Germ Cell Nuclear Factor is Not Required for the Down-Regulation of Pluripotency Markers in Fetal Ovarian Germ Cells

By Leah M Okumura

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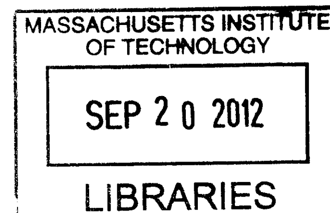
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Leah M. Okumura

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ABSTRACT

In mouse, germ cells retain expression of the pluripotency markers *Oct4* and *Nanog* longer than any other cells in the body. While somatic cells repress these markers during gastrulation, female germ cells continue to express them until around the time of meiotic initiation. It is not yet clear why pluripotency markers are down-regulated with this particular timing, nor is it understood what factors are involved in their repression.

I have examined in fetal ovarian germ cells the expression and function of *Gcnf* (germ cell nuclear factor), an orphan nuclear receptor known to regulate both *Oct4* and *Nanog* in gastrulating embryos. I have found that *Gcnf* is expressed in a female germ-cell-specific manner at the time when *Oct4* and *Nanog* are down-regulated there. *Gcnf* mutants in which the ligand binding domain is disrupted display defects after gastrulation comparable to those observed in *Gcnf*-null mutants and those lacking the DNA binding domain. In contrast, the germ cells *Gcnf* ligand binding domain mutants show no failure in repression of pluripotency markers, and other aspects of female germ cell development appear normal as well. Thus, it appears that the ligand binding domain of GCNF is not required for fetal ovarian germ cell development.

Thesis supervisor: David C. Page
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CHAPTER 1

Introduction:

Germ cell development, pluripotency, and the nuclear receptor GCNF

Germ cells provide the only physical link between generations, creating upon fertilization a new organism, which will in turn give rise to a new generation of germ cells. In this sense, germ cells can be considered the ultimate immortal cells. This introduction will provide an overview of mouse germ cell development from specification to sexual differentiation of fetal germ cells, of totipotency and pluripotency in the early embryo and in derived *in vitro* cell lines, and of the identification and function of the nuclear receptor *Gcnf* (germ cell nuclear factor), a known regulator of two key pluripotency factors, *Oct4* and *Nanog*.

I: GERM CELL DEVELOPMENT

Germ cells possess many unique properties. They are the only cells in the body that contribute DNA to the subsequent generation, ensuring the perpetuation of the species. To do so, they undergo a highly specialized type of cell division, meiosis, which halves the chromosome content of the germ cell and also introduces genetic variation through recombination, enabling the diversity underpinning the molecular mechanisms of evolution. In the course of each generation, germ cells must cycle between states of high specialization, undergoing meiosis and differentiating into sexually dimorphic, mature gametes; and very low differentiation, as they form the totipotent zygote and cells of the very early embryo, which will give rise to all the differentiated cell types of the organism.

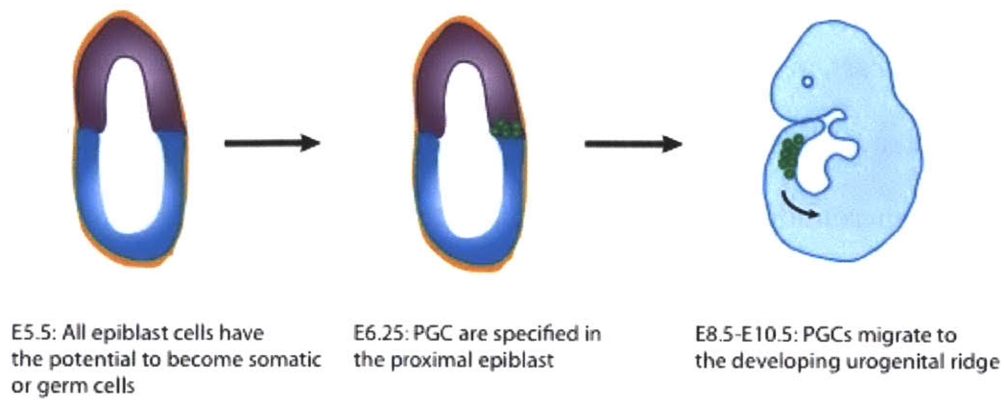
This section will provide an overview of a crucial interval in the development of mouse germ cells, from their specification after gastrulation through initiation of

the meiotic program. This time period encompasses a number of significant events in germ cell development, including their migration to the gonads, transition to a gametogenesis competent state, sexual differentiation, and the decision of whether or when to enter meiosis (summarized in Figure 1).

Specification

Germ cell identity is specified by different mechanisms in different types of organisms. These mechanisms generally fall into one of two categories, pre-formation and epigenesis. In both cases, however, the goal is the same: to prevent the future germ cells from acquiring a somatic state and to enable them to retain potential pluripotency, which will ultimately allow for the creation of an entire organism from a single cell.

Many of the commonly used model organisms specify the germ cell lineage via pre-formation. For example, in flies and worms the earliest germ cells, primordial germ cells (PGCs), are "pre-formed," specified by specialized RNA and protein in the maternal cytoplasm called germ plasm, which is deposited in the oocyte prior to fertilization. These cytoplasmic determinants are then asymmetrically distributed upon the first cell divisions, ultimately being deposited in only a handful of cells that will go on to form the germ cell lineage (reviewed in (Seydoux and Braun, 2006)). A similar mechanism can also be observed in the frog



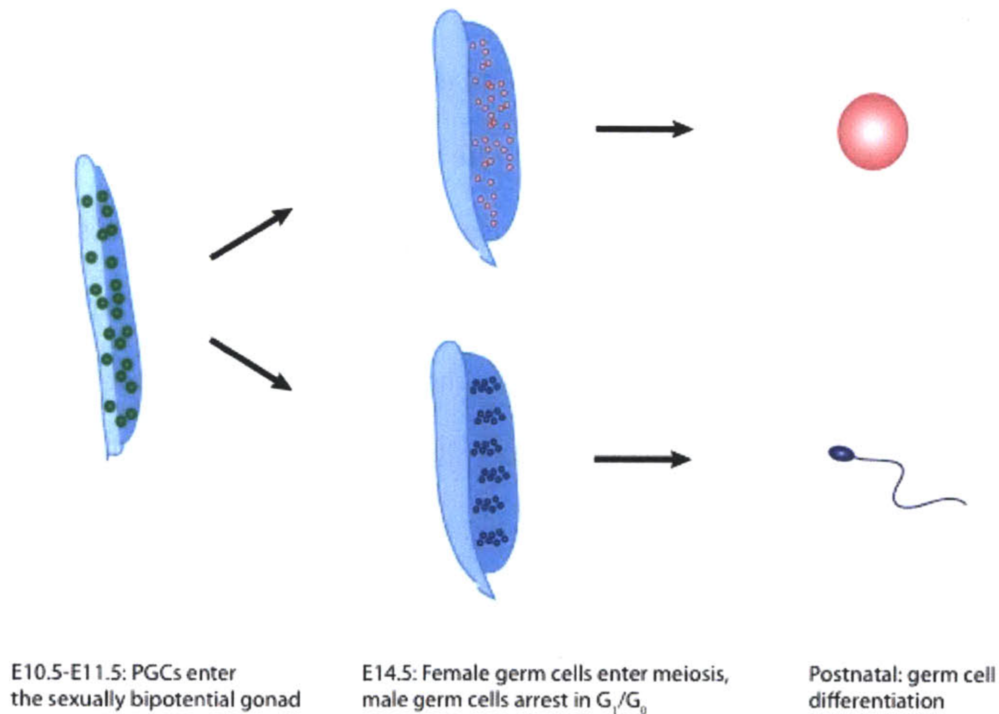


Figure 1: An overview of germ cell development. Purple: extraembryonic ectoderm. Light blue: epiblast and somatic tissue. Green: sexually bipotential germ cells. Pink: female germ cells. Dark blue: male germ cells.

Xenopus laevis and the zebrafish *Danio rerio* (reviewed in (Extavour and Akam, 2003)).

By contrast, in mouse no germ plasm has been identified in the very early embryo. Instead, PGCs are specified by induction, also called "epistasis," when signals from surrounding tissues instruct a certain set of cells to become germ cells. How mammalian PGCs are specified has long been a mystery. Although the picture is likely still incomplete, a number of key players in this process have been identified.

Mouse PGCs can be detected by around embryonic day 7.25 (E7.25) as a cluster of about 40 to 45 cells at the base of the allantois, and can be identified by the enzymatic activity of alkaline phosphatase (Ginsburg et al., 1990; Lawson and Hage, 1994). These PGCs arise from cells of the posterior proximal epiblast. BMP4 (bone morphogenic protein 4) signaling from the extraembryonic ectoderm (ExE) induces a small subset of the most proximal epiblast cells to express PRDM1 (PR domain containing 1, with ZNF domain; also called Blimp1) and PRDM14 (PR domain containing 14), the earliest known markers of germ cell precursors, both of which are required for proper PGC formation (Ohinata et al., 2005; Yabuta et al., 2006; Yamaji et al., 2008). *Bmp4* mutants suffer a complete failure to express both PRDM1 and PRDM14, indicating that in the absence of BMP4, PGC specification is disrupted at or before its earliest known event (Ohinata et al., 2009).

Only competent epiblast cells that receive enough BMP4 signal will become PGCs. Positive instructive signal from the ExE is counterbalanced by a number of inhibitory signals from the anterior visceral endoderm (AVE), restricting the area in

which cells can be induced to become PGCs. In the absence of *Smad2* (MAD homolog 2), which is required for the formation of AVE, the number of cells expressing *Prdm1* is greatly expanded , whereas in the absence of *Bmp8b* (bone morphogenic protein 8b), which restricts AVE development, *Prdm1* expression is impaired (Ohinata et al., 2009).

Together, the integration of these signaling molecules across the epiblast results in a small subset of proximal epiblast cells expressing *Prdm1* and *Prdm14*, which will eventually become the PGCs. Both *Prdm1* and *Prdm14* are required for proper PGC specification. In *Prdm1* mutants, fewer PGCs with active alkaline phosphatase (AP), a standard marker of mouse PGCs, can be detected than in wild-type embryos, and those PGCs that are present do not migrate through the hindgut, fail to up-regulate a number of PGC specific genes, and, most importantly, fail to repress expression of somatic genes (Kurimoto et al., 2008; Ohinata et al., 2005). In *Prdm14* mutants, cells positive for AP activity can be detected initially, but then are lost (Yamaji et al., 2008).

By E6.5, a very small population of cells has successfully been induced as PGC precursors. These cells then proliferate, and within a day, by E7.5, all PRDM1-expressing cells also express a number of germ cell-specific genes such as *Dppa3* (developmental pluripotency associated, also known as Stella or PGC7) and *Nanos3*, and display the AP activity characteristic of PGCs (Yabuta et al., 2006). Within the next half a day or so, pluripotency markers such as *Oct4* (also called Oct3 or Pou5f1), *Nanog*, and *Sox2* (SRY-box containing gene 2) are repressed in the

neighboring somatic cells and can be used to identify PGCs as well (Rosner et al., 1990; Yabuta et al., 2006; Yamaguchi et al., 2005).

Migration and arrival "home"

In mouse, as in many other species, PGCs are specified in a location distant from the one where they will develop. Shortly after their specification, PGCs begin a long journey toward their future home, the developing gonads. This journey will take the PGCs from their site of specification, the base of the allantois, into the endoderm of the developing hindgut, and through the dorsal mesentery until they reach the primitive gonads at around E10.5 to E11.5.

Although PGCs have long been visualized along their path by staining for AP activity or, more recently, using live imaging of an GFP-tagged *Oct4* transgene (Molyneaux et al., 2001), surprisingly little is understood about the mechanisms underlying this journey. Parsing the genetic network responsible for migration has been difficult, as loss of many of the genes that might be involved in migration; for example loss of *Nanos3* (Suzuki et al., 2008; Tsuda et al., 2003) or *Dnd1* (Youngren et al., 2005), causes apoptosis of the germ cells, leaving it ambiguous as to whether these genes affect PGC migration or their survival along the way.

One gene for which there is strong evidence for a role in PGC migration is the Kit receptor, encoded by the *W* locus, along with kit ligand (*kitl*), encoded by the *Steel* locus. Defects in gonadal germ cell numbers in *kitl* mutants were first noticed in *W/W* mice over half a century ago (Mintz and Russell, 1957). It was later noted

that germ cells in embryos homozygous for another *kitl* mutation, W^e, were retained in the hindgut at E10.5, a time at which wild-type germ cells have begun entering the gonads, indicating that *kit* is required for germ cells to move from the hindgut to the gonads (Buehr et al., 1993). Supporting this conclusion, when *kitl*-deficient germ cells were prevented from undergoing apoptosis by deletion of the pro-apoptotic gene *Bax* (BCL2-associated X protein), they were again retained in the hindgut, demonstrating that *kitl* is in fact required for the migration itself (Runyan et al., 2006).

When germ cells finally arrive at the gonads, they undergo a number of changes, in morphology, epigenetics, and gene expression. It is still unclear how germ cells know that they have arrived in the gonad and what factors, if any, cause the many changes in the germ cells, or whether these changes are intrinsically programmed in the germ cells to occur after a certain number of cell divisions, for example.

During the course of their migration, the PGCs proliferate, so that between E10.5 and E11.5 a total of approximately 3000 PGCs arrive in the somatic gonads (Tam and Snow, 1981). At this time, the germ cells are sexually monomorphic, with no known morphological or molecular differences between male and female germ cells. Upon their arrival, germ cells undergo a number of changes in morphology, epigenetics, and gene expression. Some of the most easily noted changes are that germ cells lose their motility, even when transplanted to *in vitro* culture systems (Donovan et al., 1986); change their shape, becoming more rounded in appearance with a larger nuclear diameter; and begin to aggregate into clumps (Pepling and

Spradling, 1998). These changes in gonadal germ cells compared to their migrating PGC counterparts point toward the likelihood of a differentiation event that occurs in germ cells as they reach the gonads. Although the signals inducing this event are not yet understood, evidence indicates that the germ-cell-specific gene *Dazl* (deleted in azoospermia-like) plays a key role in the differentiation (Gill et al., 2011).

In addition to these easily observed morphological changes, the germ cells undergo numerous molecular changes. Firstly, they undergo substantial DNA de-methylation between E11.5 and E12.5 (Hajkova et al., 2002). Based on the timing, this de-methylation is thought to be an active process, rather than a DNA replication-dependent, passive process. It likely serves to reset the germ cells' epigenetic status in preparation for the formation of gametes, but it may also play an additional role in enabling the expression of a number of germ cell specific genes, including *Mvh* (mouse vasa homolog, also known as *Ddx4*), *Dazl*, and *Gcna1* (germ cell nuclear antigen 1).

GCNA1 is a reliable and highly specific marker of germ cells whose antigen has not yet been identified (Enders and May, 1994). However, its function is unknown and no mutants have been characterized. Its expression is activated in both male and female germ cells upon arrival in the gonad and is maintained through the diplotene stage of meiotic prophase in both sexes, although intensity of expression varies during that time (Enders and May, 1994).

DAZL is an RNA-binding protein and a member of the DAZ family of proteins, which includes *Boll* (boule-like), the homologue of the *Drosophila* germ cell gene, *Boule*. It is still unclear what regulates *Dazl* expression, but like GCNA, it is first

detectable in germ cells of both sexes upon their arrival in the gonads (Seligman and Page, 1998). Studies on a pure genetic background indicate that *Dazl* is required for fertility in both males and females. *Dazl*-deficient germ cells of both sexes fail to undergo sex-specific differentiation after reaching the gonad, and instead retain many PGC-like characteristics (Gill et al., 2011). In both sexes the germ cells eventually die; in females this occurs around the time of birth, whereas in males it occurs earlier, just several days after the germ cells reach the gonad (Lin and Page, 2005). The reasons for this cell death are not known.

A third gene whose expression is activated when germ cells reach the gonads is the *mouse Vasa homolog* (*Mvh*) (Tanaka et al., 2000). VASA is a well-conserved RNA binding protein found specifically in germ cells in many species. In *Drosophila*, vasa protein localizes to polar granules in the developing oocyte, and its presence is required for the formation of pole cells and, consequently, germ cells (Schupbach and Wieschaus, 1986). In mice, however, *Mvh* is not required for germ cell specification, and in fact its expression is not activated until the primordial germ cells arrive at the gonad, when it is expressed in germ cells of both sexes (Toyooka et al., 2000). In males mutation of *Mvh* leads to a severe decrease in germ cell number by E12.5, and by E13.5, those that remain fail to express OCT4, which is normally maintained until after birth (Tanaka et al., 2000). Interestingly, this is a sex-specific defect, and females appear normal in both fertility and fecundity. Like *Dazl* and GCNA1, what drives the expression of *Mvh* upon arrival at the gonad is unclear. However, culture of embryonic germ (EG) cells with somatic gonadal cells can induce *Mvh* expression in the EG cells, indicating that there may be an

intercellular interaction between germ and somatic cells that activates its expression (Toyooka et al., 2000).

Sex determination and differentiation

Upon settling in the gonads, male and female germ cells are indistinguishable from one another, and the immediate changes described above occur in both sexes. The first sexually dimorphic characteristics do not appear until a day or two later, at E12.5. Sex determination of germ cells is based not on the intrinsic sex chromosome content of the germ cells, but rather on the extrinsic somatic environment in which germ cells find themselves (reviewed in (McLaren, 1995)). Although the germ cells themselves are morphologically indistinguishable, the somatic gonads surrounding them are distinct.

In the XY gonad at E10.5, around the time the first germ cells begin to arrive, the sex-determining Y-chromosome gene *Sry* is first expressed in what will later become the Sertoli cells of the testis. Expression of *Sry* triggers expression of *Sox9*, which directs Sertoli cell development and eventually results in the coordination of testis development (for a more in-depth discussion of somatic sex determination see (Jakob and Lovell-Badge, 2011).

In contrast, XX gonadal cells carry no Y chromosome and therefore do not express *Sry*. In its absence, *Sox9* is not up-regulated, the testis pathway is not initiated, and instead of Sertoli cells, granulosa cells develop, leading to the formation of an ovary. It was long thought that this was a passive or "default"

pathway if testis development was not initiated, but it is now clear that several genes including *Wnt4*, *Rspo1*, and *Foxl2* are required for ovarian development (reviewed in Pask 2011).

Male and female germ cells arrive in the somatic gonads sexually indifferent, but diverge soon thereafter, continuing down different pathways depending on whether they are housed in a male testis or a female ovary. At E13.5, male germ cells, which are enclosed in testis cords formed by somatic cells, begin to enter a G_1/G_0 cell cycle arrest. This action is not synchronous, nor is it patterned throughout the testis, but by E15.5 all testicular germ cells are arrested (Western et al., 2008). They will maintain this cell cycle arrest until shortly after birth, when they exit the arrest and commence mitotic cycling, establishing a cohort of spermatogonial stem cells which support spermatogenesis throughout life.

Fetal ovarian germ cells, however, rather than arresting, set in motion a flurry of activity, including the down-regulation of pluripotency markers, which have been maintained since germ-cell specification nearly a week prior; entry into meiotic prophase, which testicular germ cells will undertake only after birth; and the expression of markers of oogenesis.

Until recently it was unknown what the extrinsic signals were that propelled germ cells down a female or male pathway. One prominent theory postulated the existence of a meiosis inhibiting substance (MIS) in the testis (McLaren, 1995). This possibility was particularly intriguing since mis-migrating germ cells in both male and female embryos that settled in the nearby adrenal gland, rather than the gonad, can enter meiosis. It has since been found that a key regulator of at least one aspect

of germ cell sexual differentiation, meiosis, is the Vitamin A metabolite, retinoic acid (RA), which induces expression of the gene *Stra8* (stimulated by retinoid acid gene 8) in fetal ovarian germ cells (Baltus et al., 2006; Bowles et al., 2006; Koubova et al., 2006). The RA degrading enzyme CYP26b1 eliminates RA in the fetal testis, preventing testicular germ cells from expressing *Stra8* (Bowles et al., 2006; Koubova et al., 2006; Li et al., 2009; Trautmann et al., 2008).

Wild-type ovarian germ cells initiate meiosis around E13.5. They undergo pre-meiotic DNA replication to attain 4C DNA content and proceed through the initial stages of the first meiotic prophase (McLaren, 1984). Female germ cells arrive in the diplotene stage beginning around E16.5 and a diplotene arrest is maintained until ovulation, which can be weeks to months later. Upon ovulation, oocytes continue through meiosis I and arrest again at metaphase II. Female meiosis is not completed until fertilization occurs.

Stra8, which is first expressed at the anterior tip of the ovary around E12.5, has been shown to be required for the initiation of meiosis in fetal ovarian germ cells (Baltus et al., 2006; Koubova et al., 2006). In *Stra8*-mutant embryos, female germ cells fail to undergo premeiotic DNA replication, do not load the meiosis-specific cohesin REC8, and do not undergo synapsis of homologous chromosomes. In short, *Stra8* mutant germ cells fail to enter meiosis (Baltus et al., 2006).

Embryonically, *Stra8* transcript is restricted to female germ cells; it is not expressed in fetal male germ cells, which do not undergo meiotic initiation until after birth (Menke et al., 2003). This differential expression has been found to be due to differences in RA availability in female versus male fetal gonads. RA signaling

has been shown to be required for *Stra8* expression in the fetal ovary, and exogenous RA is sufficient to induce *Stra8* in fetal testicular germ cells, where *Stra8* would not otherwise be expressed. Koubova et al. cultured fetal ovaries *ex vivo* with a retinoic acid receptor (RAR) antagonist, which blocks RA signaling, and observed complete prevention of *Stra8* expression in the germ cells (Koubova et al., 2006). Somatic cells in the fetal testis express the cytochrome P450 enzyme CYP26b1, which is thought to degrade RA in the testis therefore making it unavailable to germ cells (Bowles et al., 2006; Koubova et al., 2006; Li et al., 2009; Menke and Page, 2002; Trautmann et al., 2008). Germ cells of fetal testes cultured with either exogenous RA or with ketoconazole, which inhibits activity of cytochrome p450s including CYP26b1, and those of *Cyp26b1* mutants do express *Stra8* and initiate an early meiotic program, including expression of SYCP3 (Bowles et al., 2006; Koubova et al., 2006; Li et al., 2009; Trautmann et al., 2008). Interestingly, however, deficiency of CYP26b1 activity is not sufficient to drive male germ cells completely through meiosis prematurely (J Koubova, unpublished).

Another hallmark of female germ cell development is the down-regulation of pluripotency markers. Both male and female primordial germ cells express pluripotency markers such as OCT4 and NANOG from the time of specification until after they colonize the gonads (Pesce et al., 1998; Yamaguchi et al., 2005). Male germ cells will maintain this expression until after birth. However, female germ cells down-regulate these markers around the time of meiotic entry, so that by E16.5 they are undetectable.

We have little information about the down-regulation of pluripotency markers. It was recently found that *Dazl* is required for the differentiation of PGCs upon their arrival in the gonad (Gill et al., 2011). *Dazl* mutant germ cells fail to undergo sex specific differentiation, which in females includes down-regulation of pluripotency markers. However, as *Dazl* controls a number of other events including RA-responsiveness, meiotic entry, and oogenesis (Gill et al., 2011; Lin et al., 2008), it is probable that it is more of a "master regulator," and its effect on pluripotency markers may be indirect. The factors downstream of *Dazl* that control this process have not yet been identified.

Furthermore, very little is known about the suppression of pluripotency markers in fetal ovarian germ cells. It is still uncertain what significance the suppression of pluripotency markers has in the development of germ cells. Is it required for the initiation of meiosis or oogenesis, or are these separable, independent processes? Is it required for the epigenetic reprogramming of the oocyte that enables it to form a totipotent zygote? These questions have not yet been addressed.

II: PLURIPOTENCY *IN VIVO* AND *IN VITRO*

All sexually reproducing, multicellular organisms begin life as a single cell, the zygote, formed by the joining of egg and sperm. This unique cell has the extraordinary capability to give rise to all of the different cell types in the body, a characteristic called totipotency. At some time during development, the timing of

which varies by species, dividing cells lose their their totipotency, eventually becoming unipotent, meaning they are able to give rise to only a single cell type, or terminally differentiated, meaning they are unable to further divide and create new cells.

Understanding how cells lose or, in the case of germ cells, regain their developmental potential and what the important molecular regulators are of this process is key in understanding embryonic development. These subjects have long been studied both *in vivo* in the developing embryo and, more recently, *in vitro* in multiple cell lines such as embryonic stem cells, embryonic germ cells, embryonal carcinoma cells, and more recently, induced pluripotent stem cells. The totipotency cycle and derivation of *in vitro* cell lines from various *in vivo* pluripotent cell types are summarized in Figure 2.

Totipotency and pluripotency in the early mouse embryo

In the mouse, all cells of the cleavage stage embryo are equivalent and have the capacity to give rise to any developmental lineage, including the extraembryonic tissues. This totipotency is retained until the formation of the blastocyst around E3.5, when the first differentiation event of embryogenesis takes place. The blastocyst is composed of an outer layer of cells (the trophectoderm, or TE) surrounding a fluid-filled cavity (the blastocoel) and a mass of cells (the inner cell mass, or ICM). The TE will ultimately give rise to the placenta, while the ICM forms the embryonic tissues. Until the formation of the blastocyst, the homeodomain

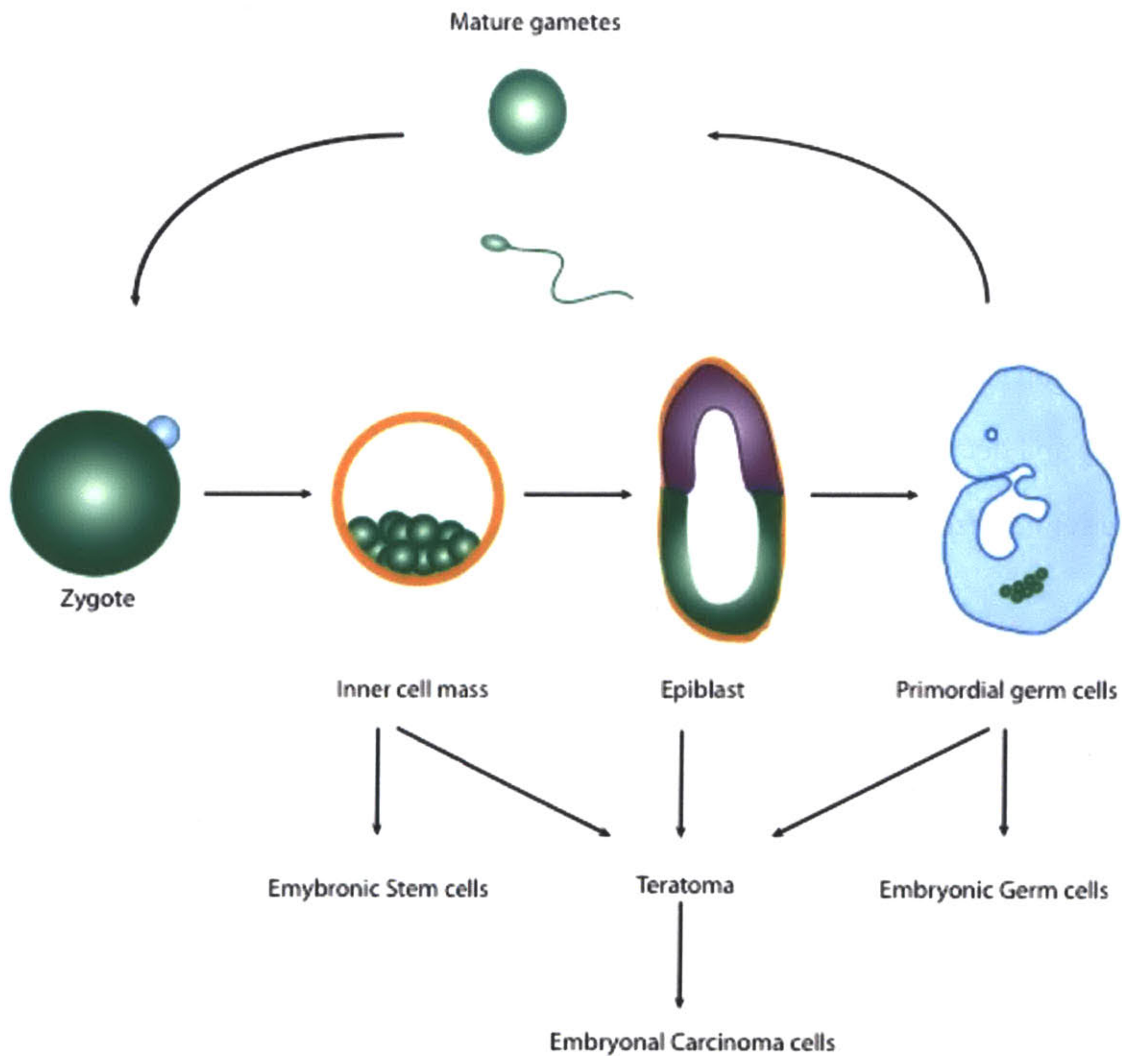


Figure 2: The totipotent cycle. Cells shown in green are part of the totipotent cycle. The pluripotency of the inner cell mass, epiblast, and primordial germ cells will be discussed in more detail in this section, as will ES, EC, and EG cells.

transcription factor OCT4 (also called Pou5f1, or Oct4), is expressed in all cells of the developing embryo. After formation of the blastocyst, however, OCT4 is restricted to the ICM and is undetectable in the cells of the TE (Palmieri et al., 1994; Rosner et al., 1990). OCT4 is required for the specification of ICM cells. *Oct4* mutant embryos die around the time of implantation and are composed of only TE-like cells (Nichols et al., 1998).

In the late morula stage, when the cells of the embryo form a compact ball just prior to blastocyst formation, expression of the homeobox transcription factor CDX2 in the outer cells of the morula promotes TE fate (Strumpf et al., 2005). The absence of CDX2 results in aberrant expression of OCT4 in the TE and embryonic lethality prior to implantation. These phenotypes suggest that CDX2 enables trophectoderm-committed cells to overcome an ICM-like fate and to suppress *Oct4*. Thus, it is likely that *Cdx2* and *Oct4* repress one another, establishing a network that enables the differentiation and/or maintenance of two mutually exclusive cell types.

When the TE and ICM differentiate, the cells of the embryo are no longer considered totipotent. Neither population is able to give rise to all of the cell types required for the formation of a complete organism, since TE cells cannot from an ICM and vice versa. However, the ICM is still pluripotent, meaning that it is capable of forming cells of any of the three germ layers of the embryo: endoderm, mesoderm, and ectoderm.

The second differentiation event to take place in the early embryo occurs in the ICM of the blastocyst, when the primitive endoderm and the epiblast are formed. The primitive endoderm, also called the hypoblast, forms a layer of cells between

the epiblast and the blastocoel. This layer of cells will go on to form the extraembryonic endoderm and eventually the yolk sac. The primitive endoderm loses its pluripotency and has the capacity to give rise to only a few cell types. During this differentiation step, *Oct4* is down-regulated in the hypoblast but maintained in the epiblast, which will give rise to the embryo proper (Palmieri et al., 1994). Formation of the epiblast requires the transcription factor NANOG, which is expressed in the entire ICM but maintained only in the epiblast (Chambers et al., 2003; Mitsui et al., 2003). In the absence of *Nanog,* embryos develop with only TE and primitive endoderm and fail to form the epiblast (Chambers et al., 2003; Mitsui et al., 2003).

The epiblast maintains pluripotency and expression of *Nanog* and *Oct4* until implantation and gastrulation respectively, when the germ layers of the embryo are formed. After these times, both *Oct4* and *Nanog* are silenced and cannot be detected in the developing somatic cells (Chambers et al., 2003; Mitsui et al., 2003; Palmieri et al., 1994; Rosner et al., 1990). The only cells that escape somatic fate and continue to express these key pluripotency regulators after E8.5 are the primordial germ cells, which maintain expression of OCT4 and NANOG for at least another week (Pesce et al., 1998; Yamaguchi et al., 2005), longer than any other cells in the body. Germ cells provide a fascinating opportunity to study the regulation of pluripotency, since they are the only cell type that is required both to be highly specialized and also to maintain the ability to give rise to a complete organism upon fertilization.

Pluripotent cell lines

In vivo, pluripotency is only a transient state that, except in the germ cells, is lost by the end of gastrulation, when somatic cells have committed to various germ layer fates. However, several embryo-derived cell lines can be maintained *in vitro* in a pluripotent state indefinitely. These include embryonal carcinoma cells, embryonic stem cells, and embryonic germ cells. All of these cell types can be maintained and manipulated in culture and can be reintroduced into early embryos where, as pluripotent cells, they are able to contribute to any and all germ layers to form a developmentally normal adult mouse.

Embryonal carcinoma (EC) cells were the first pluripotent cells to be established *in vitro* (Kleinsmith and Pierce, 1964), and are derived from teratomas, germ-cell-derived tumors that contain a diverse array of differentiated cell types deriving from all three germ layers. Teratomas arise spontaneously in the ovary or testis as a result of aberrant germ cell development. They can also form when an early embryo or primordial germ cell is transplanted to a site outside the uterus. Teratomas are sustained by pluripotent stem cells—EC cells—which can be isolated and cultured *in vitro*. These cells most closely resemble cells of the embryonic ectoderm, or epiblast. EC cells in culture can be coaxed to differentiate along different developmental pathways when treated with certain compounds, and thus provide a model for early embryonic development. For example, treatment with retinoic acid induces differentiation of neuronal and glial cells (Jones-Villeneuve et al., 1982), whereas treatment with DMSO can induce differentiation into cardiac and

skeletal muscle or epithelium (McBurney et al., 1982). Furthermore, EC cells pass the most stringent test for pluripotency: they are able to incorporate into the ICM and form chimeras when injected into a developing blastocyst (Brinster, 1974; Mintz and Illmensee, 1975; Papaioannou et al., 1975). However, despite being able to contribute to all other tissues, EC cells rarely contribute to the germline, possibly due to abnormalities attained while in a cancerous teratoma state (reviewed in (Martin, 1980)).

In contrast, embryonic stem (ES) cells readily contribute to both somatic tissues and the germ cell lineage. When whole blastocysts or isolated ICMs were cultured in conditioned media, colonies of cells arose that could be passaged and expanded, and would proliferate indefinitely (Evans and Kaufman, 1981; Martin, 1981). Despite their derivation from different origins, ES cells share many characteristics with EC cells, including the morphological appearance of colonies and cells, growth rate, the ability to differentiate into multiple cell types, and the ability to form teratomas when injected into mice. Also like EC cells, ES cells can be reintroduced to the blastocyst and become incorporated into the ICM, ultimately contributing to all tissues. ES cells also readily contribute to the germ line of chimeric mice (Bradley et al., 1984), and thus have been a valuable tool for generating transgenic mice.

The third type of embryo-derived stem cells are embryonic germ (EG) cells, which are derived from PGCs. Prior to E12.5, PGCs transplanted to an adult testis capsule can give rise to teratomas (Stevens, 1966). Teratomas, in turn, are a source of EC cells which, when injected into a blastocyst, can be incorporated into the ICM.

However, when isolated PGCs themselves are directly injected into a blastocyst, they do not contribute to the developing embryo, suggesting there is a reversible restriction of developmental potential in the PGCs. Initial attempts to establish immortal cell lines from PGCs failed, as the cells would eventually cease proliferation and die (Dolci et al., 1991; Godin et al., 1991; Matsui et al., 1991). Addition of basic fibroblast growth factor to the media enabled the PGCs to continue to proliferate and form cell lines called EG cells that closely resemble ES and EC cells (Matsui et al., 1992; Resnick et al., 1992). PGCs from about E8.5 to E12.5 are able to establish EG cell lines. Beyond E12.5, attempts have failed, suggesting that some change occurs in the germ cells at this time preventing them from undergoing the transformation to EG cells. At E12.5 germ cells *in vivo* begin differentiating and soon display the first sexually dimorphic characteristics, including drastic cell cycle events: the initiation of meiosis in females and entry into a G1/G0 arrest in males. These events may restrict the ability to derive immortal cell lines beyond this time. In culture, EG cells can differentiate into multiple cell types and when transplanted into mice give rise to teratomas that contain a wide variety of tissues (Matsui et al., 1992; Resnick et al., 1992). In addition, they can contribute to the ICM to generate chimeric mice, including the germ line (Stewart et al., 1994).

All three of these embryo-derived stem cell types (EC, ES, and EG cells) share a number of commonalities at the molecular level. Each of these cell lines expresses the glycoprotein SSEA1 and exhibits TNAP (tissue non-specific alkaline phosphatase) activity. In addition, they all express the transcription factors OCT4, SOX2, and NANOG, which have proven to be key regulators of pluripotency, as well

as the nuclear protein DPPA3 (also called STELLA, or PGC7) (reviewed in (De Miguel et al., 2010)). All of these markers also label PGCs and cells of the ICM, highlighting some of the similarities between pluripotent cell lines and their *in vivo* counterparts.

Regulation of pluripotency *in vivo* and *in vitro*

The derivation and maintenance of pluripotent mouse ES cells requires the complex interaction of multiple signaling pathways in careful balance, including LIF, WNT, and BMP signaling. (For a more complete review of these pathways, see (Boiani and Scholer, 2005)). Ultimately, these signaling cascades lead to transcriptional changes, effected by a core network of transcription factors, including OCT4, NANOG, and SOX2.

In vivo, the POU family homeodomain transcription factor OCT4 is expressed in all cells of the embryo from the zygote to the morula, as well as in ICM cells of the blastocyst, the epiblast, and the germ cells (Palmieri et al., 1994; Pesce et al., 1998; Rosner et al., 1990). In somatic cells, OCT4 is down-regulated during gastrulation, around E8.0. In female germ cells, OCT4 is expressed until around E14.5, when it is down-regulated. It is later re-expressed in maturing oocytes (Pesce et al., 1998). In addition, it is found in all three embryo-derived pluripotent cell lines described above. It has long been known as a critical factor for maintaining pluripotency. *Oct4 -/-* embryos are able to attain a blastocyst-like state, but one made up entirely of trophectoderm, with no proper ICM cells. Mutant blastocysts are unable to give rise to ES cells when cultured (Nichols et al., 1998).

These results indicate that *Oct4* is required for formation of the ICM and derivation of ES cells. However, conditional deletion of *Oct4* in early germ cells results in the death, rather than differentiation, of PGCs during their migration to the future gonad (Kehler et al., 2004), suggesting a different role or set of target genes for *Oct4* in PGCs compared to other pluripotent cell types.

Also critical for maintaining pluripotency is the transcription factor NANOG, named for the mythical Celtic land of eternal youth, Tir nan Og. *Nanog* mutant embryos fail to generate epiblast or extraembryonic ectoderm, and ES cells cannot be derived from the ICM (Mitsui et al., 2003). Consistent with these findings, *Nanog* deficient ES cells fail to maintain pluripotency and instead differentiate into extraembryonic endoderm-like cells. Interestingly, over-expression of NANOG in cultured wild-type ES cells releases them from the requirement of LIF for maintaining pluripotency (Mitsui et al., 2003). NANOG is expressed in ICM and epiblast cells as well as ES, EC, and EG cells. NANOG is also expressed in migrating PGCs. Like OCT4, it remains expressed in germ cells after they reach the gonads, being down-regulated in females around the time of meiotic initiation (Yamaguchi et al., 2005). Unlike OCT4, however, NANOG is not re-expressed in adult oocytes. Conditional knockdown of *Nanog* in PGCs results in apoptosis, rather than differentiation, of the germ cells, suggesting that, like *Oct4*, *Nanog* is required for survival and not pluripotency of migrating germ cells (Yamaguchi et al., 2009).

The Sox family transcription factor SOX2 has also been shown to play a key role in pluripotency maintenance in the early embryo and in ES cells. Embryos mutant for *Sox2* die shortly after implantation, failing to maintain the epiblast. In

addition, ES cells cannot be derived from the ICM, instead differentiating into trophoblast-like cells when cultured, similar to *Oct4* mutants (Avilion et al., 2003). It is expressed in cells of the late morula, the ICM, epiblast and extraembryonic ectoderm, and PGCs. Unlike OCT4 and NANOG, SOX2 is also found in the extraembryonic ectoderm and stem cells of the neural lineage (Avilion et al., 2003). Like OCT4, SOX2 is down-regulated around the time of meiotic entry in ovarian germ cells, and re-expressed in postnatal oocytes (Western et al., 2005). Its function in primordial germ cells has not yet been investigated.

Oct4, *Nanog*, and *Sox2* form a core regulatory network in pluripotent stem cells. OCT4 and SOX2 can interact with one another on the promoters of target genes (Yuan et al., 1995). OCT4 and NANOG appear to function via independent pathways, as deletion of one does not affect expression of the other (Chambers et al., 2003; Mitsui et al., 2003), but they likely have coordinated functions within pluripotent cells. None of the three is sufficient to maintain pluripotency in the absence of any one or both of the others.

It is interesting to note that all three of these transcription factors are expressed in primordial germ cells and at least two, *Oct4* and *Nanog* are required for PGC survival. Their expression in germ cells is maintained until after the germ cells colonize the gonads. OCT4, NANOG, and SOX2 remain expressed in fetal ovarian germ cells until entry into meiosis, when all three, as well as other pluripotency markers, are down-regulated. The coordination of these events is not well studied, and neither the underlying mechanism nor the consequences of it are well understood.

III: THE NUCLEAR RECEPTOR GCNF

GCNF is a divergent member of the nuclear receptor superfamily that is well-conserved throughout vertebrates. Originally cloned based on the homology of its DNA binding domain to those of other nuclear receptors, it has no known ligand and appears to act only as a transcriptional repressor. A number of GCNF-target genes have been identified *in vitro*, and a handful have been corroborated by *in vivo* evidence. The best-studied of these targets is the pluripotency regulator *Oct4*. GCNF has been shown to bind directly to the promoters of *Oct4* and *Nanog*, and is required for their suppression in differentiating ES and EC cells as well as in the embryo during gastrulation. Our preliminary data showing the expression of *Gcnf* in fetal ovarian germ cells led us to hypothesize that it might also be required for suppression of *Oct4* and *Nanog* in female germ cells at the time of meiotic entry.

GCNF is a nuclear receptor family member

Gcnf (germ cell nuclear factor, also called retinoic acid receptor-related testis receptor, neuronal cell nuclear factor, or Nr6a1) was originally cloned from mouse and human cDNA libraries based on the similarity of its DNA binding domain to that of other known nuclear receptors, a group that includes receptors such as glucocorticoid and estrogen receptors as well as retinoic acid and retinoid X receptors (Bauer et al., 1997; Chen et al., 1994; Hirose et al., 1995). It has since been identified in chimpanzee, frog, zebrafish, chicken, pig, cattle, and rat . It is well-

conserved throughout vertebrates, with about 98% amino acid identity between the mouse and human orthologs, and about 84% identity between mouse and frog orthologs. (Joos et al., 1996; Lei et al., 1997; Susens and Borgmeyer, 1996).

Like other nuclear receptors, GCNF has a modular structure consisting of an N-terminal domain, a DNA binding domain (DBD), a hinge region, and a ligand binding domain (LBD). The N-terminal domain, also called the A/B domain, is not well conserved among nuclear receptors, nor is does it show high homology among GCNF orthologs. In other nuclear receptors the N-terminal domain often has trans-activating function when the receptor binds its ligand. However, GCNF has no known ligand, and no trans-activating function has been shown for its N-terminal domain.

The DBD, also called the C domain, is the region of highest similarity between GCNF and other paralogous nuclear receptors, with mouse GCNF showing about 61% identity with mouse Retinoid X Receptor (RXR) and Retinoic Acid Receptor (RAR) (Hirose et al., 1995). The DBD of GCNF has been shown to bind as a monomer or homodimer to the nuclear receptor consensus element TCA[AG(G/T)TCA]$_2$, and as a homodimer to an extended half site TCAAGGTCA and a direct repeat of the core motif AGGTCA with no intervening nucleotides (Borgmeyer, 1997; Cooney et al., 1998; Greschik et al., 1999; Yan et al., 1997). The latter response element, called DR0, is unique among nuclear receptor response elements, which are typically either direct or inverted repeats with 1 to 5 intervening nucleotides (e.g. DR5).

The hinge region and ligand binding domain (LBD) of GCNF, also called the D and E domains, respectively, have been found to contain residues important for binding of co-repressors (Fuhrmann et al., 2001; Yan and Jetten, 2000).

The LBD is the largest domain, and in most nuclear receptors is associated with binding of ligands and dimerization with other nuclear receptors. No ligand has yet been identified for GCNF, and it is possible that none exists. Its LBD is relatively divergent from that of other nuclear receptors. Most significantly, it lacks the conserved AF2 domain typically required for transcriptional activation. Therefore GCNF may act only as a transcriptional repressor; thus far all of its direct targets confirmed *in vivo* have been repressed by GCNF (Fuhrmann et al., 2001; Gu et al., 2005b; Lan et al., 2003b).

In vitro studies have identified a number of potential direct targets of GCNF in mouse. These include *Gdf9* (growth differentiation factor 9), *Bmp15* (bone morphogenic protein 15), *Prm1* (protamine 1) and *Prm2* (protamine 2), *Oct4*, *Nanog*, *Ppard* (peroxisome proliferator-activated receptor beta, also called Pparb, Pparb/d, or Nr1c2), *Gpd2* (glycerol phosphate dehydrogenase 2, mitochondrial), *Hcrt* (hypocretin, also called orexin), *Mos* (Moloney sarcoma oncogene), and *Elp* (endozopine-like peptide, also called Dbil5) (Fuhrmann et al., 2001; Gu et al., 2005b; He et al., 2008; Hummelke et al., 1998; Lan et al., 2003b; Rajkovic et al., 2004; Tanaka et al., 2010; Valentin et al., 2000; Zilz and Cooper, 2004). Of these, expression of the first six is known to be disrupted in *Gcnf*-mutant mice; the others have not been analyzed in *Gcnf* mutants. For each of these targets, GCNF has been

shown via electrophoresis mobility shift assays to bind directly to either a DR0 or extended half site motif in the promoter.

Exactly how GCNF exerts its transcriptional effects is not well understood. However, it has been shown to interact with several co-repressors *in vitro*. NCoR1 (nuclear co-repressor 1) and NCoR2 (nuclear co-repressor 2, also called SMRT) are well characterized nuclear receptor co-repressors. Each has been shown to interact with the LBD of GCNF *in vitro*, and NCoR1 has additionally been shown to require certain residues in the hinge region of GCNF for binding (Fuhrmann et al., 2001; Yan and Jetten, 2000). Both NCoR1 and NCoR2 recruit histone deacetylases, which cause transcriptional silencing (reviewed in (Watson et al., 2012)). GCNF has also been shown to interact with a testis-specific protein TNRIP-1 (testis-specific nuclear receptor-interacting protein-1), which contains a CoRNR box motif often found in nuclear receptor co-repressors (Zhang et al., 2008). The function of TNRIP-1 is not known. The human protein RAP80 (receptor associated protein 80, called Uimc1 in mouse) has also been found to interact with GCNF (Yan et al., 2002), but like TNRIP-1, its function is not well understood.

Expression and function of *Gcnf* in mice

Gcnf was originally cloned from mouse testis cDNA libraries (Chen et al., 1994; Hirose et al., 1995). Consistent with this, Northern blot analysis shows that in mouse, *Gcnf* transcript is most abundant in testis, and transcript has been found in the gonads of every species in which a *Gcnf* ortholog has been detected (Braat et al.,

1999; Chen et al., 1994; Hirose et al., 1995; Joos et al., 1996; Lei et al., 1997; Susens and Borgmeyer, 1996; Zhang et al., 1998). In mammals two transcripts have been identified: a longer transcript of about 7.5kb and a shorter one of about 2.4kb. The coding regions of both transcripts are identical; they differ only in the length of their 3' untranslated regions (Yang et al., 2003). The significance of this difference is not clear. The longer transcript is produced in testicular germ cells at high levels, but also at low levels in lung, liver, and brain (Hirose et al., 1995). It is also the transcript produced during embryonic development (Susens et al., 1997). The shorter transcript has been found only in round spermatids, post-meiotic spermatogenic cells of the testis (Hirose et al., 1995).

Reports of protein expression in particular cell types of the testis are somewhat conflicting. Using immunostaining of adult testis sections, Bauer and colleagues were able to visualize GCNF protein localized only to the sex body of pachytene spermatocytes, containing the condensed sex chromosomes. Although Western blotting with the same antibody indicated that the protein was also present in round spermatids, it was not visible in those cells by immunostaining (Bauer et al., 1998). Lan and colleagues, using immunostaining with a different antibody, detected GCNF protein only in post-meiotic spermatogenic cells, the round as well as elongating spermatids. They did not detect any protein in pachytene spermatocytes; however, Western blots were not performed with this antibody (Lan et al., 2003a). Finally, a third group using yet another antibody showed GCNF protein expression in the XY body of pachytene spermatocytes, but also increasing GCNF expression throughout the nucleus during the pachytene stage. They also

showed strong expression in round spermatids, but markedly decreased expression in elongating spermatids (Yang et al., 2003). The differences among these studies may be due to the use of different antibodies, which likely recognize different antigens that may be inaccessible at various stages of spermatogenesis. Given that transcript and protein were detected in both pachytene spermatocytes and round spermatids in multiple studies by two different research groups (Bauer et al., 1998; Yang et al., 2003; Zhang et al., 1998), it is likely that GCNF is expressed at least in these two cell types. No one has reported either transcript or protein in spermatogonia, which comprise the stem cell and transit amplifying cell populations in the testis.

Despite the strong expression of GCNF during these stages, it is still unclear what, if any, role GCNF plays during spermatogenesis. Two potential spermatogenic target genes have been identified: *Prm1* (protamine 1) and *Prm2* (protamine 2). Protamines are small proteins that ultimately replace histones during post-meiotic spermatid differentiation. GCNF binds to DR0 motifs in both of these promoters *in vitro* (Hummelke and Cooney, 2004; Hummelke et al., 1998). Interestingly, although they are not translated until later, protamine genes are transcribed in round spermatids (Kleene, 1996), when GCNF protein is, by all accounts, present. Given that GCNF is thought to act only as a transcriptional repressor, and not as an activator, it is not certain how it might affect the expression of *Prm1* and *Prm2*.

In the ovary *Gcnf* transcript and protein are both detected in oocytes of primary follicles and later. At least the longer 7.5kb message is present at these stage; it is not clear whether the shorter 2.4kb message is also present. Neither

transcript nor protein is present in primordial follicle oocytes, the least developed stage of postnatal oogenesis (Katz et al., 1997). Conditional mutagenesis of *Gcnf* in primary follicles using a *Zp3* (zona pellucida glycoprotein 3) promoter-driven Cre to delete the DNA binding domain of GCNF resulted in decreased fertility due to disruption of paracrine signaling between the developing oocyte and the surrounding ovarian somatic cells (Lan et al., 2003b). Elevated levels of *Bmp15* (bone morphogenic protein 15) and *Gdf9* were observed in mutants. Both of these gene products are secreted from the oocyte and involved in paracrine signaling to somatic cells. In addition, GCNF was found to bind to DR0 elements in the promoters of both *Bmp15* and *Gdf9*, suggesting that GCNF directly represses both of these targets *in vivo* (Lan et al., 2003b).

In addition to being expressed in developing oocytes, GCNF is also found in mature, unfertilized oocytes, fertilized ova, and all early embryonic cells through the morula stage. In the blastocyst, expression is restricted to the inner cell mass (Lan et al., 2003a). No function has been ascribed to *Gcnf* during these stages of development. *Gcnf* mutants develop normally until several days post-implantation, and *Gcnf -/-* ES cells can be generated and have normal morphology, indicating that *Gcnf* is not required during pre-implantation development (Chung et al., 2001; Fuhrmann et al., 2001).

Gcnf is also expressed in the post-implantation embryo. At E6.5, *Gcnf* can be detected throughout the embryo. At E7.5 it is expressed in all embryonic tissues, and at E8.5 it is restricted to the neural folds and the posterior end of the embryo (Chung et al., 2001; Fuhrmann et al., 2001; Susens et al., 1997). Between E7.5 and

E8.5 *Gcnf* is necessary for the repression of *Oct4* and *Nanog* in somatic cells during gastrulation (Fuhrmann et al., 2001; Gu et al., 2005b). In the absence of *Gcnf*, embryos fail to down-regulate these two key pluripotency regulators, which have been identified as direct targets of GCNF-mediated repression. In addition, *Gcnf* is induced by retinoic acid treatment of ES and EC cells, where it also represses *Oct4* and *Nanog* (Fuhrmann et al., 2001; Gu et al., 2005b).

By E9.5, when *Gcnf* expression is restricted to the developing nervous system, mutants display morphological defects including a failure to turn, defects in somitogenesis, and a protrusion of the tailbud through the yolk sac, and these embryos die around E10.5 (Chung et al., 2001; Susens et al., 1997). Consistent with *Gcnf* expression in the developing nervous system, *Gcnf* mutants display defects in neural development, including disrupted establishment of the isthmic organizer, a signaling center that induces midbrain and hindbrain development (Chung et al., 2006). A number of genes are misregulated in the neural development of *Gcnf* mutants, but as none of them have been established as direct targets of GCNF, it is unclear how GCNF is involved here.

OVERVIEW

I am interested in studying the connections between germ cells and pluripotent cells. There are many similarities, particularly in gene expression, between germ cells and the pluripotent cells of the early embryo, as well as between germ cells and cultured pluripotent stem cell lines. I am particularly interested in how germ cells regulate the expression of pluripotency markers. The down-

regulation of pluripotency markers in female germ cells during the onset of meiosis is intriguing. Little is known about the mechanisms underlying this process, or its purpose. This thesis sets out to examine the function of one potential regulator of pluripotency markers in germ cells, *Gcnf*.

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CHAPTER 2

GCNF is not required for regulation of pluripotency markers in fetal ovarian germ cells

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Author contributions: Leah Okumura designed, performed and analyzed all experiments except the high-throughput RNA sequencing which was done by Jacob Mueller and Mark Gill. Mary Goodheart performed blastocyst injections to generate *Gcnf* mutant mice. David Page provided helpful discussions throughout the project and assisted with the writing of the manuscript.

ABSTRACT

Germ cells are the only cells in the body that contribute directly to the subsequent generation. As such they must maintain the ability to generate an entire organism after fertilization despite differentiation into highly specialized cell types, oocytes and sperm. During development, germ cells are the last cell type in the body to silence expression of the pluripotency genes *Oct4* and *Nanog*. In mouse, somatic cells achieve this feat around embryonic day (E) 8.0, while germ cells maintain their expression until about E14.5. The nuclear receptor GCNF is required for suppression of *Oct4* and *Nanog* in the somatic cells of the embryo and in embryonic stem (ES) cells, however whether it functions in silencing germ cell expression of Oct4 and Nanog is unknown. Here we demonstrate that *Gcnf* transcript is present in fetal ovarian germ cells when *Oct4* and *Nanog* are silenced at E14.5. However, conditional mutation of *Gcnf* using a ubiquitous, tamoxifen-inducible Cre indicates that *Gcnf* is not required for the down-regulation of pluripotency markers in this context, nor is it required for meiotic initiation or early oogenesis. These results suggest that the suppression of *Oct4* and *Nanog* in germ cells may occur via a mechanism different from that used in somatic cells during gastrulation or in ES cells.

INTRODUCTION

Germ cells, which give rise to sperm and oocytes, are some of the most highly specialized cells in the body, and are the only cells that undergo meiosis as well as mitosis. At the same time they are the only physical link between generations and must, therefore, maintain the ability to generate an entire organism upon fertilization. In this sense, they are immortal and retain the potential for pluripotency throughout development.

Several factors have been found to be essential for pluripotency in both the early embryo and *in vitro* stem cell culture, including the POU family transcription factor OCT4 and the homeobox protein NANOG. *Oct4* is expressed in the inner cell mass (ICM) of the blastocyst, where it is required for pluripotency maintenance. In its absence, ICM cells differentiate into a trophectoderm-like state and embryos are inviable (Nichols et al., 1998). *Nanog* is expressed in the epiblast when it differentiates from the hypoblast and is also required for pluripotency maintenance in the early embryo. *Nanog* mutants fail to form an epiblast, and embryos die shortly after implantation (Mitsui et al., 2003).

Both *Oct4* and *Nanog* are down-regulated in somatic cells during gastrulation, but continue to be expressed in primordial germ cells (PGCs), along with several other pluripotency markers including *Sox2* and *Dppa3* (also known as *Stella*) (Sato et al., 2002; Yabuta et al., 2006). Pluripotency gene expression is maintained in germ cells until after they migrate to the developing gonads(Pesce et al., 1998; Rosner et al., 1990). In particular, *Oct4* and *Nanog* are both required for

survival of PGCs. Conditional mutation of *Oct4* or *Nanog*, specifically in PGCs, results in apoptosis (Kehler et al., 2004; Yamaguchi et al., 2009).

In females, germ cell expression of pluripotency markers is maintained until after the germ cells reach the developing gonads. They are then down-regulated starting around E14.5, coincident with the entry into meiosis (Pesce et al., 1998; Yamaguchi et al., 2009). Interestingly, some of these markers, including *Oct4* and *Sox2* are re-expressed in the postnatal oocyte (Avilion et al., 2003; Pesce et al., 1998). It is not understood whether the repression of pluripotency markers during fetal development is required for meiosis or vice versa. Nor is it clear how the repression of these genes is regulated. Recent evidence indicates that the down-regulation of pluripotency genes in female germ cells depends on the gene *Dazl* (deleted in azoospermia-like); *Dazl*-mutant germ cells display extended expression of pluripotency markers (Gill et al., 2011). However, this may be an indirect consequence resulting from the failure of *Dazl*-deficient germ cells to differentiate from PGCs into gametogenesis-competent cells (Gill et al., 2011).

We set out to determine what other factors contribute to the down-regulation of pluripotency markers in fetal ovarian germ cells, particularly *Oct4* and *Nanog*. GCNF (germ cell nuclear factor, Nr6a1) is an orphan nuclear receptor known to repress *Oct4* and *Nanog* in the soma during gastrulation as well as during retinoic acid-induced differentiation of embryonic stem cells (Fuhrmann et al., 2001; Gu et al., 2005b). GCNF binds directly to DR0 sites in the promoters of both *Oct4* and *Nanog*, and is thought to repress them via an interaction with the nuclear corepressors SMRT and NCoR (Fuhrmann et al., 2001; Gu et al., 2005b; Yan and

Jetten, 2000). We hypothesized that *Gcnf* might also be required for the repression of *Oct4* and *Nanog* in female germ cells.

Using high-throughput mRNA sequencing and single molecule fluorescence *in situ* hybridization, we found that *Gcnf* is expressed in female germ cells at the time when *Oct4* and *Nanog* are repressed. In order to explore the function of *Gcnf* in fetal ovarian germ cells, we generated gene trap and conditional knockout mutant mice using embryonic stem cells targeted by the EUCOMM project in which the ligand-binding domain (LBD) of GCNF was disrupted. We found that, although the LBD of GCNF was required for *Oct4* repression during gastrulation, it was not required for *Oct4* or *Nanog* repression in the fetal ovarian germ cells. In *Gcnf* mutants, all aspects of fetal germ cell development examined were similar to those found in wild-type embryos. We conclude that *Gcnf* is not required for fetal ovarian germ cell development.

RESULTS

Expression of *Gcnf* in fetal ovarian germ cells

We first determined if *Gcnf* expression coincided with the down-regulation of the pluripotency markers *Nanog* and *Oct4* in fetal ovarian germ cells. Two different approaches were used: high-throughput mRNA sequencing and single-molecule fluorescence *in situ* hybridization. Both NANOG and OCT4 proteins can be detected at E13.5, are down-regulated at E14.5, and are undetectable by E16.5 (Pesce et al., 1998; Yamaguchi et al., 2005). If *Gcnf* were required for this down-regulation, it

should be highly expressed in germ cells at the time when *Nanog* and *Oct4* repression occurs.

First, we analyzed high-throughput mRNA sequencing data from wild-type gonads as well as gonads from *W/W^v* embryos, *kit* mutants which lack germ cells, at E12.5, E14.5, and E16.5 (Mueller, Gill, and Page, unpublished). We found that the *Gcnf* transcript is expressed in wild-type fetal ovaries as early as E12.5, with peak expression at E14.5, and a precipitous drop in expression at E16.5. *Gcnf* transcript levels are very low at all time points in wild-type fetal testes as well as in germ cell-less *W/W^v* gonads of both sexes (Figure 1A).

Second, we used single molecule fluorescence *in situ* hybridization (smFISH) to examine *Gcnf* transcript in wild-type gonadal ridges at E9.5, E10.5, and E14.5 (Figure 1B). Germ cells were identified using staining for the cell surface antigen SSEA1 as well as by their distinctive nuclear morphology, visualized by DAPI staining. We found that at E9.5, consistent with published data (Susens et al., 1997), expression of *Gcnf* mRNA was widespread and detectable in both somatic and germ cells. At E10.5 the majority of cells did not express *Gcnf*, and transcript could be detected in only a few cells, both somatic and germ cells. By E14.5, expression levels of *Gcnf* in germ cells increased dramatically, but no expression was detected in somatic cells. Taken together, these results indicate that at the time when pluripotency markers are repressed, *Gcnf* is expressed specifically in fetal ovarian germ cells.

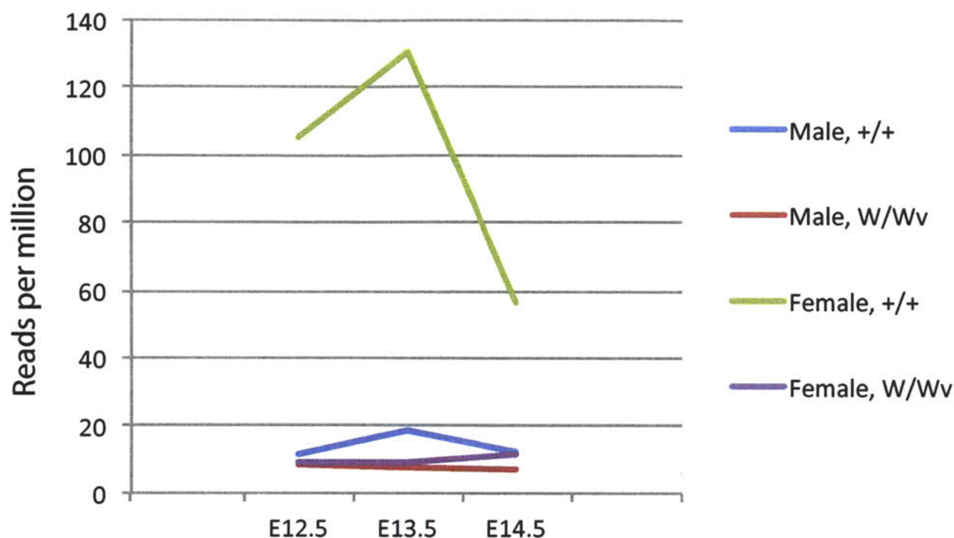
Generation and phenotypic characterization of mutant mice lacking the ligand binding domain of *Gcnf*

Gcnf is a known repressor of *Oct4* and *Nanog* both *in vivo* and *in vitro* (Fuhrmann et al., 2001; Gu et al., 2005b), but its regulation of these pluripotency markers in germ cells has not yet been explored. To determine whether *Gcnf* is required for the suppression of *Oct4* and *Nanog* in fetal ovarian germ cells, we generated *Gcnf*-mutant mice from targeted embryonic stem cells provided by the EUCOMM (European Conditional Mouse Mutagenesis) Project, in which the genomic region encoding the LBD of *Gcnf* is disrupted. This allele, referred to here as $Gcnf^{gt}$, includes a splice-acceptor β-galactosidase gene trap and a neomycin resistance (NeoR) cassette flanked by FRT sites, upstream of LoxP-flanked exon 7 (Figure 2A). Exon 7 encodes the beginning of the LBD, which has been shown to be important for GCNF's interaction with the corepressors NCoR and SMRT (Fuhrmann et al., 2001; Yan and Jetten, 2000).

We first tested whether disruption of the LBD of GCNF caused somatic defects similar to those caused by a null mutation or by deletion of the DNA binding domain (DBD), including prolonged expression of *Oct4* at E8.5 and severe morphological defects at E9.5 resulting in inviability (Chung et al., 2001; Fuhrmann et al., 2001; Lan et al., 2002). We examined mutant embryos homozygous for the *Gcnf* gene trap ($Gcnf^{gt/gt}$) as well as wild-type ($Gcnf^{+/+}$) and heterozygous ($Gcnf^{gt/+}$) littermate controls at E8.5 and E9.5. As expected, quantitative RT-PCR on whole embryos at E8.5 shows that heterozygous $Gcnf^{gt/+}$ control embryos express low

Figure 1

A.



B.

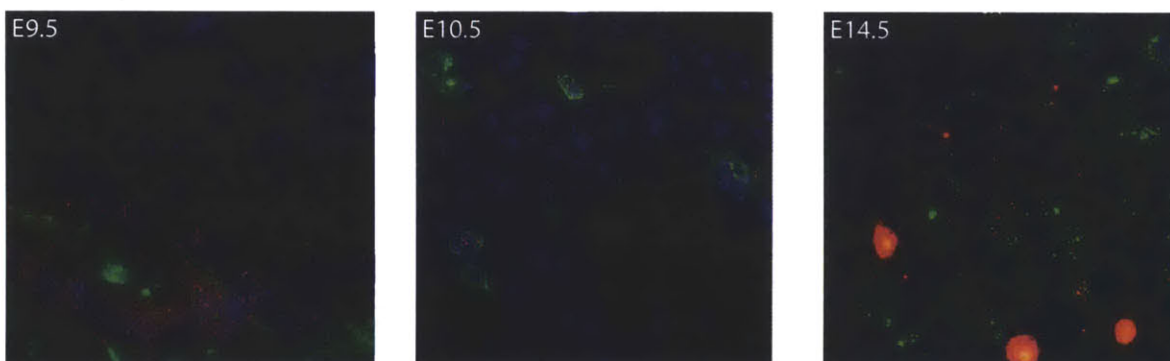


Figure 1. *Gcnf* expression in fetal ovarian germ cells. (A) Levels of *Gcnf* transcript in male and female gonads from +/+ wild-type and germ cell-less W/Wv mutant embryos at E12.5, E14.5, and E16.5, as determined by Illumina sequencing of gonadal RNA. Plotted here are average numbers of reads of *Gcnf* per million total reads from two individual biological replicates. (B) Single molecule fluorescence in situ hybridization shows *Gcnf* mRNA (red), with germ cells marked by SSEA1 (green), and nuclei marked by DAPI staining (blue).

Figure 2

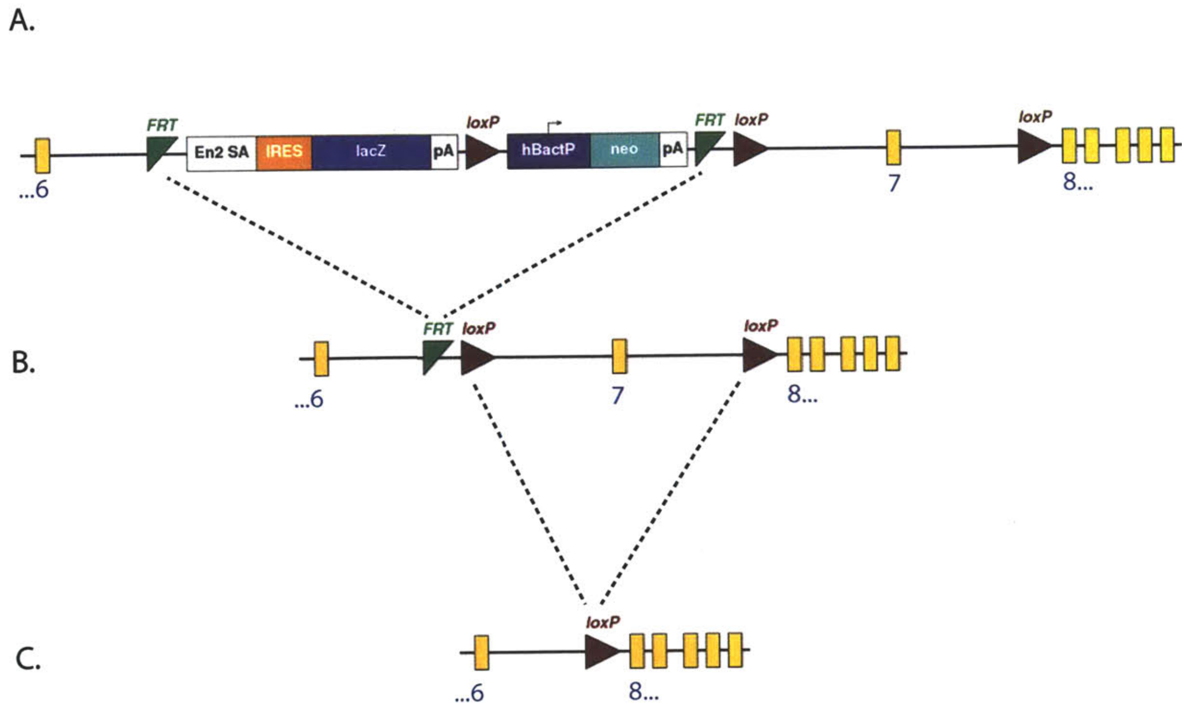


Figure 2. Targeted disruption of *Gcnf*. ES cells carrying a mutant allele of *Gcnf* were obtained from EUCOMM. (A) This allele includes a splice acceptor upstream of a LacZ reporter to create a gene trap, referred to as *Gcnf*^{gt}, followed by a neomycin resistance cassette (NeoR). The gene trap and NeoR are flanked by Frt sites. (B) After FLP-mediated recombination in vivo, the gene trap cassette is removed, leaving a true conditional knockout allele, referred to as *Gcnf*^{fl}, with LoxP sites flanking exon 7, which encodes the beginning of the ligand binding domain. (C) Upon tamoxifen-induced Cre-mediated recombination using the ubiquitous, tamoxifen-inducible Ubc-Cre-ERT2, exon 7 is deleted, resulting in a frame shift and early stop codon in exon 8.

levels of *Oct4* transcript at E8.5. In comparison, *Gcnf*$^{gt/gt}$ mutants have greatly increased levels of *Oct4* expression, suggesting that the LBD of GCNF is required for the repression of *Oct4* during gastrulation (Figure 3A). Additionally, at E9.5 *Gcnf*-mutant embryos lacking the LBD display morphological defects similar to those seen in mutants lacking the DBD. *Gcnf*$^{gt/gt}$ mutants are smaller than their wild-type littermates, and have defects in somitogenesis and morphogenesis (Figure 3B).

Because mutants lacking the LBD of GCNF recapitulate the phenotype of *Gcnf*-null mutants and those lacking the DBD, we conclude that the LBD is required for GCNF function during early embryogenesis.

Generation of *Gcnf*$^{fl/fl}$ mice and conditional deletion of *Gcnf* ligand binding domain

Female germ cells down-regulate expression of pluripotency markers around the time of meiotic initiation at E14.5 (Pesce et al., 1998; Yamaguchi et al., 2005). We next wished to determine whether *Gcnf* is required for the repression of *Oct4* and *Nanog* in female germ cells. Because *Gcnf* is required for embryonic development, and full-body mutants are inviable beyond E10.5 (Chung et al., 2001; Lan et al., 2002), we generated conditional knockout mice. Mice carrying the *Gcnf*^{gt} allele were first bred to mice carrying a ubiquitously expressed FLP recombinase, *ACTB:FLPe* (Rodriguez et al., 2000), in order to excise the gene trap and NeoR cassette. This left behind a single FRT site and LoxP sites flanking exon 7, enabling conditional deletion of exon 7 (Figure 2B). This allele is referred to as *Gcnf*^{fl}.

To create mutant embryos, we mated heterozygous *Gcnf*$^{fl/+}$ females to heterozygous *Gcnf*$^{fl/+}$ males who also carried the ubiquitously expressed, tamoxifen-inducible *Ubc-Cre-ERT2* (Ruzankina et al., 2007). Pregnant females were injected with tamoxifen twice, at E10.5 and E11.5, to induce Cre-mediated recombination throughout the embryo. This time interval was chosen for Cre induction because it is after *Gcnf* is down-regulated throughout the somatic cells of the embryo (Susens et al., 1997) but before *Gcnf* is up-regulated in female germ cells at E12.5. Cre-mediated recombination of *Gcnf*^{fl} deletes exon 7 (Figure 2C), which should cause a frameshift and an early stop codon near the beginning of exon 8, thereby disrupting translation of the ligand binding domain.

To determine the efficiency of recombination in the fetal ovary, we collected ovaries from *Gcnf*$^{fl/fl}$; *Ubc-Cre-ERT2*^{TAM} mutant embryos and *Gcnf*$^{+/+TAM}$ wild-type littermate controls at E15.5 and E16.5 and used quantitative RT-PCR (qRT-PCR) to measure levels of *Gcnf* exon 7-containing transcript. In all mutants examined, recombination was nearly complete, with an average of less than 1.5% of exon 7 transcript detectable compared to controls (Figure 4A).

To ensure that the inability to detect exon 7-containing transcript was not due to an absence of germ cells in mutant embryos we performed immunohistochemistry with a germ cell-specific marker. MVH (mouse vasa homolog) is expressed in germ cells from the time they enter the gonads between E10.5 and E11.5 (Toyooka et al., 2000). MVH staining was readily apparent in ovaries from both *Gcnf* mutants and wild-type controls (Figure 4B). In mutant

Figure 3

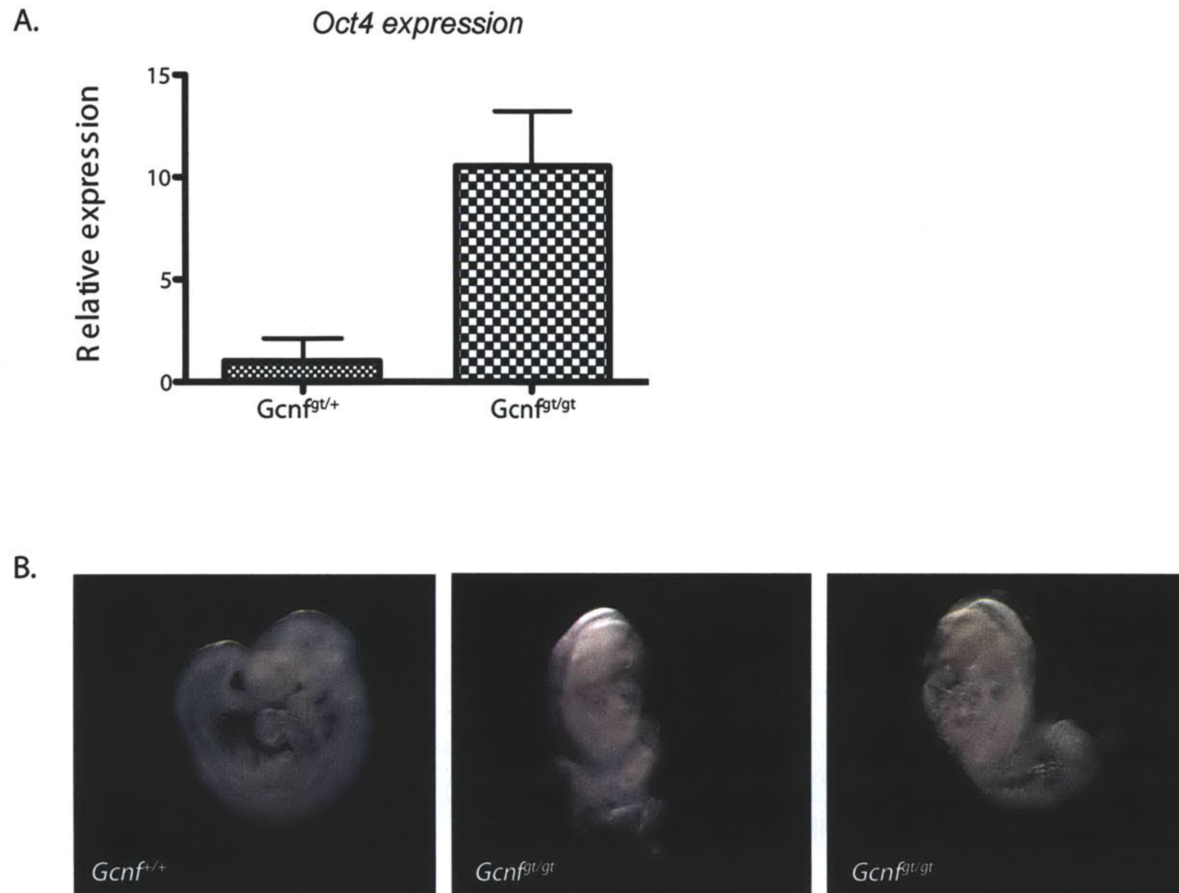
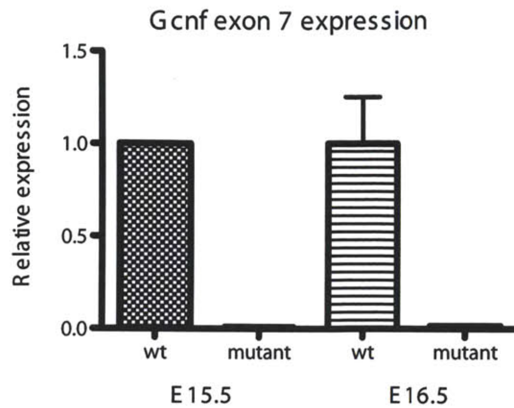
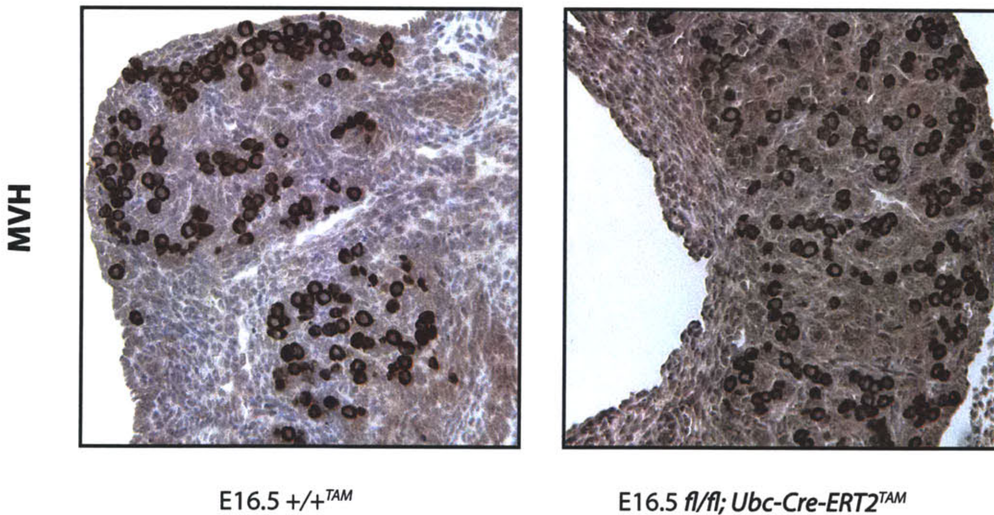


Figure 3. *Gcnf$^{gt/gt}$* mutants fail to down-regulate Oct4 and display morphological defects. (A) Quantitative RT-PCR for Oct4 on E8.5 embryos. Plotted here are average fold changes (relative to Gcnfgt/+ whole embryo, all values normalized to Hprt) of at least 4 independent biological replicates. (B) Images of Gcnf+/+ wild-type and Gcnfgt/gt mutant embryos at E9.5.

A.



B.



E16.5 +/+^{TAM} E16.5 *fl/fl; Ubc-Cre-ERT2*^{TAM}

Figure 4. *Gcnf* is efficiently deleted in mutants. (A) Quantitative RT-PCR for exon 7 of *Gcnf* in ovaries of *Gcnf*$^{fl/fl}$*; Ubc-Cre-ERT2*^{TAM} mutants and *Gcnf+/+*^{TAM} wild-type littermate controls at both E15.5 and E16.5. Plottedhere are average fold changes (relative to *Gcnf+/+*^{TAM} ovaries; all values normalized to *Hprt*) of 2 (at E15.5) or 3 (at E16.5) independent biological replicates. Error bars show standard deviation among biological replicates. (B) Immunostaining for the germ cell marker MVH in *Gcnf+/+*^{TAM} wild-type and *Gcnf*$^{fl/fl}$*; Ubc-Cre-ERT2*^{TAM} mutant ovaries at E16.5.

ovaries, germ cells appeared to be of normal number and morphology as compared to wild type ovarian germ cells.

***Gcnf* is not required for repression of *Oct4* and *Nanog* in germ cells**

Because *Gcnf* is required for the repression of *Oct4* and *Nanog* during gastrulation (Fuhrmann et al., 2001; Gu et al., 2005b), we hypothesized that it might also be required for their repression in female germ cells. Normally, both OCT4 and NANOG are expressed in female germ cells until around E14.5, and are undetectable by E15.5 (Pesce et al., 1998; Yamaguchi et al., 2005). We used immunohistochemistry to examine OCT4 and NANOG protein in ovaries from *Gcnf$^{fl/fl}$; Ubc-Cre-ERT2^{TAM}* mutant and *Gcnf$^{+/+TAM}$* wild-type littermate controls. For each embryo, we collected one ovary to examine by immunohistochemistry and extracted RNA from the other to verify by qRT-PCR whether exon 7 was efficiently deleted. As expected, both proteins were readily detectable in *Gcnf$^{+/+}$* wild-type ovarian germ cells at E13.5, but neither OCT4 nor NANOG was detectable in later *Gcnf$^{+/+TAM}$* wild-type ovaries, at E15.5 or E16.5. Mutant ovaries were indistinguishable from wild-type littermate controls, with neither OCT4 nor NANOG protein detectable at E15.5 and E16.5, indicating that both these genes are down-regulated appropriately in mutants (Figure 5). We conclude that *Gcnf* is not required for the proper down-regulation of OCT4 and NANOG.

***Gcnf* is not required for meiotic initiation or early oogenesis**

We wanted to examine whether other aspects of germ cell development (that occur during or soon after Gcnf expression) were affected in *Gcnf* mutants, namely

meiotic initiation and oogenesis. SYCP3 (synaptonemal complex protein 3) is part of the lateral element of the synaptonemal complex, which forms between homologous chromosome pairs during meiosis. Phosphorylated histone H2A.X (γH2A.X) marks sites of DNA double-strand breaks that form during meiotic prophase, an intermediate step of meiotic recombination. Both SYCP3 and γH2AX are readily detectable in wild-type female germ cells at E16.5 (Prieto et al., 2004). We used immunostaining to examine *Gcnf*$^{fl/fl}$; *Ubc-Cre-ERT2*^{TAM} mutant and *Gcnf*$^{+/+TAM}$ wild-type ovaries for SYCP3 and γH2A.X at E16.5 (Figure 6), using the germ cell marker GCNA (germ cell nuclear antigen) to identify germ cells. In mutants and wild-type littermate controls, SYCP3 was expressed in the nuclei of all germ cells (Figure 6A). Higher magnification revealed the threadlike structures of condensed chromosomes. γH2A.X was also found in all germ cell nuclei of mutants and wild-type controls (Figure 6B). Mutant and wild-type ovarian germ cells were indistinguishable from each other in staining and structure. Taken together, these results indicate that meiotic initiation and early meiotic prophase proceed normally in germ cells lacking GCNF function.

We also tested whether oogenesis was affected in *Gcnf* mutants. The Y-box protein MSY2 (also called Ybx2) marks the cytoplasm during the diplotene stage of meiosis in maturing oocytes (Yang et al., 2005; Yu et al., 2001). We looked for MSY2 expression in wild-type and *Gcnf*-mutant ovaries at E16.5 by immunofluorescence, identifying germ cells by expression of GCNA (Figure 7). All germ cells were positive for MSY2 in mutant and wild-type ovaries, indicating that early oogenesis is unaffected by absence of *Gcnf*. Taken together, these results indicate that *Gcnf* is not

Figure 5

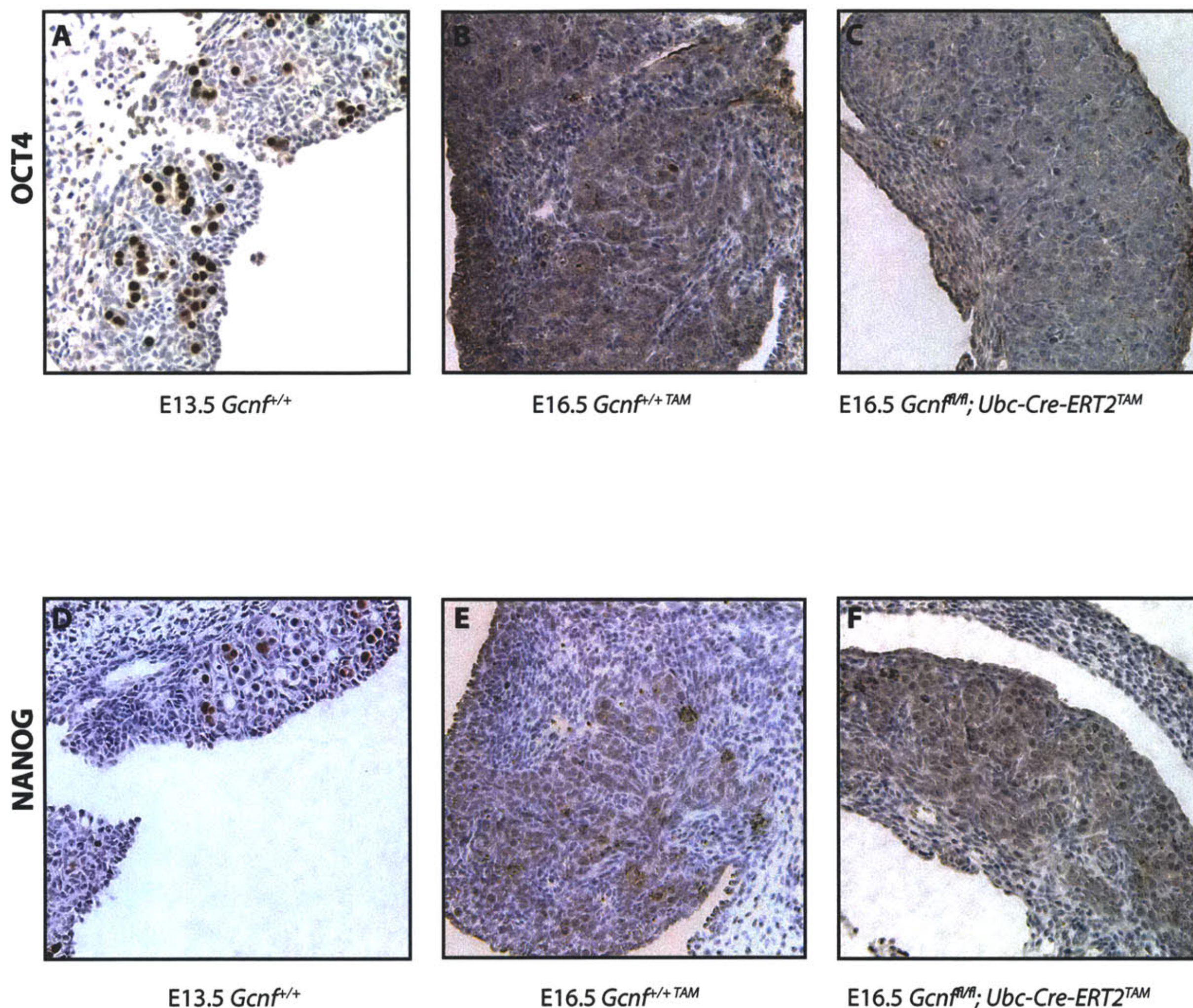


Figure 5. *Gcnf*-mutant germ cells down-regulate OCT4 and NANOG similarly to wild type. Immunostaining for OCT4 (A, B, and C) and NANOG (D, E, and F) proteins in ovary sections. At E13.5 OCT4 and NANOG are readily detectable in $Gcnf^{+/+}$ wild-type ovarian germ cells (A and D, respectively). At E16.5, as expected, OCT4 and NANOG have been down-regulated and are undetectable in $Gcnf^{+/+TAM}$ wild-type germ cells (B and E). E16.5 $Gcnf^{fl/fl}$;$Ubc\text{-}Cre\text{-}ERT2^{TAM}$ mutant germ cells do not express OCT4 and NANOG and are indistinguishable from wild type (C and F).

Figure 6

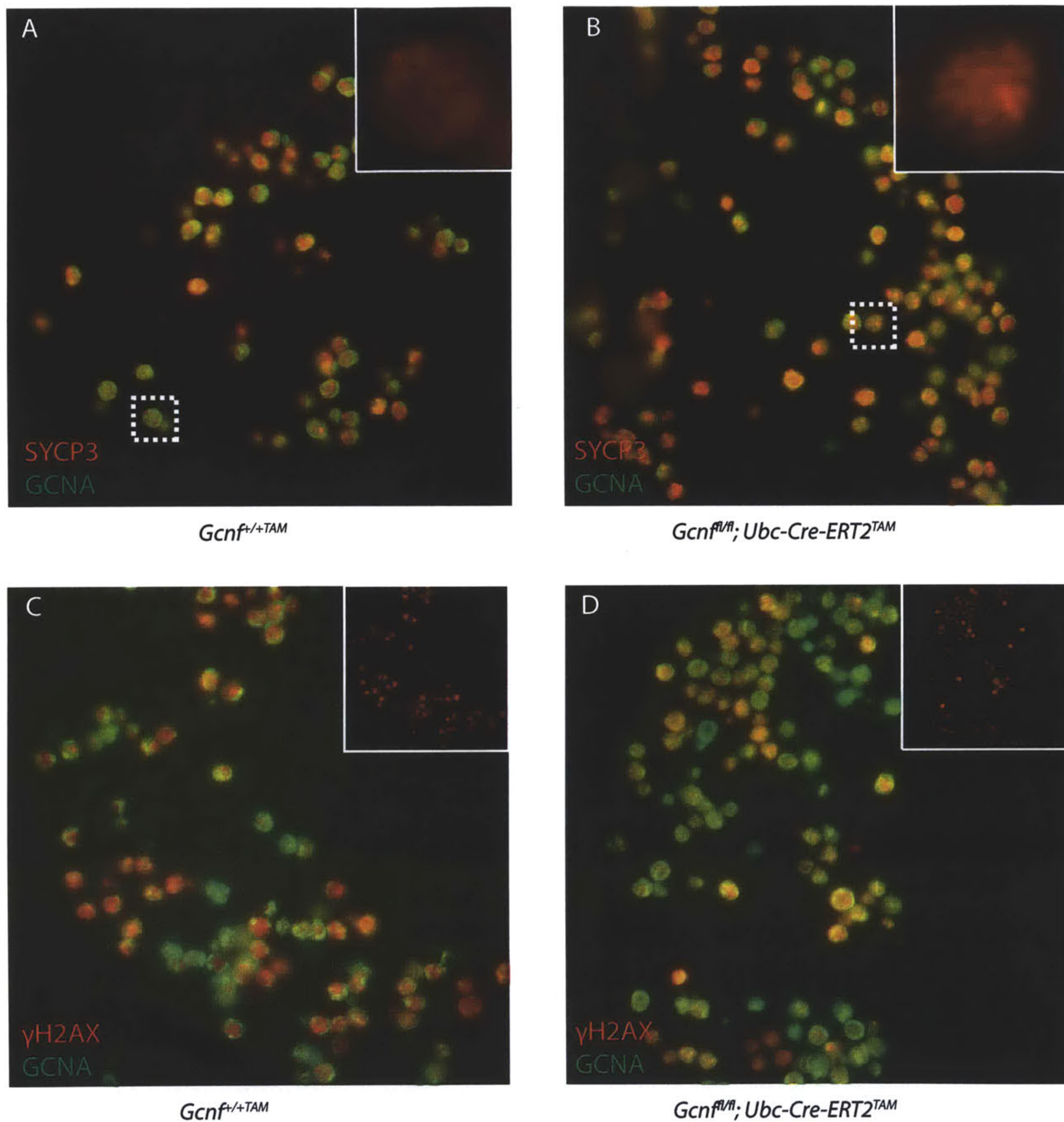


Figure 6. *Gcnf*-mutants undergo meiosis normally. (A and B) Immunofluorescence for SYCP3 (red) and GCNA (green) in *Gcnf*$^{+/+TAM}$ wild-type (A) and *Gcnffl/fl;Ubc-Cre-ERT2*^{TAM} mutant (B) ovaries at E16.5 (insets show higher magnification of the areas boxed in white, SYCP3 staining alone). (C and D) Immunofluorescence for γH2AX (red) and GCNA (green) in *Gcnf*$^{+/+TAM}$ wild-type and *Gcnf*$^{fl/fl}$*;Ubc-Cre-ERT2*^{TAM} mutant ovaries at E16.5 ; insets show lower magnification, γH2AX staining alone).

Figure 7

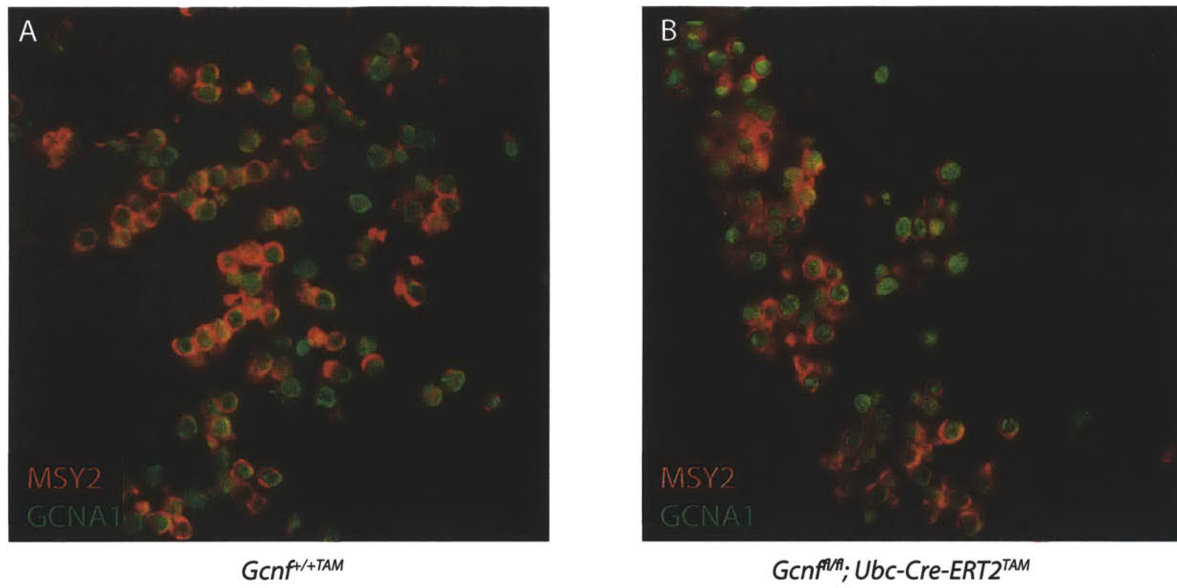


Figure 7. *Gcnf* mutants initiate oogenesis. Immunostaining for MSY2 (red) and GCNA (green) in *Gcnf*$^{+/+TAM}$ wild-type (A) and *Gcnf*$^{fl/fl}$;*Ubc-Cre-ERT2*^{TAM} mutant (B) ovaries at E16.5.

required for the early steps of female meiosis nor for early oogenesis.

DISCUSSION

The down-regulation of pluripotency markers in female germ cells around the onset of meiosis is a significant aspect of their development, and yet very little is known about this event. *Dazl* (deleted in azoospermia-like) has been shown to be required for the differentiation of germ cells upon their entry into the gonads at about E11.5, and subsequent downstream events, including the down-regulation of pluripotency markers in the female, are disrupted in *Dazl* mutants (Gill et al., 2011). Because *Dazl* controls a broad differentiation event several days before pluripotency marker repression, this is likely an indirect effect of *Dazl* function. We therefore set out to determine what molecular regulators might be more directly involved in the repression of *Oct4* and *Nanog*.

Gcnf represented a good candidate for this role because it is a known repressor of *Oct4* and *Nanog* in somatic cells, both *in vivo* and *in vitro*. *Gcnf* is expressed in the gastrulating embryo and to bind directly to response elements in both *Oct4* and *Nanog* promoters (Fuhrmann et al., 2001; Gu et al., 2005b). Here we showed that *Gcnf* is also expressed in female germ cells around the time that *Oct4* and *Nanog* are down-regulated there. Surprisingly, however, when we conditionally ablated *Gcnf* in the later embryo, we found that *Oct4* and *Nanog* regulation were unaffected in female germ cells. In addition, *Gcnf* appears to be dispensable for the initiation of meiosis and oogenesis in the female.

One explanation for this observation is leakiness of the mutant allele. However, it is unlikely that *Gcnf* was inefficiently excised in the germ cells. Each embryo provided a pair of ovaries, of which one ovary was used for RNA extraction and RT-PCR, while the other was processed for paraffin sectioning and immunostaining. Quantitative RT-PCR on individual mutant ovaries showed an average of less than 1.5% of exon 7 transcript remaining, indicating that the vast majority of cells did undergo efficient deletion of exon 7.

Another unlikely explanation is that intact GCNF protein remained by the time that *Oct4* and *Nanog* were down-regulated beginning at E14.5. Tamoxifen was administered to induce recombination of the *Gcnf*^{fl} allele beginning at E10.5. Our smFISH on wild-type gonads indicated that very few germ cells continued to express *Gcnf* transcript at that time. Any intact protein remaining by E14.5 would therefore be remaining from prior to E10.5. From E10.5 to E13.5 the germ cell population expands from about 1,000 cells at E10.5 to over 25,000 cells at E13.5 (Tam and Snow, 1981) which would severely dilute any intact GCNF protein. Therefore, although theoretically possible, it seems unlikely that enough GCNF protein would remain in every germ cell to effectively carry out its function.

Another possibility is that the DNA binding domain (DBD) is translated and functional in the absence of the ligand binding domain (LBD). Given the modular nature of the structure of nuclear receptors, including GCNF, it is quite possible that the N-terminal portion of the protein up until the beginning of the LBD, including the DBD, is, in fact, produced and could be present and properly folded. Unfortunately, we were unable to examine whether the DBD was present in female

germ cells due to the lack of appropriate antibodies. However, GCNF has been shown to interact with SMRT and NCoR, two well-characterized nuclear co-repressors (Fuhrmann et al., 2001; Yan and Jetten, 2000). Both of these co-repressors interact specifically with the LBD of GCNF, which is typical of their interactions with other nuclear receptors as well. Therefore, although we cannot formally rule out the possibility, it is unlikely that the DBD represses *Oct4* and *Nanog* in the absence of the LBD.

A more plausible explanation for our findings is that *Oct4* is regulated by a different enhancer in the germline than in the gastrulating embryo. Yeom and colleagues demonstrated that there are two enhancer elements present in the *Oct4* promoter, one proximal and one distal to the transcriptional start site, which are functionally separable (Yeom et al., 1996). The proximal enhancer (PE) drives expression of *Oct4* in the epiblast and epiblast-derived cells such as embyronal carcinoma cells. The PE is required for the expression of *Oct4* in the somatic cells of the epiblast around the time of gastrulation. The distal enhancer (DE) is necessary for expression of *Oct4* in the pre-implantation embryo as well as in the germline, even in the absence of the PE. It is unclear how these elements are differentially regulated, but it is likely that transcription factors may bind to one enhancer but not the other. GCNF is thought to perform its repressive function by competing with activating nuclear receptors for binding sites in promoters, and has been shown to bind to response elements in the proximal promoter of *Oct4*, near, though not within, the PE (Fuhrmann et al., 2001; Gu et al., 2005a). It is possible that during gastrulation, GCNF's proximity to the PE disrupts the activity of transcriptional

activators that normally bind there, resulting in the GCNF-dependent repression of *Oct4* observed in gastrulating embryos and ES cells (Fuhrmann et al., 2001). In germ cells, on the other hand, GCNF may still bind to the proximal promoter but may not be sufficient to repress *Oct4* due to its distance from the DE. In this case, some other factors, perhaps binding in or near the DE, would be responsible for repressing *Oct4* in the germline (Figure 8).

As our experiments indicate that *Gcnf* is not required *Oct4* or *Nanog* repression in the female germline, it remains unknown what factors might mediate *Dazl*'s effects in the repression of pluripotency markers in female germ cells. It will be of great interest to determine what these factors are, and whether the repression of pluripotency markers is required for the initiation of meiosis or oogenesis.

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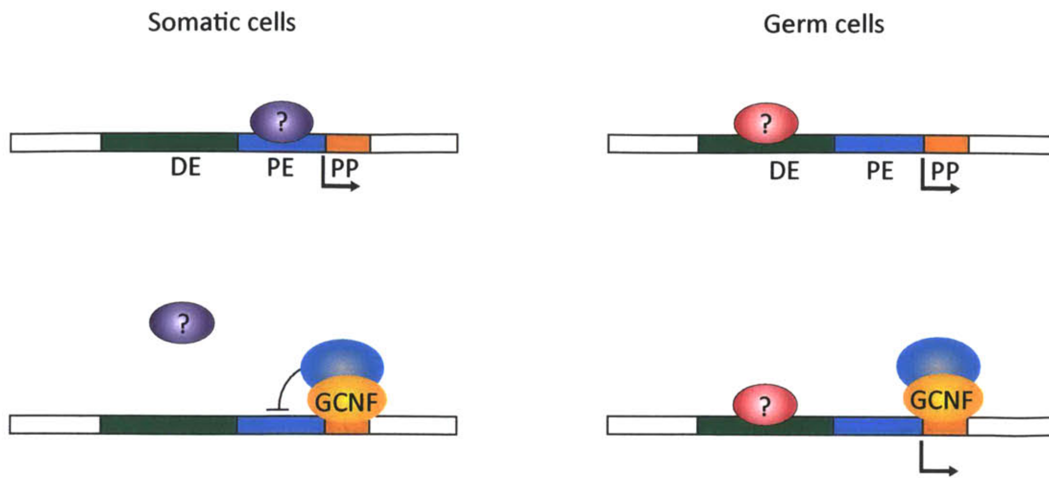

Figure 8. A proposed mechanism for GCNF function in somatic and female germ cells.

MATERIALS AND METHODS

Mice

All studies were performed on a purebred C57BL/6 background. C57BL/6 *Gcnf*$^{gt/+}$ targeted ES cells were obtained from the EUCOMM repository. The sequence of the targeting construct is available at http://www.knockoutmouse.org/targ_rep/alleles/4557/escell-clone-genbank-file ES cell clones were injected into Balb/c blastocysts and transferred to pseudopregnant CD1 females to generate chimeras. Animals were genotyped by PCR using the primers fwd: *TGCAAGGCCAGGTCTTTAAC;* rev: *AGGAGCCCTTCCAAGTTACC* which amplify the region containing the 3' LoxP site. *Gcnf*$^{fl/+}$ animals were generated by mating *Gcnf*$^{gt/+}$ to mice carrying *ACTB:FLPe* (Rodriguez et al., 2000) in order to delete the gene trap and neomycin resistance cassette. Offspring were genotyped by PCR for the 3' LoxP site as described above, as well as with the primers fwd: *TGGGGCACTGTAACAAGACC*; rev: *TGACAATCTCCTACTTGCCCTACC*, which flank the region containing the gene trap and neomycin resistance cassette. Primers for detecting presence of *Ubc-Cre-ERT2* were as follows: fwd: *GATAGTGAAACAGGGGCAATGGTGC*; rev: *TAGAGTATGGGGGGCTCAGCATCC*. All experiments involving mice were approved by the Committee on Animal Care at the Massachusetts Institute of Technology.

Gcnf mutant embryo collection

To obtain $Gcnf^{gt/gt}$ mutant embryos, $Gcnf^{gt/+}$ males and females were mated. To establish timed matings, female mice were housed with male mice overnight. Noon of the day when a vaginal plug was evident was considered E0.5. Embryos were dissected into PBS at E8.5 and E9.5 and yolk sacs were reserved for genotyping. To obtain $Gcnf^{fl/fl}$; *Ubc-Cre-ERT2* mutants, $Gcnf^{fl/+}$ females were mated to $Gcnf^{fl/+}$; *Ubc-Cre-ERT2* males, with tail samples reserved for genotyping Embryonic gonads and mesonephroi were dissected into PBS at E15.5 and E16.5. Sex of the gonads was determined by scoring the presence or absence of testicular cords.

Tamoxifen administration

To induce recombination of the $Gcnf^{fl}$ allele, tamoxifen (Sigma) was dissolved at 20mg/mL in corn oil, and administered to pregnant females via intraperitoneal injection at 5mg tamoxifen/40g mouse. Each pregnant female was injected twice, at E10.5 and E11.5.

Immunostaining

Fetal ovaries were fixed overnight at 4°C in 4% paraformaldehyde in PBS, embedded in paraffin, and sectioned. Slides were dewaxed, rehydrated, heated in Antigen Retrieval Buffer 1 (Spring Bioscience) for 8 minutes, and blocked for 30 minutes in 2.5% horse serum (colorimetric) or 3% goat serum (fluorescence). Slides were then incubated with primary antibody for 1 hour, diluted as follows:

anti-NANOG (Bethyl Laboratories, IHC-00205) 1:200; anti-OCT4 (BD Transduction Labs, 611203) 1:100; anti-MVH (Abcam, ab13840): 1:250; anti-SYCP3 (Santa Cruz, sc-74569) 1:200; anti-γH2AX (Millipore, 05-636) 1:200; anti-MSY2 (a gift from Richard Schultz) 1:200; anti-GCNA (a gift from George Enders, University of Kansas, Kansas City) undiluted. For colorimetric detection, slides were incubated with rabbit ImmPress reagent (Vector Labs) and developed using ImmPACT DAB substrate (Vector Labs), then counterstained with hematoxylin, dehydrated, and mounted using Permount (Fisher Scientific). For fluorescence detection, slides were incubated with FITC-conjugated anti-rat and Rhodamine-conjugated anti-rabbit or anti-mouse (Jackson ImmunoResearch Laboratories) at 1:250 concentration, then mounted with VECTASHIELD mounting media with DAPI (Vector Labs).

Quantitative RT-PCR

To examine *Oct4* expression in $Gcnf^{gt/gt}$ and wild-type embryos, quantitative RT-PCR (qRT-PCR) was performed on whole embryo samples. To examine *Gcnf* expression in $Gcnf^{fl/fl}$; $Ubc\text{-}Cre\text{-}ERT2^{TAM}$ and wild-type ovaries, qRT-PCR was performed on ovaries dissected away from the mesonephroi. In both cases, samples were submerged in TRIzol reagent (Invitrogen) and stored at -80°C. After genotyping of the yolk sac or tail respectively, total RNA was prepared according to the manufacturer's instructions. cDNA was transcribed from 100-200ng of RNA using SuperScript III (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed on cDNA using SYBR Green Core PCR reagents (Applied Biosystems) on an ABI9700 fast real-time PCR machine (Applied

Biosystems). Results were analyzed using the delta-delta Ct method with *Hprt* (hypoxanthine-guanine phosphoribosyltransferase) or *Actb* (actin, beta) as a normalization control. RT-PCR primers were as follows:

Gene	Forward primer sequence	Reverse primer sequence
Hprt	TCAGTCAACGGGGGACATAAA	GGGGCTGTACTGCTTAACCAG
Actb	GAAATCGTGCGTGACATCAAAG	TGTAGTTTCATGGATGCCACAG
Oct4	CAGCCAGACCACCATCTGTC	GTCTCCGATTTGCATATCTCCTG
Gcnf exon 7-8	CACCAGGCTCCACACTATCA	GATCCCTGAATGCCATGAAT

Single molecule fluorescence in situ hybridization

Fetal urogenital ridges were dissected and fixed overnight in 4% paraformaldehyde in PBS, then equilibrated in 30% sucrose, 4% paraformaldehyde in PBS before being frozen in OCT compound (Tissue-Tek). 8um sections were hybridized as described in (Raj et al., 2008), using AlexaFluor 488-conjugated anti-SSEA1 (BD Pharmigen, BD560172) to label germ cells. The probes used to visualize *Gcnf* transcript were:

tcatcctgacagaaaccgtt; attagtgcctggat caagct; gccaagtgttaaactgtcag; tgggacggaaacaggtatat; ttcgttgttcagctcgatca; agatgatcccatagtgcaag; tcttgaaaaaccccttgcag; acccgtttgttgcaaatgct; acacagttcttgtcacgact; tgacatctgttcctctgctt; atctggagacacttgagcag; tgatagccttcctgttcatg; actggtccaatgctcttgtt; cctgtccagacatgattctt; gtgattggcttcttcctcaa; ttgctctctgaagccctgtt; atgatagtgtggagcctggt; ccatttagttccacagacct; tactgatccctgaatgccat; taatgtggaggcactgacat; gctaaaaaggtgtggtatgt; aaagtggtgagtggccagaa; atcagactgtaggactgagg; ttcggctgacatcagctgat; tcaacataggtgtgcccaat; tgtcacagcatacccatctt; aagcagagcaaacagttctg; tgcctaaagagcaactcgtc; cagcttcttgatccaggcaa; ttgagagctcgcagaagaaa; aagaggcacgtgtaatcctt; attaactcctgccacgtaga; atctgcttgctgtacactgt; agtacttggctgtgacatca; tgtggagttcttcatcagag; ctccatcccttcatcactaa; ggtagatgagtcgttcaatc; cagctgatggaacttgtgat; tttcatgcatgcgtactcct; ccctgatatcttgattcagg; ttcagttgttccagctgtga; gacaaatgtaccaataccgc;

ggtttggctgatgtgtgtat; gcacatcataagatcaggaa; tgcgatgtatcggatctctg; aggggcacattcaccatctt; agcaccaccttaaagaggag; ttcaccgtacttgtcttgca.

All probes are listed 5' to 3'.

High throughput mRNA sequencing

Total RNA from E12.5, E14.5, and E16.5 gonads from wild-type and W/W^v embryos was processed using the GLOBINclear Kit (Ambion, AM1981) to remove hemoglobin mRNA. cDNA libraries for sequencing were prepared using the TruSeq RNA Sample Prep kit (Illumina). 36 base pair reads were sequenced on an Illumnia GAII sequencer. Reads were aligned using Tophat (Trapnell et al., 2009) and reads-per-million were estimated using custom Perl script (Soh and Skaletsky, unpublished).

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CHAPTER 3:

Conclusions and Future Directions

In this thesis I have explored the expression and function of *Gcnf*, which encodes an orphan nuclear receptor, in fetal ovarian germ cells. I have demonstrated that *Gcnf* is expressed specifically in the germ cells of the fetal ovary, with peak expression at E14.5, when germ cells down-regulate the pluripotency markers *Oct4* and *Nanog*. Furthermore, I examined the function of *Gcnf* in these cells and found that, despite its intriguing expression pattern, *Gcnf* is not required for the repression of *Oct4* and *Nanog* at this time, nor is it required for the initiation of either meiosis or oogenesis.

We first hypothesized that *Gcnf* might be involved in pluripotency marker regulation in female germ cells because of its known role in repressing *Oct4* and *Nanog* in the gastrulating embryo as well as in retinoic acid-treated ES and EC cells (Fuhrmann et al., 2001; Gu et al., 2005). Furthermore, recent studies in our own laboratory had demonstrated that in the absence of *Dazl*, germ cells fail to repress pluripotency markers (Gill et al., 2011), and preliminary evidence indicated that *Dazl* mutants expressed low levels of *Gcnf* relative to wild type (M. Gill, unpublished). We thought that *Gcnf* might constitute a missing link between *Dazl* and pluripotency marker regulation.

However, the characterization of *Gcnf* mutants indicated that this was not the case. Although gene-trap mutants with a disrupted ligand binding domain (LBD) failed to repress *Oct4* during gastrulation, conditional mutants in which the LBD was disrupted later in development displayed no problems repressing *Oct4* in female germ cells. Quantitative RT-PCR indicated that Cre activity in the conditional mutants was in fact quite efficient, and it is unlikely that enough GCNF protein could

have remained from prior to Cre activation to carry out its repressive function in all germ cells. We cannot rule out the possibility that the DNA binding domain (DBD) may be produced and is sufficient to repress *Oct4* and *Nanog*. However, this seems an unlikely explanation, since the co-repressors SMRT and NCoR1 have been shown to interact with the LBD of GCNF (Fuhrmann et al., 2001; Yan and Jetten, 2000), and it is unlikely that the DBD would be able to bind co-repressors in the absence of the LBD. Furthermore, characterization of the *Gcnf*$^{gt/gt}$ gene-trap mutants indicated that the LBD was necessary for *Oct4* repression in the somatic cells during gastrulation.

What seems a more likely explanation is differential regulation of *Oct4* and *Nanog* in different contexts. The *Oct4* promoter contains separable elements that are necessary and sufficient to drive its expression in epiblast cells or cells of the totipotent cycle, i.e. germ cells and the cells of the pre-implantation embryo (Yeom et al., 1996). GCNF has been shown to bind to the proximal promoter of *Oct4*, near the proximal element required for regulation in epiblast cells. It seems that GCNF is required only for this regulation, and is not sufficient for the regulation of *Oct4* in germ cells.

The expression pattern of *Gcnf* supports this idea. Although GCNF represses *Oct4* during gastrulation, there are other times during development when they are co-expressed. For example, GCNF is expressed in post-natal ovaries beginning in primary follicles, the same stage at which OCT4 resumes after having been downregulated (Lan et al., 2003a; Pesce et al., 1998). Both genes continue to be expressed through oocyte maturation, fertilization, and pre-implantation embryonic development. Additionally, once *Oct4* is repressed in the somatic cells, primordial

germ cells (PGCs) maintain its expression, along with that of a number of other pluripotency factors (Yabuta et al., 2006). In Chapter 2 our single molecule FISH results indicated that, at least at E9.5, *Gcnf* is also expressed in PGCs, indicating that although *Gcnf* is necessary for repression of *Oct4* in somatic cells, it is not sufficient for *Oct4* repression in other contexts.

One question that remains unanswered is the role of *Gcnf* in fetal germ cells. Its expression pattern is intriguing, with peak expression at E14.5, an important time during ovarian germ cell development, when germ cells undergo a number of differentiation events. In addition, both of the nuclear co-repressors with which GCNF has been found to interact, NCoR1 and SMRT, are expressed in the ovary at the same time (Mueller, Gill, and Page, unpublished), though it is unclear whether they are expressed in germ cells or somatic cells, or both.

It is possible that the expression of *Gcnf* at this time is simply coincidental, driven by factors in the germ cells. Retinoic acid (RA) is an important regulator of germ cell development and is involved in the regulation of a number of germ cell-expressed genes in the fetal ovary, including *Stra8* and *Rec8* (Bowles et al., 2006; Koubova et al., 2006). *Gcnf* has been shown to be activated by RA in ES and EC cells; perhaps the same is true in the fetal germ cells as well. It would be interesting to know whether *Gcnf* expression is elevated in *Cyp26b1-/-* testes, where germ cells are exposed to RA. However, this would require some other germ-cell specific factor, as *Gcnf* is not expressed in some places where RA is abundant, such as the limb buds.

I have shown that meiosis and oogenesis appear normal as late as E16.5 in *Gcnf* mutants. Still, it would be useful to explore whether any postnatal deficiencies

can be observed. It is possible that defects in an event initiated prenatally may not manifest until later in development. This may be particularly likely in oogenesis, where many factors are expressed early, including *Figla* (folliculogenesis specific basic helix-loop-helix), *Nobox* (NOBOX oogenesis homeobox), and *Msy2*, that are not required until after birth.

Adult female *Gcnf* mutants in which the DBD of GCNF was ablated in primary oocytes have been characterized (Lan et al., 2003b). These mice show reduced fertility, likely due to hormonal mis-regulation of the estrus cycle, secondary to elevated expression of two GCNF target genes, *Bmp15* and *Gdf9* (Lan et al., 2003b). One particularly interesting aspect of the phenotype observed was that some ovarian follicles contained more than one oocyte. This observation was not studied in further detail; it would be interesting to determine how these follicles arose. Are the oocytes in them a result of the premature completion of meiosis, or parthenogenesis? And how early does this defect occur? Our conditional mutant might allow for further study of the female phenotype.

In addition, adult male *Gcnf* mutants have not been characterized, despite the identification of several potential GCNF targets expressed specifically in male germ cells. Since *Gcnf* has been shown to be expressed in pachytene spermatocytes and round spermatids, it would be interesting to determine whether *Gcnf* mutants have any spermatogenic defects (Bauer et al., 1998; Hirose et al., 1995; Lan et al., 2003a; Yang et al., 2003; Zhang et al., 1998). It would also be useful to investigate whether the *in vitro* determined targets of GCNF can be validated *in vivo*. Of particular interest are *Prm1* and *Prm2*, genes that encode protamines, which replace histones

during the final stages of sperm differentiation, and have been identified as direct targets of GCNF (Hummelke and Cooney, 2004; Hummelke et al., 1998). To date, only repressive functions have been demonstrated for GCNF. PRM1 and PRM2 are expressed in round spermatids, as is GCNF, suggesting a possible activating role for GCNF if these targets were in fact validated *in vivo*. Alternatively, GCNF could repress the protamine genes in pachytene spermatocytes. In addition, comparing *Gcnf* wild-type versus mutant testes by high throughput mRNA sequencing could enable the identification of many more potential target genes.

Aside from *Gcnf* function, many more questions about germ cell development remain. One of these questions is why the pluripotency markers are down-regulated when they are. Fetal germ cells can be transformed *in vitro* into pluripotent EG cells only until E12.5; after this time, there seem to be some intrinsic changes within the germ cells that prevent their transformation. Even at E12.5, efficiency is quite low in female germ cells (Matsui et al., 1992; Resnick et al., 1992). Pluripotency markers, however, remain expressed in female germ cells until about E14.5, two days after their pluripotent potiential is lost (Pesce et al., 1998; Western et al., 2005; Yamaguchi et al., 2005). The role of the pluripotency markers between E12.5 and E14.5 is unclear. The apparent lack of pluripotency in germ cells during this time period is particularly surprising given that all four of the factors used to reprogram somatic cells into induced pluripotent stem cells—*Oct4, Nanog, Sox2,* and *Lin28*—are expressed during this time (Pesce et al., 1998; West et al., 2009; Western et al., 2005; Yamaguchi et al., 2005; Yu et al., 2007); Mueller, Gill, Page, unpublished).

The down-regulation of pluripotency markers is also intriguing in that it coincides with the female germ cells' entry into meiotic prophase both temporally and spatially. Germ cells express meiosis genes such as *Stra8, Sycp3* and *Dmc1* in an anterior-to-posterior wave from about E13.5 to E16.5 (Bullejos and Koopman, 2004; Menke et al., 2003). Interestingly, *Oct4* and *Sox2* are down-regulated in a very similar pattern, disappearing from the anterior tip of the ovary at about E13.5 or E14.5 and becoming undetectable at E16.5 (Bullejos and Koopman, 2004; Menke et al., 2003; Western et al., 2005).

Functional studies have not been undertaken to determine whether these two events, down-regulation of pluripotency markers and meiotic initiation, are linked. Could the repression of pluripotency markers be required for initiation of meiosis? *Dazl* mutant germ cells fail to down-regulate pluripotency markers and also fail to initiate meiosis. However, this is likely due to the role of *Dazl* as a licensing factor, required for a broad differentiation event slightly earlier in development (Gill et al., 2011). *Dazl* mutants have not elucidated which event is upstream of the other, or if they are parallel events. One way to address this question would be to use mice carrying an inducible transgene of one of the pluripotency markers. A mouse line exists in which *Oct4* can be activated by doxycycline treatment (Hochedlinger et al., 2005); however, the effects of ectopic *Oct4* expression have not yet been studied in fetal ovarian germ cells.

Alternatively, it is possible that entry into meiosis forces the repression of pluripotency markers. *Stra8* is the earliest known gene to be required for meiosis, blocking meiotic initiation prior to pre-meiotic DNA replication (Baltus et al., 2006).

In *Stra8 -/-* female germ cells, *Oct4* is still down-regulated, but not as effectively as in wild type—about half the transcript remains (S. Soh, unpublished). This suggests that meiotic initiation may play some role in pluripotency marker regulation, but there are likely other factors independent of meiosis.

A third possibility is that pluripotency markers are regulated in parallel with meiosis, and that both events are controlled by the same signaling factor(s), but independently. Retinoic acid (RA) might be a good candidate for this role, repressing pluripotency markers while activating meiosis genes such as *Stra8*. RA signaling can be blocked *in vitro* in organ cultures of whole gonads, by incubating fetal ovaries with retinoic acid receptor antagonist. In these experiments, ovaries explanted at E12.5 for 48 and cultured with antagonist exhibited increased levels of *Oct4* expression compared to ovaries cultured without antagonist (Bowles et al., 2006). However, cultures were not continued long enough to determine whether *Oct4* would, with or without antagonist, be repressed by the equivalent of E16.5, as occurs *in vivo*.

The objective of my work was to examine how plurioptency markers are regulated in fetal germ cells. Specifically, I set out to test whether the gene *Gcnf* was required for the repression of *Oct4* and *Nanog* in female germ cells. I have shown that *Gcnf* is expressed in female germ cells at this time, and while *Gcnf* is required for the repression of *Oct4* in the gastrulating embryo, it is dispensable for the regulation of *Oct4* and *Nanog* later in germ cell development. It is remains unclear what genes downstream of *Dazl* are involved in the down-regulation of pluripotency markers during female germ cell development. Many questions still persist

regarding the function, if any, of *Gcnf* in fetal germ cells, how pluripotency markers are regulated, and what the importance is of the timing of their repression.

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APPENDIX 1:

A Method for Using RNAi to Manipulate Gene Expression in Fetal Gonads

Author contributions: Aggregate cultures were designed and performed by Leah Okumura in collaboration with Mark Gill; Mark Gill performed qRT-PCR, and Leah Okumura performed immunostaining of MVH and STRA8.

Currently, the techniques available to manipulate gene expression or function in the fetal gonads are transgenics and small molecules used in *ex vivo* organ culture. Transgenic mice are expensive and time consuming to generate and maintain, and are not amenable to exploring numerous genes in parallel. In addition, many genes of interest in germ cell development are essential for embryonic development, requiring the use of conditional knockouts. The use of small molecule inhibitors or activators is limited to the drugs available and risks possible off-target effects which may confound results.

In an effort to expand options for manipulating genes in fetal gonads, we have developed a protocol for using siRNA knockdowns in *ex vivo* organ cultures and demonstrated its efficiency in knocking down *Gfp* expression in GFP-positive germ cells. Because siRNA knockdown was inefficient in intact gonads (data not shown), we adapted an aggregate culture protocol described in (Adams and McLaren, 2002) in which gonads are disassociated by trypsin, incubated with siRNA, and re-aggregated by centrifugation before culturing (Figure 1).

We dissected urogenital ridges from E12.5 embryos carrying a *Gfp* reporter driven by the *Oct4* promoter, which is expressed exclusively in germ cells at E12.5 (Anderson et al., 1999). One pair of urogenital ridges was used for each aggregate culture. Urogenital ridges were dissected into culture medium, which consisted of 10% calf serum in Dulbecco's Modified Eagle's medium with PenStrep antibiotics.

Each pair was then rinsed in PBS and incubated in 20uL of trypsin at 37°C for 10 minutes. Meanwhile, for each aggregate culture 6.25uL of siPORT NeoFX transfection reagent (Invitrogen, AM4510) was combined with 118.75uL of culture

medium and incubated for 10 minutes at room temperature. 6.25uL of 1uM siRNA was mixed with 118.75uL of culture medium, and then combined with the siPORT NeoFX medium and incubated for 10 minutes at room temperature. Trypsinization of each pair of urogenital ridges was stopped by adding 200uL of the siRNA/siPORT NeoFX mixture, and dissociated by pipetting up and down repeatedly to create a single cell suspension. This suspension was then incubated for 5 minutes at room temperature.

The suspension was reaggregated by centrifuging at 2500 RPM for 10 minutes in a tabletop centrifuge. Each pellet was then carefully transferred to a droplet of 35uL of siRNA/siPORT NeoFX mixture on the lid of a 10cm petri dish. Once all pellets were in place, the lid was inverted and placed over the petri dish, which was placed in a 15cm petri dish and the bottom of the dish was covered in sterile water. This was incubated at 37°C overnight.

The next day the siRNA/siPORT NeoFX medium was replaced with standard culture medium. Aggregates were cultured for an additional 24 hours before being collected for analysis. For RT-PCR, aggregates were put into TRIzol reagent (Invitrogen); for paraffin section and subsequent immunostaining they were incubated in Bouin's fixative or 4% paraformaldehyde overnight at 4°C.

When knocking down *Gfp*, very high efficiency was achieved, with less than 5% of transcript remaining compared to control cultures transfected with a negative control siRNA (Figure 2).

We also verified that germ cell development was not severely impacted by aggregation and culturing with the transfection reagent. We looked for presence of

germ cells using immunohistochemistry for the germ cell marker MVH (mouse vasa homolog) (Toyooka et al., 2000). Germ cells were present, though at much lower density than is observed in an intact ovary at E14.5 (Figure 3A). It is unclear whether germ cells are lost in the aggregation process or if they die during culture. The germ cells that remain after 2 days of culture do not appear apoptotic by nuclear morphology. In female aggregates, we also looked for expression of STRA8, which is required for meiotic entry (Baltus et al., 2006). STRA8 was expressed in the aggregate cultures, and appeared to recapitulate the expression pattern seen in intact ovaries, with some germ cells showing stronger, nuclear staining and others showing weaker, cytoplasmic staining (Figure 3B). These results suggest that germ cells in siRNA aggregate cultures are not grossly affected by the transfection reagents and developmental events such as meiotic initiation occur normally.

It is unclear whether the number of germ cells is low due to apoptosis; this could be determined using a TUNEL assay or by staining for activated caspase. If germ cells are in fact dying, revisions could be made in the ratio of transfection agent to medium used during the first 24 hours of culture that might abrogate apoptosis. If germ cells are simply not being retained during aggregation, additional germ cells can be isolated from donor gonads as described in (McLaren and Southee, 1997) to increase the number of germ cells in the aggregates.

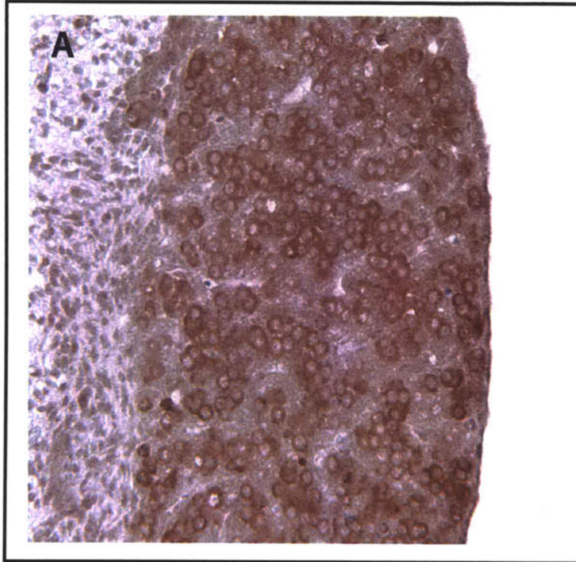
The use of siRNA in fetal gonads should prove to be a useful tool. It provides a fast, relatively inexpensive way to test gene function prior to making targeted mouse models, and allows for testing multiple genes in parallel or in combinations

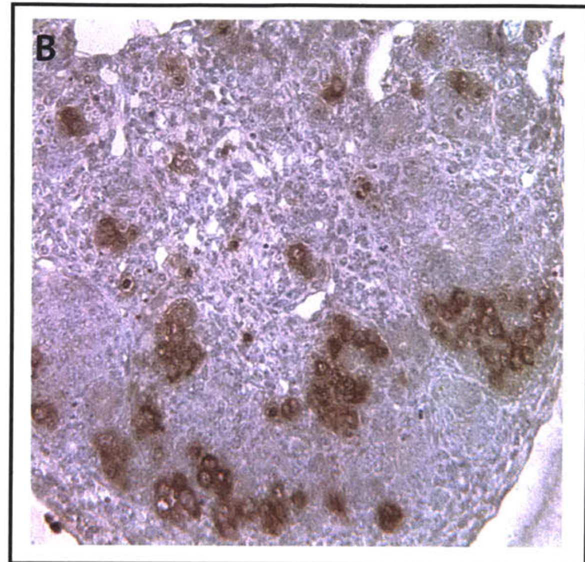
that might otherwise not be possible. Some improvements still need to be made, including improving the inclusion or retention of germ cells in the aggregates.

Intact E14.5 ovary

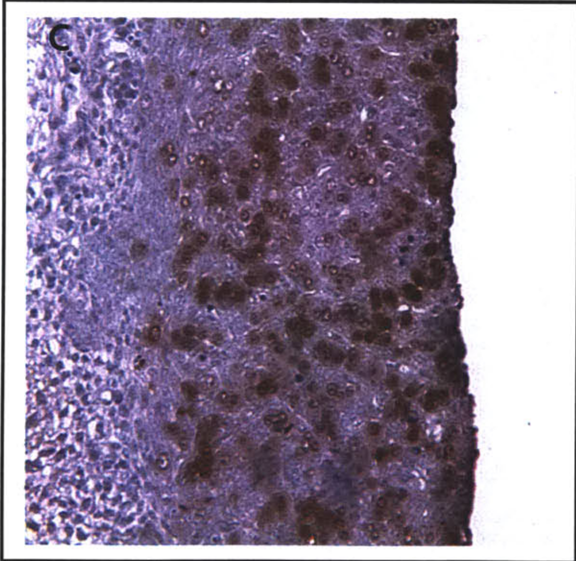
Aggregate culture

MVH





STRA8



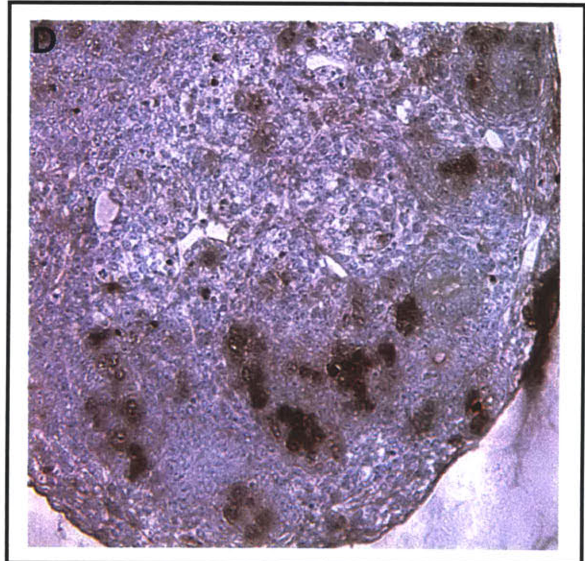


Figure 3. Immunostaining for MVH (A) and STRA8 (B) indicates that germ cells in aggregate cultures survive and undergo normal development. Immunostaining was performed as described in Chapter 2, using anti-MVH (Abcam, ab13840) and anti-STRA8 (Abcam, ab49605) each at 1:200.

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APPENDIX 2:

Generation of an Alternative Conditional Knockout Allele of *Gcnf*

Author contributions: Leah Okumura designed and performed all experiments except the replacement of the neomycin resistance cassette, which was performed by Paul Danielian, and the targeting of ES cells which was performed by the Koch Institute Transgenics Facility.

In addition to the ES cells obtained from EUCOMM, described in Chapter 2, we also generated targeted ES cells carrying a different conditional knockout allele of *Gcnf*. This allele, *Gcnf$^{DBD\text{-}fl}$*, differs from the EUCOMM allele in several respects. First, it does not contain a gene trap; instead it is a true conditional allele. Second, the exon targeted for conditional deletion is exon 4, which encodes the core of the DNA binding domain (DBD) (Susens and Borgmeyer, 2001). This exon is the same one targeted in previously generated *Gcnf* mutants (Chung et al., 2001; Lan et al., 2002). Excision of exon 4 does not cause a frameshift, and it is likely that the mutant protein would be translated and intact. However, previously reports indicate that in the absence of the DBD, GCNF is not functional and does not cause a dominant negative phenotype (Chung et al., 2001; Lan et al., 2002).

To generate the *Gcnf$^{DBD\text{-}fl}$* allele, the following steps were taken. First, the 5' end of the targeting construct was amplified from a BAC containing exon 4 of *Gcnf* using the PCR primers *fwd: TGTGTGTGGGTGGTTTATGTGC* and *rev: GGCTACTTTGCTT TCTCTACTCAGTCCTTC*. This product was cloned into the pCR-XL-TOPO vector (Invitrogen) and moved to pBluescript using BamHI and XhoI. Next, a LoxP site was inserted upstream of exon 4 by linearizing the plasmid with BfuA1 and ligating in oligonucleotides of the following sequence: 5'-gcct*ATAACTTCGTATA ATGTATGCTATACGAAGTTAT*GATGGACTTGACGGTAGGCAACC*C*-3' (italics indicate the LoxP site). Finally, the 5' arm including the LoxP site was transferred into pBS again using BamHI and PstI, and finally to pKI (Yeh et al., 2002) using BamHI and XhoI.

The 3' end of the targeting construct was amplified from the same BAC using the PCR primers *fwd:* CATTCTGCTCTTGGGAAGGACTC and *rev:* AACCACTGAGCCATT

TCTTCAGG and cloned into pCR-XL-TOPO. It was then moved to pKI-Gcnf-5' using KpnI and NotI. Lastly, the Neomycin resistance cassette of pKI-Gcnf-5'-3' was replaced with one from pL451 (NCI Frederick National Laboratory) which is flanked by FRT sites for simplified excision in ES cells. This was accomplished by transferring the fragment containing the neomycin resistance into pcDNA3.1 (Invitrogen) using BamHI and XhoI, and then into pKI-Gcnf-5'-3' using using KpnI and XhoI.

This construct was transfected into C57BL/6 ES cells at the Koch Institute ES Cell and Transgenics Facility. Colonies were screened by Southern blot. ES cell DNA was cut with KpnI, run on a 0.7% agarose gel, and transferred to BrightStar nylon membrane (Ambion). The 5' probe was generated by PCR using the primers *fwd:* TGGGCCTAGCCTTTATCTCC and *rev:* CAACAATGGCAAAA GCTGTG, and detects a fragment of about 12.5kb in targeted DNA and a fragment of about 20kb in wild-type DNA. The 3' probe was generated by PCR using the primers *fwd:* CTGCCAGTGATAAGCCTTCC and *rev:* GCCAGAGAGACCAAGTACGG, and detects a fragment of about 8.5 kb in targeted DNA and a fragment of about 20kb in wild-type DNA. Probes were radiolabeled using Amersham Readiprime DNA Labeling System (GE Healthcare Life Sciences), diluted in UltraHyb hybridization solution (Ambion), and hybridized at 42C. The same membrane was used to probe both ends.

Three clones were identified in which both the 5' and 3' ends were properly integrated as assayed by Southern blot: Plate 3, H5; Plate 4, H6; and Plate 5, B4 (Figure 1). These clones were expanded and frozen in liquid nitrogen by the KI Transgenics Facility.

Plate 5, 5′

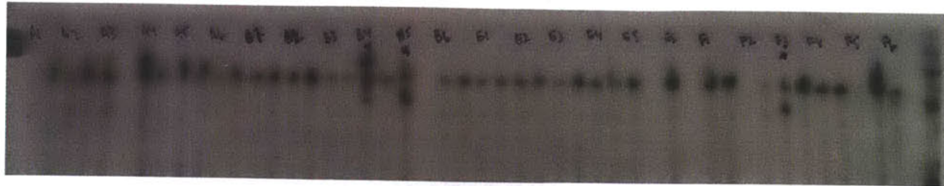

Plate 5, 3′

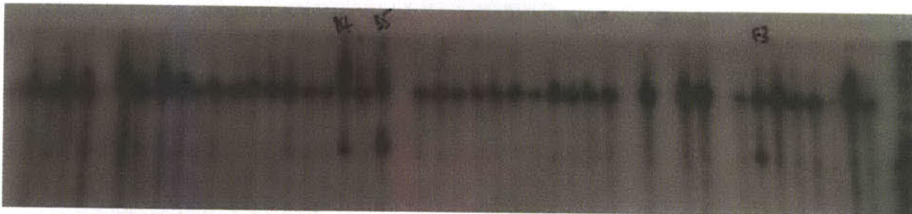

Plate 2/3, 5′

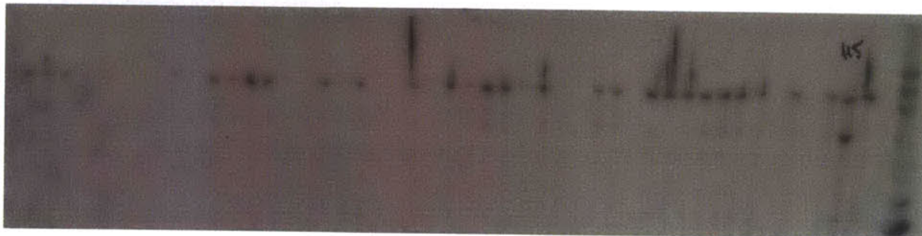

Plate 2/3, 3′

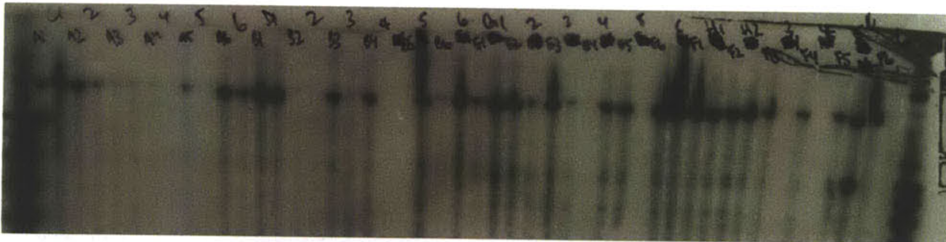

Plate 6/4, 3′

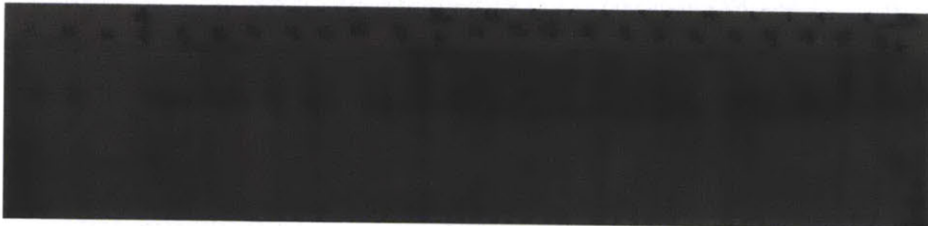

Plate 6/4, 5′

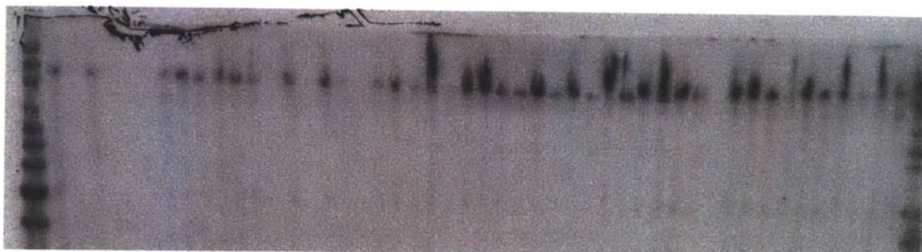

Figure 1: Targeting of *Gcnf*. Southern blots showing targeting of *Gcnf*. For both probes, wild type gives a band at approximately 20kb. The targeted allele gives a band at 12.5kb with the 5′ probe and 8.5kb with the 3′ probe. Not all correctly targeted clones maintained the 3′ LoxP site.

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