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Pluripotent Stem Cells from Testis

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1. Introduction

1.1 Importance of Spermatogonial Stem Cells (SSCs)

Spermatogonial stem cells (SSCs) are undifferentiated germ cells that balance self-renewing and differentiating divisions to maintain spermatogenesis throughout adult life. This is a productive stem cell system that produces millions of spermatozoa each day while also maintaining rigorous quality control to safeguard germline integrity. SSCs are the only adult stem cells that are capable of self renewal and that differentiate to produce haploid cells transmitting genes to next generation. In the recent years, derivation of multipotent embryonic stem (ES)-like cells, which are capable of differentiating to three germinal layers, from SSCs in the testis, has been reported. This is of immense importance in medicine, basic science and animal reproduction and overcomes ethical issues pertaining to the use of human embryos for research. Since the population of SSCs in the testis is very low (< 1%), the identification of markers that are specifically expressed in SSCs aids in their efficient isolation. The characteristics of SSCs and ES-like cells in culture, and differential expression of genes in both these cell types can provide better understanding. In the present chapter, we would describe and compare the expression of SSC-specific markers in vivo and in vitro. We will also review the in vitro culture conditions of SSCs and characteristics of ES-like cells that differentiate from SSCs. This would enhance our perceptive of these special cells that has opened new avenues for stem cell researchers.

1.2 Origin of Spermatogonial Stem Cell pool

SSCs arise from gonocytes in the postnatal testis, which originally derive from primordial germ cells (PGCs) during foetal development. PGCs are a transient cell population that is first observed as a small cluster of alkaline phosphatase-positive cells in the epiblast stage embryo at about 7–7.25 days post psot coitum (dpc) in mice (Phillips et al., 2010). The specification of PGC is dependent on the expression of BMP4 and BMP8b from the extraembryonic ectoderm (Ginsburg et al., 1990; Lawson et al., 1999; Ying et al., 2001). During the formation of the allantois, the PGCs are passively swept away from the original position before they start migrating via the hindgut to arrive at the indifferent gonad (genital ridge) between 8.5 and 12.5 dpc in mice. PGCs replicate during the migratory phase and approximately 3000 PGCs colonize the genital ridges (Bendel-Stenzel et al., 1998). In the

male gonad at about 13.5 dpc, PGCs give rise to gonocytes, which become enclosed in testicular cords formed by Sertoli precursor cells and peritubular myoid cells. Gonocyte is a general term that can be subcategorized into mitotic (M)-prospermatogonia, T1-prospermatogonia and T2-propsermatogonia (McCarrey 1993). M-prospermatogonia are located in the centre of the testicular cords, away from the basal membrane and continue proliferating until about 16.5 dpc of mouse development when they become T1-prospermatogonia and enter the G0 stage of mitotic arrest (McLaren 2003; Tohonen et al., 2003). Gonocytes resume proliferation during the first week after birth (marking their transition to T2-prospermatogonia), concomitant with migration to the seminiferous tubules basement membrane (Clermont & Perey 1957). T2-prospermatogonia that colonize the basement membrane give rise to the first round of spermatogenesis as well as establish the initial pool of SSCs that maintains spermatogenesis throughout postpubertal life (Kluin & de Rooij 1981; McCarrey 1993; Yoshida et al., 2006).

1.3 Dynamics of SSCs

The kinetics of sperm production were first described in rodents (Oakberg 1956) with the knowledge of the presence of adult stem cells, such as hematopoietic stem cells (HSCs), researchers hypothesized that germ cell differentiation in the testis required a stem cell population. The presence of a stem cell population responsible for continual sperm production in the testis was demonstrated in 1994 (Brinster & Zimmermann). They documented the first successful spermatogonial stem cell (SSC) transplantation in mice resulting in donor-derived spermatogenesis. SSCs are part of a subset of male germ cells called undifferentiated spermatogonia (Caires et al., 2010). This subset includes A_{single} (As) spermatogonia that are thought to be the SSCs and their progeny cells A_{paired} (Apr) and $A_{aligned}$ (Aal) spermatogonia (figure 1). SSCs are a self-renewing population of adult stem cells capable of producing progeny for a continual production of sperm by sexually mature males. They help in maintaining a constant supply of undifferentiated spermatogonia, which are critical to the initiation of spermatogenesis and the long-term production of sperm. One must be careful to distinguish between spermatogonial stem cells (SSC) and spermatogonia in general. The term "spermatogonia" is used collectively to refer to a continuum of cells ranging from those resting on the basement membrane (known as either $A_{isolated}$ or A_{single} spermatogonia), to proliferating sub-populations (A_{paired} to $A_{aligned}$, spermatogonia) to differentiating sub-populations [A_{1-4}, Intermediate and Type B spermatogonia]. The differentiating stages are committed to enter spermatogenesis, but whether the $A_{isolated}$ cells represent the only spermatogonia with a true stem cell nature is not yet fully clear. The differentiation of germ cells from diploid undifferentiated spermatogonia to mature and haploid spermatozoa is supported by the Sertoli cells of the seminiferous epithelium. The formation of the SSC population is dependent on the associated niche in the microenvironment in the seminiferous tubules of the testis. This niche environment supplies factors and provides interactions crucial for the survival and development of SSCs. It is usually composed of adjacent differentiated cells, the stem cells themselves, and the extracellular matrix surrounding these cells. The somatic cells produce factors, which aid in the extrinsic regulation of the SSC self-renewal/differentiation process. SSCs represent a model for the investigation of adult stem cells because they can be maintained in culture, and the presence, proliferation and the loss of SSCs in a cell population can be determined with the use of a transplantation assay.

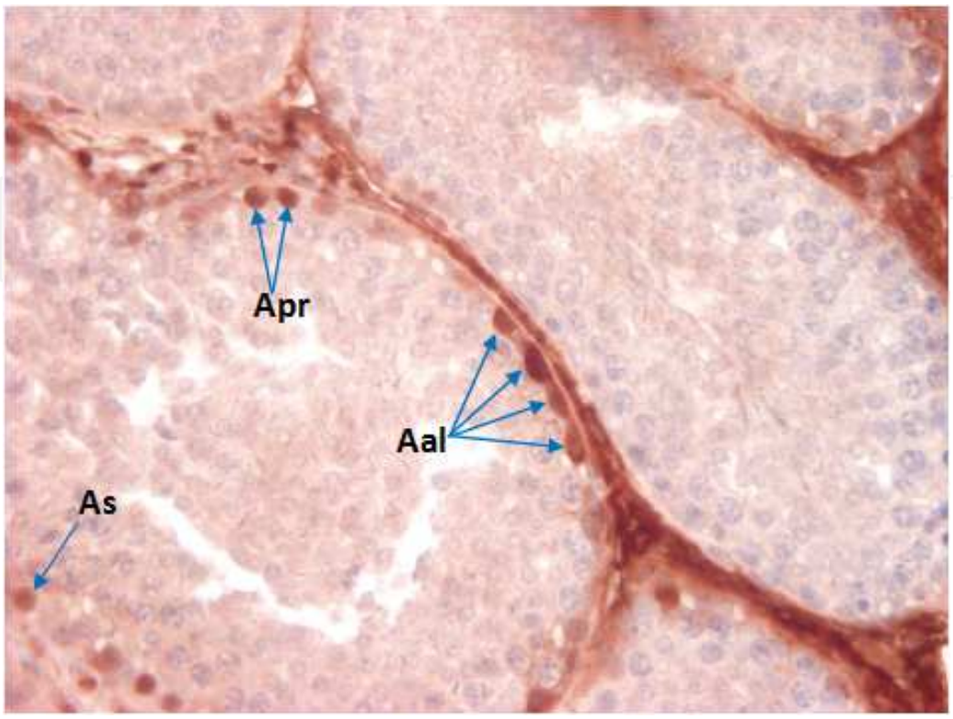


Fig. 1. Adult pig testis section stained with anti-UCHL-1 antibody. Spermatogonia are present as, A_{single} (As), A_{paired} (Apr) and $A_{aligned}$ (Aal) at the basement membrane of seminiferous tubule. The As spermatogonia are thought to be the spermatogonial stem cells (SSCs).

2. Identification of SSCs

2.1 Markers of SSCs

Exploring markers for spermatogonia help to identify the stem cell pool in the testis. Certain rodent markers for germ cells and SSCs such as VASA, DAZL, PLZF, and GFRα1are also of monkey (Hermann et al., 2007) and in other species. VASA expression marks spermatogonia in sheep (Borjigin et al., 2010), buffalo (Goel et al., 2010b) and bull (Fujihara et al., 2011) testis. It has been demonstrated that GFRα1 is a marker for mouse SSCs and probably their progeny (Meng et al., 2000; Buageaw et al., 2005; Hofmann et al., 2005; Naughton et al., 2006; He et al., 2007), and that KIT is a characteristic for the more differentiated spermatogonia, including type A_{1-4} spermatogonia (Yoshinaga et al., 1991). KIT expression is shown to mark pig gonocytes (Goel et al., 2007) and spermatogonia (Dirami et al., 1999). PLZF marks pig (Goel et al., 2007) and sheep (Borjigin et al., 2010) gonocytes and type-A spermatogonia. Because there is not a unique marker available to distinguish the SSCs and other undifferentiated spermatogonia, called A_{pr} and A_{al}, it is helpful to use two or three antibodies to characterize their phenotypes. The GFRα1 and POU5F1 antibodies stain the

same subset of mouse spermatogonia (He et al., 2007). POU5F1 also marks spermatogonia in buffalo (Goel et al., 2010b) and bull (Fujihara et al., 2011). GPR125 is also believed to be a marker for mouse spermatogonial stem/progenitor cells (Seandel et al., 2007). It is speculated that some of these markers as mentioned above will also be applicable to human spermatogonia. It has been recently shown that GPR125 may be a marker for human SSCs, as it is for mouse SSCs (He et al., 2010).

A comparison of the markers for spermatogonia and their progenitors in human and rodents indicates that these spermatogonia share many but not all phenotypes. In rodents, α6-integrin (CD49f), β1-integrin (CD29), and Thy-1 (CD90) are surface markers for mouse spermatogonial stem/progenitor cells (Shinohara et al., 1999; Kubota et al., 2003). THY-1 also marks bovine spermatogonia (Reding et al., 2010). CD9 is a surface marker for mouse and rat spermatogonial stem/progenitor cells (Kanatsu-Shinohara et al., 2004a). GFRα1 and RET are co-receptors for GDNF and markers for spermatogonial stem/progenitor cells (Buaas et al., 2004; Costoya et al., 2004; Buageaw et al., 2005; Hofmann et al., 2005; Naughton et al., 2006). In human, α6-integrin is expressed in spermatogonia and their progenitors and was used to isolate and purify human spermatogonial cells by magnetic-activated cell separation (Conrad et al., 2008). Other rodent surface markers, such as CD90, GFRα1, and CD133, were also used to select human spermatogonia by magnetic-activated cell separation (MACS) and comparable results to α6-integrin were obtained (Conrad et al., 2008). PLZF is characterized as a hallmark for mouse spermatogonial stem/progenitor cells (Buaas et al., 2004; Costoya et al., 2004). In adult monkey, the expression of PLZF is confined to the A_{dark} and/or A_{pale} spermatogonia (Hermann et al., 2007). GPR125 has been demonstrated to be expressed in mouse spermatogonia and their progenitors (Seandel et al., 2007), and it is recently reported that GPR125 is also present in human spermatogonia (Dym et al., 2009). UCHL-1 is known to express in spermatogonia of mice (Kwon et al., 2004), monkey (Tokunaga et al., 1999) and humans (He et al., 2010). UCHL-1 protein expression is present specifically in the spermatogonia of domestic animal testes such as bull (Wrobel et al., 1995; Herrid et al., 2009), pig (Luo et al., 2006; Goel et al., 2007), sheep (Rodriguez-Sosa et al., 2006) and buffalo (Goel et al., 2010b). UCHL-1 protein expression is also specific to spermatogonia of wild bovids (Goel et al., 2010a). UCHL-1 also marks spermatogonia in the testis of Indian mouse deer (*Moschiola indica*) and slender loris (*Loris tardigradus*) (unpublished data). It is therefore likely that the expression of UCHL-1 is conserved in a variety of species. Collectively, the above studies suggest that some spermatogonial markers are conserved between rodents and humans and other species. In contrast, some other rodent markers for spermatogonia and their progenitors are not applicable to human and other species. This can be illustrated by the fact that α1-integrin (CD29), a marker for rodent spermatogonial stem/progenitor cells, is not expressed in human spermatogonia but present in spermatocytes, spermatids, and spermatozoa in normal human testis (Schaller et al., 1993). Another example is that POU5F1 (Oct-4), a marker for mouse spermatogonial stem/progenitor cells (Ohbo et al., 2003; Ohmura et al., 2004; Hofmann et al., 2005), is not detected in adult human spermatogonia (Looijenga et al., 2003). POU5F1 shows a rather unique expression pattern in pig testis where spermatogonia show a weak staining, however, strong expression is present in differentiating germ cells such as spermatocytes and spermatids (Goel et al., 2008). Similarly, KIT is regarded as a marker for mouse differentiating spermatogonia (Yoshinaga et al., 1991; Schrans-Stassen et al., 1999; Dolci et

al., 2001), but it is undetected in human spermatogonia (Rajpert-De Meyts et al., 2003). Notably, some human markers for spermatogonia are also not applicable to rodents. As an example, the TSPY protein is preferentially expressed in elongated spermatids but not in spermatogonia of adult rat testis (Kido and Lau, 2006), unlike the expression pattern of the TSPY in adult human spermatogonia (Schnieders et al., 1996). Other rodent markers, including CD9 (Kanatsu- Shinohara et al., 2004b), CDH1 (Tokuda et al., 2007), neurogenin3 (Yoshida et al., 2004, 2007), RET (Naughton et al., 2006), and STRA8 (Giuili et al., 2002), were demonstrated to be expressed in spermatogonia and their progenitors; however, whether these rodent markers are present in human spermatogonia remains to be clarified. Similarly, some human markers, such as CD133 (Conrad et al., 2008), CHEK2 (also known as chk2 tumor suppressor protein) (Bartkova et al., 2001; Rajpert-De Meyts et al., 2003), and NSE (Neurone-specific enolase) (Rajpert-De Meyts et al., 2003), are also awaiting further studies to explore whether they are present in rodent spermatogonia and their progenitors. Such investigations would uncover further similarities and/or differences in spermatogonial phenotypes between human and rodents.

2.2 Functional assay of SSCs

Transplantation of isolated germ cells from a fertile donor male into the seminiferous tubules of infertile recipients can result in donor-derived sperm production. Therefore, this system represents a major development in the study of spermatogenesis and a unique functional assay to determine the developmental potential and relative abundance of spermatogonial stem cells in a given population of testis cells. The application of this method in farm animals has been the subject of an increasing number of studies, mostly because of its potential as an alternative strategy in producing transgenic livestock with higher efficiency and less time and capital requirement than the current methods such as microinjection of genes into fertilized eggs and somatic cell nuclear transfer.

Germ cell transplantation (GCT), also referred to as spermatogonial stem cell (SSC) transplantation, is a powerful technology first introduced in 1994 by Brinster and colleagues. Although initially developed using a mouse model, GCT has important applications in the study and manipulation of spermatogenesis in many species. In this method, testis cells obtained from a fertile donor male are transferred into the seminiferous tubules of infertile recipient testes, where donor-derived sperm production can occur, allowing the recipient to sire progeny (Brinster & Avarbock, 1994; Brinster & Zimmermann, 1994). In essence, donor SSCs deposited in the lumen of the recipient seminiferous tubules are allowed by the Sertoli cells to migrate to the basolateral compartment of the tubule, to proliferate, form new colonies and initiate donor-derived spermatogenesis (Nagano et al., 1999; Ohta et al, 2000). Following the original introduction of GCT in mice (Brinster & Avarbock, 1994; Brinster & Zimmermann, 1994), the technique was also successful in rats (Jiang & Short, 1995;Ogawa et al., 1999b), monkey (Schlatt et al., 2000a) and goat (Honaramooz et al., 2003). In laboratory rodents, GCT not only provides a unique opportunity for gaining a new insight into spermatogenesis and the biology of the stem cell niche, but also presents a unique functional bioassay to test the competence of putative SSCs. Furthermore, GCT also offers a new strategy for preservation of male fertility and an alternative approach for generation of transgenic animals (Brinster 2002, 2007).

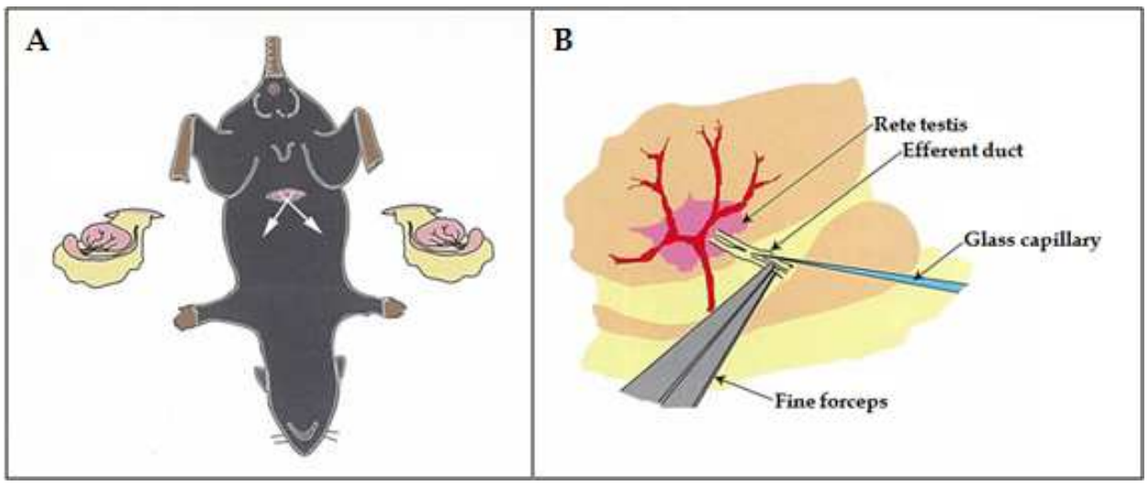


Fig. 2. Germ cell transplantation (GCT) in mice. (A) Germ cell depleted mice testes are extoriated through a ventral incision. (B) The testicular cell suspension is injected into seminiferous tubules through the efferent duct using a fine glass capillary.

Rather surprisingly, cross-species (xenogeneic/heterologous) transplantation of testis cells from donor rats and hamster into recipient mice resulted in complete rat (Clouthier et al., 1996) and hamster (Ogawa et al., 1999a) spermatogenesis. This development sparked an interest in the idea of using the laboratory mouse as a universal recipient model for testis cells from different donor species. However, GCT from genetically more distant donor species, including farm animals, into mice only resulted in colonization or proliferation of SSCs but not in complete spermatogenesis (Nagano et al., 2001, 2002; Dobrinski et al., 2000; Kim et al., 2006). We have recently shown that spermatogonia from endangered wild bovid (the Indian black buck; *Antelope cervecapra L.*) were able to colonize the recipient mice testis following GCT, however, showed no signs of proliferation or differentiation (Goel et al., 2011). This block in differentiation of donor germ cells is believed to be due to the incompatibility of donor germ cells and mouse Sertoli cells (Shinohara et al., 2003). Although GCT from nonrodent species into the mouse testis did not result in complete spermatogenesis, it is still the only available bioassay for detecting the colonization potential of SSCs in a given population of donor testis cells from any species (Dobrinski et al., 1999a, b, 2000). Interestingly, when (rather than transferring isolated testis cells into the seminiferous tubules) small fragments of testis tissue were transplanted under the back skin of recipient mice, complete donor-derived spermatogenesis was observed from a wide range of donor species, including farm animal species (Honaramooz et al., 2002, 2004; Schlatt et al., 2002b; Oatley et al., 2005; Rathi et al., 2006, 2008; Arregui et al., 2008; Ehmcke et al., 2008; Nakie et al., 2010).

3. Pluripotential ability of germ stem cells in testis

3.1 Introduction

In the years 2006 and 2007, many articles were published, where somatic skin cells could be reprogrammed to ES-like cells, the so-called induced pluripotent stem (iPS) cells. Each report on the induction of pluripotency in mouse and human skin fibroblasts used retroviral delivery of key pluripotent stem cell genes such as, Oct-4 (Pou5f1), Sox2, c-Myc, and Klf4

(Takahashi and Yamanaka, 2006; Hanna et al., 2007; Meissner et al., 2007; Okita et al., 2007; Takahashi et al., 2007; Wernig et al., 2007). In the second step, transformed iPS cells were identified and selected by expression of pluripotent markers including Nanog (Okita et al., 2007; Wernig et al., 2007) or Oct-4 (Wernig et al., 2007), or by ES-specific morphology (Meissner et al., 2007). These iPS cells had unique characteristics as they were germ-line competent and indistinguishable from ES cells derived from the embryo at the epigenetic level. Additionally, recent work has demonstrated that patient-autologous skin iPS cells can be genetically modified and used after differentiation by induction to cure a mouse model of sickle cell anemia (Hanna et al., 2007). Although this research paves the way toward stem cell therapy, it seems to be impractical for the iPS cells to be used in clinical application because of their instability and potential retroviral infections (Dym et al., 2009).

As a result, it is essential and necessary to figure out more physiological methods to induce pluripotency from adult somatic cells or adult stem cells. More recent efforts have been taken to generate iPS cells from neural stem cells using only two transcription factors, Oct-4 and either c-Myc or Klf4 (Kim et al., 2008). It has also been demonstrated that iPS cells can be produced from adult cells by non-integrating adenoviruses transiently expressing Oct-4, Sox2, Klf4, and c-Myc (Stadtfeld et al., 2008). However, it is still possible that these factors could somehow find their way into the genome of the iPS cells. Therefore, reprogramming of adult cells without the use of oncogenes would be very useful and safer as a means to produce ES-like cells. The proof of principle that spermatogonial stem cells/progenitor cells could be reprogrammed to pluripotent cells by biochemical means alone was first shown by Shinohara and colleagues (Kanatsu-Shinohara et al., 2004b). However, they could not derive ES-like cells from SSCs of adult mice. Guan et al. (2006) demonstrated that mouse adult spermatogonia, possibly the spermatogonial stem cells and/or their progeny, were able to reprogram biochemically to pluripotent ES-like cells. This was confirmed in mouse by (Seandel et al., 2007) showing that adult spermatogonia and/or their progenitors could indeed form pluripotent ES-like cells. Golestaneh and others (Conrad et al., 2008; Kossack et al., 2008; Golestaneh et al., 2009) have recently demonstrated a similar phenomenon in male germ cells and spermatogonia in the human testis. It is important to note that the SSCs/progenitor cells appear to reprogram spontaneously to pluripotency when the cells are removed from their niche and when ES cell media is added. Thus, SSCs/progenitor cells have a great potential to be used as a safe means to generate ES-like cells that eventually can be used for clinical therapies of human diseases. Human ES cells are pluripotent stem cells that have the potential to differentiate into all the types of cell lineages and tissues in the body, and thus they are ideal cell sources for cell transplantation and gene therapy. However, the major concern is the ethical issues associated with obtaining human ES cells from IVF clinics. The human iPS cells have major advantages over human ES cells because there are no ethical issues involved and, more importantly, the iPS cells appear to be similar to ES cells in morphology, proliferation, and pluripotency, as evaluated by teratoma formation and chimera contribution. In contrast, the iPS cells have some disadvantages, e.g., safety is a major concern due to the potential of cell transformation or tumor formation because of the oncogenes from the transfected iPS. It may be possible to overcome these issues by generating pluripotent stem cells without oncogene transfer directly from spermatogonial stem cells by testicular biopsy. Thus, the generation of human ES-like cells from SSCs may offer a safer means of obtaining pluripotent stem cells than from the iPS cells. The identification of human SSCs is now especially important in view of the discrepancy between the Skutella report (Conrad et al., 2008) and the report by the Reijo-

Pera group (Kossack et al., 2008). The Skutella group concluded that the ES-like cells derived from human spermatogonia (Spga) were in fact as pluripotent as true embryonic stem (ES) cells. The Reijo-Pera group noted that their cells differ from true ES cells in gene expression, methylation, and in their ability to form teratomas. Comparisons are difficult because the Skutella group used isolated Spga to get their ES-like cells, whereas the Reijo-Pera group used the entire testis biopsy without separating the Spga. It is possible that ES-like cells derived from isolated spermatogonial stem cells yield superior ES-like cells compared to using whole testis, but this remains to be determined. There are now three means to generate human pluripotent stem cells: (1) from a fertilized embryo as the traditional method; (2) from adult somatic cells through iPS technology; and (3) from adult SSCs and/or their progeny. One major advantage of the third approach is that the production of the ES-like cells is spontaneous, unlike method 2, where several genes, some cancer causing, is employed. Thus, human SSCs and/or their progeny have a great potential for cell- and tissue engineering-based medical regeneration for various human diseases. It is possible that in the near future men could be cured of their diseases using a biopsy from their own testes (Dym et al., 2009).

3.2 Culture of SSCs and induction of ES-like colonies

Kanatsu-Shinohara et al. (2004b) cultured SSCs in such a way that these cells propagated themselves, while retaining their capacity to repopulate a recipient mouse testis upon transplantation. A special medium, which was designed to culture hematopoietic stem cells, has been used for culture and contained several growth factors, including GDNF. In this culture system, a feeder layer is first formed that is composed of the contaminating somatic cells of the neonatal testis. Then, after 2 weeks and two passages, mitomycin-treated mouse embryonic fibroblasts (MEFs) are used as a feeder layer. During the first weeks of culture, the formed colonies consisted of SSCs, but, within 4-7 weeks, colonies that morphologically resembled ES cell colonies formed. Further work indicated that these colonies were indeed composed of multipotent ES-like cells. In order to maintain the multipotent character of these ES-like cells, they were subsequently cultured under a standard ES cell culture conditions in medium containing 15% fetal calf serum and LIF. Under these conditions, the cultured SSCs could not be propagated because of the lack of GDNF. ES-like colonies could only be obtained when the starting population of SSCs was derived from neonatal mice; when it was derived from older mice, ES-like colonies did not appear. However, cultures of SSCs derived from adult *p53* (*Trp53*)-null mice did produce ES-like colonies. P53 is involved in the cellular response to DNA damage and a lack of P53 increases the chances of teratoma development. Possibly, P53-deficient SSCs are more capable of undergoing the transition into ES-like cells.

An essentially similar protocol was followed by Seandel et al. (2007), except that this group used inactivated testicular stromal cells consisting of a mixture of $CD34^+$ peritubular cells, α -smooth-muscle-actin-positive peritubular cells and cells positive for the Sertoli cell marker vimentin, as a feeder layer because they had less success using MEFs. By this method, ES-like colonies only appeared after more than 3 months in culture, more slowly than reported by Kanatsu-Shinohara et al. (2004b). A substantially different approach was taken by Guan et al. (2006). Their starting material was derived from 4- to 6-week-old mice and they did not use the stem cell medium described by Kanatsu-Shinohara et al. (2004b) but simply Dulbecco's Modified Eagle's Medium (DMEM) with serum and added GDNF, in which testicular cells were initially cultured for 4 to 7 days. These cells were then sorted for the

expression of STRA8 and subsequently cultured in DMEM under various conditions, but without adding GDNF. Colonies of ES-like cells formed when LIF was added to the medium and/or when the cells were cultured on a feeder layer of MEFs. The ES-like cells were further expanded by culture on MEFs and the addition of LIF.

Hu et al. (2007) cultured germ cells of prepubertal mice under conditions that favour osteoblast differentiation and reported the emergence of cells that had characteristics of osteoblasts after several weeks in culture. In this system, there was no period of culture with added GDNF. Finally, Boulanger et al. (2007) employed no culture step at all. This group transplanted cells isolated from adult mouse seminiferous tubules, together with mammary cells, into mammary fat pads to obtain the differentiation of SSCs into mammary epithelial cells.

We have shown that gonocytes from neonatal pig testis can attain multipotency during short-term culture (Goel et al., 2009). Freshly isolated gonocytes were found to have either weak or no expression of pluripotency determining transcription factors, such as *POU5F1*, *SOX2* and *C-MYC*. Interestingly, the expression of these transcription factors, as well as other vital transcription factors, such as *NANOG*, *KLF4* and *DAZL*, were markedly upregulated in cultured cells. The formation of teratomas with tissues originating from the three germinal layers following the subcutaneous injection of cells into nude mice from primary cultures confirmed their multipotency.

Taken together, it does not seem that a very specific approach is required to obtain the transformation of SSCs into ES-like cells. This transformation can occur on different feeder layers and even without a feeder layer, if LIF is added to the culture medium. Furthermore, the culture medium also does not seem to play a decisive role in the transformation of SSCs into ES-like cells, as the groups of Kanatsu-Shinohara et al. (2004b) and Seandel et al. (2007) used a specific stem cell medium, whereas Guan et al. (Guan et al., 2006) used DMEM. All three groups added GDNF to the culture, either continuously (Kanatsu-Shinohara et al., 2004b; Seandel et al., 2007) or only at the start (Guan et al., 2006). However, to obtain the transformation of SSCs into cells of another lineage, it might not be necessary for them to become ES-like cells first. Putting the SSCs in an osteoblast-inductive environment in culture (Hu et al., 2007) or transplanting them into a mammary gland-inductive environment in vivo (Boulanger et al., 2007) might be enough for these cells to change their lineage. This rather suggests that SSCs are restricted to the spermatogenic lineage owing to the seminiferous tubular environment in which they reside. Once outside of this environment, they can switch to another lineage depending on the particular niche in which they are placed.

3.3 Gene expression in SSCs and ES-like cells

A crucial question is what changes in gene expression accompany the transition from a cultured SSC to an ES-like cell? In this respect, it is interesting to study the possible changes in the expression of those genes that can transform a fibroblast into an ES-like cell, that is *Myc, Oct4* (*Pou5f1*), *Sox2* and *Klf4* (Takahashi and Yamanaka, 2006; Wernig et al., 2007), in SSCs and in the ES-like cells derived from them. Kanatsu-Shinohara et al. (2008b) found that all four pluripotency genes are already expressed at low levels in cultured SSCs, although no NANOG (Kanatsu-Shinohara et al., 2004b) or SOX2 protein expression was found in these cells. In ES-like cells, the expression of these four genes is much increased. In addition to these pluripotency genes, the ES cell markers such as stage-specific embryonic antigen-1

(SSEA-1; FUT4) and, to a low level, Forssman antigen (GBGT1), were induced in the ES-like cells and, as in ES cells, high levels of alkaline phosphatase (AP) were also found (Kanatsu-Shinohara et al., 2004b). Guan et al. (2006) assayed expression patterns in SSCs cultured under conditions that induced these cells to become ES-like cells. In this situation, it is difficult to categorize these cells as being either SSCs or ES-like cells as they might be in an in-between state. In these SSCs/early ES-like cells, *Oct4, Nanog* and *SSEA1* were expressed (Guan et al., 2006). Indeed, the level of expression of *Nanog* and *SSEA1* suggests that these cells were already on their way to becoming ES-like cells. Seandel et al. (2007) also studied gene expression levels before and after the transition of cultured SSCs to ES-like cells. *Oct4* was present in both cell types, but *Nanog* and *Sox2* were strongly induced in ES-like cells, whereas the early spermatogonial markers *Stra8, Plzf* (*Zbtb16*), *c-Ret* and *Dazl* became inhibited. Besides these specific studies, Kanatsu-Shinohara et al. (2008b) also carried out a microarray study and found that a great many genes changed their expression levels during the transition from being a cultured SSC to an ES-like cell. Among these genes, over a 100 were induced more than 5-fold in ES-like cells as compared with cultured SSCs, and another 100 were inhibited more than 5-fold in ES-like cells. Clear differences between the patterns of genomic imprinting are also seen between cultured SSCs and ES-like cells. Kanatsu-Shinohara et al. (2004b) studied the imprinting pattern of three paternally (*H19, Meg3* and *Rasgrf1*) and two maternally (*Igf2r* and *Peg10*) imprinted regions in cultured SSCs and in the ES-like cells derived from them. Cultured SSCs show a completely androgenetic (paternal) imprinting pattern at the differentially methylated regions (DMRs) of these genes and loci; the DMRs of *H19* and *Meg3* are completely methylated and that of *Igf2r* is demethylated. By contrast, in the ES-like cells, the paternally imprinted regions are methylated to different degrees and the maternally imprinted regions (*Igf2r* and *Peg10*) are rarely methylated. Interestingly, the methylation patterns that are seen in the ES-like cells are not the same as those seen in proper ES cells, as the DMRs in ES cells are generally more methylated than those in ES-like cells, including the maternally imprinted regions. Furthermore, both Kanatsu-Shinohara et al. (2004b) and Seandel et al. (2007) reported that most of the ES-like cells obtained had a normal karyotype and that there was no evidence of clonal cytogenetic abnormalities. However, recently, Takahashi et al. (2007) did find some SSC-derived ES-like cells that were trisomic for chromosomes 8 or 11, which is a common chromosomal abnormality in ES cells.

In conclusion, the transition from cultured SSCs to ES-like cells is accompanied by extensive changes in gene expression, during which three of the four pluripotency genes (the exception being *Oct4*, which is already expressed in mouse SSCs) become expressed at higher levels, along with many other genes. Furthermore, changes occur in the genomic imprinting patterns of these cells as they undergo this transition. Although the ES-like cells acquire the expression of ES cell-specific genes, the expression pattern of these genes in ES-like cells is not identical to that seen in normal ES cells, with differences evident, for example, in the expression of brachyury, *Gdf3*, Forssman antigen, *Nog* and *Stra8*.

4. Applications of SSCs

4.1 Restoration of fertility

SSC transplantation may have application for treating male infertility in some cases. For example, high-dose chemotherapy and total body radiation treatment of cancer can cause permanent infertility. While adult men can cryopreserve a semen sample prior to their

oncologic treatment, this is not an option for pre-adolescent boys who are not yet making sperm. Using methods similar to those already established for other species, it may be possible for these young cancer patients to cryopreserve testis cells or tissue prior to cancer treatment and use those tissues to achieve fertility after they are cured (Orwig & Schlatt, 2005; Goossens et al., 2008; Hermann et al., 2009). Recently, a non-human primate model of cancer survivorship to test the safety and feasibility of SSC transplantation in a species that is relevant to human physiology has been established (Hermann et al., 2007). Although SSC transplantation is not yet ready for the human fertility clinic, it may be reasonable for young cancer patients, with no other options to preserve their fertility, to cryopreserve testicular cells (Schlatt et al., 2009). Ginsberg and co-workers have been cryopreserving testicular tissue for young cancer patients since 2008 and report that this intervention is acceptable to parents and that testicular biopsies caused no acute adverse effects (Ginsberg et al., 2010). A human SSC culture system would be particularly useful in this setting because a few SSCs could be obtained in a small biopsy and expanded to a number sufficient for transplantion therapy.

4.2 Genetic modification

4.2.1 Genetic modification of rodents

The establishment of SSCs led to the development of a new strategy for generating a genetically modified animal as an alternative or a potentially superior method to the conventional ES-based technology. First, SSCs are infected or transfected with a viral or plasmid vector carrying a drug-resistant gene. Individual clones of drug-resistant SSCs cells are selected and expanded in vitro. After DNA analysis, genetically modified SSCs are transplanted to infertile mice. In theory, half of spermatozoa that developed from the SSCs carry the transgene. Finally, recipient male mice are crossed with female mice to yield heterozygous transgenic mice with a theoretical success rate of 50%. In the conventional ES cell-based technology, the frequency of heterozygous transgenic mice depends on the properties of chimera mice. In some cases, no germline chimera can be found in the chimera population. In this respect, SSCs which are committed to spermatogenesis are advantageous. When SSCs were transfected with a plasmid vector bearing a drugresistant gene and used for the production of transgenic mice based on this method, approximately 50% of offspring derived from a drug-resistant clone maintained a transgene (Kanatsu-Shinohara et al., 2005). Mice lacking a specific gene (i.e. occludin) by homologous DNA recombination was then successfully produced (Kanatsu-Shinohara et al., 2006). Homozygous mutant mice showed signs of chronic gastritis, osteoporosis and a loss of acidic granule in the salivary glands, similar to the occludin knockout mice generated using ES cells (Saitou et al., 2000). From these finding, it was demonstrated that SSCs can be used for gene targeting in a similar manner to ES cells.

4.2.2 Application to generation of knockout animals in domestic species

The method for generating genetically modified mice based on ES cell technology has become a conventional experimental technique, and contributed to the functional analyses of many genes. Under current culture conditions, SSCs grow slower than ES cells. For example, clonal expansion from a single transfected SSCs requires mixing with non-transfected SSCs to maintain cell densities during drug selection. Thus, SSC-based transgenic technology is not yet useful as ES cell-based technology. However, SSC-based

technology is potentially applicable to animal species other than mouse, and this is the greatest advantage of this technology. At present, ES cell-based transgenesis is feasible only in mice, and cloning by somatic cell nuclear transfer is extremely difficult or impossible due to the low proliferation potential of somatic cells and the poor success rates of nuclear transfer. If SSC culture conditions specific for animal species are established, animal transgenesis may become feasible in other species. Specifically, SSCs are derived from the testes of rat, hamster and cattle (Hamra et al., 2005; Ryu et al., 2005; Aponte et al., 2008; Kanatsu- Shinohara et al., 2008c). Hamsters have been historically difficult to manipulate genetically; however, the SSC technology may provide a good animal model. Bovine SSCs may be useful for the cattle industry as an important application. In particular, rats are important experimental animals that are larger than mice and have been used in various research areas. Therefore, SSC technology will be highly beneficial if it allows unlimited transgenesis in rats. Transplantation of SSCs is an essential step in SSC-based animal transgenesis and is technically challenging. The efficiency of this method is low in large animals (Ogawa et al., 1999b). Development of technologies for xenogeneic transplantation and in vivo spermatogenesis, in addition to the determination of culture conditions, are anticipated for universal application to various species in the future. With regard to xenogeneic transplantation from rats to mice, normal rat offspring were successfully born after transferring rat spermatids and spermatozoa developed in the mouse testes into rat oocytes by in vitro microinsemination (Shinohara et al., 2006; Kanatsu-Shinohara et al., 2008a).

4.3 Regenerative therapy

Recent advances in cellular therapies have led to the emergence of a multidisciplinary scientific approach to developing therapeutics for a wide variety of diseases and genetic disorders. Although most cell-based therapies currently consist of heterogeneous cell populations, it is anticipated that the standard of care needs well-characterized stem cell lines that can be modified to meet the individual needs of the patient. Extensive research in the area of regenerative medicine is focused on the development of cells, tissues and organs for the purpose of restoring function through transplantation. The general belief is that replacement, repair and restoration of function is best accomplished by cells, tissues or organs that can provide the appropriate physiological/metabolic functions more efficiently than any mechanical devices, recombinant proteins or chemical compounds. Several cell-based strategies are currently being investigated, including cell preparations from autologous parenchyma or established cell lines, as well as cell therapies derived from a variety of stem cell sources such as bone marrow or cord blood stem cells, embryonic stem cells, as well as cells, tissues and organs from genetically modified animals. Several lines of evidence have suggested extensive proliferation activity and pluripotency of germline stem cells, including SSCs. These characteristics provide new and unprecedented opportunities for the therapeutic use of SSCs for regenerative medicine.

Guan et al. (2006) succeeded in developing a procedure for the isolation and purification of SSCs from adult mouse testis. They were able to isolate and culture these cells in culture medium containing the precise combination of cellular growth factors needed for the cells to reproduce themselves *in vitro*. These cells were characterized with regard to their molecular profiles and these were compared via molecular profiling of embryonic stem cells using a stem cell array which contains relevant genes related to stem cell metabolism. The results

indicate that SSCs share many molecular characteristics with embryonic stem cells. On the cellular level, SSCs resemble embryonic stem cells; they form an embryoid body structure after 2 weeks of culture. Stem cell potential of isolated SSCs was examined using the transplantation technique. This method allowed SSCs to recolonize the seminiferous tubules of germ cell-depleted mice and regenerate spermatogenesis. These cells are able to differentiate into various cell types of three germ layers *in vitro* (Guan et al., 2006, 2007). In contrast to ESCs, the use of SSCs for cell transplantation will allow establishment of individual cell-based therapy, because the donor and recipient are identical. In addition, any ethical problems are avoided.This approach provides an accessible *in vitro* model system for studies of mammalian gametogenesis, as well as for developing new strategies for reproductive engineering, infertility treatment and establishment of regenerative therapy.

4.4 Conservation biology

Thus far, most works on technologies of assisted reproduction in males have focused on mature spermatozoa. Successful semen collection, cryopreservation, and thawing techniques have been determined for a number of species. Advancements in the handling and storage of mature sperm have revolutionized the practice of both human and veterinary clinical reproductive medicine. In addition, these innovations have changed the nature of agriculture and agricultural economics, as artificial insemination has often supplanted natural breeding in intensive production regimens. Research on mature sperm is limited in that there are no cell culture systems that support spermatogenesis *in vitro*; therefore, sperm can only be obtained as primary cells from reproductively mature individuals. The stem cells that will produce sperm, on the other hand, are present in early neonates. Soon after birth in most species, gonocytes migrate to the basement membrane of the seminiferous cords; at this point in time the gonocytes transition into spermatogonia.

The development of reproductive technologies based on SSCs could preserve the breeding potential of males that die prior to puberty. This can be of great importance when the genetic contribution of a single individual could have significant impact on a long-term viability of a population. Examples of this would include attempts to preserve the genetic information represented in offspring of founder individuals in captive breeding programs, or attempts to propagate individuals with diseases that preclude natural mating. In addition, because SSCs can be maintained in culture, these cells are similar to ESCs in providing an opportunity for genetic manipulation that is not present in the terminally-differentiated spermatozoa. Efforts to take advantage of the attributes of SSCs have thus far focused on two technologies: spermatogonial stem cell transplantation (SSCT) and testis xenografting.

5. Conclusions

SSCs are the novel source of pluripotent stem cells that have an advantage over the iPS cells in numerous ways. Firstly, pluripotent cells from SSCs do not require the addition of any foreign genes as they are spontaneously generated in culture. Secondly, since pluripotent cells from SSCs are derived without viral transduction, they provide a safer alternative to iPS cells. Finally, SSCs and SSC-derived pluripotent cells can find immediate application in the field of regenerative medicine, fertility restoration, and genetic modification of mice and large animals and in the conservation of endangered species.

6. References

Aponte, P. M., Soda, T., Teerds, K. J., Mizrak, S. C., van de Kant, H. J. & de Rooij, D. G. (2008). Propagation of bovine spermatogonial stem cells in vitro, *Reproduction* 136(5): 543-57.

Arregui, L., Rathi, R., Megee, S.O., Honaramooz, A., Gomendio, M., Roldan, E.R. & Dobrinski, I. (2008). Xenografting of sheep testis tissue and isolated cells as a model for preservation of genetic material from endangered ungulates, *Reproduction* 136(1): 85–93.

Bartkova, J., Falck , J., Rajpert-De Meyts E, Skakkebaek, N.E., Lukas , J. & Bartek J. (2001). Chk2 tumour suppressor protein in human spermatogenesis and testicular germ-cell tumours, *Oncogene* 20(41): 5897-902.

Bendel-Stenzel, M., Anderson, R., Heasman , J. & Wylie, C. (1998). The origin and migration of primordial germ cells in the mouse, *Semin Cell Dev Biol* 9 (4): 393–400.

Borjigin, U., Davey, R., Hutton, K. & Herrid, M. (2010). Expression of promyelocytic leukaemia zinc-finger in ovine testis and its application in evaluating the enrichment efficiency of differential plating, *Reprod Fertil Dev* 22(5):733-42.

Boulanger, C. A., Mack, D. L., Booth, B. W. & Smith, G. H. *(*2007*).* Interaction with the mammary bovine spermatogonial stem cells in vitro, *Reproduction* 136 (5): 543–57.

Brinster, R.L. & Avarbock, M.R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation, *Proc Natl Acad Sci USA* 91(24): 11303–7.

Brinster, R.L. (2002). Germline stem cell transplantation and transgenesis, *Science* 296 (5576): 2174-6.

Brinster, R.L. (2007). Male germline stem cells: from mice to men, *Science* 316(5823): 404–5.

Brinster, R.L. & Zimmermann, J.W. (1994). Spermatogenesis following male germ-cell transplantation, *Proc Natl Acad Sci USA* 91(24): 11298–302.

Buaas, F.W., Kirsh, A.L., Sharma, M., McLean , D.J., Morris ,J.L., Griswold, M.D., de Rooij, D.G., Braun ,R.E. (2004). Plzf is required in adult male germ cells for stem cell self-renewal, *Nat Genet* 36(6): 647-52.

Buageaw, A., Sukhwani, M., Ben-Yehudah, A., Ehmcke, J., Rawe, V.Y., Pholpramool, C., Orwig, K.E., Schlatt, S. (2005). GDNF family receptor alpha1 phenotype of spermatogonial stem cells in immature mouse testes, *Biol Reprod* 73(5): 1011-6.

Caires, K., Broady, J. & McLean, D. (2010) Maintaining the male germline: regulation of spermatogonial stem cells. *J Endocrinol* 205(2): 133-45.

Clermont, Y. & Perey, B. (1957). Quantitative study of the cell population of the seminiferous tubules in immature rats, *Am J Anat* 100(2): 241–67.

Clouthier, D. E., Avarbock, M.R., Maika, S.D., Hammer, R.E. & Brinster, R.L. (1996). Rat spermatogenesis in mouse testis, *Nature* 381(6581): 418–21.

Conrad, S., Renninger, M., Hennenlotter, J., Wiesner, T., Just, L., Bonin, M., Aicher, W., Bühring, H.J., Mattheus, U., Mack, A., Wagner, H.J., Minger, S., Matzkies, M., Reppel, M., Hescheler, J., Sievert, K.D., Stenzl, A. & Skutella T. (2008). Generation of pluripotent stem cells from adult human testis, *Nature* 456 (7220): 344–9.

Costoya, J.A., Hobbs, R.M., Barna, M., Cattoretti, G., Manova, K., Sukhwani, M., Orwig, K.E., Wolgemuth, D.J., Pandolfi, P.P. (2004). Essential role of Plzf in maintenance of spermatogonial stem cells, *Nat Genet* 36(6): 653-9.

Dirami, G., Ravindranath, N., Pursel, V. & Dym, M. (1999). Effects of stem cell factor and granulocyte macrophage-colony stimulating factor on survival of porcine type A spermatogonia cultured in KSOM, *Biol Reprod* 61: 225-30.

Dobrinski, I., Avarbock, M.R. & Brinster, R.L. (1999a) Transplantation of germ cells from rabbits and dogs into mouse testes, *Biol Reprod* 61(5): 1331–9.

Dobrinski, I., Avarbock, M.R. & Brinster, R.L. (2000). Germ cell transplantation from large domestic animals into mouse testes, *Mol Reprod Dev* 57(3): 270–9.

Dobrinski, I., Ogawa, T., Avarbock, M.R. & Brinster, R.L. (1999b). Computer assisted image analysis to assess colonization of recipient seminiferous tubules by spermatogonial stem cells from transgenic donor mice, *Mol Reprod Dev* 53(2): 142–8.

Dolci, S., Pellegrini, M., Di Agostino, S., Geremia, R. & Rossi, P. (2001). Signaling through extracellular signal-regulated kinase is required for spermatogonial proliferative response to stem cell factor, *J Biol Chem* 276(43): 40225-33.

Dym, M., He, Z., Jiang, J., Pant, D. & Kokkinaki, M. (2009). Spermatogonial stem cells: unlimited potential, *Reprod Fertil Dev.* 21(1): 15-21.

Dym, M., Kokkinaki, M. & He, Z. (2009). Spermatogonial stem cells: mouse and humancomparisons. *Birth Defects Res C Embryo Today* 87(1): 27-34.

Ehmcke, J. & Schlatt, S. (2008). Animal models for fertility preservation in the male, *Reproduction*. 136(6): 717–23.

Fujihara, M., Kim, S.M., Minami, N., Yamada, M. & Imai, H. (2011). Characterization and In Vitro Culture of Male Germ Cells from Developing Bovine Testis, *J Reprod Dev*. 57(3): 355-64.

Ginsberg, J.P., Carlson, C.A., Lin, K., Hobbie, W.L., Wigo, E., Wu, X., Brinster, R.L. & Kolon, T.F. (2010). An experimental protocol for fertility preservation in prepubertal boys recently diagnosed with cancer: a report of acceptability and safety, *Hum Reprod* 25(1): 37-41.

Ginsburg, M., Snow, M.H. & McLaren, A. (1990). Primordial germ cells in the mouse embryo during gastrulation, *Development* 110(2): 521-8.

Giuili, G., Tomljenovic, A., Labrecque, N., Oulad-Abdelghani, M., Rassoulzadegan, M. & Cuzin, F. (2002). Murine spermatogonial stem cells: targeted transgene expression and purification in an active state, *EMBO Rep* 3(8): 753-9.

Goel, S., Sugimoto, M., Minami , N., Yamada, M., Kume, S. & Imai, H. (2007). Identification, isolation, and in vitro culture of porcine gonocytes, *Biol Reprod* 77(1): 127-37.

Goel, S., Fujihara, M., Minami, N., Yamada, M. & Imai, H. (2008). Expression of NANOG, but not POU5F1, points to the stem cell potential of primitive germ cells in neonatal pig testis, *Reproduction* 135(6): 785-95.

Goel, S., Fujihara, M., Tsuchiya, K., Takagi, Y., Minami, N., Yamada, M. & Imai, H. (2009). Multipotential ability of primitive germ cells from neonatal pig testis cultured in vitro, *Reprod Fertil Dev* 21(5): 696-708.

Goel, S., Mahla, R.S., Suman, S.K., Reddy, N. & Imai, H. (2010a). UCHL-1 Protein Expression Specifically Marks Spermatogonia in Wild Bovid Testis. *Euro J Wildlife Res* DOI: 10.1007/s10344-010-0454-1.

Goel, S., Reddy, N., Mandal, S., Fujihara, M., Kim, S.M. & Imai, H. (2010b). Spermatogonia-specific proteins expressed in prepubertal buffalo (Bubalus bubalis) testis and their utilization for isolation and in vitro cultivation of spermatogonia, *Theriogenology* 74 (7): 1221-32.

Goel, S., Reddy, N., Mahla, R.S., Suman, S.K., & Pawar, R.M. (2011). Spermatogonial stem cells in the testis of an endangered bovid: Indian Black buck (Antelope cervicapra L), *Animal Reproduction* Science DOI 10.1016/j.anireprosci.2011.05.012.

Golestaneh, N., Kokkinaki, M., Pant, D., Jiang, J., DeStefano, D., Fernandez-Bueno, C., Rone, J.D., Haddad, B.R., Gallicano, G.I. & Dym, M. (2009). Pluripotent stem cells derived from adult human testes. *Stem Cells Dev* 18(8): 1115-26.

Goossens, E., Geens, M., De Block, G. & Tournaye, H. (2008). Spermatogonial survival in long-term human prepubertal xenografts, *Fertil. Steril.* 90(5): 2019–22.

Guan, K., Wagner, S., Unsold , B. et al. (2007).Generation of functional cardiomyocytes from adult mouse spermatogonial stem cells, *Circ Res.* 100(11): 1615–25.

Guan, K., Nayernia, K., Maier, L. S., Wagner, S., Dressel, R., Lee, J. H., Nolte, J., Wolf, F., Li, M., Engel, W. & Hasenfuss, G. (2006). Pluripotency of spermatogonial stem cells from adult mouse testis, *Nature* 440(7088): 1199 -1203.

Hamra, F.K., Chapman, K.M., Nguyen, D. M.,Williams-Stephens, A. A., Hammer, R. E. & Garbers, D. L. (2005). Self renewal, expansion, and transfection of rat spermatogonial stem cells in culture, *Proc Natl Acad Sci USA* 102(48): 17430–5.

Hanna, J., Wernig, M., Markoulaki, S. Sun, C.W., Meissner, A., Cassady, J.P., Beard, C., Brambrink, T., Wu, L.C., Townes, T.M. & Jaenisch, R. (2007). Treatment of sickle cell anemia mouse model with iPS cells generated from autologous skin, *Science* 318(5858): 1920–3.

He, Z., Jiang, J., Hofmann, M.C. & Dym, M. (2007). Gfra1 silencing in mouse spermatogonial stem cells results in their differentiation via the inactivation of RET tyrosine kinase, *Biol Reprod* 77(4): 723-33.

He Z, Kokkinaki, M., Jiang, J., Dobrinski, I. & Dym, M. (2010).Isolation, characterization, and culture of human spermatogonia, *Biol Reprod* 82(2): 363-72.

Hermann, B. M., Sukhwani, M., Hansel, M. & Orwig, K. (2009). Spermatogonial stem cells in higher primates: are there differences to those in rodents? *Reproduction* 139: 479–493.

Hermann, B.P., Sukhwani, M., Lin, C.C., Sheng, Y., Tomko, J., Rodriguez, M., Shuttleworth, J.J., McFarland, D., Hobbs, R.M., Pandolfi, P.P., Schatten, G.P. & Orwig KE (2007). Characterization, cryopreservation and ablation of spermatogonial stem cells in adult rhesus macaques, *Stem Cells* 25(9): 2330–8.

Herrid, M., Davey, R.J., Hutton, K., Colditz, I.G. & Hill, J.R. (2009). A comparison of methods for preparing enriched populations of bovine spermatogonia, *Reprod Fertil Dev* 21(3): 393-9.

Hofmann, M.C., Braydich-Stolle, L., Dym, M. (2005 Mar). Isolation of male germ-line stem cells;influence of GDNF. *Dev Biol*. 279(1): 114-24.

Honaramooz, A., Behboodi, E., Blash, S., Megee, S.O. & Dobrinski, I. (2003). Germ cell transplantation in goats, *Mol Reprod Dev* 64(4): 422–8.

Honaramooz, A., Li, M.W., Penedo, M.C.T., Meyers, S. & Dobrinski, I. (2004). Accelerated maturation of primate testis by xenografting into mice, *Biol Reprod* 70(5): 1500–3.

Honaramooz, A., Megee, S.O. & Dobrinski, I. (2002). Germ cell transplantation in pigs, *Biol Reprod* 66(1): 21–8.

Honaramooz , A., Snedaker, A., Boiani , M., Schöler, H., Dobrinski, I. & Schlatt, S. (2002). Sperm from neonatal mammalia testes grafted in mice, *Nature* 418(6899): 778–81.

Hu, H. M., Xu, F. C., Li, W. & Wu, S. H. *(2007).* Biological characteristics of spermatogonial stem cells cultured in conditions for fibroblasts , *J Clin Rehab Tiss Engin Res* 11: 6611 -4.

Jiang, F.X. & Short, R.V. (1995). Male germ cell transplantation in rats: apparent synchronization of spermatogenesis between host and donor seminiferous epithelia, *Int J Androl* 18(6): 326–30.

Kanatsu-Shinohara, M., Toyokuni, S., Shinohara, T. (2004a). CD9 is a surface marker on mouse and rat male germline stem cells, *Biol Reprod* 70 (1): 70–5.

Kanatsu-Shinohara, M., Ikawa, M., Takehashi, M., Ogonuki, N., Miki, H., Inoue, K., Kazuki, Y., Lee, J., Toyokuni, S., Oshimura, M., Ogura, A. & Shinohara, T. (2006). Production of knockout mice by random and targeted mutagenesis in spermatogonial stem cells, *Proc. Natl Acad Sci USA* 103(21): 8018–23.

Kanatsu-Shinohara, M., Inoue, K., Lee, J., Yoshimoto, M., Ogonuki, N., Miki, H., Baba, S., Kato, T., Kazuki, Y., Toyokuni, S., Toyoshima M., Niwa O., Oshimura M., Heike T., Nakahata T., Ishino F., Ogura A., Shinohara T. (2004b). Generation of pluripotent stem cells from neonatal mouse testis, *Cell* 119(7): 1001 -12.

Kanatsu-Shinohara, M., Kato, M., Takehashi, M., Morimoto, H., Takashima, S., Chuma, S., Nakatsuji, N., Hirabayashi, M. & Shinohara, T. (2008a). Production of transgenic rats via lentiviral transduction and xenogeneic transplantation of spermatogonial stem cells, *Biol Reprod* 79(6): 1121–8.

Kanatsu-Shinohara, M., Lee, J., Inoue, K., Ogonuki, N., Miki, H., Toyokuni, S., Ikawa, M., Nakamura, T., Ogura, A. & Shinohara, T. (2008b). Pluripotency of a single spermatogonial stem cell in mice, *Biol Reprod* 78 (4): 681 -7.

Kanatsu-Shinohara, M., Toyokuni, S. & Shinohara, T. (2005). Genetic selection of mouse male germline stem cells in vitro: offspring from single stem cells, *Biol Reprod* 72(1): 236–40.

Kim, J.B., Zaehres, H., Wu, G., Gentile, L., Ko, K., Sebastiano, V., Araúzo-Bravo, M.J., Ruau, D., Han, D.W., Zenke, M. & Schőler, H.R. (2008). Pluripotent stem cells induced from adult neural stem cells by reprogramming with two factors, *Nature* 454(7204): 646-50.

Kim,Y., Selvaraj, V., Dobrinski, I., Lee, H., Mcentee, M.C. & Travis, A.J. (2006).Recipient preparation and mixed germ cell isolation for spermatogonial stem cell transplantation in domestic cats, *J Androl* 27(2): 248–56.

Kluin, P. M. & de Rooij, D. G. (1981). A comparison between the morphology and cell kinetics of gonocytes and adult type undifferentiated spermatogonia in the mouse, *Int J Androl* 4: 475–493.

Kossack, N., Meneses, J. & Shefi, S., Nguyen, H.N., Chavez, S., Nicholas, C., Gromoll, J., Turek, P.J., Reijo-Pera, R.A. (2009). Isolation and characterization of pluripotent human spermatogonial stem cell-derived cells, *Stem Cells* 27(1): 138–49.

Kubota, H., Avarbock, M.R. & Brinster, R.L. (2003). Spermatogonial stem cells share some, but not all, phenotypic and functional characteristics with other stem cells, *Proc Natl Acad Sci USA*100(11): 6487-92.

Kwon, J., Wang, Y.L., Setsuie, R., Sekiguchi, S., Sakurai, M., Sato, Y., Lee, W.W., Ishii, Y., Kyuwa, S., Noda, M., Wada, K. & Yoshikawa, Y. (2004). Developmental regulation of ubiquitinC-terminal hydrolase isozyme expression during spermatogenesis in mice, *Biol Reprod.* 71(2): 515-21.

Lawson, K.A., Dunn, N.R., Roelen, B.A., Zeinstra, L.M., Davis, A.M., Wright, C.V., Korving, J.P. & Hogan, B.L. (1999). Bmp4 is required for the generation of primordial germ cells in the mouse embryo, *Genes Dev* 13(4): 424–36.

Looijenga, L.H., Stoop, H., de Leeuw, H.P., de Gouveia Brazao, C.A., Gillis, A.J., van Roozendaal, K.E., van Zoelen, E.J., Weber, R.F., Wolffenbuttel, K.P., van Dekken, H., Honecker, F., Bokemeyer, C., Perlman, E.J., Schneider,D.T., Kononen, J., Sauter, G.& Oosterhuis, J.W. (2003). POU5F1 (OCT3/4) identifies cells with pluripotent potential in human germ cell tumors, *Cancer Res* 63(9): 2244-50.

Luo, J., Megee, S., Rathi, R. & Dobrinski, I. (2006). Protein gene product 9.5 is a spermatogonia-specific marker in the pig testis: application to enrichment and culture of porcine spermatogonia, *Mol Reprod Dev* 73(12): 1531-40.

McCarrey, J. (1993). Development of the germ cell, In: *Cell and molecular biology of the testis* (eds Desjardins, C. & Ewing, L. L.), (pp. 58–89), Oxford University Press , ISBN13: 9780195062694 , New York.

McLaren, A. (2003). Primordial germ cells in the mouse, Dev. Biol. 262(1): 1–15.

Meissner, A., Wernig, M. & Jaenisch, R. (2007). Direct reprogramming of genetically unmodified fibroblasts into pluripotent stem cells, *Nat Biotechnol* 25(10):1177–81.

Meng, X., Lindahl, M., Hyvönen, M.E., Parvinen, M., de Rooij, D.G., Hess, M.W., Raatikainen-Ahokas, A., Sainio K., Rauvala , H., Lakso, M., Pichel ,J.G., Westphal, H., Saarma, M. & Sariola, H. (2000). Regulation of cell fate decision of undifferentiated spermatogonia by GDNF, *Science* 287(5457): 1489-93.

Boulanger, C.A., Mack, D.L., Booth, B.W. & Smith, G.H. (2007) Interaction with the mammary microenvironment redirects spermatogenic cell fate in vivo, *Proc Natl Acad Sci USA* 104 (10): 3871 -6.

Nagano, M., Avarbock, M.R. & Brinster, R.L. (1999). Pattern and kinetics of mouse donor spermatogonial stem cell colonization in recipient testes, *Biol Reprod* 60(6): 1429–36.

Nagano, M., McCarrey, J.R. & Brinster, R.L. (2001). Primate spermatogonial stem cells colonize mouse testes, *Biol. Reprod.* 64(5): 1409–16.

Nagano, M., Patrizio, P. & Brinster, R.L. (2002). Long-term survival of human spermatogonial stem cells in mouse testes, *Fertility and Sterility* 78(6): 1225–33.

Nakai, M., Kaneko, H., Somfai,T., Maedomari, N., Ozawa, M., Noguchi, J., Ito, J., Kashiwazaki, N. & Kikuchi, K. (2010). Production of viable piglets for the first time using sperm derived from ectopic testicular xenografts, *Reproduction* 139(2): 331–5.

Naughton, C.K., Jain, S., Strickland, A.M., Gupta, A. & Milbrandt, J. (2006). Glial cell-line derived neurotrophic factor-mediated RET signaling regulates spermatogonial stem cell fate, *Biol Reprod* 74(2): 314-21.

Oakberg, E.F. (1956). Duration of spermatogenesis in the mouse and timing of stages of the cycle of the seminiferous epithelium, *Am J Anat* 99(3): 507-16.

Oatley, J.M., Reeves, J.J. & McLean, D.J. (2005). Establishment of spermatogenesis in neonatal bovine testicular tissue following ectopic xenografting varies with donor age, *Biol Reprod* 72(2): 358–64.

Ogawa, T., Dobrinski, I., Avarbock, M.R. & Brinster, R.L. (1999a). Xenogeneic spermatogenesis following transplantation of hamster germ cells to mouse testes, *Biol Reprod* 60(2): 515-21.

Ogawa, T., Dobrinski, I. & Brinster, R.L. (1999b). Recipient preparation is critical for spermatogonial transplantation in the rat, *Tissue and Cell* 31(5):461–72.

Ohbo, K., Yoshida, S., Ohmura, M., Ohneda, O., Ogawa, T., Tsuchiya, H., Kuwana, T., Kehler, J., Abe, K., Schöler, H.R. & Suda, T. (2003). Identification and characterization of stem cells in prepubertal spermatogenesis in mice small star, filled. *Dev Biol* 258(1): 209-25.

Ohta, H., Yomogida, K., Yamada, S., Okabe, M. & Nishimune, Y. (2000). Real-time observation of transplanted 'green germ cells': proliferation and differentiation of stem cells, *Dev Growth Differ* 42(2):105–12.

Okita, K., Ichisaka, T., Yamanaka, S. (2007). Generation of germline-competent induced pluripotent stem cells, *Nature* 448(7151): 313–17.

Orwig, K.E.& Schlatt, S. (2005). Cryopreservation and transplantation of spermatogonia and testicular tissue for preservation of male fertility, J Natl Cancer Inst Monogr 34: 51–6.

Phillips, B.T., Gassei, K. & Orwig, K.E.(2010). Spermatogonial stem cell regulation and spermatogenesis. *Philos Trans R Soc Lond B Biol Sci* 365(1546): 1663-78.

Rajpert-De Meyts, E., Jacobsen, G.K., Bartkova, J., Aubry, F., Samson, M., Bartek, J. & Skakkebaek, N.E. (2003). The immunohistochemical expression pattern of Chk2, p53, p19INK4d, MAGE-A4 and other selected antigens provides new evidence for the premeiotic origin of spermatocytic seminoma, *Histopathology* 42(3): 217-26.

Rathi, R., Honaramooz, A., Zeng, W., Turner, R. & Dobrinski, I. (2006). Germ cell development in equine testis tissue xenografted into mice, *Reproduction* 131(6): 1091–8.

Rathi, R., Zeng, W., Megee, S., Conley, A., Meyers, S. & Dobrinski, I. (2008). Maturation of testicular tissue from infant monkeys after xenografting into mice, *Endocrinology* 149(10): 5288–96.

Reding, S.C., Stepnoski, A.L., Cloninger, E.W. & Oatley, J.M. (2010). THY1 is a conserved marker of undifferentiated spermatogonia in the pre-pubertal bull testis, *Reproduction* 139(5): 893-903.

Rodriguez-Sosa, J.R., Dobson, H. & Hahnel, A. (2006). Isolation and transplantation of spermatogonia in sheep, *Theriogenology* 66(9): 2091-103.

Ryu, B.Y., Kubota, H., Avarbock, M.R. & Brinster, R.L. (2005). Conservation of spermatogonial stem cell self-renewal signalling between mouse and rat, *Proc Natl Acad Sci U S A* 102(40): 14302–07.

Saitou, M., Furuse, M., Sasaki, H., Schulzke, J. D., Fromm, M., Takano, H., Noda, T. & Tsukita, S. (2000). Complex phenotype of mice lacking occludin, a component of tight junction strands, *Mol. Biol. Cell* 11(12): 4131–42.

Schaller, J., Glander, H.J, Dethloff, J. (1993). Evidence of beta 1 integrins and fibronectin on spermatogenic cells in human testis, *Hum Reprod*. 8(11):1873-8.

Schlatt, S., Foppiani, L., Rolf, C., Weinbauer, G.F., Nieschlag, E. (2002a). Germ cell transplantation into X-irradiated monkey testes, *Hum Reprod.* 17 (1): 55–62.

Schlatt, S., Kim, S.S., Gosden, R. (2002b). Spermatogenesis and steroidogenesis in mouse, hamster and monkey testicular tissue after cryopreservation and heterotopic grafting to castrated hosts, *Reproduction* 124(3): 339-46.

Schlatt, S., Ehmcke, J., Jahnukainen, K. (2009). Testicular stem cells for fertility preservation: preclinical studies on male germ cell transplantation and testicular grafting, *Pediatr Blood Cancer* 53(2): 274–80.

Schnieders, F., Dörk, T., Arnemann, J., Vogel, T., Werner, M. &Schmidtke, J. (1996). Testis-specific protein, Y-encoded (TSPY) expression in testicular tissues, *Hum Mol Genet* 5(11): 1801-7.

Schrans-Stassen, B.H.G.J, van de Kant, H.J.G, de Rooij, D.G & van Pelt, A.M.M. (1999). Differential expression of c-kit in mouse undifferentiated and differentiating type A spermatogonia, *Endocrinology* 140(12): 5894-900.

Seandel, M., James, D., Shmelkov, S.V., Falciatori, I., Kim, J., Chavala, S., Scherr, D. S., Zhang, F., Torres, R., Gale, N. W. ancopoulos, G.D., Murphy, A., Valenzuela, D.M., Hobbs, R.M., Pandolfi, P.P. & Rafii, S. *(*2007*).* Generation of functional multipotent adult stem cells from GPR125+ germline progenitors, *Nature* 449 (7160): 346 -50.

Shinohara, T., Avarbock, M.R. & Brinster, R.L. (1999). beta1- and alpha6-integrin are surface markers on mouse spermatogonial stem cells, *Proc Natl Acad Sci USA* 96(10):5504-9.

Shinohara, T., Orwig, K.E., Avarbock, M.R, Brinster, R.L. (2003). Restoration of spermatogenesis in infertile mice by Sertoli cell transplantation, *Biol Reprod* 68(3): 1064–71.

Shinohara, T., Kato, M., Takehashi, M., Lee, J., Chuma, S., Nakatsuji,N., Kanatsu-Shinohara, M. & Hirabayashi, M. (2006). Rats produced by interspecies spermatogonial transplantation in mice and in vitro microinsemination, *Proc. Natl Acad. Sci. U S A* 103(37): 13624–8.

Stadtfeld, M., Nagaya, M., Utikal, J., Weir, G. &Hochedlinger, K. (2008). Induced pluripotent stem cells generated without viral integration, *Science* 322(5903): 945–46.

Takahashi, K., Tanabe, K., Ohnuki, M., Narita, M., Ichisaka, T., Tomoda, K., Yamanaka, S. (2007). Induction of pluripotent stem cells from adult human fibroblasts by defined factors, *Cell* 131(5): 861–72.

Takahashi, K. & Yamanaka, S. (2006). Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors, *Cell* 126(4): 663 -76.

Takehashi, M., Kanatsu-Shinohara, M., Miki, H., Lee, J., Kazuki, Y., Inoue, K., Ogonuki, N., Toyokuni, S., Oshimura, M., Ogura, A. & Shinohara, T. (2007). Production of knockout mice by gene targeting in multipotent germline stem cells, *Dev Biol* 312(1): 344 -52.

Tohonen,V., Ritzen, E. M., Nordqvist, K., Wedell, A. (2003). Male sex determination and prenatal differentiation of the testis, *Endocr Dev* 5: 1-23.

Tokuda, M., Kadokawa, Y., Kurahashi, H. & Marunouchi, T. (2007). CDH1 is a specific marker for undifferentiated spermatogonia in mouse testes, Biol Reprod 76(1): 130-41.

Tokunaga, Y., Imai, S., Torii, R. & Maeda, T. (1999). Cytoplasmic liberation of protein geneproduct 9.5 during the seasonal regulation of spermatogenesis in the monkey (Macaca fuscata), *Endocrinology* 140(4): 1875-83.

Wernig, M., Meissner, A., Foreman, R., Brambrink, T., Ku, M., Hochedlinger, K., Bernstein, B. E. & Jaenisch, R. (2007). In vitro reprogramming of fibroblasts into a pluripotent ES-cell-like state, *Nature* 448(7151): 318 -24.

Wrobel, K.H., Bickel, D., Kujat, R. & Schimmel, M. (1995). Configuration and distribution of bovine spermatogonia. *Cell Tissue Res.* 279(2): 277-89.

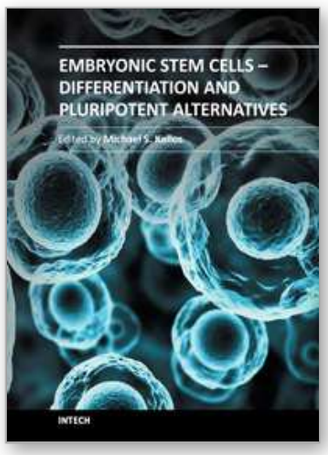
Ying, Y., Qi, X. & Zhao, G. Q. (2001). Induction of primordial germ cells from murine epiblasts by synergistic action of BMP4 and BMP8B signaling pathways, *Proc Natl Acad Sci USA* 98(14): 7858–62.

Yoshida, S., Sukeno, M. & Nabeshima, Y. (2007). A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis, *Science* 317(5845): 1722-6.

Yoshida, S., Sukeno, M., Nakagawa, T., Ohbo, K., Nagamatsu, G., Suda, T. & Nabeshima, Y.(2006) The first round of mouse spermatogenesis is a distinctive program that lacks the self-renewing spermatogonia stage, *Development* 133(8): 1495–1505.

Yoshida, S., Takakura, A., Ohbo, K., Abe, K., Wakabayashi, J., Yamamoto, M., Suda, T. & Nabeshima, Y. (2004). Neurogenin3 delineates the earliest stages of spermatogenesis in the mouse testis, *Dev Biol* 269(2): 447-58.

Yoshinaga, K., Nishikawa, S., Ogawa, M., Hayashi, S., Kunisada, T., Fujimoto, T. & Nishikawa, S. (1991). Role of c-kit in mouse spermatogenesis: identification of spermatogonia as a specific site of c-kit expression and function, *Development* 113(2): 689-99.



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The ultimate clinical implementation of embryonic stem cells will require methods and protocols to turn these unspecialized cells into the fully functioning cell types found in a wide variety of tissues and organs. In order to achieve this, it is necessary to clearly understand the signals and cues that direct embryonic stem cell differentiation. This book provides a snapshot of current research on the differentiation of embryonic stem cells to a wide variety of cell types, including neural, cardiac, endothelial, osteogenic, and hepatic cells. In addition, induced pluripotent stem cells and other pluripotent stem cell sources are described. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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