Spermatogonial stem cell regulation and spermatogenesis

Bart T. Phillips†, Kathrin Gassei† and Kyle E. Orwig*

Department of Obstetrics, Gynecology and Reproductive Sciences, Magee-Women Research Institute, University of Pittsburgh School of Medicine, 204 Craft Avenue, Pittsburgh, PA, USA

This article will provide an updated review of spermatogonial stem cells and their role in maintaining the spermatogenic lineage. Experimental tools used to study spermatogonial stem cells (SSCs) will be described, along with research using these tools to enhance our understanding of stem cell biology and spermatogenesis. Increased knowledge about the biology of SSCs improves our capacity to manipulate these cells for practical application. The chapter concludes with a discussion of future directions for fundamental investigation and practical applications of SSCs.

Keywords: spermatogonial stem cells; spermatogenesis; fertility

1. INTRODUCTION
Spermatogonial stem cells (SSCs) are at the foundation of spermatogenesis and male fertility. Similar to other tissue-specific stem cells, SSCs are rare, representing only 0.03 per cent of all germ cells in rodent testes (Tegelenbosch & de Rooij 1993). This is because SSCs are heavily outnumbered by the differentiating spermatogonia, spermatocytes, spermatids and sperm that they produce (detailed below). SSCs are defined like all other stem cells, by their ability to balance self-renewing divisions and differentiating divisions. This balance maintains the stem cell pool and meets the proliferative demand of the testis to produce millions of sperm each day. Studies of SSCs are complicated because these cells are few in number and no unique identifying characteristics have been reported to date. We will review experimental tools used to study SSCs and summarize current knowledge about the characteristics and regulation of these adult tissue stem cells. We will focus primarily on rodent models, which have generated the majority of data about SSCs and the spermatogenic lineage.

2. ORIGIN OF THE SPERMATOGONIAL STEM CELL POOL
SSCs arise from gonocytes in the postnatal testis, which arise 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 coitum (dpc). PGC specification 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 out of the embryo before they start migrating via the hindgut to arrive at the indifferent gonad 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-prospermatogonia (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 G0 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 maintain spermatogenesis throughout postpubertal life (Kluin & de Rooij 1981; McCarrey 1993; Yoshida et al. 2006).

3. THE SPERMATOCYTOGENIC CYCLE
Spermatogonial lineage development is a complex process, but occurs in an orderly manner, referred to as the spermatogonial cycle (Clermont 1972), which is divided in a species-specific number of stages or cell associations (i.e. 12 stages in the mouse (Oakberg 1956a,b) and 14 stages in the rat (Leblond & Clermont 1952a,b)). This synchronized spermatogenetic development may be facilitated by incomplete
cytokinesis during mitotic divisions that lead to maintenance of cytoplasmic bridges among germ cells. Proteins and messenger RNAs are exchanged via the cytoplasmic bridges and may help in coordinating the synchronized development of germ cell clones (Braun et al. 1989). Each stage is characterized by a combination of the types of spermatogonia, spermatocytes and spermatids that synchronously proceed through the spermatogenic process (figure 1). For example, the basement membrane of a stage V seminiferous tubule depicted in figure 1 is mostly filled with preleptotene primary spermatocytes (blue cells of a large clone). By stage VI, these spermatocytes migrate off the basement membrane and will be replaced by spermatogonia. Thus, stage V can be distinguished from stage VI by the presence or absence of spermatocytes on the basement membrane. The duration of each stage is precisely timed, and the complete spermatogenic cycle was determined to be around 8.6 days in the mouse (Oakberg 1956b), and 12.8 days in the rat (Hilscher et al. 1969). One complete cycle (12 stages) of the mouse seminiferous epithelium is depicted in figure 1.

4. SPERMATOGONIAL LINEAGE DEVELOPMENT

In order to understand the regulation of spermatogonial stem cells, it is important to understand them in the context of the spermatogenic lineage that they produce. Spermatogonia are primitive diploid germ cells, located on the basement membrane of the seminiferous tubules. Three types of spermatogonia were initially described based on their nuclear morphology (Roosen-Runge & Giesel 1950; Clermont & Leblond 1953; Monesi 1962). Type A spermatogonia were considered the most primitive because heterochromatin is absent from the nucleus, a general characteristic of undifferentiated cells. The nuclei of intermediate type spermatogonia contain a small amount of heterochromatin and type B spermatogonia contain a large amount of heterochromatin, indicating a more differentiated state.

Histological staining of whole-mount preparations of seminiferous tubules provided additional level of detail about spermatogonial morphometry compared with tissue sections alone and broadened the knowledge of the spermatogonial cell types in the testis. To facilitate the following discussion, figure 1 depicts one compete cycle of the mouse seminiferous epithelium and represents whole-mount perspective as well as corresponding cross-section and longitudinal section perspectives. Figure 1 traces the development of three putative clones (green, red and blue) through the spermatogenic process (figure 1). Chains of 4–16 Aal spermatogonia (see green clone in figure 1, stages IX, X and XI) or (ii) remains connected by an intercellular cytoplasmic bridge and produces a chain of four Aal spermatogonia at the next division (differentiating division, see red clone in figure 1, stages IX and X). Further cell divisions lead to the formation of chains of 8, 16 and sometimes 32 Aal spermatogonia (see red clone in stages XII and I and blue clone in stage VII, figure 1). Chains of 4–16 Aal are generally considered committed to the differentiation process. Thus, the stem cell pool includes Aa and at least some Apr spermatogonia. Some have argued that stem cell potential may extend to larger clones (e.g. Aa4 or beyond; (Yoshida et al. 2007a; Morimoto et al. 2009)), but this is difficult to confirm experimentally. Note that while each clone can be observed in histological sections as well as in whole-mount preparations of seminiferous tubules, clone size can only be observed in the whole-mount preparations (figure 1).

Aa, Apr and smaller chains of four Aal spermatogonia are evenly distributed along the seminiferous epithelium (Huckins 1971a,b; Tegelenbosch & de Rooij 1993). Larger chains of Aa (8, 16 and 32) become differentiating A1 spermatogonia between stages IV and VIII of the seminiferous epithelium (there is no cell division at this transition, see blue clone in figure 1, stages VII and VIII) and these give rise to A2 spermatogonia at stage IX (see blue clone in figure 1, stage IX). Thus, in contrast to undifferentiated spermatogonia, differentiating spermatogonia (A1, A2, A3, A4, intermediate and B) divide in a synchronized manner and are found at specific stages of the seminiferous epithelium (for detailed description see Oakberg 1971). B spermatogonia give rise to primary spermatocytes that progress into meiosis. Two meiotic divisions lead to the formation of secondary spermatocytes and haploid spermatids respectively, which undergo 16 steps of morphological changes to finally become spermatozoa ready to be released from the seminiferous epithelium (Oakberg 1956a).

An alternative to the Aa model of SSC self-renewal described above is the A0/A1 model (Clermont & Bustos-Obregon 1968; Dym & Clermont 1970; Clermont & Hermo 1975). This model is very similar to the Adark and Apale model that has been used to describe stem cell activity in non-human primates (Clermont & Leblond 1959; Clermont & Antar 1973). Briefly, A0 spermatogonia were observed as singles or pairs of cells that were present at all stage of the seminiferous epithelium. Mitotic figures were rarely observed in these cells, so they were considered 'reserve stem cells' not contributing to steady-state spermatogenesis. These reserve stem cells are only activated when spermatogenesis is destroyed by toxic insult (i.e. radiation). The 'active stem cell' pool is comprised of A1–A4 spermatogonia. When A4 spermatogonia divide, they give rise either to new A1 spermatogonia (self-renewal) or to intermediate spermatogonia (differentiation). While there continues to be vigorous debate about the merits of the Aa versus the A0/A1 models, the Aa model is currently favoured by most investigators in the field and will be the basis.
for further discussion of spermatogonial self-renewal in this review.

5. EXPERIMENTAL TOOLS FOR STUDYING SPERMATOGONIAL STEM CELLS

As discussed above, experimental investigation of SSCs is complicated because these cells are rare and are difficult to distinguish from the differentiating progeny that they produce. Whole-mount analyses of seminiferous tubules help in distinguishing $A_s$ from $A_{pr}$ and $A_{al}$ spermatogonia, but there is continuing debate about whether the stem cell pool is restricted to $A_s$ or might be expanded to include $A_{pr}$ and some $A_{al}$ (Nakagawa et al. 2007; Yoshida et al. 2007a). Thus, the only way to definitively identify an SSC is by observing its biological capacity to produce and maintain spermatogenesis in a transplant paradigm.

6. SPERMATOGONIAL STEM CELL TRANSPLANTATION

A technique for transplanting SSCs was first described by Brinster and colleagues in 1994 (Brinster & Avarbock 1994; Brinster & Zimmermann 1994). Briefly, germ cells are isolated from the testes of donor animals and transplanted into the testicular seminiferous tubules of infertile recipients, where they produce normal colonies of spermatogenesis and functional sperm (figure 2). Infertility of recipients is because of genetic mutation (i.e. $W$ mutant mice, (Ogawa et al. 2000)) or induced experimentally (e.g. busulphan treatment (Brinster & Zimmermann 1994)). In mice, these studies are facilitated by the availability of transgenic donors (e.g. $lacZ$ and $GFP$) with germ cells that can be readily identified in the testes of non-transgenic recipients. By definition, only a stem cell can produce and maintain a colony of spermatogenesis and

Figure 1. Mouse spermatogenic clone development by stage. The mouse spermatogenic cycle contains twelve stages (I–XII). Each stage is temporally unique, and the stages in the diagram represent the relative time each stage lasts in the mouse. Each stage in the diagram is shown in cross-sectional, longitudinal and whole-mount perspectives (labelled in stage VII). Three putative spermatogonial clones are highlighted in blue, red and green. The dotted lines in the whole-mount perspective indicate the planes of the cross section and longitudinal section views. For example, in stage VII, the red cell is in the vertical line and therefore appears in the cross-sectional view. A green cell is in the horizontal line, so is observed in the longitudinal section view. The development of three putative clones (blue, red and green) through one cycle of the seminiferous epithelium is shown. Stage VII: $A_{al,16}$ (blue); $A_{pair}$ (red); $A_{single}$ (green); stage VIII: $A_1$ (clone of 16) (blue); $A_{pair}$ (red); $A_{single}$ (green); stage IX: $A_2$ (clone of 32) (blue); $A_{pair}$ (red); $A_{single}$ (green); stage X: $A_2$ (clone of 32) (blue); $A_{1,4}$ (red); $A_{pair}$ (green); stage XI: $A_3$ (clone of 64) (blue); $A_{1,4}$ (red); $A_{single}$ (x2) (green); stage XII: $A_3$ (clone of 64) (blue); $A_{1,4}$ (red); $A_{single}$ (x2) (green); stage I: $A_4$ (clone of 128) (blue); $A_{2,8}$ (red); $A_{single}$ and $A_{pair}$ (green); stage II: intermediate spermatogonia (clone of 256) (blue); $A_{2,8}$ (red); $A_{single}$ and $A_{pair}$ (green); stage III: intermediate spermatogonia (clone of 256) (blue); $A_{2,8}$ (red); $A_{single}$ and $A_{pair}$ (green); stage IV: Type B Spermatagonia (clone of 512) (blue); $A_{2,8}$ (red); $A_{single}$ and $A_{pair}$ (green); stage V: Type B Spermatagonia (clone of 512) (blue); $A_{2,8}$ (red); $A_{single}$ and $A_{pair}$ (green); stage VI: primary spermatocytes (lifting off the basement membrane) (blue); $A_{2,8}$ (red); $A_{single}$ and $A_{pair}$ (green).
each colony arises from the clonogenic proliferation and differentiation of a single SSC (Dobrinski et al. 1999; Zhang et al. 2003; Kanatsu-Shinohara et al. 2006). Therefore, the SSC transplantation technique provides a quantitative functional assay to characterize stem cell activity in any donor cell population. SSC transplantation remains the gold standard method for identifying SSCs, but this approach can be technically challenging. In addition, SSC transplantation is a retrospective assay with an inherent two to three months timeframe between transplant and analysis. To accelerate investigations of SSCs, Nagano and co-workers recently suggested that the SSC culture system (described below) may provide a shorter term, in vitro assay for SSCs (Ye h et al. 2007). However, culture does not assess regenerative activity.

7. DISSECTING THE MOLECULAR PHENOTYPE OF SPERMATOGONIAL STEM CELLS

Fluorescence-activated cell sorting (FACS), combined with SSC transplantation is a powerful tool that has enabled investigators to systematically characterize cell surface molecules of SSCs. This experimental approach is patterned after similar studies to characterize and enrich haematopoietic stem cells (Spangrude 1989; Smith et al. 1991; Osawa et al. 1996). Briefly, a heterogeneous testis cell suspension is stained with a fluorescent-conjugated antibody that recognizes a cell surface antigen. Marker+ and marker− cells are fractionated by FACS and each fraction is transplanted into the seminiferous tubules of infertile recipient mice to determine the relative stem cell activity. The first application of this approach for characterizing SSCs was reported by Shinohara and co-workers, who demonstrated that SSCs specifically bind to laminin-coated plates. The laminin-binding cells were enriched for β1-integrin, making this surface molecule a candidate for enriching SSCs (Shinohara et al. 1999). Subsequent transplantation of magnetic-activated cell-sorted (MACS) and FACS-sorted testis fractions indicated that SSCs express β1-integrin and α6-integrin, but are negative for αv-integrin and the c-KIT receptor tyrosine kinase (Shinohara et al. 1999, 2000). Based on several similar studies, mouse SSCs can now be described by the cell surface phenotype, α6-Integrin (CD49d)+, β1-Integrin (CD29)+, THY-1 (CD90)+, CD9+, GFRα1+, CDH1+, αv-Integrin (CD51), c-KIT (CD117)+, major histocompatibility complex class I (MHC-I)+, CD45 (Shinohara et al. 1999, 2000; Kubota et al. 2003; Kanatsu-Shinohara et al. 2004b; Buageaw et al. 2005; Fujita et al. 2005; Hofmann et al. 2005b; Lo et al. 2005; Tokuda et al. 2007, table 1). Using combinations of positive and negative markers, it is now possible to achieve significant enrichment (100- to 200-fold) of mouse SSCs (Shinohara et al. 2000; Kubota et al. 2003). However, it should be noted that none of these markers are
## Table 1. Germ cell markers in the rodent testis.

<table>
<thead>
<tr>
<th>Germ cell markers in the rodent testis</th>
<th>Experimental evidence</th>
<th>Transplantable SSC?</th>
<th>Germ cell type</th>
<th>A1–4</th>
<th>In</th>
<th>B</th>
<th>Spc</th>
<th>RS</th>
<th>ES</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-kit</td>
<td>Mu, RT–PCR, ISH, IHC, Tr</td>
<td>no</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>GCNA1</td>
<td>WB, IHC</td>
<td>not tested</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>VASA (MvH)</td>
<td>ISH, WB, IHC, KO</td>
<td>not tested</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>EE2 antigen</td>
<td>WB, IHC, KO</td>
<td>not tested</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>DAZL</td>
<td>RT–PCR, NB, ISH, KO, IHC</td>
<td>not tested</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Stra8</td>
<td>RT–PCR, ISH, IHC, WM, TG, MACS, Tr</td>
<td>yes</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>α6-integrin (CD49F)</td>
<td>FC, MACS, Tr</td>
<td>yes</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>β1-integrin (CD29)</td>
<td>FC, MACS, Tr</td>
<td>yes</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Epcam</td>
<td>IHC, MACS</td>
<td>not tested</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Pou5f1 (Oct4)</td>
<td>IHC, WM, TG, FC, Tr, ISH</td>
<td>yes</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>GFR-α1</td>
<td>TG, ISH, IHC, MACS, Tr, WM</td>
<td>yes</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>CD24</td>
<td>FC</td>
<td>not tested</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Thy1 (CD90)</td>
<td>FC, TR</td>
<td>yes</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Nanos2</td>
<td>ISH, KO, WB, RT–PCR, Tg, IHC, Tr, WM</td>
<td>yes</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>Nanos3</td>
<td>NB, ISH, KO, WB, RT–PCR, Tg, IHC, WM</td>
<td>not tested</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

(Continued.)
exclusive to SSCs and no marker or combination of markers has produced a pure population of SSCs. Also, while FACS and MACS sorting followed by transplantation are powerful tools for characterizing the cell surface phenotype of SSCs, this approach has limited utility for characterizing cytoplasmic or nuclear markers.

Genetic mouse models in which GFP expression is driven by a promoter from a putative SSC gene provide an alternative approach for characterizing SSCs. For example, Schöler and co-workers reported that the OCT-4 transcription factor is expressed by gonocytes and type A spermatogonia of newborn, pup and adult mouse testes (Pesce et al. 1998). This group subsequently characterized an 18 kb promoter/enhancer fragment of the OCT-4 gene that directed faithful expression of lacZ and GFP transgenes (Yeom et al. 1996; Yoshimizu et al. 1999). The Oct-4–GFP mouse is a valuable tool that enabled FACS-based isolation and transplantation of Oct4 expressing germ cells from a heterogeneous testis cell suspension (Ohbo et al. 2003; Ohmura et al. 2004). Stem cell activity was significantly enriched in the Oct4 expressing (GFP+ ) population compared with the Oct4 negative (GFP−) population of mouse testis cells (Ohmura et al. 2004). Interestingly, gonocytes and pre-spermatogonia from neonatal mice with an Oct4–EGFP+/c-Kit− phenotype had a greater repopulation capacity than Oct4–EGFP+/c-Kit+ cell fractions (Ohbo et al. 2003). These data suggest that there is molecular heterogeneity among pre-spermatogonia. This observation is consistent with reports

Table 1. (Continued.)

<table>
<thead>
<tr>
<th>germ cell markers in the rodent testis</th>
<th>experimental evidence</th>
<th>transplantable SSC?</th>
<th>germ cell type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>As</td>
</tr>
<tr>
<td>CD9</td>
<td>FC, IHC, MACS, Tr</td>
<td>yes</td>
<td>X</td>
</tr>
<tr>
<td>EGR3</td>
<td>IVC, IHC</td>
<td>not tested</td>
<td>X</td>
</tr>
<tr>
<td>Ngn3</td>
<td>ISH, TG, WM, IHC, Tr</td>
<td>yes (approx. 10% of SSCs)</td>
<td>X</td>
</tr>
<tr>
<td>PLZF</td>
<td>Mu, KO, Tr, WM, FC, ISH, IHC</td>
<td>yes</td>
<td>X</td>
</tr>
<tr>
<td>RBM</td>
<td>RT–PCR, IHC</td>
<td>not tested</td>
<td>X</td>
</tr>
<tr>
<td>Sox-3</td>
<td>KO, IHC</td>
<td>not tested</td>
<td>X</td>
</tr>
<tr>
<td>TAF-4B</td>
<td>KO, IHC</td>
<td>not tested</td>
<td>X</td>
</tr>
<tr>
<td>Bcl6b</td>
<td>siRNA</td>
<td>not tested</td>
<td>X</td>
</tr>
<tr>
<td>Numb</td>
<td>NB, WB, IHC</td>
<td>not tested</td>
<td>X</td>
</tr>
<tr>
<td>Lrp4</td>
<td>WB, IHC</td>
<td>not tested</td>
<td>X</td>
</tr>
<tr>
<td>Ret</td>
<td>IHC, MACS, Tr</td>
<td>no</td>
<td>X</td>
</tr>
<tr>
<td>Sohlh1</td>
<td>KO, RT–PCR, IHC, IHC</td>
<td>not tested</td>
<td>X</td>
</tr>
<tr>
<td>Sohlh2</td>
<td>RT–PCR, IHC</td>
<td>not tested</td>
<td>X</td>
</tr>
<tr>
<td>CDH1 (CD1324)</td>
<td>IHC, WM, MACS, Tr</td>
<td>yes</td>
<td>X</td>
</tr>
<tr>
<td>GPR125</td>
<td>TG, FC, IVC, Tr</td>
<td>yes</td>
<td>X</td>
</tr>
<tr>
<td>Nucleostemin</td>
<td>TG, IHC, FC, Tr, IVC, Tr, siRNA</td>
<td>yes</td>
<td>X</td>
</tr>
<tr>
<td>UTF1</td>
<td>RT–PCR, IHC</td>
<td>not tested</td>
<td>X</td>
</tr>
<tr>
<td>Lin28 (Tex17)</td>
<td>IHC, WM, WB, siRNA</td>
<td>not tested</td>
<td>X</td>
</tr>
</tbody>
</table>

As, A single spermatogonia; Apr, A paired spermatogonia; Aal, A aligned spermatogonia; A1–4, differentiating type A1 to A4 spermatogonia; In, intermediate type spermatogonia; B, type B spermatogonia; Spc, spermatocytes; RS, round spermatids; ES, elongated spermatids; Mu, mutant mouse; TG, transgenic mouse; KO, Knockout mouse; Tr, germ cell transplantation; IHC, immunohistochemistry; WM, whole mount immunostaining; FC, flow cytometry (including FACS); IVC, in vitro culture; WB, Western blot; NB, Northern blot; ISH, in situ hybridization; RT–PCR, reverse transcriptase–PCR; siRNA, in vitro knockdown experiment using siRNA; MACS, magnetic-activated cell sorting.

*As determined by the spermatogonial stem cell transplantation assay.

**Phil. Trans. R. Soc. B** (2010)
suggested that some gonocytes/pre-spermatogonia establish the initial pool of SSCs, while other gonocytes/pre-spermatogonia differentiate to produce the first round of spermatogenesis (Kluin & de Rooij 1981; Yoshida et al. 2006).

Transgenic and conditional knock-in approaches were recently used to demonstrate that neurogenin 3 (Ngn3) is expressed by the earliest spermatogonia (Yoshida et al. 2004), including at least 11 per cent of transplantable SSCs (Nakagawa et al. 2007). The fact that Ngn3 was not expressed by all transplantable stem cells in that study provides additional evidence that there may be heterogeneity among SSCs. A conditional knock-in approach was also used to demonstrate that Nanos2 is expressed by SSCs (Sada et al. 2009). Finally, transgenic models suggest that Stra-8 (stimulated by retinoic acid-8) is expressed by undifferentiated spermatogonia, including SSCs (Giuli et al. 2002; Guan et al. 2006; Sadate-Ngatchou et al. 2008), although the transplant data in the Stra-8 studies were limited.

Knock-out models have also been used to demonstrate that specific genes/proteins are required for SSC function. Male mice carrying the luxoid (lu) mutation are subfertile and show abnormal sperm development. Progression of infertility is caused by gradual loss of SSCs (Buaas et al. 2004). The mutation was shown to affect the Zfp145 locus, which encodes the transcriptional repressor PLZF (promyelocytic leukaemia zinc-finger). PLZF is expressed during embryogenesis and plays a crucial role during limb and axial skeletal patterning. Targeted disruption of Zfp145 resulted in a testicular phenotype similar to that of luxoid mutant mice (Costoya et al. 2004). In the testsis, PLZF expression is restricted to A0, A1 and undifferentiated spermatogonia, including SSCs as demonstrated by transplantation experiments of testicular cells from luxoid or PLZF−/−/mice that failed to initiate donor-derived spermatogenesis in recipient mice (Buaas et al. 2004; Costoya et al. 2004). A possible role for PLZF in spermatogonia could be the maintenance of an undifferentiated state (Filipponi et al. 2007), similar to the role suggested for Plzf in haematopoietic precursor cells (Reid et al. 1995).

Similar knock-out and over-expression studies implicate gial cell line-derived neurotrophic factor (GDNF) and its receptor GFRα1 in stem cell self-renewal (Meng et al. 2000). GDNF signalling has since been shown to be required for in vitro expansion of SSCs, and it has been demonstrated that a combination of GDNF and soluble GFRα1 is most favourable for the self-renewal of SSCs in vitro (Kubota et al. 2004a,b; see below). Finally, knock-out studies implicate Sox3 in the differentiation of the earliest germ cells (Raverot et al. 2005). The latter study indicated that Ngn3 expression is dependent on SOX3 and suggested that SOX3 may act directly or indirectly through Ngn3 to regulate spermatogonial differentiation (Raverot et al. 2005).

In addition to data derived from flow cytometry, genetic models and transplantation, immunohistochemistry in tissue sections or intact seminiferous tubules (whole mount) has been widely used to investigate the expression patterns of various proteins in the male germ lineage. In this context, a candidate SSC marker would be expressed by cells located on the basement membrane of seminiferous tubules and be co-expressed with confirmed markers of SSCs. This histochemical approach is most convincing in whole-mount preparations of seminiferous tubules in which it is possible to correlate marker expression with clone size (i.e. A0, A1, A4, A4d). Several established markers of stem, progenitor and differentiating spermatogonia are listed in figure 3. Here we define progenitors as undifferentiated spermatogonia that are committed to differentiate. An example of this approach is shown in figure 4 for the putative SSC marker, Spalt-like 4 (SALL4). SALL4 is a zinc finger transcription factor that is expressed in the inner cell mass of the late blastocyst in a pattern similar to OCT4 and SOX2 (Elling et al. 2006). In vitro, SALL4 stimulates embryonic stem (ES) cell proliferation (Sakaki-Yumoto et al. 2006) and maintains pluripotency by repressing trophoderm differentiation (Yuri et al. 2009), possibly by binding the Oct-4 proximal promoter (Zhang et al. 2006) and by interacting with NANOG (Wu et al. 2006). Thus, SALL4 is an important stemness factor and together with OCT-4, SOX2 and NANOG constitutes a tightly regulated transcription circuit important for stem cell pluripotency (Lim et al. 2008; Yang et al. 2008). Postnatally, Sall4 expression is restricted to the gonads and is expressed by isolated spermatogonia (Wang et al. 2001; Sall4 was identified as testis-expressed gene 20 (Tex20) in that paper). Co-stained whole-mount seminiferous tubules (figure 4) indicated that SALL4 is expressed by single, paired and aligned cells on the seminiferous tubule basement membrane and overlaps with consensus SSC markers, PLZF (figure 4a–c) and GFRα1 (figure 4d–f). However, these whole-mount immunohistochemistry results highlight the heterogeneity among undifferentiated spermatogonia, including A0 spermatogonia (see figure 4f with examples of SALL4+/GFRα1− and SALL4−/GFRα1+ A0 spermatogonia). GFRα1 appears to have the most restricted expression (limited to singles, pairs and chains of four), while PLZF and SALL4 are also expressed by larger chains of 8 and 16 A4d spermatogonia.

Similar observations of molecular heterogeneity among undifferentiated A0, A1 and A4d spermatogonia have been reported in several recent studies (Tokuda et al. 2007; Grisanti et al. 2009; Sada et al. 2009; Suzuki et al. 2009; Zheng et al. 2009). The functional significance of this heterogeneity remains to be determined. Through the combination of FACS and MACS analyses, transplantation, genetic models and histochemical approaches, the phenotype of rodent SSCs is beginning to emerge. A list of putative SSC and undifferentiated spermatogonia markers is provided in table 1 along with the experimental evidence used to characterize each marker.

8. THE SPERMATOGONIAL STEM CELL NICHE
SSCs reside within a specialized microenvironment called ‘niche’ that regulates testicular homeostasis by balancing SSC self-renewal and differentiation. A
stem cell niche is comprised of cells, extracellular matrix components, and local soluble factors present in the vicinity of the stem cell that regulates cell fate. The structural basis for the SSC niche in the mammalian testis is the basal compartment of the seminiferous tubules that is composed of Sertoli cells and peritubular myoid cells (Dadoune 2007) (figure 5). Together, Sertoli and peritubular myoid cells secrete the basement membrane components to which the SSCs are connected via adhesion molecules (Tung et al. 1984). Sertoli cells are polarized columnar epithelial cells that support SSCs and differentiating germ cells by providing nutrients and mediating external signals in order to support spermatogenesis (Griswold 1998). The importance of Sertoli cells for germ cell differentiation is demonstrated by the transplantation of normal Sertoli cells into the testis of infertile mutant recipients with a Sertoli cell defect and successful initiation of spermatogenesis by recipient-derived spermatogonia (Kanatsu-Shinohara et al. 2003b, 2005b). Tight junctions between adjacent Sertoli cells constitute a protective blood–testis barrier (BTB) that divides the seminiferous epithelium into basal and adluminal compartments (figure 5a) and plays an important role in the regulation of germ cell differentiation (Cheng & Mruk 2002). The BTB maintains a selective substance flow between luminal fluid, blood plasma and interstitial fluid, thereby creating an immune-privileged environment for haploid germ cells in the adluminal compartment of the seminiferous tubules.

Along the length of the tubule, SSCs are thought to be localized in areas adjacent to the interstitial space (Chiarini-Garcia et al. 2003). Undifferentiated spermatogonia are observed predominantly in tubule areas adjacent to vasculature (Yoshida et al. 2007b; figure 5a).

The SSC niche mediates endocrine and paracrine signals that regulate self-renewal and differentiation (figure 5b). A key regulator of the SSC niche is GDNF which is secreted by Sertoli cells and acts through Ret receptor tyrosine kinase and GFRα1 co-receptor, which form a receptor complex on the surface of A1, A1pair and A1al (Meng et al. 2000). Downstream signalling pathways that are activated by GDNF in undifferentiated spermatogonia are the PI3K/Akt pathway, members of the Src kinase family and the Ras/Erk1/2 pathway (Braydich-Stolle et al. 2007; Oatley et al. 2007; He et al. 2008). GDNF is thought to act through these pathways to regulate SSC self-renewal.

Targeted disruption of the Ets variant gene 5 (Etv5) results in defective maintenance of the SSC pool, whereas spermatogonial differentiation appears to be unaffected by this mutation (Chen et al. 2005). The transcription factor Etv5 is expressed in Sertoli cells and loss of Etv5 appears to impair the ability of Sertoli cells to support spermatogonia, possibly by disrupted BTB function as indicated by decreased Claudin-5 (CLDN5) levels in mutant mice (Morrow et al. 2009). In Sertoli cells, Etv5 is upregulated by FGF2 in vitro, which is important for SSC renewal in culture (Kanatsu-Shinohara et al. 2003a; Kubota et al. 2004a; Yoon et al. 2009). Therefore, in addition to a direct effect of FGF2 on SSCs, an indirect paracrine effect of FGF2 on Sertoli cells appears possible.

**Figure 3.** Genes expressed by stem, progenitor and differentiating spermatogonia. The A1, seen at the top of the diagram, is responsible for self renewal and differentiation. Self-renewal is represented here by the A1pair dividing to form two A1. Differentiation is indicated by colour change (from dark to light) and the lengthening chain of germ cells. Genes are listed with their expression at the given stages of spermatogonial development. While stem cell activity is considered to reside in the pool of A1 spermatogonia, the tapered triangle on the left indicates that stem cell activity may extend to A1pair and some A1al spermatogonia.
The importance of peritubular myoid cells for spermatogonia maintenance has long been discussed. New data now suggest a role for the peritubular cell product colony-stimulating factor 1 (CSF1) on SSC maintenance (Oatley et al. 2009). Csf1 was found to be expressed in interstitial Leydig and peritubular myoid cells, whereas the Csf1 receptor (Csf1r) was highly enriched in THY1⁺ cell fractions from pre-pubertal and adult mouse testis.

9. SSC CULTURE

SSC culture provides a new approach for investigating the molecular mechanisms and cell-signalling pathways that regulate SSC function. While methods for maintaining and amplifying pluripotent ES and embryonic germ cells in culture are routine, methods for culturing adult tissue stem cells (including SSCs) had been more difficult to establish. However, tremendous progress culturing mouse and rat SSCs has been reported during the past 5–6 years (Kanatsu-Shinohara et al. 2003a; Kubota et al. 2004a,b; Hamra et al. 2005; Ryu et al. 2005). Rodent SSCs can now be maintained for a very long time (perhaps indefinitely) with a significant amplification in numbers. Stem cell activity in these cultures was confirmed by SSC transplantation, as diagrammatically represented in figure 2. The doubling time for mouse SSCs was determined to be 5.6 days (Kubota et al. 2004b), while the doubling time for rat SSCs is 3–4 days (Hamra et al. 2005) or 11 days (Ryu et al. 2005).

Several factors were critical to the establishment of long-term SSC cultures. First, methods to fractionate testis cell populations (FACS or MACS sorting and/or differential attachment and replating) resulted in the enrichment of SSCs and the removal of somatic cells that promote germ cell differentiation. Second, development of a serum-free, defined medium facilitated the discovery of essential growth factors. Specifically, GDNF is necessary to maintain and expand rodent SSCs in culture (Kanatsu-Shinohara et al. 2003a; Kubota et al. 2004b). The trophic effects of GDNF in both mice and rats is enhanced by the addition of soluble GFRα1 (the receptor for GDNF) and FGF2 (Kubota et al. 2004a; Ryu et al. 2005).

Unlike mouse ES cells, the additions of leukaemia inhibitory factor (LIF) and foetal bovine serum (FBS) to cultures are superfluous and detrimental, respectively, in SSC cultures (Kubota et al. 2004b). Third, STO or mouse embryonic fibroblast (MEF) feeder cells are usually required. Whereas Shinohara’s group has demonstrated that mouse SSCs can also be maintained in feeder-free conditions (Kanatsu-Shinohara et al. 2005a), SSC cultures are usually established from mouse pup testes (5–12 days postpartum) because SSCs are enriched at this stage of development. However, SSC cultures can be established from neonate (Kanatsu-Shinohara et al. 2003a) and adult mouse testis cells (Kubota et al. 2004a). Immortalized SSC lines have been established by the introduction of a retroviral telomerase gene (Feng et al. 2002) or treatment with the SV40 large T-antigen (Hofmann et al. 2005a). Evidence that each of these immortalized cell lines is spermatogonial-like is based primarily on genetic or immunocytochemical data, but transplantation data are lacking.

Figure 4. Immunofluorescent co-staining of adult mouse whole-mount seminiferous tubules. (a) SALL4 labels undifferentiated Aa, Apr and Aal spermatogonia. (b) PLZF labels undifferentiated Aa, Apr and Aal spermatogonia. (c) Merged picture from (a,b). SALL4 and PLZF are mostly co-expressed in undifferentiated spermatogonia. (d–f) Co-staining of SALL4 and GFRα1 reveals heterogeneity within the population of undifferentiated spermatogonia. GFRα1 expression appears more restricted than SALL4 or PLZF. Scale bar, 50 μm.
containing on Oct-4-targeted shRNA. The treatment caused a significant reduction in OCT-4 expression and reduced colonizing activity in the transplant assay by sixfold. Thus, through genetic manipulation and transplantation of SSC cultures, studies will continue to unravel regulatory pathways required for SSC self-renewal and differentiation.

10. FUTURE DIRECTIONS
We have attempted to review the current state of knowledge and research in the biology of SSCs, focused primarily on the rodent model. Many areas of research are only beginning to be thoroughly investigated in SSCs, such as the molecular regulation of stem cell fate decisions and SSC heterogeneity. Recent progress characterizing, manipulating and culturing SSCs has opened the door to new experimental approaches for fundamental investigation and possible practical applications discussed below.

In vitro derivation of haploid gametes (elongated spermatids or sperm) may help to overcome spermatogenic barriers in infertile men. Feng et al. (2002) reported the production of spermatocytes and spermatids from a stable mouse SSC line, though the fertilization potential of these cells was not tested. Haploid male germ cells have also been generated by differentiation of ES cells in mice (Toyooka et al. 2003; Geijsen et al. 2004; Nayernia et al. 2006) and humans (Kee et al. 2009). Two studies demonstrated that in vitro, ESC-derived spermatids were competent to fertilize mouse eggs, generating blastocysts (Geijsen 2004) and live progeny (Nayernia et al. 2006), respectively. However, because of the epigenetic reprogramming that occurs during in vitro germ cell development, the epigenetic regulation of in vitro gametogenesis must be carefully assessed before clinical applications ensue (Georgiou et al. 2007).

Generation of haploid germ cells from primary SSC cultures has not yet been reported, but this approach may have epigenetic advantages over ESC-derived gametes. Furthermore, progress establishing human SSC cultures will be an important experimental tool in a species where transplantation is not an option for characterizing SSCs.

In addition to trying to drive SSCs towards their typical biological end, there is evidence that SSCs are a source of pluripotent stem cells (Kanatsu-Shinohara et al. 2004a; Guan et al. 2006; Seandel et al. 2007). The ability to derive pluripotent stem cells from adult tissues, with the consent of the donor, may have some advantages over other approaches to pluripotency. However, more details are needed to understanding the genetic, epigenetic constitution of these cells, as well as their developmental potential.

Testicular tissues or testicular cell suspensions (containing SSCs) can be cryopreserved and may provide an avenue for preservation of valuable strains or species. Honaramooz et al. (2002) recently demonstrated that testicular tissues from newborn mice, pigs or goats can be grafted under the skin of immune-deficient mice and generate complete spermatogenesis. This approach has now been reported

Stable SSC culture provides a valuable tool for dissecting mechanisms that regulate SSC renewal and differentiation. GDNF is required for SSC renewal in vitro (Kubota et al. 2004a) and in vivo (Meng et al. 2000). Through withdrawal and/or addition of GDNF to SSC cultures, two groups have now demonstrated that GDNF action is mediated by Src family kinases acting through PI3 kinase/Akt-dependent pathways (Braydich-Stolle et al. 2007; Oatley et al. 2007). In addition, microarray analysis identified genes that are regulated by GDNF withdrawal in SSC cultures. The importance of three of these genes (Bcl6b, Erm and Lhx1) was confirmed by transfecting SSC cultures with siRNAs specific for each gene. siRNA treatment caused decreased clump formation in vitro and decreased colonization of recipient testes after transplantation (Oatley et al. 2006, 2007).

Transfection of SSC cultures with siRNA, as described above, enables temporary knockdown of the target gene. To achieve stable knockdown of a target gene, short hairpin RNAs (shRNAs) can be coupled with lentiviral vectors. Dann et al. (2008) recently treated cultured SSCs with a lentiviral vector

Figure 5. SSC niche. The SSC (dark blue) is diagrammed in its physical niche (a) surrounded by Sertoli cells (orange) and differentiating germ cells (light blue) within the seminiferous tubule. Niche components outside the tubule itself include myoid cells (green), blood vessels (red) and Leydig cells (yellow). The components of the niche and the some factors known to be provided by each are shown in (b). While some factors are known to act directly on the SSC, such as GDNF, others, like testosterone are important for spermatogenesis but may not act on the SSC.

Phil. Trans. R. Soc. B (2010)
for several species (Oatley et al. 2004, 2005; Snedaker et al. 2004; Zeng et al. 2006; Kim et al. 2007; Arregui et al. 2008; Rodriguez-Sosa et al. 2010) and may allow germline preservation for endangered species or valuable domestic strains. Alternatively, valuable germlines can be preserved by freezing testis cell suspensions (containing SSCs) for future SSC transplantation. The proof in principle for this approach is already established for mice, rats, goats and dogs (Brinster & Avarbock 1994; Brinster et al. 2003; Honaramooz et al. 2003; Ryu et al. 2003; Kim et al. 2008).

SSC transplantation may have application for treating some cases of male infertility. 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). We have recently established 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 (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 transplant therapy.

Progress studying SSC origins, regulation and activity over the past half century, has laid the foundation to pursue the clinical and veterinary options described in the preceding paragraphs. The field of SSC biology has grown substantially in the past two decades, fuelled in part by development of the SSC transplantation technique (Brinster & Avarbock 1994; Brinster & Zimmermann 1994), which impacted fundamental investigations as well as clinical application. Growth was also fuelled by the explosive development of the pluripotent stem cell and regenerative medicine fields. The next half century should bring many new discoveries about the biology and regenerative potential of SSCs that parallels the development of the haematopoietic stem cell field in the 1980s and 1990s.

The authors would like to thank Dr Brian Hermann for critically reviewing the chapter. B.T.P. produced the artwork in figures 1, 2, 3 and 5. K.E.O. is supported by NIH grants HD055475 and HD008610 and the Magee-Womens Research Institute and Foundation.

REFERENCES


Kanatsu-Shinohara, M., Toyokuni, S. & Shinohara, T. 2004b CD9 is a surface marker on mouse and rat male germline stem cells. Biol. Reprod. 70, 70–75. (doi:10.1095/biolreprod.102.028067)


Rodriguez-Sosa, J. R., Foster, R. A. & Hahnel, A. 2010 Development of strips of ovine testes after xenografting...


Sakaki-Yumoto, M. et al. 2006 The mouse homolog of SALL4, a causative gene in Okihiro syndrome, is essential for embryonic stem cell proliferation, and cooperates with Sall1 in oncogenic transformation in vivo. Cell 1241, 1239–1248. (doi:10.1016/j.cell.2006.03.007)


