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Review Article

Spermatogonial stem cells are a promising and pluripotent cell source for regenerative medicine

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Abstract: Regenerative medicine has been shown to hold enormous potential to treat traumatic and degenerative diseases, and substantial advancements have been made in the recent decades. In particular, different cell types were evaluated in basic research and preclinical studies on cell-based therapy applications. Despite the extraordinary achievements made in experimental studies and clinical practice, a considerable number of obstacles, such as the cellular source, ethical and safety issues, hinder further clinical applications. Spermatogonial stem cells (SSCs) are gradually becoming the research focus of cell-based regenerative medicine owing to their unique merits over other types of stem cells, particularly the lack of ethical concerns and lower immunogenicity. In addition, SSCs have been successfully induced to differentiate into other cell types under different appropriate conditions in compelling studies. Based on these properties, we systemically reviewed the development of SSCs as an attractive cell source for cell-based regenerative medicine.

Keywords: Spermatogonial stem cells, transdifferentiation, regenerative medicine, cell therapy, degenerative disease

Introduction

Regenerative medicine can be narrowly defined as the repair, replacement or replenishment of degenerative/damaged cells and tissues compromised by trauma, tumors, infections, and other complications with genetically similar young and functional cells to restore cellular function [1, 2]. Thus, various types of pluripotent stem cells, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs), have been extensively evaluated in basic studies and preclinical trials. Nevertheless, each type has pros and cons as an available source for regenerative therapies to combat a variety of diseases. When generating stem cell types, the most serious concerns lie primarily in ethical issues, tumorigenicity, accessibility, immunogenicity, and viral contamination risk, and these factors have bedeviled the clinical use of these cells [3-8]. Therefore, an alternative stem cell source is needed to circumvent these concerns, and spermatogonial stem cells (SSCs) could be an emerging stem cell type for clinic therapy.

In this review, we systematically elucidate the characteristics of SSCs. In addition, this review discusses in detail the current advancements in the pluripotency and differentiation of SSCs in the regenerative medicine field. Finally, we will outline the prospects of utilizing SSCs for cell-based treatment in future medicine.

Characteristics of SSCs

SSCs are a subpopulation of type A spermatogonia located at the basement membrane of the seminiferous tubules, and they play a pivotal role in spermatogenesis, which is a delicately orchestrated process of continuous sperm cell production that generates billions of haploid spermatozoa daily [9, 10]. Spermatogonia are a subtype of undifferentiated male germ cells that give rise to spermatocytes [9]. The subtypes of spermatogonia in rodents usually include A-single (As), A-paired (Apr), A-aligned (Aal), A1-A4, intermediate, and type B. Although SSCs originate in spermatogonia, they are different from the spermatogonia subpopulations (e.g., Apr and Aal) with respect to their morphological features and lack of intercellular

bridge. By definition, SSCs should belong to type As spermatogonia and act as actual stem cells [10]. In general, SSCs can divide asymmetrically to give rise to SSCs to maintain the stem cell pool or generate the other daughter cells to initiate spermatogenesis. The process of spermatogenesis occurs in the seminiferous tubules, where SSCs both self-renew and differentiate into spermatogonia, spermatocytes, spermatids, and eventually mature spermatozoa. In the testis, although SSCs account for only approximately 0.03% of the total germ cells, they give rise to millions of sperms after undergoing a precise and complicated procedure [11-13]. More strikingly, spermatogenesis usually occurs throughout the mammalian lifetime, which mainly transmits genetic information to the next generation. Due to the cell diversity and complexity of testis tissue, unique phenotypic markers of SSCs have not been previously used to identify and isolate SSCs and some existing markers are slightly different in various species. For instance, GPR125, CD49f, PLZF, UCHL1, GFRA1 and THY1 are expressed in human and rodent SSCs. However, human SSCs do not express certain phenotypic markers, such as Oct-4 and KIT, which are specific to rodent spermatogonia and SSCs, and these markers are likely associated with distinct species-specific properties of SSCs. Regardless of the lack of typical markers for SSC identity and biology, several markers are able to identify SSCs [14].

Because spermatogenesis is an ongoing process throughout the lifetime of an adult male, SSC division produces genetically identical daughter cells. One daughter cell has the same stem cell identification, while the other undergoes differentiation and forms mature sperm [15]. The maintenance of SSC stemness, self-renewal and cell number regulation in the complicated male germline system remains to be explored. Together with their surroundings, SSCs construct a complicated and precise microenvironment termed the “niche”. The surrounding cells and tissues, including Sertoli cells, peritubular myoid cells and testis mesenchyme, secrete GDNF, LIF, FGF2, CXCL12 and other key factors, whose receptors are mainly detected on SSCs, and they play a major role in the maintenance of SSC self-renewal [16, 17]. The interaction between SSC factors and their receptors activate specific signaling pathways in SSCs, including GFRA1/RET and PI3K-Akt pathway in GDNF, MAPK pathway in FGF2 and CXCL12-CXCR4 pathway, which could maintain the self-renewal activity and inhibit differentiation signals. Once a shortage of factors or blocking of their downstream occurs, the number of SSCs in the testis is exhausted and the in vitro expansion is terminated rapidly. However, the overexpression of these factors or activation of their pathway might result in the accumulation of undifferentiated cells and the appearance of teratomas [18-24]. These factors not only make sense individually and jointly in vitro but construct the complicated niche in testis. Further exploration of the niche and its regulatory mechanism clarifies the dynamics of SSCs in the testis. And additional information on SSC niches would help us to establish a more efficient long-term SSC culture in vitro, which is the foundation of research and further applications.

As for biological property, SSCs were responsible for spermatogenesis and sustain male fertility and the consequent offspring. Unfortunately, there were many people experience infertility as a result of chemotherapeutic/radiotherapeutic treatment for cancers, hematological or immunological diseases and abnormal spermatogenesis processes and spermatogenesis microenvironment. Given the status of SSCs in the testis, whether SSC transplantation could restore fertility should be determined. Such research has been performed in many infertile animal models, which revealed that the transplanted SSCs migrated to the seminiferous tubules of the recipient, colonized the epithelium and underwent a series of self-renewal and differentiation so that permanent spermatogenesis was established eventually. Thus, SSC-based transplantation might be an efficient approach for people suffering from irreversible testis destruction, azoospermia and oligospermia. The cell transplantation rescued not merely in acquired infertility, some congenitally infertile mice also benefited from that [25]. Considering the loss of SSCs during the preparation and exploration of the maintenance of the SSCs in the niche, researchers successfully developed a long-term culture of SSCs in vitro prior to transplantation, which not only increased the number of SSCs for transplantation but also provided a firm foundation for further research [26-29]. Although SSCs

have been maintained in vitro for a longer time, SSCs are still inaccessible to patients undergoing treatment for destroyed testis tissues in some cases. With the advent of cryopreservation, SSC suspensions or testicular biopsies could be preserved for a long period for autologous SSC transplantation, especially for prepubertal boys [30]. Simultaneously, cryopreservation could also diminish biological metabolism, thereby stopping enzymatic and chemical reactions, which would maximally protect the origin of SSCs. In this way, SSCs or tissues could be cryopreserved before the destructive therapy and used to transplant the cells back into the testis when propagation is needed. Some transplantation techniques have been successful in animal models but this delicate and arduous process is still needed to be optimized before SSC transplantation can be considered a clinical tool [31-34].

Recently, increasing studies have demonstrated that mouse and human SSCs possess pluripotency and are able to differentiate into all cell lineages from the three germ cell layers. In addition, SSCs can become embryonic stem (ES)-like cells, further giving rise to somatic cells for regenerative medicine. Most importantly, compared to other types of stem cells, SSCs avoid many of the abovementioned concerns and thus may represent a novel source for cell-based therapy [35, 36]. These studies revealed some new therapeutic prospects for human SSCs to generate various mature and functional cells for regenerative medicine, and these prospects will be reviewed below.

Pluripotency of SSCs

Early in the last century, teratomas appeared spontaneously, uncommonly and, almost exclusively in the gonads. Utilizing gene-editing mice with a high frequency of teratomas, researchers found that the tumors in newborns were frequently associated with broken seminiferous tubules after postmortem. Simultaneously, direct continuity was observed between the seminiferous epithelium and tumor cells, which also made it appear as if the tumors had originated within the tubules. Cumulative evidence indicated that there might be a subpopulation of cells in seminiferous tubules with the potential to form teratomas [37]. In other words, these cells might hold pluripotency. To identify the origin of the teratoma, several attempts have been made and a few morphologically and phenotypically ES-like cells were ultimately obtained. What remains to be determined is whether these ES-like cells are capable of being induced into other cell lineages under specific conditions.

Considering the cell proliferation and differentiation in testis, SSC has received considerable attention. Subsequently, a series of comprehensive studies on SSC properties in vitro and in vivo provided compelling evidence that SSCs may possess pluripotency potential. In 2004, ES-like cells were developed from neonatal mouse testes in primary culture with trophic factors. The ES-like cells not only show a morula-like structure similar to ESCs but also express markers identical to those of ESCs. Under the conditions used for the differentiation of ESCs, ES-like cells were capable of giving rise to various cell types. Moreover, these cells could produce teratomas after inoculation into mice [38], which is the first report of the generation of pluripotent ES-like cells from the SSCs of neonatal mice, and this finding strongly indicates that SSCs have the potential to be induced into ES-like cells. Afterwards, numerous studies were conducted to optimize the transition from the neonatal testis to ES-like cells, which also had a proven pluripotency [39-41]. More notably, SSCs from adult mouse testes shared similar characteristics to those from neonatal testes and were successfully induced into a multipotent state, which showed great potential for regenerative therapy in humans. Referring to the fertility preservation by testicular biopsies, it is important to establish human pluripotent stem cells from biopsies. Fortunately, in 2009, Kossack N et al isolated human multipotent germline stem cells (hMGSCs) from a testis biopsy. In addition to the expression of human pluripotency markers SSEA4, TRA1-81, OCT4 and SOX2, hMGSCs also were positive for the hESC marker TNAP. Furthermore, the cells form embryoid bodies that contain derivatives of all three germ layers, maintain a normal XY karyotype and express high levels of telomerase. Although the culture conditions needed to be optimized, these data provided important insights for future clinical applications [42].

At the present, although the factors that trigger the transition of SSCs into other types of stem cells have not been identified in further investigations of SSCs, and this controversy regarding the transition remains, this particular

phenomenon has been observed. Namely SSCs can be transitioned into other stem cell states. Considering the epigenetic reprogramming roadmap in generation of iPSCs from somatic cells, thus it postulated that SSCs in vitro can be converted into ES-like cells by cellular reprogramming. This reprogramming process might revert SSCs into a more primitive state. When adding some transcriptional factor cocktails similar to somatic reprogramming, SSCs will escape from the original developing landscape and convert to ES-like cells in vitro with low efficiency. In addition, epigenetic factors usually participate in the reprogramming event. Liu et al found that some important promoters essential for the maintenance and differentiation of ESCs is enriched in SSCs with histone H3-lysine4 and -lysine 27 trimethylations [28]. The chemical modification bestowed the ESC-like promoter chromatin to multipotent adult spermatogonial-derived stem cells (MASCs). For the enhancers, the core pluripotency circuitry is activated absolutely in MASC concomitant with loss of germ cell-specific gene expression and initiation of embryonic-like program [43-45]. These epigenetic modifications might provide novel approaches for increasing SSC reprogramming efficiency and eliminating exogenous transcriptional factors. However, the existence of intermediate process such as indirect generation of iPSCs or ES-like cells may influence the potential application in the clinic due to low efficiency and tumorigenicity through the intermediate transition [46-48]. Thus, it is necessary to develop a new method to overcome these issues. Alternatively, SSCs themselves show pluripotent. In the testis, SSCs interacted physiologically within the niche which secretes some bioactive factors, leading to spermatogenesis, and inhibits differentiation into other lineages. In contrast, when cultured without Sertoli cells or injected into blastocysts, SSCs might lose their properties in the testis and easily differentiate into other types of cells, implicating that an appropriate condition might evoke the potential multipotency of SSCs. Thus, the high plasticity and potential of SSCs is likely to serve as a promising option for developing treatment strategies for regenerative medicine.

Therapeutic potential of SSCs

As previously mentioned, SSCs, a type of stem cells, also possessed the unique characteristics and pluripotency. Compared to other types of stem cells, clinical application of SSCs can bypass ethical concerns, immune rejection and risk of tumorigenesis. Thus, SSCs are particularly appealing for regenerative medicine and are currently the most promising stem cells in preclinical and clinical research. Considering the high plasticity, SSCs may thus propose as an ideal cell source for cell-based therapy in various systems. Next, we will systematically discuss the advancement of the conversion of SSCs into specific cell types using a two-step approach, direct differentiation and the possible clinical applications. The special inductions methods are listed in **Table 1**.

Neuronal differentiation of SSCs

Central nervous system (CNS) injuries and degenerative disorders are currently considered irreversible and devastating and have high morbidity and mortality, and lead to neurological complications and eventually to serious disability and even death. These injuries themselves and therapeutic strategies have perplexed neurologists for a long period. To date, no curative treatment approaches can be used for these diseases caused by cell necrosis or loss of neurons. Therefore, seeking an ideal alternative cell source for replenishment of neurons is crucial for effectively treating neurological diseases. Because the unique advantages and pluripotential of SSCs to give rise to neurons, SSCs could serve as a promising candidate type for neural regeneration.

Early in 2006, ES-like cells from SSCs were proven to possess pluripotency and could be induced to undergo neural-type differentiation. Although the cells from neural differentiation expressed neural transcription factors at the mRNA level and exhibited a neuron-like morphology, the specific mechanism of transition still needs to be explored. With further investigation of ES-like cells from SSCs and reprogramming approaches, some new achievements have been reported [43]. Strikingly, multipotent adult germline stem cell (maGSC)-derived neurons can further selectively segregate into GABAergic, glutamatergic, serotonergic, and tyrosine hydroxylase-positive phenotypes. In addition, these converted neuron-like cells from maGSCs could spontaneously generate neural function in neural networks, which demonstrated that the differentiation of maG-

Table 1. Specific differentiation of SSCs and their inducing methods

Origin	Inducing methods	Target cell	reference
maGSCs from mice SSCs	20 ng/ml Noggin for 8 days. ITSFn medium containing 5 μg/ml insulin, 50 μg/ml human apotransferrin, 30 nM sodium selenite, and 2.5 μg/ml fibronectin for 5 days. DMEM/Ham's F12 medium supplemented with 25 μg/ml insulin, 100 μg/ml apo-transferrin, 30 nM sodium selenite, 20 nM progesterone and 10 ng/ml FGF2 for multipotent neural precursors. 10 ng/ml FGF2 and 20 ng/ml epidermal growth factor (EGF), followed by a 4-day-propagation in the presence of 10 ng/ml FGF2 and 10 ng/ml platelet-derived growth factor (PDGF) for glial precursors.	Neurons and glia	[49]
ES-like cells from neonatal mouse SSCs	DMEM/F12 medium with 5% FBS for 2 days to form EB. DMEM/F12 with 5% FBS and 1 μM RA and 300 nM Shh for 8 days to neuron-like cells. Cells were cultured in serum-free DMEM/F12 and neurobasal medium (1:1) supplemented with N2B27-I for 18 days to motor neuron-like cells.	motor neuron-like cells	[50]
maGSCs from cultured adult SSCs	For NSC: DMEM/F12 with GlutaMAX supplemented with 1× N2 1× NEAA, 50 μM β-ME, bFGF 20 ng/ml EGF 10 ng/ml for 10 to 18 days. For neural differentiation: DMEM/F12 with GlutaMAX, 20 μg/ml insulin, 20 μg/ml human apotransferrin, 6.3 ng/ml progesterone, 16 μg/ml putrescine, 30 nM sodium selenite, and 50 μg/ml BSA combined 1:1 with Neurobasal medium supplemented with 1× B27 with vitamin A 1× L-glutamine, and 50 μM β-ME.	Neurons and glia	[51]
ES-like cells from SSCs	Neurobasal medium added N2 and B27 supplements, 20 ng/ml bFGF and 10 ng/ml EGF.	Oligoprogenitor Cells	[52]
ES-like cells from SSCs	Differentiation day 0 (DD0): Glasgow-MEM with 5% KSR, 0.1 mM NEAA, 0.1 mM pyruvate and 0.1 mM β-ME. DD1: Matrigel was added. DD7: DMEM/F-12 with GlutaMax, N-2 supplement. DD10: DMEM/F-12 with 10% ES-FBS, GlutaMax, N-2 supplement, 0.5 μM retinoic acid and 30 ng/ml BDNF.	Retinal Ganglion Cells	[54]
Chicken SSCs	DMEM with 10% FBS, 2% chicken serum, 2 μmol/mL L-glutamine, 1 μmol/mL sodium pyruvate, 5.5×10^{-2} μmol/mL β-ME, 5 ng/mL hSCF, 10 ng/mL bFGF, 4×10^{-2} ng/ mL hIL-2, 10 ng/mL IGF and 100 μg/mL gentamicin sulfate for 2 days; DMEM plus 10% FBS without LIF for additional 2 days; DMEM with 10% FBS, 5.5 10-2 μmol/mL β-ME, 1×10^{-4} μmol/mL desamethasone, 10 μmol/mL β-sodium glycerophosphate and 0.05 mg/mL vitamin C for osteoblasts. DMEM plus 10% FBS, 5.5×10^{-2} μmol/mL β-ME, 1 nmol/mL retinoic acid (RA) and 5.0 μmol/mL IBMX for neuron-like cells. DMEM plus 10% FBS, 5.5×10^{-2} μmol/mL β-ME, 1 nmol/mL dexamethasone, 0.01 mg/mL insulin and 0.5 μmol/mL IBMX for adipocytes.	Osteoblasts Neuron-like cells adipocytes	[56]
$CD49f^{+}$ SSCs	serum-free DMEM:F12 (1:1) containing 20 ng/ml EGF and 1 ng/ml bFGF supplement, 1% B27 and N2 supplement, and 1.0 μmol/l all-trans retinoic acid for 8 days	Dopaminergic neuron cell-like cells	[55]
Mice SSCs	OECCM with 15 ng/ml GDNF, 5 μM RA, 250 ng/ml SHH, 1 ng/ml TGFβ3 and 100 ng/ml FGF8α.	Dopaminergic Neurons	[57]
Human SSCs	OECCM containing 15 ng/ml GDNF, 5 μM RA, 250 ng/ml SHH, 1 ng/ml TGFβ3, 100 ng/ml FGF8α, 1 mM VPA, 10 μM SB, and 1 μM forskolin for 4 days. This medium was supplemented with 250 ng/ml SHH, 1 ng/ml TGFβ3, 100 ng/ml FGF8α and maintained for another 3-4 weeks.	Functional DA neurons	[58]
Rat SSCs	OECCM-GDNF + 5 μM RA + 10 μM SB431542 + 0.5 mM IBMX + 250 ng/ml SHH + 1 ng/ml TGFβ3 + 20 ng/ml FGF2	Spinal Cord Neurons	[59]
mGSCs from chicken SSCs	DMEM supplemented with 20% FBS, 1% NEAA, β-mercaptoethanol 50 μmol/L, 10 μmol L^{-1} 5-aza-2'-deoxycytidine, and 10^{-5} M IBMX	cardiomyocytes	[60]
mGS cells from neonatal mouse	Cells were cultured on OP-9 stromal cell layers in α-minimum essential medium containing 10% FCS and 5×10^{-5} M 2-mercaptoethanol.	Cardiac cells and Endothelial cells	[61]
maGSCs from mouse SSCs	Endothelial growth medium supplemented with 50 ng/ml VEGF.	Endothelial Cells	[67]
Mice SSCs	DMEM/F12 supplemented with 0.5% FBS, 50 ng/ml Nodal, 50 ng/ml Wnt3a, and 20 ng/ml bFGF to hepatic stem-like cells. Hepatocyte culture medium (HCM) plus EGF supplement and 20 ng/ml hepatocyte growth factor (HGF) for 5 days to immature hepatocytes. HCM supplemented with 10 ng/ml HGF, 10 ng/ml Oncostain M and 10^{-4} mM dexamethasone for another 5-10 days to matured cells.	hepatocyte-like cells	[69]
Human SSCs	DMEM/F12 containing 10% KSR, 100 ng/ml Activin A 50 ng/ml Wnt3a and 20 ng/ml bFGF for 10 days. HCM plus EGF supplement and 20 ng/ml hepatocyte growth factor for 5-10 days. HCM supplemented with 10 ng/ml HGF, 10 ng/ml oncostain M and 10^{-4} mM dexamethasone for another 10 days.	hepatocytes	[70]
Human SSCs line	Human SSC line and mouse liver mesenchymal cells were placed in PBS with collagen type I, incubated at 37 °C for 1 h-1.5 h, and cultured with DMEM/F12 medium. After incubated with DMEM/F12 overnight, the cells in collagen gel were grafted under the renal capsule.	Hepatocytes	[71]

SCs into neurons could likely occur via the transition of SSCs [49-52]. Based on previous achievements, Kim and colleagues further modified the approach and found that CD24, a neural precursor marker, plays a pivotal role in the neural differentiation of maGSCs. Moreover, upregulation of CD24 expression in maGSCs by paracrine factors could elicit maGSC neural differentiation, implicating that neural precursor proteins might contribute to the neural differentiation of SSCs to some extent. Therefore, it is critical to supply some exogenous factors to develop an efficient approach for accelerating differentiation through upregulating CD24 [53]. Two-step differentiation was also applied as a potential treatment for glaucoma. Tin-Lap Lee et al utilized ES-like cells from SSCs as intermediates and successfully converted them into retinal ganglion cells (RGCs), which present morphological and functional characteristics [54]. After transplantation into a glaucoma model, the survival of RGCs from donor was detected in the model. Although the recovery of function was not mentioned in this manuscript, the accessibility of RGCs provided a promising approach to treat glaucoma [54].

Given the deficiency of the two-step differentiation of SSCs and reprogramming, it needs to be determined whether SSCs can be directly converted into the desired cell type. Regarding the neural differentiation of SSCs, Li and his colleagues first reported that chicken SSCs can be directly induced into neuron-like cells under special conditions (retinoic acid (RA) and 3-isobutyl-1-methylxanthine (IBMX)) and the converted cells have an acquired neuron biochemical phenotype, morphological features and cellular characteristics as demonstrated by toluidine blue and immunohistochemical staining assays [55, 56]. However, the advanced function of neuron-like cells was not involved. Bird SSCs and mammalian SSCs have the same characteristics [56]. Specifically, the survival of grafts and recovery of function were emphasized. Following transplantation into a Parkinson disease (PD) rat model, rat SSCs survived and differentiated into neurons in the ventral tegmental area (VTA) of the model. Concomitantly, partial behavioral recovery was observed, which signified an important breakthrough in SSC therapy for PD [57]. Based on these achievements, our group also made an effort to direct differentiation. Considering the contribution of olfactory ensheathing cells (OECs) in neural cell development, differentiation, survival, and growth, we utilized OEC-conditioned medium (OECCM) and several defined growth factors (DFGs) (i.e., GDNF, SHH, TGFβ3, and FGF8α) to accomplish a highly efficient conversion of SSCs into dopaminergic (DA) neurons [58]. These derived neurons not only acquired morphological properties but also formed synapses, possessed electrophysiological activity and secreted dopamine. Significantly, the conversion was mediated through the PI3K/Akt pathway and the p21/Nolz1 cascade, resulting in cell cycle exit and fate determination, which illuminated the molecular mechanism of neural development from SSCs. We successfully converted hSSCs into DA neurons in vitro under OECCM and DFGs according to a previously mentioned protocol, which indicated that this method is much closer to clinical application [58]. Notably, the induced hSSCs could survive, migrate, and differentiate into DA neurons after being transplanted into the mouse striatum, resulting in a functional improvement in PD model animals [59]. The direct conversion from SSCs to DA neurons partially improved the safety and provided a novel cell source for future applications. For spinal cord injury, we efficiently induced SSCs to differentiate into functional spinal cord neurons in vitro [60]. Remarkably, in addition to morphological features and biochemical identification, almost all neurons exhibited functions similar to the functions of genuine neurons, such as synapse formation, neuronal-specific calcium influx, and electrophysiology. This is the first report that SSCs could reproducibly generate large amounts of functional spinal cord neurons, and this system could offer a novel treatment for spinal cord injury [60]. The direct conversion of stem cells in the damaged zone of the spinal cord is still being explored for clinical applications.

Cardiovascular system

Cardiovascular diseases are usually accompanied by the degeneration and loss of cardiomyocytes, smooth muscle cells, and endothelial cells. Once cell apoptosis occurs, cardiomyocytes are gradually replaced by non-functional scar tissue, leading to cardiac dysfunction. Thus, exogenous replenishment of cardiomyocytes is becoming the most prospective approach for the efficient treatment of heart disease.

The two-step conversion occurred diffusely in cardiomyocyte differentiation investigations. Following the establishment of maGSCs from SSCs, cardiomyocytes were successfully converted, and these converted cells not only exhibited morphological and molecular features but also exerted some functional properties. Similar to native cardiomyocytes, the differentiated cardiomyocytes exhibited a positive response to β-adrenergic stimulation or Ca2+ blockers [61-64]. Similar to previous reports, many achievements of SSC conversion have been made in nonmammalian areas. For instance, Im et al found that chicken maGSCs could be converted into cardiomyocytes with the expression of cardiac-specific markers, such as sarcomeric alpha actinin, alpha-cardiac actinin and conexin-43, which are crucial for the synchronized contraction of the heart. Critically, the converted cells also express cardiac troponin T, a kind of molecule to regulate muscle contraction [65].

In regenerative medicine, vascular smooth muscle cells (VSMCs) and endothelial cells (ECs), which participate in angiogenesis, have also received considerable attention for the treatment of the cardiovascular system [66]. In 2011, utilizing FACS-mediated selection and VEGF-mediated lineage induction, functional and proliferating ECs were yielded from SSCs [67, 68]. These ECs possessed a typical 'cobblestone' morphology and expressed several endothelial lineage markers. More importantly, a few vascular endothelial cells were detected 4 weeks after the transplantation of maGSCs into the hearts [61, 67, 68]. Although the generation of cardiomyocytes and vascular endothelial cells from SSCs was successful, the conversion efficiency is insufficient for future clinical applications. Therefore, a constant optimization for cell differentiation is a major problem that needs to be solved at this stage.

Due to the difficulties of the two-step conversion process, more efforts should be made to induce SSC pluripotency and establish a direct conversion for cardiomyocytes, VSMCs and ECs. Moreover, the approach of efficiently strengthening the integration of the transplanted cells into the surrounding cardiomyocytes and vascular endothelial cells needs to be further investigated.

Hepatocyte differentiation

Although traditional liver transplantation has achieved enormous success in terminal liver disorders, the stem cell-based strategy is a potentially alternative option considering the rarity of liver donors worldwide [69].

In 2013, after an unremitting endeavor, SSCs were first induced to transdifferentiate directly into therapeutic stem-like cells termed as hepatocyte-stem-like cells [69]. These cells could be further induced to have a hepatocyte phenotype and acquired similar mature hepatocyte properties without any in vitro interventions as demonstrated by morphological, phenotypic and functional characteristic assays. Moreover, the conversion event of SSCs into hepatocytes occurs through the cross-talk of the ERK1/2 and Smad2/3 signaling pathways. Additionally, the inactivation of cyclin A, cyclin B and cyclin E also contributes to the conversion of SSCs [70]. Subsequently, in 2016, the same group achieved the transition from human SSCs to hepatocytes [71]. This is the first report of direct differentiation of human SSCs to mature and functional hepatocytes via hepatic cell induction from stem cells to small cells to mature cells. During differentiation, the expression of beta-Catenin, Hnf4a, Foxa1 and GATA4 was upregulated in converted SSCs. Functional assays indicated that the SSC-derived hepatic-like cells were capable of producing albumin, removing ammonia, and up-taking and releasing indocyanine green, suggesting that these cells possessed the functions of genuine hepatic cells [71]. With the progressive illumination of the molecular mechanism underlying hepatocyte differentiation, effectively and broadly available cellular therapeutics for the treatment of different types of liver disease were have achieved great development. A liver injury model was generated by treating mice with carbon tetrachloride to simulate the proper environment for human SSC transplantation. Hepatic mesenchymal cells were chosen as feeder cells for SSC transdifferentiation. Both types of cells were transplanted under mouse renal capsules. The derived cells were detected in the injured liver and expressed specific hepatocyte markers, including ALB, AAT, CK18, and CYP1A2 [72]. Moreover, these cells acted as mature hepatocytes, and significant improvement was seen in the liver tissues of mice with

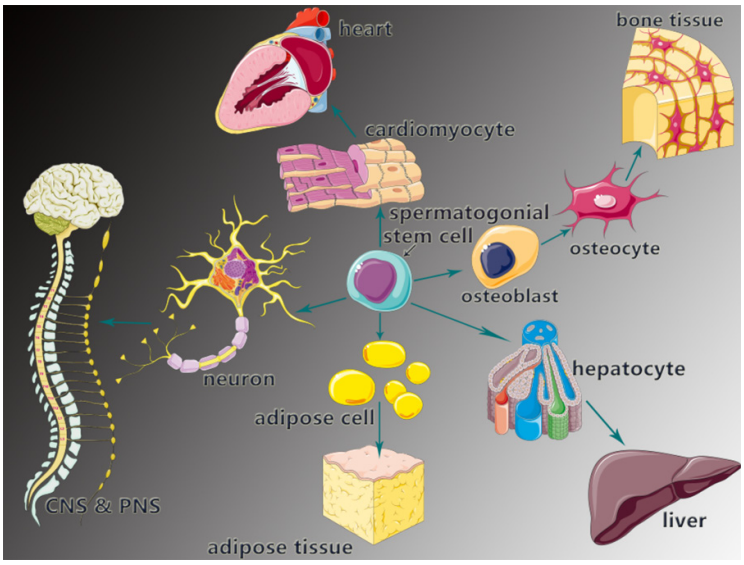

Figure 1. Schematic representation of transdifferentiation of SSCs into some specific cell types directly via different inductions in vitro and in vivo.

human SSCs [72]. This achievement indicated that cells derived from SSCs could migrate to the desired destination through vessels and improve the injured tissue, which provided a novel theory for the treatment of liver injury.

Other types of differentiation

In addition to the aforementioned achievements regarding the differentiation of SSCs, there were some other cell types generated from SSCs through direct differentiation. Li B et al found that chicken SSCs can be directly induced into osteoblasts after 15 and 21 days under special conditions. After a series of examinations and identifications, the converted cells were confirmed to gain the characteristics of genuine osteoblasts. In addition, SSCs from pigs were successfully induced into adipocytes by the addition of hexadecanol and insulin to the media. These SSC-derived adipocytes possessed the phenotypic and functional properties of wild-type adipocytes as characterized by biochemical staining, qRT-PCR, immunocytochemistry and karyotyping assay, and they survived for 22 days [56, 73]. These findings imply that the application of SSC-based therapies cover a vast array of regenerative medicine disciplines.

Taken together, great progress has been made for indirect or direct SSC differentiation, suggesting that SSCs have great potential for use in clinical therapy. However, these achievements are insufficient when compared with those of well-defined stem cells. Therefore, considerably efforts are still needed before the clinical application of SSCs.

Prospects

In this review, we focus on the current status of research on SSCs and their multipotent potential in regenerative medicine. Taking into account all the great advantages of SSCs over the other types of stem cells previously described, there is no doubt that SSCs represent an alternative cell source for regenerative medicine and can shed light on translational medicine.

The presented studies have revealed that by utilizing different methods, gene transfections of exogenous transcription factors, SSCs can give rise to certain terminally differentiated cell types through specific strategies as shown in **Figure 1**. Unfortunately, these methods still face formidable challenges, such as tumorigenesis by gene modification, differentiation uncertainty when transplanted into recipients. Although the latest advances can provide a vital clue to solving some persistent issues, several puzzles remain regarding SSC-based therapy for clinical applications. As an emerging and available cell source, SSCs have great potential to directly and efficiently produce other cell types of interest, which avoids the abovementioned problems to some extent. However, studies concerning the differentiation of SSCs are currently in their nascent stage. Thus, the next step for SSC research is to explore an approach to efficiently and sufficiently obtain desired cell types. In addition, persistent maintenance in recipients and interactions with the native terminal cells still need to be further strengthened. Given the clarification of the SSC niche, some biomaterials have been considered, and they could be utilized by loading essential factors with a controlled-release mechanism to simulate the testis niche. The existence of biomaterials will ensure a sufficient number of SSCs and interactions between the SSCs with the surroundings. We

believe that a large amount of experimental data has been accumulated, and could answer these questions. Further SSC developments will certainly lead to better clinical applications earlier than expected.

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