

# Transplantation directs oocyte maturation from embryonic stem cells and provides a therapeutic strategy for female infertility

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Ten to 15% of couples are infertile, with the most common causes being linked to the production of few or no oocytes or sperm. Yet, our understanding of human germ cell development is poor, at least in part due to the inaccessibility of early stages to genetic and developmental studies. Embryonic stem cells (ESCs) provide an *in vitro* system to study oocyte development and potentially treat female infertility. However, most studies of ESC differentiation to oocytes have not documented fundamental properties of endogenous development, making it difficult to determine the physiologic relevance of differentiated germ cells. Here, we sought to establish fundamental parameters of oocyte development during ESC differentiation to explore suitability for basic developmental genetic applications using the mouse as a model prior to translating to the human system. We demonstrate a timeline of definitive germ cell differentiation from ESCs *in vitro* that initially parallels endogenous oocyte development *in vivo* by single-cell expression profiling and analysis of functional milestones including responsiveness to defined maturation media, shared genetic requirement of *Dazl*, and entry into meiosis. However, ESC-derived oocyte maturation ultimately fails *in vitro*. To overcome this obstacle, we transplant ESC-derived oocytes into an ovarian niche to direct their functional maturation and, thereby, present rigorous evidence of oocyte physiologic relevance and a potential therapeutic strategy for infertility.

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## INTRODUCTION

One of the earliest events in human development is the setting aside, or specification, of the cells that are destined to form the germ cells, the oocytes and sperm, of the adult (1). Germ cell specification occurs in the first 3 weeks of development. By the fourth gestational month in the female, approximately 1000–2000 germ cells migrate from a position outside the embryo into the genital ridge, proliferate to a population of five to seven million and then enter meiosis and differentiate to oocytes (1). By the fifth gestational month, gradual oocyte loss begins so that, by birth, there are approximately one million oocytes in ovarian follicles. In the adult, oocytes

may then encounter one of several fates: they may remain quiescent, may be recruited for further development and ovulation or may apoptose and die. Over time, without regeneration, the oocyte population is depleted in women until less than a thousand remain and menopause ensues (2–4). Approximately, 90% of women experience menopause in their early 50s. Ten percent may experience menopause prior to 46 years (often termed early menopause), and 1% may experience menopause at an age <40 years (frequently termed premature menopause or premature ovarian failure) (5,6).

Given these facts, the differentiation of oocytes from embryonic stem cells (ESCs), for studies of the genetic,

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epigenetic and environmental factors that affect oocyte development, is merited. Ultimately, the differentiation of functional oocytes from autologous stem cells, such as induced pluripotent stem cells (iPSCs), potentially provides a means to understand infertility and treat women with premature ovarian failure, reproductive aging and/or poor oocyte quality (7). However, critical aspects of ESC-derived germ cell development, especially oocyte maturation, have not been well-documented. Indeed, although several studies have demonstrated that human ESCs can differentiate into cell types of somatic and germ lineages including germ cell precursors, or primordial germ cells (PGCs) (8–13), germ cell differentiation has been largely limited to the earliest stages.

In contrast to humans, *in vitro* differentiation of putative ESC-derived oocytes and follicle-like structures has been reported in mice (14–18). However, the studies have been difficult to reproduce, and validation of ESC-derived germ cell identity, as frequently assessed by expression profile analysis of ESC-derived populations, has not been straightforward owing to the heterogeneity of ESC-derived cultures and similarity of genes expressed by ESCs and germ cells/oocytes (7,8,19). Moreover, physiologic relevance and functional significance have been difficult to define *in vitro* because very few germ cells are formed, and correlations of genetic and functional requirements between endogenous development and ESC-derived differentiation are lacking (20).

Oocyte enclosure in ovarian follicles shortly after birth in mice is essential for complete functional oocyte maturation through interaction with neighboring somatic granulosa cells in the ovary (21). In the absence of ovarian somatic cells and follicle formation, as in ectopic oocytes of the adrenal gland (22) or testes (23), the oocytes rapidly degenerate and are lost by 3–4 weeks after birth. In addition to the requirement of ovarian follicle formation and development for endogenous oocyte maturation *in vivo*, functional *ex vivo* maturation of oocytes has required fetal-to-newborn-stage ovary organ culture or transplantation (24–29). Historically, transplantation into an appropriate stem cell niche has been necessary to confirm germ and somatic cell identity and function (30–33).

In this study, we differentiated mouse ESCs to germ cells. We then characterized and compared the differentiated germ cells to undifferentiated ESCs at the single-cell level and further examined functional properties associated with endogenous oogenesis, such as their responsiveness to defined maturation media, genetic requirements for germline formation and maturation and entry into meiosis. Then, to test the function of oocytes differentiated *in vitro* and promote further maturation, we transplanted ESC-derived germ cells into a synchronized ovarian niche (Fig. 1A). Finally, we examined the feasibility of using the methods developed to construct a human fetal ovarian niche for promoting human oocyte development.

## RESULTS

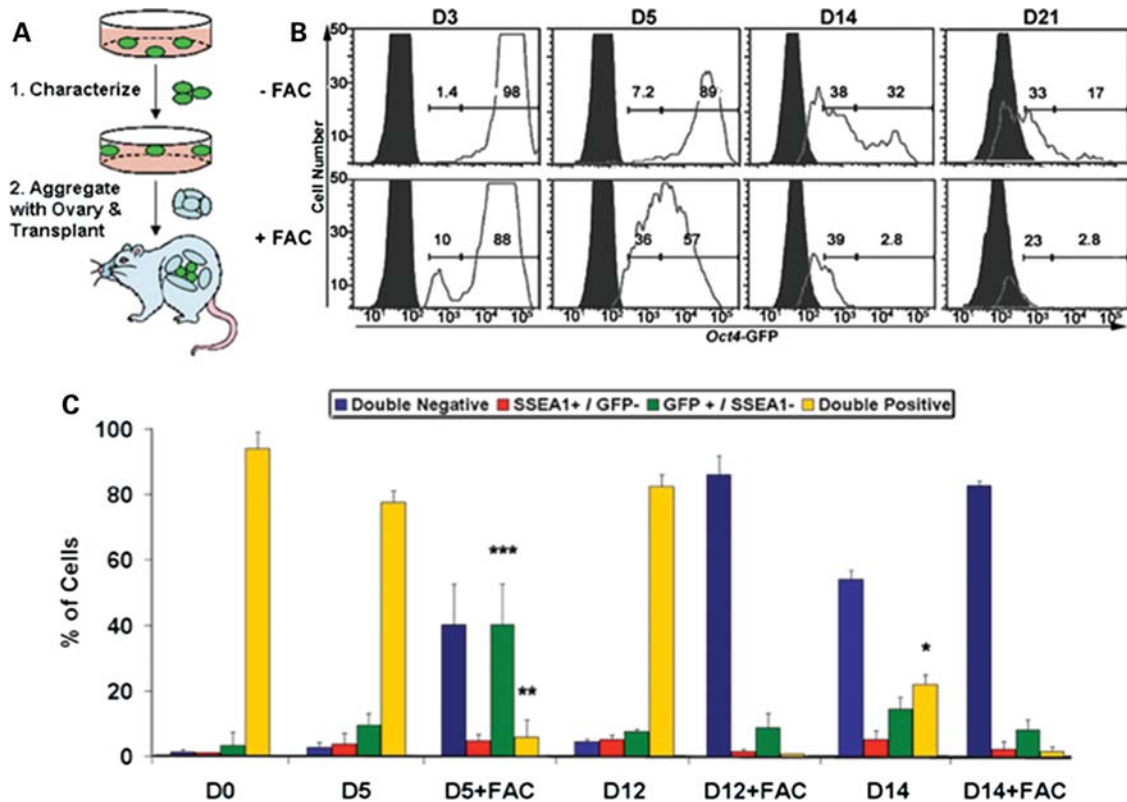
### Identification of germ cells and oocytes *in vivo* and *in vitro*

We differentiated mouse ESCs, derived from transgenic mice carrying a germ cell reporter ( $\Delta$ PE *Oct4* promoter—GFP

(34)), *in vitro* and sought to develop an effective strategy to identify putative ESC-derived germ cell differentiation and oocyte maturation based on flow cytometry analysis. However, a major challenge of developing effective methods to differentiate and isolate germ cells *in vitro* is the lack of markers to distinguish germ cells and oocytes from ESCs. To overcome this challenge, we turned to an examination of endogenous oocytes *in vivo* to find a suitable method. Although the *Oct4*-GFP reporter is expressed in early embryos and in the germline (35), oocytes from transgenic embryos expressed the GFP reporter at a low level of intensity relative to undifferentiated ESCs, PGCs from the genital ridges of either sex or pro-spermatogonia from fetal testes by flow cytometry analysis (Supplementary Material, Fig. S1A). In addition, 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 14.5 (e14.5) as reported previously (36) and confirmed by flow cytometry (Supplementary Material, Fig. S1B). Therefore, flow cytometry analysis was used to identify subpopulations of cells based on *Oct4*-GFP+ and SSEA1– status, in addition to a low intensity of GFP expression.

After determining that *Oct4*-GFP intensity, combined with SSEA1 expression on the cell surface, was diagnostic of 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 characterized 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 endogenous PGC culture and meiotic progression in the absence of feeder layer support (37). The FAC cocktail comprised anti-apoptotic (38), germ cell specification (39) and meiotic induction factors (40), including bone morphogenetic protein 4 (BMP4), retinoic acid, cytochrome p450, 26 (CYP26) inhibitor (R115866), stromal cell-derived factor 1 (SDF1), stem cell factor (SCF), basic fibroblast growth factor (bFGF), *n*-acetyl-cysteine and forskolin. We observed that, whereas almost all undifferentiated ESCs expressed *Oct4*-GFP at high intensity, cells with low-intensity GFP expression were formed by day 14 of differentiation along with a reduction in the percentage of cells expressing GFP (Fig. 1B). In addition, low-intensity GFP+ cells were increased in numbers and expedited, with the appearance of the low-intensity population by day 3 of differentiation, following FAC media treatment (Fig. 1B).

We also note that, concomitant with 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). Together, these observations indicated that the pattern of putative germ cell differentiation is similar in timeline to endogenous maturation of PGCs to an oocyte developmental program on e13.5. Moreover, 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 corresponding decrease in double-positive cells



**Figure 1.** ESC-derived germ cell identification. (A) In this study, mouse ESCs were differentiated as 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 number). (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. FAC media induced a significant elevation in the percentage of GFP+/SSEA1- and reduction in double-positive populations by day 5. Data are represented as mean ± SD ( $n = 3$ ); \* $P < 0.01$  to D12, \*\* $P < 0.01$  to D0, \*\*\* $P < 0.05$  to D0.

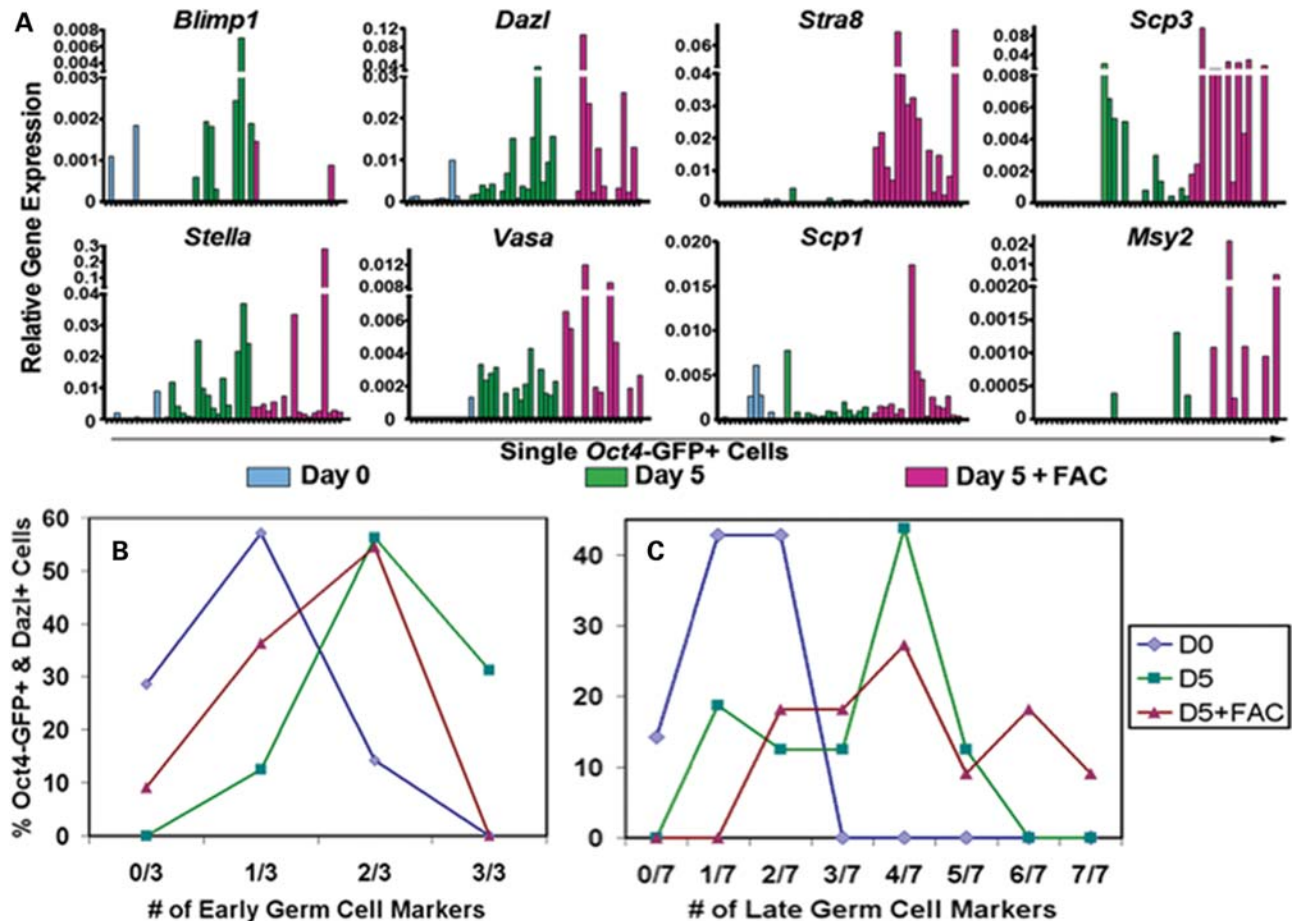
(Fig. 1C). These results indicated that germ cell differentiation and oocyte maturation, via this methodology, occurred along an endogenous developmental timeline.

### Single-cell analysis of ESC-derived germ cell identity and maturation

Our knowledge of ESC differentiation to specific lineages is derived from analysis of populations of differentiated cells. For example, in the case of studies of germ cell differentiation from ESCs, there are no reports of single-cell analysis that would allow us to distinguish gene expression profiles of individual ESCs from germ cells and determine the extent of germ cell maturation. Here, we examined gene expression in single cells that were differentiated for 5 days and isolated by fluorescence-activated cell sorting (FACS). Several findings emerged from this analysis. First, we observed that transcripts such as *Oct4*, *Stella*, *Nanos3* and *Vasa*, which are expressed early in germ cell development, were elevated in the double-positive population, whereas *Stra8* (stimulated by retinoic acid gene 8) and *Gdf9*, markers of later stages such as

meiotic entry and oocyte maturation, respectively, were increased in the GFP+/SSEA1- population (Supplementary Material, 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 (Supplementary Material, Fig. S2A). These 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.

Second, when we analyzed gene expression in GFP+ single cells following 5 days of differentiation in both the absence and presence of FAC media, we observed that the number of cells expressing markers diagnostic of germ cells was greater in the differentiated population than in undifferentiated ESCs. This included markers of PGCs (early genes: *Blimp1*, *Stella*, *Dazl* and *Vasa*) and meiotic germ cells (late genes: *Stra8*, *Scp1*, *Scp2*, *Scp3*, *Gcnf*, *Mlh1* and *Msy2*). Although



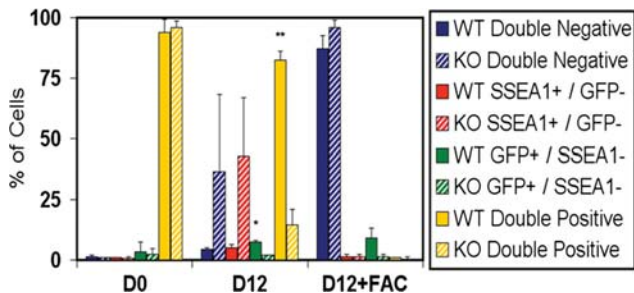
**Figure 2.** Characterization of ESC-derived germ cell identity and maturation. (A) *Oct4*-GFP<sup>+</sup> ESC-derived germ cell identity was confirmed by single-cell quantitative RT-PCR expression profiling of pre-meiotic and meiotic germ cell transcripts before and after 5 days of *in vitro* differentiation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

some undifferentiated GFP<sup>+</sup> ESCs did express detectable levels of germ cell marker transcripts (Fig. 2A and Supplementary Material, Fig. S2B), a greater percentage of GFP<sup>+</sup> 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 downregulated in endogenous PGCs by the time of oogenesis (41) (Supplementary Material, Fig. S3).

Third, we noted that at the single-cell level, a single *Oct4*-GFP<sup>+</sup> cell following differentiation was more likely to express multiple germ cell marker transcripts in the same cell than an undifferentiated GFP<sup>+</sup> ESC. For example, none of the *Oct4*-GFP<sup>+</sup> ESCs that also expressed *Dazl* transcript was found to express more than two additional early or late germ cell transcripts, with only 14% of ESCs expressing two early markers (Fig. 2B and C). In contrast, 87% of GFP<sup>+</sup> and *Dazl*<sup>+</sup> cells from day 5 of differentiation without FAC media expressed two to three additional early germ cell

markers (Fig. 2B), and 70% contained three to five late germ cell markers (Fig. 2C). Similar to cells from EBs without FAC media, 55% of *Oct4*-GFP<sup>+</sup> and *Dazl*<sup>+</sup> cells from EBs with FAC media expressed two additional early germ cell markers (Fig. 2B). Furthermore, 81% of GFP<sup>+</sup> and *Dazl*<sup>+</sup> cells from EBs with FAC media contained more than two late markers with up to seven markers expressed together in the same cell, which is consistent with functional FAC media induction of GFP<sup>+</sup> germ cell maturation (Fig. 2C).

Finally, our analysis of single cells indicated that in addition to expressing a greater number of germ cell markers within the same cell, single-*Oct4*-GFP<sup>+</sup> cells from day 5 EBs contained significantly elevated levels of most germ cell transcripts analyzed compared with undifferentiated *Oct4*-GFP<sup>+</sup> ESCs (Supplementary Material, Fig. S4). To summarize, undifferentiated ESCs occasionally expressed germ cell transcripts, but differentiated ESC-derived germ cells did so more frequently,



**Figure 3.** ESC-derived germ cell development requires *Dazl*. The percentage of early (*Oct4*-GFP+ and SSEA1+; yellow) and late (*Oct4*-GFP+ and SSEA1-; green) ESC-derived germ cell populations significantly decreased, and GFP- somatic cells increased, by flow cytometry analysis following *Dazl* null (KO, striped bars) ESC differentiation for 12 days (-/+ FAC media) compared with *Dazl* WT (solid bars) lines, suggesting *in vitro* ESC-derived germ cell commitment, or maturation, along an endogenous developmental timeline with a shared genetic program. Data are represented as mean  $\pm$  SD ( $n = 3$ ); \* $P < 0.05$  GFP+/SSEA1- WT to KO, \*\* $P < 0.05$  double-positive WT to KO.

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 date, studies of germ cell differentiation from ESCs have been limited in genetic analysis. To further inspect *in vitro* ESC-derived germ cell identity and maturation, ESC lines, containing the  $\Delta$ PE *Oct4*-GFP reporter, were obtained from mice carrying a *Dazl* (*Deleted in azoospermia-like*) null mutation and differentiated alongside wild-type (WT) 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 (34,42,43). As expected, *Dazl* null (knockout—KO) ESC lines displayed a significantly reduced percentage of double-positive PGCs (15% KO compared with 83% WT) and GFP+/SSEA1- germ cells (2% KO compared with 8% WT without FAC; 2% KO compared with 9% WT with FAC) in comparison with WT 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).

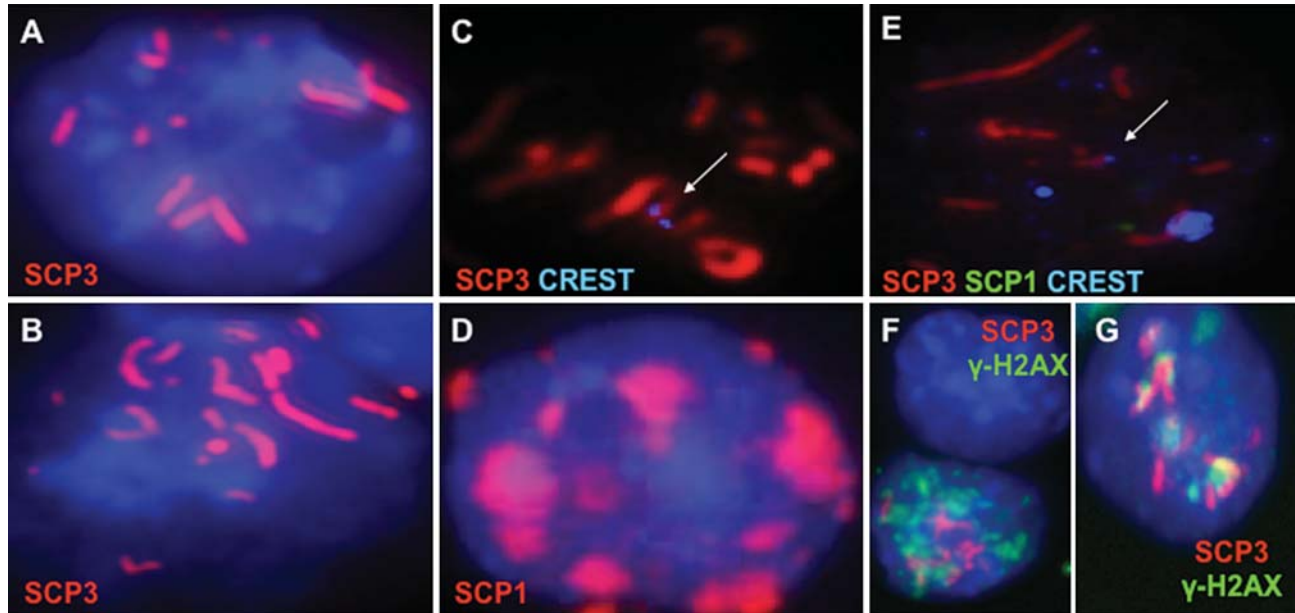
Hence, the functional genetic requirement of *Dazl* for germ cell maturation was shared between endogenous germ cell development *in vivo* and ESC-derived germ cell differentiation *in vitro*. Moreover, analysis of the *Dazl* null phenotype also suggested ESC-derived commitment, or maturation, to a sex-specific developmental program by 12 days of *in vitro* differentiation without FAC media, thereby paralleling the manifestation of the *Dazl* null phenotype by e12.5 *in vivo* and corresponding to a timeline of endogenous PGC commitment to an oocyte fate in the absence of signals from the fetal testis (22,44–46).

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

Our results above indicated that we had devised a strategy to isolate and characterize ESC-derived germ cells, that single cells had distinguishing characteristics of germ cells and that the genetic requirements for germ cell development *in vitro* paralleled those of germ cell development *in vivo*. However, a landmark event in germ cell development is the initiation and progression through meiosis. Thus, we next examined whether ESC-derived oocytes entered and progressed through meiosis. As shown, we observed that approximately 1–3% of *Oct4*-GFP+ cells initiated meiosis, as evidenced by synaptonemal complex protein (SCP) expression and chromosomal localization (Fig. 4). However, we detected only partial chromosomal alignment of SCP3, and although other meiotic proteins such as SCP1 were expressed in the GFP+ cells, nuclear localization of SCP1 was focal and indicative of limited meiotic progression (Fig. 4D). We did observe, however, that CREST centromere staining was occasionally co-localized telocentrically with the elongated SCP structures confirming chromosomal alignment (Fig. 4C and E). In addition, 10% of the GFP+ cells with partial SCP3 alignment also expressed nuclear  $\gamma$ -H2AX, a marker of meiotic DNA double-strand breaks, 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 and G) (47).

In order to optimize meiotic progression, we used an alternative ESC line that carried a meiotic reporter, *Stra8*-GFP, to further examine differentiation in experiments that paralleled those described above (Supplementary Material, Fig. S5A–C). Given the role of *Stra8* in meiosis, a greater percentage of the *Stra8*-GFP+ cells had initiated meiosis (up to 10–15%), but these cells also exhibited a block in meiotic progression (Supplementary Material, Fig. S5D). Furthermore, whereas meiotic cells were detected by day 15 of differentiation, similar in timeline to endogenous oogenesis, the maximum percentage of GFP+ germ cells in meiosis with expression and partial chromosomal localization of SCP3 was unexpectedly observed by day 5 of ESC differentiation in both cell lines (Fig. 5A and B). Nevertheless, the analysis of ESC-derived germ cells at these earlier time points of differentiation, or after treatment with FAC-supplemented media, did not significantly improve the extent of meiotic progression for either line. In addition, alternative approaches, based on previous studies of endogenous germ cell entry into meiosis (44,45,48), also were unable to overcome the block to meiotic progression, including the co-culture of the differentiating EBs with female genital ridge from e11.5 embryos and overexpression of the intrinsic regulator of meiotic entry and progression, *Dazl* (data not shown).

Instead, we observed the most significant enhancement of meiosis *in vitro* when ESCs were differentiated in co-culture with dissociated e5.5 embryos. This may reflect timing *in vivo*, as endogenous PGCs are specified from the epiblast at this stage. We observed that e5.5 embryo co-culture induced a significant increase in the percentage of ESC-derived germ cells initiating meiosis compared with non-co-culture controls on day 5 of differentiation (14% of *Oct4*-GFP+; 30% of



**Figure 4.** ESC-derived oocytes enter meiosis but are limited in meiotic progression *in vitro*. (A–C) Immunofluorescence staining 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 and E) CREST centromere staining co-localized with SCP3 and confirmed SCP3 alignment on some, but not all, chromosomes (arrows). (F and G)  $\gamma$ -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) and (E). Magnification is  $\times 630$ .

*Stra8*-GFP+) (Fig. 5C). However, as in previous experiments with alternative strategies, the extent of ESC-derived germ cell progression through prophase I of meiosis was not improved by the e5.5 embryo co-culture.

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

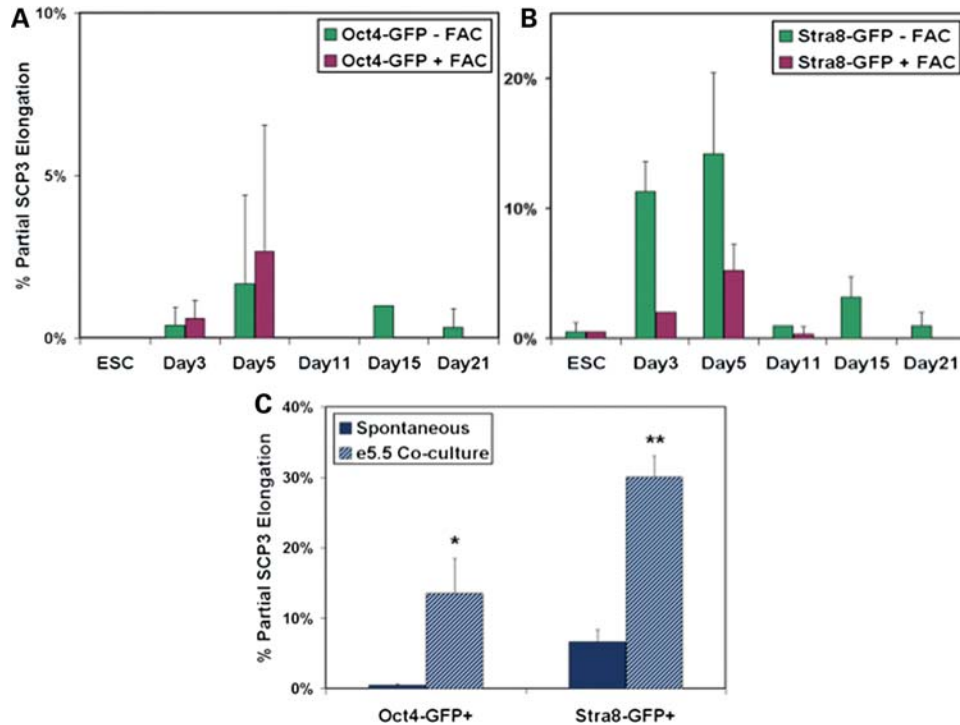
Since meiotic progression was incomplete and follicle formation was not observed *in vitro*, we next sought to test whether we could achieve oocyte maturation via transplantation (Figs 6 and 7). Ovarian follicle formation occurs just after birth in mice which have 21 days of gestation, and re-aggregated newborn ovaries support robust endogenous ovarian follicle development and oocyte maturation following transplantation under the kidney capsule (49) (Fig. 7A). 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 WT newborn ovarian tissue. We then transplanted the co-aggregates under the kidney capsule of recipient mice for 3 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 *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. 6A–F) and others at a primordial/primary stage (Figs 6H and 7B)

of development. The primary follicles consisted of an ESC-derived oocyte that recruited a single layer of endogenous cuboidal granulosa cells, surrounded by a basement membrane, with robust *Oct4*-GFP expression above background staining of endogenous WT oocytes (Fig. 6G and I). In addition, the oocytes contained a single large germinal vesicle nucleus and were 10–20  $\mu$ m 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. 7C–H) and expressed the germ cell marker TRA98 and peri-nuclear Balbiani body-like oocyte marker GM130, but did not express the stem cell/progenitor marker SSEA1 (Fig. 7F–H) (50).

To further examine GFP+ oocyte origin, we isolated GFP+ cells by FACS, following transplantation, genotyped them at 50 polymorphic single-nucleotide polymorphism (SNP) markers across the mouse genome and detected homozygous parental ESC strain sequence for every marker analyzed but did not detect WT newborn ovary donor or recipient mouse strain sequences, thereby confirming an ESC origin of the *Oct4*-GFP+ oocytes (Fig. 7I).

Finally, to determine the potential of developing a human ovarian niche for future studies of human ESC-derived germ cell transplantation, we obtained human fetal ovaries (after approval by the appropriate institutional review board) and transplanted intact or re-aggregated tissues, as described above, into immuno-deficient mice. Following 2 months of transplantation, grafts were analyzed for oocyte survival and development in ovarian follicles (Fig. 8). Intact human fetal ovaries supported the survival of oocytes in primordial-stage follicles. Interestingly, after dissociation and re-aggregation, the human fetal ovaries were still able to support oocyte



**Figure 5.** Embryo co-culture improves the initiation of meiosis but confirms an *in vitro* block to ESC-derived oocyte maturation. (A and B) ESCs containing the *Oct4*-GFP or *Stra8*-GFP reporter were differentiated up to 21 days in standard differentiation media or in media supplemented with FAC. GFP+ germ cells were isolated by FACS and examined for meiotic entry and progression by SCP3 protein expression and elongated chromosomal localization. The maximum percentage of cells entering meiosis was observed by 5 days of differentiation from both cell lines; however, meiotic progression remained partial, and complete progression was not detected for any time point, cell line or treatment. Data are represented as mean  $\pm$  SD ( $n = 3$ ). (C) ESCs were differentiated in suspension for 5 days as EBs without or with dissociated e5.5 embryo co-culture, and GFP+ cells were analyzed for meiosis. The e5.5 embryo co-culture induced a significant increase in the percentage of ESC-derived germ cells that entered meiosis from both cell lines. However, e5.5 embryo co-culture did not improve the extent of meiotic progression which remained partial. Data are represented as mean  $\pm$  SD ( $n = 2$ ). \*\*\* $P < 0.05$ .

survival and organization into primordial follicles. Thus, we demonstrated the feasibility of physiologic human ovarian niche transplantation for future studies of human ESC-derived oocyte maturation.

## DISCUSSION

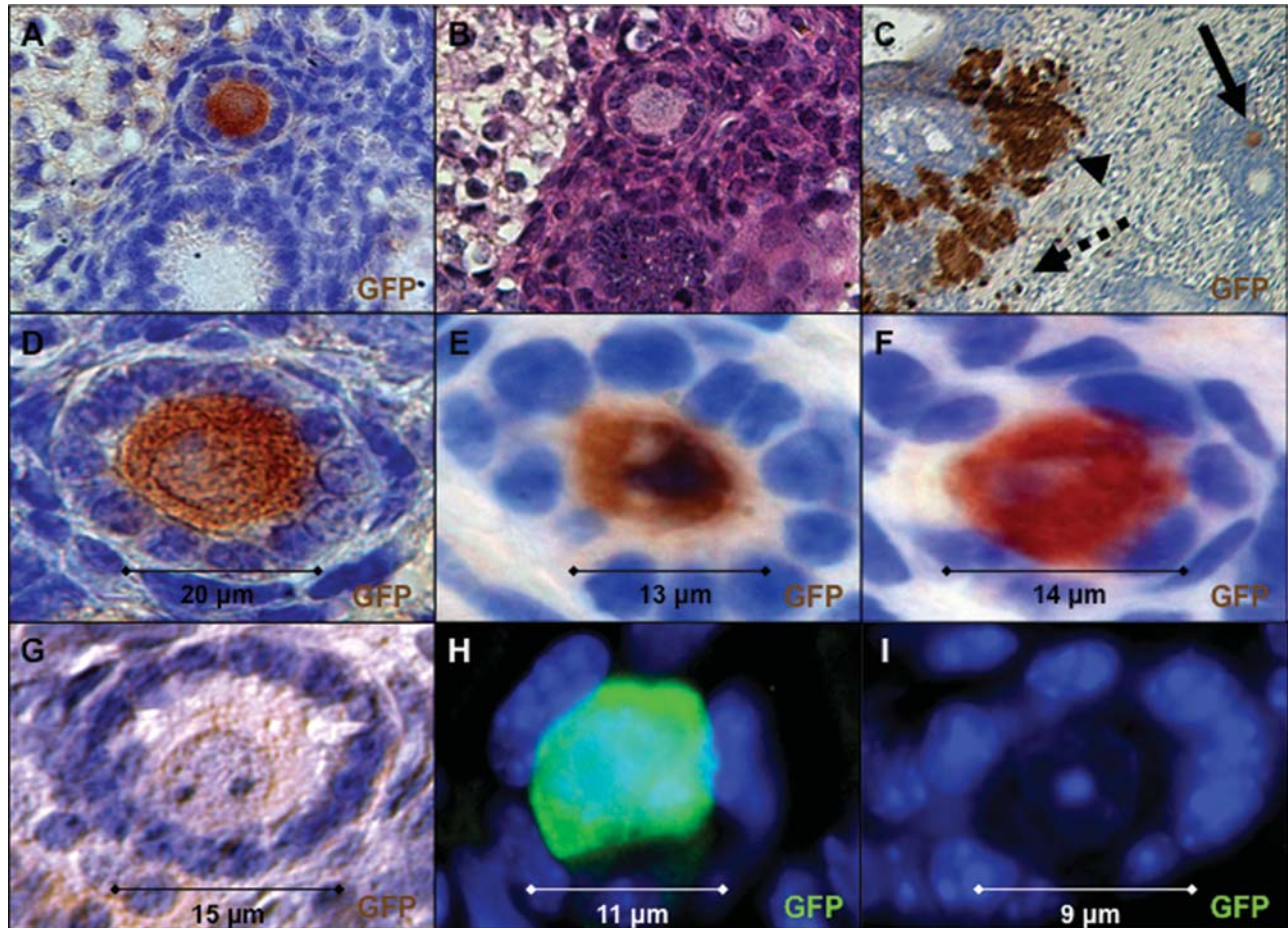
This work documents *bona fide* oocyte differentiation from ESCs by describing functional genetic, biochemical and cellular events that define endogenous oocyte development *in vivo*. The evidence for authentic germ cell differentiation and maturation in this study includes gene expression analysis at the single-cell level, ability of ESC-derived germ cells to respond to defined maturation media, a shared requirement of ESC-derived and endogenous germ cells for the function of the *Dazl* gene and finally, the ability of ESC-derived oocytes to contribute to folliculogenesis by transplantation into an ovarian niche. We suggest that the methods and results outlined above may provide a platform for the development of new strategies for regenerative medicine in the arena of reproductive health.

We first present a novel method for the effective isolation of germ cells and oocytes on the basis of properties of endogenous germ cell development. In contrast to several studies that used SSEA1 for positive selection and enrichment of early-stage ESC-derived PGCs (11,13,17,51), we used SSEA1 for

negative selection to allow the isolation of *Oct4*-GFP+ and SSEA1- post-PGC-stage germ cells that exhibited properties of meiosis and maturation. In addition, this method may be useful for the isolation of later stage human ESC-derived germ cells because, similar to mouse, SSEA1 expression is substantially reduced in germ cells of the human fetal ovary by the end of the first trimester upon oocyte initiation of meiosis (52). Furthermore, SSEA1- selection will help eliminate the possibility of teratoma formation following transplantation due to the ectopic reprogramming of human ESC-derived PGCs (53).

With this method, we were able to isolate and examine single putative germ cells to confirm germ cell identity and maturation. To our knowledge, this is the first report of ESC-derived germ cell analysis at the single-cell level and discrimination of ESC and germ cell gene expression profiles. Single-cell expression profiling confirmed that an authentic ESC-derived germ cell expresses a more coordinated and extensive set of germ cell-specific markers compared with an undifferentiated ESC that may randomly mis-express an occasional marker during culture.

Furthermore, we expect that ESC-derived germ cells should possess the ability to respond to endogenous environmental cues, defined by germ cell maturation FAC media, which induced the accelerated maturation of germ cells and oocytes by day 5 of *in vitro* differentiation. For the first



**Figure 6.** 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 ( $\times 200$ ). (B) Hematoxylin and eosin 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) ( $\times 100$ ). (D) High magnification of (A) ( $\times 630$ ). (E and F) Additional ESC-derived oocytes in primary follicles with robust GFP+ immunoperoxidase staining above background endogenous GFP– oocytes (G) derived from WT newborn ovary in the same section ( $\times 630$ ). (H) *Oct4*-GFP+ ESC-derived oocytes in primordial/primary follicles were also detected by immunofluorescence stain for GFP above background staining of WT oocytes (I) from newborn ovary located adjacent to (H) ( $\times 630$ ). (A–G) Blue is hematoxylin counter stain. (H and I) Blue is DAPI.

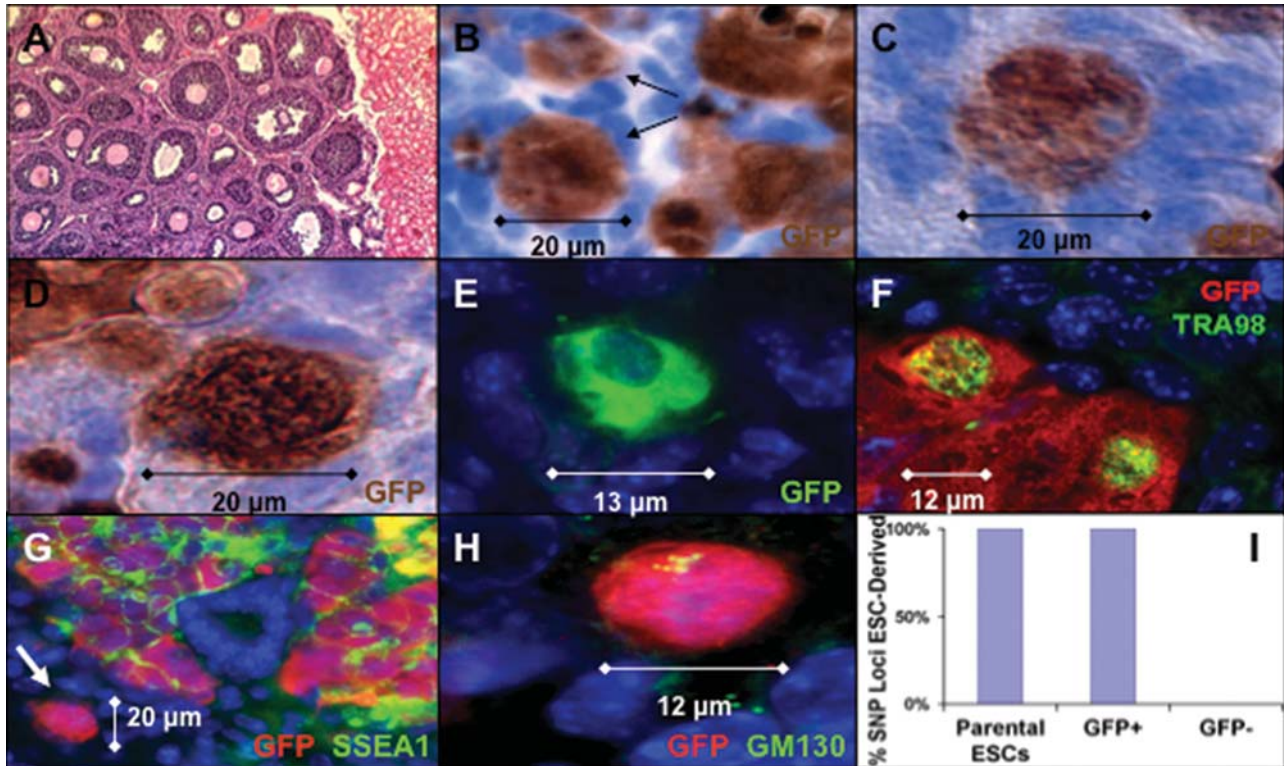
time, defined media containing multiple factors were used to accomplish ESC-derived germ cell maturation. Previous studies had differentiated mouse ESCs in defined media containing a single factor, such as retinoic acid (15,17,33,51) or BMPs (9,32,54). However, endogenous germ cells require multiple factors, such as SDF1 and SCF signaling, from somatic cells in the genital ridge (55–58) and ovary (25,59,60). In addition, the CYP26 inhibitor, R115866, was added to the cocktail in this study to increase the potency of retinoic acid and prevent its metabolism (44,45). Of note, retinoic acid has not yet been used in studies of germ cell differentiation from human ESCs. Retinoic acid may be of particular importance in promoting human germ cell survival and meiotic maturation, comparable with mouse (40,61), since endogenous human germ cells also express *STRA8* in the human fetal ovary by the end of the first trimester (62).

Another critical component of this study is the emphasis on correlating *in vivo* and *in vitro* requirements for germ cell development, such as the function of *Dazl*. Undifferentiated ESCs expressing both *Oct4*-GFP and SSEA1 were not affected

by a homozygous genetic null mutation in the *Dazl* gene, even though ESCs express robust levels of *Dazl*, which was consistent with its germ cell-specific function. In contrast, the percentage of *Dazl* null ESC-derived germ cells was significantly reduced by 12 days of differentiation. Thus, ESC-derived germ cells shared a functional genetic program requirement and developmental timeline with endogenous germ cells, further confirming germ cell identity and maturation. Similarly, *DAZL*, or other germ cell-specific transcript, knockdown by RNAi in human ESCs, or differentiation of human iPSCs from patients with congenital infertility, including Turner syndrome, will assist in the validation of human ESC-derived germ cell differentiation.

Meiosis is a unique functional hallmark of endogenous germ cell development, and efficient ovarian follicle formation may require the prior completion of fetal oocyte progression through prophase I of meiosis (63). Similar to endogenous fetal oocytes, ESC-derived oocytes also entered meiosis. We detected SCP3 and  $\gamma$ -H2AX expression and localization to the chromosomes, and SCP1 was expressed in the nucleus,





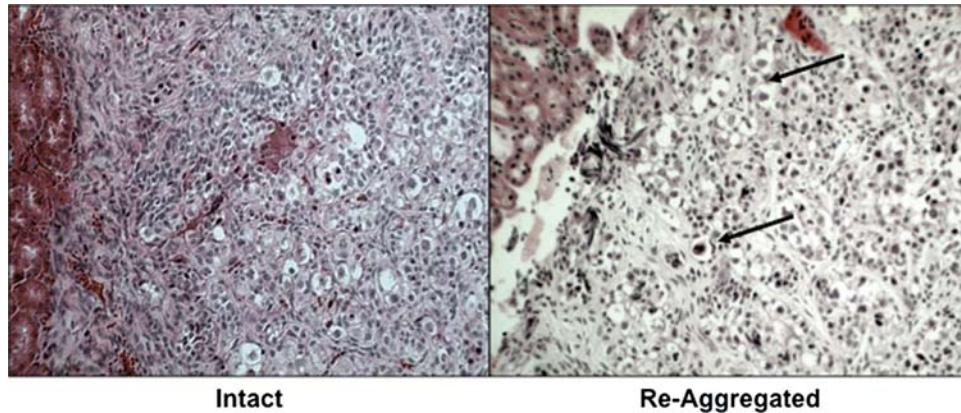
**Figure 7.** ESC-derived oocyte characterization following transplantation. (A) Hematoxylin and eosin stain of newborn ovarian tissue following re-aggregation without ESCs and transplantation revealed robust oocyte maturation and folliculogenesis adjacent to kidney tissue ( $\times 100$ ). (B) ESC-derived oocytes (arrows) enclosed in primordial/primary-stage follicles were detected by immunoperoxidase staining for GFP following transplantation. (C and 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) ( $\times 200$ ). (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, whereas 0% of GFP- cell loci were homozygous. (A–D) Blue is hematoxylin counter stain. (E–H) Blue is DAPI. (B–F and H)  $\times 630$ .

indicating that ESC-derived germ cells could enter and progress through early stages of meiotic prophase I. However, SCP3 chromosomal alignment remained partial, and SCP1 was not elongated, confirming an *in vitro* block to ESC-derived meiotic progression (20). Incomplete meiotic progression was surprising since endogenous PGCs enter and progress completely through meiotic prophase I according to a germ cell-intrinsic timing mechanism (22,37,46,64), and suggested improper germ cell specification *in vitro*. Encouragingly, e5.5 (germ cell specification-stage) embryo co-culture resulted in a significantly increased percentage of ESC-derived germ cells initiating a meiotic program. However, progression through prophase I remained partial, revealing that additional factors, possibly related to germline specification, are needed to direct complete *in vitro* ESC-derived oocyte meiosis.

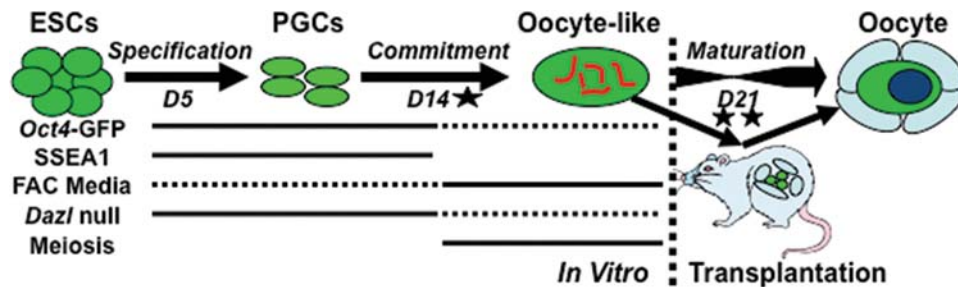
Finally, we also noted an *in vitro* block to ovarian follicle formation (Fig. 9). Soon after birth in mice, meiotic oocytes are surrounded by somatic ovarian granulosa cells to form follicles that are essential for functional oocyte maturation. In fact, endogenous granulosa cells require oocyte-independent activation of critical signaling pathways to direct sex determination of the ovary for subsequent oocyte survival and development in follicles (65–69). Thus, we co-aggregated and transplanted ESC-derived meiotic oocytes with newborn mouse ovarian tissue containing somatic granulosa cells that

are primed to direct follicle formation and oocyte development. Transplantation into an appropriate endogenous niche has traditionally been used as a litmus test of cell type identity and function. For example, transplantation to germ cell-depleted testis was used to determine endogenous and ESC-derived spermatogonial stem cell potential and to achieve functional spermatogenesis (30,32,33). However, before this study, transplantation to the ovary had not been used to test the physiologic relevance of ESC-derived oocytes. Indeed, following transplantation, ESC-derived oocytes integrated into the ovarian niche, recruited somatic granulosa cells from the mouse ovary and directed follicle formation and development to the primary follicle stage (Fig. 9).

Considerable work remains before safe and effective clinical translation can be realized. ESC-derived oocytes in primary follicles will need to be further matured, fertilized and shown to support the development of healthy offspring with respect to karyotype and epigenetic reprogramming. To promote further maturation, ESC-derived oocytes can be transplanted for longer periods of time to allow for the development of antral-stage follicles, or, alternatively, the primordial-to-primary-stage follicles that were generated in this study can be isolated and matured *in vitro*, comparable with isolated endogenous oocytes in primordial follicles that later supported the production of offspring (70). Furthermore,



**Figure 8.** Development of a human ovarian niche via human fetal ovary re-aggregation and transplantation. Intact or re-aggregated 20 week human fetal ovaries were transplanted under the kidney capsule for 2 months and analyzed for the support of oocyte survival and development in ovarian follicles by hematoxylin and eosin staining. Oocytes in primordial-stage follicles were present in both samples, indicating that, similar to mouse newborn ovaries, human fetal ovaries supported the re-organization of follicles following re-aggregation and transplantation and may be utilized for the direction of human ESC-derived oocyte maturation in follicles via transplantation. Arrows show clusters of oocytes in primordial follicles ( $\times 100$ ).



**Figure 9.** 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); 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).

clinical treatment for female infertility will also require the use of autologous stem cells, such as iPSCs, for the derivation of genetically related germ cells (13). Human iPSC-derived germ cells could then be matured via co-aggregation with human ovarian tissue and transplantation to a site preferably less invasive than the kidney capsule in order to direct oocyte maturation (71,72). As described in this study, the human fetal ovarian niche may be suitable for this purpose. However, an autologous niche would also be advantageous for clinical translation, including transplantation into the adult ovary of the patient (73,74) or co-aggregation with iPSC-derived somatic granulosa cells. Along with many scientific challenges, the future derivation of mature gametes from human stem cells for clinical purposes will be accompanied by several ethical and policy issues (75).

The clinical relevance of research involving stem cell-derived oocytes is unquestionable (7). 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 USA alone, nearly 10 000 fresh and 5000 frozen embryo transfers were performed using donor oocytes (Centers for Disease Control and Prevention, 2005 Artificial Reproductive Technology

Report: National Summary; <http://apps.nccd.cdc.gov/ART2005/nation05.asp>). This number of cycles is most certainly an underestimate of the true clinical need for functional oocytes, because not all couples with infertility on the basis of ovarian failure or diminished ovarian reserve will choose to pursue oocyte donation. 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. 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 understand better some of the causes of abnormal offspring in spontaneous conceptions occurring among the general population.

## MATERIALS AND METHODS

### ESC culture and differentiation

Transgenic  $\Delta$ PE *Oct4*-GFP, *Dazl* WT and null, ESC (XX) lines were derived from C57BL/6-FVB/N mice as described

(34), and the gene trap *Stra8*-GFP ESC (XY) line was obtained from the Canadian Mouse Mutant Repository (clone ID 339H10). Undifferentiated ESCs were cultured on irradiated mouse embryonic fibroblasts in standard ESC media containing Dulbecco's modified Eagle's medium with high glucose and 2 mM L-glutamine, 1 mM sodium pyruvate, 100  $\mu$ M non-essential amino acids, 15% fetal bovine serum (all Invitrogen), 100  $\mu$ M 2-mercaptoethanol (Sigma) and 1000 U/ml of LIF (Millipore); plus 165  $\mu$ g/ml of 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) and without LIF on ultra-low attachment plates (Corning). Alternatively, ESCs were differentiated in FAC media consisting of standard EB media supplemented with a germ cell factor cocktail: mouse SCF 100 ng/ml, mouse SDF1 20 ng/ml, mouse bFGF 20 ng/ml, mouse BMP4 50 ng/ml (all R&D Systems), *N*-acetylcysteine 1 mg/ml, Forskolin 5  $\mu$ M, retinoic acid 1  $\mu$ M (all Sigma) and CYP26 inhibitor R115866 1  $\mu$ M (Johnson & Johnson).

#### Flow cytometry and FACS

ESCs, EBs and fetal gonads were dissociated to single cells with 0.25% Trypsin (Invitrogen) for 5–10 min at 37°C, or first treated with 1 mg/ml each of Collagenase IV and Dispase (both Invitrogen) for 20 min at 37°C prior to trypsinization for adult tissues, and re-suspended in standard media. Cells were strained through a 40  $\mu$ m filter (BD Biosciences) 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 (1:20, Abcam) for 20 min on ice, washed, incubated with APC-conjugated anti-mouse IgM secondary antibody (1:200, Jackson Immunoresearch) for 20 min 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) microfluidics system for RT-PCR, as described (34). In brief, we pre-amplified samples by treating single cells (Fig. 2A and Supplementary Material, Fig. S2B) or 50 cells (Supplementary Material, Fig. S2A) per sample per time point following the manufacturer's protocol (Fluidigm Corporation) using TaqMan gene expression assays (Applied Biosystems) as indicated in the respective figures. Then, 2.25  $\mu$ l of pre-amplified cDNA was mixed with 2.5  $\mu$ l of 2 $\times$  Universal Master Mix (Applied Biosystems) and 0.25  $\mu$ l of sample loading buffer (Fluidigm Corporation) and loaded into the sample inlets of the 96  $\times$  96 Dynamic Array (Fluidigm Corporation). For each probe, the reaction mix contained 2.5  $\mu$ l of 2 $\times$  TaqMan Gene Assay and 2.5  $\mu$ l of assay loading buffer (Fluidigm Corporation) for loading into the assay inlets on the Dynamic Array. Each

sample had two 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 20  $\mu$ l of hypo-extraction buffer (pH 8.2) [30 mM Tris, pH 8.2, 50 mM sucrose, 17 mM citric acid, 5 mM EDTA, 0.5 mM DTT and 1% protease inhibitor cocktail (all Sigma)] for 30 min at room temperature. Then, 60  $\mu$ l of 100 mM sucrose was added, and the cell suspension was spread onto slides pre-coated with 1% paraformaldehyde (USB Corporation) and 0.15% Triton X-100 (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), rabbit anti-SCP1 (1:500 Abcam), goat anti-SCP1 (1:50 Santa Cruz), human CREST (1:100, Antibodies Incorporated) and mouse anti- $\gamma$ -H2AX (1:500, Millipore). Slides were subsequently incubated with secondary antibodies for 30 min, 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).

#### Ovarian tissue aggregation and kidney capsule transplantation

Newborn ovaries were dissected from WT CD-1 female pups (Charles River), dissociated to single-cell suspensions by 10 min 0.25% trypsinization and pipetting 10–20 times and re-suspended in standard EB media. The ovarian cell suspension from four female pups per graft was then mixed with or without 100 000 ESC-derived cells post-FACS sort and 0.2 mg/ml of phytohemagglutinin (Sigma). Cell suspensions were pelleted into grafts at 10 000g for 1 min and incubated overnight in standard EB media on CM cell culture inserts (Millipore) at 37°C. 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](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) for 30 min, permeabilized in 0.1% Triton X-100 (Sigma) for 5 min, blocked with 10% chicken serum in TBST overnight and incubated with primary antibody in TBST with 1% serum for 1 h at room temperature. Primary antibodies included anti-GFP rabbit monoclonal (1:1000, Abcam), anti-TRA98 rat monoclonal (1:500, B-Bridge), anti-SSEA1 mouse monoclonal (1:100, Abcam or Developmental Studies

Hybridoma Bank) and anti-GM130 mouse monoclonal (1:100, BD Biosciences). After washing in TBST, slides were incubated with secondary antibody for 30 min at room temperature. For immunofluorescence, secondary antibodies included chicken anti-rabbit, rat and mouse (1:1000, Invitrogen), and coverslips 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 min at room temperature before permeabilization. Slides were blocked overnight with 10% goat serum (Abcam) in TBST and incubated with primary antibody for 1 h, biotinylated goat anti-rabbit secondary antibody (1:200, Vector Labs) for 30 min, Vectastain ABC solution (Vector Labs) for 30 min, ImmPact DAB substrate (Vector Labs) for 1–5 min, 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), and was then analyzed for the sequence of 50 SNPs that were polymorphic between C57BL/6-FVB/N and CD1-BALB/C mouse strains by the Jackson Laboratory Genome Scanning Service. The SNP panel contained three markers on chromosomes 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 12, two markers on chromosomes 11, 13, 14, 15, 16, 17, 18 and 19, and one marker on the X chromosome. SNP sequences at each locus were compared with the parental ESC line and to each of the four mouse strains using a modified Amplifluor fluorescent PCR-based system.

### Statistical analysis

Data are represented as mean  $\pm$  standard deviation (SD). Statistical significance was determined with Excel (Microsoft) using an unpaired Student's *t*-test with two-tailed distribution of two-sample unequal variance. Significance of values in Supplementary Material, Fig. S4 was determined by Prism (GraphPad Software) two-way ANOVA with Bonferroni post-test.

### SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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