Germ-line immortality

Martin M. Matzuk*

Departments of Pathology, Molecular and Cellular Biology, and Molecular and Human Genetics, and Program in Developmental Biology, Baylor College of Medicine, Houston, TX 77030

Major advances in stem cell research have occurred over the last decades. Progress has included the generation of lines of human and mouse embryonic stem (ES) cells and the identification and purification of stem cells for multiple independent lineages. Recent studies by Brinster and colleagues in this issue of PNAS (1) also suggest that the reproductive potential of an organism can be prolonged indefinitely by using germ-line stem cells. It even appears that eggs and sperm can develop from cultured mouse ES cells (2–4). Although gametes derived *in vitro* have yet to prove their developmental potential, these studies suggest that ES cells and germ-line stem cells share many characteristics.

In most mammalian females, meiosis initiates during *in utero* development, such that no oogenic stem cells exist in the postnatal ovary, thereby limiting the reproductive lifespan of a woman. However, the reproductive lifespan of a man is in theory without limits. Although this reproductive potential of males has long been known, the further characterization and identification of spermatogonial stem cells is only a recent advance.

Three major requirements were needed to advance spermatogonial stem cell research, including (*i*) an *in vivo* assay for stem cell function, (*ii*) knowledge of stem cell markers, and (*iii*) a method to maintain the stem cells continuously in culture. Similar to his pivotal studies to optimize egg culture and transgenesis (reviewed in refs. 5 and 6), Brinster's group has systematically made giant leaps toward these goals.

Table 1. Pathway of differentiation in males

Markers	ES cells	→	PGCs	→	Gonocytes	→	Spermatogonial stem cells	→	Differentiated spermatogonia
Kit	+		+		+/–		– (low)		+
Thy-1	+		?		+		+ (low)		–
Oct4	+		+		+		+		–
Plzf	+ (low)*		+*		+		+		–
GCNA1	–		+†		+		+		+
TNAP	+ (high)		+ (high)		–		+ (low)/–		–
RET	+ (low)*		?		?		+		–
GFRα1	+ (low)*		?		?		+ (low)		–
NCAM	+		?		+		+		?

Markers that are known to be expressed (+) or absent (–) in many of the pathway cells are listed. GCNA1, germ cell nuclear antigen 1; TNAP, tissue-nonspecific alkaline phosphatase; NCAM, neural cell adhesion molecule.
*mRNA levels.
†Postmigratory PGCs only.

Spermatogonial Transplantation

A decade ago, Brinster and Zimmerman (7) and Brinster and Avarbock (8) published in PNAS methods for spermatogonial transplantation. These studies were a major breakthrough in the development of an assay for *in vivo* stem cell function. Male mice lacking spermatogenesis were chosen as recipients. Wild-type mice or transgenic mice carrying a round spermatid-expressed β-galactosidase (*lacZ*) gene (or, more recently, either ROSA26 or actin-enhanced green fluorescent protein transgenic mice) have served as stem cell donors. After enzyme digestion of the donor tubules, the germ cells of perinatal to 4-week-old testes (along with trypan blue dye) were injected with a 40-μm glass pipette into the tubules of the recipient males. The process was surprisingly efficient, with up to 100% of the injected testes containing donor cells.

Stem Cell Markers Emerge

With transplantation methodology in hand, Brinster and colleagues (9, 10) moved to develop methods to enrich for spermatogonial stem cells. Similar to the relatively small populations of other adult stem cells, spermatogonial stem cells are rare ($\approx$1 in 3,000 adult testis cells). Fluorescence-activated cell sorting (FACS) was used to identify cell-surface markers for the spermatogonial stem cells (9, 10). Through a meticulously conducted series of experiments, populations separated by FACS (and, now, magnetic-activated cell sorting) are transplanted back into mice. The first major finding was that the Kit tyrosine kinase positive populations of testis cells did not have increased stem cell activity (9). This finding was a surprise because ES cells, primordial germ cells (PGCs), gonocytes, and differentiated spermatogonia are Kit$^+$ (Table 1). This result is consistent with findings that Kit ligand is necessary for embryonic germ cell formation but not for proliferation of postnatal day 2 spermatogonia or germ-line stem cells in culture (11).

In the search for markers strongly associated with stem cell function, Brinster's group (10) showed that Thy-1, expressed on ES cells (12), is also present on spermatogonial stem cells. However, whereas many adult stem cells, including mammary gland stem cells (13) and cardiac stem cells (14), express stem cell antigen 1 (Sca-1), spermatogonial stem cells are Sca-1$^-$ (10). Thus, spermatogonial stem cells could be sorted as MHC-I$^-$ Thy-1$^+$ Kit$^-$ Sca-I$^-$ α6-integrin$^+$ αv-integrin$^{-/\mathrm{dim}}$ (9, 10).

Spermatogonial Stem Cells Keep Going and Going . . .

The third major advance in the spermatogonial stem cell field is presented by Kubota *et al.* (1) in this issue of PNAS. For the entire adult stem cell field, it has been extremely difficult to generate stable lines of stem cells that can propagate *in vitro*. Kubota *et al.* now show that mouse spermatogonial stem cells from several strains can be maintained continuously in culture for 6 months and reconstitute normal spermatogenesis in an infertile host. There were three keys to their success. First, by using the markers described above, stem cells were separated from contaminating testes cells that interfere with proliferation. Second, the stem cells were grown on a mitotically inactive layer of STO fibroblasts. Third, the stem cells could be cultured in a well defined serum-free medium; absence of serum was critical because serum supports the proliferation of other cells, including fibroblasts, but deters growth of the stem cells. This finding contrasts with the long-term culture of germ-line stem cells from only one mouse strain (DBA/2) by Kanatsu-Shinohara *et al.* (11), who used 1% bovine serum and mitotically inactive mouse embryonic fibroblasts. [Interestingly, 40 years ago, Brinster demonstrated that a serum-free medium including BSA was necessary for egg culture (reviewed in ref. 6).] Depending on the strain of mice from which they

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*E-mail: mmatzuk@bcm.tmc.edu.

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were derived, the cells grew in serum-free conditions with the addition of glial cell line-derived neurotrophic factor (GDNF) or also in the presence of soluble GDNF-family receptor α-1 (GFRα1) and basic fibroblast growth factor. The requirement for GDNF and a ligand-binding soluble receptor component was not surprising because *Gdnf* $^{+/-}$ mice lose their spermatogonia (15), GDNF can stimulate self-renewal of spermatogonia (15, 16), and the spermatogonial stem cells express three GDNF receptors [RET, GFRα1, and neural cell adhesion molecule (1)]. Similar to *in vivo*, the cultured cells doubled every ≈5.6 days and continued to have spermatogenic potential in Brinster's transplantation assay (7, 8), indicating that the culture conditions are nearly perfect for maintaining the cells in a viable, proliferating, and self-renewing state. Unlike a previously described Kit$^+$ transformed spermatogonial cell line (17), or eggs or sperm from ES cells (2–4), these transplanted spermatogonial stem cells give rise to offspring, suggesting that their chromosome complement is stable under Kubota *et al.*'s (1) culture conditions.

Insights and Implications

It is amazing that transplanted spermatogonial stem cells can migrate from the seminiferous tubule lumen to the basement membrane, recapitulating gonocyte migration during development. This finding might suggest that gonocytes and spermatogenic stem cells have the ability to "sense" similar migratory environmental cues. These cues are apparently conserved because spermatogonal stem cells from dog, cow, mouse, rat, macaque, baboon, and human, injected into the seminiferous tubules of immunodeficient mice, can migrate to their niche apposed to the basement membrane and continue to proliferate within a microenvironment separated from that of their origin by 100 million years of evolution.

With regard to spermatogonial stem cell self-renewal, Braun and coworkers (18) and Pandolfi and coworkers (19) showed that promyelocytic leukemia zinc finger (Plzf) is essential. Without Plzf, spermatogonial stem cells fail to self-renew, and the mice eventually lack spermatogenesis. Plzf likely functions by recruiting Polycomb family members and subsequent histone deacetylases. Thus, the ability of Plzf to control the epigenetic state of the stem cell dictates the rate of spermatogonial stem cell self-renewal and the timing of differentiation, both processes likely influenced by the GDNF signaling pathway.

Although not true of other species, spermatogonial stem cells from rat are not only competent to undergo all of the steps of spermatogenesis in the seminiferous tubule of a mouse but also determine the rate, performing the process in 52 days (the rate of rat spermatogenesis) as compared with 35 days (the rate of mouse spermatogenesis) (20). This finding is similar to ovarian folliculogenesis, where the state of the female germ cell (oocyte) dictates the rate of folliculogenesis (21). Thus, the germ cells, but not the somatic cells (i.e., Sertoli cells in male and granulosa cells in female), control the timing of spermatogenesis and folliculogenesis.

One beauty of the Kubota *et al.* (1) work is that the culture conditions are simple and defined. This result will allow the group to manipulate the culture conditions further to address unanswered questions. For example, what levels of spermatogonial differentiation occur during the cell culture? If spermatogonial differentiation does occur, can it be manipulated to regulate the entrance of the cells into meiosis and possibly spermiogenesis? Which testis cells [i.e., Sertoli cells (intratubule cells), myoid cells (which line the seminiferous tubule wall), and/or Leydig cells (which lie between tubules)] and/or which characteristics of the basement membrane contribute the necessary stimuli for migration and population of the spermatogonial stem cells? Although Sertoli cells are necessary for spermatogenesis *in vivo*, diffusible factors from elsewhere in the testis are likely to be critical for spermatogonial stem cell renewal. Finally, because there are >100 identified genes that play key roles in mammalian spermatogenesis, many of which are germ cell-specific (22), can gene targeting correct an infertile man's germ-line defect in his own spermatogonal stem cells? Clearly, a robust culture system, as described by Kubota *et al.* (1), combined with an established transplantation technology will provide the foundation for gene targeting, and with extension to other species will fuel a major advance in genetic modification for farm animals, endangered species, and primates, including humans.

The current study by Kubota *et al.* (1) also has important relevance for testicular cancer, stem cell potential, and male contraception. Germ cell tumors are the most common cancers in young men; yet, unlike ES cells, the transplanted spermatogonial stem cells fail to form tumors. This finding indicates that germ cell cancers derive from a precursor germ cell population [e.g., ES cells, PGCs, or gonocytes]. The close resemblance of cultured spermatogonial stem cells, PGCs, and ES cells [e.g., morphological appearance as well as Oct4 and alkaline phosphatase activity (see Table 1)] raises the possibility that the spermatogonial stem cells might be converted to ES cells by *in vitro* manipulation and would then have multipotential capability, including tumorigenicity. Likewise, as we understand how to maintain spermatogonial stem cells in a state of self-renewal in culture, then we might pursue *in vivo* maintenance of this state to prevent differentiation, thereby inducing a contraceptive effect. Future experiments, including studies to profile gene expression patterns of spermatogonial stem cells, ES cells, PGCs, gonocytes, and germ cell cancers, will provide targets for healthcare. Clearly, Brinster's group will continue to lead the technological advances and address key biological questions in stem cell research and reproduction.

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