > 12 mo
5	No substiantial improvement despite of a treatment in neurological/functional status for the 3 mo before study enrollment
6	Severe disability defined as subject confined to a wheelchair/required to have home nursing care/needing assistance with activities of daily living
7	Expectation that the patient will receive standard post-treatment care and attend all visits
8	Signing in the written informed consent form for confirming to that know the treatment to be applied and to be willing by their parents/a surrogate
	Exclusion criteria
1	Presence of any other clinically significant medical/psychiatric condition, or laboratory abnormality, for which study participation would pose a safety risk in the judgment of the investigator/sponsor or history within the past year of drug/alcohol abuse
2	Recently diagnosed severe infection (meningitis, *etc.*)/development of liver, kidney/heart failure/sepsis or skin infection at the *i.v.* infusion site or positive for hepatitis B, C/HIV
3	History of uncontrolled seizure disorder
4	History of cerebral neoplasm, or cancer within the past 5 yr, with the exception of localized basal or squamous cell carcinoma
5	Having clinic symptoms that formation of white sphere number ≥ 15000/μL or platelet count ≤ 100.000/μL
6	Serum aspartate aminotransferase and serum alanine aminotransferase > 3× upper limit of normal/creatinine > 1.5× upper limit of normal
7	Participation in an another investigational stem cell study before treatment
8	The patient/parents decides to abandon the treatment or the patient death

HIE: Hypoxic-ischemic encephalopathy; HIV: Human immunodeficiency virus.

Procedure

Ethical considerations and consent: UCs were supplied from the Good Manufacturing Practice facility of LivMedCell (Istanbul, Turkey). In line with the approval of an institutional regulatory board (LivMedCell), various donors donated the UCs after they were informed about the purpose of the study and gave written informed consent. Postnatal UCs were obtained from full-term pregnant women who donated UCs[6-8].

UC processing and quality control

Phosphate-buffered saline (Invitrogen/Gibco, Paisley, United Kingdom) was used to wash the UCs. Tissue samples were cut into pieces of 5-10 mm^3 in the form of explants following the removal of blood vessels. The explants were placed into dishes and cultured under humanized culture conditions at 37 °C with 5% CO_2 until the migration of cells. When the resulting cells reached 70% to 80% confluency, they were collected and subjected to characterization tests at passage 3. The standards of the Turkish Medicines and Medical Devices Agency were followed to carry out quality control and quality assurance to produce these cells[6-8].

Characterization of WJ-MSCs by flow cytometry

Expressed surface antigens were analyzed by flow cytometry, which revealed that the cells were consistently positive for CD44, CD73, CD90, and CD105 and negative for the hematopoietic lineage markers of CD34, CD45, and Human Leukocyte class II DR antigens. The telomerase activities of WJ-MSCs were found to remain stable during the culture process with a large and flat cellular morphology[6-8].

Cell differentiation and karyotying procedure

Some stem cell expressions and the differentiation markers of TERT, SOX2, POU5F1, CD44, ZFP42, VIM, ICAM1, THY1, VCAM1, BMP2, RUNX-1, and NES were identified. These cells were confirmed to have trilineage (chondrocytes, osteoblasts, and adipocytes) differentiation capacity by differentiation analyses. Karyotyping studies

Table 2 Study population

		Frequency	Percent
Age	1.00	1	16.7
	6.00	2	33.3
	7.00	1	16.7
	9.00	1	16.7
	12.00	1	16.7
Sex	F	4	66.7
	M	2	33.3
Cause of hypoxia	Acute meningitis	1	16.7
	Cardiac arrest after an orthopedic surgery	1	16.7
	Cardiac arrest due to long QT syndrome	1	16.7
	Cardiac arrest, unknown etiology	1	16.7
	Drowning in water	1	16.7
	Foreign body aspiration	1	16.7
Duration of Hypoxia	25.00	1	16.7
	30.00	3	50.0
	45.00	1	16.7
	75.00	1	16.7
Comorbidity	Long QT syndrome	1	16.7
	no	4	66.7
	Osteogenesis imperfecta	1	16.7
Duration Between Hypoxia & First SCT	6.00	3	50.0
	9.00	1	16.7
	32.00	1	16.7
	72.00	1	16.7

SCT: Stem cell therapy.

showed no numerical or structural chromosomal abnormalities for these cells[6-8].

Pre-transplantation process: The final WJ-MSC preparations to be used for implantation were collected from passage 3 of cultures and kept in normal saline at final densities of 1×10^6 in 3 mL, 1×10^6 in 20 mL, 1×10^6 in 30 mL[6-8].

WJ-MSC transplantation and surgical procedures: Before initiating treatment, patients were examined by a physician team consisting of a pediatrician and a pediatric neurologist, as well as experts in neurosurgery, anesthesia and reanimation, and physical therapy and rehabilitation. Before the implantation procedure of WJ-MSCs, patients were evaluated for contraindications to sedoanalgesia or general anesthesia as well as severe infectious diseases like sepsis, and the procedure was then performed when they were stable[6-8].

In the procedure, allogeneic WJ-MSCs were administered IT, intramuscularly (IM), and IV, respectively, in the operating room by the same physician team (Kabatas S, Kaplan N, Can H, and Genç A), following the standard protocol of the MSC treatment trial (Table 3). IT administration of WJ-MSCs was performed through a lumbar puncture as described by previous studies[9]. IM administration of WJ-MSCs was performed under the guidance of ultrasound for each muscle, while IV infusion was slowly administered in 30 min. Following the completion of the procedure, patients were transferred to intensive care unit (1st level). A day later, patients were transferred to Neurosurgery Department for follow-up and initiated on physical therapy and rehabilitation. Patients did not perform exercises during the days of stem cell adminis-

Table 3 Administration schedule

Rounds	Route	WJ-MSC
Round 1	IT	1×10^6/kg in 3 mL
	IV	1×10^6/kg in 30 mL
	IM	1×10^6/kg in 20 mL
Round 2 (2nd week)	IT	1×10^6/kg in 3 mL
	IV	1×10^6/kg in 30 mL
	IM	1×10^6/kg in 20 mL
Round 3 (4th week)	IT	1×10^6/kg in 3 mL
	IV	1×10^6/kg in 30 mL
	IM	1×10^6/kg in 20 mL
Round 4 (6th week)	IT	1×10^6/kg in 3 mL
	IV	1×10^6/kg in 30 mL
	IM	1×10^6/kg in 20 mL

WJ-MSC: Wharton's jelly-derived mesenchymal stem cells; IT: Intrathecal; IV: Intravenosus; IM: Intramuscular.

tration. The same protocol was followed before and after every administration.

Pretreatment neurological examination

Patients were evaluated before treatment with a comprehensive examination by a physician team consisting of medical and rehabilitation doctors. Each step of the neurological and functional evaluation was documented in detail. Patients were evaluated for spasticity with the Modified Ashworth (MA) scale and for quality of life with the Wee Functional Independence Measure (Wee FIM) scale based on the statements of their parents[10].

Safety evaluation criteria

The safety criteria for the procedure were determined as follows: Any evidence of infection, headache, fever, pain, allergic reactions or shock, leukocytosis, an elevated level of C-reactive protein, and perioperative complications (wound site infections, analgesia, and anesthesia-related complications) during 7-14 d post-treatment. The safety criteria for WJ-MSC administration were determined as follows: Any evidence of infection, development of cancer, neuropathic pain, and worsening neurological status during the 1-year follow-up[6,8].

Follow-up assessment of treatment success

For follow-up assessment of treatment success, patients were evaluated neurologically and functionally in detail. They were evaluated for spasticity with the MA Scale and for quality of life with the Wee FIM Scale[11,12]. Moreover, patients were also evaluated for secondary infections, neuropathic pain, urinary tract infections, or decubitus ulcers.

Statistical analysis

The non-parametric tests of Friedman Test and Wilcoxon Signed Ranks Test were employed to measure the change in the pre-treatment and post-treatment Wee FIM and MA Scale scores of patients. As the number of data was not sufficient for parametric tests, nonparametric tests were carried out.

RESULTS

Safety of procedure and adverse events

The procedure was well tolerated by patients with no severe adverse events associated with the procedure. Three patients developed only early complications such as low-grade fever, mild headache associated with IT injection, and muscle pain associated

with IM injection, all of which were transient and disappeared within 24 h (Table 4). No other adverse events or safety issues were reported during the 1-year follow-up period.

Wee FIM scale score

Despite a slight increase in the post-treatment 4-mo and 12-mo Wee FIM Motor scores of patients, the increase in their cognitive scores continued throughout the post-treatment follow-up period.

The analysis as shown in Table 5 revealed that participants had a statistically significant difference in their pre-treatment and post-treatment Jee FIM Motor scores ($\chi^2 = 23.444$, $P < 0.001$). The differences between binary measurements were determined by the Wilcoxon signed-rank test. As a result of the analysis, there was no significant difference between pretest and one-week posttest scores (z = 0.000, $P > 0.05$); between one-week posttest and one-month posttest scores (z = 0.000, $P > 0.05$); between one-month posttest and two-month posttest scores (z = -1.414, $P > 0.05$); between two-month posttest and four-month posttest scores (z = -2.070, $P < 0.05$); and between the four-month posttest and 12-mo posttest scores (z = -1.633, $P > 0.05$). On the other hand, when the pretest score and subsequent measurements were compared, no significant difference was observed between the pretest score and one-week posttest (z = 0.000, $P > 0.05$), one-month posttest (z = 0.000, $P > 0.05$), two-month posttest (z = -1.414, $P > 0.05$) 12-mo posttest (z = -2.041, $P < 0.05$) scores whereas there was a significant difference between the pretest score and four-month posttest (z = -2.236, $P < 0.05$) scores. In other words, while there was no difference in the Wee FIM Motor scores of the patients until the 4th postoperative month, a significant increase was observed in the 4th month (Figure 1 and Table 5).

The analysis as shown in Table 6 revealed that participants had a statistically significant difference in their pre-treatment and post-treatment Wee FIM cognitive scores ($\chi^2 = 28.255$, $P < 0.001$). The differences between binary measurements were determined by the Wilcoxon signed-rank test. As a result of the analysis, no significant difference was observed between pretest and one-week posttest scores (z = -1.000, $P > 0.05$); between one-week posttest and one-month posttest scores (z = -1.841, $P > 0.05$); between two-month posttest and four-month posttest scores (z = -2.041, $P < 0.05$); and between the four-month posttest and 12-mo posttest scores (z = -2.264, $P < 0.05$), whereas the difference between the one-month posttest and two-month posttest scores was significant (z = -2.070, $P < 0.05$). On the other hand, when the pretest score and subsequent measurements were compared, no significant difference was observed between the pretest score and one-week posttest (z = -1.000, $P > 0.05$), one-month posttest (z = -1.826, $P > 0.05$) scores, whereas there was a significant difference between the pretest score and two-month (z = -2.023, $P < 0.05$), four-month (z = -2.207, $P < 0.05$) and 12-mo posttest (z = -2.201, $P < 0.05$) posttest scores. While there was no difference in the Wee FIM Cognitive scores of the patients until the second postoperative month, a significant increase was observed in the second month (Figure 1 and Table 6).

MA scale score

Patients had a continuous decrease in their MA scale right and left scores throughout the follow-up period, which was indicative of improvement.

The analysis as shown in Table 7 revealed that patients had a statistically significant difference in their pre-treatment and post-treatment macrophage activation syndrome (MAS) right scores ($\chi^2 = 29.439$, $P < 0.001$). The differences between binary measurements were determined by the Wilcoxon signed-rank test. As a result of the analysis, no significant difference was observed between pretest and one-week posttest scores (z = -1.841, $P > 0.05$) and between two-month posttest and four-month posttest scores (z = -1.857, $P > 0.05$), whereas there were significant differences between the one-week posttest and one-month posttest scores (z = -2.214, $P < 0.05$); between the one-month posttest and two-month posttest scores (z = - 2.041, $P < 0.05$); and between four-month posttest and 12-mo posttest scores (z = -2.264, $P < 0.05$). In other words, while there was no significant difference in patients' MAS Right values in the first post-treatment week, the values started to decrease significantly in parallel with the recovery after the first week (Figure 2 and Table 7).

The analysis as shown in Table 8 revealed that patients had a statistically significant difference in their pre-treatment and post-treatment MAS left scores (z = 29.000, $P < 0.001$). Wilcoxon Signed Ranks Test was performed between the binary measurements to identify the differences between variables. As a result of the analysis, no significant difference was observed between pretest and one-week posttest scores (z = -1.342, $P > 0.05$) and between two-month posttest and four-month posttest scores (z = -1.841, $P >$

Table 4 Early and late complications of the procedures

Complications		Patient No. 1 Administration				Patient No. 2 Administration				Patient No. 3 Administration				Patient No. 4 Administration				Patient No. 5 Administration				Patient No. 6 Administration			
		1st	2nd	3rd	4th	1st	2nd	3rd	4th	1st	2nd	3rd	4th	1st	2nd	3rd	4th	1st	2nd	3rd	4th	1st	2nd	3rd	4th
Early	Infection	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Fever	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	+	+	-	-	-	-	-	-
	Pain	-	-	-	-	-	-	-	-	+	-	+	-	+	+	-	-	+	-	-	-	-	-	-	-
	Headache	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
	Increased level of C-reactive protein	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Leukocytosis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Allergic reaction or shock	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Perioperative complications	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Late	Secondary infections	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Urinary tract infections	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Deterioration of neurological status	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Neuropathic pain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Carcinogenesis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

–: Not present; +: Present.

0.05), whereas there were significant differences between the one-week posttest and one-month posttest scores (z = -2.041, $P < 0.05$); between the one-month posttest and two-month posttest scores (z = - 2.032, $P < 0.05$); and between four-month posttest and 12-mo posttest scores (z = -2.264, $P < 0.05$). In other words, while there was no significant difference in patients' MAS Left values in the first post-treatment week, the values started to decrease significantly in parallel with the recovery after the first week (Figure 2 and Table 8).

DISCUSSION

HIE is one of the leading causes of death and long-term neurological impairment in the pediatric population. Today, there is a limited number of treatment options for

Table 5 Friedman test results regarding the change in the Wee Functional Independence Measure Motor scores of the patients before and after the operation

	n	Mean	SD	Mean rank	χ^2	df	*P* value
Pre-test	6	8.00	0.00	2.50	23.444	5	0.000
Post-test 1 wk	6	8.00	0.00	2.50			
Post-test 1 mo	6	8.00	0.00	2.50			
Post-test 2 mo	6	8.33	0.52	3.17			
Post-test 4 mo	6	9.67	0.82	4.92			
Post-test 12 mo	6	10.50	1.52	5.42			

SD: Standard deviation.

Table 6 Friedman test results regarding the change in the Wee Functional Independence Measure cognitive scores of the patients before and after the operation

	n	Mean	SD	Mean rank	χ^2	df	*P* value
Pre-test	6	5.50	0.55	1.67	28.255	5	0.000
Post-test 1 wk	6	5.67	0.82	1.83			
Post-test 1 mo	6	7.17	1.60	2.75			
Post-test 2 mo	6	8.33	2.25	3.83			
Post-test 4 mo	6	10.17	3.06	4.92			
Post-test 12 mo	6	13.00	2.83	6.00			

SD: Standard deviation.

Table 7 Friedman test results regarding the change in the macrophage activation syndrome right scores of the patients before and after the operation

	n	Mean	SD	Mean rank	χ^2	df	*P* value
Pre-test	6	18.67	4.72	5.83	29.439	5	0.000
Post-test 1 wk	6	16.83	6.15	5.17			
Post-test 1 mo	6	14.00	7.62	3.92			
Post-test 2 mo	6	12.17	6.94	2.92			
Post-test 4 mo	6	10.17	6.65	2.17			
Post-test 12 mo	6	7.67	6.12	1.00			

SD: Standard deviation.

HIE, *e.g.*, cooling. Different therapeutic approaches have been used to treat and improve functional and neurological outcomes of HIE patients. Among these approaches, MSCs come to the fore as a potential treatment option for ischemic brain injury caused by HIE[13]. MSCs are the most frequently used regenerative cells in clinical trials since they have a relatively safe profile, ease of isolation, and ability to reduce cell apoptosis, ameliorate oxidative stress and inflammation, and recover energy failure[14]. Results of a meta-analysis of preclinical studies on HIE showed the potential of treatment with mesenchymal stromal cells for improving neurological functions[15].

Furthermore, although regenerative cells are characterized by a low level of immunogenicity, autologous transplantation probably appears to be linked with a lower risk of immune rejection and infection development[16]. Allogeneic stem cell

Table 8 Friedman test results regarding the change in the macrophage activation syndrome left scores of the patients before and after the operation

	n	Mean	SD	Mean rank	χ^2	df	*P* value
Pre-test	6	16.33	5.13	5.58	29.000	5	0.000
Post-test 1 wk	6	15.67	5.89	5.25			
Post-test 1 mo	6	13.17	7.33	4.08			
Post-test 2 mo	6	11.33	6.31	2.92			
Post-test 4 mo	6	9.17	5.74	2.17			
Post-test 12 mo	6	7.00	5.33	1.00			

SD: Standard deviation.

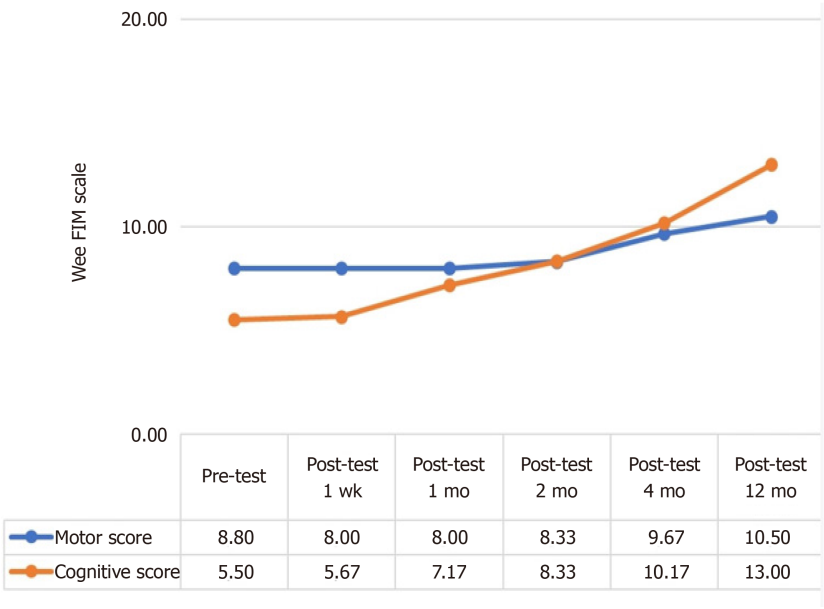
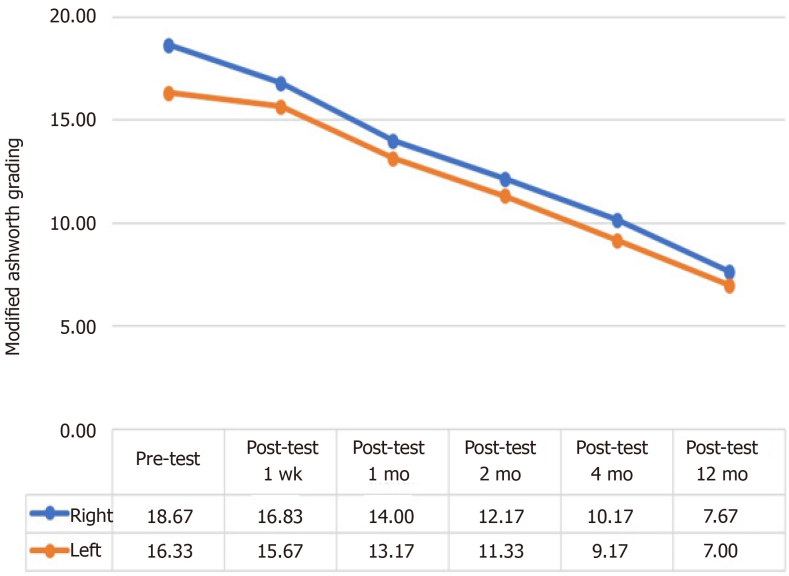


Figure 1 Change in the mean pretest and posttest the Wee Functional Independence Measure Motor and cognitive scores of the patients.
Wee FIM: Wee Functional Independence Measure.

transplantation might provide significant advantages in terms of practicability[17]. In recent years, the UC comes to the fore as the most commonly used tissue to harvest regenerative[18,19]. In preclinical studies, UC-MSCs administration has been suggested to enhance axonal regeneration and nerve fiber remyelination and promote sensorimotor functions with better long-term neurological outcomes[20-23]. Stem cell transplantation for ischaemic stroke, a Cochrane review, evaluated three small trials conducted on adults[24]. There is one Cochrane review on MSC-based therapies for the prevention and treatment of bronchopulmonary dysplasia in preterm infants[25]. There are also early phase trials on the use of cord blood cells and MSCs or (or the combination of both) for severe intraventricular hemorrhage (NCT02274428), bronchopulmonary dysplasia, and HIE[26,27].

Despite the promising potential of stem cells and progenitor cells for HIE in experimental and clinical pilot studies, cell therapy in humans still remains in the initial stage[28]. The study by Miao *et al*[9] reported that 47 patients (47%), including 12 patients with spinal cord injury, 11 patients with cerebral palsy, 9 patients with post-brain infarction syndrome, 9 patients with post-traumatic brain syndrome, 3 patients with motor neuron disease, and 3 patients with spinocerebellar ataxia had an improvement in their functional indices a year after the intrathecal administration of UC-MSCs[9]. Preclinical studies have shown substantial favorable effects of intraarterial, intracisternal, intratracheal, intravenous, or intracerebral administrations of allogeneic WJ-MSCs[29]. The present study demonstrated that both triple-route and multiple administrations of allogeneic WJ-MSCs were safe and improved the



	Pre-test	Post-test 1 wk	Post-test 1 mo	Post-test 2 mo	Post-test 4 mo	Post-test 12 mo
Right	18.67	16.83	14.00	12.17	10.17	7.67
Left	16.33	15.67	13.17	11.33	9.17	7.00

Figure 2 Change in the mean pretest and posttest macrophage activation syndrome right and left scores of the patients.

functional status of patients.

The current study is the largest trial of both triple route and multiple implantations of allogeneic WJ-MSC in pediatric patients with HIE and the first to evaluate allogeneic WJ-MSC therapy in this population regardless of our previous pilot study [6]. With a dose of 1 × 10^6/kg for each route, patients developed mild adverse reactions, all of which were transient and disappeared with 24 h. Three patients developed only early complications such as low-grade fever, mild headache associated with IT injection, and muscle pain associated with IM injection, all of which were transient and disappeared within 24 h.

Patients with chronic HIE usually exhibit functional deterioration but pediatric patients included in this study exhibited a constant functional improvement through the 12-mo follow-up. HIE-related impairments usually show a bimodal recovery pattern. The majority of HIE survivors first exhibit a little spontaneous recovery, for instance, improvement in the motor system during the first months. However, they experience a significant deterioration in functional status a year after the onset of HIE. This is of note as there is a limited number of treatment options for patients with chronic HIE.

In the current study, functional gains were seen, though were modest in magnitude. Although moderate, patients included in the present study showed functional improvements. Despite that, a 2.5-point increase in the Wee FIM Scale motor scores of chronic HIE patients are of great value (Table 5). Although patients had a low increase in their Wee FIM motor scores, they achieved an increase of 7.5 points in their post-treatment cognitive scores (Table 6). This continuous improvement through the 12-mo follow-up indicates the broad effects of MSC on brain functions. There is a need for larger, placebo-controlled studies to verify these results; however, these results also support that this treatment can be used as a promising approach to improve the functions of patients with chronic HIE. Treatment-specific outcome measures may be the subject of future studies to obtain more detailed estimates of behavioral improvements in the neural systems of patients. In the present study, the percentage of patients who achieved an excellent functional improvement (Wee FIM scale total score = 126) increased from 10.71% (at baseline) to 12.03% at 1 mo and to 18.65% at 12 mo post-treatment (Tables 5 and 6). Preclinical studies conducted with animal models of HIE have shown a significant improvement in the functions of treatment groups with MSCs. The potential of MSC therapies to treat neurologic conditions is associated with their ability to restore energy failure, inhibit the inflammatory response, and enhance neurogenesis as well as angiogenesis in the hypoxic brain area. Our study is in line with preclinical studies on HIE in terms of continuous improvement in functions through the 12-mo follow-up[15].

The present study has several strengths. The study population consisted of chronic HIE patients with substantial functional impairments, who have a limited number of treatment options. However, optimization of regenerative cells requires considering

several factors. Some examples of these factors are the source to derive stem cells, processing of cells, number of passage, frequency, dose, timing, and administration route, all of which have an effect on treatment efficacy[30]. Moreover, various laboratory processing techniques, including cell expansion medium, oxygen tension, number of passage, the use of cryopreserved or fresh cells, also have an effect on the therapeutic potential of regenerative cells[31-33]. Since the use of multiple passages may damage cellular functions, fewer passages should be preferred as much as possible[34,35]. In the present study, the cells used in infusion were allogeneic so the requirement for immunosuppressive therapy was excluded when compared with autologous cell therapies. This comparatively immune-privileged feature of MSC makes it possible to use this approach in a large pediatric population with HIE. The safety of the treatment was evaluated by including both triple-route and multiple administrations of WJ-MSC in the study protocol. The limit of cell culture was set at 3 passages, which provided an important advantage considering that the use of multiple passages may damage cellular functions of MSCs such as differentiation, proliferation, viability, and homing. The safety evaluation also included comprehensive laboratory tests through a 1-year follow-up.

The study also has several limitations, which are not including a control group to compare behavioral gains since the study was designed as a safety study, and not studying the mechanism of action. Stem cell therapies to improve outcomes of patients with chronic HIE are likely to act through multiple mechanisms, including the release of growth factors and anti-inflammatory effects, and probably exosomes. Future studies can focus on this. Patients with HIE can benefit from restorative therapies to a maximum extent when they combine the treatment with training, which was not given to patients included in this study. The present study showed that both triple-route and multiple administrations of WJ-MSC were safe in the pediatric HIE population suffering from substantial functional impairments. The results of this study also demonstrate the functional benefit of WJ-MSC therapy, which should be verified in controlled studies. Collectively, the results of this study support future studies to evaluate both triple-route and multiple administrations of WJ-MSC in pediatric HIE with its mechanism of action.

CONCLUSION

Stem cell therapies appear to be a potential treatment option for brain injury resulting from HIE. In recent years, stem cell therapies, especially WJ-MSCs therapy, have led to the development of novel treatment protocols for ischemic brain injury. However, many unanswered questions on stem cell therapies still remain. There is a need for much effort to devote to thoroughly elucidating how stem cell therapy works, what paracrine mediators are important, when and what type of therapy should be used, and which patients are eligible candidates for this treatment. Therefore, there is a need for further preclinical studies to optimize the treatment protocol as well as multicenter clinical trials to confirm safety and efficacy.

ARTICLE HIGHLIGHTS

Research background

Hypoxic-ischemic encephalopathy (HIE) is one of the leading causes of death and long-term neurological impairment in the pediatric population.

Research motivation

Despite a limited number of treatments to cure HIE, stem cell therapies appear to be a potential treatment option for brain injury resulting from HIE.

Research objectives

The present study investigated the efficacy and safety of stem cell-based therapies in pediatric patients with HIE.

Research methods

Wharton's jelly-derived mesenchymal stem cells (WJ-MSCs) were intrathecally, intramuscularly, and intravenously administered to participants at a dose of 1×10^6

/kg for each administration route twice monthly for 2 mo. In different follow-up durations, the effect of WJ-MSCs administration on HIE as well as the quality of life and prognosis of patients was investigated, and patients were evaluated for neurological, cognitive functions, and spasticity using the Wee Functional Independence Measure Scale and Modified Ashworth Scale to determine the associated adverse reactions.

Research results

Three patients developed only early complications which were transient and disappeared within 24 h. The treatment was evaluated to be safe and effective as demonstrated by magnetic resonance imaging examinations, electroencephalographies, laboratory tests, and neurological and functional scores of patients. Patients exhibited significant improvements in all neurological functions during the 12-mo follow-up period.

Research conclusions

Multiple triple-route WJ-MSC administrations were found to be safe for pediatric HIE patients, indicating neurological and functional improvement.

Research perspectives

More comprehensive randomized and placebo-controlled studies can be conducted to further support the results of this study.

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