

Multipotency of Spermatogonial Stem Cells and Potential Applications in Regenerative Medicine

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ABSTRACT

Adult stem cells show excellent perspectives for applications in the field of regenerative medicine. In the last two decades, great strides have been made in spermatogonial stem cell research showing the potentiality of these stem cells in organ regeneration. Spermatogonial stem cells, derived from post-natal or adult mice, not only differentiate into cell types other than the spermatozoa when injected in different environments *in vivo*, but they are also capable of converting spontaneously into pluripotent stem cells that are similar to mouse embryonic stem cells *in vitro*. The pluripotent spermatogonial stem cells proliferate extensively and can be maintained for long period *in vitro*. Moreover, these cells can be frozen and thawed several times without change in karyotype and loss of viability, and can also be induced to differentiate into multiple lineages without ethical constraints. In this chapter, we will provide an update on the potential application of spermatogonial stem cells in regenerative medicine.

Keywords: Germline cell-derived pluripotent stem cells; Spermatogonial stem cells; Differentiation; Tissue regeneration

INTRODUCTION

Tissue-specific adult stem cells are a small pool of cells that are capable of replenishing all specialised cells of that tissue. They fulfill the definition of stem cells in that they are capable of self-renewal whilst maintaining the ability to differentiate. Adult stem cells have several advantages over stem cells of embryonic origin: ethical concerns and controversies over the use of embryos for stem cell generation are bypassed, and immunological rejection is avoided in autologous transplantation. Tissue stem cells may be multipotent or unipotent, and different stem cell types with tissue renewal capacity may reside within the same tissue [1]. Tissues that are generally composed of one cell type like the epidermis (with basal cells generating only keratinocytes) or the testis (with generation of spermatocytes) contain unipotent stem cells. In this chapter, we will discuss the findings on the stem cells derived from the testis, termed spermatogonial stem cells, and the interest they roused lately in the field of regenerative medicine.

SPERMATOGENESIS

Spermatogenesis is a complex process and begins postnatally in mammals. During fetal development, primordial germ cells migrate to the genital ridges during which they replicate. In the male gonad, the primordial germ cells give rise to the gonocytes which enter the G0 mitotic arrest and proliferate in the testis only after birth to give rise to the first round of spermatogenesis and establish the initial pool of Spermatogonial Stem Cells (**SSCs**) [2]. SSCs are a relatively rare population of cells (0.3% of all germ cells in rodents) found on the basement membrane of the seminiferous tubules and ensure spermatogenesis throughout the adult life in most mammals. SSCs are self-renewing and show unipotent differentiation into the spermatogenic lineage *in vivo*. SSCs reside in a special microenvironment called “niche” which provides the right cues, including growth factors, adhesion molecules, extracellular matrix, mechanical support and vascularization hence allowing the SSCs to maintain the balance between self-renewal and differentiation.

Due to their rarity and the difficulty to distinguish them from the progenitor cells, it is complicated to study SSCs. The only functional assay to determine the presence of SSCs is by performing transplantation. This technique was first described by Brinster and colleagues in 1994 in mice [3,4]. Germ cells isolated from donor testes were transplanted into the testicular seminiferous tubules of busulfan-treated recipient infertile mice. Sperm production resumed and fertility was restored. This approach is technically challenging but remains the best approach to identifying SSCs.

ISOLATION AND CULTURE OF SSCS

A stable *in vitro* expansion system is the prerequisite to get sufficient cells to perform basic and pre-clinical research. SSC isolation and culture procedures have been described for humans as well as for rodents and zebrafish [5,6]. Phenotypic markers have been used to enrich for SSCs from whole testicular tissue and a comparison of these markers in mouse and human SSCs

has been described by Guo et al. [5]. For example, the surface molecule GPR125 has enabled the enrichment of both mouse and human SSCs using Magnetic-Activated Cell Sorting (**MACS**). Mouse and human SSCs share several other markers like Integrin alpha-6, Glial Cell Line-Derived Neurotrophic Factor (**GDNF**) receptor- $\alpha 1$ (GFR- $\alpha 1$), THY1/CD90 that allows SSC isolation [5]. SSCs have been sorted using these markers prior to *in vitro* culture to reduce contaminating somatic cells, but till now no SSC-specific surface markers have been found to isolate pure SSCs from tissue.

Long-term culture of SSCs has been established only in mouse and rat. Nagano et al. were the first to report an *in vitro* method for culturing SSCs using feeder cells [7]. *In vitro* maintenance of SSCs was successively improved by their co-culturing on feeder cells and/or in defined medium containing various growth factors, such as GDNF, GFR- $\alpha 1$, basic Fibroblast Growth Factor (**bFGF**), Epidermal Growth Factor (**EGF**), and Leukemia Inhibitory Factor (**LIF**) [8]. SSCs could thus be grown *in vitro* for extended periods of time without affecting their stemness. Alternatively, Knockout Serum Replacement (**KSR**) which is a defined cell culture supplement has been used in order to reduce cytokine supplementation in the culture medium [9]. Attempts have been made at culturing human SSCs for long-term, but have been not as successful as for the mouse SSCs, probably due to their different growth requirements [5,10]. The ability to culture and propagate SSCs from humans *in vitro* for long period will provide a remarkable leap towards their clinical applications.

CLINICAL APPLICATIONS OF SSCS

In vitro culture can provide an unlimited supply of SSCs, and one of the main applications for human health is for fertility restoration following various testicular injuries. Infertility can be induced by germinal epithelial damage following anti-cancer (chemotherapy, radiotherapy) treatments in humans, in particular in pre-pubertal boys [11]. With the improvement in survival rate of cancer patients in the recent years, infertility caused by oncological treatments has become a relevant concern for cancer survivors [11]. One of the proposed solutions is fertility restoration through SSC transplantation. SSCs should be harvested before treatment initiation and cryopreserved until the patient is ready to autologously receive the transplant at any age. The transplanted suspensions need to be devoid of contaminating cancer cells and this may be possible by sorting only SSCs from the testicular tissue taken from the patients. Thus, rigorous screening is required prior to cryopreserving and banking the SSCs. Apart from fertility restoration, SSC studies are important for other clinical applications, such as cell-based organ regeneration therapy, as described below.

PLASTICITY OF SPERMATOGONIAL STEM CELLS

SSCs were previously regarded as unipotent stem cells as they can only give rise to spermatids within the mammalian testis. Recently, however, several studies have demonstrated that SSCs can transdifferentiate *in vivo* into somatic cell types when combined with the appropriate

mesenchyme [12]. Transdifferentiation is a process whereby a differentiated cell of one lineage converts into a differentiated cell of another lineage without reverting to stem cell progenitor status. During this conversion, the cell loses the function of the original cell type and acquires a new final cell type-dependent function. However, not all markers of the cell of origin are lost, as shown by our previous large-scale cDNA profiling. For example, SSCs, upon pluripotency acquisition and induced differentiation (described below), invariably expressed serine/threonine kinase receptor associated protein (Strap), testis expressed 9 (tex9), zinc finger protein 330 (zfp330) compared to mouse embryonic stem cells, showing that these cells retain patterns of gene expression reminiscent of their origin [13].

Work from several groups has shown the highly plastic behaviour of SSCs upon environmental change and their transdifferentiation into cells of the host tissue. The first indication of the ability of SSCs to alter their cell fate upon interaction with the mammary gland environment during pregnancy came in 2007 when Boulanger et al. injected adult testicular cells isolated from seminiferous tubules mixed with mammary epithelial cells into the epithelium-divested mammary fat pads [14]. Analysis of mammary outgrowths showed that testicular cells effectively interacted with mammary epithelial cells to form a regenerated mammary gland capable of fully functional differentiation [14]. SSCs were successively demonstrated to transdifferentiate following exposure to an inductive mesenchyme [15]. Freshly isolated SSCs/spermatogonial progenitors were recombined with urogenital mesenchyme prepared from day 16.5 fetuses and grafted under the renal capsule of syngeneic male hosts. Four weeks after grafting, 70% of urogenital mesenchyme-recombined SSCs transdifferentiated into prostatic epithelium. Tissue recombinants resulting from neonatal uterine mesenchyme or dermis and SSCs also gave rise to mesodermal derivative and ectodermal derivative/epidermis, respectively. Another proof of SSC transdifferentiation potential was provided by the work of Ning et al. who transplanted murine SSCs derived from 7-10 days old male pups into the bone marrow of busulfan-treated female mice in order to investigate whether SSCs could transdifferentiate into bone marrow-like cells [16]. Strikingly, twelve weeks after transplantation, donor-derived cells not only expressed surface markers characteristic of hematopoietic stem cells *in vivo*, but also adopted hematopoietic cell function. More than 6% of Y chromosome-positive cells were found in the bone marrow of female recipients. These results suggested that SSCs were induced to differentiate into bone marrow-like cells by paracrine factors released in the bone marrow environment. Direct differentiation of mouse SSCs into renal parenchymal cells *in vivo* has also been demonstrated. SSCs were directly injected in the renal parenchyma of whole-body irradiated adult female mice to investigate the differentiation potential of these cells [17]. Three months after SSC injection, histological analysis revealed that SSCs had migrated to the basement membrane and integrated the kidney tubules. Moreover, using organ culture of metanephric kidney, Heer et al., showed that mouse SSCs were primed *in vitro* with conditioned media from human kidney fibroblasts showed renal differentiation *ex vivo* [18]. More recently, Zhang and colleagues demonstrated that SSCs

can directly transdifferentiate to hepatic stem-like cells *in vitro*. The SSC-derived hepatic stem-like cells were small and capable of responding to differentiation stimuli to become mature and functional hepatocyte-like cells *in vitro* [19]. Thus, SSCs are highly plastic and may locally respond to extrinsic factors and transdifferentiate into derivatives of the three germ layers *in vivo*.

PLURIPOTENCY OF SPERMATOGONIAL STEM CELLS

The property of SSCs that rendered them most attractive for organ regeneration is the acquisition of pluripotency *in vitro*. Various groups have shown that SSCs isolated from neonatal mice testis can reproducibly and spontaneously give rise pluripotent Embryonic Stem (**ES**)-like cells when cultured for long-term (4-7 weeks) *in vitro* [8,20,21]. These pluripotent SSCs, termed herein germline cell-derived pluripotent stem cells (GPSCs, alias mGS or MaGSCs), are morphologically similar to ES cells. Characterization of GPSCs showed that these cells express key pluripotency factors such as Oct4 (octamer-binding transcription factor 4), c-Myc, Sox2 (SRX (sex determining region Y)-box 2), Lin28 which are also expressed in the starting cell type, SSCs. Nanog is expressed only after pluripotency acquisition. There are several advantages of using GPSCs over ES cells. First, GPSCs are obtained *in vitro* from SSCs isolated from neonatal or adult testis (at least up to 7 weeks of age) without particular procedures, thus avoiding the ethical concerns over embryo use for the generation of pluripotent stem cells as in the case of ES cells [20-23]. Second, GPSCs can be propagated for long period *in vitro* without changes in karyotype. Third, GPSCs can differentiate into derivatives of the three germ layers *in vitro* (described below) and produce teratomas upon injection in immune-compromised mice. Fourth, when injected into blastocysts, GPSCs contribute to the development of several organs in chimeric animals and show germline transmission. Moreover, GPSCs can be readily frozen and thawed without loss of cell viability compared to other adult stem cells, and represent thus, a promising stem cell source in the regenerative medicine field, as described below.

GPSC-DERIVED CARDIOMYOCYTES

To explore the differentiation potential of GPSCs, these cells were cultured as “hanging drops” used for embryonic stem cell differentiation [20]. The expression of a panel of lineage-specific genes was examined to determine whether GPSCs could differentiate into derivatives of the three germ layers. Brachyury expression showed that the mesoderm, from which cardiac cells arise, formed in the embryoid bodies and cardiac transcription factors islet-1, GATA binding protein 4 (Gata4), Homeobox protein Nkx-2.5 (Nkx2.5) and Myocyte-specific enhancer factor 2c (Mef2c) were expressed at early time points, while later on, cardiac-specific genes such as α -myosin heavy chain, ventricular isoform 2 of myosin light chain and atrial natriuretic factor were found highly expressed. After 10 days of differentiation, spontaneously contracting areas were observed in the embryoid bodies, and showed sarcomeric striations and stained positive for α -sarcomeric actinin sarcomeric Myosin heavy chains (**MHC**) and cardiac troponin T. The GPSC-derived cardiomyocytes were functional in that they contained L-type calcium channels which responded to cardioactive

drugs [24]. Moreover, analysis of cell-to-cell coupling in the synchronously contracting clusters showed that pan-cadherin (adherens junction) and connexin 43 (gap junctions) were present among cardiomyocytes. These cells also responded to β -adrenergic stimulation or Ca²⁺ blockers and had an intact calcium cycling [24]. Guan et al. further demonstrated that GPSCs, when injected intramyocardially in mice, proliferated after transplantation, but did not form teratomas. *In vivo*, these cells differentiated spontaneously into vascular endothelial and smooth muscle cells but not into mature cardiomyocytes [24]. Directing the differentiation of GPSCs into cardiomyocytes *in vitro* might greatly improve their *in vivo* differentiation. Interestingly, in another study, Baba et al., showed that Fetal liver kinase 1 (Flk1)⁺ cells sorted from differentiating GPSCs could give rise to mature cardiomyocytes and endothelial cells *in vitro*, and that transplantation of GPSC-derived Flk1⁺ cells into an ischemic heart failure mouse model improved cardiac function and enhanced angiogenesis around the ischemic area [25,26]. Thus, GPSCs may be a promising source of cells for cardiac repair.

GPSC-DERIVED HEPATOCYTES

Several adult stem cells, such as those derived from bone marrow and adipose tissue, have been used to obtain hepatocyte-like cells [27]. Recently, encouraging results have been obtained from GPSCs, too. During the initial embryoid body-mediated differentiation of GPSCs, the phenotypic characteristics of hepatocytes were found by analyzing the early hepatic marker, alpha-fetoprotein, and the late marker cytokeratin 18 expression [20]. GPSCs were induced into hepatocyte-like cells by Loya et al. and our group [13,28]. GPSCs were cultured as embryoid bodies to induce endoderm formation and then further cultured *in vitro* under conditions promoting hepatocyte differentiation. The GPSC-derived hepatocyte-like cells expressed several hepatic markers such as alpha-fetoprotein and albumin. We further demonstrated that 82.68% of the cells in the embryoid bodies expressed GFP under a hepato-specific promoter [13]. These cells were metabolically active as shown by albumin and haptoglobin secretion, urea synthesis, glycogen storage, and indocyanine green uptake. Our large-scale gene expression analysis further showed that mouse GPSCs was similar to mouse embryonic stem cells during hepatocyte differentiation, and that *in vitro*, GPSC-derived hepatocytes were fetal-like [13]. Streckfuss-Bomeke et al. used another system to increase the efficiency of hepatic differentiation. GPSCs were co-cultured with OP9 stroma cells in the presence of Activin A and this resulted in a high percentage of GPSC-derived hepatic progenitors (51% showing alpha-fetoprotein positivity and 61% positive for Delta-like homolog 1) and mature hepatic-like cells (26% showing positivity for albumin) [29]. Recently, we sorted Liv2-positive hepatic precursors from GPSC-derived embryoid bodies cultured in hepatocyte differentiation media, and transplanted these cells in the liver parenchyma of female mice subjected to partial hepatectomy [30]. Liv2-sorted cells showed regional and heterogeneous engraftment in the injected lobe of mice and approximately 50% of cells were positive for Y chromosome one month after cell injection. This study showed for the first time that GPSCs-derived hepatocytes could engraft *in vivo* in the mouse liver. Further studies are needed to assess their functionality *in vivo* in mice models of hepatic diseases.

GPSC-DERIVED RENAL TUBULAR CELLS

Renal tubular cells can also be generated from GPSCs as recently shown by our group [31]. GPSCs were induced to differentiate into mesoderm, from which the urogenital system arises during development in mammals, through embryoid body formation. The GPSCs were then further cultured in conditioned media derived from mouse primary renal tubular cells containing Glial Cell-Derived Neurotrophic Factor (**GDNF**) and retinoic acid in order to promote tubular specification. GPSC-derived tubular-like cells formed and expressed renal tubular cell-specific markers, such as cadherin-16/ KSP. These GPSC-derived tubular cells were functional *in vitro* and were capable of forming tight junctions, characteristic of epithelial cells. The functionality of the GPSC-derived tubular cells were assessed *in vivo* by injecting these cells intravenously in female mice following renal Ischemia/Reperfusion Injury (**IRI**) and unilateral nephrectomy. Our results showed that the GPSC-derived tubular cells could home to the injured kidney and protect mice against both ischemia reperfusion injury-induced acute and chronic kidney damage [31]. The Y chromosome-positive GPSC-derived tubular cells engrafted in the renal parenchyma and showed long-term engraftment in the kidney. Thus, GPSC-derived tubular cells were functional and offered protection against kidney injury.

GPSC-DERIVED NEURONS

The idea of using stem cells to treat neurodegenerative diseases is very attractive. Several candidate cell types have been tested for cell therapy and functional recovery in animal models of neurodegenerative diseases [32]. GPSCs have also been tested for their neurogenic activity. Streckfuss-Bomeke et al. differentiated GPSCs into neural precursors *in vitro* under specific culture conditions [33]. After 10-18 days in culture, cells changed morphology and gave rise to small, elongated, sharp-edged and flattened tightly-packed rosettes that were characteristics of neural progenitors. A stage-wise differentiation program was used to guide the GPSCs towards the formation of functional neurons. Quantitative analyses revealed that 43% of the differentiated GPSCs stained positive for neuronal markers SNAP25 and/or β III-tubulin, 33.4% for the astrocyte-specific marker GFAP, 11.7% for the oligodendrocyte progenitor marker NG2 and 20.2% for the oligodrocyte marker O1 [33]. The electrophysiological recordings of passive and active membrane properties and postsynaptic currents revealed that the GPSC-derived cells were functional. Glaser et al. also showed that GPSC-derived neurons could generate active functional networks, which used both GABAergic synaptic transmission and engaged in synchronized oscillatory activity [34]. GPSC-derived oligodendrocytes completed their maturation and were able to ensheath host axons in organotypic slice cultures of myelin-deficient rat cerebellum. The *in vivo* engraftment of GPSC-derived neurons and their ability to effectively replace damaged ones still needs to be investigated.

GPSC-DERIVED VASCULAR AND HEMATOPOIETIC CELLS

GPSCs were tested for their ability to differentiate into vascular endothelial cells and smooth muscle cells *in vitro* [35]. Briefly, GPSCs were induced to undergo mesodermal differentiation which was confirmed by the expression of Brachyury, Flk1 and Mesoderm Posterior BHLH Transcription Factor 1 (Mesp1) in the embryoid bodies. Further differentiation induced the formation of vascular endothelial cells and vascular smooth muscle cells. Only 7% of the GPSC-derived cells expressed Vascular Endothelial (**VE**)-cadherin and CD31. GPSC-derived vascular smooth muscle cells expressed specific markers such as myocardin and alpha-Smooth Muscle Actin (**α -SMA**) and contained intracellular fibril structure characteristic of vascular smooth muscle control cells. The authors found that the vascular differentiation process of GPSCs followed a developmentally appropriate sequence of transcription factor expression which demonstrated the potentiality of GPSCs for vascular research and therapy [35].

GPSCs were also examined for their hematopoietic progenitor potential *in vitro* and *in vivo* by Yoshimoto and colleagues [36]. GPSCs were co-cultured with OP9 stromal cells, and four days later, 50% of the differentiated cells expressed the mesodermal marker, Flk1. FLk1-positive GPSC-derived cells were sorted and plated on OP9 cells, and three days later, small round hematopoietic cells were found that had round, nucleated cell morphology reminiscent of primitive erythrocytes and were positive for anti-embryonic hemoglobin. Further differentiation *in vitro* showed the presence of enucleated red blood cells which were the definitive erythrocytes. Moreover, GPSCs, when cultured on OP9 cells with another cocktail of growth factors, differentiated into cells of the lymphoid lineage including CD19+ B cells and CD4+CD8+ T cells *in vitro*. GPSCs could also give rise to clonogenic hematopoietic progenitors; Burst-Forming Uniterythroid (**BFU-E**), Colony-Forming Unit Granulocyte-Macrophage (**CFU-GM**), and CFU-Mix were observed. *In vivo*, intra-bone marrow injection of GPSC-derived hematopoietic cells showed that these cells localised to the bone marrow cavity of immunodeficient mice. These data show the multipotent differentiation capacity of GPSCs both *in vitro* and *in vivo*.

CONCLUSION

The mouse SSCs and GPSCs are well characterized and their culture established. Attempts to culture human SSCs in the long-term to derive human GPSCs has given controversial results, and to date, there are no SSC-derived cells with strong pluripotent characteristics to be described as human GPSCs. Thus, the most immediate future research objectives are to find an efficient method for human SSC isolation, develop culture conditions which will enable long-term culture of human SSCs for human GPSCs derivation, improve *in vitro* differentiation protocols to obtain functional cells with optimal engraftment and rescue *in vivo*, and cryopreserve testicular tissue in biobanks for future isolation and transplantation of SSCs.

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