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Human stem cell research and regenerative medicine—present and future

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Introduction: Stem cells are cells with the ability to grow and differentiate into more than 200 cell types.

Sources of data: We review here the characteristics and potential of human embryonic stem cells (hESCs), induced pluripotent stem cells (iPSCs) and adult stem cells (ASCs).

Areas of agreement: The differentiation ability of all stem cell types could be stimulated to obtain specialized cells that represent renewable sources of functional cells useful for cell-based therapy.

Areas of controversy: The proof of functional differentiated cells needs to be investigated in more detail using both *in vitro* and *in vivo* assays including animal disease models and clinical studies.

Growing points: Much progress has been made in the ASCs-based therapies. Meanwhile hESCs and iPSCs have dramatically emerged as novel approaches to understand pathogenesis of different diseases.

Areas timely for developing research: A number of new strategies become very important in regenerative medicine. However, we discuss the limitations of stem cells and latest development in the reprogramming research.

Keywords: stem cells/pluripotency/reprogramming/cell therapy/regenerative medicine

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Introduction

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Stem cells have the capacity to divide and give rise to identical daughter stem cells (symmetrical division) or to differentiate into specific cells of somatic tissues (asymmetrical division).[1] Commonly, stem cells are derived from two main sources: (i) early embryos (embryonic stem cells, ESCs) and (ii) adult tissue (adult stem cells, ASCs). Thomson

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et al.[2] proposed that the essential characteristics of ESCs should include the following: (i) derivation from the pre-implantation embryo, (ii) prolonged proliferation in their pluripotent state and (iii) stable developmental potential to form derivatives of all three embryonic germ layers (Fig. 1). The main advantages of ESCs, compared with ASCs, are their ability to proliferate for long period under *in vitro* conditions and their potential for differentiation into a broad range of cell types.[3]

Among ASCs, the haematopoietic stem cells (HSCs) were first discovered in the 1960s, followed by the discovery of stromal stem cells (also called mesenchymal stem cells (MSCs) (for review see ref. 4). Since then stem cells have been found in many tissues and organs including epidermis, liver and bone.[4] The main role of ASCs is believed to be replacement of damaged and injured tissue. Due to their immunomodulatory ability and capacity for self-renewal and differentiation into tissues of mesodermal origin, MSCs are ASCs that are most often used in clinical studies as possible new therapeutic agents for the treatment of autoimmune or degenerative diseases. MSCs are multipotent, self-renewable cells that can be found in almost all postnatal organs and tissues.[5,6] MSCs are most frequently isolated from bone marrow (BM), but the source tissue for isolation of MSCs can also be every tissue in which MSCs are present including adipose tissue, umbilical cord blood and compact bone.[6] MSCs show variable levels of expression of several

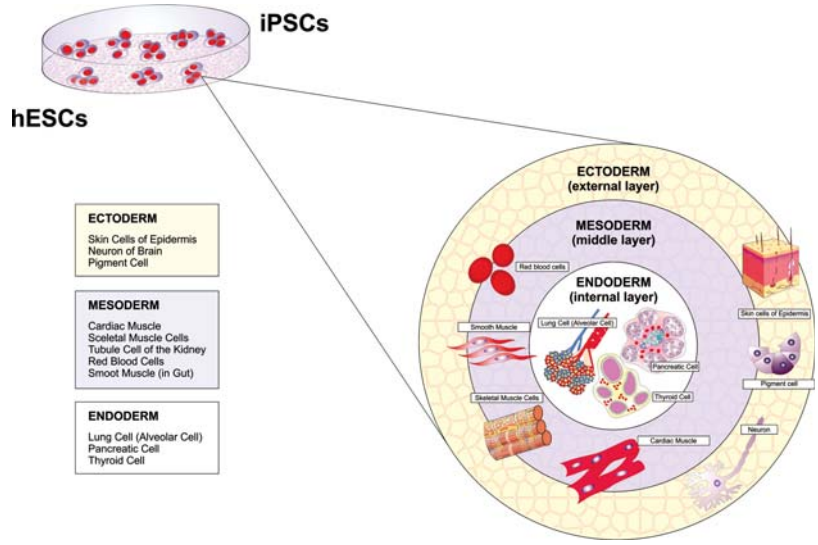

Fig. 1 Differentiation potential of hESCs and iPSCs. Both stem cell types are pluripotent and have potential to grow under *in vitro* conditions for unlimited time and differentiate into any cell type of human body. iPSCs are patient specific and frequently used to study reprogramming mechanisms or pathogenesis of inherited diseases.

molecules: CD105 (SH2), CD73 (SH3/4), stromal antigen 1, CD44, CD166 (vascular cell adhesion molecule), CD54/CD102 (intracellular adhesion molecule) and CD49 (very late antigen) and lack the expression of surface markers characteristic for HSCs (CD14, CD34, CD45 and CD11a/LFA-1), erythrocytes (glycophorin A) and platelet and endothelial cell (CD31).[6] Non-embryonic, or "adult" stem cells have been identified in many organs and tissues.[5] Prevailing models assume the existence of a single quiescent population of stem cells residing in a specialized niche of a given tissue. These tissue-specific stem cells have been identified in the BM, brain, skin, skeletal muscle and many other tissues.[5] Depending on the origin, these ASCs exhibit the potency to differentiate into multiple or specific cell types[5] (Fig. 2).

Stem cells can also be derived from extra-embryonic tissues (amnion, chorion, placenta, umbilical cord; for review see 7. Amnion and

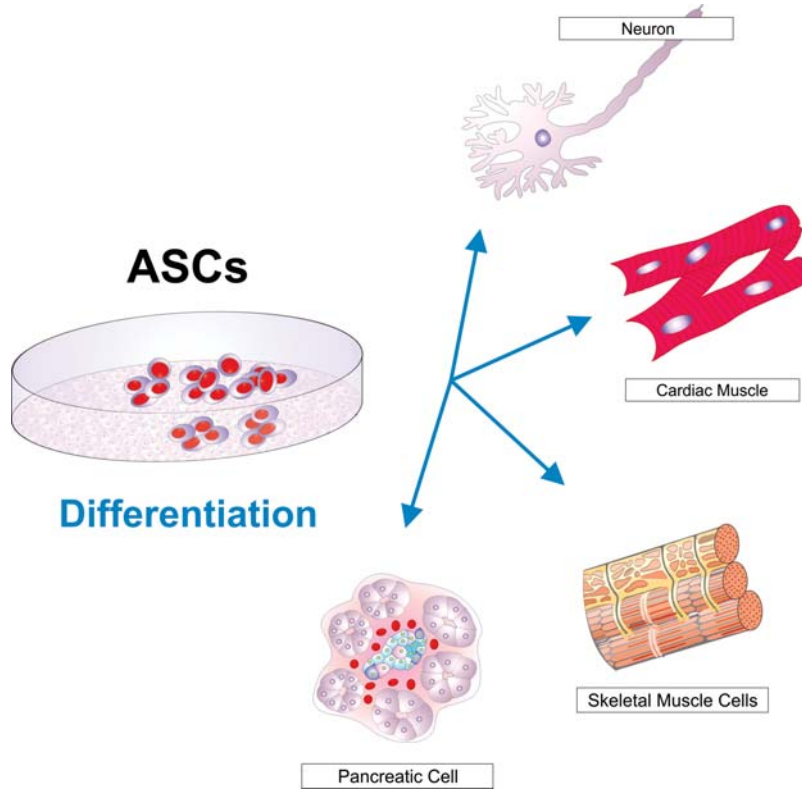

Fig. 2 Differentiation potential of ASCs. ASCs are multipotent and have limited differentiation potential. Using new technologies and methods (identification of growth factors or reprogramming) ASCs could be grown and manipulated in a plastic dish to keep them multipotent or obtain various mature and functional cell types for the treatment of debilitating human diseases.

chorion contain stromal cells that display characteristics and differentiation potential similar to BM-derived MSC are able to differentiate into adipocytes, endothelial cells, hepatocytes, osteocytes, myocytes and neurons.[7] After transplantation into ischaemic mouse brain, they survived and differentiated into neural lineages, predominantly astrocytes. Placental-derived stem cells have the capacity to differentiate into ectodermal, mesodermal and endodermal cell types, while umbilical cord matrix stem cells (UCMSCs) have similar capacity like stromal cells. After transplantation, UCMSCs enhanced muscle regeneration in mouse model of severe muscle damage and promoted blood vessel formation and neurological function in animal models of ischaemic brain disease.[7] The advantage of stem cells derived from extra-embryonic tissues is the efficient isolation from tissues normally discarded at birth avoiding ethical concerns that plague the isolation of human embryonic stem cells (hESCs).

A scientific breakthrough achieved by Yamanaka in 2006 showed that it was possible to reprogramme somatic cells back to the pluripotency stage by transducing a few key transcription factors.[8] These cells are named induced pluripotent stem cells (iPSCs) and exhibit morphology of ESCs, express ESC markers, have normal karyotype, express telomerase activity and maintain the developmental potential to differentiate into derivatives of all three primary germ layers (Fig. 1).

Although stem cells can be derived from different tissues, BM and umbilical cord derived stem cells are the only currently available sources for stem cell therapy (SCT).

Possible application of stem cell therapy

Stem cells could be stimulated *in vivo* or *in vitro* to develop various numbers of specialized cells that represent sources of cells useful for transplantation in cell-based therapy of genetic and degenerative diseases (Table 1).

SCT for the treatment of diabetes mellitus

The potential of ESCs, iPSCs and eventually MSCs to differentiate into insulin-producing beta cells could be the new and effective therapeutic approach for the treatment of diabetes mellitus type 1.[9] Although much work remains to be done, considerable progress has been achieved in defining conditions necessary for beta cell differentiation from ESCs and iPSCs.[9] Differentiated cells are characterized

Table 1 Therapeutic potential of stem cells

Tissue type	Cell lineage	Disease application
Endocrine	Islet beta cells	Diabetes
Blood	Lymphoid cells	Immune deficiencies
	Erythrocytes	Sickle cell anaemia
Liver	Hepatocyte	Liver failure
Renal	Podocytes and tubular cells	Acute and chronic renal failure
Neural	Oligodendrocyte	Spinal cord injury
	Dopamine neuron	Parkinson's disease
	Motor neuron	Motor neuron disease
Musculoskeletal	Skeletal muscle	Muscular dystrophy
Eye	Retinal epithelium	Retinal degeneration or injury
Cardiovascular	Cardiomyocyte	Myocardial infarction
	Endothelial	Stroke
		Ischaemic limb disease

Different tissue and cell lineage types could be obtained from undifferentiated stem cells, progenitor cells or somatic cells using reprogramming technology.

by the expression of the beta-cell transcription factors *PDX1*, *NKX6-1* and *MAFA*.[9] However, differentiated cells have an impaired ability to respond to glucose stimulation *in vitro*.[9] It seems that complete differentiation of insulin-producing beta cells must occur *in vivo*.[10] One to three weeks after engraftment into a mouse, the cells acquire the ability to respond to glucose and begin to secrete human insulin and C-peptide in response to high glucose.

In vivo hyperglycaemia is an important factor for BM-derived MSCs differentiation into insulin-producing beta cells capable of normalizing hyperglycaemia in a diabetic animal model.[6,11] Due to their differentiation ability, capacity for self-renewal and capability for regulation of immune response, MSCs are, in experimental models, used in the therapy of diabetes mellitus type 1 in experimental models.[10] This disease is immune mediated and MSCs could be used as new therapeutic agents for the treatment particularly due to their immunomodulatory ability.[6] MSCs are able to alter immune response through cell-to-cell contact or through producing soluble factors[6] and altering dendritic cells secretion profile, which results in increased production of anti-inflammatory cytokine IL-10 and decreased production of inflammatory cytokines IFN-γ and IL-12. MSCs inhibit T cell proliferation by engagement of the inhibitory molecule programmed death 1 (PD-1) to its ligands PD-L1 and PD-L2 and by producing anti-inflammatory cytokines TGF beta and IL-10 increasing at the same time immunosuppressive T regulatory cells and inhibiting proliferation and IgG secretion of B-cells.[6]

SCT for the treatment of chronic myeloid and chronic lymphocytic leukemia

Chronic myeloid leukaemia (CML) and HSCs transplantation (HSCT) have been closely linked together with success and failure over the past 50 years. Bone marrow ablation by high-dose chemo/radiotherapy and substitution by healthy donor BM cells with immunological control of malignant disease have been the basic principles of HSCT during past decades. The frequency of HSCT declined rapidly when the specific BCR/ABL tyrosine kinase inhibitor (TKI) imatinib appeared. Today, a balanced view prevails. Risk assessment of both, disease risk and transplant risk (graft-versus-host disease, GVHD), has become standard.[12] Allogeneic HSCT remains the first-line approach for patients with CML in accelerated phase or blast crisis and it is the standard of care for patients with failed first-line therapy and a low-risk HSCT. It is the best option for all patients with failed second-line TKIs, with mutations T315I or with progressive disease. It can always be considered in situations with limited resources.[12] Autologous stem cell transplantation (auto-HSCT) could be a novel therapeutic approach that should minimize the risk of GVHD after stem cell transplantation. The long-term follow-up of patients with CML having received auto-HSCT show that this therapy is very efficient to debulk the disease, restore Ph-negative haematopoiesis and is able to sustain molecular responses in the majority of patients resulting with long-term survival rates.[13]

SCT for the treatment of liver disease

Numerous studies have concentrated on investigating the ability of a variety of stem cells that can be readily isolated from the patient with liver failure to give rise to personalized, immunologically matched hepatocytes or other functional hepatic elements (sinusoidal endothelial cells, stromal cells, etc.).[14] Depending on the source of cells and aetiology of disease, the outcome will differ significantly in terms of numbers and how hepatocytes are being generated. Umbilical cord blood HSCs more consistently generate higher levels of hepatocytes following transplantation than HSCs isolated from BM or from peripheral blood. However, HSCs with the highest hepatocytic potential are adult BM-derived $CD34^{+}Lin^{-}CD38^{-}$ cells.[15]

MSCs appear to be able to exert beneficial effects in a wide range of liver injuries and liver diseases.[14] The transplantation of MSCs stimulates the host's liver to repair itself without the donor cells actually having to persist long term within the recipient.[14] Several studies clearly demonstrated that secretion of factors that stimulate regeneration of endogenous parenchymal cells were likely to play important

roles in promoting tissue recovery.[16–18] These findings led to the question of whether MSCs can actually generate hepatocytes or if all the effects they produce are simply mediated through their paracrine effects, through release of soluble factors. Because MSCs are quite amenable to genetic modification, they could be harvested from the patient's own marrow, even if the liver disease is the result of an underlying genetic defect.[14] Autologous MSCs could be genetically corrected, propagated *in vitro*, differentiated into hepatocytes thus generating sufficient numbers of cells to achieve meaningful levels of engraftment and then transplanted into the patient.[14]

Clinical use of BM-derived cells for liver repair or regeneration is still in its infancy. The results of the study which observed the effects of the infusion of autologous BM-derived $CD133^+$ cells in patients who were undergoing partial hepatectomy for liver cancer were promising.[14] Patients that received the infusion of BM cells (which likely contained both HSCs and endothelial progenitor cells (EPCs) exhibited 2.5-fold higher mean proliferation rates when compared with a group of three consecutive patients who did not receive BM cells.[19] Infusion of autologous $CD34^+$ cells via either the portal vein or the hepatic artery was also shown to transiently improve serum bilirubin and albumin for more than 60 days in five patients suffering from cirrhosis.[20] However, the infusion of $CD34^+$ cells via the hepatic artery resulted in hepatorenal syndrome and death of one patient, with the remaining three patients showing no evidence of significant clinical improvement.[20] In contrast, infusion of BM-derived MSCs via a peripheral vein were found to be well tolerated and have a definite therapeutic effect for two of the four patients in the trial.[21] In other study, 24 week follow-up of patients with cirrhosis that were treated by portal vein infusion of autologous whole, unselected BM cells, revealed some improvement in Child-Pugh score, albumin and showed biopsy evidence of an increase in hepatocyte turnover.[14]

All results obtained in these clinical studies must be interpreted with caution because of limited number of patients enrolled in each trial and the lack of appropriate controls. Furthermore, the precise mechanism of observed clinical improvement is still unknown because autologous cells were used in these trials and there was no way for the investigators to assess the actual engraftment, persistence or differentiation potential of these transplanted cells.

SCT for the treatment of neural diseases

Stem cells can also be used for the generation of neural tissues and this raises new possibilities for the therapy of neurodegenerative disorders

including Alzheimer's disease, Huntington's disease, Parkinson's disease or spinal cord injury (SCI, 22). The first clinical application of hESCs in the treatment of central nervous system disorders has already started and consists of transplantation of newly generated oligodendrocytes for the treatment of SCI.[22–25] Our group and others have developed efficient protocols for production of relatively pure isolation of oligodendrocytes via targeted differentiation of hESCs or ependymal stem cells.[23,25–27] Similar strategy was described using neural stem cells (NSCs). These cells can be extracted directly from foetal or adult nervous tissue via the dissection and digestion of brain regions of interest. When allowed to differentiate spontaneously after removal of growth factors or mitogens in serum-free media, NSCs are able to generate oligodendrocytes, neurons and astrocytes in an approximate ratio of 1:5:25.[22] It is very encouraging that NSCs have already entered the clinical studies.[22] Stem Cells Inc. (Palo Alto, CA) completed the first FDA-approved clinical trial in patients with neuronal ceroid lipofuscinosis (NCL) in January 2009. The open-labelled, dose-escalating phase I study that enrolled six patients in the advanced stages of infantile or late infantile NCL showed that transplanted NSCs had long-term survival, efficiently protected neurons improving clinical picture of all treated patients.[22]

Here, we would like to underline that there are many optimistic studies[22,28–30] which report that MSCs could be transdifferentiated or converted into NSCs or neurons. However, the proof of functional neurons derived from MSCs has not been provided yet. Besides the opened question about transdifferentiation, it is possible that the main role of MSCs is to provide trophic support to damaged neurons, thus resulting in clinical improvement in patients with diabetic polyneuropathy and Parkinson's disease.[30]

Stem cells and cardiac regenerative therapy

The morbidity and mortality associated with cardiac diseases are mostly secondary to permanent loss of myocardial tissue, thus making SCT a potentially crucial treatment for repair of cardiac function.[31] Embryonic stem cells, MSCs, as well as resident cardiac progenitor cells, skeletal myoblasts and EPCs used in cardiac regenerative therapy are capable of either regenerating into viable cardiac tissue or of supporting the process of regeneration.[31–33]

When MSCs are exposed to the DNA demethylating agent (5-asacytidine), they express specific cardiac genes, adopt myotube morphology, produce intercalated disks and have other functions associated with cardiac myocytes.[32] Although MSCs have the potential

to become mature beating cardiac myocytes, their role in clinical cardiac regenerative therapy is still uncertain. While it remains unclear whether or not these cells can integrate into the electromechanical system of myocytes *in vivo*, it is likely that they facilitate myocyte regeneration through a paracrine cytoprotective influence, through myocardial remodelling, reduction of infarct size scar formation and stimulation of angiogenesis.[33] Several mediators including hypoxia inducible factor-1 alpha, hepatocyte growth factor-1, MSC factor, vascular growth factor and insulin-like growth factor-1, have been postulated as potential factors associated with the MSC paracrine effects on cardiac remodelling after intramyocardial, intracardiac, intravenous or intracoronary MSC transplantation.[30,31]

Several studies have already examined the possible applications of stem cells in cardiac regeneration therapy.[34,35] In a randomized, double-blind, placebo-controlled, multicentre trial, autologous mononuclear BM-derived progenitor cells intracoronary infused early after myocardial infarction improved global left ventricular function and prevented ventricular end-systolic expansion 4 months after the event.[34] In another study,[35] intramyocardial injection of autologous BM-derived mononuclear cells in patients with chronic ischaemia resistant to medications resulted in an improvement in myocardial perfusion. However, the main limitation for MSCs therapy of cardiac diseases are possible side effects that are noted in preclinical studies such as creation of encapsulated intramyocardial ossifications and calcifications, the appearance of arrhythmias, sarcomas and teratomas.[36] Therefore, additional trials evaluating the benefit and side effects of MSC infusion, either directly into the heart or intravenously, in a variety of cardiac diseases are anticipated.

The future: iPSCs

Stem cells are able to proliferate and differentiate into various cell types, create mixtures of cells representing tissues created under *in vitro* conditions. Disease-specific hESCs and iPSCs are now available and are used to study pathogenesis of inherited and sporadic disorders. For instance, disease-specific hESC lines have been derived to study Duchenne and Becker muscular dystrophies, Huntington disease, fragile-X syndrome, adrenoleukodystrophy and neurofibromat osis-1.[4,8,22] Moving from cells alone in a culture dish towards the more physiological condition of multiple cell types being able to interact to maintain homeostasis in the face of toxic exposure will lead us toward useful and correct predictions of *in vivo* toxicities.[37] Embryonic stem cells represent an alternative toxicological model to predict

developmental cardiotoxicity or neurotoxicity demonstrating that this is an excellent model to increase the safety of novel drugs.[37] In addition, patient-specific iPSCs generated from humans with specific diseases maintain some of the programming characteristic of that disease.[8] This implies that patient-specific iPSCs or iPSCs obtained from a wide variety of people encompass the broad spectrum of metabolic abilities, drug susceptibilities, resistance or susceptibility to disease and are very useful for the testing of new biological agents or drugs in order to find the most effective therapeutic agent for the treatment of iPSC-derived-patient's disease.[37] Several laboratories have already derived iPSCs from patients suffering from Huntington's and Parkinson's disease, juvenile diabetes, muscular dystrophy, Down syndrome, familial dysautonomia and LEOPARD syndrome, thus recapitulating the cell abnormalities in a plastic dish as they are seen in patients.[8,38] When the cultured iPSCs obtained from patients with familial dysautonomia and LEOPARD syndrome were exposed to experimental drugs for these diseases, the 'symptoms' were partially alleviated in culture.[38] This principle may result in the development of new drugs and should be applied to many other diseases for which currently there are no efficient therapies.

Animal disease models and pilot clinical studies showed that although SCT could be effective, there are several limitations that should be solved in future before their clinical application becomes a reality and these include immune rejection, risk for malignant transformation and medical ethics. The potential risk for malignant transformation of ESCs and MSCs is connected to their undifferentiated status or chromosal instability.[6] Innovative technologies of reprogramming and derivation of iPSCs address these concerns. Derived from patient's own somatic cells, iPSCs eliminate the potential for immune rejection representing an ethically acceptable alternative to the use of human embryos for ESCs derivation. Key advantage of iPSCs compared with ASCs is the possibility of repairing disease-causing mutations by homologous recombination, a technology that has been used with limited success in ASCs because of notorious difficulties in growing them outside the body.[8,37] Promising experiments in mice suggest that the treatment of genetic disorders sickle cell anaemia and haemophilia A with iPSCs is feasible.[39] Skin cells from the mice were first reprogrammed into iPSCs, the disease-causing mutation was subsequently fixed in iPSCs by gene targeting, and the repaired cells were then coaxed into blood-forming progenitors. These, now healthy, progenitors were transplanted back into anaemic mice, where they produced normal red blood cells and cured the diseases.[39] In principle, this approach could be applied to any disease in humans for which the underlying mutation is known and that can be treated by cell

transplantation. However, despite successes in animal models, iPSCs are not yet ready for transplanting into patients. Most iPSCs have been generated with integrating vectors, which may not get silenced efficiently or could disrupt endogenous genes, which pose potential impediments for the use of human iPSCs in cell therapy.[38] In addition, iPSCs-derived haematopoietic progenitor cells have been shown to undergo premature senescence and iPSCs therapy can lead to tumour formation.[40] For instance, an increased propensity of iPSC-derived neural cells to form tumours after transplantation into the brains of immunocompromised mice has been noted.[40] It will thus be critical to further improve the transgene-free approaches for derivation of new patient-specific iPSC lines.

Researchers must evaluate different types of original cells and induction methods to determine the best combination for generating the safest iPSCs. At minimum, researchers need to focus on safety of iPSCs therapy in light of the potential for cancer formation. Therefore, removal of the c-Myc transgene from reprogramming cocktail and the use of synthetic mRNA to reprogramme human fibroblasts to pluripotency are new approaches for generating safe iPSCs. The use of synthetic mRNA overcomes the innate antiviral immune response and showed superior conversion efficiency and kinetics than the established viral protocols. This strategy completely eliminates the risk of genomic integration and insertional mutagenesis inherent to DNA-based methodologies.[40]

Conclusion

This review has discussed a number of already established SCT and new stem cell approaches and strategies. As mentioned, some types of stem cells (especially HSCs and MSCs) are routinely used or become more and more important in regenerative medicine. During past decade, much progress has been made in the ASCs-based therapies using animal models, preclinical and clinical trials. Meanwhile hESCs and especially iPSCs have dramatically emerged as potential novel approaches to understand and treat devastating and otherwise incurable diseases. However, iPSC therapy should not be considered as substitution for hESCs therapy. Despite numerous technical advances in the reprogramming technology, iPSCs are not yet ready for transplanting into patients. Relatively little is known about iPSCs molecular and functional equivalence to hESCs, which could affect their potential therapeutic utility. Addressing this question will require a careful analysis of the genomic and epigenomic integrity of human iPSCs. Further studies are necessary to develop optimized growth and differentiation

protocols and reliable safety assays to evaluate the potential of stem cells and their derived specialized cells for the broader application in regenerative medicine and drug development.

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