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Deriving multipotent stem cells from mouse spermatogonial stem cells: a new tool for developmental and clinical research

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In recent years, embryonic stem (ES) cell-like cells have been obtained from cultured mouse spermatogonial stem cells (SSCs). These advances have shown that SSCs can transition from being the stem cell-producing cells of spermatogenesis to being multipotent cells that can differentiate into derivatives of all three germ layers. As such, they offer new possibilities for studying the mechanisms that regulate stem cell differentiation. The extension of these findings to human SSCs offers a route to obtaining personalized ES-like or differentiated cells for use in regenerative medicine. Here, we compare the different approaches used to derive ES-like cells from SSCs and discuss their importance to clinical and developmental research.

Introduction

Spermatogonial stem cell (SSC) research has experienced an enormous boost since 1994, when a functional assay for these cells was first developed (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994). More recently, culture systems have been developed for the long-term culture and in vitro propagation of SSCs (Kanatsu-Shinohara et al., 2003; Kanatsu-Shinohara et al., 2005; Kubota and Brinster, 2006), which have lead to the discovery that SSCs can be induced to become multipotent cells again, able to differentiate into various differentiated cell lineages. In fact, these multipotent cells resemble embryonic stem (ES) cells in their differentiation capacity and in the morphology and growth characteristics of the colonies they form in culture, and, as such, in this review they will be referred to as ES-like cells. These findings have brought excitement and enthusiasm to the field, and several reports have commented on the possible uses of these SSC-derived ES-like cells, including in regenerative medicine (Cyranoski, 2006; de Rooij, 2006; Kanatsu-Shinohara and Shinohara, 2006; Nayernia, 2007; Nayernia, 2008). However, the ways in which SSCs are transformed into ES-like cells and how these cells are induced to differentiate differ considerably between the various groups that are studying SSCs.

In this review, we describe and compare the methods that these groups have used and the results they have obtained, with the aim of informing developmental researchers about the usefulness of these various methods in an otherwise rather confusing and complex field. We describe how recent results show that SSCs can be propagated in culture and discuss how SSCs will enable studies into the mechanisms that govern how stem cells that are already dedicated to a specific lineage can dedifferentiate and return to a multipotent state. We also discuss how they can transdifferentiate into stem cells of another lineage.

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In articles on the production of ES-like cells from SSCs, each group has used different names for the cultured SSCs and ES-like cells that have been formed. For the sake of clarity, we use only the latter terms in this review. All data available to date on the derivation of ES-like cells from SSCs derive from experiments in mice; these data will therefore be interpreted in the context of our current knowledge of spermatogonial multiplication and stem cell renewal in rodents.

Spermatogonial stem cells

SSCs reside on the basal membrane of the seminiferous tubules and are single cells (Fig. 1). Upon their division, the daughter cells can move away from each other to lead to stem cell renewal, or they can stay together as a pair of so-called A_{pr} spermatogonia that are connected by an intercellular cytoplasmic bridge (Fig. 2). This latter event represents the first step in the differentiation process that ultimately leads to the formation of spermatozoa. After the formation of a pair of A_{pr} spermatogonia, there are nine to ten further divisions, which lead to spermatogonial clones of increasing length (see Figs 1 and 2). Then, spermatocytes form that leave the basal membrane and take up a position closer to the lumen of the tubules. Although it was recently suggested that pairs and short chains of spermatogonia can also have stem cell properties (Nakagawa et al., 2007), this is still the prevailing scheme of spermatogonial multiplication and stem cell renewal (for a review, see de Rooij, 2001).

SSCs can be morphologically distinguished in whole-mounts of seminiferous tubules and can be counted (e.g. Tegelenbosch and de Rooij, 1993), but since 1994 (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994) it has also become possible to determine the presence of stem cells by a functional test, the SSC transplantation assay. In this assay, cell suspensions that contain SSCs are transplanted into the testes of recipient mice, in which endogenous spermatogenesis has been abolished, for example, by treating them with a cytostatic drug or by irradiation (Brinster and Avarbock, 1994; Brinster and Zimmermann, 1994; Creemers et al., 2002). This method makes it possible to detect the presence of functional stem cells in a cell suspension and to compare stem cell numbers after various treatments or culture periods. Recently, a much less laborious assay, based on the in vitro formation of SSC colonies, has been developed (Yeh et al., 2007).

The isolation of SSCs

The first step when working with SSCs in vitro is to isolate these cells. In the mouse, only about 0.03% of the germ cells are stem cells (Tegelenbosch and de Rooij, 1993). Therefore, an attempt is usually made to purify SSCs from isolated germ cells. Surprisingly, the various groups working on the plasticity of SSCs have used widely different SSC purification approaches. Kanatsu-Shinohara et al. (Kanatsu-Shinohara et al., 2004), for example, have used the testes of neonatal mice to purify SSCs, in which the only germ cell types present are the earliest types of spermatogonia, including SSCs.

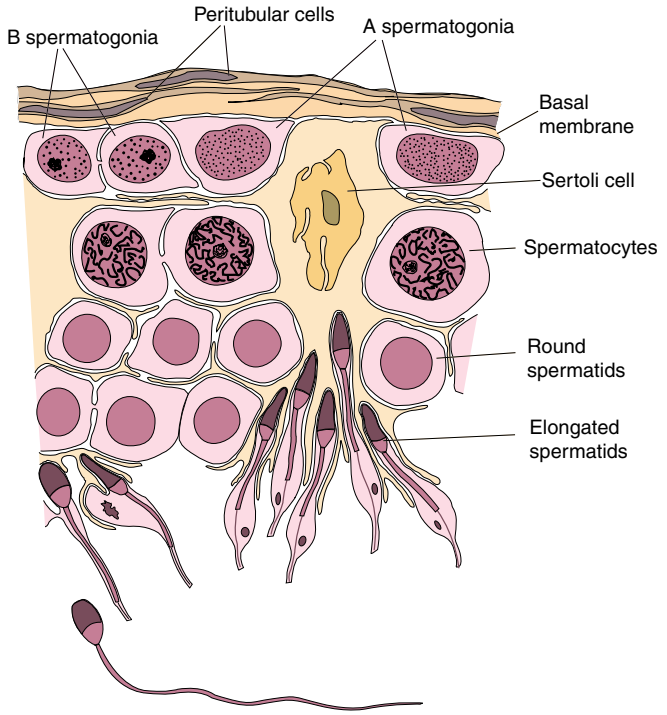

Fig. 1. Spermatogonial stem cell location and divisions. Schematic of a magnified cross-section through a mouse seminiferous tubule, showing the basal membrane of the seminiferous tubule and the position of spermatogonia in relation to this membrane. The spermatogonial stem cells (A_s spermatogonia) are in a similar position to the other spermatogonia, on the basal membrane and surrounded by Sertoli cells. Also shown are primary spermatocytes, Sertoli cells and, more towards the lumen of the tubule, round spermatids. Nearest the lumen are elongated spermatids that will soon be shed into the lumen as spermatozoa. Modified with permission from Gilbert et al. (Gilbert et al., 2003).

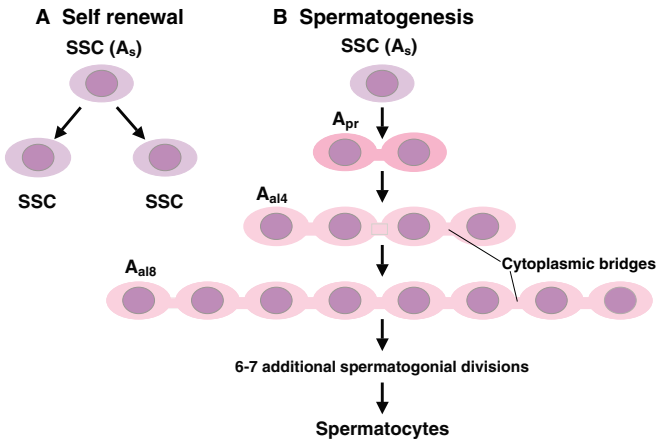

Fig. 2. A scheme of spermatogonial multiplication and stem cell renewal in rodents. Spermatogonial stem cells (SSCs, purple) can either (**A**) self renew by forming two new single cells (A-single or A_s spermatogonia) or (**B**) can differentiate, in which case the daughter cells stay together to form a pair of spermatogonia (A-paired or A_{pr} spermatogonia; pink) that are linked by a cytoplasmic bridge. The A_{pr} spermatogonia divide to form a chain of four spermatogonia (A-aligned or A_{al}; A_{al4}) that subsequently form a chain of eight spermatogonia (A_{al8}). After a total of nine to ten divisions, spermatocytes form. Spermatocytes give rise to spermatids following meiotic division, and the spermatids then transform into spermatozoa, which exit the seminiferous tubules through the lumen.

Guan et al. (Guan et al., 2006) have used testes of 4- to 6-week-old mice as the starting material, which have the full range of germ cell types, and have purified SSCs from this tissue by sorting for cells expressing STRA8 (stimulated by retinoic acid gene 8). As STRA8 is expressed by all premeiotic cells, this selection is for all types of spermatogonia, which will render a less pure population of SSCs than that obtained by Kanatsu-Shinohara et al. (Kanatsu-Shinohara et al., 2004). Seandel et al. (Seandel et al., 2007) isolated a cell suspension that contains SSCs by isolating germ cells from testes of 3- to 35-week-old mice. Clearly, this procedure renders a much less pure SSC population than that which uses neonatal mouse testes. Hu et al. (Hu et al., 2007) isolated germ cells from 6- to 8-day-old mouse testes. As these testes only contain spermatogonia and possibly early spermatocytes, the degree of purity of the cells obtained is comparable to that obtained by Guan et al. Finally, Boulanger et al. (Boulanger et al., 2007) removed the interstitial cells from seminiferous tubules prepared from adult mouse testes and just made a cell suspension of the tubules.

In none of these studies, therefore, was a serious attempt made to purify just the SSCs. In fact, this would have been difficult to achieve as there are no specific markers for SSCs. The marker that comes closest is GFRα1, one of the receptors for glial cell line derived neurotrophic factor (GDNF). GDNF is produced by Sertoli cells and is probably the most important growth factor involved in the regulation of SSC renewal and proliferation (Meng et al., 2000). GFRα1 is only expressed in SSCs and in the pairs of spermatogonia that result from the differentiating divisions of these cells, and therefore the percentage of SSCs must be high in the cell suspensions sorted for its expression. Hofmann et al. and Buageaw et al. showed the feasibility of SSC purification by making use of GFRα1 as a membrane marker, but no figures for the efficiency of the purification were given (Hofmann et al., 2005; Buageaw et al., 2005). In all events, the conclusion of all these results must be that the purity of the starting population of SSCs is not of crucial importance for producing ES-like cells.

The culture and induction of ES-like colonies from SSCs

Kanatsu-Shinohara et al. cultured SSCs in such a way that these cells propagated themselves, while retaining their capacity to repopulate a recipient mouse testis upon transplantation (Kanatsu-Shinohara et al., 2004). A special medium was used, designed to culture hematopoietic stem cells, to which several growth factors, including GDNF, were added. 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 only colonies that formed consisted of SSCs, but, within 4-7 weeks, colonies formed that morphologically resembled ES cell colonies. 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 subsequently had to be cultured under 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. (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. (Kanatsu-Shinohara et al., 2004). A substantially different approach was taken by Guan et al. (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. (Kanatsu-Shinohara et al., 2004) 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 added LIF.

Hu et al. (Hu et al., 2007) cultured germ cells of prepubertal mice under conditions that favor 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. (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.

Taken together, it does not seem that a very specific approach is required to obtain the transformation of SSCs into ES-like cells (see Table 1). This transformation can occur on different feeder layers and even without a feeder layer, provided that 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. (Kanatsu-Shinohara et al., 2004) and Seandel et al. (Seandel et al., 2007) used

Table 1. An overview of the different protocols for deriving ES-like cells from cultured mouse spermatogonial stem cells (SSCs) and the differentiation potential of the ES-like cells obtained

Starting material	Initial culture protocol	Second culture	Differentiation potential	References
Germ cells from neonatal mouse testes	Culture in stem cell medium + GDNF on MEFs for 4-7 months	Standard ES cell medium	Teratoma, EBs and chimaeras form with differentiation/contribution to endodermal, mesodermal and ectodermal derivatives. Using ES cell differentiation protocols, differentiation into hematopoietic cells, neurons, glial cells, vascular cells and myocytes was achieved	(Kanatsu-Shinohara et al., 2004; Baba et al., 2007)
Germ cells of 4- to 6-week-old mice, purified for STRA8 expression	DMEM + serum + GDNF, 4-7 days	ES cell-like colonies when cultured + LIF and/or on MEFs	Teratomas and EBs. Both form endodermal, mesodermal and ectodermal derivatives. Cardiomyocytes formed from EBs	(Guan et al., 2006; Guan et al., 2007)
Germ cells of 3-week to 8-month-old mice	Culture in stem cell medium + GDNF on inactivated testicular somatic cells for at least 3 months	On MEFs in ES cell medium	Teratoma and EBs. Both form endodermal, mesodermal and ectodermal derivatives. Chimaera formation upon transplantation into blastocyst	(Seandel et al., 2007)
Germ cells from 6- to 8-day-old mouse testes*	IMDM + serum for 3 days	Culture under osteoblast-promoting conditions (+ DMSO and FGF2)	Osteoblast formation	(Hu et al., 2007)
Cell suspension from adult seminiferous tubules*	Not applicable	Not applicable	Mammary epithelial cell differentiation upon inoculation together with mammary cells in mammary fat pad in vivo	(Boulanger et al., 2007)

DMEM, Dulbecco's Modified Eagle's Medium; DMSO, dimethylsulfoxide; EB, embryoid body; ES cell, embryonic stem cell; FGF2, fibroblast growth factor 2; GDNF, glial cell line derived neurotrophic factor; IMDM, Iscove's Modified Dulbecco's Medium; LIF, leukemia inhibitory factor; MEFs, mouse embryonic fibroblasts; STRA8, stimulated by retinoic acid gene 8.
*Two studies in which direct transdifferentiation of SSCs into other cell lineages was reported.

a specific stem cell medium, whereas Guan et al. (Guan et al., 2006) used DMEM. All three groups did add GDNF to the culture, either continuously (Kanatsu-Shinohara et al., 2004; 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.

Gene expression in SSCs and ES-like cells

An important 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. (Kanatsu-Shinohara et al., 2008) found that all four pluripotency genes are already expressed at low levels in cultured SSCs, although no NANOG (Kanatsu-Shinohara et al., 2004) or SOX2 protein expression was found in these cells. In ES-like cells, the expression of these four genes is much increased (Table 2). In addition to these pluripotency genes, the ES cell markers 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., 2004). Guan et al. (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) (Table 2). 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. (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 (Table 2).

Besides these specific studies, Kanatsu-Shinohara et al. (Kanatsu-Shinohara et al., 2008) 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. (Kanatsu-Shinohara et al., 2004) 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. and Seandel et al. reported that most of the ES-like cells obtained had a normal karyotype and that there was no evidence of clonal cytogenetic abnormalities (Kanatsu-Shinohara et al., 2004; Seandel et al., 2007). However, recently, Takahashi et al. (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

Table 2. Gene expression in cultured SSCs, SSC-derived ES-like cells, ES cells and in cells that are possibly intermediate between SSCs and ES-like cells

	Gene/protein expression levels			
Gene/protein	Cultured SSC	SSC/early ES	ES-like cells	ES-cells
AP	–*	ns	+*	+*
brachyury (*T*)	ns	ns	+‡	(+)‡
CD9	+*	ns	+*	+*
Cripto (*Tdgf1*)	–*	ns	+*	+*
Dazl	+‡	ns	(+)‡	ns
mAb EE2 reactivity	+*	ns	+*	+*
EpCAM (TACSTD1)	+*	ns	+*	+*
Eras	–*	ns	+*	+*
Esg1 (*Dppa5a*)	–*	+†	+*† (+)‡	+*†‡
Forssman antigen	–*	ns	(+)*	+*
Gdf3	ns	ns	(+)‡	+‡
Gro1 (*Cxcl1*)	ns	ns	+†	+†
α6-integrin GS2	+*	ns	+*	ns
β1-integrin GS2	+*	ns	+*	ns
c-KIT	–‡	ns	–* (+)‡	ns
KLF4, *Klf4*	(+)§	ns	+§	+§
MYC, *Myc*	(+)§	ns	+§	+§
MYCN	(+)§	ns	+§	+§
NANOG, *Nanog*	– pr*, (+) mRNA§	+ (mRNA)†	+*†‡	+*†‡
OCT3/4, *Oct3/4* (*Pou5f1*)	+* (+)‡§	+†	+*†‡	+*†‡
Nog	ns	ns	+‡	(+)‡
Plzf (*Zbtb16*)	+‡	ns	(+)‡	ns
Ras1 (*Rasd1*)	ns	ns	+†	+†
c-Ret	+‡	ns	(+)‡	ns
Rex1 (*Zfp42*)	+*	+†	+*† (+)‡	+*†‡
SOX2, *Sox2*	– pr, (+) mRNA§	ns	+‡§	+‡§
SSEA-1 (FUT1)	–*	+†	+*	+*
STRA8, *Stra8*	+‡	+†	(+)‡	+†
Utf1	+*	+†	+*	+*†
Zfp57	+*	ns	+*	+*

AP, alkaline phosphatase; mAb, monoclonal antibody; pr, protein.
+, expression is present; –, no expression; (+), expression (very) weak; ns, not studied.
*(Kanatsu-Shinohara et al., 2004).
†(Guan et al., 2006).
‡(Seandel et al., 2007).
§(Kanatsu-Shinohara et al., 2008).

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* (see Table 2).

Differentiation of SSC-derived ES-like cells

Kanatsu-Shinohara et al. (Kanatsu-Shinohara et al., 2004), Guan et al. (Guan et al., 2006) and Seandel et al. (Seandel et al., 2007) were all able to derive teratomas from the ES-like cells they obtained from cultured SSCs. In these teratomas, derivatives of all three embryonic germ layers were found. When ES-like cells were cultured using the 'hanging drop' method, embryoid bodies (EBs) formed that also gave rise to ectodermal-, mesodermal- and endodermal-derived tissues (Table 1).

Furthermore, Kanatsu-Shinohara et al. (Kanatsu-Shinohara et al., 2004) cultured the SSC-derived ES-like cells on an OP9 stromal feeder layer that supports the differentiation of mesodermal cells, such as hematopoietic or muscle cells. Within 10 days, different cell types were identified, including hematopoietic cells, vascular cells and spontaneously beating cardiomyocytes. Some ES-like cells were transferred onto gelatin-coated dishes to promote the differentiation of neural lineages and formed neurons or glial cells. Dopaminergic neurons were also found in these cultures, albeit at low frequency. In addition, Baba et al. (Baba et al., 2007) found that ES-like cells cultured in this way have the potential to differentiate into cardiomyocytes and endothelial cells.

Guan et al. (Guan et al., 2006) applied the 'hanging drop' method to induce ES cell differentiation. Differentiation into mesodermal lineages (e.g. cardiac, skeletal muscle and vascular cells) was confirmed by the expression of the early mesoderm marker brachyury (*T*), as well as of lineage-specific genes and proteins. In addition, single cardiomyocytes were isolated from beating areas of the cultures. These cells showed sarcomeric striations when stained for α-sarcomeric actinin, sarcomeric MHC and cardiac troponin T. Expression of the gap-junction protein connexin 43 (GJA1) in cardiac clusters indicated that cell-to-cell contacts had been made in these cultures and that cells were in communication with each other. Patch-clamp electrophysiological studies of these single cardiomyocytes showed spontaneous action potentials (Guan et al., 2007). The differentiation of SSC-derived ES cell-like cells into vascular endothelial and smooth muscle cells was confirmed by the expression of genes that encode proteins specific for these cell types. In ES-like-cell-derived EB outgrowths, cells were present that bore the characteristics of endothelial cells. Neuroectoderm differentiation in these EBs was confirmed by the expression of nestin, a marker for neuroepithelial precursors. Seandel et al. (Seandel et al., 2007) also derived EBs from SSC-derived ES-like cells and stimulated their differentiation into endodermal, ectodermal and mesodermal lineages. This group showed the presence of cytokeratin (KRT1)-positive cells derived from ectoderm and of brachyury-positive or skeletal-muscle-myosin (MYH2)-positive cells derived from mesoderm, as well as the presence of spontaneously beating cardiomyocytes.

Kanatsu-Shinohara et al. and Seandel et al. also microinjected ES-like cells into blastocysts to investigate whether these cells could contribute to chimeras in vivo (Kanatsu-Shinohara et al., 2004; Seandel et al., 2007). Indeed, these cells contributed widely to the tissues of the embryo. Guan et al. microinjected cultured SSCs into recipient mouse seminiferous tubules, before these cells made the transition to becoming ES-like cells, and found that they were still able to colonize the testis of a recipient mouse with spermatogenic cells (Guan et al., 2006). Surprisingly, after microinjection in blastocysts cultured SSCs were also able to contribute to the tissues of the embryo. These data are in conflict with those of Kanatsu-Shinohara et al. (Kanatsu-Shinohara et al. 2004; Kanatsu-Shinohara et al., 2008), who were not able to show the colonization of blastocysts by cultured SSCs. However, in the protocol of Guan et al., ES-like colonies form relatively early during culture and so some of the SSCs transplanted into the blastocysts might have already taken some steps towards the ES-like state.

All three groups showed that cultured SSCs, before their transition into becoming ES-like cells, can colonize a recipient mouse testis in the transplantation assay. The intriguing question then is whether or not the SSC-derived ES-like cells are still able to revert to the spermatogenic lineage. Kanatsu-Shinohara et al. transplanted the ES-like cells into the testes of *W*/*W* ($Kit^{-/-}$) recipient mice (Kanatsu-Shinohara et al., 2004). These mice lack endogenous spermatogenesis because of a deficiency of the c-KIT receptor, which is necessary for primordial germ cell development and for the differentiation of SSCs. In all cases, teratomas formed upon ES-like cell transplantation and no normal spermatogenesis was initiated. Thus, although the ES-like cells can differentiate into many lineages, it is unlikely that they can revert to becoming spermatogenic cells.

Finally, in two studies, SSCs were placed in an environment that favors tissue-specific differentiation. Boulanger et al. (Boulanger et al., 2007) injected a mixture of mouse testis cells and mammary cells into the mammary fat pad and obtained differentiation of the SSCs into cells that have mammary epithelial progenitor cell properties; mammary epithelial cells of SSC origin also formed. Hu et al. (Hu et al., 2007) cultured germ cells from 6- to 8-day-old mouse testes under conditions that favor osteoblast differentiation [the cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) plus dimethylsulfoxide and FGF2]. In this culture, cells appeared that stained for both collagen I and for AP activity in the supernatant (collagen I and AP are markers of osteoblasts). AP levels were low early on in culture and increased gradually. Thus, these two studies suggest that SSCs can transdifferentiate into other cell lineages. As in the mammary gland experiment, no teratomas were found. As such, this transdifferentiation event might not proceed via the formation of ES-like cells; if this were the case, teratomas would probably have formed.

In conclusion, the SSC-derived ES-like cells can differentiate into a great many cell lineages, either by their being directly cultured according to differentiation protocols developed for ES cells, or via the formation of EBs by these cells. In addition, some studies suggest that SSCs can transdifferentiate into non-spermatogenic cell types when placed directly into an environment that stimulates a particular differentiation pathway.

Origin of the ES-like cells

An important question that is raised by these studies is the origin of the ES-like cells that are derived from cultured testicular cells. In order to answer this question, Seandel et al. (Seandel et al., 2007) used knock-in mice bearing a GPR125-β-galactosidase fusion protein under the control of the native *Gpr125* promoter. *Gpr125* encodes an orphan adhesion type G protein-coupled receptor, and in the testis is only expressed in spermatogonia and early spermatocytes. Subsequently, this group found β-galactosidase staining in the testis-derived ES-like cells, as well as in the teratomas, EBs and chimeric mice partly formed by the transplantation of these ES-like cells into blastocysts. This clearly shows that the ES-like cells produced are of germ cell origin. A second question that arises is whether, in addition to the SSCs that maintain normal spermatogenesis, there exists in the testis a

residual small population of multipotent cells that might originate from the fetal testis, from which the ES-like colonies form in culture. This question was recently answered by Kanatsu-Shinohara et al., who showed that in a cultured SSC line derived from one cultured SSC, a colony of ES-like cells could arise alongside normal SSCs (Kanatsu-Shinohara et al., 2008). The ES-like cells that formed on this occasion could also be propagated further as ES-like cells in an appropriate ES medium. After this event, the SSC line was cultured for another 6 months without forming another ES-like colony. This indicates that ES-like cells can derive anew from normal cultured SSCs and that each SSC apparently has a small chance to convert from being a cultured SSC to an ES-like cell. Unfortunately, as the molecular mechanisms that bring about this transition are as yet completely unknown, there are no clues as to how we can influence the occurrence of this chance event. These findings indicate that the formation of ES-like cells from germ cells does not depend on the presence of a specific, residual population of potentially multipotent germ stem cells, other than the normal SSCs that remain after birth. The data rather suggest that all SSCs in principle have the potential to become multipotent again. However, in the normal seminiferous epithelium, this capacity never becomes apparent and then only rarely under the culture conditions that are presently used for SSCs.

Conclusions

The findings discussed above offer a number of new research tools and pose new questions to be answered. The transition from being a stem cell that is dedicated to a specific lineage to being a multipotent stem cell, and the regulation of this step, are of considerable, fundamental interest. This process can now be studied using the SSC culture system. However, the rate of progress in this direction will depend on our finding a way to enhance the likelihood that this transition will occur because, in the current protocols, this transition is still a relatively rare event, as described by Kanatsu-Shinohara et al. (Kanatsu-Shinohara et al., 2004; Kanatsu-Shinohara et al., 2008) but not by the other groups; in their experiments, it was probably a rare event too. A first step in finding ways to enhance the chance that a cultured SSC will become an ES-like cell has been provided by Kanatsu-Shinohara et al. (Kanatsu-Shinohara et al., 2008) in their comparison of gene expression patterns between SSCs and the ES-like cells derived from them. This approach might provide clues about how to stimulate this transition.

Another important point is whether the results obtained in mice can also be achieved in other mammalian species, particularly in humans. If applicable to human SSCs, these techniques could offer new opportunities in the field of regenerative medicine. However, for these possibilities to be realised, new differentiation protocols will have to be developed and ways found to prevent the formation of teratomas. In addition, spermatogonial multiplication and stem cell renewal in primates differ from those in non-primate mammals and are not yet fully understood (de Rooij and Russell, 2000; Ehmcke and Schlatt, 2006). Human SSCs might be different in nature and might be regulated in different ways, and they might also have different levels of susceptibility to making the transition to an ES-like cell.

From the discussions above, it is clear that the method used to isolate SSCs is not of crucial importance, and Kanatsu-Shinohara et al. (Kanatsu-Shinohara et al., 2004) and Seandel et al. (Seandel et al., 2007) used roughly the same culture protocol to generate ES-like cells, with some differences in feeder layers and in the time taken for ES cell-like cells to emerge (Fig. 3). The culture protocol of Guan et al. (Guan et al., 2006) was different in that GDNF was only added during the first 4-7 days of culture and a simple medium was then used with added serum. Taken together, from these studies one can speculate that the time for ES-like colonies to develop depends on the resemblance of the culture system to the original SSC niche. It took the longest in the Seandel et al. study (Seandel et al., 2007), in which testicular peritubular and Sertoli cells were used as a feeder layer and GDNF was added, and the least time in the Guan et al. method (Guan et al., 2006), in which no MEFS or feeder layer was provided, and GDNF was given only at the start of the cultures. Conversely, Kanatsu-Shinohara et al. (Kanatsu-Shinohara et al., 2004) found that ES-like colonies failed to form when the SSCs from neonatal mice were cultured in ES medium right from the start.

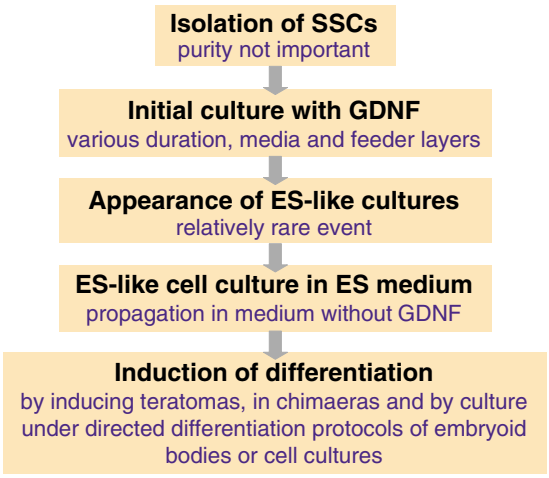

Fig. 3. A summary of the procedures involved in obtaining ES-like cells from spermatogonial stem cells (SSCs).

The transition from cultured SSCs to ES-like cells is accompanied by extensive changes in gene expression. Cultured SSCs express many germ cell-specific genes. The ES-like cells that originate from the SSCs show high levels of expression of the pluripotency genes (*Oct4*, *Sox2*, *Myc* and *Klf4*), as well as of their encoded proteins. Furthermore, germ cell-specific genes are downregulated. The important question is how the expression of the pluripotency genes is regulated. As noted by Kanatsu-Shinohara et al. (Kanatsu-Shinohara et al., 2008), as the pluripotency genes are already expressed in cultured SSCs, one has to assume that in these cells there is a mechanism through which the expression of these genes is kept at a low level. Furthermore, upon transition into ES-like cells, the expression of the germ cell-specific genes has to be shut down.

The three studies in which SSCs were cultured and then ES-like cells appeared all demonstrate the multipotent character of the ES-like cells, which can give rise to derivatives of all three germ layers, teratomas, EBs and to chimaeras (upon injection into blastocysts). Using ES cell differentiation protocols or via EB formation, SSCs have also been differentiated into a myriad of cell types. Interestingly, differences also exist in the gene expression patterns and epigenetic marks of SSC-derived ES-like cells as compared with those of ES cells and induced pluripotent cells (iPS) (Kanatsu-Shinohara et al., 2004; Kanatsu-Shinohara et al., 2008). It will be of fundamental importance to study the differences in the differentiation patterns between these cell types because particular ES-like cell types might be more suitable for producing a particular differentiated cell type.

In two studies, a transition of SSCs into other cell lineages was reported without being preceded by culture and by the transition of SSCs into ES-like cells (Boulanger et al., 2007; Hu et al., 2007). These findings will have to be confirmed and studied in further detail to establish more precisely how the transition of SSCs into cells of another lineage takes place. In itself, for regenerative medicine purposes, it would be of great advantage if SSCs could be made to differentiate into other cell lineages without becoming multipotent ES-like cells first, as these ES-like cells have the potential to form teratomas.

Remarkably, so far all the data published on the plasticity of SSCs have been obtained in mice. We still have to wait to see whether the conclusions drawn for mouse SSCs also hold true for other mammals, especially humans. Needless to say, the implications will be enormous if SSCs from humans could be used to produce ES-like cells that could be made to differentiate into a tissue a patient needs. There have been statements that this has already been achieved in humans (e.g. http://goliath.ecnext.com/coms2/summary_0199-5356645_ITM), but, so far, these have not been substantiated by publications in peer-reviewed international journals.

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