Modeling germ cell differentiation

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1. Germline differentiation of embryonic stem cells

1.1. Early germline fate commitment and PGC development

Endowed with the task of transmitting the genetic information to the next generation, germ cells are without doubt critical cells for any species that multiplies through sexual reproduction. Recognizing the vital importance of germ cells to species survival, in many lower organisms including *Drosophila Melanogaster*, *Xenopus Laevis* and the Zebrafish (*Danio Rerio*) germ cell formation is rigidly programmed. In these species, germ cell specification occurs through the localization of so-called "germ plasm" to the posterior pole of the unfertilized egg (Baughman and Geijsen, 2005; Jin and Xie, 2006; Strome and Lehmann, 2007). Following the first cleavage divisions, cells that receive the germ plasm are destined to become the germline stem cells. Such a pre-determination model of germ cell specification assures that germ cells are set-aside during the earliest steps of embryonic development, protecting them from the lineage specification and differentiation events that craft the body plan of the embryo. In egg-laying species, which have limited control over the embryo's extra-embryonic milieu, the specification of germ cells through pre-localized germ-plasm may protect the germline against environmental influences. The inheritance of germ plasm

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in lower organisms is sufficient to install a germ cell identity, but the specific mechanisms by which this occurs remain unknown.

The term "germ plasm" was first coined by August Weismann in a 1893 book of the same title, although in Weismann's view germ plasm was the carrier of hereditary information, reminiscent of our current understanding of genetic information (Weismann, 1893). Currently the term "germ plasm" refers to a large complex of proteins and RNA that visually polarizes the oocyte. The molecular components of germ-plasm are best understood in Drosophila, where a molecular cascade of interacting proteins and RNAs has been identified (Lehmann and Ephrussi, 1994; Mahowald, 2001; Rongo et al., 1997; Rongo and Lehmann, 1996). The keystone component of *Drosophila* germ-plasm assembly is Oskar (Ephrussi et al., 1991; Kim-Ha et al., 1991). Oskar RNA becomes localized to the posterior pole of the egg where it's translation leads to the assimilation of additional germ plasm components. Mis-localization of Oskar RNA or protein to the distal pole results in germ cell formation at the distal site (Ephrussi and Lehmann, 1992). Many of the Drosophila germ plasm components are conserved and play an essential role in mammalian germ cell formation as well, but it is interesting to note that the key factor in Drosophila, Oskar, has no known orthologs in any other species. As mentioned, in many species, including mammals, orthologs of other *Drosophila* germ plasm components have been identified and have been shown to play a role in germ cell development. However, no higher order germ plasm complexes have been identified in the mammalian oocyte. Unlike the rigid specification of germ cells in lower organisms, mammalian segregation of germline and somatic cells occurs later in embryonic development and is guided by extracellular cues (Baughman and Geijsen, 2005; Saitou et al., 2002). Mammalian germ cell specification is a plastic process and occurs after the embryo has implanted into the uterus. Recent work from the laboratory of Dr. Azim Surani has identified Blimp-1 (Prdm1) as an early marker of the first primordial germ cells that can be detected in a small cluster of 6–10 cells in the proximal epiblast at E6.5 of murine embryonic development (Ohinata et al., 2005; Saitou et al., 2005). These cells migrate to the base of the allantois where at E7.5 they express the germ cell specific marker gene Stella (Dppa3; Saitou et al., 2002). The functional significance of both these early germ cell markers is unclear, but Blimp-1 knockout studies have demonstrated an essential role for this gene in germ cell determination (Ohinata et al., 2005; Saitou et al., 2005). Blimp-1 is a transcriptional repressor containing a SET/PR domain. In the absence of Blimp-1 expression, early PGCs fail to suppress Hox gene expression, resulting in the activation of a somatic differentiation program. Recently, Blimp-1 was shown to interact with Prmt5, an arginine-specific histone methyltransferase, which mediates symmetrical dimethylation of arginine 3 on histone H2A and/or H4 tails (Ancelin et al., 2006). The Blimp-1 mediated arginine methylation may act to suppress the expression of genes associated with somatic differentiation to preserve germ cell pluripotency during a developmental stage in which strong morphogen gradients act to pattern the early embryo. A second marker, Stella, marks the early PGCs at the base of the allantois at E7.5 (Saitou et al., 2002; Yabuta et al., 2006). While Stella is a highly specific molecular marker for these early germ cell precursors, the function of this gene in germ cell development is obscure. The early expression of Stella suggested a role in germ cell specification, but germ cell development proceeds normally in Stella knockout mice (Bortvin et al., 2004; Payer et al., 2003). Stella-deficient animals are viable and both sexes are fertile and demonstrate no defects in germ cell development. However, litters from Stella-deficient females are small and further analysis of second-generation embryos revealed that early development is impaired and most embryos fail to develop to the blastocyst stage. Impaired development could not be rescued by crossing the Stella-deficient females with wild-type males, demonstrating that Stella is an important maternal factor that plays a critical role during preimplantation development (Payer et al., 2003). While the molecular role of Stella is not fully understood, a recent report suggests that Stella may act to protect the maternal genome from demethylation shortly after fertilization (Nakamura et al., 2007).

Regardless of its function, Stella remains the most faithful molecular marker for early germ cell fate commitment. Unlike Blimp-1, which is also expressed in the developing primitive endoderm, Stella expression is restricted to developing PGCs and thus can be used to track the development of these cells both *in vitro* and *in vivo* (Payer et al., 2006). In vivo, Stella-positive cells migrate from the base of the allantois through the hindgut to the gonadal ridge, where the cells partner with the developing somatic supporter cells of the gonad. It is the somatic cells that drive sex specification in this initially bi-competent gonad. The Sry gene, located on the Y-chromosome, plays a critical role in this process. Sry is expressed by the developing Sertoli cells and induces the formation of testicular cords between E11.5 and E12.5 where the germ cells commit to the male lineage and enter mitotic arrest to become gonocytes (DiNapoli and Capel, 2008). In the absence of Sry expression, germ cells undergo female sexual differentiation and initiate meiosis. Retinoic acid (RA) was recently shown to play a determining role in the initiation of female sex determination (Koubova et al., 2006). At E11.5, RA signaling induces the expression of Stra8 in the developing female gonad. Stra8 was initially identified as a male germ cell specific marker that is expressed upon initiation of differentiation of spermatogonial stem cells, but recent data indicates that Stra8 marks differentiation of germ cells of either sex at different stages. In the male gonad, Stra8 expression is repressed by the expression of Cyp26(b1), a

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CYP-family enzyme capable of RA degradation (Koubova et al., 2006). The specific expression of Cyp26(b1) in the developing male gonad acts to remove RA, thereby blocking premature initiation of meiosis in male germ cells.

While conventional genetic analysis has helped elucidate some of the molecular events underlying germ cell specification, migration, and sex differentiation, many of the factors guiding these processes are not well understood. The limited numbers of early germ cells and the relative inaccessibility of the developing murine embryo severely hamper the investigation of these processes. The recent development of *in vitro* culture methods for the generation of germ cells from embryonic stem cells eliminates some of these restrictions and allows for the first time robust molecular and biochemical analysis of these processes.

1.2. In vitro germ cell differentiation

1.2.1. Female germ cell development

In 2003, Hübner and colleagues demonstrated that *in vitro* cultures of differentiating embryonic stem cells (ES cells) yielded structures resembling primordial follicles and even oocyte-like cells (Hubner et al., 2003). Using transgenic ES cells expressing a GFP fluorescent reporter gene under the control of a germ-cell specific Oct4 promoter, they demonstrated that monolayer differentiation of ES cells resulted in the induction of GFP expression in about 40% of the cells after 8 days in culture. Some of these cells subsequently lift off in clusters containing both GFP-positive germ cells that express Vasa protein and somatic cells. Further culture of these cell clusters under growth factor conditions that favor oocyte development results in the formation of primordial follicle-like structures containing large cells resembling oocytes. Oocyte differentiation of ES cells was subsequently reported by two other groups using slightly different methods. Lacham-Kaplan *et al.* allowed ES cells to differentiate into so-called embryoid bodies (EBs; Lacham-Kaplan et al., 2006). EBs form when ES cells are dissociated and allowed to form aggregates in the absence of the growth factors LIF and Bmp4 that normally mediate ES cell pluripotency. Under these conditions ES cells in the newly formed EB will initiate a differentiation program that recapitulates early embryonic development (Doetschman et al., 1985; Leahy et al., 1999). Within the context of the developing EB, all three germ layers form and generate more differentiated derivatives. Thus, EB differentiation is a tractable *in vitro* model for the study of cell differentiation, with the caveat that the spatial organization of the normal embryo and the extraembryonic tissues are lacking.

Lacham-Kaplan employed a modified EB differentiation protocol, in which the EBs are grown in conditioned media from cell cultures of newborn testes (Lacham-Kaplan et al., 2006). Under these conditions, structures formed containing oocyte-like cells that expressed Vasa protein as well as Oct4, Dazl and Stella, molecular markers of germ cell development. The large oocyte-like cells were surrounded by granulosa-like cells, and expressed the oocyte markers Figα, ZP3 and Stra8. However, a Zona Pellucida, a structural hallmark of maturing oocytes, did not form around these cells. Moreover, the authors reported a wide variation in cell size, indicating that while these germ cells appear to initiate a molecular program of germline differentiation, *in vitro* oocyte maturation is impaired. This suspicion was reconfirmed by Novak and colleagues, who analyzed the expression of markers of meiosis in these putative oocytes (Novak et al., 2006). The authors demonstrated that while murine ES cells can form follicle-like structures that express the meiotic regulator SYCP3 specifically in the developing germ cells, nuclear distribution of SYCP3 protein is abnormal. As a consequence, the typical chromosomal synapses, which are mediated by SYCP3 and precede meiotic recombination, are not observed. Thus, while genes associated with the early onset of meiosis are expressed in oocyte-like cells, indicating the initiation of germ cell development along the female lineage, the lack of guidance by the proper environmental clues apparently precludes normal progression into meiosis and oocyte maturation.

The derivation of oocyte-like cells has recently been reported from alternative cell sources such as porcine skin (*in vitro*) and bone marrow (*in vivo*; Dyce et al., 2006; Johnson et al., 2005). The oocyte identity of these cells has been based largely on cell morphology and/or the expression of molecular markers. To date, no functional demonstration of fertilization or embryonic development has been reported from such cells, raising skepticism about their true identity and function.

1.2.2. Male germ cell development

Complementing the reports of oocyte differentiation from ES cells in vitro, two laboratories have demonstrated differentiation along the male lineage. Interestingly, whereas monolayer differentiation appears to promote formation of oocyte-like cells, three-dimensional EB formation appears to be the method of choice to drive germ-line development to the male lineage, for unclear reasons. Noce and co-workers used a lacZ knock-in ES cells and vasa-lacZ+ cells to

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identify early germ cell precursors within developing EBs (Toyooka et al., 2003). When vasa-GFP$^+$ cells were mixed with dissociated embryonic gonadal tissue and transplanted into the testes of recipient mice in which endogenous germ cells were ablated, GFP-positive cells colonized the seminiferous tubules and mature ES-cell derived sperm formed. The ability of this sperm to fertilize oocytes and generate offspring was not reported. We independently observed male gametogenesis from ES cells using the cell surface antigen SSEA-1 to identify and isolate primordial germ cells from developing EBs (Geijsen et al., 2004). Using a protocol of brief exposure of the cells to Retinoic Acid, we were able to demonstrate the derivation of EG-like colonies from our EB cultures, which displayed the erasure of imprints, a functional property that is the exclusive domain of developing primordial germ cells. Prolonged culture of EBs resulted in the emergence of a rare population of haploid cells that could be isolated using an antibody directed against primitive spermatocytes. When injected into oocytes, these cells generated diploid blastocysts, but never supported full mouse development.

The above studies on the derivation of male and female germ cell from ES cells demonstrate that the formation of PGCs from ES cells is robust, but further germ cell differentiation is inefficient and the functional quality of the resulting gametes is unclear (Daley, 2007). In fact, the inability to generate live pups was a common thread in all of the early studies on *in vitro* germ cell development and suggested that the complex interactions with somatic cells and endocrine environments cannot be fully recapitulated in an *in vitro* system. Yet, in a provocative study, Engel and colleagues recently reported live offspring from ES-derived sperm (Nayernia et al., 2006). Using a GFP reporter for Stra8, the Engel group differentiated the pluripotent F9 teratocarcinoma line for 2 months and identified GFP$^+$ cells that reportedly repopulated germ-cell depleted recipient testes. However, in this in vivo environment the cells failed to develop into functional sperm as the sperm showed structural abnormalities and reduced motility. While the sperm supported embryo formation after being injected into oocytes, no live pups were born. Again, the lack of live offspring suggests that functional gametes were not formed. More remarkable, however, are the claims from this group that ES-derived male gametes isolated from ES cell cultures using comparable selection strategies generated live offspring (Nayernia et al., 2006). The achieve this, the group sequentially selected Stra8-GFP$^+$ cells followed by cells expressing a dsRed reporter driven by the protamine promoter, thus identifying cells at a later stage in spermiogenesis. Upon injection of protamine-dsRed$^+$ cells into oocytes, 65 embryos developed to the blastocyst stage, out of which 12 animals were born. Curiously, only a few of the offspring harbored the transgene as demonstrated by Southern blot analysis. These data, while promising, are unfortunately inconclusive, since the authors failed to demonstrate unequivocally that the offspring carry a full haploid contribution from the ES-derived sperm. Genome-wide polymorphism analysis of the offspring would have provided an answer, but was not reported by the authors.

Early germ cell specification, differentiation and the formation of primordial germ cells that display imprint erasure are robustly recapitulated during in vitro differentiation of ES cells, and has been shown to occur in human ES cells as well (Clark et al., 2004). But to date, live offspring have never been demonstrated conclusively from ES derived gametes, generated fully or even partially *in vitro*. Current data suggests that proper progression through meiosis critically depends on signals from the somatic microenvironment and the in vitro environment appears insufficient to support the generation of mature functional gametes.

2. Applications, practical and ethical challenges

While the derivation of live offspring from ES cells appears a distant prospect, the possibility of partial, or complete in vitro culture protocols to create functional germ cells poses new ethical questions to the field of assisted reproductive technologies. The initial reports on the *in vitro* generation of germ cells sparked hope and concern alike that human babies could one day be generated whose parentage traced to a cell line. Recently, the Hinxton Group (www.hinxtongroup.org), an international Consortium that studies the ethical and legislative challenges of pluripotent stem cell research, convened to evaluate the current state of the field and it's future potential implications. The group has outlined a set of recommendations that can be used by the general public, scientists and policy makers to decide on appropriate measures to ensure that the scientific progress this technology offers doesn't contrast with societal values.

The ability to drive germline differentiation of ES cells offers a valuable tool to study development and disease. ES cell cultures are readily scalable, making otherwise rare germ cell populations amenable for studies of mammalian germ cell development. The availability of reporter cell lines will allow the prospective identification and interrogation of specific stages of the germ cell differentiation process and may aid in determining at what stage *in vitro* differentiation goes awry. Such tools will also aid high throughput screens for factors that either augment or impair germ cell differentiation, which may lead to insights into the molecular basis of infertility and the development of novel contraceptives.

Mammalian fertility is not well understood, partly because infertility is incompatible with the propagation of mutant mouse strains. Key players in the germ cell differentiation process are often found by accident, as a byproduct of the study of gene function in a different organ. The ability to study germ cell development *in vitro* uncouples the experimental system from the germ cell reproductive task, and for the first time allows a directed effort to uncover critical pathways that direct germ cell specification and differentiation.

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