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INVITED REVIEW

Sperm Biology

# Spermatogonial stem cells: progress and prospects

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Twenty years ago, the transplantation of spermatogonial stem cells (SSCs) from a mouse to other recipient mice was shown to be feasible, which clearly demonstrated the functional identity of SSCs. Since then, several important new findings and other technical developments have followed, which included a new hypothesis on their cell kinetics and spermatogonial hierarchy in the testis, a culture method allowing their self-renewal and proliferation, a testis tissue organ culture method, which induced their complete differentiation up to sperm, and the *in vitro* induction of germ cells from embryonic stem cells and induced pluripotent stem cells. These advancements reinforced or advanced our understanding of this unique cell. Nonetheless, there are many unresolved questions in the study of spermatogonial stem cells and a long road remains until these cells can be used clinically in reproductive medicine. *Asian Journal of Andrology* (2015) 17, 771–775; doi: 10.4103/1008-682X.154995; published online: 18 May 2015

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## INTRODUCTION

Spermatogonial stem cells (SSCs) play a number of pivotal roles in spermatogenesis. Firstly, SSCs are the cells that spermatogenesis starts from. Once SSCs have committed to differentiate, the subsequent cellular processes progress in a strictly ordered manner up to sperm formation in the seminiferous tubules. Secondly, SSCs sustain spermatogenesis throughout life owing to their function as stem cells. In other words, SSCs maintain themselves by self-renewal, which facilitates the continuation of spermatogenesis. Thirdly, disruption of spermatogenesis, whether physiological such as in seasonal breeders or due to pathological insult, can be regulated or restored by SSCs. Along with these roles, SSCs must maintain the integrity of DNA so it can be effectively transmitted to the next generation. Thus, SSCs are not just one type of tissue stem cell, but could be called stem cells ensuring the continuation of life.

To produce adequate numbers of daughter cells which are destined to undergo the differentiation process of spermatogenesis while maintaining themselves in a certain range as a rather small population, SSCs have to achieve a delicate balance between commitment to differentiation and self-renewal. This balance is maintained not only by SSCs themselves but also requires support from the surrounding somatic cells. In addition to germ cells, the testis contains many different kinds of somatic cells, including Sertoli, Leydig, peritubular myoid and immune cells, as well as vascular cells (Figure 1). Each cycle of spermatogenesis and its sequential progression are made possible by delicate cooperation among these somatic cells. The number of SSCs has been estimated to be as low as 0.03% of all germ cells in the rodent testis.<sup>1</sup> This fact, along with the lack of specific markers for identifying them, has hampered the study of SSCs. These difficulties, however, are being overcome with technological advancements in this and related areas of science that have taken place over the last two decades. In this review, we summarize the characteristics of SSCs, give a historical overview and describe recent progress in SSC research, and finally discuss prospects of future research.

## SSCS *IN SITU* IN THE TESTIS

The SSCs along with other spermatogonia reside inside the basal lamina of the seminiferous tubules. In other words, they are considered to be present on a two-dimensional plane. Thus, a whole mount technique, which can provide a view of almost half of the area of the peripheral plane of a seminiferous tubule, is superior for their observation compared to regular histological thin-sectioning. Using this whole mount method, classical studies on spermatogonia were performed to clarify the cell kinetics of spermatogonia, which comprise different subtypes including type A, Intermediate, and B. Based on their numbers, the morphology of the nucleus, especially the amount and appearance of heterochromatin, and mitotic cell cycles, it is clear that the SSCs are a subset of the type A spermatogonia. Among type A spermatogonia, more immature subgroups were classified and collectively named undifferentiated type A spermatogonia. These undifferentiated type A spermatogonia include  $A_{\text{single}}$  ( $A_s$ ),  $A_{\text{paired}}$  ( $A_{pr}$ ), and  $A_{\text{aligned}}$  ( $A_{al}$ ); this classification is based on cyst formation by inter-cellular cytoplasmic bridges (Figure 2). In addition to morphological techniques, studies using <sup>3</sup>H-thymidine labeling revealed the unique cell kinetics of these spermatogonia.<sup>2</sup> The differentiating spermatogonia undergo synchronous successive cell division based on a fixed schedule, while undifferentiating spermatogonia, especially  $A_s$  and to a lesser extent  $A_{pr}$ , are flexible in that regard. The cell cycle length was also different in each group. These data qualitatively distinguished undifferentiated type A spermatogonia from differentiating spermatogonia, which is difficult to do based on morphology alone. Then, along with other data accumulated by morphological observation, the  $A_s$  model was established, proposing that  $A_s$  spermatogonia are the only cells functioning as SSCs in the testis.<sup>3,4</sup> Other cells, including  $A_{pr}$  and  $A_{al}$  spermatogonia, were considered to have committed to differentiate toward sperm formation. This  $A_s$  model has been regarded as plausible and accepted as a likely hypothesis to establish the identity of SSCs. On the other hand, another mechanism for maintaining the number

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of stem cells in a particular organ, the niche theory, was proposed in 1978.<sup>5</sup> It was later demonstrated in *Drosophila* gonads that the stem cell niche actually exists and controls the fate of stem cells.<sup>6-8</sup> Based on these findings, along with the prevailing  $A_s$  model, it was generally assumed that each SSC exists at a particular location on the basal lamina and is responsible for the continuation of spermatogenesis in the surrounding area, at least under stable conditions.

This assumption was challenged in 2007 by a report demonstrating that clones of each SSC fluctuate markedly in size even under a steady state condition.<sup>9</sup> To our surprise, the majority of those clones disappear sooner or later and the vacant area left behind is occupied by neighboring clones. Time-lapse observation of GFR $\alpha$ 1-positive cells, a receptor of GDNF (explained below) and a marker for the subset of undifferentiated spermatogonia, revealed that they are actively migrating over a large area on the basal lamina and do not stay at a certain point. It was also found that the breakage of inter-cellular bridges took place more often than thought. This means that  $A_s$  spermatogonia are produced by their duplication but also from  $A_{pr}$  or  $A_{al}$  spermatogonia by fragmentation. More correctly, it was observed that pure duplication, namely the production of two  $A_s$  by cell division, was rather rare and the majority of  $A_s$  resulted from the fragmentation of  $A_{pr}$  and  $A_{al}$ .<sup>10</sup> These observations naturally necessitated a new model for the cell kinetics and hierarchy of spermatogonia including SSCs. In fact, the same research group proposed a new biophysical model, which faithfully predicted the fate of GFR $\alpha$ 1-positive spermatogonia in not only a steady-state but also in a postinsult regeneration state, by adopting the rates of cell division and fragmentation as parameters.<sup>10</sup> Simply put, not only  $A_s$  spermatogonia but also  $A_{pr}$  and  $A_{al}$  have the potential to become SSCs and actually work as SSCs in the mouse testis. It appears that SSCs are not a fixed entity but a differentiation state which could be lost or regained according to their physical status, whether they occur singly, are connected with other sibling germ cells, or become free again to be single.

Many critical questions regarding the nature of SSCs remain to be answered. For instance, it is not clear how they are maintained in small populations in the testis which are controlled in a robust and flexible manner. It is true, however, that research on SSCs has been providing and will continue to provide cutting-edge information on the biology of tissue stem cells in general.

### IN VITRO PROPAGATION OF SSCS

Now, it is possible to culture and propagate SSCs of mice and some other species. It had been known for many years that germ cells were difficult to culture, so the development of culture systems for these cells was a historic achievement in germ cell biology. We would like to mention that, prior to this achievement, there were two important earlier breakthroughs that significantly contributed to the development of these culture methods: establishment of the spermatogonial transplantation technique (in 1994), and identification of GDNF as a key growth factor for the proliferation of SSCs (in 2000).

In 1994, Ralph L. Brinster and his colleagues reported that testis cells from a donor mouse could be dissociated into single cells and injected in the seminiferous tubules of recipient mice, which were pretreated with busulfan to ablate endogenous spermatogenesis. Certain cells in the injected population, namely SSCs, settled to the basal lamina and formed colonies of spermatogenesis in the seminiferous tubules of host testes.<sup>11</sup> The number of the spermatogenic colonies was proportional to the number of cells transplanted and they continued expanding in the subsequent period.<sup>12</sup> Thus, this method, called spermatogonial transplantation, became a novel and unique

functional assay for SSCs, with each colony in the transplanted testis corresponding to a single SSC or a unit of SSCs. With this method, it became possible to study functional aspects of SSCs, whose identity was still unclear, with no molecular markers available. Following the development of this spermatogonial transplantation method, several important and interesting studies utilizing this method were reported. For instance, surface markers of SSCs were identified using fluorescence-activated cell sorting (FACS) analysis. The sorted cells were tested by spermatogonial transplantation to determine whether they could form spermatogenic colonies in recipient mouse testes. With this strategy, several surface markers for SSCs were identified, including  $\alpha$ 6-integrin (CD49f),<sup>13,14</sup>  $\beta$ 1-integrin (CD29),<sup>13</sup> Thy1 (CD90)<sup>15</sup> and CD9.<sup>16</sup> Another interesting advance using the transplantation method was interspecies transplantation. It was shown that rat SSCs could actually develop spermatogenic colonies in mouse testis, while cells of other animals phylogenetically more distant did not form spermatogenic colonies in the mouse testis.<sup>17,18</sup>

In 2000, the important discovery was reported that glial cell line-derived neurotrophic factor (GDNF) is key regulatory factor for the self-renewal of SSCs. Transgenic mice which overexpress GDNF showed hyper-proliferation of spermatogonia, while mice with a hemi-defect of GDNF showed spermatogonial depletion.<sup>19</sup> This crucial finding soon led to the development of culture medium for the propagation of mouse SSCs *in vitro*.

In 2003 and 2004, two groups of researchers independently reported a culture method for spermatogonial stem cells using GDNF and other factors in the culture media.<sup>20,21</sup> One of them, Shinohara's team, named the cultured SSCs germline stem (GS) cells.<sup>20</sup> Mouse GS cells proliferated exponentially and their karyotypic, genetic, and epigenetic states could be stably maintained. The stem cell characteristics of the GS cells were then tested with the transplantation method, which had played a critical role in the identification of SSCs. When GS cells were tested with the transplantation technique, they produced spermatogenic colonies in the recipient testes, which was the only and definite proof of their identity as SSCs. In addition, sperm derived from these transplanted GS cells were functional and normal, producing offspring.

Through studies involving SSC culture along with the transplantation methods, several genes were identified which delineate SSCs. Those include GFR $\alpha$ 1 (GDNF family receptor  $\alpha$  1), nanos-2 and -3,<sup>22</sup> PLZF (promyelocyte leukemia zinc-finger factor),<sup>23</sup> inhibitor of differentiation 4 (Id4),<sup>24</sup> and Pax-7,<sup>25</sup> which are useful both for identification of SSCs as well as studies of the molecular control of their behavior and functions.

The culture method has been modified and applied to other species, including the rat, hamster, and rabbit, and successfully allowed for propagation of SSCs from these various species.<sup>26-29</sup> Human SSCs have also been tested for *in vitro* propagation by several researchers. However, up till now, the culture conditions do not seem optimal for their successful maintenance and proliferation. In fact, the medium formulation has not yet been optimized even for mouse GS cells. At present, the factors necessary for successful culture have been reported to be GDNF and FGF2.<sup>30</sup> There are reports of trying to improve the culture conditions by modifying the medium formula.

It is known that germ cells are pluripotent because they can give rise to a teratoma or teratocarcinoma. This unique characteristic was initially attributed specifically to primordial germ cells (PGCs). PGCs were also converted to ES-like pluripotent cells, embryonic germ (EG) cells, under culture conditions with Kit ligand, basic fibroblast growth factor, and leukemia inhibitory factor. On the other hand, it was not

clear if SSCs also maintain such pluripotent potential. In 2004, it was reported that GS cells were converted to ES-like cells during culturing. This conversion is very rare in practice but has important implications for germ cell carcinogenesis. It is noteworthy that GS cells cannot be converted to iPS cells by Yamanaka factors. There appears to be different kinds of signal transduction machinery or transcriptional compositions which are dominant in the germ cells. Recently, it was reported that the conversion of GS cells to the pluripotent state became highly efficient by down-regulating Dmrt1 and p53 together. Down-regulating Dmrt1 seemed to induce Sox 2 and Oct4 expression, which conferred pluripotency to the cell.<sup>31</sup> It is important to know the exact mechanism of such conversion between uni-potent SSCs and the pluripotent ES-state, in that this would allow us to manipulate these important cells in a reliable manner.

### IN VITRO SPERMATOGENESIS WITH AN ORGAN CULTURE METHOD

Studies on *in vitro* spermatogenesis date back to organ culture experiments about a century ago.<sup>32</sup> In 1937, it was reported that spermatogenesis proceeded up to the pachytene stage of meiosis in testis tissues of a newborn mouse placed on a blood clot.<sup>33</sup> In the 1960s, organ culture methods advanced and various conditions were extensively examined. However, it was not possible to promote spermatogenesis beyond the pachytene stage.<sup>34,35</sup> Thereafter, cell culture methods, instead

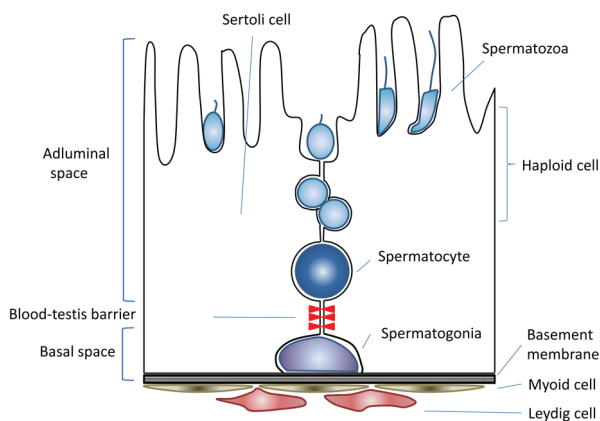


Figure 1: Schematic view of the seminiferous epithelium.

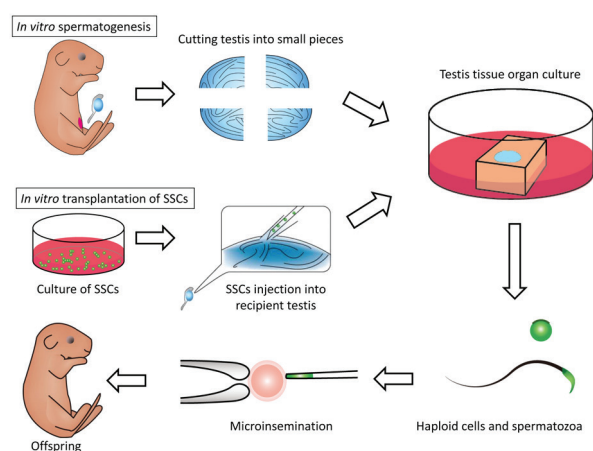


Figure 3: Schematic view of organ culture and *in vitro* transplantation of SSCs for *in vitro* spermatogenesis.

of organ culture, were used with new concepts and devices, including immortalized germ cell lines,<sup>36</sup> the production of Sertoli cell lines for use as feeder cells,<sup>37</sup> and bicameral chamber methods.<sup>38-40</sup> Despite such endeavors, progress has been limited and it had been impossible to produce fertility-competent haploid cells from SSCs *in vitro*.<sup>39,40,41</sup> After achieving the successful cultivation of SSCs and GS cells, we and others tried to promote their differentiation to sperm *in vitro*. However, we did not observe meiotic progression of the cells, let alone production of haploid cells. We then decided to re-evaluate organ culture methods based on the idea that the histological architecture of the testis, seminiferous tubules in particular, is necessary for the progression of spermatogenesis. We adopted the classical air-liquid interphase method that Takeshi Miura *et al.* and Emil and Anna Steinberger used for the testes of eels and rats, respectively.<sup>34,35,42</sup> Using mice, we successfully replicated the results reported by the Steinbergers but were not able to make spermatogenesis progress beyond the pachytene stage of meiosis. When we used KnockOut™ Serum Replacement (KSR) instead of FBS, however, the efficiency of spermatogenesis improved markedly and it culminated in sperm production. The method faithfully produced haploid cells, up to sperm in some cases, which were functional and give rise to offspring by micro-insemination<sup>43</sup> (Figure 3).

Then a question arose: does the spermatogenesis taking place in the cultured tissue really originate from SSCs? In other words, does it really encompass the whole process of spermatogenesis, from SSCs to sperm? We addressed this issue by devising a new experimental technique: the combination of the organ culture method and transplantation of GS cells into seminiferous tubules of the host testis. With this technique,

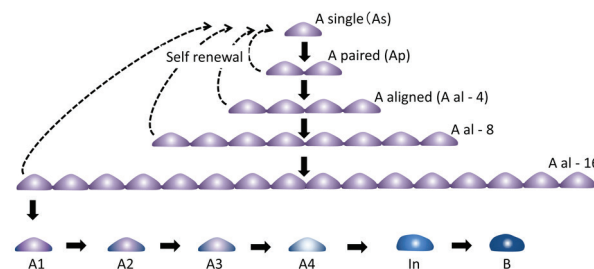


Figure 2: Schematic view of self-renewal and differentiation of SSCs. Solid and broken arrows indicate differentiation and self-renewal, respectively.

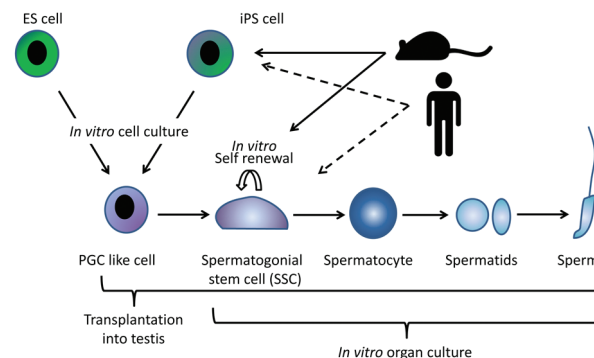


Figure 4: Schematic view of recent progress in the study of *in vitro* spermatogenesis. In mice, PGC-like cells can be induced from ES and iPS cells under *in vitro* condition. Transplantation of PGC-like cells into the recipient mouse testis induces their differentiation up to sperm. On the other hand, SSCs can be induced to differentiate into sperm by organ culture *in vitro* (solid arrows). In future, a therapeutic strategy for infertile patients could be possible through same strategies, especially with *in vitro* culture method.



named *in vitro* transplantation, we found that GS cells initially floating in the seminiferous tubule migrated toward the basement membrane to colonize it. They proliferated and formed colonies of spermatogenesis and differentiated into sperm in the cultured tissues<sup>44</sup> (Figure 3). This was the first experiment to produce functional sperm from authentic SSCs totally *in vitro*.

We also tried to reconstruct the testicular histological architecture from cells singly dissociated enzymatically. We succeeded in inducing such a reconstruction using the subcutaneous space of nude mice as an incubator.<sup>45</sup> In that experiment, testes of neonatal mice were digested with enzymes to produce a cell suspension, which was injected under the back subcutis of nude mice. The injected suspensions formed a quasi-testis structure which received its vascular supply from the host mouse. When we mixed GS cells in the dissociated testis cell suspension, some GS cells were incorporated into the newly formed seminiferous tubule and underwent spermatogenesis up to round spermatid formation, and viable offspring were produced with these round spermatids by micro-insemination.<sup>45</sup> Thus, we wondered whether the same reconstruction would be possible under culture conditions, although a vascular supply could not be available. In the initial step, the enzymatically dissociated cells of the neonate mouse testis were cultured under suspension conditions to induce aggregation. Formed cell-aggregates were then transferred to the top of agarose gel and they were cultured according to our organ culture method. In 2 weeks, tubular structures emerged. The tubules that were formed contained Sertoli cells which expressed Sox9. Leydig cells, which expressed  $\beta$ -HSD, were observed between the tubules. There were also some germ cells in the reconstructed tubules and they differentiated up to meiotic cells.<sup>46</sup> This means that it is possible to build the testicular architecture *de novo* from singly dissociated immature testicular cells and expect spermatogenesis to proceed up to, at least, the meiotic phase under culture conditions. This will certainly be a useful method for the study of testicular organogenesis and spermatogenesis. The combination of cells from different sources, not only germ cells but also different kinds of somatic cell, will be possible for testis reconstruction. When those cells become available from induced pluripotent stem cells (iPSCs) or other accessible cell sources,<sup>47,48</sup> testicular tissue fragments could be rebuilt *in vitro* from those cells without depending on the testis of individuals as a cell source in the future, which will make this method more useful and even practical for application to the study of human spermatogenesis.

### INDUCTION OF SSCS FROM ESCS/IPSCS

The origin of SSCs traces back to primordial germ cells (PGCs) in the embryonic stage, while PGCs originate from a small population of cells in the epiblast, which comes from the inner cell mass (ICM) of the blastocyst. Thus, it should be possible to induce PGCs or SSCs from ES cells (ESCs), which are derived from ICM and can be introduced back into ICM to take part in the subsequent embryogenesis, if we can exactly replicate the embryonic developmental process under culture conditions. Based on such an idea, Hayashi and Saitou successfully produced PGCs, although they cautiously call them "PGC-like cells (PGCLCs)," from ESCs *in vitro*.<sup>49</sup> They meticulously mimicked the developmental process; thus, ES cells were induced first to "epiblast-like cells (EpiLCs)" before becoming PGCLCs. The PGCLCs differentiated into sperm when transplanted into the seminiferous tubules of a recipient mouse. The sperm were functional and produced offspring by micro-insemination. The same germ cell induction was also achieved from not only ESCs but also from iPSCs. This means that somatic cells were artificially converted through iPSC cells to germ cells. The same

research team also succeeded in producing oocytes from iPSC cells.<sup>50</sup> In addition, this conversion from ES/iPSC cells to PGCs became highly efficient with a direct reprogramming method, the introduction of a set of transcription factors.<sup>51</sup> These recent research developments are encouraging to elucidate the mechanisms of germ cell development and gametogenesis in the near future. At the same time, conversion from somatic to germ cells would arouse ethical concerns anticipating future clinical applications.

### CONCLUSION

The study of SSCs has markedly advanced over the last two decades, especially in the last decade. Their identity in the testis along with their proliferation/differentiation control mechanism became clearer. They can be maintained *in vitro* and induced to differentiate up to sperm with an organ culture method. In addition, ES/iPSC cells were induced to become PCGs *in vitro* (Figure 4). Conversion of somatic cells up to sperm totally *in vitro* would be possible in the near future. However, these results were mostly obtained using the mouse as a model animal. Future studies may be focused on species other than mice, including humans. Studies on human SSCs and human spermatogenesis are now needed to reinforce our knowledge of male reproductive physiology and pathology. Based on such new information, we will be able to establish a sophisticated management method for male fertility control and treatments for male infertility patients.

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### COMPETING FINANCIAL INTERESTS

None.

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