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Embryonic stem cell-derived oocyte development in follicles by transplantation into an endogenous ovarian niche

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Embryonic stem cell-derived oocyte development in follicles by transplantation into an endogenous ovarian niche

by

Cory Robert Nicholas

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Sciences

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO
Dedication and Acknowledgements

I dedicate my thesis to my beautiful wife and soul-mate, Tina (aka Gypsy), whose love inspired this work and continues to inspire my dreams, and who passionately twirls them into reality; To my Mom and Dad, Brother, Citto and Giddo, Grandma and Papa, and Family, whose light has guided, grounded, and nurtured, who have always stressed the importance of education and unconditionally provided me with every advantage to excel; To my Friends, whose peace offers positivity and support in good times and bad. I am truly blessed and eternally grateful for the love in my life.

I thank my advisor, Dr. Renee Reijo Pera, for her dedication to understanding human development and disease, generous financial support, brilliant scientific guidance, and for believing that we can accomplish any ambitious scientific endeavor. I thank my thesis committee, Drs. Michael German, Harold Bernstein, and Arnold Kriegstein, for their guidance and generous commitment of time and effort to mentor and ensure my success. I also thank all of my mentors at UCSF, Stanford, UC Berkeley, Sugen, Inc., and beyond who have fostered my scientific skills and interests. Last but certainly not least, I would like to thank my colleagues in the Reijo Pera lab for invaluable assistance and unforgettable fun. It was an honor and pleasure to work with them.

I especially acknowledge mice whose heroic sacrifice made this work possible.

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Embryonic stem cell-derived oocyte development in follicles by transplantation into an endogenous ovarian niche

by

Cory Robert Nicholas

Abstract

The oocyte (egg) is the mother of all cells, propagating genetic and epigenetic information, integrating paternal components, and reprogramming the fertilized embryo to generate offspring. However, the oocyte is mysteriously short-lived. Female reproductive potential is limited in mammalian species including humans where cessation of fertility is reached by mid-age, and 10-15% of couples are infertile due to female factors in half of these instances. Because they are restricted in number, differentiation of oocytes from embryonic stem cells (ESCs) will facilitate analyses of the genetic, epigenetic, and environmental factors affecting oocyte development and contributing to infertility and/or birth defects. The differentiation of functional oocytes from autologous embryonic stem cells will ultimately enable the understanding, potential treatment, and/or prevention of infertility in women. Additionally, ESC-derived oocytes may be used as nuclear transfer recipients to study cellular reprogramming and early embryo development.

ESCs can differentiate into cell types of three somatic germ layers and the germline. Although putative germ cells with oocyte-like characteristics were previously reported to spontaneously differentiate from mouse ESCs in vitro, functional analyses and
correlation to endogenous oocyte development *in vivo* have been limited. In this study, I developed germ cell-specific reporter and surface marker strategies to isolate and characterize mouse ESC-derived germ cells. Using these strategies, I established a developmental timeline and genetic program of female germ cell differentiation from ESCs *in vitro* that initially paralleled endogenous oocyte development *in vivo*. However, ESC-derived oocyte maturation eventually failed, as complete meiotic progression and ovarian follicle formation were not detected.

To overcome this *in vitro* maturation bottleneck, I examined endogenous mouse fetal oocyte development following transplantation to develop a synchronized ovarian niche that was competent to direct oocyte maturation. I then transplanted ESC-derived oocytes into this ovarian niche and analyzed transplants for oocyte maturation in ovarian follicles. Indeed, ESC-derived oocytes functionally integrated into the ovarian niche, recruited endogenous somatic granulosa cells, and successfully developed to the primary follicle stage. In summary, this study provides rigorous evidence of physiologically relevant ESC-derived oocyte identity and function, and presents a potential clinical strategy for treatment of infertility through ovarian niche-based transplantation.
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Chapter 1

Introduction

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**Instructing an embryonic stem cell-derived oocyte fate:**

**lessons from endogenous oogenesis**

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Abstract

Female reproductive potential is limited in the majority of species due to oocyte depletion. Because functional human oocytes are restricted in number and accessibility, a robust system to differentiate oocytes from stem cells would enable a thorough investigation of the genetic, epigenetic, and environmental factors affecting human oocyte development. Also, the differentiation of functional oocytes from stem cells may permit the success of human somatic cell nuclear transfer for reprogramming studies and for the production of patient-specific embryonic stem cells (ESCs). Thus, ESC-derived oocytes could ultimately help to restore fertility in women. Here, we review endogenous and ESC-derived oocyte development, and we discuss the potential and challenges for differentiating functional oocytes from ESCs.
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I. Introduction

Stem cells are unique in their ability to undergo both self-renewal and differentiation. Embryonic stem cells (ESCs) are derived from the inner cell mass of the preimplantation embryo and retain their pluripotency, or the capacity to develop into any cell type of somatic ectodermal, mesodermal, or endodermal lineages (1). In addition, ESCs can develop into the germ cell lineage, or germline, as shown by mouse blastocyst injection and by in vitro differentiation of mouse and human ESCs (2-8). Several reports have recently documented primordial germ cell- (PGC), sperm-, and oocyte-like cell development following mouse and human ESC differentiation, and one study observed mature and functional mouse ESC-derived sperm that were capable of producing offspring (2-8). However, functional ESC-derived oocytes have not yet been reported. In this review, we summarize the strategies described to differentiate ESC-derived oocyte-like cells in the context of endogenous oocyte development.

II. Endogenous Oocyte Development

A. GERMLINE SPECIFICATION

The germline is formed, or specified, by different mechanisms in non-mammalian and mammalian species. In non-mammalian species, the germline is specified through germ plasm inheritance from the maternal oocyte just after fertilization (9). The germ plasm is an oocyte cytoplasm that is composed of RNAs and RNA binding proteins including Oskar, Vasa, Tudor, Aubergine, Nanos, and Pumilio (10). In Drosophila, the germ cells...
are the first cell type to be specified in the early embryo following the inheritance of the maternal germ plasm (11). The components of the maternal germ plasm are thought to silence transcription in nascent germ cells and thereby repress the activation of somatic developmental programs in order to direct germ cell commitment and development (12). Similar mechanisms of germ plasm inheritance-based specification occur in *Caenorhabditis elegans* (*Worm*), *Danio rerio* (*Zebrafish*), and *Xenopus laevis* (*Frog*) (13).

In contrast to non-mammalian species, the mammalian germline is specified through inductive signaling during gastrulation (14). In mice, Bmp-4 and Bmp-8b signaling from the extra-embryonic ectoderm induces the formation of PGCs in the proximal epiblast of embryonic day 5.5 to 6 (e5.5-6) embryos by Alk-2 receptor activation and Smad-1/5 signaling (15-20). Of note, Bmp factors have been used to induce *in vitro* germ cell specification from ESCs as discussed below (3, 5). Although initiated by different mechanisms, non-mammalian and mammalian germ cells have much in common. Mammalian germ cells lack definitive germ plasm but still contain homologues of germ plasm components such as Nanos and Pumilio (21-25). These components, in addition to epigenetic regulation, are thought to similarly suppress somatic development and activate germ cell-specific programs. Mammalian germ cells have additional mechanisms to preserve germ cell identity in the midst of somatic cell embryonic development. The early mammalian PGCs are set aside in the extra-embryonic tissue and are therefore physically segregated from the somatic cells during early embryogenesis. Shortly after specification, Oct-4, Sox-2, Stellar, Fragilis, Alkaline Phosphatase, E-Cadherin, and
Blimp-1 positive PGCs move out of the epiblast and become clustered at the base of the allantois in the extra-embryonic mesoderm on e7 (26, 27) (Figure 1). Blimp-1 inhibition of somatic transcription factor expression, and epigenetic modification mediated in part by Blimp-1/Prmt-5, is thought to represent an initial event driving germ cell commitment (28-30).

B. PRIMORDIAL GERM CELL (PGC) MIGRATION

PGC migration is another process shared among many species during germ cell development (31). After specification, PGCs migrate from their extra-gonadal origin to the developing gonad, or genital ridge, where they interact with gonadal somatic cells that will later determine their commitment to a sex-specific developmental program and maturation into functional gametes. By e9 in mice, PGCs have migrated back into the embryonic endoderm and then migrate up the hindgut toward the genital ridges where they begin colonization on e10. During this time, RNA binding proteins, cell adhesion proteins, tyrosine kinase receptors, and G protein-coupled receptors facilitate PGC migration. PGCs express the c-Kit receptor while the cells of the hindgut express the corresponding Kit ligand (Scf). c-Kit signaling between PGCs and hindgut cells is thought to promote PGC survival, motility, and proliferation (32, 33). In addition, PGCs express the Cxcr-4 receptor that binds the chemokine ligand Sdf-1. Sdf-1 is expressed along the migratory route of the hindgut and genital ridge and provides a guidance signal for PGC migration (34, 35). PGCs also express β1/2 Integrin that is thought to interact with the extra-cellular matrix along their journey (36). Furthermore, PGC-PGC interactions are important for PGC migration and colonization. PGCs express the Cx43
gap-junction protein during migration, and PGCs up-regulate E-Cadherin upon leaving the gut and colonizing the genital ridge. E-Cadherin mediated cell-cell adhesion may support PGC aggregation and migration arrest in the developing gonad (37, 38). During migration and early colonization, PGCs also express Nanog and the cell surface markers Ssea-1, Ema-1, and Tg-1 (39-41) (Figure 1). Indeed, antibodies to Ssea-1 have been used to identify and isolate PGC-like cells following ESC differentiation (6, 42, 43).

C. GONAD COLONIZATION & OOCYTE COMMITMENT
The genital ridges have formed by e10 in mice and are colonized by PGCs shortly thereafter. Upon entry to the genital ridge, the gonadal PGCs, or gonocytes, begin expressing Dazl, Vasa, and Gcna, proliferate two to three more times, and begin to down-regulate Ssea-1 and Alkaline Phosphatase (44) (Figure 1). Then, germ cells in male mice enter mitotic arrest as pro-spermatogonia from e12.5 to e14.5 while germ cells in female mice are induced to enter meiotic prophase as primitive oocytes beginning on e13.5. Interestingly, the sex chromosome complement in the gonadal somatic cells, rather than in the germ cells, determines germ cell commitment to a sex-specific developmental program. Retinoic acid (RA) is a meiotic induction signal expressed by the gonadal neighboring mesonephroi of both sexes beginning on e12.5 but is only able to induce Stra-8 expression and the subsequent meiotic entry of germ cells in the fetal ovary (45) (Figure 2). Germ cells in the fetal testis are protected from RA exposure and are inhibited from entering meiosis due to factors expressed by somatic cells in the fetal testis that include the Cyp-26 mediated degradation of RA (46, 47). Male germ cells do not enter meiosis until puberty (48).
In summary, PGCs appear committed to an oocyte fate by default unless they are in a male environment. Thus, XY PGCs in a fetal ovary will enter meiosis and develop as XY oocytes, and XX PGCs in a fetal testis will enter mitotic arrest and develop as XX pro-spermatogonia (49, 50). Also, PGCs isolated before e12.5 from the fetal gonads of either sex will enter meiosis when cultured in vitro (51). However, while germ cells isolated after e12.5 from fetal ovary will enter meiosis, germ cells isolated after e12.5 from fetal testis will not enter meiosis because they are already committed to a male-specific developmental program (52). In addition, germ cells isolated before e13.5 from fetal ovary will not enter meiosis when mixed with fetal testis, but germ cells isolated after e13.5 from fetal ovary are already committed to an oocyte fate and will still enter meiosis even in a fetal testis environment (53). Therefore, it appears that PGCs commit to a sex-specific developmental program on e12.5 and e13.5 in fetal testis and fetal ovary, respectively. The importance of gonadal environments for germ cell sex determination and development has prompted the use of fetal and newborn gonadal tissue in transplantation and co-culture systems to promote ESC-derived germ cell maturation (5, 42).

In contrast to the default oocyte developmental program of germ cells, the developmental program of gonadal somatic cells appears to be male dominant (54). In the developing ovary, oocytes are required for follicle formation, and an absence of oocytes results in an absence of follicles (55). However, seminiferous tubules will form in the developing testis in the presence or absence of male germ cells (56). Also, the lack of meiotic
oocytes in the ovary can result in ovarian sex reversal, the formation of testicular-like tubules, and differentiation of Sertoli-like cells from granulosa cells (57). Thus, meiotic oocytes are required to direct ovarian follicular maturation (58) (Figure 2).

D. EPIGENETIC REPROGRAMMING

In order to accomplish its future fate as an oocyte or sperm, a developing germ cell must erase the epigenetic program obtained in early embryogenesis, remove the genomic imprints inherited from the previous generation, and re-establish parental imprints in a sex-specific manner during spermatogenesis or oogenesis. This erasure and remodeling of epigenetic marks is termed epigenetic reprogramming and refers to DNA modifications, which do not alter the sequence of DNA. Three main types of epigenetic mechanisms are known to exist and include DNA methylation, histone modification, and RNA-mediated silencing (59).

1. DNA Methylation

Of the different epigenetic mechanisms, DNA methylation is the most widely studied and best-characterized epigenetic modulator. The methylation of DNA is mediated by a family of DNA methyltransferases (Dnmts), which catalyze the transfer of a methyl group to the 5’-position of cytosine residues in CpG dinucleotides located near or within promoter regions and/or the first exon of genes. DNA methylation of CpG dinucleotides inhibits the binding of transcription factors and is involved in recruiting methyl-CpG-binding proteins, both of which prevent the transcription of genes (60). Besides being associated with the epigenetic silencing of genes, DNA methylation has also been shown
to be important for normal embryonic development, X chromosome inactivation, and genomic imprinting, whereby mono-allelic expression of a subset of genes varies according to maternal or paternal origin (61, 62).

Two waves of erasure and establishment of DNA methylation patterns occur during early embryogenesis (63). At the morula stage of development, the methylation of DNA is almost completely erased, with the exception of maternal and paternal DNA imprints. DNA methylation is subsequently re-established during a period of *de novo* methylation, which takes place at the time of implantation (64). Additionally, all cells in the early female embryo randomly inactivate one of the X chromosomes, in part by DNA methylation, by e6.5 (65). The second wave of erasure, unlike the first, affects only the germ cell population and is genome-wide, including the erasure of imprinted loci. Imprinting erasure is a gradual process that begins between e10 and e11.5, depending on the gene, as migrating germ cells colonize the genital ridge (66, 67). Moreover, the inactive X chromosome that was randomly inactivated in the early female embryo is reactivated in female germ cells during migration and as they enter the genital ridge (68, 69). Global re-methylation of DNA is thought to coincide with imprint re-establishment, which occurs after PGCs have committed to a sex-specific developmental program (70). Sex-specific DNA methylation patterns are re-established during male and female gametogenesis, albeit at different times. In the male germline, pro-spermatogonia become methylated at paternal imprint loci starting on e15, while oocytes do not initiate maternal imprint methylation until after birth (71). Methylation analysis of the maternally imprinted genes, *Snrpn*, *Igf2r*, *Mest* and *Peg3* demonstrated that maternal imprints are
established in a gene-specific manner during the non-dividing phase of oocyte maturation (72).

2. Histone Modification

A recent study demonstrated that the methylation of DNA is inhibited by interactions between certain DNMTs and histone modifications, suggesting that the modification of histones is able to control the DNA methylation machinery (73). Histones are the main protein components of chromatin and are comprised of two classes, core histones and linker histones. Core histones are unique in that they have the capacity to undergo post-translational modifications, including methylation, acetylation, phosphorylation, ubiquitination, sumoylation, citrullination, and ADP-ribosylation, which alter their interaction with DNA and nuclear proteins. While the methylation of DNA involves the addition of methyl groups to cytosine bases in genomic DNA, histone methylation is associated with the addition of methyl groups to certain lysine residues. An increase in the methylation of H3-K27 and H3-K4 as well as a reduction in H3-K9 methylation, for instance, results in gene expression and chromatin organization modifications characteristic of post-specification PGCs (74, 75).

Although chromatin remodeling in female germ cells is not as dramatic as the DNA-protein structure changes that occur during the final stages of male germ cell development (76), certain histone modifications are known to be essential for achieving meiotic and developmental competency in the oocyte (77). While phosphorylation is important for histone regulation during spermatogenesis (78), acetylation appears to be
the predominant type of histone modification in oogenesis. Indeed, maturing oocytes are characterized by a global decrease in acetylation, which might influence gene expression during oocyte growth and development (79). In oocyte meiotic prophase, the somatic histone H1 is replaced by H1Foo (H1 histone family, oocyte-specific; formerly named H1oo), a maternal histone variant that is thought to play a role in chromatin condensation and transcriptional repression. This notion is supported by data showing that chromatin in mouse oocytes has a high H1Foo content during periods of low transcriptional activity and a loss of H1Foo expression is observed as soon as zygotic gene activation takes place (80).

3. RNA-mediated Silencing

Recently, it was suggested that the methylation of DNA might occur in a sequence specific a manner. This specificity may be mediated through small RNAs that are able to direct DNA methylation to target loci (81). In the germline, this class of small RNAs is called Piwi-interacting RNAs (piRNAs) for the Piwi proteins that bind the piRNAs (82). piRNAs have been identified in both mammalian and non-mammalian species and differ from microRNAs (miRNAs) and small interfering RNAs (siRNAs) in several ways. Therefore, piRNAs represent a third, distinct small RNA pathway (83).

Although the majority of studies have been conducted on piRNAs in male germ cells (84), recent findings suggest that there are a large number piRNAs corresponding to mRNAs or retrotransposons in growing oocytes. In addition, it was also demonstrated that oocyte piRNAs were bound to Piwi proteins and that retrotransposon transcript levels
were elevated in Piwi mutant oocytes, suggesting that piRNA pathways suppress retrotransposons in mouse oocytes (85, 86). Based on these observations and other studies, which demonstrated that piRNAs are involved in DNA methylation-dependent repression of retrotransposons in fetal male germ cells undergoing de novo methylation, it has been suggested that the piRNA system provides a germline defense against Transposable Elements (TE) activity (87, 88). By interacting with the DNA methylation machinery and histone-modifying proteins, Piwi-piRNA complexes are thought to have the ability to detect the synthesis of TE transcripts during gametogenesis (86, 87).

Altogether, these studies suggest that DNA methylation, histone modification, and RNA-mediated silencing are important for the epigenetic reprogramming of the oocyte. Although a thorough analysis of epigenetic reprogramming in ESC-derived germ cells has been lacking thus far, one study reported imprint methylation erasure at two loci, one showed imprint erasure at one locus, and another found abnormal imprint methylation at three loci (6, 7, 43). Moreover, Tilgner et al. also demonstrated that changes in certain histone modifications, namely the methylation of H3-K4, H3-K9, and H3-K27, in putative germ cells differentiated from ESCs might be associated with PGC identity (43). Future studies should enhance our understanding of how each of these epigenetic mechanisms is cooperatively regulated to achieve PGC formation and development.

**E. MEIOSIS**

Mammalian oogenesis is characterized by three main developmental stages, the initiation of meiosis in the fetus, follicle formation during the perinatal period and oocyte growth
and maturation in the adult (89). PGCs in the fetal mouse ovary begin to enter meiosis asynchronously from e13.5 to e16.5 in an anterior to posterior wave that follows a similar pattern of RA-induced Stra-8 expression. By e16.5, all of the female germ cells are thought to have entered meiosis (45, 90). These primitive oocytes then progress through the sequential leptotene, zygotene, pachytene, and diplotene stages of meiotic prophase I. The majority of oocytes are in zygotene by e15.5-16.5, pachytene by e17.5, and arrest in diplotene around the time of birth (91). During zygotene, the synaptonemal complex component Scp-3 protein begins to elongate axially along the synapsing sister chromatids and then exhibits complete axial localization and alignment by the pachytene stage (92). Scp-1 protein elongates transversely along the synapsing homologous chromosomes during pachytene, and Scp-1 is down-regulated when oocytes arrest in diplotene (93). The premature loss of Scp-1 and accelerated diplotene progression can increase the rate of follicle assembly suggesting that meiotic prophase I must be completed prior to the initiation of folliculogenesis (94) (Figure 2). Upon ovulation in the adult, the oocytes complete meiosis I and arrest in metaphase II until fertilization. Despite the importance of meiotic prophase I progression and arrest for proper oocyte maturation, thorough characterization of ESC-derived oocyte-like cell meiotic progression has not been reported aside from one report of abnormal meiosis (95).

The primitive oocytes begin to undergo atresia shortly after their entry into meiosis. They are at their peak in numbers on e12-13 following PGC mitosis, but begin to undergo substantial atresia through apoptosis during meiotic prophase in the fetal ovary with only 30% of the rodent oocytes surviving to birth and forming primordial follicles (96).
oocytes continue to undergo atresia after birth or are matured and ovulated until the oocyte pool is exhausted and menopause ensues (97). It is thought that atresia is necessary to eliminate aberrant meiotic or aneuploid oocytes and to allow for follicle formation (98). Atresia may also be caused by limited nutrients and, similar to nurse cells in invertebrates, might be required to support the health of the surviving oocytes; thereby sacrificing quantity for quality (99).

F. FOLLICULOGENESIS & OOCYTE MATURATION

Oogenesis is a complex process that is tightly regulated by both endocrine and locally produced factors. From follicle formation to ovulation, extensive crosstalk between the arrested meiotic oocyte and the granulosa cells is required for proper oogenesis (100). The oocyte directs granulosa cell proliferation while the granulosa cells regulate oocyte maturation (101). During fetal development, the oocytes develop in groups, or cysts, and are connected, as in spermatogenesis, by intercellular bridges (102). The intercellular bridges enable the sharing of RNAs and proteins to facilitate oocyte maturation. The oocyte cysts are then surrounded by pre-granulosa somatic cells during meiotic prophase and later break down at birth in mice. This break down allows for the infiltration of pre-granulosa cells and formation of primordial follicles consisting of a single oocyte surrounded by a single layer of flattened granulosa cells (103). TNFα signaling is thought to be important for cyst break down and primordial follicle assembly while estrogen and progesterone inhibit follicle assembly (104, 105) (Figure 2). During this time, the expression of certain transcription factors is essential for further maturation (106). Oocyte expression of Figla is required for primordial follicle formation at birth.
Figla is also required later in development for the expression of zona pellucida genes and the formation of the oocyte coat necessary for oocyte maturation, sperm binding, and fertilization (108).

At puberty in mice, cyclical primordial follicle recruitment is initiated whereby sets of primordial follicles enter a three week growth and maturation phase. Oocyte expression of the cKit receptor promotes oocyte growth and maturation through interaction with granulosa cells expressing the Kit ligand (109, 110). Granulosa cells then turn cuboidal in shape, forming primary follicles, and this primordial to primary follicle transition requires oocyte-specific expression of the transcription factors Sohlh-1/2, Lhx-8, and Nobox (111, 112). Then, primary follicles transition to secondary follicles as two or more granulosa cell layers develop around the growing oocyte, and theca somatic cells surround the granulosa layer. Induced by Nobox, Gdf-9 and Bmp-15 signaling from the oocyte to the surrounding follicular cells is required for secondary follicle development and stimulates granulosa and theca cell proliferation (113, 114). The subsequent secondary to antral follicle transition is characterized by the formation of a fluid filled cavity, or antrum, within the granulosa layer. Fsh and Lh gonadotropin signaling, from the pituitary to receptors on granulosa and theca cells, stimulates extensive secondary and antral follicle growth and induces steroidogenic enzyme expression (115, 116). The steroidogenic enzymes expressed in theca cells convert cholesterol into androgen, and androgen is then used by granulosa cells expressing aromatase to produce estrogen. Oocytes reach maturity during the antral follicle stage, and mature mouse oocytes of approximately 70-80 μm in size are capable of resuming meiosis upon ovulation.
Though follicle-like aggregates expressing steroidogenic enzymes and producing estrogen have been generated from differentiating ESCs, the characterization of stepwise primordial to antral follicle maturation has not been reported (8, 95).

G. HUMAN OOGENESIS

Despite certain differences in ovarian physiology between rodents and humans, the molecular mechanisms mediating human oogenesis have often been extrapolated from studies in these model organisms, predominantly the mouse (117).

1. Meiotic Entry in the Fetal Ovary

Meiosis is initiated in the human fetal ovary between 11 and 12 weeks of gestation (118), while meiotic entry begins at approximately 13.5 dpc in the mouse ovary (68, 119) (Figure 3). Analogous to the mitotic cell cycle, the two stages of meiosis, meiosis I and meiosis II, are organized into prophase, metaphase, anaphase and telophase. Moreover, prophase I of meiosis is further subdivided into distinct phases similar to mouse meiosis, as briefly discussed above. In the leptotene stage of prophase I, the chromosomes begin to condense, forming thin strands of DNA within the nucleus. By the end of the zygotene phase, homologous chromosomes have aligned during a process termed synapsis. Once synapsed, the chromosomes undergo genetic recombination, whereby homologous chromosomes exchange genetic material in the pachytene stage. Following the completion of homologous recombination, the primary oocytes progress to the diplotene stage of prophase I and undergo protracted arrest known as dictyate/diplonema (89). As
each oocyte enters this quiescent state, a single layer of pre-granulosa cells surrounds the
oocytes, forming primordial follicles.

2. Follicle Formation in the Perinatal Period

While the first follicles are formed in the second trimester of pregnancy during human
fetal development (120), follicle formation occurs immediately after birth in the mouse
(103)(Figure 3). Although the exact signal(s) that triggers the formation of primordial
follicles is unknown, previous studies suggest that initial recruitment is mediated by the
interplay of various stimulatory and inhibitory hormones as well as locally produced
growth factors (121). Interestingly, primordial follicles do express receptors for follicle
stimulating hormone (FSH), but are considered to be independent of gonadotropin
support until the antral stage of development. Certain studies in rodents, however,
suggest that the development of early follicles is under the influence of gonadotropins
(115, 122). Recently, it was postulated that decreased estrogen availability in the human
and the sudden decline of maternal estrogens at birth in the mouse might initiate the
recruitment process (105). Given the difference in developmental timing between humans
and mice and the finding that the oocyte may regulate its own fate during follicle growth,
it is possible that the recruitment signal may actually originate from the oocyte itself
(123). Nevertheless, a dramatic loss of oocytes, termed atresia, also occurs in both the
human and mouse ovary at the time of follicle formation. In humans, it has been
estimated that this loss may reduce the number of oocytes by as much as 80% or more.
Thus, the newborn ovary contains only a small fraction of the total oocytes that entered
meiosis in the fetal ovary (120).
3. Oocyte Growth & Maturation in the Adult

In the human, primordial follicles remain quiescent for several years until recruited to become primary follicles (124). In order to resume and complete the first meiotic division, a primary oocyte has to undergo extensive hormone-dependent growth and maturation. Although the signal(s) that recruits an arrested oocyte into the pool of growing follicles remains unclear, it is known that the majority of follicles that initiate growth degenerate during this process (124). Interestingly, the first wave of oocyte growth actually occurs in the sexually immature female, but without the necessary endocrine support, these follicles are unable to further grow and mature. Upon sexual maturation, however, circulating levels of the pituitary gonadotropins, FSH and luteinizing hormone (47), are sufficient to maintain the advanced stages of follicle development and ovulation. In response to a mid-cycle surge of LH, one oocyte, on average in the case of the human, completes the growth phase and is ovulated each month. The time from the initiation of growth to ovulation is approximately 85 days in humans, while only two weeks in mice (125)(Figure 3).

Besides gonadotropin support, other locally produced factors such as estrogen are thought to play a role in the regulation of oocyte growth and maturation (126). It is the granulosa cells, the somatic cellular compartment that is in direct contact with the oocyte, and the theca cells, which reside in the stromal tissue between follicles that assist the secondary oocyte during the maturation process. Moreover, several studies suggest that the oocyte can influence both the proliferation and differentiation of granulosa cells through the
production of growth factors (127, 128). This suggests that communication between an oocyte and somatic cells is bi-directional and that the oocyte may be involved in the regulation of its own development (123).

During the final stages of oocyte maturation, the chromatin organization and the microtubule network of the oocyte drastically change to coincide with the acquisition of meiotic competence (129). As the oocyte resumes meiosis I, the chromosomes condense and one half of the chromosomes remain in the oocyte while the other half is segregated to a small bleb of cytoplasm called the first polar body. Following chromosome segregation, the oocyte, now known as an egg, arrests in metaphase II of meiosis II until fertilized or it degenerates. The fusion between a sperm and the egg plasma membrane at fertilization triggers the resumption of meiosis II, whereby sister chromatids segregate to either the egg or the second polar body. Once the second meiotic division is complete, it is the remaining sister chromatids, together with the chromosomes contributed by the sperm that form a zygote (89).

H. GERM CELL DEVELOPMENT EX VIVO

In contrast to adult mouse testicular spermatogonial stem cells which can be extensively propagated in vitro and can generate haploid cells, mouse PGCs have been difficult to culture and mature in vitro and undergo rapid apoptosis (130-132). However, culturing PGCs on a feeder layer with Lif, Scf, and bFgf can stimulate their survival and proliferation (133). Recently, a cocktail of soluble growth factors and compounds including Lif, Scf, bFgf, Bmp-4, Sdf-1, Nac, forskolin, and RA was reported to sustain
PGC survival and proliferation in the absence of feeder layer support (134). In addition, PGCs were able to enter and progress through meiotic prophase I when meiosis inhibiting Lif was omitted from the media (51) (Figure 2). Nevertheless, the advances to date in PGC culture methods are still only able to extend PGC survival for a limited time and highlight the importance of gonadal somatic cells in sustaining PGC survival and maturation.

Greater success has been achieved in maturing fetal germ cells through co-culture and/or transplantation with gonadal somatic cells. In fact, PGCs from as early as e8.5 have been reported to colonize the testis of infertile newborn mouse recipients, enter spermatogenesis, and produce healthy offspring following oocyte micro-insemination (135). Conversely, PGCs have been much more difficult to mature as oocytes. Intact mouse female genital ridge from e12.5 can be transplanted under the kidney capsule, and follicles can be isolated following transplantation and cultured in vitro, yielding mature oocytes that produce healthy offspring (136). However, intact e12.5 female genital ridge cultured in vitro without transplantation generated oocytes that were unable to resume meiosis, highlighting the importance of transplantation for maturing pre-meiotic PGCs (137). Intact ovaries from e16.5 or from newborn pups, on the other hand, could successfully produce mature and functional oocytes without transplantation (138-140). In the absence of granulosa cells, however, isolated e16.5 oocytes could not grow beyond 25μm, emphasizing the requirement of ovarian somatic cells for oocyte maturation (141). Furthermore, a recent report isolated PGCs from e12.5 female genital ridge, re-aggregated the germ cells with fetal ovarian somatic cells of the same stage, and
transplanted the aggregates under the kidney capsule to generate mature oocytes in 20% of the transplants (142). In a separate report of fetal ovary dissociation and culture, only fetal germ cells from committed e13.5 ovary or later could develop follicles while follicles could not be detected in pre-committed e12.5 female genital ridge cell cultures (143). Notably, both reports showed that isolated fetal ovarian germ cells required interaction with similar stage ovarian somatic cells for maturation, and that prenatal female germ cells could not be matured when mixed with ovarian somatic cells from earlier or later stages of development (Figure 2).

Thus, it appears that pre-committed and pre-meiotic fetal ovarian germ cells are unable to completely mature in vitro and require transplantation to produce mature oocytes. Moreover, in contrast to PGC isolation and testicular transplantation for sperm maturation, isolated PGCs and fetal oocytes require stage-matched ovarian somatic cells to coordinate oocyte maturation. The requirements of transplantation and synchronized fetal germ cell-somatic cell crosstalk present challenges for robust ex vivo PGC- and ESC-derived oocyte development. Successful transplantation has not yet been reported in studies of oocyte differentiation from ESCs.

I. PLURIPOTENCY

Oocytes can be fertilized to produce offspring, form teratomas in humans and mice following parthenogenetic activation, and reprogram terminally differentiated somatic nuclei following somatic cell nuclear transfer (144, 145). Mature gametes are ultimately totipotent, but nascent PGCs require reprogramming during germ cell development to
regain pluri/totipotency. For example, epiblast cells after e5 and PGCs from various stages are not able to directly reconstitute blastocysts and generate chimeras (146, 147).

Yet, PGCs can be prematurely, or ectopically, reprogrammed to pluripotency in rare cases \textit{in vivo}. Spontaneous gonadal and extra-gonadal teratocarcinomas containing cell types derived from three somatic germ layers have been detected in humans and mice and can yield embryonal carcinoma cell (ECC) lines when cultured \textit{in vitro} on a feeder layer (148). ECCs are pluripotent as shown by their \textit{in vitro} differentiation potential and, in some cases, chimera contribution (149-151). These tumors and cell lines originate from germ cells as observed following e12.5 genital ridge transplantation (152).

In fact, mouse and human PGCs can be directly reprogrammed to embryonic germ cell (EGC) lines when artificially cultured \textit{in vitro} on a feeder layer with Lif, Scf, and bFgf (133, 153, 154) (\textbf{Figure 2}). EGCs, like ECCs, are also pluripotent and can generate chimeras and teratomas (155, 156). EGCs have been derived from PGCs of e8.5 to e13.5 embryos, and those EGC lines from post-migratory stage PGCs exhibit imprint erasure similar to their imprint status \textit{in vivo} (157). However, EGCs do not accurately reflect PGC methylation status since EGC lines derived from pre-erasure PGCs may lack DNA methylation imprints. Moreover, EGCs derived from post-migratory PGCs may be hyper-methylated at some imprinted loci, suggesting that artificial culture conditions and/or intrinsic timing mechanisms might induce aberrant methylation patterns (67, 158, 159). In addition to differences in somatic developmental potential, reprogrammed EGCs differ from PGCs in their germline developmental potential as well. PGCs efficiently
enter meiosis \textit{in vivo} and \textit{in vitro}, but reprogrammed EGCs lose their ability to directly and efficiently mature as germ cells (160). Similar to ESC differentiation, PGCs must be re-specified from EGCs during differentiation in order to develop as germ cells.

EGC line derivation has not been reported after e12.5-e13.5 in mice, which corresponds to a culmination of PGC de-methylation, imprint erasure, and commitment to a sex-specific developmental program (161). As such, perhaps the e13.5 stage, and not e7.25, marks PGC commitment to the germline when germ cells are no longer able to be converted into somatic cells or germ cells of the opposite sex. However, the recent finding that adult mouse and human testicular spermatogonial stem cells can be reprogrammed \textit{in vitro} to pluripotent stem cells challenges this hypothesis (162-164). Thus, it appears that post-specification mammalian germ cells may possess the potential to be artificially reprogrammed throughout the entirety of germ cell development until the naturally reprogrammed totipotent state of mature gametes is restored.

\section*{III. Oocyte Differentiation from ESCs}

\subsection*{A. OVERVIEW \& CHALLENGES}

1. ESC \& PGC Similarities

Analogous to ECC and EGC lines, ESCs derived from the inner cell mass are pluripotent and can contribute to the three primary somatic germ layers and the germline (151, 165, 166). Similar to oocytes and EGC/ECCs, ESCs can also reprogram somatic cells to an embryonic-like state following cell fusion (167-169). However, it is the similarity
between ESCs, particularly mouse ESCs, and PGCs that makes germ cells difficult to identify and isolate following ESC differentiation. Mouse ESCs express many of the same markers also expressed by PGCs and mature germ cells (170) (Figure 1). The striking similarity in expression profiles suggests that mouse ESCs may actually be derived from PGC precursors in the early embryo rather than an undifferentiated inner cell mass, or that inner cell mass-derived ESCs may transition to a germ cell-like state during derivation and culture under artificial conditions (171). Yet, mouse ESCs may not be bonafide PGCs because they do not appear to efficiently enter meiosis upon Lif withdrawal and instead generate a variety of somatic cell types. Human ESCs, in contrast, exhibit a different colony morphology and lack the expression of many early germ cell markers including Ssea-1 and Vasa, but do express Dazl, Nanos, and Pumilio (2) (Figure 1). Global gene expression profiling has shown human ESC lines to be distinct from the inner cell mass (172). In fact, human ESC lines may be more closely related to epiblast since they are similar to mouse epiblast derived stem cells, and human ESCs may have different germ cell developmental potential than mouse ESCs (173).

2. Alternative Stem Cell Lines

Epiblast stem cell (EpiSC) lines have been derived directly from the epiblasts of mouse embryos; however, in contrast to mouse ESCs, mouse EpiSCs do not express germ cell markers suggesting a non-germ cell origin or differences in culture environment (174, 175). Although an assessment of their germ cell developmental potential through germline transmission in vivo is not possible since EpiSCs are unable to reconstitute blastocysts, preliminary evidence indicates that in vitro germ cell differentiation from
EpiSCs can be induced by Bmp-4 (175). While investigations of mouse EpiSC-derived oogenesis have not yet been reported, future studies of oocyte differentiation from EpiSCs may be relevant for the human ESC system considering that human ESCs and mouse EpiSCs have similar growth factor requirements and colony morphologies.

A regenerative medicine approach for infertility may ultimately be possible if functional gametes are generated from stem cells. The clinical utility of stem cell-derived oocytes would require autologous stem cells for the treatment of non-germ cell autonomous, and a combination of this with gene therapy for germ cell autonomous, causes of infertility. Additionally, oocyte differentiation from autologous stem cells would be useful for drug screening and toxicity testing. In contrast to men who have testicular spermatogonial stem cells into adulthood that can yield pluripotent autologous stem cells, it is thought that women do not have functional adult germline stem cells as evidenced by oocyte depletion and limited reproductive potential (163, 164). This dogma was recently challenged, but the existence of functional adult female mouse or human germline stem cells in the bone marrow or peripheral blood remains controversial (176-180). There is also a conflicting body of evidence regarding the existence of functional adult germline stem cells in the ovary (181-186). Analogous to ESCs, if adult female germline stem cells do exist, future studies of adult stem cell-derived oocyte commitment, meiotic progression, follicular maturation, epigenetic reprogramming, and function, in both model and human systems, are needed to assess their clinical utility (187).
Aside from hematopoietic and ovarian adult germline stem cells, fetal porcine skin stem cells and adult rat pancreatic stem cells were reported to differentiate into oocyte-like cells in follicle-like aggregates that expressed germ cell markers (188, 189). In the case of porcine skin stem cell differentiation, the oocyte-like cells could bind sperm and support the development of parthenogenetic embryo-like structures, however, meiotic progression and fertilization were not reported (188). Nevertheless, the optimization of this protocol to achieve functional oocytes and the extension of these findings to adult porcine and human skin stem cells could have clinical implications.

Additionally, the reprogramming and induction of pluripotent stem cells (iPSCs) from adult human fibroblasts by the transient overexpression of defined exogenous transcription factors enables the production of autologous stem cells (190, 191). Although human iPSC-derived germ cell differentiation has yet to be reported, human iPSCs share properties with human ESCs and may have similar germline developmental potential. In addition, human iPSCs are similar to mouse iPSCs which support germline transmission following blastocyst injection (192). However, the viral integration of transcription factors may limit the clinical relevance of current human iPSC protocols. Recent reports of non-integrating adenoviral- and non-viral-based induction of mouse iPSCs suggest that application of these modified protocols to human cells should be possible (193, 194).

From an ethical perspective, iPSC and adult stem cell approaches would be optimal in comparison to autologous stem cells derived from parthenogenetic or somatic cell nuclear
transfer (SCNT) embryos that require embryo destruction for stem cell derivation. Parthenogenetic-derived HLA homozygous pluripotent human stem cells have been generated and are similar to human ESCs, but are not practical from a clinical perspective based on the requirement for oocytes, which are limited or absent in infertile women (195). SCNT for therapeutic cloning and ESC derivation has been successful in many species including primate, but the derivation of human ESC lines from SCNT embryos has proven unsuccessful to date due to the limited availability of human oocytes (196, 197). The differentiation of functional oocytes from human ESCs could provide a virtually unlimited source for human SCNT and the successful derivation of nuclear donor-autologous ESCs for subsequent oocyte differentiation. Furthermore, SCNT-derived ESCs would avoid genetic modification that is currently required for human iPSC generation.

3. ESC-derived Embryonic Germ Cells (EGCs)

Pluripotency can be artificially maintained in vitro by culturing mouse ESCs with Lif or human ESCs with bFgf and feeders (1). The artificial nature of this system poses a significant challenge for generating germ cells considering that endogenous PGCs may be reprogrammed when removed from their somatic niche in vivo and cultured in vitro on feeders with similar factors. Lif and bFgf are usually withdrawn during differentiation, but persisting feeders and newly derived fibroblasts or other cell types may provide new sources of Lif, bFgf, and/or Scf that could reprogram ESC-derived germ cells into EGCs. The lack of an appropriate and synchronized gonadal somatic niche may also contribute to EGC conversion. Therefore, the artificial nature of both the undifferentiated and
differentiated ESC culture systems may result in an ESC to germ cell to EGC loop that reduces the efficiency of ESC-derived oocyte differentiation (Figure 2). Furthermore, the persistence of EG-like cells in differentiating ESC cultures presents a significant risk of teratoma formation upon transplantation and is detrimental to ESC-based therapeutic applications of any ESC-derived cell type (198, 199).

4. X Chromosome Reprogramming

It seems logical to assume that gender would dictate the use of XX ESC lines in generating ESC-derived oocytes. However, mouse XX ESC lines are more difficult to derive than XY ESC lines, frequently develop aneuploidy in culture through the loss of one X chromosome, and are less efficient in chimera contribution (200, 201). Both X chromosomes are active in undifferentiated mouse XX ESCs due to the transiently X active state in the inner cell mass. Therefore, inappropriate X chromosome gene dosage may be problematic for prolonged ESC culture (202). In contrast, the majority of human XX ESC lines contain an inactive X chromosome corresponding to the random X inactivation in the epiblast, but this inactivation may not be stable during culture (203, 204). The instability of the X chromosome in XX ESCs coupled with the requirement for X chromosome inactivation and reactivation in developing XX germ cells may render XX ESCs more difficult to utilize in oocyte differentiation (69, 205). It is therefore not surprising that many of the ESC-derived oocyte studies have used XY ESCs based on the observation that XY PGCs can generate XY oocytes in an XX environment. However, the differentiation of fully-functional oocytes will require XX ESCs since XY oocytes do not support pre-implantation embryo development due to meiosis II impairment (206).
5. Differentiation Strategies

There have been 18 reports of germ cell differentiation from ESCs as discussed below; 12 of which used mouse ESCs. Of these mouse reports, five studied oocyte-like differentiation (8, 42, 95, 207, 208), four investigated sperm-like differentiation (5-7, 209), one observed both sperm- and oocyte-like differentiation (210), and two reported PGC-like development (211, 212). Three prior human ESC studies reported both the spontaneous and induced differentiation of germ cells from human ESCs (2-4). During the preparation of this review, three more reports were published that document the isolation and further characterization of human ESC-derived germ cells, and these studies are discussed in brief below (43, 213, 214) (Table 1 and 2).

B. ESC-_DERIVED GERM CELL DIFFERENTIATION

1. Adherent vs Suspension Culture

Undifferentiated ESCs grow as colonies with defined borders and are compact cells with prominent nucleoli. ESC differentiation is routinely achieved by withdrawing the pluripotency maintaining factors from the culture media, removing the feeder layer, and allowing the ESCs to spontaneously differentiate into a heterogeneous mixture of cell types representing the three somatic germ layers and the germline. ESCs are primarily differentiated as either embryoid bodies (EBs) in suspension, using hanging drops or untreated low-attachment plates, or as adherent monolayer cultures. EBs are three dimensional structures that may resemble the early embryo in vivo (215). In contrast, adherent cultures form a monolayer of cells that is much different from the
undifferentiated ESC colony morphology. The cells in the adherent monolayer quickly overgrow the culture plate and form multiple layers. Hubner et al. first reported an adherent differentiation protocol to derive oocyte-like cells from mouse ESCs (8). Subsequently, Nayernia et al. also used adherent differentiation to generate mature sperm-like cells from mouse ESCs (7). In contrast, Toyooka et al. and Geijsen et al. differentiated mouse ESCs as EBs to produce sperm-like cells, and Clark et al. differentiated human ESCs as EBs to study germ cell formation (2, 5, 6).

2. Specification Methods

Spontaneous differentiation of ESCs into a diverse mixture of cell types is poorly understood and is most likely related to the unknown mixture of factors in the serum and media. To help direct the differentiation of a specific cell type, factors can be added to the differentiation media, with or without serum, to generate more homogeneous cultures. Because Bmp factors induce germ cell specification in the epiblast, Bmps were added to the differentiation media to increase the efficiency of germ cell differentiation from ESCs. Toyooka et al. was the first to differentiate sperm-like cells from mouse ESCs using Bmp-4 to induce the germline, and fetal mouse testis aggregation and testis capsule transplantation to promote sperm maturation (5). To identify and isolate germ cells, knock-in XY ESC lines were generated with GFP or LacZ inserted into the Vasa locus. Notably, Bmp-4, but not Bmp-8b, increased the efficiency and rate of germ cell differentiation in cultures of suspension EBs with Bmp expressing feeders (5). A seven fold greater efficiency of germ cell induction was achieved on day one of differentiation compared to three to seven days without Bmp-4. Curiously, adding up to 500 ng/ml of
Bmp-4 directly to the differentiation media did not have an effect on germ cell
differentiation. Also, Kee et al. added a cocktail of Bmp factors (100 ng/ml each of
Bmp-4, Bmp-7, and Bmp-8b) to human EB differentiation cultures. While Bmp-4 alone
stimulated modest germ cell induction, all three factors together had an additive effect
and increased germ cell induction by at least five fold on day two of differentiation (3).

In addition, Lacham-Kaplan et al. differentiated XY mouse ESCs in EB suspension
cultures with newborn mouse testicular cell conditioned media and observed an induction
of ovarian follicle-like structures that were not detected in the absence of the conditioned
media (207). The testicular cells were previously shown to secrete factors important for
PGC and oocyte development including Bmp-4, Scf, Lif, Gdf-9, and Igf-1.

3. Enrichment Methods

Geijsen et al. was the first to utilize RA as a germ cell enrichment factor following mouse
ESC differentiation (6). To isolate germ cells, ESCs were spontaneously differentiated as
EBs in suspension for up to seven days and sorted for the germ cell surface marker Ssea-
1 using immuno-magnetic beads. However, in addition to being expressed by migrating
and gonad colonizing PGCs, Ssea-1 is expressed on the surface of undifferentiated mouse
ESCs. Therefore, germ cells and persisting ESCs, with similar expression profiles, would
be isolated from EBs. But, RA is a potent inducer of ESC differentiation through the
inhibition of Lif signaling pathways and can also stimulate PGC proliferation in vitro
(132, 216, 217). Thus, EB-derived Ssea-1+ cells were re-plated on feeders and cultured
for seven days in the presence of 2 µM RA in order to distinguish between these two cell
types and enrich for germ cells. Undifferentiated ESC cultures treated with RA extinguished Ssea-1 and Alkaline Phosphatase expression and presumably differentiated toward somatic cell lineages, while EB-derived Ssea-1+ cells continued to express Alkaline Phosphatase with up to 10% of cells remaining Ssea-1+. Since the Ssea-1+ populations also expressed PGC transcripts including Oct-4 and Dazl, these cells most likely represented ESC-derived PGCs. Furthermore, Ssea-1+ and RA enriched populations could be expanded with Lif, Scf, and bFgf and reprogrammed into EGC lines. The ESC-derived EGC lines exhibited imprint erasure at the H19 and Igf2r loci, and the extent of erasure corresponded to the duration of differentiation. Geijsen et al. also differentiated XY ESCs as EBs for up to 26 days, and meiotic germ cells were identified by FACS analysis for the spermatocyte/spermatid marker FE-J1 and for DNA content. Of note, round haploid spermatid-like cells isolated by FACS from day 20 EBs were injected into recipient oocytes, and 20% of the injected oocytes progressed to the blastocyst stage.

Because of its multi-faceted role in stimulating PGC proliferation, meiotic entry, and ESC differentiation, RA has subsequently been used by several groups to enrich and mature germ cells following ESC differentiation. Remarkably, Nayernia et al. differentiated XY mouse ESCs in the presence of RA to produce sperm. ESCs modified with a Stra-8 promoter driving a GFP reporter, and a Protamine-1 promoter driving RFP, were differentiated as adherent cultures (7). After ten days in 10 μM RA, 60% of the differentiating cells were GFP+. Following three more days of differentiation in 1 μM RA, 30% of the cells developed as haploid RFP+ motile sperm-like cells that were
released into the supernatant. In addition, RFP+ sperm from the supernatant were then injected into recipient oocytes. Astonishingly, 12 offspring were generated from the ESC-derived sperm, and one pup also developed RFP+ sperm. However, the offspring exhibited developmental defects and died by five months of age. These defects correlated with the abnormal methylation of imprinted loci in offspring and were likely attributed to the aberrant methylation patterns observed in the ESC-derived sperm-like cells.

4. Human ESC-derived Germ Cell Identification & Isolation

Germ cells were originally identified in human ESC-derived cultures by assessing the expression of germ cell-specific markers during differentiation (Table 1). Clark et al. detected the inner cell mass/germ cell markers Oct-4, Stellar, and Nanos, and the gonocyte marker Dazl, in undifferentiated cultures of three different human ESC lines (H9 (XX), HSF-1 (XY), and HSF-6 (XX)) (2) (Figure 1). However, neither Vasa nor the later stage germ cell markers Scp-1 (meiotic prophase), Gdf-9 (oocyte), or Tektin-1 (sperm) were detected in undifferentiated cultures. Upon differentiation as EBs for up to 14 days, the early germ cell markers expressed in undifferentiated ESCs decreased and presumably became confined to emerging germ cells while the expression of Vasa and the later stage germ cell markers were induced during differentiation. Additionally, meiotic markers Scp-1 and Scp-3 were detected after 14 days of differentiation, although chromosomal alignment indicative of meiotic prophase I progression was not observed. Also, Kee et al. similarly observed an increase in the expression of Vasa and Scp-3 markers during three days of H9 EB differentiation that was further induced by Bmp...
treatment (3). Chen et al. found Vasa and Gdf-9 expression in NTU ESC lines after 14 to 21 days of differentiation as well (4).

Likewise, West et al. detected germ-like cells during BG01 (XY) human ESC differentiation for up to 30 days (213). Oct-4 and Vasa double positive cells were identified by day 10 of adherent differentiation and were induced by Mefs and bFgf. Germ-like cells expressed additional markers including Dazl and meiotic stage Daz, Scp-3, and Mlh-1. Although Scp-3 and Mlh-1 expression was localized to the nucleus, chromosomal localization was not reported. Also, spermatocyte and/or oocyte germ cell identities were not determined.

In a recent report, Tilgner et al. isolated putative PGCs by FACS sorting for Ssea-1 following up to three weeks of adherent differentiation of H9 (XX) and hES-NCL1 (XX) human ESCs (43). The Ssea-1 positive cells expressed germ cell markers including Vasa, Scp-3, and Scp-1. The authors further reported that a small sub-population of (XX) Ssea-1 positive cells may be haploid based on DNA content cell cycle analysis. This finding was surprising since the Y chromosome is required for human sperm production, and oocytes do not complete meiosis II until fertilization. Furthermore, nuclear localization of Scp-3 was not detected. Tilgner et al. then showed imprint methylation erasure at one of two loci in the Ssea-1 positive population. In addition, histone modifications were assessed with permissive H3K4me2 enriched at the promoters of germ cell markers, as well as at somatic cell marker promoters, in the Ssea-1 positive cells. Similarly, repressive H3K9me2 was reduced at both germ and somatic cell marker
promoters, while repressive H3K27me3 was reduced and elevated at the promoters of germ and somatic cell markers, respectively. These findings suggest that the putative hESC-derived PGCs may have begun the epigenetic reprogramming process.

Bucay et al. recently reported a modified protocol for the induction of putative PGCs and Sertoli cells from human H9 or HSF6 (XX) ESCs (214). Passaging ESCs in smaller clusters and with less frequent media changes resulted in an increased percentage of Cxcr-4 positive cells that were enriched for Blimp-1 and c-Kit transcript expression after isolation by FACS sorting for Cxcr-4. ESCs were then differentiated in adherent cultures for up to 10 days, and Dazl, Vasa, and Acrosin germ cell marker expression was detected. Sertoli cell marker expression was also observed, and Sertoli-like cells appeared to cluster with germ-like cells in differentiating cultures.

5. Mouse ESC-derived Germ Cell Isolation & Oocyte Commitment

Hubner et al. differentiated mouse ESCs, genetically modified with a germ cell-specific Oct-4 promoter driving GFP reporter construct, as an adherent monolayer (8) (Table 2). GFP was also expressed in undifferentiated ESCs but was expected to disappear in differentiating somatic cells and primarily persist in developing germ cells during ESC differentiation. Indeed, the percentage of GFP+ cells was found to initially decrease to 25% after four days of adherent differentiation and then increase to 40% after seven days of differentiation. Differentiating cell cultures were also analyzed for c-Kit and Oct-4-GFP expression by FACS in order to identify germ cells. Although c-Kit is expressed by somatic cells as well, double marker analysis was used to help distinguish cKit+/GFP-
somatic cells from cKit+/GFP+ migratory stage germ cells with low Vasa expression and cKit-/GFP+ post-migratory germ cells with high Vasa expression. Adherent differentiation was continued, and the formation of GFP+ and Vasa+ colonies was observed by day 12. Small aggregates expressing Vasa, that predominantly lacked Oct-4-GFP expression, then detached from the adherent culture and were found in the supernatant. Vasa+/GFP- aggregates were thought to represent post-migratory germ cells that down-regulated Oct-4 expression upon entry into meiotic prophase. By day 16, nuclear Scp-3 was detected in ESC-derived oocyte-like cells; however, distinct axial chromosomal alignment of Scp-3 or Scp-1 was not reported.

Subsequently, Novak et al. reproduced the Hubner protocol and also found follicle-like aggregates, and elevated levels of estrogen, in the supernatant after 12 days of adherent mouse ESC differentiation (95). Between day 14 and 16 of differentiation, 40% of the cells within the aggregates were Scp-3 positive; however, Scp-3 nuclear localization was variable. Moreover, instead of the long axial core chromosomal alignment of Scp-3, only short filamentous structures were observed in the aggregates suggestive of an abnormal meiotic prophase. Furthermore, the expression of other meiotic markers, including Scp-1 and Scp-2, were not detected, and chromosome synapsis was lacking as shown by centromeric Crest and chromosome 1 FISH staining. Therefore, the ESC-derived oocyte-like cells produced by the Hubner protocol appeared defective and not capable of proper progression through meiotic prophase I.
Recently, Qing et al. reported an alternative method to differentiate oocyte-like cells from mouse ESCs in two separate steps (42). The first step incorporated RA treatment to enrich and detect PGCs, and the second step involved the co-culture of EBs with ovarian granulosa cells and conditioned media to promote oocyte maturation (see maturation section below). First, XY mouse ESCs were differentiated as EBs in suspension for four days. Then, similar to the Geijsen and Hubner sorting methods, EBs were FACS sorted for the germ cell markers Ssea-1 and cKit. Double positive cells represented 25% of the cells in the EBs and were re-plated on feeders in the presence of 2 μM RA for seven days to differentiate persisting ESCs and enrich for ESC-derived germ cells. After RA treatment, alkaline phosphatase positive germ cell colonies were observed in double positive cell cultures but rarely detected from double negative cell populations.

Similar to the Nayernia protocol, Kerkis et al. used a RA differentiation protocol to produce sperm- and oocyte-like cells from mouse ESCs (210). XY mouse ESCs were differentiated in suspension as EBs for four days without RA and four more days with 0.1 μM RA. Cells on the periphery of the EBs appeared to have a different morphology and were positive for germ cell markers including Ssea-1, Oct-4, Dazl, Vasa, Stra-8, Scp-1, Scp-3, and Zp-3. Although these transcripts were also expressed in undifferentiated ESCs, the expression of Gdf-9 and Acrosin was low to absent in ESCs and increased during differentiation.

Using a Gdf-9 promoter driving GFP reporter construct, Salvador et al. reported the identification of oocyte-like cells in cultures of XX mouse ESCs (208). After
differentiation for one day on feeders or in suspension without LIF, GFP+ oocyte-like cells were detected in the supernatant and expressed Gdf-9 and Zp-3. Surprisingly, the addition of Lif to the culture media increased the number of GFP+ cells by three fold. However, follicle-like structures were not detected, and the oocyte-like cells quickly degenerated, which was suggested to be due to an inability of the ESC-derived oocytes to properly execute meiosis (208).

As an alternative to genetic modification and immuno-staining methods for isolating germ cells, a recent report by Saiti et al. used Percoll and Nycodenz gradients to isolate putative PGCs following mouse EB differentiation for up to five days (212). Cells isolated from both the EBs, and genital ridge positive controls, expressed germ cell markers and were viable, suggesting that density gradient isolation might represent a feasible and clinically relevant approach for obtaining ESC-derived germ cells.

6. Mouse ESC-derived Oocyte-like Cell Maturation

From day 12 to day 26 of the Hubner et al. protocol (above), the floating ESC-derived aggregates developed into morphologically visible follicle-like structures that contained putative oocytes (Table 2). These follicular structures also expressed Gdf-9, steroidogenic enzymes, and produced estrogen. On day 26, the follicles released oocytes of 50-70 μm in diameter that expressed Zp-2/3 and Figla. Upon extended culture, pre-implantation stage embryos were observed and were likely the result of parthenogenetic oocyte activation. Notably, both XX and XY mouse ESC lines produced oocyte-like cells.
After detecting putative ESC-derived PGCs, Qing et al. co-cultured day four EBs with granulosa cells from newborn mouse ovaries in adherent cultures, or with granulosa cell conditioned media in suspension culture, to promote oocyte-like cell maturation (42). Ten days later, EBs cultured with granulosa cells expressed Figla, Gdf-9, Zp-1, Zp-2, and Zp-3, while EBs cultured with conditioned media or CHO cells did not express these markers. EBs re-plated with granulosa cells developed large cells, up to 25 μm in size, and expressed Vasa, Scp-3, and Gdf-9. However, follicular structures were not detected.

In Kerkis et al., new aggregates developed after 9-11 days of further differentiation that were different from EBs by morphology and FE-J1 positive expression. Electron microscopy revealed the formation of spermatid-like structures similar to endogenous sperm acrosome, midpiece, and axoneme morphologies. In addition, putative follicle-like structures were observed in the same EB cultures, but in different aggregates than the sperm-like cells. Although only detected at low frequency, an oocyte-like cell was reported to express Dazl. A putative blastocyst-like structure was also shown. However, parthenogenetic activation of an oocyte-like cell should not be excluded.

Similarly, in Lacham-Kaplan et al., further differentiation for two to three weeks in testicular conditioned media resulted in an increased number and size of EB-derived follicle-like structures. These follicle-like cell clusters contained putative oocyte-like cells of up to 35 μm in diameter and expressed Stra-8, Figla, and Zp-3.
However, in all of the above reports of mouse ESC-derived oocyte-like cell maturation, oocyte functionality, or their ability to be fertilized and produce offspring, was not demonstrated. Furthermore, the meiotic progression of ESC-derived oocyte-like cells was not assessed in these reports. Although Novak et al. characterized the meiotic progression of oocyte-like cells produced using the Hubner et al. protocol, the authors detected abnormal progression through meiotic prophase I and/or meiotic arrest (95).

C. FUTURE REQUIREMENTS TO ACHIEVE FUNCTIONALITY

Many challenges need to be overcome in order to achieve robust and functional oocyte differentiation from ESCs. The low efficiencies of ESC-derived oocyte-like cell maturation reported to date may be unavoidable since most endogenous oocytes undergo atresia during development. However, the optimization of methods to direct ESC-derived germ cell specification, oocyte commitment, and oocyte maturation may increase efficiencies and enable functional oocyte production.

1. Germ Cell Specification

Germ cells are one of the first cell types specified in the early embryo and should also be one of the first cell types to develop in vitro during ESC differentiation. However, germ cell differentiation from ESCs has been difficult. Optimal conditions for ESC-derived germ cell differentiation must be determined including the use of XY and XX ESC lines with robust germline developmental potential, adherent versus suspension differentiation culture, and the kinetics of germ cell differentiation, which may be considerably slower
for the human ESC system (Figure 3). The addition of factors, including Bmps, in the differentiation media to induce the specification of ESC-derived germ cells has increased the efficiency as well as the rate of germ cell differentiation and may help to synchronize ESC-derived germ cell populations (3, 5, 208, 213). Also, serum-free differentiation media, in combination with defined factors, may assist in directing homogenous germ cell differentiation from ESCs by removing unknown factors in the serum that result in heterogeneous non-germ cell fates.

Following endogenous germ cell specification in the early embryo, germ cells are set aside from the embryo proper and are isolated from initial somatic cell differentiation. Perhaps germ cells specified from ESCs must also be isolated and removed from heterogeneous cultures during the early stages of in vitro ESC differentiation to ensure proper development. Several germ cell surface marker (Ssea-1, c-Kit, Cxcr-4) and fluorescent reporter (Oct-4, Vasa, Stra-8, Protamine-1, Gdf-9) strategies have been developed and utilized in the isolation of ESC-derived germ cells by FACS or MACS sorting, but additional germ cell-specific markers and reporters may be needed (5, 6, 8, 42, 43, 208, 214, 218). Also, because ESCs express germ cell markers, enrichment methods, such as RA treatment, may be required to distinguish ESC-derived PGCs from undifferentiated ESCs (6, 7, 42, 210).

Previous studies have confirmed ESC-derived germ cell identity by gene expression analysis of differentiation cultures and/or isolated germ cell populations. While an important first step, heterogeneous ESC-derived populations may contain germ and
somatic cell types at different stages of development. Thus, future studies should incorporate single-cell gene expression analysis, and global microarray profiling, to help accurately characterize the identity of ESC-derived germ cells. Additionally, future studies should include extensive epigenetic analysis of methylation and imprint patterns, X chromosome status, and histone modifications in ESC-derived cells to expand on the epigenetic studies reported to date (6, 7, 43).

Finally, the genetic requirements for in vitro ESC-derived germ cell specification and differentiation should be assessed to provide convincing evidence of germ cell identity and to compare ESC-derived germ cells to those that develop in vivo. As such, genes essential for endogenous germ cell development can be perturbed via genetic-null ESC derivation, or shRNA knockdown, to determine germ cell identity and function during ESC differentiation. Conversely, genes required for endogenous germ cell development can be over-expressed in ESCs to direct germ cell specification, commitment, maturation, and/or rescue of genetic null/knockdown phenotypes during differentiation.

2. Oocyte Commitment

Following specification, ESC-derived PGCs must then commit to an oocyte developmental fate and avoid artificial reprogramming to EGCs or commitment to a sperm developmental program. Endogenous oocyte commitment occurs in the fetal ovary, or female genital ridge, and subsequent meiotic entry is induced by RA signaling. Consequently, co-culture with fetal ovary may help to induce the oocyte commitment of ESC-derived PGCs. However, co-culture should not be necessary since endogenous
oocyte commitment, meiotic entry, and progression can occur rather efficiently as a
default developmental program when PGCs are cultured in vitro in the absence of fetal
testicular tissue and Lif. It is therefore quite surprising that ESCs and ESC-derived germ
cell-like populations, which express PGC markers, have not been reported to progress
through meiotic prophase I (95). The addition of RA to the differentiation media can
induce meiosis in ESC-derived germ cells (7). RA may also be required to prevent in
vitro ESC-derived germ cell reprogramming to EGCs and promote persisting ESC
differentiation (6).

Because a fetal testis environment can prevent oocyte commitment and meiotic entry in vivo, differentiating ESC cultures should be monitored for the expression of Sertoli cell markers, including RA metabolizing Cyp26b1, and initiation of Sry-mediated testicular development. If expressed, the Cyp26 inhibitor, R115866, could be added to the differentiation media to inhibit RA metabolic activity. Notably, Sertoli-like cells were recently identified following ESC differentiation (214).

Nevertheless, previous reports showed nuclear Scp3 localization indicative of meiotic entry, however, chromosomal alignment of Scp3 and Scp1, marking chromosome synapsis and meiotic progression, has yet to be documented (2, 8, 95, 213). In addition to synaptonemal complex analysis, future studies should examine ESC-derived germ cells for other markers of meiotic progression including proper cohesin, DNA repair, and recombination nodule complex formation. Considering that meiotic progression to diplotene arrest may be required for subsequent follicle formation, it will be of utmost
importance to optimize ESC-derived germ cell specification and/or oocyte commitment protocols to overcome the current obstacles to proper meiotic entry and progression during ESC differentiation.

3. Oocyte Maturation

Given the success of ESC-derived sperm and endogenous PGC maturation via transplantation, and the particular importance of ovarian somatic cells in supporting endogenous oocyte development, appropriately staged ovarian granulosa cells may be required for the maturation of committed ESC-derived oocytes using co-culture and/or transplantation methods. A human granulosa cell-based maturation system would be challenging but may be possible to develop using aborted human fetal ovarian tissue, human granulosa cell lines, and/or autologous stem cell-derived granulosa cells. In addition to steroidogenic enzyme expression and estrogen production, the follicular maturation of ESC-derived oocytes must then be characterized for sequential progression through primordial to antral follicle stages by morphological analysis and by the appropriate expression of genes involved in oocyte-granulosa cell crosstalk (8). ESC-derived oocytes should also be examined for the expression of transcription factor networks, including Sohlh, Figla, and Nobox, required for follicle formation and maturation, and for the epigenetic re-establishment of maternal imprints.

Ultimately, the complete maturation and function of mouse ESC-derived oocytes must be determined by analyzing germinal vesicle break down (GVBD) and completion of meiosis I, fertilization and completion of meiosis II following intra-cytoplasmic sperm
injection (ICSI), the support of pre- and post-implantation embryo development, and the derivation of ESC lines following fertilization or SCNT. Human ESC-derived oocytes can be examined for GVBD, embryo cleavage following parthenogenetic or SCNT activation, and ESC line derivation.

IV. Clinical Significance of Stem Cell-derived Oocytes

If stem cell-derived oocytes could someday be safely utilized for reproductive purposes, a large and diverse group of infertile women would have the opportunity to bear genetically-related offspring. Common causes of infertility that could be treated using stem cell-derived oocytes include premature ovarian failure, reproductive aging associated with delayed childbearing, and poor oocyte quality which can occur even in young women.

A. PREMATURE OVARIAN FAILURE

Premature ovarian failure is typically defined by amenorrhea (absence of menses), hypoestrogenism, and hypergonadotropinsim in a woman who is under 40 years of age at the time of onset (219). Although the incidence of premature ovarian failure is not known with certainty, it has been estimated to occur at a frequency of approximately 1%. Because some women with apparent premature ovarian failure may ovulate or occasionally conceive after the diagnosis is established, the term “primary ovarian insufficiency” has been suggested to more accurately describe the condition (220).
Primary ovarian insufficiency is a heterogeneous disorder which may result from a decrease in the initial primordial follicle number, an increase in follicle destruction, or a failure of the follicle to respond to gonadotropin stimulation (220). Known genetic causes include absence or structural abnormalities of the X chromosome (such as monosomy X or deletions on the X chromosome), Fragile X Syndrome, structural rearrangements of autosomes, point mutations of autosomal genes which are required for oogenesis and folliculogenesis, and abnormalities of pleiotropic Mendelian genes which may disturb ovarian function along with perturbation of other organ systems (e.g. galactosemia). Multiple autoimmune etiologies for primary ovarian insufficiency have also been described, and environmental causes are just beginning to be explored as well. For example, in the mouse, in-utero exposure to the environmental contaminant bisphenol A has been convincingly associated with oocyte aneuploidy in female offspring (221). Ovarian function may also be lost via surgical removal of the ovaries due to ovarian cancer or as a result of chemotherapy for non-gynecologic malignancy. Many causes of ovarian failure remain idiopathic. Research involving stem cell-derived oocytes may not only provide a potential reproductive option for women with primary ovarian insufficiency, but may also lead to a better understanding of its causes.

B. REPRODUCTIVE AGING

Childbearing at an advanced maternal age is strongly associated with an increase in the risk of fetal aneuploidy. An age-associated increase in aneuploidy has also been noted in human oocytes (222) and in biopsied human embryos (223). Multiple factors could potentially account for genetic abnormalities in the oocyte, and it is likely that
abnormalities may arise during multiple stages of oogenesis (89). Stem cell-derived oocytes may eventually allow a better understanding of age-associated aneuploidy as well as a potential option for the creation of euploid oocytes.

C. POOR OOCYTE QUALITY

Fertility specialists commonly encounter infertile women with poor oocyte quality and resulting poor embryo development, particularly in couples who were characterized as having “unexplained” infertility prior to in vitro fertilization. In some cases, the problem with oocyte quality may be defined by recurrent low rate of oocyte maturation. In other cases, the recurrent problems with oocyte quality are characterized on the basis of abnormal morphological appearance of the oocytes and resulting embryos which develop with high rates of fragmentation and arrest prior to the blastocyst stage. There are no suitable techniques for improving oocyte quality either in the clinic or in the embryology laboratory. Problems with oocyte quality are poorly understood, and it is hoped that stem cell-derived oocytes may offer some understanding and eventually a treatment option for these infertile couples as well.

D. CLINICAL NEED

Currently, the options available for childbearing to women who lack reproductively competent oocytes are limited to oocyte donation, embryo donation, or adoption. In 2005 in the United States alone, nearly 10,000 fresh and 5,000 frozen embryo transfers were performed using donor oocytes (CDC website: 2005 ART Report: National Summary). This number of cycles is most certainly an underestimate of the true clinical need for
functional oocytes, as not all couples with infertility on the basis of ovarian failure or diminished ovarian reserve will choose to pursue oocyte donation. For some couples, oocyte donation is not an acceptable option because of an ethical or other personal objection to combining genetic material from a spouse (intended father) with an individual chosen only as a source of genetic material. Other couples are unable to afford oocyte donation. Oocyte donation is very expensive due to the need for extensive screening of the donor, ovarian stimulation with injections of gonadotropin, oocyte retrieval, and financial compensation given to the donor. As an alternative to oocyte donation, some couples may be willing to consider embryo donation (i.e. receipt of cryopreserved embryos from a couple who has completed their family) (224). However, few embryos are donated for reproductive purposes in part because couples may be reluctant to allow their embryos to be donated to another couple who will then become the legal parents of the resulting children. Clearly, more options are needed to allow women who lack reproductively competent oocytes to have genetically-related offspring, and stem cell-derived oocytes could someday fulfill this need.

Although much work is needed before stem cell-derived oocytes could be used safely for reproductive purposes, there is a clear clinical need for such an option. Research utilizing oocytes derived from stem cells could also provide valuable insight into the processes of both normal and abnormal oocyte development. It is quite plausible that this research will not only enhance our ability to address infertility, but will also help us to better understand some of the causes of abnormal offspring in spontaneous conceptions.
occurring among the general population. The clinical relevance of research involving stem cell-derived oocytes is unquestionable.

V. Conclusions

From their formation in the early embryo to their ultimate acquisition of functionality in the adult, germ cells must complete a long journey with many genetic and epigenetic requirements. Human ESC-derived PGC-like cells and mouse ESC-derived oocyte-like cells have been generated in vitro by several groups, however, their ability to appropriately enter and progress through meiosis, support complete follicle formation and maturation, undergo proper epigenetic reprogramming, and support the development of offspring has not yet been demonstrated. In contrast, mouse ESC-derived haploid sperm have been produced but contain epigenetic abnormalities that have been passed to offspring (7). Thus, there is a long road ahead until the potential for functional ESC-derived oocytes is realized, and progress will undoubtedly depend on a greater understanding of endogenous oocyte development.

Acknowledgements

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<td>Oocyte-like cells but no follicular structures detected</td>
<td>FE-J1+, haploid sperm-like cells and DAZL+ oocyte-like cells</td>
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Figure Legends

Figure 1. Germ Cell Marker Expression

A list of 20 germ cell markers expressed during different stages of oocyte development that are commonly analyzed during embryonic stem cell (ESC) differentiation to detect the presence of germ cells and determine the extent of ESC-derived germ cell maturation. The pre-natal timeline refers to days of mouse embryonic development. The dashed lines represent decreased expression. Notably, the transcript of every marker examined can also be detected in undifferentiated mouse ESCs (unpublished data). The plus signs indicate expression levels in undifferentiated mouse ESCs, and underlined genes are expressed in undifferentiated human ESCs.

Figure 2. Specification, Commitment, and Maturation

A summary of hallmark events during endogenous mouse oocyte development, and a timeline of embryonic development and embryonic stem cell (ESC) differentiation: Primordial germ cells (PGCs) are specified in the proximal epiblast by signals from the extra-embryonic ectoderm including BMPs (e5.5 in mice, day 1-10 in EB). PGCs in the female genital ridge/fetal ovary become committed to an oocyte fate and enter meiosis in response to retinoic acid (e13.5 in mice, day 10-20 in EB). PGCs in the fetal testis are inhibited from entering meiosis, commit to a sperm fate, and enter mitotic arrest. LIF can also inhibit the meiotic entry of isolated PGCs in culture. Prior to e13.5, PGCs cultured *in vitro* on a feeder layer with LIF, bFGF, and SCF can be reprogrammed to pluripotent embryonic germ cells (EGCs, dashed arrow). Meiotic oocytes block testicular gonad development, and meiotic progression to diplotene arrest, including the down-regulation
of SCP-1, promotes follicle formation and subsequent maturation in the presence of synchronized pre-granulosa somatic cells (birth, day 20-30 in EB). Oocyte expression of the transcription factor FIGLA is required for primordial follicle formation. Follicle formation is also mediated in part by TNFα stimulation of oocyte cyst break down and is inhibited by the maternal hormones estrogen and progesterone.

**Figure 3. Developmental Timeline of Human and Mouse Oogenesis**

A comparison of the hallmarks of (A) human and (B) mouse oogenesis, including meiotic entry of germ cells in the fetal ovary, the formation of primordial follicles during the perinatal period, and the growth and maturation of primary, secondary, and antral follicles in the adult. Two phases of ovarian follicle recruitment, the initial recruitment of primordial follicles in the perinatal period and the cyclic recruitment of antral follicles in the sexually mature adult, occur in humans and mice, albeit at different times of development. Both recruitment stages are associated with atresia, which is thought to eliminate aberrant oocytes.
### Figure 1. Nicholas et al.

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**++/+ Oct-4**
**+++ Stella**
**+++ AP**
**++ Blimp-1**
**nd ECadherin**

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<th>+++ Ssea-1</th>
<th>+++ eKit</th>
<th>+++ Cxcr-4</th>
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**mESC Expression:**

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<td>+ Zp-1-3</td>
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**___ = hESC expression**
Chapter 2

Genetic modification and homogeneous expansion of embryonic stem cells with reporter constructs

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ABSTRACT

Genetic modification of human embryonic stem cells is essential for studies of gene function and differentiation. The expression of transgenes may direct tissue-specific differentiation and aid in the identification of various differentiated cell types. Stable genomic integration of transgenes is optimal because human embryonic stem cell differentiation can span several days to weeks and include numerous cell divisions, and establishing homogeneous modified cell lines will facilitate research studies. Herein we provide a method for producing and expanding human embryonic stem cell lines from single cells that have been isolated by fluorescence-activated cell sorting following genetic modification by lentivirus vectors. Using this method, we have established eGFP-expressing human embryonic stem cell lines that are pluripotent, contain a diploid chromosomal content, and stably express eGFP following more than two months of routine culture and in vivo differentiation.
INTRODUCTION

Differentiation of human embryonic stem cells (hESCs) to specific cell types provides an unprecedented opportunity to study normal human development and disease progression \textit{in vitro}. Additionally, hESC-derived cells may provide novel therapeutic opportunities for the treatment of common diseases. However, for the promise of hESCs to be realized, it will be necessary to obtain pure populations of cells differentiated from hESCs. This necessitates the development of reliable methods to genetically modify hESCs using ubiquitous and tissue-specific reporters for the identification and isolation of specific, differentiated cell types. In addition, genetic modification of hESCs will facilitate studies of gene function via gene silencing and over-expression.

Genetic modification protocols, optimized for many cell types including mouse embryonic stem cells, are often inefficient or unreliable in hESCs. Thus, a number of reports have documented strategies to improve the reliability of hESC genetic modification using lipofection, electroporation, and lentivirus transduction.

The spontaneous differentiation of hESCs is robust and yields a heterogeneous cell population comprised of many different cell types representing the three somatic germ layers and the germline (2). However, the differentiation of a specific cell type from hESCs can be inefficient. For example, germ cells represent less than 1% of all cells in differentiated embryoid bodies (EBs). To facilitate further analysis of specific cell types, we sought to generate genetically modified hESC lines derived from single cells to ensure that heterogeneous EBs would be homogeneous for their transgene integration. With this approach, all cells in a rare population of a specific cell type would be more likely to express the transgene at similar levels. This technical note details a
method for single-cell isolation and expansion of genetically modified hESCs using FACS sorting.

METHODS AND RESULTS

HESC Culture

Routine culture of H9 cells (WA09, http://stemcells.nih.gov/research/registry/) of hESCs was performed largely as described (225). Embryoid bodies (EBs) were generated as described (27) using Ultra Low Cluster 6-well plates (Corning).

Production and Titering of Self-Inactivating Lentivirus

The self-inactivating lentivirus vectors, FUGW and 199, express eGFP using the constitutive human Ubiquitin-C and PGK promoters respectively (226)(Fig. 1). Virus was generated using either of these transfer vectors in combination with the packaging vector Δ8.9, and a VSVG envelope vector (227) as described (228). Briefly, 293T cells (ATCC) were tri-transfected using Lipofectamine 2000 (Invitrogen) or calcium phosphate precipitation at 37°C for 6 or 10 hours respectively. Virus was harvested 72 hours following transfection, and viral titering was performed as described. Viral titers of 3-5 x 10^6 infectious particles/ml were typically achieved without concentration. If necessary, virus can be concentrated by ultra-centrifugation.

Lentivirus Transduction of hESCs

Two days after splitting cells, one well of H9 hESCs was treated with collagenase and scraped into 2 ml of KSR media. The cell suspension was transferred to a 50 ml conical centrifuge tube, and 2 ml of virus stock supplemented with polybrene (6 μg/ml final concentration, Sigma) was added to the cell suspension at a multiplicity of infection of
approximately 5 infectious particles/cell as defined by titering on NIH 3T3 cells. The mixture was incubated for two hours at 37°C with gentle mixing every 30 minutes. Cells were pelleted, and the infection was repeated with fresh virus. Following a second infection, cells were washed with PBS, re-suspended in 3 ml of KSR media, and plated into one well of a 6-well plate containing fresh MEFs. Cells were fed with one-half volume of fresh KSR media daily until eGFP expression was readily detectable by microscopy, typically two to four days following infection.

Bright-field and fluorescent images taken four days after transduction demonstrated that individual H9 colonies had an expected patchwork pattern of infected cells (Fig. 2A). Stable transduction efficiency, quantified by FACS analysis as the percentage of eGFP positive cells 10 days following infection, ranged from 10 – 25% (Fig. 2B). Modifications to the above infection method have subsequently improved our transduction efficiency to 40 – 60%. For this, a collagenase IV and dispase solution (both 1 mg/ml, 37°C for 15-20 minutes; Invitrogen) was used to remove hESC colonies without feeder cells. Colonies were pipetted to physically break them into smaller clumps, pelleted, washed with PBS, and re-suspended in 1 or 2 ml of KSR media. 0.5 ml of the cell suspension was transferred to a sterile 2.0 ml microfuge tube, 1 ml of virus stock with polybrene was added, and the tube was incubated on a rotator for two hours at 37°C. The cells were pelleted, re-infected, and re-plated. While high transduction efficiencies are clearly feasible, they may not be optimal for genetic modification because they are more likely to produce cell lines with multiple, and possibly deleterious, integration events. Moreover, high infection efficiency is not necessary when using FACS for isolation of individual transduced cells.
FACS Isolation and Expansion

The purpose of this report is to present a method to generate hESC lines following FACS sorting and re-plating of individual genetically modified cells (Fig. 3A). Three days after passing lentivirus infected H9 hESCs, cells were incubated for 5 minutes at 37°C in 0.05% Trypsin-EDTA (Invitrogen), disrupted by gentle pipetting, and incubated for an additional 2 minutes at 37°C. Cells were then harvested, passed through a 40-μm cell strainer (Becton Dickinson), and transferred to a sterile FACS tube (either on ice or at room temperature) for sorting. Prior to sorting, the cell sorter was sterilized with 5 ml of 70% ethanol followed by a flush of 20 ml of 0.2 micron filtered pyrogen free water. EGFP positive cells were collected in one ml KSR media on ice, washed once with sterile PBS, and re-suspended in KSR media.

The eGFP positive cells were counted, diluted in KSR media, and a range of plating densities was screened for re-plating efficiency in 1 ml/well of KSR media in 12-well plates seeded with 60,000 MEFs/well. We found that 100 cells/well plated yielded roughly 5 colonies/well (Table 1). To test handling conditions, 100 FACS-isolated single hESCs were plated per well following different incubation periods between isolation and re-plating. While we expected single-cell plating efficiency to decrease with increasing time between isolation and re-plating, a delay of up to two hours following single-cell isolation did not adversely affect re-plating efficiency (Table 1).

Based on the post-sorting plating efficiency (Table 1), we plated 20 cells/well to obtain, on average, a single colony per well for expansion and establishment of cell lines. Colonies became visible within 5-7 days of re-plating. The majority of wells had no colonies, and one third of the wells contained a single colony (Table 2). To avoid the
expansion of oligoclonal colonies, only colonies from wells with a single colony were expanded to generate cell lines. Single colonies were treated with collagenase for 5 minutes at 37°C, washed with PBS, harvested using a 20 μl pipette, dispersed into several small cell clumps, and transferred to 12-well plates seeded with MEFs. One ml of fresh KSR media was added to each well and pipetting was used to further disrupt the colonies. One ml of fresh KSR media/well was provided every three days. After an additional 5-7 days, multiple colonies were visible in some of the wells and ready for passage. Cells were treated with collagenase for 5 minutes, and mechanically scraped as during routine passage. The entire well was transferred to a single well of a fresh 6-well plate seeded with MEFs and fed with 3 ml/well of KSR media. Media was changed daily, and cells were passed at a ratio of 1:1 after 3-5 days. Cells were subsequently passed 1:1 every three days for one to three additional passages and then routinely passed at a ratio of 1:3 every three days. Of the initial colonies, 40 to 60 % were successfully expanded (Table 2).

**Stable Transgene Expression**

Established cell lines following single-cell plating demonstrated homogeneous eGFP expression by fluorescence microscopy (Fig. 3B). They were continuously passed for 8 weeks, or approximately 20 passages following FACS-isolation, and regularly analyzed by FACS to evaluate eGFP expression stability. Approximately 85% of cells continued to express eGFP at stable levels over an 8-week period (Fig. 4A), without significant change in mean fluorescence intensity. Based on visual inspection of colonies in the fluorescent microscope, we suspected that the bulk of the 15% eGFP-negative population represented feeder cell contamination. Indeed, when we switched to a new collagenase
and dispase treatment method to harvest the hESCs relatively free of underlying MEFs, greater than 95% of the hESCs were eGFP positive by FACS analysis (Fig. 4A). However, these data do not eliminate the possibility that a small fraction of the hESCs lose their eGFP expression.

As an independent method to assay for eGFP expression stability, individual H9-eGFP cell lines were differentiated into embryoid bodies (EBs) and analyzed for eGFP expression by FACS analysis. Greater than 90% of the cells continued to express the transgene (Fig. 4B), demonstrating extensive, but not necessarily complete maintenance of GFP expression throughout the cells of the EB. Fluorescence microscopy demonstrated maintained EGFP expression in EBs following 45 days of differentiation (Fig. 4C).

**Comparative Genomic Hybridization (CGH)**

Recent reports have documented aneuploidy in hESCs when using trypsin for routine passaging (229, 230). While we did not use trypsin for routine passaging, we did use trypsin to obtain single cells for FACS sorting and expansion. Thus, the chromosomal content of derived H9-eGFP cell lines was determined using Comparative Genomic Hybridization (CGH) analysis. Individual cell line DNA was labeled, hybridized to the HumArray 3.1 containing 2464 BAC clones spotted in triplicate, and normalized to differentially labeled normal reference DNA as described (http://cc.ucsf.edu/microarray/protocols/index.html). CGH demonstrated that 4/11 (36%) of the H9-eGFP cell lines analyzed had acquired chromosomal abnormalities (Table 2). In particular, we observed isolated trisomy of either chromosome 17 or chromosome 1. The remaining 7 cell lines, 64%, exhibited diploid chromosomal content (Fig. 5A-C).
Analysis of Pluripotency and Differentiation

To determine if the derived H9-eGFP cell lines retained marker expression patterns consistent with undifferentiated hESCs, the cells were transferred to chamber slides (Nunc) containing 50,000 feeders/well. Cells were cultured for 24 or 48 hours, fixed, and incubated with primary antibodies according to manufacturer’s instructions (ES Cell Marker Sample Kit protocol; Chemicon) followed by incubation with APC conjugated secondary antibodies (Jackson ImmunoResearch). The derived H9-eGFP cell lines expressed SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, and lacked SSEA-1 expression, consistent with undifferentiated hESCs (Fig. 6A-B).

Pluripotent hESCs differentiate into cell types representative of the three somatic germ layers. To assay for differentiation potential in vitro, EBs were generated and subjected to quantitative RT-PCR expression analysis to detect the induction of neural cell adhesion molecule (NCAM), kinase domain receptor (KDR), and alpha-fetoprotein (AFP), markers of ectoderm, mesoderm, and endoderm lineages, respectively using methods essentially as described. In brief, total RNA was prepared using the RNeasy Mini Kit (Qiagen), and cDNA was produced from 500 ng of RNA with SuperScript II RNase H- Reverse Transcriptase (Invitrogen) using random primers. Amplification specificity was confirmed by melt curve analysis and gel electrophoresis. Our undifferentiated genetically modified cell lines lacked the expression of differentiation markers, and the expression of these markers increased following EB differentiation suggesting pluripotency (Fig. 6C).

An additional test for pluripotency is the ability of hESCs to form teratomas. For this assay, recipient two-month old nude mice were anesthetized, and two grafts
(approximately 200,000 cells/graft) were implanted under the kidney capsule per mouse. Teratomas were harvested two months post-transplant and evaluated histologically and by FACS analysis. An example of teratoma formation is shown following the transplantation of one of our cell lines (Fig. 6D). More than 80% of the cells from the isolated teratomas analyzed by FACS continued to express eGFP following differentiation in vivo, illustrating extensive transgene expression stability (Fig. 6D). Regarding the GFP-negative cells observed in FACS analysis of disrupted teratomas, our techniques did not allow us to distinguish between contaminating murine cells and failure to maintain GFP expression in the hESC-derived cells.

Additionally, the teratomas were comprised of tissues representing the 3 somatic germ layers, including squamous epithelium and neural tissue (ectoderm), smooth muscle and cartilage (mesoderm), and glandular epithelium (endoderm), demonstrating the pluripotency of the derived H9-eGFP cell lines (Fig. 6E-G). Of note, both normal and trisomy lines expressed markers of pluripotency, differentiated in vitro, and formed teratomas in standard assays (Fig. 6).
DISCUSSION

In this report, we describe a straightforward method for FACS-based single-cell isolation and expansion of genetically modified hESCs following lentivirus transduction. The protocol offers an alternative strategy to using antibiotic selection (231-235) or mechanical single-cell isolation (236, 237) for the generation of specifically modified hESC lines. FACS sorting is a high throughput, common, and robust technology that provides advantages over mechanical isolation. Additionally, antibiotic selection entails feeder-free or antibiotic resistant feeder culture, both of which may not be optimal for hESC growth. FACS sorting technology has been used previously to enrich and expand genetically modified hESC polyclonal pools but not for the isolation and expansion of individual cells following hESC genetic modification (238).

During the preparation of this manuscript, Sidhu and Tuch (239) reported the use of FACS sorting to derive homogeneous hESC lines. Our method extends this and other prior work by providing a protocol that directly combines efficient lentivirus transduction of hESCs with FACS-based isolation of single cells and the expansion of homogeneous hESC cell lines, and by providing a detailed analysis of the steps contributing to cloning efficiency (Table 2).

The genetic modification of hESCs has value for a number of reasons. First, lines with constitutive fluorescent reporters such as those reported here can be directly used to track the fate of differentiated and undifferentiated hESCs, such as in vitro co-culture or in vivo transplantation experiments. Second, a similar strategy could be used for the production of hESCs that carry tissue-specific reporters. For example, hESCs would be transduced with lentivirus carrying a constitutive fluorescent reporter in tandem with the
tissue-specific reporter followed by single cell FACS sorting and expansion. In addition, individual hESC lines could be produced for gene silencing and over-expression, with both constitutive and regulated expression (240).

As with any individual cell selection process, multiple derived lines can be used in future experiments to avoid aberrant results due to a single cell line with deleterious transgene integration. Our low plating density of 20 single cells/well of a 12-well dish, and the expansion of derived colonies only from wells containing a single colony was chosen to improve our chance of generating clonal cell lines. However, it is possible that some of our individual H9-eGFP cell lines are oligoclonal, the result of more than a single hESC expanding in the same location on the feeder cells.

The H9-eGFP cell lines that we report stably expressed eGFP over two months of routine culture, as long as was studied, and following freeze/thaw cycles. Moreover, the eGFP transgene was expressed at high levels throughout EB differentiation in vitro and teratoma formation in vivo. Our method allows one to generate stably transduced hESC cell lines that retain their pluripotency, transgene expression, and chromosomal content following isolation and expansion from single cells. However, we note that karyotypically abnormal lines can also be produced. Thus, it is imperative that chromosomal content be assessed following the generation of genetically modified hESC cell lines.

ACKNOWLEDGEMENTS

We thank Amander Clark for helpful discussions, technical assistance, and support, Valentin Stein for generously providing us with the FUGW, Δ8.9 and VSVG lentivirus
vectors, Shuwei Jiang for cell sorting, Roanna Medina for kidney capsule grafting assistance, Margaret Mayes and Tania Zaitsev for tissue sections, and Randy Davis and Anthony Lam for CGH analysis. We also thank members of the Reijo Pera and Leavitt laboratories for their general assistance, and Susan Fisher for initial conversations regarding single-cell hESC plating.
Table 1. Single-Cell Plating Efficiency.

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<th>Pre-Sort</th>
<th>Post-Sort</th>
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<tr>
<td>45 minutes&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>1 hour&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>2 hours&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
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<tr>
<td>Average # of hESC Colonies&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.2</td>
<td>4.3</td>
</tr>
<tr>
<td>Range</td>
<td>2 - 17</td>
<td>1 - 9</td>
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<sup>a</sup> Time from single cell isolation to re-plating following FACS sort

<sup>b</sup> Average number of colonies from 12 separate platings of 100 single cells/well
### Table 2. Overall Efficiency.

<table>
<thead>
<tr>
<th>Sub-lines Analyzed</th>
<th>FACS Efficiency (^a)</th>
<th>Colony Formation Efficiency (^b)</th>
<th>Colony Expansion Efficiency (^c)</th>
<th>Diploid Chromosomal Content (^d)</th>
<th>Overall Efficiency (^e)</th>
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<tr>
<td>11</td>
<td>5 %</td>
<td>33 %</td>
<td>50 %</td>
<td>64%</td>
<td>0.53 %</td>
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\(^a\) 5 colonies were established per 100 single hESCs plated

\(^b\) 20 single cells plated per well yielded one colony in only one third of the wells

\(^c\) Approximately half of established colonies were expanded into sub-lines

\(^d\) 7 out of 11 expanded sub-lines retained a normal chromosomal content

\(^e\) 0.53% of all cells plated generated diploid hESC sub-lines following modification
Figure Legends

Figure 1. Self-inactivating lentivirus transfer vector maps. (A) FUGW and (B) 199. WRE: Woodchuck hepatitis virus post-transciptional regulatory element; cPPT or HIV-1 FLAP: central PolyPurine Tract element

Figure 2. Lentivirus transduction of hESCs. (A) Fluorescent (top) and bright-field (bottom) images of H9 colonies 4 days following FUGW transduction (magnification X100). (B) FACS histogram 10 days following FUGW transduction (x: FL1=GFP; y: cell #) shows that 15% of the cells express eGFP.

Figure 3. Isolation and expansion of genetically modified hESCs. (A) General strategy for FACS sorting and expansion following lentivirus transduction. FACS-sorted cells were plated at 20 cells/well, and only colonies from wells with a single colony were harvested to generate cell lines. (B) Bright-field (top) and fluorescent (bottom) images of FUGW cell lines expanded for 3 weeks following single cell isolation by FACS sorting (magnification X100).

Figure 4. Stable eGFP transgene expression. (A) FACS analysis to monitor eGFP expression in derived H9-eGFP cell lines. For the initial 8-week time course following single-cell sorting and re-plating, cells were harvested using trypsin. Cells were frozen after 8 weeks and thawed at 35 weeks. The 35-week FACS time point used collagenase/dispace treatment, a modification that minimizes the MEF contamination relative to when trypsin is used to harvest cells. (B) FACS analysis of EB cultures
following 4 days of differentiation shows that 95% of the cells express eGFP (x: FL1=GFP; y: cell #). (C) Bright-field (top) and fluorescent (bottom) images of a FUGW cell line following 45 days of EB differentiation (magnification X100).

**Figure 5.** Comparative genomic hybridization. (A) An H9-eGFP hESC cell line with trisomy of chromosome 17. (B) An independent H9-eGFP hESC cell line with a normal chromosomal content. Note that the second X chromosome of the female H9 line is readily seen when compared to normal male reference sample DNA. (C) The parental H9 hESC line prior to genetic modification and FACS sort is diploid.

**Figure 6.** Derived cell lines retain pluripotency. 199 cell lines (A) and a FUGW cell line (B) stained for SSEA-1, TRA-1-60, and TRA-1-81 by immuno-fluorescence (94); DAPI (blue) provides a nuclear stain. I.F. = immunofluorescence. Magnification X200 for A and B. (C) An H9-eGFP FUGW cell line after EB differentiation for 4 days was assayed for somatic cell marker expression by quantitative RT-PCR. Expression was normalized to GAPDH and fold change is indicated relative to undifferentiated hESCs. (D) Teratomas from an H9-eGFP FUGW cell line. Upper panel shows gross image and size; lower panel demonstrates that more than 80% of the teratoma cells were GFP-positive (x: FL1=GFP, y: cell #). (E-G) Teratoma sections stained with hematoxylin:eosin represent tissues derived from three somatic germ lineages: (E) ectoderm (squamous epithelium), (F) mesoderm (smooth muscle), and (G) endoderm (glandular epithelium, magnification X400 for E-G).
Figure 3 - Revised

A.

B. FUGW #2  FUGW #3  FUGW #10
Figure 4 - Revised

A

% GFP Positive

199V #3
199V #9

Weeks Post Sorting

B

FUGW #3 95%

C

Image of the FUGW #3 cell line showing a 95% GFP expression.
Chapter 3

Characterization of a germ cell-specific Deleted in AZoospermia-Like (DAZL) reporter in transgenic mice and embryonic stem cells
Characterization of a Dazl-GFP Germ Cell-Specific Reporter

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Summary: In this study, we characterized the promoter activity of a 1.7 kb sequence in the 5’ flanking region of the mouse Deleted in Azoospermia-Like (Dazl) gene. We found the 1.7 kb sequence sufficient to drive robust germ cell-specific expression of GFP in adult mouse testis and lower levels of expression in adult ovary and in fetal and newborn gonads of both sexes. This expression pattern was confirmed in two independently-derived transgenic mouse lines. In adult testis, Dazl-GFP exhibited a developmentally-regulated, stage-specific expression pattern during spermatogenesis. GFP was highly expressed in spermatocyte stages, with strongest expression in pachytene spermatocytes. Weaker expression was observed in round and elongating spermatids, as well as spermatogonial cells. In the fetal gonad, GFP transcript was detected by e12.5 in both sexes; however, GFP fluorescence was only detected during later embryonic stages. In addition, we produced mouse embryonic stem cell lines harboring the Dazl-GFP reporter and used this reporter to isolate putative germ cell populations derived from mouse embryonic stem cells following embryoid body differentiation and fluorescence activated cell sorting.
INTRODUCTION

The development of an *in vitro* system to robustly isolate germ cells that are differentiated from embryonic stem cell (ESC) lines would greatly facilitate the study and understanding of germ cell development. Such a system would be especially useful for the study of human germ cell development, where germ cell specification and commitment occur during early fetal stages that are inaccessible. Because ESCs can spontaneously differentiate to form a heterogeneous mixture of various tissues, and because specific germ cell surface antigens to allow isolation are lacking, validated germ cell-specific reporters represent a promising strategy for identification and isolation of ESC-derived germ cells. In fact, recent studies have documented the isolation of germ cells from mouse ESCs (5-8, 42, 95, 210), and two of these studies utilized germ cell-specific reporters to identify and isolate sperm. However, functional oocytes have yet to be isolated following mouse ESC differentiation, and the isolation of human germ cells following ESC differentiation has not yet been reported, thus necessitating the development of additional differentiation and identification strategies.

Here, we sought to characterize the germ cell specificity of a mouse *Dazl* promoter sequence driving the expression of GFP, and to then use this reporter to isolate germ cells following ESC differentiation. The *DAZ* (*Deleted in AZoospermia*) gene family of *BOULE, DAZL, and DAZ* encodes RNA binding proteins that are exclusively expressed in germ cells and are required for germ cell differentiation across non-mammalian and mammalian species (241-244). In particular, the autosomal *DAZL* gene is expressed...
throughout germ cell development from embryonic stage primordial germ cells (PGC) to mature gametes in adults (244-248). Mouse Dazl null mutants are sterile in both sexes, exhibiting a near complete lack of mature germ cells by birth and reduced germ cell numbers in mouse embryos (249, 250). Consequently, defining regulatory elements of the mouse Dazl gene represents an opportunity to design a pan-germ cell-specific reporter that would be expressed during essentially all stages of post-specification germ cell development in both sexes. The evolutionary conservation of the DAZ family may also allow the extension of the mouse DAZL reporter to a human system and similarly facilitate the isolation of germ cells following their differentiation from cultures of human ESCs (251).

RESULTS

Production of Dazl-GFP Transgenic Mice

A Dazl-eGFP reporter was constructed using a 5’ 1.7 kb sequence located upstream of the mouse Dazl translational start site that also included the 5’ UTR. The 1.7 kb sequence was chosen based on limited flanking sequence available at the time and based on the size of several promoter constructs required for germ cell-specific expression. The elements required for such expression in spermatocytes or spermatids were usually present within ~2 kb or less from the coding region. The putative promoter fragment was cloned upstream of the coding region for enhanced green fluorescent protein (eGFP), which was followed by the SV40 poly-adenylation sequence. To produce transgenic
mice, the reporter vector was linearized, purified, and then micro-injected into the maternal pro-nucleus of fertilized mouse FVB/N oocytes. The micro-injected oocytes were transferred into the oviducts of pseudopregnant female recipients, which then gave birth to 35 pups; 12 of these 35 pups were identified as founder transgenic mice based on genomic PCR analysis for the Dazl-eGFP transgene. Nine of these founders were subsequently mated with wild-type FVB/N mice, and six of nine (66%) founders transmitted the transgene to offspring to generate the Dazl-eGFP lines. For comparison, the 1.7 kb Dazl sequence was also linked to a coding sequence for humanized renilla hrGFP followed by the SV40 poly-adenylation sequence, and this construct was also used to generate lines of transgenic mice, termed Dazl-hrGFP mice.

**Transgene Expression**

Testes from mice bearing the Dazl-eGFP transgene were analyzed for eGFP expression by fluorescence microscopy at two to three months of age. Green fluorescence above wild-type was detected in three of six (50%) lines; these lines were designated Dazl-eGFP transgenic lines 5, 10, and 26 (Fig. 1A; line 5 and 26 data not shown). The Dazl-eGFP line (or line eGFP) in this report refers to line 10 which contained robust testicular transgene expression. Line 26 originally displayed high levels of transgene expression as well but was later observed to undergo transgene silencing upon colony expansion. Nevertheless, line 26 was characterized in parallel with line 10 and found to have an identical transgene expression pattern both before and after silencing, despite lower levels of expression after silencing. Line 5 exhibited very weak transgene expression and was
not characterized further. *Dazl*-hrGFP transgenic mouse line 7-2 also showed high levels of transgene expression in the testis and was designated transgenic line *Dazl*-hrGFP (or line hrGFP) (Fig. 1A). Transgene expression could not be detected above background in intact ovarian tissues (data not shown). However, weak GFP transgene expression was detected above background in individual oocytes from disrupted adult ovarian tissues (Fig. 1B). Adult gonadal transgene expression was also examined by flow cytometry for GFP fluorescence and was detected above background in both adult testis and ovary. 18.81% of cells exhibited eGFP fluorescence in transgenic line *Dazl*-eGFP adult testis while only 0.34% of cells were fluorescent in transgenic adult ovary (Fig. 1C). Similarly, 18.18% of cells were hrGFP positive in transgenic line *Dazl*-hrGFP testis compared to 0.78% positive in line hrGFP ovary (data not shown).

To confirm these results, RT-PCR was performed to assay GFP transcript levels in the adult gonads of each mouse line. GFP expression was detected in adult testis and ovary from line *Dazl*-eGFP and -hrGFP (Fig. 2A; line eGFP data shown). Expression of GFP transcript was also detected in newborn and fetal (e12.5, e14.5, and e16.5 dpc) gonads from both sexes, resembling the expression pattern of endogenous *Dazl* transcript which has been detected as early as e11.5 in the fetal gonad and may be expressed at even earlier stages of embryonic development (Fig. 2B) (252). A similar expression profile for hrGFP was obtained when the gonads from the *Dazl*-hrGFP transgenic mouse line were analyzed by RT-PCR (data not shown).
Surprisingly, eGFP and hrGFP fluorescence from fetal and newborn transgenic gonads was not readily detectable above background by fluorescence microscopy, despite the presence of GFP transcript during these stages. Faint GFP fluorescence was identified above background in testes from e14.5 and e16.5 dpc; however, GFP fluorescence could not be detected in e12.5 testes or in ovary from any stage (data not shown). Furthermore, fetal gonads from e12.5 and e17.5 were analyzed by flow cytometry, and eGFP fluorescence was only observed above background in later stage testes (0.76%) and ovaries (0.03%) from e17.5 (Fig. 3). Likewise, low levels of hrGFP fluorescence were only identified above background in e17.5 stage fetal testes (0.11%) and ovaries (0.02%) from Dazl-hrGFP transgenic mice (data not shown). Therefore, Dazl-GFP reporter transcriptional activity was initiated at the primordial germ cell stage in the gonads as early as e12.5, but translational activity was not detected until after germ cells committed to a sex-specific program as pro-spermatogonia/oocytes. However, we can not rule out alternative or additional mechanisms responsible for the absence of GFP protein in gonocytes such as transcript instability and targeted degradation.

**Gonad-Specific Expression**

Next, a panel of adult tissues from the transgenic lines was examined in comparison to wild-type mice to determine whether transgene expression was confined to the gonad. By fluorescence microscopy, GFP fluorescence was only identified above background in the testis of transgenic mice (Fig. 4A; line hrGFP data shown). Similar expression patterns were observed in line eGFP (data not shown). Transgene expression was next
assayed by fluorometry in a panel of tissues isolated from the Dazl-hrGFP mice. Again, GFP expression could only be detected in the testis of transgenic mice when compared to tissues from wild-type mice (Fig. 4B). Additionally, RT-PCR analysis confirmed the expression of GFP transcript in the testes and ovaries of line hrGFP transgenic mice (Fig. 4C; line eGFP data not shown). GFP transcript was also present in kidney tissue; however GFP protein was not detected above background in the kidney by fluorometry.

**Germ Cell-Specific and Stage-Specific Expression**

To determine whether transgene expression from the Dazl promoter sequence was restricted to germ cells, adult testes from the transgenic lines were enzymatically disrupted into single cell suspensions and fluorescence activated cell sorted (FACS) into GFP positive and negative populations. Total RNA was isolated from each population and then analyzed by quantitative RT-PCR for the relative expression of germ and somatic cell marker transcripts. As illustrated in Fig. 1C, approximately 18% of testicular cells were GFP positive in transgenic line eGFP when compared to testicular cells from age matched wild-type mice. The germ cell transcripts, *Dazl*, *Vasa*, and *Scp*-3, were expressed at similar levels in both the GFP positive and negative testis cell populations isolated from mouse line eGFP (Fig. 5A). The detection of germ cell-specific transcripts in the GFP negative population suggested that the Dazl-GFP transgene is not expressed in some germ cells of the testis and was indicative of stage-specific GFP expression in germ cells during spermatogenesis. In contrast, *Sox*-9 and *Lhr*, markers for Sertoli and Leydig somatic cells respectively, were greatly enriched in
the GFP negative population illustrating that testicular somatic cells lack robust GFP transgene expression (Fig. 5B). Thus, transgene expression from the Dazl promoter sequence was restricted to the germline with possible germ cell- and stage-specific regulation in the testis.

Transgene expression also appeared to be germ cell-specific in adult ovary. Faint GFP fluorescence above background was only detected in oocytes following the disruption of ovarian tissue (Fig. 1B). In addition, transgenic adult ovaries and e17.5 embryonic testes that were analyzed by flow cytometry in figures 1C and 3 were also FACS sorted into GFP positive and negative populations and examined by RT-PCR for endogenous Dazl transcript expression. Notably, Dazl transcript was only detected in the GFP positive population and was not detected in the GFP negative population suggesting that GFP positive cells from adult ovary and embryonic testis are germ cells and that Dazl reporter activity is restricted to germ cells (Fig. 5C).

Subsequently, testis sections from transgenic lines eGFP and hrGFP were immuno-stained for GFP to confirm the results obtained by RT-PCR. Indeed, GFP expression was restricted to germ cells within the seminiferous tubules of transgenic mice (Fig. 6A, line eGFP data shown). However, germ cell staining was not uniform, as some tubules did not stain positive for GFP, and others exhibited partial staining. Consistent with RT-PCR analysis (Fig. 5A), these immuno-staining results showed the Dazl-GFP reporter to be highly regulated during specific epithelial cycle stages of sperm development. Similar results were obtained with transgenic line hrGFP (data not shown). GFP expression
could not be detected above background in transgenic adult ovary sections by immunostaining (data not shown).

Overall, Dazl-GFP expression in transgenic adult testis was primarily detected from three main stages - pachytene spermatocytes, round spermatids, and elongating spermatids, with strongest expression in mid-pachytene spermatocytes (Fig. 6D,E). Initial expression was found in early pachytene spermatocytes of epithelial cycle stage II, followed by strong expression in mid-pachytene spermatocytes and spermatids of stage VI-VII. Transgene expression decreased in late pachytene spermatocytes and elongating spermatids of stage VIII to IX and completely disappeared by stage XII. Expression was not detected in leptotene or zygotene spermatocytes. Weak staining was observed in some spermatogonial cells, but further analysis is needed to determine the specific subtypes that express the Dazl-GFP transgene, potentially including spermatogonial stem cells.

Sertoli cells lining the basement membrane of tubules did not appear to be stained (Fig. 6E). Additionally, although interstitial Leydig cells appeared to be positive, negative control slides also illustrated Leydig cell staining (Fig. 6B,C). This result, combined with the above RT-PCR analysis showing a 20-fold increase in LHR transcript level in GFP negative testicular cells, demonstrated that the interstitial cell staining was non-specific. Hence, GFP transgene expression, driven by the 1.7kb mouse Dazl promoter, was germ cell-specific in adult testis. However, we cannot eliminate the possibility of low basal
transgene expression in a subset of testicular somatic cells or other non-testicular somatic tissues that were not analyzed above.

**Germ Cell Differentiation from Embryonic Stem Cells**

Following the validation of germ cell specificity in transgenic mouse gonad, mouse ESC lines were genetically modified to contain the *Dazl*-eGFP reporter transgene by electroporation and antibiotic selection. Three independent *Dazl*-eGFP clonal ESC lines were generated, which contained low (line 2), medium (line 17), and high (line 8) levels of transgene expression in undifferentiated ESC cultures (Fig. 7A). *Dazl* reporter expression in undifferentiated ESC cultures was anticipated because *Dazl* transcripts have previously been detected at high levels in ESCs (6), and *Dazl* protein was also found in pre-implantation embryos (252). We reasoned, as confirmed by results below (Fig. 7), that *Dazl* reporter expression should disappear in differentiating somatic cells and only persist in the germ cell lineage following ESC differentiation, if expression recapitulates that observed *in vivo*.

*Dazl*-eGFP ESC lines were differentiated in embryoid body suspension cultures for 1, 4, 9, or 15 days (Fig. 7B). Then, GFP positive and negative populations were isolated by FACS and analyzed by quantitative RT-PCR to determine germ cell or somatic cell identities (Fig. 7C). As expected, the percentage of eGFP positive cells in embryoid bodies declined dramatically in all three transgenic reporter lines during a 15 day time-course of differentiation (Table 1).
Analysis of somatic cell markers *Ncam*, *Afp*, and *Kdr* (markers of ectoderm, endoderm, and mesoderm, respectively) revealed a general decrease of somatic cell marker transcript levels in the eGFP positive population compared to the negative population over the time-course (Fig. 7C). In contrast, germ cell marker analysis of *Dazl*, *Vasa*, and *Scp-3* demonstrated a gradual increase in the fold change of all germ cell marker transcript levels in the eGFP positive population over the 15 day time-course of differentiation (Fig. 7C). Thus, *Dazl*-eGFP reporter expression becomes restricted to the germline during mouse ESC differentiation and can be used to identify and isolate the putative ESC-derived GFP positive germ cell populations.

**DISCUSSION**

We report the characterization of two independently-derived transgenic mouse lines containing an eGFP or hrGFP reporter downstream of a 5’ sequence flanking the mouse *Dazl* gene. The 1.7kb 5’ sequence is sufficient to direct germ cell-specific expression of the GFP transgene in transgenic mice and following ESC differentiation. High levels of transgene expression are detectable in germ cells from adult testis, and low levels of transgene are expressed in oocytes from adult ovary and in fetal and newborn gonads of both sexes. Specifically, the *Dazl* reporter is active in adult testis from pachytene spermatocyte to elongating spermatid stages, with strongest reporter expression observed in mid-pachytene spermatocytes, and in some spermatogonial cells. While the expression of GFP transcript can be detected in primordial germ cell stage e12.5 gonads, the
expression of GFP protein (via fluorescence) could only be found at low levels in fetal gonad from e14.5 to e17.5. The low levels of fluorescence observed at these later embryonic pro-spermatogonia/oocyte stages mirror the low levels of GFP protein and fluorescence expressed in adult spermatogonia and oocytes, respectively. This is in stark contrast to the robust transgene expression of GFP protein in pachytene spermatocytes from adult testis and is surprising given that e17.5 oocytes are also predominantly in a pachytene stage of meiotic prophase but do not robustly express GFP protein. As a result, our study highlights the regulated expression of Dazl during meiosis in the testis; other studies have suggested that this expression may be required for progression through meiotic prophase (253, 254).

However, Dazl is clearly required for pre-meiotic embryonic germ cell development in both males and females (249, 250). Notably, previous reports have found strong endogenous pre-meiotic Dazl expression (245-247, 255, 256). Thus, the Dazl-GFP transgene protein expression observed here does not parallel endogenous protein expression patterns as previously reported and demonstrated by mouse gene disruption.

Robust spermatogonia, oocyte, and primordial germ cell protein expression may require additional regulatory sequence from the mouse Dazl locus. A sex-specific expression pattern was also observed in Scp-1 reporter transgenic mice, where 1.8 kb of 5’ flanking sequence was sufficient to drive transgene expression in testis but not in ovary (257). Additionally, sex-specific transcriptional regulatory elements were identified in Gdf-9 reporter transgenic mice including a 5’ flanking E-box sequence required for ovary
expression and a 3’ flanking sequence that can repress testis expression (113). Translational regulatory elements may also be required for endogenous expression patterning. The 3’ UTR is essential for post-transcriptional regulation of many transcripts and developmental processes in mammalian and non-mammalian species (258). In mice, Dazl protein can bind to sequences in the 3’ UTR and can activate the translation of Vasa and SCP-3 transcript in pre-meiotic germ cells (259, 260). Dazl transcript also contains an identical sequence in its 3’ UTR and may undergo self-regulation to activate pre-meiotic translation. Similarly in mice, the Nanos-2 3’ UTR functions as a translational repressor element in ovary but is an enhancer element in testis (25). Likewise in drosophila, c.elegans, and zebrafish, Nanos and Dazl translation is regulated by the 3’ UTR (261-263). Therefore, the accurate recapitulation of endogenous expression patterns of the Dazl reporter transgene, and other germ cell-specific reporters, may require regulatory elements from the 3’ UTR and from additional 5’ and/or 3’ flanking sequence.

Following the validation of germ cell specificity in transgenic mice, we used the Dazl-GFP reporter to identify and isolate putative germ cells that were differentiated from mouse ESCs. Recent studies have characterized germ cell reporter transgenic mice containing Oct-4 (264), Blimp-1 (29), Fragilis (265), Stella (211), Tnap (266), Vasa (267), Stra-8 (7), SCP-1 (268), Alf (269), Gdf-9 (270), Zp-3 (270), Msx-2 (270), Acrosin (7), and Protamine (271) reporters, and mouse ESC-derived germ cell populations utilizing Oct-4 (8), Stella (211), Vasa (5), Stra-8 and Protamine-1 (7) reporters. However, these reporters have limitations in that they may be expressed in somatic and
germ cells, may be sex-specific, and/or may be limited in expression to brief stages of germ cell development. Although we sought to use the Dazl-GFP transgene to report various stages of ESC-derived male and, especially, female germ cell development, GFP fluorescence was difficult to detect in oocytes and absent or below detection in primordial germ cells. Thus, the optimal use of the Dazl-GFP reporter presented here may be in the study of male germ cell development in vitro or in vivo in mice, and possibly in human stem cell systems. Additionally, the transgene may be useful for identifying and characterizing cis- and trans-elements that confer sex-specific and germ cell stage-specific regulation on the Dazl gene.

**METHODS**

**Construction of pDazl-GFP**

A 1.707 kb genomic fragment located at positions -15 to -1722 relative to the start codon for the Dazl open reading frame, and encompassing 176 bp of 5’ UTR in the first exon, was amplified from C57BL/6 mouse genomic DNA by PCR (pfu-turbo, Stratagene, Inc.). The PCR primers used to amplify the genomic fragment (5’-ttcgggtggtaaaacctcg-3’ and 5’-tcttcctttcgagaattccag-3’) were based on sequence information originally obtained from the Celera mouse genome data base. Limited to 4 kb of available 5’ sequence flanking Dazl, multiple primer pairs were originally designed to amplify 1.5 to 3 kb of the promoter region. The 1.7 kb PCR product was the only amplicon successfully amplified and was purified from a 1% agarose gel and then ligated into pCR Blunt (Invitrogen, Inc). The 1.7 kb fragment was excised from pCR blunt using Nsi I and Not I, and then
ligated into the Nsi I and Not I sites of phrGFP (Stratagene, Inc.) to generate pDazl-hrGFP. Then, the 1.7kb fragment was excised from pDazl-hrGFP using Nsi I and Pst I, and ligated into the Pst I site of pEGFP-1 (Clontech) to generate pDazl-eGFP.

**Production of Dazl-GFP Transgenic Mice**

The plasmid containing the Dazl-hrGFP transgene was digested with Nsi I and Mlu I to yield a 2.82 kb fragment free of most vector sequences, and which contained the Dazl 5’-flanking DNA driving the hrGFP expression cassette. The gel-purified transgene fragment was injected into the pronuclei of fertilized one-cell B6SJL F2/J mouse eggs, which after culturing to the two-cell stage, were transferred to the oviduct of day 1 pseudopregnant B6CBA F1/J recipients. Transgenic mice were identified by dot blot analysis of DNA isolated from a tail biopsy using a 32-P-labeled hrGFP probe. Among the 47 animals born from injected eggs, 14 were identified as transgenic. Transgenic lines were analyzed for the expression of hrGFP in testicular germ cells by viewing squash-preps of seminiferous tubules under a fluorescent microscope. Expression of hrGFP above wild-type levels was detected in ~57% of the lines analyzed. Transgenic line 7-2 expressed the highest relative levels of hrGFP in testicular germ cells, and therefore, was further maintained as a homozygous line that produced normal numbers of offspring/litter (Mean litter size = 6.5, SD±1.7, n=15 litters). Dazl-eGFP transgenic mice were produced by similar methods (see results). Transgenic Dazl-eGFP founders and offspring were identified by PCR on tail-tip genomic DNA using Platinum Taq DNA Polymerase (Invitrogen) with primers: (F- GCCTATTGGCTGTAGCACGTCACG, R- CTTCAGCTCGATGCGGTTACGACG).
RT-PCR

Gonads and somatic tissues were dissected and digested using Collagenase IV/Dispase (1 mg/mL, Invitrogen) and DNasel (0.1%, Roche) for 30 minutes at 37C for adult gonads; or using 0.25% Trypsin (Invitrogen) and DNasel (0.1%, Roche) for 10-15 minutes at 37C for newborn/fetal gonads and embryoid bodies. Total RNA was prepared using the RNeasy Mini Kit (Qiagen), and cDNA prepared with the SuperScript III Kit (Invitrogen). RT-PCR and quantitative RT-PCR were performed with iQ SYBR Green Supermix (BioRad) on a MyiQ system (BioRad) or with Power SYBR Green PCR Master Mix (Applied Biosystems) on a 7300 Real Time PCR System (Applied Biosystems). For relative expression analysis, sample Ct values were normalized to UbiquitinB by subtracting the UbiquitinB Ct and then by calculating 2 raised to the negative power of the difference in Ct values. For fold change analysis, UbiquitinB Ct values were again subtracted from sample Ct values, and the difference in the GFP- samples was then subtracted from the difference in the GFP+ samples. The fold change of GFP+ over GFP– was calculated as 2 raised to the negative power of this difference.

Primers:
F/R:eGFP, AGCTGACCCTGAAGTTCATCTG/TATAGACGTGTTGGCTGTTGTAGT; UbiquitinB, GCGGTTTGTGCTTTTCATCAC/GGCAAAGATCAAGCTCTGCT; Dazl, AGGCTCAGTAAAAGAAGTGAAGATAA/AGTACATAAATTTTGTTTCCTGATTG; Vasa, CTAGGAAGACCAAATAGTGAATCTGAC/TCCAGAACCTGTTACTACTT; SCP3, AGAAATGTATACCAAAGCTTCTTTCA/TTAGATAGTTTTCTTGTTCCTCA; Sox9, CACGGAACAGACTACACTACATCTCTCT/CTTCTCGCTTCAGAT; CAACTTT; Lhr, TTTCCAAAATATGTGAAAGGCAC/CAACACCCCTAAGGAGCAT.
AG;Ncam,GAGGTGACCCCAGATTCA/GAGGTGACCCCAGATTCAGA/TCTGGCTCATCAAACTGCAC;Afp,GAA
GCAAGCCCTGTGAACTC/AGCTTGGCAGAGGCGATGAG;Kdr,GCCGGTGGTGAC
AGATATCTT/GTGAACAGAGGCGATGA.

Flow Cytometry and FACS Sorting

Mouse tissue and embryoid bodies were digested to single cell suspensions as above. Cells were washed and re-suspended in PBS (Invitrogen) with 1% BSA (Sigma) or in differentiation media, filtered through 40µm nylon cell strainer (BD Biosciences), and analyzed/sorted for GFP on MoFlo (DakoCytomation) or Aria (BD Biosciences) machines. For embryonic gonad analysis, embryonic heads were first genotyped to identify transgenic embryos using the ZR Genomic DNA II kit (Zymo Research) prior to processing and analysis. Embryonic sex was determined by gonad morphologies.

Fluorometric Analysis

Relative levels of fluorescence were measured in lysates prepared from various mouse tissues using a FL600 fluorescence microtiter plate reader (BioTek, Inc.) equipped with filter wheel sets for maximal excitation at 485 nm and maximal emission at 516 nm, as previously described (272).

Immuno-histochemistry

Adult testes were fixed in 4% Para-formaldehyde (Sigma) overnight at 4C, paraffin embedded, and sectioned (5µm). Briefly, slides were deparaffinized and rehydrated, blocked in 10% Hydrogen peroxide (Sigma) for 20 minutes, permeabilized with 0.1%
Triton-X100 (Sigma) for 5 minutes, blocked in 4% Normal horse serum (Sigma), incubated overnight at 4C with 1:200 Anti-GFP JL-8 antibody (Clontech), incubated for 30 minutes at room temperature with 1:200 Biotinylated horse anti-mouse IgG secondary antibody (Vector Labs), 30 minutes at room temperature with VECTASTAIN ABC Standard (Vector Labs), 3 minutes DAB (Vector Labs), counterstained with Mayer’s hematoxylin (Sigma) for 30 seconds, and coverslips mounted with Prolong Gold (Invitrogen).

**Mouse Embryonic Stem Cells**

Mouse embryonic stem cell lines were maintained in Knockout DMEM (Invitrogen) ES media with 10% FBS (Hyclone), 1% NEAA and 1% L-glutamine (both Invitrogen), 0.001% BME (Sigma), 1000 U/mL LIF (Chemicon), and passaged with 0.05% Trypsin (Invitrogen) on MEFs or 0.1% Gelatin (Sigma). To generate transgenic ESC lines, Dazl-GFP reporter plasmids were linearized and purified by alcohol precipitation. 30µg of DNA were electroporated into 20 million ESCs (F1-2.1.10B mouse ESC line from 129XCastaneus – gift from Barbara Panning) at 500µF, 250V, 500C. Transfected cells were replated onto gelatin coated plates and selected with 250µg/mL Geneticin (Invitrogen) for one week following transfection for 48 hours. After selection, colonies were picked and expanded separately on MEFs. For embryoid body differentiation, mouse ESCs were passaged into differentiation media (same as knockout ES media but without LIF) and transferred to Costar ultra low attachment plates (Corning) for suspension culture.
ACKNOWLEDGEMENTS

This research was supported in part by the National Institutes of Health (RO1 HD047721; U54HD055764 as part of the Specialized Cooperative Centers Program in Reproduction and Infertility Research) and the California TRDRP (California TRDRP and the National Institutes of Health 14RT-0159;15DT-0187). We thank Dr. Joanna Gonsalves for eGFP cloning assistance, and Dr. Fredrick Moore, Dr. Nigel Killeen, and Jason Dietrich from the UCSF Transgenic/Targeted Mutagenesis Core for generating the Dazl-eGFP transgenic mice. Additionally, we thank Margaret Mayes and Amarjeet Grewall for histology services, Shuwei Jiang and Patty Lovelace for FACS services, and members of the Reijo Pera laboratory for helpful conversations during this study.
Table 1
Percentage *Dazl*-eGFP expression by FACS following differentiation.

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<sup>a</sup> – *Dazl*-eGFP mouse embryonic stem cell clones

<sup>b</sup> – Days of embryoid body differentiation
FIGURE LEGENDS

Fig. 1. *Dazl*-GFP Transgene Expression in Adult Testis and Ovary.
A. Brightfield and fluorescent microscopy pictures of adult testes from wild-type (WT), *Dazl*-eGFP (eGFP), and *Dazl*-hrGFP (hrGFP) transgenic mouse lines (100X).  B. Brightfield and fluorescent microscopy pictures of oocytes from wild-type (WT) and transgenic (line eGFP and hrGFP) adult mouse ovary (200X).  C. Flow cytometry analysis of adult gonads from wild-type (WT) and transgenic (line eGFP) mice showing the percentage of GFP positive cells (x axis: GFP, y axis: side scatter).

Fig. 2. GFP Transcript Expression in Adult, Newborn, and Fetal Gonad.
A. RT-PCR for GFP and *UbB* control transcripts in wild-type (WT) and transgenic line eGFP testis and ovary from adult mice.  B. RT-PCR for GFP and *UbB* control transcripts in wild-type (WT) and transgenic line eGFP testis (M) and ovary (F) from adult (Ad), newborn (Nb), and e12.5, e14.5, or e16.5 embryonic stage mice. Reverse transcriptase (RT) negative controls and *Dazl* positive controls are included.

Fig. 3. GFP Fluorescence in Fetal Gonad.
Flow cytometry analysis of e12.5 and e17.5 testes (M) and ovaries (F) from wild-type (WT) and transgenic (line eGFP) embryos (x axis: GFP, y axis: side scatter).

Fig. 4. Gonad-Specific Expression.
A. Brightfield and fluorescent microscopy pictures of adult testis, kidney, liver, and spleen from wild-type and transgenic line hrGFP mice (100X).  B. Fluorometric
absorbance analysis for hrGFP in a panel of adult tissues from wild-type (striped bars) and transgenic mice (solid bars) illustrating testis-specific transgene expression (n=3). C. RT-PCR analysis for hrGFP and UbB control transcripts in adult tissues from wild-type (WT) and line hrGFP mice with reverse transcriptase (RT) negative controls. (Lv-Liver, He-Heart, Lu-Lung, Si-Small Intestines, Li-Large Intestines, Pa-Pancreas, Sp-Spleen, Ki-Kidney, Te-Testis, Ov-Ovary).

**Fig. 5. Germ Cell-Specific Expression.**

A. Adult testes were analyzed for Dazl, Vasa, and Scp-3 germ cell transcripts by quantitative RT-PCR analysis of FACS isolated GFP negative (grey bars) and GFP positive (green bars) populations from transgenic line eGFP, normalized to UbB (n=4).

B. FACS isolated GFP negative and positive populations from transgenic testes were also analyzed for Sox-9 and Lhr somatic cell transcripts by quantitative RT-PCR analysis, normalized to UbB (n=4).

C. FACS sorted populations from transgenic line eGFP adult ovary and e17.5 testis analyzed by RT-PCR for Dazl and UbB transcripts.

**Fig. 6. Germ Cell-Specific and Meiotic Stage-Specific Expression.**

A. Immuno-histochemistry (IHC) staining of an adult testis section from transgenic line eGFP with GFP antibody (100X).

B. Secondary antibody only control staining of a testis section from line eGFP (100X).

C. An adult testis section from wild-type mice stained with GFP antibody (200X).

D. An overview of Dazl-eGFP expression in line eGFP testis by IHC illustrating tubules in different stages of the epithelial cycle (200X).
E. Strongest GFP expression was observed in mid-pachytene spermatocytes, and was also detected at lower levels in round and elongating spermatids, but was not detectable in Sertoli cells and spermatocyte stages prior to pachytene (630X).

Fig. 7. *Dazl*-eGFP Mouse Embryonic Stem Cell Differentiation.

A. Brightfield and fluorescent microscopy pictures of transgenic clonal ESC lines 2, 8, and 17 (100X).  
B. *Dazl*-eGFP ESCs were differentiated for up to 15 days as embryoid bodies and subsequently FACS sorted to isolate eGFP positive and negative populations (100X).  
C. Germ cell (*Dazl, Vasa, Scp-3*) and somatic cell (*Ncam, Afp, Kdr*) marker transcript levels in the eGFP positive population from transgenic ESC line 8 embryoid bodies were analyzed by quantitative RT-PCR and compared to transcript levels in the eGFP negative population (x axis: length of differentiation in days, y axis: fold change of GFP+/GFP- normalized to *UbB*, n=2).
Fig. 1.
Fig. 2.

a

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110
Fig. 3.

WT

Dazl-eGFP

e12.5 M  e12.5 F  e17.5 M  e17.5 F

0%  0%  0%  0%

0.01%  0%  0.76%  0.03%
Fig. 4. (a) WT and Dazl-hrGFP tissues staining. (b) Absorbance 485/590 bar graph showing differences between WT and Dazl-hrGFP. (c) Gel images for UbB and hrGFP expression in various tissues.
Fig. 5.

a

![Histogram of gene expression levels for DAZL, VASA, and SCP3 with GFP+ and GFP- groups.]

b

![Bar graphs showing relative expression levels for SOX9 and LHR with GFP+ and GFP- groups.]

c

![Gel electrophoresis images for Dazl and UbB in Adult Ovary and E17.5 Testis with GFP+ and GFP- groups.]
Fig. 7.

(a) 

(b) 

mESCs 

mEBs 

(c) 

Fold Change (GFP+GFP−) 

NCAM  AFF  KDR  DAZL  VASA  SCP-3 

D1  D4  D9  D15
Chapter 4

Development of an ovarian niche that promotes endogenous oocyte maturation in ovarian follicles
Intact ovarian cords protect germ cells from the mesonephros and promote oocyte development

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ABSTRACT

The development of germ cells in cysts, or clusters, is conserved from flies to humans. Although germline cysts and testicular cords are required for spermatogenesis and male fertility, the requirement of cyst and ovarian cord formation for mammalian oocyte (egg) development and female fertility has not been determined. Here, we use fetal mouse gonad re-aggregation and transplantation to examine the role of intact germ and somatic cell cord-like structures in oocyte development. Germ cells from disrupted female gonad prior to embryonic day e13.5 did not survive following transplantation, which was associated with an absence of ovarian cord formation. Furthermore, transplanted ovaries from e13.5 to e16.5 developed with a reduced number of oocytes when re-aggregated with the mesonephros. However, exclusion of the mesonephros before re-aggregation and transplantation rescued oocyte survival and development in ovarian follicles. Thus, we demonstrate that intact ovarian cords promote oocyte development through protection from the mesonephros.

Keywords: ovary, ovarian cord, cyst, oocyte, follicle, mesonephros, mouse, germ, transplant, development
Introduction

Ovarian follicles, consisting of an oocyte (egg) and somatic granulosa cells, are essential for oocyte survival and maturation; however, the role of pre-follicle fetal ovarian structures in mammalian oocyte development has not been well defined (100, 273). Prior to follicle formation, germ cell precursors of several species have been observed to develop in cysts (274, 275). Germline cyst formation occurs by incomplete cytokinesis during mitosis, resulting in the connection of daughter cells by intercellular bridges. In *Drosophila*, cyst formation and intercellular bridge-mediated transport of organelles and RNA determine oocyte fate and fertility (276-278).

Mouse primordial germ cells (PGCs) also develop in cyst-like clusters following colonization of the genital ridge and subsequent cell division. The maximum number of germ cell clusters is detected on embryonic day e13.5 just prior to commitment, or maturation, of PGCs to an oocyte developmental program and successive entry into meiosis (102). Upon commitment, oocyte clusters become organized into poorly defined ovarian, or ovigerous, cord-like structures containing oocytes and pre-granulosa somatic cells (273). Shortly after birth, ovarian cords and cysts break down into follicles characterized by oocyte apoptosis, the organization of granulosa cells around surviving oocytes, and basement membrane remodeling (99, 279, 280). Follicle formation therefore requires intricate synchronization of oocyte precursor germ cells and pre-granulosa somatic cells in pre-follicle fetal ovarian structures (142, 143).
Although ovarian cyst and cord break down has been implicated in follicle formation (99), it is not yet known whether cyst and/or cord formation has an important role in mammalian oocyte development before follicle formation. Previously, intercellular bridges were found to be dispensable for female fertility in mammals (281); however, germ cell cyst formation was not obstructed in mice lacking bridges, and, therefore, the requirement of cyst and cord formation for oocyte development could not be determined. Hence, we utilize a fetal ovary re-aggregation and transplantation system to directly perturb ovarian cysts and cords, and we demonstrate the significance of intact ovarian cord formation and maturation in promoting oocyte survival and development.

Materials and methods

Gonad re-aggregation and kidney capsule transplantation

The transplantation system was adapted from a previous report of successful oocyte maturation following newborn ovary re-aggregation and transplantation under the kidney capsule (282). Fetal to newborn stage female gonads with or without the mesonephros were dissected from wild type CD-1 mice (Charles River) and dissociated to single cell suspensions by a 10 minute 0.25% trypsinization followed by pipetting 10-20 times and re-suspension in standard media (DMEM, 10% FBS, 1% L-glutamine; Invitrogen). For e11.5 to e13.5 stages, genomic DNA was isolated from somatic tissue of each embryo and genotyped for Sry to confirm the gender (Zymo) (283). The gonadal cell suspension from 4 embryos or pups was then mixed with 0.2mg/mL of phytohemagglutinin (Sigma). Cell suspensions were pelleted at 10,000 g for 1 minute, and pellets were incubated overnight in standard media on CM cell culture inserts (Millipore) at 37 °C. Pellets, or
grafts, were transplanted under the kidney capsule of bi-laterally ovariectomized CB.17 SCID recipient mice (Charles River) according to the protocol (#16146) approved by the Stanford University Administrative Panel on Laboratory Animal Care and as described: (http://mammary.nih.gov/tools/mousework/Cunha001/Pages/Written_Method.html).

**Meiotic cell spread and immunofluorescence**

Following transplantation, tissues were dissociated as above and re-suspended in 20μL of hypo-extraction buffer pH-8.2 (30mM Tris pH 8.2, 50mM Sucrose, 17mM Citric Acid, 5mM EDTA, 0.5mM DTT, and 1% protease inhibitor cocktail (all Sigma)) for 30 minutes at room temperature. 60μL of 100mM Sucrose was then added, and the cell suspension was spread onto slides pre-coated with 1% Paraformaldehyde (USB Corporation) and 0.15% Triton-X100 (Sigma) in PBS pH-9.2, and dried overnight at room temperature. Slides were blocked in 4% chicken serum (Abcam) and incubated overnight at 4°C with primary antibody in TBST (tris buffered saline (TBS), 1% BSA, and 0.1% Tween-20 (all Sigma)) and 1% serum. Primary antibodies included rabbit anti-SCP3 (1:1000, Abcam, ab15092) and rabbit anti-SCP1 (1:500, Abcam, ab15090). Slides were subsequently incubated with Alexa Fluor chicken anti-rabbit (1:1000, Invitrogen) secondary antibodies for 30 minutes, and cover slips were mounted with Prolong Gold Antifade with DAPI (Invitrogen).

**Immunofluorescence and TUNEL**

Grafts were fixed overnight in 4% Paraformaldehyde (USB Corporation), embedded in paraffin, and sectioned in 5 to 8 μm intervals. In brief, sections on slides were de-
paraffinized, re-hydrated, antigens unmasked by incubating in Target Retrieval Solution (Dako) at 95°C for 30 minutes, permeabilized in 0.1% Triton-X100 (Sigma) for 5 minutes, blocked with 10% chicken serum in TBST overnight, and incubated with primary antibody in TBST with 1% serum for 1 hour at room temperature. Primary antibodies included anti-VASA rabbit polyclonal (1:500, Abcam, ab13840), anti-TRA98 rat monoclonal (1:500, B-Bridge, 73-003), anti-FOXL2 goat polyclonal (1:100, Abcam, ab5096), active anti-Caspase2 (ab2251) or anti-Caspase3 (ab13847) rabbit polyclonal (both 1:100, Abcam), anti-CD31 PECAM rabbit polyclonal (1:20, Abcam, ab28364), anti-Laminin rabbit polyclonal (1:200, Abcam, ab11575), anti-SOX9 rabbit polyclonal (1:100, Abcam, ab3697), anti-WNT4 goat polyclonal (1:100, Abcam, ab15699), and anti-GFP rabbit monoclonal E385 (1:1000, Abcam, ab32146). After washing in TBST, slides were incubated with secondary antibody for 30 minutes at room temperature. Secondary antibodies included Alexa Fluor chicken anti-rabbit, rat, and goat (1:1000, Invitrogen), and cover slips were mounted with Prolong Gold Antifade with DAPI (Invitrogen). The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay was performed prior to co-immunofluorescence according to the manufacturer’s instructions (Roche Diagnostics).

**Fluorescence activated cell sorting (FACS)**

*Oct4-GFP* gonads were dissociated to single cells with 0.25% Trypsin (Invitrogen) for 5-10 minutes at 37 °C and re-suspended in standard media. Cells were strained through a 40μm filter (BD Biosciences) and then analyzed on a BD-FACSaria cell sorting system (BD Biosciences). Post sorting, 10 to 20 thousand GFP+ germ cells were re-plated per
well on a 24-well PET 0.4μm cell culture insert (BD Falcon) in standard media for three days. The GFP+ germ cells were then trypsinsized and co-aggregated with dissociated wild type e17.5 or P2 ovarian tissue and transplanted as described above.

**Statistical analysis**

Data are represented as mean +/- standard deviation (s.d.). Statistical significance was determined by unpaired one-tailed Student’s t-Test of unequal variance. Following transplantation, grafts from at least three separate transplants per fetal/newborn stage were sectioned and sampled by counting oocytes with nuclei in follicles on every tenth section by H&E stain. Follicle number per graft was normalized per section or normalized per milligram (mg) of graft tissue. For meiotic entry and progression, 100 cells from each of three separate transplants per sample were analyzed for meiotic chromosome synapsis. For oocyte survival, the numbers of TRA98+ oocytes were counted in three separate samples per time point. For apoptotic markers, at least 100 TRA98+ oocytes were assessed for co-expression of active Caspase2/3 or TUNEL.

**Results**

**Intact ovary maturation promotes oocyte development**

To disrupt intact gonad structure, fetal to newborn stage mouse female gonads with the mesonephros were dissociated to single cells, re-aggregated, and transplanted under the kidney capsule of bilaterally ovariectomized immuno-deficient mouse recipients. Using this system, we constructed a timeline of gonadal stage competence to support oocyte
development. Female gonads from e11.5 to post-natal P2 pups were then analyzed for the presence of oocyte-containing follicles by hematoxylin and eosin staining following re-aggregation and transplantation for three weeks. Follicles containing oocytes with visible nuclei were counted on every 10th section, and the average follicle number per graft (n=3 grafts per gonadal stage) was normalized either per section (Fig. 1) or per weight (mg) of graft tissue (Fig. S1). No significant difference was found between normalization methods.

We observed that the number of oocytes in follicles detected following re-aggregation and transplantation increased with the age of embryos used as ovary donors (Fig. 1A). Oocytes that were not enclosed in follicles were never detected by VASA immunostaining analysis of any ovarian stage following three weeks of transplantation (data not shown). In contrast to significant folliculogenesis following transplantation of intact e12.5 or e14.5 female gonad controls, ovarian follicles were not detected from re-aggregated e11.5 or e12.5 female gonads (Fig. 1B). Rare oocytes in follicles were observed from re-aggregated e13.5 ovaries, and follicle number increased from e14.5 to P2, with a significant elevation from e15.5 to e16.5 (Fig. 1B). The absence of follicles observed before e13.5 was therefore not solely the result of the mechanical gonadal re-aggregation method, as oocyte development occurred following re-aggregation and transplantation of later stage ovaries that were treated identically. The results of the re-aggregation time course indicated that pre-e13.5 stage gonadal cells were not yet competent to support oocyte development following disruption. Thus, we concluded that
intact ovarian structures from post-e13.5 stages were important for oocyte survival and maturation.

**Intact ovaries are not required for oocyte meiotic entry or progression**

The time at which female germ cells became competent to develop as oocytes after re-aggregation corresponded to the time of female sex determination and PGC commitment to an oocyte developmental program (52). Soon after their commitment, oocytes begin to enter meiotic prophase I from e13.5 to e16.5 (90, 91), representing an early hallmark of fetal oocyte development and also paralleling the increase in oocyte development observed in our re-aggregation timeline.

Consequently, we examined pre-e13.5 stage germ cells for meiotic entry and progression as a measure of oocyte commitment, and we observed that re-aggregated germ cells were still competent to commit to an oocyte developmental program and enter meiosis following transplantation (Fig. 2A). Re-aggregated or intact e12.5 female genital ridges transplanted for three days and assayed by immunofluorescence for expression and localization of synaptonemal complex protein markers of chromosome synapsis displayed an almost equivalent percentage of meiotic cells. Both samples contained oocytes with markers of zygotene to diplotene stages of meiotic prophase such as chromosomal alignment of SCP3 (Synaptonemal Complex Protein 3; 59% in intact and 51% in re-aggregated), and markers of pachytene to diplotene stages such as chromosomal localization of SCP1 (45% in intact and 33% in re-aggregated) (Fig. 2B).
Thus, the complete absence of oocytes and follicles from re-aggregated e12.5 gonads was not due to lack of entry into meiosis.

To determine the extent of meiotic progression, we examined e12.5 gonads after five days of transplantation and detected only a small percentage of cells that stained positive for elongated SCP3 and SCP1 for either re-aggregated or intact control genital ridges (6% in intact and 14% in re-aggregated for SCP3; 4% in intact and 2% in re-aggregated for SCP1) (data not shown). These results indicated that germ cells from both intact and re-aggregated e12.5 gonads entered and progressed through meiotic prophase I to dictyate arrest as oocytes and subsequently down-regulated the expression of SCP proteins. Down-regulation of SCP1 upon successful meiotic progression of oocytes has been suggested to facilitate follicle formation (94). Therefore, PGC commitment to oogenesis and oocyte progression through meiotic prophase I did not appear to be affected by re-aggregation with the mesonephros, although we cannot eliminate the possibility of defects in DNA mismatch or double strand break repair. In addition, neither complete sex reversal to spermatogenesis nor ectopic PGC reprogramming and teratoma formation following gonad disruption were ever observed (data not shown).

**Intact ovarian cord formation and maturation promote oocyte survival before follicle formation**

Although oocytes entered and progressed through early stages of meiosis in the three to five day transplants from re-aggregated e12.5 female genital ridge, the oocytes did not survive at three weeks of transplantation (Fig 1). We hypothesized that re-aggregation
directly impaired oocyte survival before ovarian follicle formation, resulted in oocyte loss after follicle formation, or indirectly led to oocyte degeneration subsequent to an obstruction in the formation of follicles. Endogenous ovarian follicle formation occurs just after birth in mice. Follicles were not detected from either intact or re-aggregated e12.5 gonads after only five to seven days of transplantation, comparable to e17.5 to e19.5 pre-natal stages; however, by day twelve of transplantation, many primordial and primary follicles were identified from the intact e12.5 female gonad (Fig. 2C). In contrast, ovarian follicles were not observed from the re-aggregated e12.5 gonad by day twelve (Fig. 2C).

To further investigate oocyte loss, we characterized the decline in oocyte number by TRA98 oocyte marker immunostaining of intact and re-aggregated e12.5 female gonads after transplantation for five, seven, and twelve days. While intact transplants exhibited gradual oocyte loss during the time course, analogous to endogenous oocyte death during follicle formation (99), re-aggregation with the mesonephros, in contrast, resulted in accelerated oocyte loss and significant reduction in oocyte number by day seven of transplantation, with no oocytes surviving to day twelve (Fig. 3A). Thus, analysis of oocyte number before (day 7) and after (day 12) the time of follicle formation revealed that oocytes in transplants from re-aggregated gonads were being lost before follicle formation.

To determine the mechanism of oocyte loss after re-aggregation, we examined markers of apoptosis following transplantation of intact or re-aggregated e12.5 female gonads and
detected evidence of Caspase-independent apoptosis. Although activated Caspase2 and 3 apoptotic pathways have been previously implicated in oocyte death (284-290), TRA98+ oocytes expressing active Caspase2 or Caspase3 were not detected following re-aggregation (Figs. 3B, S2). However, 2% of oocytes expressed active Caspase2 in intact gonad (Fig. S2), and active Caspase3 was observed in some FOXL2+ granulosa cells from both samples (Fig. 3B). In contrast, we detected no TRA98/TUNEL double positive oocytes in the intact gonads after five days of transplantation, while 1.7% of the TRA98+ oocytes were TUNEL+ in the re-aggregated gonads (Fig. S2). On day 7, we detected 2% and 2.7% TUNEL+ oocytes in intact and re-aggregated gonads, respectively. Therefore, TUNEL+, Caspase-independent apoptosis may play a role in re-aggregation induced oocyte death, but other mechanisms of cell death or loss, such as necrosis and/or autophagy, are likely to be involved in oocyte reduction/depletion as well. Additionally, oocyte differentiation cannot be excluded.

In addition to disparity between oocyte numbers during the transplantation time course, we observed a striking difference in ovarian cord formation that correlated with oocyte loss in re-aggregated gonads (Fig. S3). In transplants of intact gonads, oocytes and granulosa cells co-localized in large clusters or cords (Fig. 3B). However, ovarian cord formation was significantly disrupted in transplants from re-aggregated e12.5 female gonads. Clusters of TRA98+ oocytes and FOXL2+ granulosa cells were not observed at any stage examined after transplantation. Instead, individual TRA98+ oocytes were dispersed throughout the re-aggregated gonad after five to seven days of transplantation and were not detected by day twelve (Fig. 3B). FOXL2+ granulosa cells were also
detected throughout the re-aggregated samples and not confined to discrete cord-like structures containing oocytes. Granulosa cells were still present on day twelve of transplantation despite the absence of TRA98+ oocytes in line with prior observations (56, 291). Thus, re-aggregation disrupted ovarian cord formation and was associated with a lack of oocyte survival before follicle formation.

**Intact ovarian cords protect oocytes from the mesonephros and support oocyte development**

We next explored overall ovarian structure following transplantation, and we observed that re-aggregation of e12.5 female gonads with the mesonephros resulted in the disorganization of ovary structure and corresponded to the absence of ovarian cords and subsequent oocyte loss. In intact gonad transplants, cells expressing PECAM (Fig. 4A) and Laminin (Fig. 4B), markers of endothelial cells and basement membrane, respectively, were predominantly separated from oocytes by intact ovarian cords. In re-aggregated gonads, in contrast, cells expressing PECAM and Laminin were instead uniformly interspersed along with oocytes and granulosa cells by day 5 of transplantation (Figs. 4A, B). Complete sex reversal was not observed, but testicular SOX9 expression was detected in somatic cells following re-aggregation, suggesting partial activation of a male developmental program, in addition to ovarian cell WNT4 expression in both transplants (Figs. 4C, S4). Thus, these results correlated ovarian cord disruption and oocyte loss to ovary disorganization and partial sex reversal after e12.5 female gonad re-aggregation with the mesonephros and transplantation.
Exclusion of the mesonephros prior to gonad re-aggregation and transplantation rescued oocyte loss and resulted in considerable oocyte survival and development. Previous reports suggested that ovarian cells antagonize the migration of testicular cells, including endothelial cells, from the mesonephros and reinforce ovary sex determination and development (292, 293). To investigate an antagonistic role of the mesonephros in our disrupted ovaries, we removed the mesonephros before e13.5 gonad re-aggregation and transplantation for twelve days. Oocyte survival and development increased following re-aggregation of gonads without the mesonephros (1.8 follicles/section), in comparison to re-aggregated transplants with the mesonephros (0.1 follicles/section), and confirmed that the mesonephros restricted ovary development (Figs. 4D, S5). However, the number of oocytes in transplants re-aggregated without the mesonephros was still reduced compared to intact gonad transplant controls (5.1 follicles/section) (Figs. 4D, S5).

The incomplete rescue of re-aggregated oocyte survival and development in the absence of the mesonephros suggested a role for additional factors in the intact ovarian cords that promote oocyte development. To investigate further, we used a transgenic Oct4-GFP mouse model (36) to purify e12.5 germ cells from inhibitory mesonephros factors, and from stimulatory factors in the intact cords, by fluorescence activated cell sorting. Following isolation of GFP+ germ cells and culture in vitro for three days to induce oocyte entry into meiosis (78% efficiency), meiotic oocytes were re-aggregated with permissive e17.5 or P2 ovaries and transplanted. However, we were unable to detect GFP+ oocyte survival and development even though many GFP- oocytes in follicles from e17.5 and P2 ovaries were observed (data not shown). Thus, we confirmed that intact
ovarian cords promote oocyte development in the absence of the mesonephros, in addition to protecting oocyte survival in the presence of the mesonephros.

**Discussion**

In the present study, we demonstrate that intact ovarian cord formation and maturation promote oocyte development and protect oocytes from the mesonephros. The female embryonic gonad with mesonephros was not competent to support oocyte survival and development when disrupted prior to ovary sex determination and cord formation. Furthermore, oocyte competence to survive and mature following ovary re-aggregation paralleled the duration of oocyte development within intact fetal ovarian cords prior to their disruption. Pre-ovarian cord stage genital ridge re-aggregation triggered oocyte loss before follicle formation, which was independent of meiotic chromosome synapsis defects or Caspase pathway activation, and was correlated with ovarian cord formation impairment and disorganization. Removal of the mesonephros partially rescued oocyte survival in re-aggregated gonads and confirmed a role for intact ovarian structures in protecting oocytes from detrimental mesonephros influence (Table 1).

Based on these findings, we suggest a model in which intact ovarian cord structures provide factors that support oocyte development and protection from toxic factors produced outside of the cords that impair oocyte survival (Fig. 5). A notable aspect of intact ovary maturation is the development of oocytes in clusters which may facilitate cell contact and/or paracrine signal support of oocyte survival. In a previous study, intercellular bridges were not required for oocyte cyst formation, survival, and female
fertility, although their absence resulted in significant peri-natal oocyte loss (281). However, germ cell contacts have also been reported to occur by cadherin protein adhesion in vivo and in vitro as early as the PGC migratory stage and may account for oocyte cyst-like clustering and survival in the absence of intercellular bridge support (37, 294, 295). Although re-aggregation-mediated disruption of intercellular bridges may have contributed to the reduction in oocyte numbers following transplantation, we attribute the complete absence of oocyte survival to the additional perturbation of ovarian cyst and cord structures. Furthermore, ovarian cords are comprised of pre-granulosa somatic cells, and pre-granulosa-oocyte interactions through cell contacts and/or paracrine factors may promote oocyte survival as well (296). Cord organization of granulosa cells in close physical proximity to oocytes may also facilitate cyst break down and follicle formation (56, 99, 280), and the results presented here support this notion.

We also demonstrate that intact ovarian cords protect oocytes from deleterious factors present in the mesonephros, ensuring oocyte survival and development (Fig. 5). Although the mesonephros has traditionally been referenced as a facilitator of female ovary maturation and oocyte meiosis (47, 297, 298), we identified a restrictive role of the mesonephros in oogenesis. In addition to the presence of meiotic oocytes (58), WNT4, R-spondin1, and Follistatin somatic cell signaling factors were required for ovary sex determination and repressed testicular endothelial and somatic cell migration from the mesonephros (292, 293, 299-301). Female mice containing genetic null mutations in these factors developed disorganized ovaries with partial sex reversal, SOX9 mis-expression, and oocytes that entered meiosis but did not survive; analogous to the
phenotype reported in this study. The unexplained oocyte loss in these mutant mice therefore may be an indirect consequence of ovary disorganization downstream of the mutation. In the current study, although ovarian somatic cell signaling pathways were not mutated, gonad disruption was sufficient to prevent re-organization of ovary structure and protection of oocytes from restrictive factors, including those of the mesonephros.

In conclusion, we report that intact ovarian cord structures that develop by e13.5 are important for oocyte development and protect oocytes from the mesonephros. Thus, we emphasize the significance of intact ovary organization during mid to late gestation for oocyte survival and maturation. Human oocytes also develop in cyst- and cord-like structures (302), and genetic mutations and/or environmental toxins affecting human fetal ovary organization could impair oocyte survival and result in premature ovarian failure. Additionally, understanding intact fetal ovarian structure and function will facilitate the maturation of oocytes from other potential sources such as stem cells.

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Table 1. Summary of analysis and results following transplantation.

<table>
<thead>
<tr>
<th>Days of Transplantation</th>
<th>Analysis</th>
<th>Intact Gonad</th>
<th>Re-AgG Gonad</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Meiosis</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>5</td>
<td>Ovarian Cord Formation</td>
<td>+++</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>Oocyte Survival</td>
<td>+++</td>
<td>+/-</td>
</tr>
<tr>
<td>12 to 21</td>
<td>Oocyte Development in Follicles</td>
<td>+++</td>
<td>- a</td>
</tr>
</tbody>
</table>

*Oocyte survival and development were partially rescued by removing mesonephros*
Figure Legends

Fig. 1. Female gonad re-aggregation prior to ovary maturation impaired oocyte development. (A) Female mouse gonads from e11.5 (embryonic day) to P2 (post-natal day) were disrupted by re-aggregation, transplanted under the kidney capsule for three weeks, and sections were examined by H&E stain for oocyte development in follicles. 100X magnification. (B) The average number of oocytes in follicles per graft, normalized to the number of sections counted, was determined following three weeks transplantation of intact or re-aggregated gonad. Error bars represent s.d. (n=3, 150 sections/graft).

* = p<0.05 between e16.5 and e15.5 re-aggregated. ** / *** = p<0.05 between e12.5 / e14.5 intact and re-aggregated, respectively.

Fig. 2. Gonad re-aggregation did not affect oocyte meiotic entry or progression but disrupted oocyte survival and further development. (A) Intact or re-aggregated (re-agg) e12.5 female gonads were transplanted for three days, and germ cells were subsequently analyzed for entry and progression through stages of meiosis prophase I by SCP3 and SCP1 immunostaining of chromosome synapsis. Blue is DAPI. 630X magnification. (B) Intact or re-aggregated e12.5 gonads on day three of transplantation were quantified for the percentage of cells in meiotic prophase I as determined by SCP chromosomal alignment localization by immunofluorescence. Error bars represent s.d. (n=3, 100 cells/graft). No statistically significant difference was detected. (C) By twelve days of transplantation, many primordial and primary follicles were observed from the intact e12.5 gonad but none were detected from the re-aggregated gonad by H&E stain. 200X magnification.
**Fig. 3.** Re-aggregation impaired ovarian cord structure and induced Caspase-independent oocyte loss before follicle formation. (A) The numbers of surviving oocytes per graft were determined by TRA98 immunofluorescence following five, seven, and twelve days of intact or re-aggregated (re-agg) e12.5 female gonad transplantation. Error bars represent s.d. (n=3) * / ** = p<0.05 between d7 / d12 re-aggregated and intact, respectively. (B) Ovarian cord structure was examined by TRA98 germ cell and FOXL2 granulosa somatic cell immunofluorescence over twelve days of e12.5 female gonad transplantation. Oocytes and granulosa cells clustered together in defined ovarian cord-like structures (dashed lines) following five to seven days of intact gonad transplantation, and ovarian follicles were detected by day twelve. Re-aggregated gonads did not form ovarian cords or follicles during the time course, and TRA98+ oocytes were not detected after day seven in re-aggregated transplants. Active Caspase3 was not detected in oocytes. 100X magnification.

**Fig. 4.** Oocyte loss was associated with ovary disorganization and partial sex reversal following re-aggregation with the mesonephros. (A,B) Immunofluorescence revealed TRA98+ oocytes and FOXL2+ granulosa cells in ovarian cord-like structures (dashed lines) primarily sequestered from PECAM+ endothelial cells (A) and Laminin+ basement membrane (B) following five days of intact e12.5 female gonad transplantation. Along with disrupted ovarian cord formation, re-aggregation with the mesonephros also resulted in a uniform distribution of PECAM+ and Laminin+ cells amongst dispersed oocytes and granulosa cells. 100X magnification. (C) Cells expressing WNT4, an ovarian
determinant, were detected in both samples, but testicular determinant, SOX9, was only expressed in somatic cells following re-aggregation with the mesonephros. 100X magnification. (D) Oocyte survival and development were partially rescued when e13.5 gonads were re-aggregated without the mesonephros (meso) and transplanted for twelve days, in contrast to a lack of oocyte development after re-aggregation and transplantation with the mesonephros; however, the number of oocytes in follicles was still reduced compared to intact gonad transplants. 100X magnification.

**Fig. 5.** Intact ovarian cord formation and maturation protect oocytes from the mesonephros and promote oocyte survival and development in follicles. Prior to ovary sex determination on e13.5, germ cells located in cysts in the e12.5 female genital ridge are not yet competent to survive and form follicles when re-aggregated (re-agg) in the presence of the mesonephros. However, ovary maturation on e13.5 and development of ovarian cord clusters containing oocytes and somatic granulosa cells are sufficient for some oocyte survival and follicle formation upon re-aggregation. By e16.5, ovarian cord-enclosed meiotic oocyte clusters and granulosa somatic cells are now primed to undergo robust follicle formation and oocyte development in follicles when re-aggregated. Oocyte (green) and granulosa cell (blue) contacts and paracrine factors support oocyte survival (+), and intact ovarian cords facilitate the protection of oocytes from re-aggregation induced death (94) mediated by harmful factors (-) from the mesonephros (M).
Fig. 2. A

e12.5
Intact

e12.5
Re-Agg

Zygotene  Pachytene  Diplotene  Pachytene  Diplotene

SCP3

SCP1

B

% Chromosomal Alignment

0  10  20  30  40  50  60  70

SCP3  SCP1

Intact  Re-Agg

C

e12.5
Intact  e12.5  Re-Agg
Fig. 3.

A

TRAB+ Oocyte #

Day 5 Day 7 Day 12

intact ReAggl

B

e12.5 Intact

e12.5 Re-Agg

Day 5 Day 7 Day 12

TRA86 FOXL2 aCaspase3
Fig. 5.
Supplementary Figure Legends

**Fig. S1.** Female gonad re-aggregation before ovary maturation impaired oocyte development. The average number of oocytes in follicles per graft, normalized to graft weight (mg), was determined following three weeks transplantation of intact or re-aggregated gonads. Error bars represent s.d. (n=3; p < 0.05). * Significance between e16.5 or e17.5 and e15.5 re-aggregated. ** / *** Significance between e12.5 / e14.5 intact and re-aggregated, respectively.

**Fig. S2.** Re-aggregation induced oocyte loss was Caspase-independent. The percentage of TRA98+ oocytes (100 cells/graff) undergoing apoptosis was assayed by TUNEL and active Caspase2 or Caspase3 co-immunofluorescence following five to seven days of transplantation. Intact = solid bars. Re-aggregated = striped bars.

**Fig. S3.** Re-aggregation impaired ovarian cord formation. Low magnification (40X) analysis of ovary structure by TRA98 germ cell and FOXL2 granulosa somatic cell immunofluorescence after five days of e12.5 female gonad transplantation revealed the striking difference in ovary organization between intact and re-aggregated gonads. Intact gonad oocytes and granulosa cells clustered together in defined ovarian cord-like structures (dashed lines), in contrast to re-aggregated gonads which did not form ovarian cords containing oocyte clusters. Active Caspase3 was not detected in oocytes.
Fig. S4. Testicular SOX9 was only expressed in somatic cells following re-aggregation with the mesonephros. Cells expressing WNT4, an ovarian determinant, were detected in both intact and re-aggregated gonads after five days of transplantation, but testicular SOX9 was only expressed in somatic cells following re-aggregation with the mesonephros along with a disruption of ovarian cord formation and structure in re-aggregated transplants. 100X magnification.

Fig. S5. Oocyte loss was partially rescued by removing the mesonephros prior to ovary re-aggregation and transplantation. Oocyte survival and development in follicles were partially rescued when e13.5 gonads were re-aggregated without the mesonephros (meso) and transplanted for twelve days, in contrast to a lack of oocyte development after re-aggregation with the mesonephros; however, the number of oocytes in follicles was still reduced compared to intact gonad transplant controls.
Fig. S1.
Fig. S2.
Fig. S3.

e12.5 Intact  e12.5 Re-Agg
Fig. S4.
Fig. S5.

- e13.5 Intact +meso
- e13.5 Re-Agg +meso
- e13.5 Re-Agg -meso

Follicle # / graft section
Chapter 5

Embryonic stem cell-derived oocyte maturation in follicles directed by transplantation into an endogenous ovarian niche
Embryonic stem cell-derived oocyte maturation in follicles by transplantation into an ovarian niche

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SUMMARY

Transplantation of stem cells into an appropriate niche has traditionally been a definitive measure of stem cell potential and function. Although embryonic stem cells (ESCs) have been shown to differentiate to female germ cells in vitro, functional analyses and correlation of ESC differentiation to endogenous oocyte (egg) development have been limited. Here, we establish a timeline and genetic program of female germ cell differentiation from ESCs in vitro that initially parallel endogenous oocyte development in vivo; however, ESC-derived oocyte maturation ultimately fails in vitro. We then transplant ESC-derived germ cells into a synchronized ovarian niche to direct the successful maturation of ESC-derived oocytes in ovarian follicles. Thus, we provide rigorous evidence of ESC-derived oocyte physiologic relevance and function by integration and development in an endogenous ovarian niche via transplantation.

Short Title: ESC-derived oocyte maturation

Key Words: Embryonic stem cell, germ, oocyte, follicle, ovarian niche, transplantation
INTRODUCTION

Mammalian oocytes (eggs) are restricted in number and accessibility in all species including humans (273). Consequently, the differentiation of oocytes from embryonic stem cells (ESCs) will facilitate analysis of the genetic, epigenetic, and environmental factors affecting oocyte development. In addition, new differentiation strategies potentially provide a means to understand infertility and treat women with premature ovarian failure, reproductive aging, and/or poor oocyte quality (303).

Although human ESC-derived oocyte differentiation has not yet been reported, spontaneous in vitro differentiation of putative ESC-derived oocytes and follicle-like structures have been reported in mice (8, 42, 207, 208, 210). However, their physiologic relevance and function have been difficult to define in vitro (95), and validation of ESC-derived germ cell identity has not been straightforward due to the heterogeneity of ESC-derived cultures and similarity of genes expressed by ESCs and germ cells (2, 171, 303). Moreover, the correlation of ESC-derived germ cell differentiation in vitro to fundamental parameters of endogenous oocyte development in vivo has been lacking.

Specifically, functional ex vivo maturation of endogenous oocytes has required fetal to newborn stage ovary organ culture or transplantation (136, 137, 139-142). Indeed, transplantation into a stem cell niche has historically been necessary to provide rigorous evidence of germ and somatic cell identity and function (304, 305). Therefore, we characterized the timeline and genetic program of germ cell differentiation from ESCs in vitro, in comparison to endogenous oogenesis, and then transplanted ESC-derived
oocytes into a synchronized ovarian niche to examine their physiologic relevance and to
direct their functional maturation in ovarian follicles (Fig. 1A).

MATERIALS AND METHODS
ESC culture and differentiation
Transgenic ΔPE Oct4-GFP, Dazl WT and null, ESC (XX) lines were derived from
C57BL/6-FVB/N mice as described (306), and the gene trap Stra8-GFP ESC (XY) line
was obtained from the Canadian Mouse Mutant Repository (Toronto, CAN; clone ID
#339H10). Undifferentiated ESCs were cultured on irradiated mouse embryonic
fibroblasts in standard ESC media containing Dulbecco’s modified eagle medium with
high glucose and 2mM L-glutamine, 1mM sodium pyruvate, 100μM non-essential amino
acids, 15% fetal bovine serum (all Invitrogen, CA, USA), 100 μM 2-mercaptoethanol
(Sigma, MO, USA), and 1000U/mL LIF (Millipore, MA, USA); plus 165μg/mL G418
(Invitrogen) for the Stra8-GFP gene trap line only. ESCs were differentiated in
suspension as EBs by culturing in standard EB media with the same components as ESC
media but with 20% fetal bovine serum (Hyclone, UT, USA) and without LIF on ultra-
low attachment plates (Corning, NY, USA). Alternatively, ESCs were differentiated in
FAC media consisting of standard EB media supplemented with a germ cell factor
cocktail: mouse SCF 100ng/mL, mouse SDF1 20ng/mL, mouse bFGF 20ng/mL, mouse
BMP4 50ng/mL (all R&D Systems, MN, USA), N-acetylcysteine 1mg/mL, Forskolin
5μM, Retinoic Acid 1μM (all Sigma), and CYP26 inhibitor R115866 1μM
(Johnson&Johnson, PA, USA).
Flow cytometry and fluorescence activated cell sorting

ESCs, EBs, and fetal gonads were dissociated to single cells with 0.25% Trypsin (Invitrogen) for 5-10 minutes at 37 degrees, or first treated with 1mg/mL each of CollagenaseIV and Dispase (both Invitrogen) for 20 minutes at 37 degrees prior to trypsinization for adult tissues, and re-suspended in standard media. Cells were strained through a 40µm filter (BD Biosciences, CA, USA) and then analyzed on a BD-FACSARia cell sorting system (BD Biosciences). For flow cytometry analysis of SSEA1 expression, dissociated cells were first re-suspended in phosphate buffered saline (PBS, Invitrogen) with 1% bovine serum albumin (BSA, Sigma), incubated with mouse anti-SSEA1 antibody MC480 (1:20, Abcam, MA, USA) for 20 minutes on ice, washed, incubated with APC conjugated anti-mouse IgM secondary antibody (1:200, Jackson ImmunoResearch, PA, USA) for 20 minutes, and washed again before sorting. GFP and SSEA1 positive gates were based on non-transgenic control ESC lines and secondary antibody only control staining, respectively.

Quantitative gene expression analysis

Gene expression analysis was performed using the BioMark Dynamic Array (Fluidigm Corporation, CA, USA) microfluidics system for RTPCR, as described (306). In brief, we pre-amplified samples by treating single cells (Figs. 2A, S2B) or 50 cells (Fig. S2A) per sample per time point following manufacturer’s protocol (Fluidigm Corporation) using Taqman gene expression assays (Applied Biosystems, CA, USA) as indicated in the respective figures. 2.25µL of pre-amplified cDNA was mixed with 2.5µL of 2x Universal Master Mix (Applied Biosystems), 0.25µL Sample Loading Buffer (Fluidigm
Corporation), and loaded into the sample inlets of the 96 x 96 Dynamic Array (Fluidigm Corporation). For each probe, the reaction mix contained 2.5µL 2x Taqman Gene Assay and 2.5µL Assay Loading Buffer (Fluidigm Corporation) for loading into the assay inlets on the Dynamic Array. Each sample had 2 technical replicates. Average CT values were calculated and normalized to Gapdh.

**Meiotic cell spread and immunofluorescence**

Following ESC, EB, or tissue dissociation, cells were re-suspended in 20µL of hypo-extraction buffer pH-8.2 (30mM Tris pH 8.2, 50mM Sucrose, 17mM Citric Acid, 5mM EDTA, 0.5mM DTT, and 1% protease inhibitor cocktail (all Sigma)) for 30 minutes at room temperature. 60µL of 100mM Sucrose was then added, and the cell suspension was spread onto slides pre-coated with 1% Paraformaldehyde (USB Corporation, OH, USA) and 0.15% Triton-X100 (Sigma) in PBS pH-9.2, and dried overnight at room temperature. Slides were blocked in 4% chicken serum (Abcam) and incubated overnight at 4 degrees with primary antibody in TBST (tris buffered saline (TBS), 1% BSA, and 0.1% Tween-20 (all Sigma)) and 1% serum. Primary antibodies included rabbit anti-SCP3 (1:1000, Abcam), rabbit anti-SCP1 (1:500 Abcam), goat anti-SCP1 (1:50 Santa Cruz Biotech, CA, USA), human CREST serum (1:100, Antibodies Incorporated, CA, USA), and mouse anti-γ-H2AX JBW301 (1:500, Millipore). Slides were subsequently incubated with secondary antibodies for 30 minutes, and cover slips were mounted with Prolong Gold Antifade with DAPI (Invitrogen). Secondary antibodies included chicken anti-rabbit, goat, mouse (1:1000, Invitrogen), and human (1:250, Aves Labs, OR, USA).
**Ovarian tissue aggregation and kidney capsule transplantation**

Newborn ovaries were dissected from wild type CD-1 female pups (Charles River, MA, USA), dissociated to single cell suspensions by 10 minute 0.25% trypsinization and pipetting 10 to 20 times, and re-suspended in standard EB media. The ovarian cell suspension from 4 female pups per graft was then mixed with or without 100,000 ESC-derived cells post-FACS sort and 0.2mg/mL of phytohemagglutinin (Sigma). Cell suspensions were pelleted into grafts at 10,000xg for 1 minute and incubated overnight in standard EB media on CM cell culture inserts (Millipore) at 37 degrees. Grafts were transplanted under the kidney capsule of bi-laterally ovariectomized CB.17 SCID recipient mice (Charles River) according to the protocol approved by the Stanford University Administrative Panel on Laboratory Animal Care and as described in detail: (http://mammary.nih.gov/tools/mousework/Cunha001/Pages/Written_Method.html).

**Graft and tissue immunohistochemistry**

Tissues were fixed overnight in 4% Paraformaldehyde (USB Corporation), embedded in paraffin, and sectioned. In brief, sections on slides were de-paraffinized, re-hydrated, antigens unmasked by boiling in Target Retrieval Solution (Dako, CA, USA) for 30 minutes, permeabilized in 0.1% Triton-X100 (Sigma) for 5 minutes, blocked with 10% chicken serum in TBST overnight, and incubated with primary antibody in TBST with 1% serum for 1 hour at room temperature. Primary antibodies included anti-GFP rabbit monoclonal E385 (1:1000, Abcam), anti-TRA98 rat monoclonal (1:500, B-Bridge, CA, USA), anti-SSEA1 mouse monoclonal MC480 (1:100, Abcam or Developmental Studies Hybridoma Bank), and anti-GM130 mouse monoclonal (1:100, BD Biosciences).
washing in TBST, slides were incubated with secondary antibody for 30 minutes at room temperature. For immunofluorescence, secondary antibodies included chicken anti-rabbit, rat, and mouse (1:1000, Invitrogen), and cover slips were mounted with Prolong Gold Antifade with DAPI (Invitrogen). For immunoperoxidase, slides were treated as above but with additional blocking in 10% hydrogen peroxide (Sigma) for 20 minutes at room temperature before permeabilization. Slides were blocked overnight with 10% goat serum (Abcam) in TBST and incubated with primary antibody for 1 hour, biotinylated goat anti-rabbit secondary antibody (1:200, Vector Labs, CA, USA) for 30 minutes, Vectastain ABC solution (Vector Labs) for 30 minutes, Impact DAB substrate (Vector Labs) for 1 to 5 minutes, counterstained with Mayer’s hematoxylin, and mounted with Prolong Gold Antifade (Invitrogen).

**Genetic analysis post-transplantation**

After 3 weeks of transplantation, grafts were harvested, dissociated, and Oct4-GFP positive and negative cells isolated by FACS. Genomic DNA was prepared from these populations, and from the parental Oct4-GFP ESC line (DNeasy, Qiagen, CA, USA), and was then analyzed for the sequence of 50 SNPs (single-nucleotide polymorphisms) that were polymorphic between C57BL/6-FVB/N and CD1-BALB/C mouse strains by the Jackson Laboratory (ME, USA) Genome Scanning Service. The SNP panel contained 3 markers on chromosomes 1,2,3,4,5,6,7,8,9,10, and 12, 2 markers on chromosomes 11,13,14,15,16,17,18, and 19, and 1 marker on the X chromosome. SNP sequences at each locus were compared to the parental ESC line and to each of the 4 mouse strains using a modified Amplifluor fluorescent PCR-based system.
Statistical analysis

Figs. 1, 3 data are represented as mean +/- standard deviation s.d. (n=3). Statistical significance was determined by Excel (Microsoft) using an unpaired Student’s t-Test with two-tailed distribution of two-sample unequal variance. Fig. S4 significance was determined by Prism (GraphPad Software) 2-way ANOVA with Bonferroni posttest.

RESULTS

ESC-derived oocyte identification

We differentiated mouse ESCs, derived from germ cell ΔPE Oct4 promoter driving GFP reporter transgenic mice (306), *in vitro* and identified putative ESC-derived germ cell differentiation and oocyte maturation by flow cytometry analysis. Although the Oct4-GFP reporter is expressed in early embryos and the germline of these mice (264), oocytes from the ovaries of transgenic embryos were uniquely found to express the GFP reporter at a low level of intensity in contrast to high intensity GFP expression by undifferentiated ESCs, primordial germ cells (PGCs) from the genital ridges of either sex, or prospermatogonia from fetal testes (Fig. S1A). Additionally, the cell surface marker, SSEA1 (Stage-Specific Embryonic Antigen 1), is expressed by undifferentiated mouse ESCs and PGCs but is not expressed in fetal or adult gonads after embryonic day e14.5 as reported previously (307) and confirmed by flow cytometry (Fig. S1B). Therefore, flow cytometry analysis of ESC-derived cultures was used to distinguish Oct4-GFP+ and SSEA1-oocytes from double-positive PGCs or ESCs, in addition to a low intensity of GFP expression in oocytes.
Having determined that Oct4-GFP intensity, combined with SSEA1 expression on the cell surface, could distinguish female germ cells in vivo, we differentiated ESCs containing the Oct4-GFP reporter in suspension as embryoid bodies (EBs) for up to 21 days in vitro and analyzed EBs by flow cytometry for oocyte differentiation. EBs were cultured in either standard differentiation media or in media containing a germ cell maturation factor cocktail (FAC) adapted from a report of PGC culture and meiotic progression in the absence of feeder layer support (134). The FAC cocktail, which included BMP4, retinoic acid, CYP26 inhibitor (R115866), SDF1, SCF, bFGF, n-acetyl-cysteine, and forskolin, was comprised of anti-apoptotic, germ cell specification, and meiotic induction factors. While almost all undifferentiated ESCs expressed Oct4-GFP at high intensity, cells with low intensity GFP expression were detected by day 14 of differentiation along with a reduction in the percentage of cells expressing GFP compared to non-transgenic control cultures (Fig. 1B). Additionally, putative oocyte maturation could be induced by FAC media which expedited the appearance of the low intensity population by day 3 of differentiation (Fig. 1B).

Furthermore, in parallel to the appearance of low intensity Oct4-GFP+ cells, the percentage of GFP+ / SSEA1- cells increased by day 14 of differentiation and was accompanied by a significant decrease in the percentage of double-positive cells as well as an increase in double-negative cells (Fig. 1C). We noted that this pattern is similar in timeline to endogenous maturation of PGCs to an oocyte developmental program on e13.5. Again, the ESC-derived cultures were responsive to FAC media, which induced a
significant elevation in the percentage of GFP+ / SSEA1- and double-negative cells by day 5 of differentiation along with a concomitant decrease in double-positive cells (Fig. 1D). These results indicated that germ cell differentiation and oocyte maturation occurred in ESC-derived cultures along an endogenous developmental timeline as identified by flow cytometry.

**Characterization of ESC-derived germ cell identity and maturation**

To verify ESC-derived germ cell identity and oocyte maturation, Oct4-GFP ESCs were differentiated for 5 days, and germ cells were isolated by fluorescence activated cell sorting (FACS), based on parameters defined above for endogenous oocyte development, and analyzed for gene expression. We observed that germ cell transcripts, expressed early in germ cell development in PGCs including Oct4, Stella, Nanos3, and Vasa, were elevated in the double-positive population while Stra8 and Gdf9, markers of later events such as meiotic entry and oocyte maturation, respectively, were increased in the GFP+ / SSEA1- population (Fig. S2A). Double-negative cells, in contrast, expressed only minimal levels of germ cell markers. Conversely, somatic cell markers Kdr and Sox1 were elevated in the double-negative population and minimally expressed in the GFP+ germ cell populations (Fig. S2A). Thus, our results confirmed that the ESC-derived Oct4-GFP+ populations were enriched for germ cells, and that double-positive ESC-derived PGCs differentiated toward either a GFP+ / SSEA1- oocyte fate with low GFP intensity along an endogenous timeline of germ cell development or toward a double-negative somatic cell fate.
In order to dissect the composition of the Oct4-GFP+ population and distinguish ESC-derived germ cells from undifferentiated ESCs, we analyzed gene expression in GFP+ single cells from day 5 of differentiation, in both the absence and presence of FAC media, in comparison to single GFP+ undifferentiated ESCs. The gene expression markers assayed included those expressed in PGCs (early genes: Blimp1, Stella, Dazl, and Vasa), and genes expressed in meiotic germ cells (late genes: Stra8, Scp1, Scp2, Scp3, Gcnf, Mlh1, and Msy2). Although some undifferentiated GFP+ ESCs did express detectable levels of germ cell marker transcripts (Figs. 2A, S2B), a greater percentage of GFP+ cells expressed germ cell markers following 5 days of differentiation with or without FAC media for each marker studied with the exception of Blimp1 in FAC media, which is known to be down-regulated in endogenous PGCs by the time of oogenesis (308) (Fig. S3).

We also noted that, at the single cell level, a single Oct4-GFP+ cell following differentiation was more likely to express multiple germ cell marker transcripts in the same cell than an undifferentiated GFP+ ESC. For example, none of the Oct4-GFP+ ESCs that also expressed Dazl transcript was found to express more than 2 additional early or late germ cell transcripts, with only 14% of ESCs expressing 2 early markers (Fig. 2B, C). In contrast, 87% of GFP+ and Dazl+ cells from day 5 of differentiation without FAC media expressed 2 to 3 additional early germ cell markers (Fig. 2B), and 70% contained 3 to 5 late germ cell markers (Fig. 2C). Unlike cells from EBs without FAC media, only 55% of Oct4-GFP+ and Dazl+ cells from EBs with FAC media expressed a maximum of 2 additional early germ cell markers (Fig. 2B). Instead, 81% of
GFP+ and Dazl+ cells from EBs with FAC media contained more than 2 late markers with up to 7 markers expressed together in the same cell, which is consistent with FAC media induction of germ cell maturation (Fig. 2C).

In addition to expressing a greater number of germ cell markers within the same cell, single Oct4-GFP+ cells from day 5 EBs contained significantly-elevated levels of most germ cell transcripts analyzed compared to undifferentiated Oct4-GFP+ ESCs (Fig. S4). To summarize, undifferentiated ESCs occasionally expressed germ cell transcripts, but differentiated ESC-derived germ cells did so more frequently, expressed more germ cell markers within the same cell, and also expressed those markers more robustly than undifferentiated ESCs, especially following differentiation in FAC media.

**Genetic requirement of Dazl for ESC-derived germ cell development**

To further inspect in vitro ESC-derived germ cell identity and maturation, ESC lines, containing the ΔPE Oct4-GFP reporter, were obtained from mice carrying a Dazl (Deleted in Azoospermia-Like) null mutation and differentiated alongside wild type lines. Dazl null mice are sterile and begin to exhibit a reduction in germ cell numbers in the pre-committed embryonic genital ridge by e12.5 in both sexes with significant germ cell loss by e14.5 in the post-committed fetal ovary or testis (249, 250, 306). As expected, Dazl null (KO) ESC lines displayed a significantly reduced percentage of double-positive PGCs (15% KO compared to 83% WT) and GFP+ / SSEA1- germ cells (2% KO versus 8% WT without FAC; 2% KO versus 9% WT with FAC) in comparison to wild type lines by day 12 of differentiation (Fig. 3). Notably, we did not observe a Dazl null
phenotype after only 7 or 10 days of differentiation without FAC treatment (data not shown). Hence, the genetic requirement of Dazl was shared between endogenous germ cell development in vivo and ESC-derived germ cell differentiation in vitro. Moreover, analysis of the Dazl null phenotype also suggested ESC-derived commitment, or maturation, to a sex specific developmental program by 12 to 14 days of in vitro differentiation without FAC media; thereby paralleling the timeline of endogenous PGC commitment to an oocyte fate in the absence of signals from the fetal testis (46, 47, 52, 309).

**ESC-derived oocyte maturation is limited in vitro**

As committed oocytes rapidly enter meiosis in vivo, and Oct4-GFP+ populations were enriched for germ cells, including oocytes, following differentiation in vitro, we next assessed ESC-derived oocyte entry and progression through meiosis. In fact, we observed meiotic entry in 1-3% of Oct4-GFP+ cells as evidenced by partial synaptonemal complex protein elongation by immunohistochemistry for SCP3 (Synaptonemal Complex Protein 3) (Fig. 4). However, nuclear localization of other proteins such as SCP1 was rarely observed in the GFP+ cells and was focal rather than elongated (Fig. 4D).

Thus, in order to optimize meiotic progression, we obtained an alternative ESC line that contained a GFP reporter knocked into the mouse Stra8 locus. Then, we examined differentiation in experiments that paralleled those described above (Fig. S5A-C). Due to the expected role of Stra8 in meiosis, a greater percentage of Stra8-GFP+ cells were meiotic (10-15%), but these cells also exhibited incomplete SCP3 alignment and an
absence of SCP1 elongation (Fig. S5D). We did observe, however, that CREST centromere staining was occasionally co-localized with the elongated SCP3 structures indicating chromosomal alignment (Fig. 4C, E). In addition, 10% of the GFP+ cells with partial SCP3 alignment also expressed nuclear γ-H2AX in a punctate pattern that co-localized with regions of the SCP3 coated chromosomes and confirmed a leptotene-zygotene-like stage of meiotic DNA double strand breaks and synapsis (Fig. 4F, G) (310). From these experiments, we conclude that a fraction of ESC-derived germ cells can enter meiotic prophase during in vitro differentiation, but importantly, meiotic progression is limited. Moreover, we did not observe ovarian follicle formation at any point during the in vitro differentiation time course and differentiation in FAC-supplemented media did not induce follicle formation, improve the efficiency of meiotic entry, or extent of meiotic progression for either ESC line (data not shown).

**ESC-derived oocyte maturation following ovarian niche transplantation**

Since follicle formation was not observed during in vitro differentiation, we sought to provide a synchronized ovarian niche via transplantation to direct ESC-derived oocyte development. Ovarian follicle formation occurs just after birth in mice which have 21 days of gestation, and re-aggregated newborn ovaries support robust endogenous ovarian follicle development and oocyte maturation following transplantation under the kidney capsule (282) (Fig. 6A). Furthermore, our data indicated that in vitro ESC-derived germ cell differentiation initially followed an endogenous timeline. Thus, to synchronize ESC-derived oocyte differentiation with the newborn ovarian niche, we differentiated Oct4-GFP ESCs for 21 days in vitro without FAC media, isolated GFP+ germ cells by FACS,
and co-aggregated the cells with dissociated wild type newborn ovarian tissue. We then transplanted the co-aggregated graft under the kidney capsule of recipient mice for three weeks. Five out of eight grafts grew in size and contained ovarian tissue following transplantation. Indeed, we observed ESC-derived GFP+ cells in two of the grafts. From these grafts, 23 ESC-derived \textit{Oct4}-GFP+ oocytes were detected out of 100,000 cells transplanted for an efficiency of 0.023%.

We observed ESC-derived oocytes enclosed in ovarian follicles with some reaching the primary follicle stage (Fig. 5A-F) and others at a primordial/primary stage (Figs. 5H, 6B) of development. The primary follicles consisted of a single layer of cuboidal granulosa cells, surrounded by a basement membrane, and enveloped oocytes with robust \textit{Oct4}-GFP expression above background staining of endogenous wild type oocytes (Fig. 5G, I). Additionally, the oocytes contained a single large germinal vesicle nucleus and were 10-20 micrometers in size; equivalent to the size of endogenous primordial to primary follicle stage oocytes. We also observed GFP+ oocytes that were not yet completely enclosed in follicles (Fig. 6C-H) and expressed the germ cell marker TRA98 and perinuclear Balbiani body-like oocyte marker GM130, but did not express the stem cell/progenitor marker SSEA1 (Fig. 6F-H) (311).

To further examine GFP+ oocyte origin, we isolated GFP+ cells by FACS following transplantation, genotyped them at 50 polymorphic SNP (single-nucleotide polymorphism) markers across the mouse genome, and detected homozygous parental ESC strain sequence for every marker analyzed but did not detect wild type newborn
ovary donor or recipient mouse strain sequences; thereby confirming an ESC origin of the Oct4-GFP+ oocytes (Fig. 6I).

DISCUSSION

In this study, we demonstrate similarities between ESC-derived and endogenous oocyte development that encompass developmental timeline, genetic program, and entry into meiosis. Comparable to endogenous mouse PGC commitment and maturation toward an oocyte fate on e13.5, Oct4-GFP mouse ESCs expressed the reporter at a lower level of intensity and down-regulated the expression of SSEA1 by day 14 of in vitro differentiation. ESC-derived single cell expression profiling and responsiveness to germ cell maturation FAC media also confirmed germ cell identity and maturation, and reduced the possibility of false positive identification due to Oct4-GFP+ ESCs that express some germ cell-specific transcripts. In addition, germ cell maturation from ESCs required Dazl, a member of the highly conserved Deleted in Azoospermia gene family, by day 14 of differentiation and corresponded to the requirement of Dazl for germ cell development in vivo by e14.5. Furthermore, ESC-derived oocytes entered meiosis; however, we observed that oocyte maturation was restricted in vitro with respect to a bottleneck in ESC-derived meiotic progression and follicle formation (Fig. 7).

Thus, we directed successful mouse ESC-derived oocyte maturation in ovarian follicles by transplantation into a physiologic ovarian niche (Fig. 7). The integration of ESC-derived oocytes into the niche and interaction with ovarian somatic cells also confirmed endogenous oocyte-comparable identity, function, and potential. Transplantation
represents an important step towards an effective clinical strategy for the treatment of infertility whereby autologous stem cell-derived oocytes could be matured via co-aggregation with ovarian tissue and transplantation to a site preferably less invasive than the kidney capsule (312). However, considerable work remains before safe and effective clinical translation can be realized.

First, the teratoma potential of persisting pluripotent stem cells and PGCs must be eliminated. In the same grafts containing ESC-derived oocytes within follicles, we also observed teratoma formation with Oct4-GFP expression in either uniform embryonal carcinoma-like or primitive neural rosette-like patterns. Teratoma formation may have resulted from undifferentiated pluripotent stem cells that persisted in the absence of FAC media treatment, or from ESC-derived PGCs, since endogenous PGCs can also form ectopic teratomas in vivo (148). Second, the efficiency of ESC-derived oocyte maturation should be improved. Inefficiency may be attributed to incomplete in vitro ESC-derived progression through meiotic prophase I to dictyate arrest as observed here and in a prior report (95). Finally, a demonstration that ESC-derived oocytes can support the development of healthy offspring is needed once the efficiency of oocyte maturation is improved. Nevertheless, mouse ESC-derived oocyte maturation via ovarian niche transplantation provides critical support for physiologically relevant, endogenous oocyte-comparable function and sets the stage for human ESC-derived oocyte development and future academic and clinical utility.

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FIGURE LEGENDS

Fig. 1. Embryonic stem cell (ESC)-derived germ cell identification. (A) In this study, mouse ESCs were differentiated as embryoid bodies (EBs), and putative ΔPE *Oct4*-GFP reporter positive germ cells were first isolated and characterized for germ cell identity and development *in vitro*. To promote oocyte development, ESC-derived germ cells were subsequently aggregated with ovarian tissue and transplanted. (B) GFP high and low intensity populations were identified above non-transgenic control EBs (solid peaks) by flow cytometry following *Oct4*-GFP ESC differentiation *in vitro* with an increase in the percentage of low intensity cells by day 14, similar to the endogenous levels of *Oct4*-GFP expression observed in fetal ovaries of transgenic mice (D = day). Differentiation in germ cell maturation FAC media induced a rapid elevation in the percentage of low intensity cells by day 3 of EB culture. Histogram percentages of high intensity (gated on GFP+ ESCs) and low intensity (gated below GFP+ ESCs and above GFP- control) populations (X-axis: *Oct4*-GFP intensity, Y-axis: Cell #). (C) The percentage of the *Oct4*-GFP and SSEA1 double-positive population decreased significantly by day 14 of differentiation along with an increase in the percentage of GFP+ / SSEA1- and double-negative populations, analogous to endogenous PGC maturation. (D) FAC media induced a significant elevation in the percentage of GFP+ / SSEA1- and reduction in double-positive populations by day 5. (C,D) Data represented as mean +/- s.d. (n=3); * p < 0.01 to d12, ** p < 0.01 to d0, *** p < 0.05 to d0.

Fig. 2. Characterization of ESC-derived germ cell identity and maturation. (A) *Oct4*-GFP+ ESC-derived germ cell identity was confirmed by single cell quantitative
RTPCR expression profiling of pre-meiotic and meiotic germ cell transcripts before \textit{in vitro} differentiation and after 5 days of differentiation (-/+) FAC media. Pre-meiotic germ cell transcripts were enriched in GFP+ cells from 5 day EB cultures, and meiotic transcripts were elevated following differentiation in FAC media, compared to ESCs. Relative expression ($2^{-\Delta Ct}$ relative to \textit{Gapdh}). (B,C) The percentage of GFP+ single cells expressing \textit{Dazl} and additional early (B) (\textit{Blimp1, Stella, Vasa}) or late (C) (\textit{Stra8, Scp1, Scp2, Scp3, Gcnf, Mlh1, Msy2}) germ cell markers by RTPCR were plotted according to the number of markers expressed within the same cell. GFP+ cells expressed more early and late marker transcripts in the same cell following differentiation than undifferentiated ESCs. GFP+ ESCs (blue diamond) did not express more than 2 additional early or late markers, but GFP+ cells following 5 days of differentiation contained up to 3 additional early markers (without FAC; green square) (B), or 5 (without FAC; green square) and 7 (with FAC; purple triangle) late meiotic transcripts (C) within the same cell.

\textbf{Fig. 3. ESC-derived germ cell development requires \textit{Dazl}.} The percentage of early (\textit{Oct4-GFP+ and SSEA1+}; yellow) and late (\textit{Oct4-GFP+ and SSEA1-}; green) ESC-derived germ cell populations significantly decreased, and GFP- somatic cells increased, by flow cytometry following \textit{Dazl} null (KO, striped bars) ESC differentiation for 12 days (-/+ FAC media) compared to \textit{Dazl} wild type (WT, solid bars) lines, suggesting \textit{in vitro} ESC-derived germ cell commitment, or maturation, along an endogenous developmental timeline with a shared genetic program. Data represented as mean +/- s.d. (n=3); * p < 0.05 GFP+ / SSEA1- WT to KO, ** p < 0.05 double positive WT to KO.
Fig. 4. ESC-derived oocyte maturation is limited in vitro. (295) Immunofluorescence stain confirmed Oct4-GFP+ ESC-derived germ cell meiotic entry and progression by SCP3 nuclear localization and partial chromosomal alignment. (D) SCP1 localized to the nucleus but was focal instead of elongated, indicating that in vitro ESC-derived meiotic progression was limited. (C,E) CREST centromere staining co-localized with SCP3 and confirmed SCP3 alignment on some, but not all, chromosomes (arrows). (F,G) γ-H2AX stain revealed a punctate expression pattern in the nuclei of GFP+ cells that also co-localized with regions of chromosomal SCP3 and demonstrated the initiation of DNA double-strand breaks and synapsis. Blue is DAPI except (C,E). Magnification is 630X.

Fig. 5. ESC-derived oocyte maturation in ovarian follicles directed by transplantation into an ovarian niche. (A-D) An Oct4-GFP+ ESC-derived oocyte enclosed in a primary stage follicle. (A) Immunoperoxidase stain for GFP expression following transplantation (200X). (B) H&E stain on adjacent section. (C) Low magnification image of (A) showing GFP+ oocyte (solid arrow), GFP+ clusters (arrowhead), and isolated single GFP+ oocytes not completely enclosed in follicles (dashed arrow) (100X). (D) High magnification of (A) (630X). (E-F) Additional ESC-derived oocytes in primary follicles with robust GFP+ immunoperoxidase staining above background endogenous GFP- oocytes (G) derived from wild type newborn ovary in the same section (630X). (H) Oct4-GFP+ ESC-derived oocytes in primordial/primary follicles were also detected by immunofluorescence stain for GFP above background
staining of wild type oocytes (I) from newborn ovary located adjacent to (H) (630X). Blue is hematoxylin counter stain. (H,I) Blue is DAPI.

Fig. 6. ESC-derived oocyte characterization following transplantation. (A) H&E stain of newborn ovarian tissue following re-aggregation without ESCs and transplantation revealed robust oocyte maturation and folliculogenesis adjacent to kidney tissue (100X). (B) ESC-derived oocytes (arrows) enclosed in primordial/primary stage follicles were detected by immunoperoxidase staining for GFP following transplantation. (C-D) Oct4-GFP+ ESC-derived oocytes not completely enclosed in follicles. (E) Pre-follicle stage GFP+ ESC-derived oocytes identified by immunofluorescence. (F) Oct4-GFP+ and TRA98+ ESC-derived oocytes not completely enclosed in follicles. (G) GFP and SSEA1 co-stain demonstrated double-positive clusters and isolated Oct4-GFP+ / SSEA1- ESC-derived oocytes (arrow) (200X). (H) Oct4-GFP+ ESC-derived oocytes expressed GM130 in Balbiani body-like structures. (I) The ESC origin of Oct4-GFP+ oocytes was confirmed by SNP genotyping of FACS isolated cells following transplantation with 100% of GFP+ cell loci homozygous for parental ESC strain sequence, while 0% of GFP- cell loci were homozygous. (A-D) Blue is hematoxylin counter stain. (E-H) Blue is DAPI. (B-F,H) 630X.

Fig. 7. Ovarian niche transplantation surmounts the in vitro ESC-derived oocyte maturation bottleneck. The characterization of germ cell differentiation by several methods demonstrates that mouse ESCs differentiate in vitro to cells with oocyte-like properties along an endogenous timeline and genetic program, including the hallmarks of
PGC commitment (single star) and oocyte meiotic entry; however, *in vitro* oocyte maturation is limited (dashed lines represent reduced levels). Transplantation (double star) into an ovarian niche is required to overcome this *in vitro* bottleneck and direct physiologic maturation of ESC-derived oocytes in ovarian follicles (d=days).
Figure 1.

A
1. Characterize
2. Aggregate with Ovary & Transplant

B
- FAC
+ FAC

C
D

% of Cells

D0  D5  D12  D14

D0  D5+FAC  D12+FAC  D14+FAC
Figure 2.

A

Relative Gene Expression

B

% Oct4-GFP+ & Daz1+ Cells

C

% Oct4-GFP+ & Scp1+ Cells

Day 0

Day 5

Day 5 + FAC

# of Early Germ Cell Markers

# of Late Germ Cell Markers
Figure 3.

% of Cells

D0  D12  D12+FAC

WT Double Negative
KO Double Negative
WT SSEA1+ / GFP-
KO SSEA1+ / GFP-
WT GFP+ / SSEA1-
KO GFP+ / SSEA1-
WT Double Positive
KO Double Positive
Figure 4.
Figure 6.
**SUPPLEMENTARY MATERIAL**

**Fig. S1. Endogenous fetal oocytes express ΔPE Oct4-GFP at low intensity and lack SSEA1 expression.** (A) *Oct4*-GFP was expressed at high intensity in transgenic ESCs, female/male genital ridge at e12.5, and e15.5 fetal testis but was expressed at low intensity in oocytes from post-committed e15.5 ovary by flow cytometry analysis. The high intensity GFP+ gate was based on transgenic ESC and e12.5 PGC intensity. The low intensity GFP+ gate was set below the high intensity gate and above non-transgenic GFP- cells (solid peak). (B) Undifferentiated ESCs and PGCs from e12.5 genital ridge were SSEA1+, but e15.5 ovary and testis lacked robust SSEA1 expression. Solid peak represents ESCs stained with only secondary antibody. X-axis: fluorescence intensity. Y-axis: cell number.

**Fig. S2. Confirmation of *Oct4*-GFP+ ESC-derived oocyte maturation.** (A) Transcripts expressed beginning in pre-meiotic germ cells (*Oct4, Stella, Nanos3, and Vasa*) were enriched in the double-positive population while markers of meiotic entry and oocyte maturation (*Stra8 and Gdf9*) were elevated in GFP+ / SSEA1- cells by quantitative RTPCR analysis. Somatic cell markers (*Kdr and Sox1*) were robustly detected in the double-negative population with minimal levels observed in GFP+ germ cells. *Hoxa1* was expressed in endogenous somatic and germ cells and was also detected in all ESC-derived populations. (B) *Oct4*-GFP+ ESC-derived germ cell maturation was confirmed by single cell quantitative RTPCR expression profiling of meiotic germ cell transcripts before *in vitro* differentiation and after 5 days of differentiation (+/-) FAC media. Meiotic
transcripts were elevated following differentiation in FAC media compared to ESCs and non-FAC treated cultures. Relative expression ($2^{\Delta Ct}$ relative to Gapdh).

**Fig. S3.** Single *Oct4*-GFP+ cells are more likely to express germ cell marker transcripts following differentiation. Although a fraction of the undifferentiated GFP+ ESCs expressed germ cell markers, a greater percentage of GFP+ cells expressed germ cell transcripts following differentiation by quantitative RTPCR analysis. Relative expression ($2^{\Delta Ct}$ relative to Gapdh).

**Fig. S4.** Single *Oct4*-GFP+ cells are enriched for germ cell transcripts following differentiation. Transcript levels were increased following 5 days of differentiation by quantitative RTPCR analysis and were elevated with significance following differentiation in FAC media, compared to undifferentiated GFP+ ESCs. Transcript levels were normalized ($2^{\Delta Ct}$ relative to Gapdh), and single cell values were averaged for each population. Significance of D5+FAC to D0 ESC (p=0.0048). Significance of D5+FAC to D5 (p=0.0071).
Fig. S5. *Stra8*-GFP ESC-derived meiotic entry. (A,B) Similar to *Oct4*-GFP ESC flow cytometry analysis, *Stra8*-GFP is expressed in most cells before and throughout the differentiation time course with a reduction in SSEA1 expression by day 14 of differentiation and by day 7 in FAC media. X-axis: GFP intensity. Y-axis: SSEA1 intensity. (C) *Stra8*-GFP+ cells isolated from day 14 EBs by FACS were enriched for meiotic marker transcripts by RTPCR analysis (*Stra8*, *Scp3*, *Scp1*, normalized to *UbiquitinB*). (D) *Stra8*-GFP+ ESC-derived germ cell meiotic entry and early progression demonstrated by immunofluorescence staining for SCP3 showing nuclear localization and partial SCP3 chromosomal alignment. (E) Endogenous pachytene spermatocyte control illustrating complete SCP3 and SCP1 chromosomal alignment with telocentric CREST centromere staining. (F) Endogenous leptotene-zygotene spermatocyte controls with nuclear SCP3 and punctate γ-H2AX expression. (G) Endogenous pachytene spermatocyte controls showing complete SCP3 chromosomal alignment and γ-H2AX localization to the XY body. (D,F,G) Blue is DAPI. (57) magnification is 630X.

Fig. S6. ESC and ovary immunostaining controls. (A,B) TRA98 immunofluorescence stain of undifferentiated ESCs (A) and newborn ovary (B) showing robust oocyte-specific expression. (C,D) GFP and SSEA1 co-immunofluorescence stain of transgenic *Oct4*-GFP undifferentiated ESCs (C) and adult ovary (D) confirming a lack of SSEA1 expression by oocytes. Blue is DAPI. Magnification is 200X.
Figure S2.

A

Relative Gene Expression

Oct4
Stella
Nanos3
Vasa
Strai
Gdf9
Hoxa1
Kdr
Sox1

Double Negative   GFP+/SSEA1+   Double Positive

B

Relative Expression

Scp2
Gcnf
Mlh1

Single Oct4-GFP+ Cells

Day 0    Day 5    Day 5 + FAC
Figure S3.
Figure S4.
Figure S6.
Chapter 6

Conclusions
Rationale and Summary of Aims

Just prior to the onset of this study, germ cell differentiation from mouse ESCs was reported by three separate laboratories, and our laboratory reported evidence of human ESC-derived germ cells. Mouse ESCs were observed to both differentiate toward the germ lineage and undergo maturation as egg or sperm. In contrast, the extent of germ cell development from human ESCs was confined to germ cell precursor, or pre-meiotic primordial germ cell, differentiation. Thus, there appeared to be a solid foundation in the literature to extend protocols successful for mouse ESC-derived gamete development to the maturation of oocytes from human ESCs.

Consequently, I initially pursued oocyte maturation from human ESCs both in vitro and following transplantation into a human fetal ovarian niche, using the mouse ESC system as a positive control. Expecting human oocyte maturation to be a rare event and seeking a method to identify oocytes of human ESC origin following transplantation, I developed a system to genetically modify undifferentiated human ESCs with germ cell-specific and/or constitutive fluorescent reporters using lentivirus transduction followed by homogeneous expansion of modified lines from single cells to facilitate the detection of ESC-derived oocytes (Chapter 2). However, upon differentiation of human and mouse ESCs in parallel, I found that published protocols for mouse ESC-derived oocyte maturation in vitro were not reproducible.

In contrast to reported spontaneous differentiation of putative oocytes in follicle-like aggregates in suspension from mouse ESCs, I instead observed occasional oocyte-like
cells by morphology in the adherent monolayer (Figure 1A). However, these oocyte-like cells did not grow in size, and they neither expressed Oct4-GFP nor other oocyte-specific or granulosa cell-specific markers definitively above background levels of expression in oocyte-negative controls (Figure 1B). Furthermore, pilot transplantation studies of intact or re-aggregated human fetal ovarian tissue grafted under the mouse kidney capsule for two to four months resulted in no oocyte maturation past the primordial follicle stage in contrast to robust antral follicle maturation following mouse ovary transplantation (Figure 2). Therefore, these results indicated that a solid foundation of mouse ESC-derived oocyte maturation did not exist, and the prolonged timeline for human oocyte maturation and ovarian niche development under the mouse kidney capsule was not practical to pursue given the time constraints of this thesis project and lack of a positive control in the mouse system.

Closer inspection of the initial report of mouse ESC-derived oocyte maturation revealed a lack of functional analysis that was limited to *in vitro* characterization. Moreover, since this initial report, only one other group has reported the generation of oocytes from mouse ESCs with this protocol, and the oocytes exhibited abnormalities at an early stage of meiosis. Subsequent reports have also relied heavily on morphological and gene expression, rather than functional, analysis to confirm oocyte differentiation from mouse ESCs. Thus, I focused my thesis work on demonstrating functional properties, in addition to gene expression profiles, of mouse ESC-derived oocyte maturation in comparison to landmarks of endogenous oocyte development, and on transplantation; a definitive measure of physiologic cell identity, function, and potential.
To determine whether mouse ESCs have the potential to differentiate toward a germ cell fate and undergo functional maturation as oocytes, my aims were:

1. To develop and utilize germ cell-specific reporters and surface antigens to identify and characterize germ cell differentiation from ESCs
2. To examine endogenous oocyte development from transplanted embryonic ovaries and construct a robust ovarian niche promoting oocyte maturation
3. To direct functional ESC-derived oocyte maturation in ovarian follicles by transplantation into the endogenous ovarian niche

**Aim 1 Summary: To develop and utilize germ cell-specific reporters and surface antigens to identify and characterize germ cell differentiation from ESCs**

While ESC-derived oocyte maturation was not definitively or reproducibly demonstrated *in vitro*, several groups, including our own, have identified cells with gene expression profiles indicative of early stage ESC-derived primordial germ cell (PGC) differentiation. However, it was difficult for us to conclusively distinguish PGCs from undifferentiated ESCs that also express several germ cell-specific markers (Chapter 1). Thus, I sought a germ cell-specific marker that was not expressed in ESCs and accordingly developed additional reporter strategies including *Dazl*-GFP, *Gdf9*-GFP, *Vasa*-LacZ, *Oct4*-GFP, and *Stra8*-GFP reporter constructs.

*Deleted in AZoospermia-Like (Dazl)* is a highly conserved member of the DAZ family and is required for fertility in many species. Furthermore, *Dazl* is expressed almost
continuously from post-migratory PGCs to mature gametes in both sexes and represented an optimal reporter strategy for identification of germ cells at different stages of development following ESC differentiation. As such, we genetically modified mouse ESCs to contain 1.7 kb of 5’ sequence flanking the Dazl gene, and including the 5’ UTR, driving a GFP reporter. Following differentiation and isolation of putative GFP+ germ cells, GFP+ populations expressed many germ cell-specific transcripts suggesting a germ cell identity (Chapter 3). However, this reporter strategy was problematic on two counts. Undifferentiated ESCs expressed the reporter at high intensity, and more importantly, characterization of transgenic mice containing this reporter revealed a testicular-specific expression pattern not suitable for reporting ESC-derived oocyte maturation (Chapter 3).

Consequently, we developed mouse ESC lines with alternative germ cell reporter constructs. Gdf9 is required for female fertility and is expressed primarily by oocytes during folliculogenesis beginning at the primary follicle stage. Moreover, Gdf9 is not robustly expressed in undifferentiated ESCs. ESCs were modified with a 3.3 kb 5’ sequence flanking the Gdf9 gene driving a GFP reporter construct and differentiated. GFP+ cells were identified and isolated by FACS, and indeed, GFP+ populations were enriched for Gdf9 transcript compared to the GFP- population (Figure 3). However, GFP+ populations were not enriched for other germ cell markers including Dazl and Vasa, suggesting the reporter to be non-specific (Figure 3). Interestingly, Gdf9 is also expressed in testicular and somatic cells, although it has been used by several groups to confirm ESC-derived oocyte maturation. Perhaps Gdf9 germ cell-specificity is not sufficient to report oocyte differentiation, or the reporter sequence does not faithfully
recapitulate endogenous expression patterns as in the case of the *Dazl* reporter described above.

Next, we focused our efforts on developing a *Vasa* reporter. Like *Dazl*, *Vasa* is highly conserved, specifically expressed in post-migratory germ cell to adult gamete stages, required for fertility in many species, and expressed at a lower level than *Dazl* in undifferentiated ESCs. I obtained an ESC line and mice with LacZ knocked into the *Vasa* locus and confirmed germ cell-specific expression in the gonads of these mice. Furthermore, ESCs and derivatives could be analyzed by flow cytometry for reporter activity by conversion of a synthetic substrate to fluorescein in the presence of LacZ expression. Following differentiation, LacZ+ populations were enriched for *Vasa* transcript as expected; however, other germ cell markers including *Oct4* and *Dazl* were not enriched (Figure 4). Therefore, like the *Gdf9* reporter, the *Vasa*-LacZ reporter line also appeared non-germ cell-specific and was perhaps related to non-specific conversion of substrate to fluorescein.

In contrast, GFP+ populations isolated by FACS following differentiation of ESCs modified with the delta PE *Oct4*-GFP reporter were enriched for both Oct4 transcript and other germ cell specific markers including pre-meiotic and meiotic *Blimp1, Stella, Dazl, Vasa, Stra8, Scp1-3, Gcnf, Mlh1, and Msy2* transcripts (Figure 4 and Chapter 5). Additionally, an ESC line containing GFP knocked into the *Stra8* locus differentiated into GFP+ populations enriched for *Stra8* and other meiotic transcripts (Chapter 5). Thus, our results indicated that *Oct4* and *Stra8* reporters could be used to identify and isolate
putative germ cell populations. However, both reporters were also robustly expressed in undifferentiated ESCs, which was unexpected for *Stra8* given its role in meiosis, allowing the possibility of an ESC identity rather than a germ cell identity of GFP+ populations following differentiation.

To distinguish ESC-derived germ cells from undifferentiated ESCs and to identify oocyte maturation, we combined these germ cell-specific reporters with the stem cell surface marker, SSEA1. SSEA1 is expressed on the surface of undifferentiated ESCs and PGCs of both sexes but is not expressed by maturing oocytes or sperm after e14.5 days of mouse gestation. Hence, flow cytometry analysis was used to distinguish GFP+ and SSEA1- germ cells undergoing maturation to oocyte or sperm fates from GFP and SSEA1 double positive ESCs or PGCs. Indeed, GFP+ and SSEA1- populations emerged by day 14 of ESC differentiation, similar in timeline to endogenous oogenesis (Chapter 5). In addition to the disappearance of SSEA1 expression, oocyte maturation is correlated to a reduction in levels of *Oct4* transcript, and I also observed a corresponding decrease in the intensity of *Oct4*-GFP expression by day 14 of ESC differentiation.

To confirm germ cell identity and characterize the extent of ESC-derived germ cell maturation, we examined the expression profiles of single GFP+ cells isolated by FACS following ESC differentiation in comparison to single GFP+ undifferentiated ESCs. Indeed, GFP+ cells after differentiation were more likely to express germ cell-specific markers, expressed more markers within a single cell, and expressed these markers at elevated levels compared to undifferentiated ESCs; confirming germ cell identity.
(Chapter 5). In addition, GFP+ cells following 12-14 days of ESC differentiation required the Dazl gene for survival and maturation, similar to endogenous germ cells that require Dazl function by e12-14.5 of gestation, while undifferentiated GFP+ ESCs with a genetic null mutation of the Dazl gene did not require Dazl for their derivation, expansion, or proliferation/survival. Lastly, GFP+ cells after differentiation entered into meiosis in contrast to undifferentiated ESCs whose meiotic entry was not detected (Chapter 5). Thus, I demonstrated that Oct4 and Stra8 reporters, combined with SSEA1, could be used to identify authentic ESC-derived germ cell populations, including oocytes, as confirmed by both single cell expression profiling and functional parameters of endogenous oogenesis including developmental timeline, genetic program, and entry into meiosis.

**Aim 2 Summary: To examine endogenous oocyte development from transplanted embryonic ovaries and construct a robust ovarian niche promoting oocyte maturation**

ESC-derived germ cells entered meiosis, however, progression through prophase I of meiosis was limited in vitro and complete oocyte progression to dictyate arrest and formation of ovarian follicles was not detected. To overcome this bottleneck, I turned to the study of endogenous oocyte development in the embryonic gonad in vivo. On e13.5, endogenous PGCs of either sex undergo sex determination or commitment to a female fate and mature as oocytes in the absence of a testicular environment whether developing in the ovary, ectopically in other organs, or in vitro. Upon commitment to oogenesis, oocytes immediately begin to enter and progress through prophase I of meiosis and support the formation of primordial follicles in the presence of ovarian somatic granulosa cells. In contrast to spermatogenesis where the testicular somatic environment is
dominant irrespective of germ cell status, the presence of meiotic oocytes in the ovary serves to reinforce ovary sex determination by inhibiting testicular development. In addition to meiotic oocytes, ovarian somatic pre-granulosa cell signaling pathways are required for inhibition of testicular differentiation, oocyte survival, and development in ovarian follicles. Oocytes isolated from the ovary ectopically or in vitro do not form ovarian follicles and subsequently degenerate. Therefore, the ovarian follicle is essential for oocyte survival and maturation, and folliculogenesis depends on the synchronization of oocyte-autonomous development with the non-oocyte autonomous development of ovarian granulosa cells.

Since ESC-derived oocytes could enter meiosis, I hypothesized that non-oocyte autonomous lack of an appropriate ovarian somatic cell environment, or niche, and/or presence of testicular cells were responsible for restricting complete ESC-derived oocyte meiotic progression and follicle formation in vitro. Furthermore, purified endogenous PGCs and nascent oocytes required re-aggregation with same-stage ovarian tissue and transplantation under the kidney capsule to promote oocyte development. Accordingly, I constructed a timeline of re-aggregated fetal and newborn ovary development in follicles following transplantation to determine the optimal ovarian niche for promoting ESC-derived oocyte maturation.

Indeed, oocyte maturation following re-aggregation and transplantation corresponded to the increasing age of ovary donors (Chapter 4). In contrast to intact gonad transplants with robust oocyte maturation, pre-ovary disruption and re-aggregation of e11.5 and
e12.5 female genital ridge with the mesonephros completely impaired oocyte survival and development following transplantation. Rare oocytes in follicles developed in re-aggregated e13.5 to e15.5 transplants with a significant increase in oocyte maturation observed after e15.5. The maximum numbers of oocytes in follicles were detected following transplantation of re-aggregated newborn stage ovaries.

Re-aggregation did not impair pre-e13.5 PGC commitment to oogenesis, result in spermatogenesis, or pluripotent embryonic germ cell reprogramming, since re-aggregated germ cells could enter and progress through meiosis prophase I and initially develop as oocytes. Closer examination of oocyte survival following a time course of genital ridge transplantation revealed that re-aggregation induced accelerated oocyte loss before follicle formation. Hence, I suspected that disruption of intact pre-follicle ovarian structures was responsible for the impairment of oocyte development.

Before ovary sex determination and maturation on e13.5, PGCs develop in clusters or cysts. Upon ovary maturation, oocyte cysts become organized with pre-granulosa cells in poorly defined ovarian cord structures. Ovarian cord and cyst breakdown at the time of birth in mice is then required for ovarian follicle formation. Thus, I hypothesized that intact ovarian cord formation is required for oocyte survival and development, and that pre-ovary genital ridge re-aggregation obstructed cord formation and impaired oocyte survival and subsequent development in follicles.
Further inspection of oocyte and granulosa cell organization in transplants indeed confirmed that re-aggregation with the mesonephros impaired cord formation and corresponded to oocyte loss, ovary disorganization, and partial sex reversal. This phenotype was reminiscent of oocyte loss secondary to testicular endothelial and steroidogenic cell infiltration from the mesonephros in mice containing genetic null mutations in components of the ovarian somatic cell sex determination signaling pathway. Consequently, I removed the mesonephros prior to gonad re-aggregation and transplantation. Mesonephros exclusion, indeed, resulted in a partial rescue of oocyte survival and development in follicles (Chapter 4).

In summary, intact ovarian cord formation protects oocytes in the presence of the mesonephros and also promotes oocyte development in the absence of the mesonephros. These results were surprising given that the mesonephros has traditionally been thought of as facilitator of ooogenesis. Although, they provide an explanation for testicular masculinization and oocyte loss following disruption of ovarian cell signaling pathways and emphasize intact pre-follicle stage ovary structure and organization in promoting oogenesis. These results also agree with previous reports of fetal gonad re-aggregation and culture or transplantation: one group only observed follicle formation from post e13.5 stage ovaries, while another group observed rare oocyte survival and development in follicles from e12.5 genital ridge without the mesonephros. Nevertheless, re-aggregated newborn ovary transplants supported the most robust endogenous oocyte maturation and were subsequently used as an ovarian niche to direct ESC-derived oocyte maturation.
Aim 3 Summary: To direct functional ESC-derived oocyte maturation in ovarian follicles by transplantation into the endogenous ovarian niche

Mouse ESCs differentiated to oocytes; however, oocyte maturation was restricted in vitro. To direct further maturation of oocytes in follicles, ESC-derived germ cells were transplanted into a synchronized ovarian niche. Ovarian follicle formation occurs at birth in mice which have 21 days of gestation, and re-aggregated newborn ovaries supported robust oocyte development following transplantation. Furthermore, ESC-derived oocyte differentiation paralleled the developmental timeline of endogenous oogenesis. Accordingly, Oct4-GFP+ germ cells, including oocytes, were isolated by FACS following 21 days of differentiation, co-aggregated with newborn ovarian tissue, and transplanted under the kidney capsule in order to synchronize ESC-derived oocytes with the ovarian niche.

Remarkably, Oct4-GFP+ ESC-derived oocytes were observed in primordial to primary stage follicles following transplantation (Chapter 5). ESC-derived oocytes functionally integrated into the niche, recruited endogenous granulosa cells, and supported folliculogenesis; thus demonstrating physiologically relevant oocyte identity, maturity, and functionality. Similar to endogenous PGCs and nascent oocytes, ESC-derived oocytes required an ovarian niche and transplantation for further maturation. In addition, single nucleotide polymorphism (SNP) genotyping of GFP+ germ cells, including oocytes, isolated by FACS after transplantation confirmed ESC origin rather than fusion of GFP+ cells with wild type oocytes from the newborn ovary. However, ESC-derived
oocyte maturation in follicles was only detected at very low efficiency (0.023%) following transplantation and precluded additional studies examining further maturation and fertilization. I hypothesized that low efficiencies were either the result of non-oocyte autonomous sub-optimal synchronization of newborn ovarian niche somatic cells with *in vitro* ESC-derived oocytes or an oocyte autonomous intrinsic abnormality with respect to meiosis.

Although ESC-derived oocyte differentiation initially followed an endogenous timeline of oogenesis *in vitro*, progression through meiosis was eventually restricted to an early leptotene-zygotene-like stage of prophase I corresponding to e12-14.5 days of gestation. Perhaps, therefore, an earlier stage fetal gonadal niche would be more synchronized to direct efficient ESC-derived oocyte maturation. In chapter 4, results indicated that re-aggregation impaired oocyte development from these earlier gonadal stages; however, oocytes enclosed in intact ovarian cord structures were able to mature. Additionally, removal of the oocyte-toxic mesonephros increased oocyte survival and development. Moreover, *Oct4*-GFP+ ESC-derived germ cells appeared to develop in cyst-like clusters during *in vitro* differentiation, resembling endogenous intact germline cysts. Thus, work is ongoing to co-aggregate intact ESC-derived germ cell clusters with intact e13.5 stage ovarian tissue without the mesonephros to promote more efficient oocyte maturation through improved stem cell-niche synchronization and understanding of endogenous pre-follicle oogenesis (Figure 5).
Alternatively, an ESC intrinsic defect preventing efficient progression through meiosis may be responsible for inefficient oocyte maturation regardless of ovarian niche synchronization. Endogenous PGCs and nascent oocytes enter and efficiently progress through meiotic prophase I completely \textit{in vitro} (80\% efficiency). Moreover, isolated migratory stage PGCs, or PGCs that mis-migrate to the adrenal gland and never come in contact with the fetal gonad, can still enter and progress through prophase I, suggesting that the fetal ovarian niche is not necessary for successful oocyte meiosis. Hence, I further investigated \textit{in vitro} $Oct4$-GFP and $Stra8$-GFP ESC-derived oocyte meiosis following a thorough time course of differentiation, the addition of extrinsic and intrinsic factors, and early embryonic cell co-culture to promote meiotic progression.

In contrast to gene expression profiling and the genetic requirement of $Dazl$, which suggested an endogenous developmental timeline, I detected ESC-derived germ cell meiotic entry by day 3 of differentiation (Figure 6). The highest percentages of meiotic cells were observed by day 5 (2\% for $Oct4$-GFP ESCs; 14\% for $Stra8$-GFP ESCs), much earlier than endogenous oocyte meiotic entry on e13.5 days of mouse gestation. However, meiotic progression was still limited at these early timepoints, and germ cell maturation factor cocktail (Chapter 5) treatment did not increase the efficiency of meiotic entry or extent of meiotic progression. In addition, treatment of differentiation cultures with other extrinsic factors, including anti-LIF antibody to neutralize LIF-mediated inhibition of meiosis, Brefeldin to inhibit testicular Sertoli cell signaling-mediated inhibition of meiosis, and 5-Azacytidine and Trichostatin A to de-methylate germ cell
DNA and inhibit histone deacetylation, respectively, and promote meiotic gene activation, did not affect ESC-derived meiotic entry or progression.

Because extrinsic factor treatment had no effect on meiosis, I examined germ cell intrinsic factors. Interestingly, maximum ESC-derived meiotic entry occurred by day 5 of differentiation, however, Dazl function was not required for ESC-derived germ cell survival until day 12 (Chapter 5). In contrast, Dazl has been well characterized in vivo as a master regulator of pre-meiotic germ cell survival and is required for meiotic entry and progression. Therefore, I questioned whether delayed expression and function of Dazl in ESC-derived germ cells was responsible for restricted meiosis, and ESCs were consequently modified with a Dazl over-expression construct driven by a constitutive promoter. However, Dazl over-expression did not improve meiotic entry or progression at any timepoint during the differentiation time course (data not shown).

Based on these results and understanding of the literature, meiosis appeared to follow an intrinsic clock, or cell-autonomous program, established during germ cell formation, or specification, in the early embryo. I next hypothesized that germ cells were incorrectly specified from ESCs in vitro resulting in faulty meiosis. Although BMP4 was present in the factor cocktail described above and plays an essential role in germ cell specification in vivo from the proximal epiblast on e5.5, many details and mechanisms of germ cell specification still remain unknown. Thus, I differentiated ESCs in co-culture with dissociated e5.5 embryos for 3 days to direct proper germ cell specification and subsequent meiosis (Figure 7). In fact, the e5.5 embryo co-culture induced a substantial
increase in the percentage of ESC-derived germ cells entering meiosis compared to non-
co-culture or e11.5 female genital ridge co-culture differentiation (17% for Oct4-GFP; 33% for Stra8-GFP). However, e5.5 embryo co-culture did not result in extended meiotic progression, and ESC-derived meiosis was still restricted. Nonetheless, these results suggested an in vitro ESC-derived germ cell intrinsic defect related to faulty specification. Elucidation of endogenous germline specification will undoubtedly assist in promoting complete ESC-derived oocyte meiotic entry, progression, and efficient maturation in ovarian follicles.

Conclusion

In summary, this work demonstrates that mouse ESCs can generate bona fide oocytes with functional properties when directed by a physiologic ovarian niche and transplantation. ESC-derived oocyte differentiation displayed hallmarks of endogenous oocyte development with respect to developmental timeline, genetic program, entry into meiosis, and folliculogenesis through the recruitment of endogenous somatic granulosa cells. Thus, oocytes, with endogenous-comparable identity and function, can be differentiated from mouse ESCs. This work also identifies two major challenges to efficient ESC-derived oocyte maturation: development of a synchronized ovarian niche with intact ovarian cord structure, and proper specification of in vitro ESC-derived germ cell programs to complete meiosis (Figure 8). Future work will address these challenges and pursue ESC-derived oocyte fertilization and support of healthy offspring. It is my sincerest hope that this study provides a solid foundation for the maturation of oocytes from human ESCs and application to understanding and treating infertility in women.
Figure Legends

Figure 1. ESC-derived oocytes in follicle-like aggregates are not reproducible *in vitro*. (A) Structures resembling oocytes with germinal vesicles in follicle-like aggregates were occasionally observed by morphology in adherent cultures following monolayer differentiation of mouse ESCs; however, analysis of transcript expression (B) revealed a lack of germ cell and granulosa cell marker enrichment in these isolated structures (Follicle +) indicating false positive identification by morphology alone.

Figure 2. Human fetal ovary transplantation did not support oocyte maturation. Human fetal ovaries from 20 weeks of gestation were transplanted under the mouse kidney capsule intact or following re-aggregation (re-agg). Primordial ovarian follicles were present and survived, but oocyte maturation to the primary follicle stage was not detected even in intact ovaries after 4 months of transplantation, identifying a major challenge for human ovarian niche-based oocyte maturation through transplantation.

Figure 3. Characterization of a *Gdf9*-GFP reporter in mouse ESCs. ESCs containing a *Gdf9*-GFP reporter were differentiated and GFP+ populations isolated by FACS. The reporter successfully labeled cells expressing *Gdf9*, but these cells did not express other germ cell-specific markers suggesting this reporter to be non-specific and not suitable as an ESC-derived germ cell reporter.

Figure 4. Characterization of a *Vasa*-LacZ reporter in mouse ESCs. ESCs with LacZ knocked into the *Vasa* locus were differentiated and LacZ+ populations isolated by FACS
for analysis of transcript expression. Like the *Gdf9* reporter, LacZ+ cells were enriched for *Vasa* transcript but not other germ cell-specific markers in contrast to Oct4-GFP+ cells which expressed many germ cell-specific markers. Fold change is GFP+/GFP-.

**Figure 5. Development of an intact ovarian niche to promote robust oocyte maturation.** ESC-derived oocyte maturation was inefficient following dissociation of EBs and newborn (P2) ovaries with trypsin, re-aggregation, and transplantation (left). Work is ongoing to develop an earlier, more synchronized ovarian niche (e13.5) without the mesonephros and to leave ovarian structures intact by collagenase/dispase treatment to dissociate EBs and ovaries prior to re-aggregation in order to promote efficient ESC-derived oocyte maturation (15).

**Figure 6. Characterization of ESC-derived meiosis.** ESCs containing the *Oct4*-GFP or *Stra8*-GFP reporter were differentiated up to 21 days in standard differentiation media or in media supplemented with a germ cell maturation factor cocktail (FAC). GFP+ cells were isolated by FACS and examined for meiotic entry and progression by SCP3 protein expression and chromosomal elongation localization. Maximum meiotic entry was observed by 5 days of differentiation; however, meiotic progression was partial and complete progression was not detected for any timepoint, cell line, or treatment.

**Figure 7. ESC differentiation with e5.5 embryo co-culture enhanced the competence of ESC-derived germ cells to enter meiosis.** ESCs were differentiated in suspension for 3 days as EBs without or with dissociated e5.5 embryos or e11.5 female genital ridge co-
culture, and GFP+ cells were analyzed for meiosis. The e5.5 embryo co-culture induced a remarkable increase in the percentage of ESC-derived germ cells that entered meiosis. However, co-culture did not improve the extent of meiotic progression which remained partial. E11.5 gonad co-culture had no effect on meiotic entry or progression.

**Figure 8. A model of strategies to overcome inefficient ESC-derived oocyte maturation.** Challenges to efficient ESC-derived oocyte maturation include oocyte-autonomous and non-autonomous ovarian niche components that contribute to a maturation bottleneck. A fetal ovarian stage niche may be more synchronized with ESC-derived oocytes; however, intact fetal ovarian structures and exclusion of the mesonephros are important for promoting oocyte survival and development. In addition, ESC-derived oocyte meiosis is abnormal due to presumptive defects in *in vitro* germ cell specification. E5.5 embryo co-culture may improve ESC-derived oocyte meiotic entry; however, meiotic progression remains a mystery.
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8


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