***In vivo* and *in vitro* differentiation of male germ cells in the mouse**

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Abstract

Primordial germ cells appear in the embryo at about day 7 after coitum. They proliferate and migrate towards the genital ridge. Once there, they undergo differentiation into germ stem cells, known as 'A spermatogonia'. These cells are the foundation of spermatogenesis. A spermatogonia commit to spermatogenesis, stay undifferentiated or degenerate. The differentiation of primordial germ cells to migratory, postmigratory and germ stem cells is dependent on gene expression and cellular interactions. Some of the genes that play a crucial role in germ cell differentiation are *Steel*, *c-Kit*, *VASA*, *DAZL*, *fragilis*, *miwi*, *mili*, *mil1* and *mil2*. Their expression is stage specific, therefore allowing solid identification of germ cells at different developmental phases. In addition to the expression of these genes, other markers associated with germ cell development are nonspecific alkaline phosphatase activity, the stage specific embryonic antigen, the transcription factor *Oct3/4* and β 1- and α 6-integrins. Commitment of cells to primordial germ cells and to A spermatogonia is also dependent on induction by the bone morphogenetic protein (BMP)-4. With this knowledge, researchers were able to isolate germ stem cells from embryonic stem cell-derived embryoid bodies, and drive these into gametes either *in vivo* or *in vitro*. Although no viable embryos were obtained from these gametes, the prospects are that this goal is not too far from being accomplished.

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Introduction

Three studies published in recent months have dramatically changed the outlook on infertility and animal biotechnology, as the message they delivered is that male and female gametes can be produced *in vitro* from embryonic stem cells (Hubner *et al.* 2003, Toyooka *et al.* 2003, Geijsen *et al.* 2004). All three studies have used embryonic stem cells to establish a population of primordial germ cell-like cells, which, either by transplantation into the testes or by extending cultures, established differentiation into male or female gametes.

The experiments included in these three studies were based on years of thorough investigation of the *in vivo* development and differentiation of primordial germ cells (PGCs) and germ stem cells (GSCs). By following the path embryonic cells undergo during embryogenesis (Fig. 1), and during pre- and postnatal development to specify as germ cells, researchers have been successful in obtaining sperm and oocytes, as described below.

This review summarizes this path and highlights the most crucial steps embryonic cells undergo to become male germ cells with some references to female germ

cells. Markers used to identify PGCs and GSCs during their progress towards spermatogenesis are also detailed.

***In vivo* differentiation of germ cells**

Following germ cell differentiation through the published literature is rather confusing as to which cell type authors refer to by their specific terminology. Most studies agree that PGCs are the initial cells identified as germ cells. From then on, it becomes a bit confusing. Germ cells before commitment to spermatogenesis are termed pre-migratory PGCs, postmigratory PGCs, gonocytes, A spermatogonia, GSCs and premeiotic germ cells. In the mouse, PGCs appear at the same time as the three embryonic layers: endoderm, mesoderm and ectoderm. PGCs start migrating from the primitive streak into the allantois and then into the adjacent embryonic endoderm (Anderson *et al.* 2000). Attempts to identify germ-cell precursor cells at the embryonic stage in mice failed. PGCs are visible around 7 days post-coitum (dpc) in the extra-embryonic mesoderm; they migrate through the allantois at 8dpc to the hindgut and are referred to as migratory PGCs. From the hindgut, they move to reach the gonadal

Figure 1 The path that embryonic cells follow during embryogenesis and pre- and postnatal development to specify as germ cells.

ridge at 9.5–11 dpc (Anderson *et al.* 2000). At this location, they are referred to as postmigratory PGCs. The testis structure is established while the PGCs proliferate and differentiate into gonocytes, which are mitotically quiescent in mice until day 2 after birth. Gonocytes reach the basement membrane of the seminiferous tubuli or remain in the lumen and degenerate. Those that survive differentiate into GSCs, known in the male also as 'A spermatogonia' (De Rooij 2001). A spermatogonia undergo multiple cell divisions in stages specified as spermatogonia A1–A4 and differentiation into spermatocytes that continue their meiosis into spermatids. The latter complete their differentiation process into sperm.

The general hypothesis until recently has been that females do not retain GSCs for oogenesis and follicle formation throughout adult life, as males do for spermatogenesis. Recent studies have demonstrated that this hypothesis is not correct. Juvenile mouse and adult mouse and human ovaries possess mitotically active germ cells, allowing continued renewal of the follicle pool in adult ovaries (Bukovsky *et al.* 2004, Johnson *et al.* 2004). Hence, it can be postulated that a similar process of germ cell differentiation happens in males and females.

Markers of PGCs and GSCs

The recent development of microarray expression of data for genes involved in mitosis and meiosis, gamete formation and germline development (Schultz *et al.* 2003) has enabled the identification of genes that are stage specific to differentiating PGCs and GSCs. The general trend of markers expressed at different stages of PGCs and GSCs is illustrated in Fig. 2. The specification of PGCs involves the synergistic action of bone morphogenetic proteins (BMPs), members of the transforming growth factor type β superfamily (Kierszenbaum & Tres 2001). These proteins bind to receptor complexes signaling to transcription factors to target specific genes. Of this family, BMP4, which is produced from the extraembryonic mesoderm, is associated with PGC formation (Ying & Zhao 2001). BMP-4 is also produced by Sertoli cells very early in postnatal life, and is downregulated just before puberty (Pellegrini *et al.* 2003). BMP-4 affects PGCs and oogonia via the *Alk3* and the *R-Smad* receptors on these cells (Pellegrini *et al.* 2003). Therefore, its addition to embryonic stem (ES) cell and to GSC cultures supports their development into differentiated germ cells (Hubner *et al.* 2003,

Toyooka *et al.* 2003, Geijsen *et al.* 2004). At the same time, a putative interferon-inducible gene family, *fragilis*, *fragilis* 2 and *fragilis* 3, code for a transmembrane protein that is apparently associated with the acquisition of germ cell competency by the epiblast cells (Lange *et al.* 2003). This cluster of genes is expressed in epiblast cells after BMP-4 stimulation. Proliferation of PGCs is gonadotropin-dependent and is mediated by *Akt/PTEN* signaling (Moe-Behrens *et al.* 2003).

PGCs express *c-Kit* at relatively high levels. In PGCs as well as in hematopoietic cells, this expression is related to a single DNaseI-hypersensitive site (HS2) absolutely necessary for its activity (Cairns *et al.* 2003). The *c-Kit* proto-oncogene plays a dual role in the control of male fertility in mice via two products. The first *c-Kit* the transmembrane tyrosine kinase receptor for stem cell factor (SCF), which is expressed and functional in differentiating spermatogonia of the postnatal testis. The second, *tr-kit*, an intracellular protein, which is specifically accumulated during spermatogenesis through the use of an alternative intronic promoter, is able to trigger mouse egg activation when injected into mature metaphase II (MII) oocytes. With the onset of meiosis, *c-Kit* expression ceases, and *tr-kit* is expressed in postmeiotic stages of spermatogenesis, mainly at the early and late spermatid stages (Sandlow *et al.* 1999). The white spotting locus gene encodes the transmembrane receptor of the cytokine stem cell factor/Kit ligand (KL), which plays an important role as a regulator of proliferation, survival and migration of PGCs (Pesce *et al.* 1997, Rossi *et al.* 2000). Estrogen stimulates the *Steel* gene transcription, increasing the production of KL and consequently inducing PGC growth (Rossi *et al.* 2000).

Expression of *fragilis* is increased in the migratory PGCs, inducing expression of other germ cell-specific genes such as *stella* (Sato *et al.* 2002) and the VASA homolog (*Mvh*) (Toyooka *et al.* 2000). VASA, which is maintained until postmeiotic germ cells are formed, is a cytoplasmic protein, a product of the VASA homolog (*Mvh*) gene induced by the somatic cells of the genital ridge (Toyooka *et al.* 2000). Mutation in the *Mvh* gene leads to deficiency in proliferation and differentiation of PGCs.

Other genes that were identified in PGCs and germ cells belong to the *piwi* family, *miwi* and *mili*, which regulate PGC production and spermatogenesis (Kuramochi-Miyagawa *et al.* 2001). Two genes, *mil1* and *mil2*, showing similarities to the human interferon-induced transmembrane protein gene (*Ifitm*), were also identified in PGCs (Tanaka & Matsui 2002). *mil1* starts to be expressed continuously in differentiating PGCs at about 6.5–7.5 dpc, and *mil2* expression appears in gonadal PGCs during differentiation at about 9.5–13.5 dpc.

Members of the deleted in azoospermia (*DAZ*) gene family, which are also used as markers for germ cell identification, are expressed exclusively in germ cells. One of the protein products of the *DAZL* gene is

Figure 2 A general description of PGC- and GSC-specific marker expression during their development. The graphs demonstrate a trend only and do not represent quantity. Points marked at the bottom of the box represent zero or very low expression. Points marked at the top part of the box represent high expression.

expressed throughout most of the life of germ cells and is required for the development of PGCs and for the differentiation and maturation of germ cells from PGCs onwards (Yen 2004).

It has been also found that PGCs have a high level of tissue nonspecific alkaline phosphatase (TNAP) activity (MacGregor *et al.* 1995). However, it is not clear whether this activity is required for the survival of these cells.

Other markers for PGCs include the stage-specific embryonic antigen (*SSEA1*) (Fox *et al.* 1981), and the transcription factor *Oct3/4* (Yeom *et al.* 1996). PGCs are the only cells expressing *Oct3/4* after postgastrulation, which has a role in their totipotent phenotype (Pesce & Scholer 2000).

The differentiation of PGCs into GSCs results in a marked change in markers, including a decrease in alkaline phosphatase activity and a decrease in *c-Kit* and *SSEA-1* expression (Sandlow *et al.* 1999). GSCs gain additional surface markers such as β 1- and α 6-integrin (Shinohara *et al.* 1999). *C-Kit* expression appears again in differentiated spermatogonia A (Sandlow *et al.* 1999).

The arrival of PGCs in the genital ridge stimulates proliferation of other epithelial and mesenchyme cells to form the undifferentiated gonad composed of two compartments. The first is that of epithelial cells containing the PGCs, and the other is a stromal compartment containing fibroblasts and blood vessels. A morphologic distinction in female and male gonads is obvious about 12–12.5 dpc. Although a few genes that appear in both male and female gonads have been connected to gonadal establishment and sex differentiation (Merchant-Larios & Moreno-Mendoza 2003), attention has been given to the *Sry* gene located at the Y chromosome (Koopman *et al.* 1990). The exact pathway by which *Sry* affects differentiation to a male gonad is elusive and is probably an outcome of gene expression and cellular interactions. Following sex determination, male germ cells enter a mitotic arrest that is maintained until after birth. Female germ cells enter meiotic prophase. Recently, the *Stra8* gene has been identified as an early molecular marker of female germ cell differentiation (Menke *et al.* 2003). This gene is upregulated in germ cells of XX gonads prior to meiotic entry and is not expressed in male embryonic germ cells. This is in conjunction with upregulation of the meiotic gene *Dmc1* and downregulation of *Oct 3/4*.

From embryonic stem (ES) cells to gametes *in vitro*

Restoration of a complete spermatogenesis process in culture is important to understand the biologic events related to cell proliferation and cell differentiation. Even more important is the hope of providing a treatment to many infertile individuals. Cocultures of germ cells with Sertoli cells aid a limited number of cells to undergo meiosis and become round spermatids (Parks *et al.* 2003). The latter can be directly injected into oocytes to result in viable embryos and live offspring (Kimura & Yanagimachi 1995, Parks *et al.* 2003). However, a critical requirement for successful *in vitro* spermatogenesis is maintaining germ-cell viability *in vitro* for long periods of time, and this implies greater limitations on initiation of spermatogenesis from PGCs or GSCs. To examine the ability of PGCs to progress through spermatogenesis, researchers have been concentrating on developing methods to enrich this cell population *in vivo* and *in vitro* (McCarrey *et al.* 1987,

DeFelici 1998, Mayanagi *et al.* 2003a,b) in conjunction with the optimization of prolonged culture systems. Mouse PGCs are generally difficult to maintain *in vitro* for longer than a week even when cultured within urogenital complexes. Longer maintenance of these complexes and an increase in PGC number has been accomplished by the replacement of bovine and equine serum with rodent serum (Mayanagi *et al.* 2003b). Interestingly, more PGCs can be obtained when gonads originate from male mice. Studies have also shown that 17β -estradiol (E2) or mycoestrogen zearalnone (ZEA) significantly increase, in a dose-dependent manner, the number of PGCs cultured on STO fibroblasts in the presence of gonadal somatic cells (Moe-Behrens *et al.* 2003).

Methods to separate GSCs from testicular tissue and maintain them for prolonged periods *in vitro* have been also explored (Kanatsu-Shinohara *et al.* 2003, Nagano *et al.* 2003). As they are a rare cell population within the testis, enrichment (McLean *et al.* 2002 2003, Shinohara *et al.* 2000a) and identification (Schrans-Stassen 1999, Shinohara *et al.* 1999 2000b, Van der Wee *et al.* 2001) of GSCs are imperative. A purified population of A spermatogonia may survive a prolonged culture period when cocultured on monolayers of Sertoli cells producing stem cell factor (SCF) (Van der Wee *et al.* 2001, Feng *et al.* 2002). *In vitro* addition of SCF to *c-Kit*-expressing A(1)–A(4) spermatogonia from prepubertal mice, or to immortal A spermatogonia (Feng *et al.* 2002), stimulates their progression into the mitotic cell cycle and significantly reduces apoptosis in these cells (Yan *et al.* 2000, Dolci *et al.* 2001).

The use of PGCs and GSCs in transplantation studies to restore fertility has been initiated with varied degrees of success (Parks *et al.* 2003, Nayernia *et al.* 2004), leading scientists to push boundaries even further by using ES cells as the starting point for germ cell differentiation. Embryoid bodies (EBs), established from ES cells, consist of tissue lineages typical of the early embryo. Recently, researchers have been able to show that mouse and human ES cell-derived EBs include cells that express markers specific to germ cells (Hubner *et al.* 2003, Toyooka *et al.* 2003, Clark *et al.* 2004, Geijsen *et al.* 2004). In two separate studies, scientists succeeded in isolating these spontaneously differentiated cells and driving their development further in spermatogenesis *in vivo* or *in vitro*. Toyooka *et al.* (2003) isolated PGCs from male knock-in embryonic stem cells in which GFP or *LacZ* was inserted adjacent to the *VASA* gene homolog. Cells expressing GFP or *LacZ* also expressed *Mvh*, indicating their differentiation into germ stem cells. The appearance of these cells was observed in 5–7-day-old Ebs, and their concentration increased after exposure to BMP-4 secreted by trophectoderm cells. After isolation and transplantation into testicular tubuli, these cells were able to progress in spermatogenesis and produce fully differentiated sperm.

In the second study, spermatogenesis up to haploid cells was accomplished *in vitro* (Geijsen *et al.* 2004).

Like Toyooka *et al.* (2003), Geijsen *et al.* (2004) also isolated germ cells from EBs. Addition of retinoic acid to these EBs resulted in manifestation of a cell population with positive *SSEA-1* expression that stained positive for alkaline phosphatase, indicating them to be PGC-like cells. The *in vitro* differentiation of germ cells identified by Geijsen *et al.* (2004) was confirmed by the positive expression of markers such as *Oct3/4*, *Piwil2* and *DAZL*. The *Sry* gene, specific to the male germ cell, was also expressed in some of the cells. This specification to a male germline was confirmed by the identification of upregulation of acrosin and heparin in the highly specified cells, suggesting that the default phenotype of female germ cells was suppressed in their cultured EBs. The differentiated cells underwent limited meiosis to produce a small number of haploid cells. These cells were able to fertilize mature oocytes by intracytoplasmic injection, and 20% of the embryos developed to blastocysts *in vitro*.

In another study (Hubner *et al.* 2003), oocytes were obtained *in vitro* from male or female ES cells with no specific or significant modification to culture conditions, suggesting that germ-cell differentiation from ES cells is a spontaneous event. It can be postulated that the observations in this study were detected purely because of the extended culture time of 26 days, not previously used by other researchers. Hubner and colleagues (2003) used ES cells with a restricted germ cell *Oct3/4* and GFP marker for their study. These cells were cultured in a common ES culture medium. After 4 days, germ cell-specific *Oct3/4* was detected in some cells. About 40% of cells expressed germ cell *Oct3/4* at 7 days in culture. These cells were separated from the rest and were examined for the expression of *c-Kit* and *VASA*. Three germ cell-type cells were identified: 1) cells positive for *Oct3/4* and *c-Kit* with little expression of *VASA* that resemble premigratory germ cells (PGCs); 2) cells expressing *Oct3/4* and *VASA*, but not *c-Kit*, that resemble early postmigratory germ cells (PGCs and gonocytes); 3) cells with negative *Oct3/4* expression, negative for *c-Kit* expression and positive for *VASA* expression, that resemble postmigratory cells about to enter prophase I. Although, in this study, ES cells were not allowed to form EBs in culture, defined aggregates formed during the extended culture time were collected and cultured in new plates. These formed structures were similar to ovarian follicles producing oocytes. Hubner *et al.* (2003) proposed that male and female ES cells develop into germ cells with the female phenotype because of the absence of appropriate *Sry* expression. This lack of *Sry* expression may have been affected by the lack of appropriate stimulant for the production of male germ cells. In relation to the other two studies, this may be BMP-4 and/or retinoic acid. The oocytes underwent spontaneous activation and formed blastocysts.

These three recent studies have proved that gametes can be obtained in culture, and that the prospect of overcoming all types of sterility is more realistic than was thought. The fact that no viable embryos were produced

in any of these studies has raised speculation regarding the future of such developments. It is now remains to determine whether germ cells produced *in vitro* are capable of producing healthy offspring.

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