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Oocyte differentiation is genetically dissociable from meiosis in mice

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Oogenesis is the process by which ovarian germ cells undertake meiosis and differentiate to become eggs. In mice, *Stra8* is required for the chromosomal events of meiosis to occur, but its role in differentiation remains unknown. Here we report *Stra8*-deficient ovarian germ cells that grow and differentiate into oocyte-like cells that synthesize zonae pellucidae, organize surrounding somatic cells into follicles, are ovulated in response to hormonal stimulation, undergo asymmetric cell division to produce a polar body and cleave to form two-cell embryos upon fertilization. These events occur without premeiotic chromosomal replication, sister chromatid cohesion, synapsis or recombination. Thus, oocyte growth and differentiation are genetically dissociable from the chromosomal events of meiosis. These findings open to study the independent contributions of meiosis and oocyte differentiation to the making of a functional egg.

Oogenesis is the process by which an ovarian germ cell becomes a female gamete, an egg. A functional egg is capable of undergoing fertilization and giving rise to a developing embryo, and it should contribute to that embryo exactly one copy of each chromosome. The former capability is achieved through growth and differentiation of the cell, and the latter is achieved through the chromosomal mechanics of meiosis. These cellular and chromosomal events of oogenesis are closely coordinated, but whether they are interdependent remains unknown.

In mice, early stages of germ cell development unfold identically in XX and XY embryos. In both sexes, primordial germ cells (PGCs) arise from the epiblast and migrate to the gonad[1]. There, both XX and XY PGCs transition to become gametogenesis-competent cells (GCCs). Both XX and XY GCCs are competent to respond to signals from the gonadal soma to initiate meiosis and undergo sexual differentiation[2,3].

The first morphological difference between germ cells in XX and XY gonads appears when meiotic chromosome condensation—a defining feature of prophase of meiosis I—occurs in ovarian (XX) germ cells during fetal development[1,4]. (Testicular germ cells do not enter meiotic prophase until well after birth.) Thus, the fetal onset of meiotic prophase marks the onset of oogenesis. Indeed, fetal entry into meiotic prophase commonly serves as a proxy for subsequent oocyte growth and differentiation, and the absence of meiotic prophase is conventionally taken as evidence that fetal germ cells have adopted a spermatogenic fate[1,4–10]. This interpretation assumes that fetal meiotic initiation is necessary for oocyte growth and differentiation to occur—an assumption that has not been tested before the studies reported here.

Meiotic initiation is governed by the retinoic acid–responsive gene *Stra8* (refs. 11,12). Upon receiving the retinoic acid signal and expressing *Stra8*, ovarian germ cells replicate their chromosomes and enter prophase of meiosis I (hereafter referred to as 'meiotic prophase'). Oocytes then progress through this prophase, arresting at its diplotene stage. A few days later, these cells differentiate to become primordial oocytes while remaining arrested in meiotic prophase. Throughout the reproductive life of the female, prophase-arrested primordial oocytes are recruited to differentiate into full-grown oocytes. (Meiosis is not resumed until ovulation and is not completed until fertilization.) Thus, in mice, oocytes grow and differentiate only after reaching the diplotene stage of prophase. Oocyte growth and differentiation during arrest in meiotic prophase are conserved in humans, frogs, flies and worms, among other animals[13,14]. In rats, the timing of primordial follicle formation can be altered by manipulating chromosome desynapsis (a hallmark of progression to diplotene and completion of prophase)[15]. Collectively, these observations are consistent with meiotic initiation and prophase being required for oocyte differentiation, which explains why many investigators have held this view.

Recent advances in embryonic stem (ES) and induced pluripotent stem (iPS) cell technologies have motivated efforts to derive oocytes *in vitro* from these and other undifferentiated cell types. Several laboratories have reported deriving cells that morphologically resemble oocytes[16–22]. Some reports of *in vitro*–derived oocyte-like cells did not directly assess the state of the chromosomes, drawing criticism from meiosis researchers[23,24]. In other cases, the oocyte-like cells reportedly reduced their DNA content to 1C *in vitro* in the absence of fertilization[22]; normally, this reduction would occur in female germ cells only upon fertilization. These difficulties in achieving proper coordination of oocyte differentiation and meiosis *in vitro* may reflect the underlying biology of oogenesis; they could be taken as evidence that differentiation and meiosis are regulated independently.

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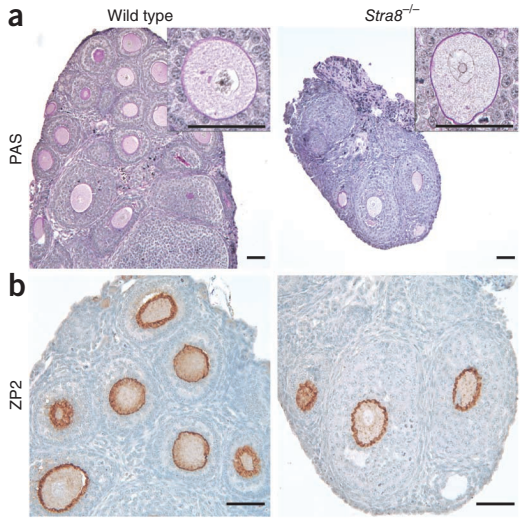

Figure 1 *Stra8*-deficient ovarian germ cells differentiate into oocyte-like cells. (**a**) Representative photomicrographs of sections from wild-type and *Stra8*-deficient ($n = 9$) ovaries at P21 stained with PAS and hematoxylin. Insets show higher magnification of individual germ cells. (**b**) Immunohistochemical staining of sections of P21 wild-type and *Stra8*-deficient ovary for the zona pellucida protein ZP2; sections are counterstained with hematoxylin. Scale bars, 50 μm. For a developmental time course, see also **Supplementary Figure 4**.

In this study, we investigated the functional relationship between meiosis and oocyte differentiation *in vivo*. We found that *Stra8*-deficient germ cells, which do not initiate meiosis, can differentiate into oocyte-like cells. Morphological and functional analyses showed that *Stra8*-deficient oocyte-like cells can synthesize zonae pellucidae, organize surrounding somatic cells into follicles, be ovulated in response to hormonal stimulation, undergo asymmetric cell division to produce polar bodies and cleave to form two-cell embryos upon fertilization. Direct analysis showed that these oocyte-like cells had developed without entering meiotic prophase or even undergoing premeiotic chromosome replication. We conclude that oocyte growth and differentiation can be genetically dissociated from premeiotic chromosome replication and the subsequent chromosomal events of meiosis. We postulate the existence of a *Stra8*-independent pathway, yet to be identified, that governs oocyte growth and differentiation.

RESULTS

Our previous studies of *Stra8*-deficient ovarian germ cells had been conducted in mice of mixed genetic background (not inbred)[11]. To ensure the reproducibility of our findings, we backcrossed the *Stra8* mutant allele onto an inbred genetic background (C57BL/6) and characterized the meiotic defect in these mice (**Supplementary Fig. 1**). Corroborating our earlier findings[11], C57BL/6 *Stra8*-deficient ovarian germ cells did not properly assemble the synaptonemal complex, did not properly localize meiotic cohesin REC8 (ref. 25) and did not form the DNA double-strand breaks (DSBs) that are essential for meiotic recombination[26]. These findings demonstrated that *Stra8* is necessary for meiotic prophase in C57BL/6 females, which we employed in all subsequent experiments.

As we reported previously, fetal and postnatal loss of germ cells is accelerated in *Stra8*-deficient females: ovaries from these mice had reduced numbers of germ cells relative to wild-type mice at birth and were devoid of germ cells by 6 or 8 weeks of age (**Supplementary Fig. 2**)[11]. Nonetheless, some *Stra8*-deficient germ cells evidently survived embryonic development, despite the meiotic initiation block at embryonic day (E) 13.5 to E14.5 (ref. 11). We confirmed this by comparing ovarian histology in wild-type and *Stra8*-deficient C57BL/6 mice between E14.5 and E16.5 (**Supplementary Fig. 1d**). To corroborate our previous conclusion[11] that some *Stra8*-deficient germ cells survive postnatally, we stained postnatal day (P) 2 ovarian sections for MVH (mouse vasa homolog; DDX4) protein, a marker of germ cells; we observed MVH-positive germ cells in both wild-type and *Stra8*-deficient ovaries (**Supplementary Fig. 3**). We then examined the oogenic potential of these surviving germ cells.

Stra8-deficient oocyte-like cells

If entry into meiotic prophase is required for oocyte growth and differentiation, then the latter processes should not occur in *Stra8*-deficient ovarian germ cells. To test this hypothesis, we compared the ovarian histology of wild-type and *Stra8*-deficient females.

In a wild-type ovary, during the first postnatal week, a cohort of germ cells was recruited to grow and differentiate synchronously. Unexpectedly, in *Stra8*-deficient ovaries, the surviving germ cells also began to grow and differentiate on a timetable similar to that of the first cohort in wild-type ovaries (**Supplementary Fig. 4**).

We next examined histological sections of *Stra8*-deficient ovaries at P21, when, in wild-type ovaries, growth and differentiation of the first cohort of oocytes were pronounced (**Fig. 1a**). In wild-type ovaries, we observed full-grown oocytes with large nuclei, called germinal vesicles. The perimeters of these large cells stained brightly using periodic acid–Schiff (PAS) reagent, indicating the presence of an oocyte-specific glycoprotein coat called the zona pellucida. Examining sections of ovaries from nine different *Stra8*-deficient mice, we found that all contained germ cells whose size and morphology were comparable to those of the wild-type oocytes. Like wild-type oocytes, these large *Stra8*-deficient germ cells featured germinal vesicles and zonae pellucidae, the latter of which we confirmed by staining sections for the zona pellucida protein ZP2 (**Fig. 1b**).

To examine the *Stra8*-deficient germ cells in greater detail, we generated electron micrographs of these oocyte-like cells in large antral follicles of P21 *Stra8*-deficient females. This imaging showed ultrastructural characteristics of the *Stra8*-deficient cell that were indistinguishable from those of wild-type oocytes (**Fig. 2**)[27]. Cytoplasmic processes from cumulus cells traversed the zona pellucida, terminating at the surface of the *Stra8*-deficient oocyte-like cell. The cytoplasm of the oocyte-like cell contained structures typical of wild-type oocytes, including cortical granules, lattice structures and oval vacuolated mitochondria (**Fig. 2**).

Stra8-deficient cells have not entered meiotic prophase

Despite the evidence that, as a population, germ cells in *Stra8*-deficient fetal ovaries do not initiate meiosis and progress through meiotic prophase (**Supplementary Fig. 1**)[11], the small number of germ cells surviving postnatally raised the possibility that these individual *Stra8*-deficient survivors had differentiated as oocytes after entering meiotic prophase. We excluded this possibility through a double-mutant (epistasis) experiment involving *Stra8* and *Dmc1* (**Fig. 3**). *Dmc1*-deficient ovarian germ cells die perinatally because *Dmc1* is required for the repair of the DSBs that arise during meiotic prophase[28–30]. This death of *Dmc1*-deficient germ cells does not occur if DSB formation is prevented, for example, in mice lacking a gene (*Spo11*) required for meiotic DSB formation[31–33]. We reasoned that, if the surviving *Stra8*-deficient germ cells had entered meiotic prophase and formed meiotic DSBs, then no double-mutant (*Stra8*- and *Dmc1*-deficient)

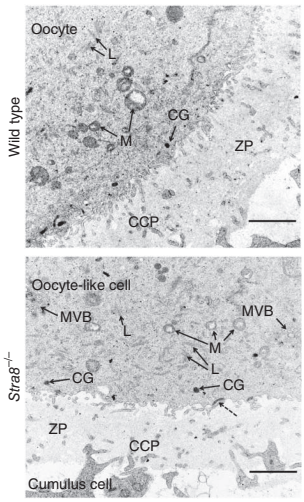

Figure 2 A *Stra8*-deficient oocyte-like cell displays the ultrastructural features of a wild-type oocyte. Electron micrographs of a wild-type oocyte and a *Stra8*-deficient oocyte-like cell in large antral follicles at P21. For both the wild-type oocyte and the *Stra8*-deficient oocyte-like cell, the cytoplasm contained vacuolated mitochondria (M), multivesicular bodies (MVB), lattice structures (L) and cortical granules (CG). Cytoplasmic processes from the cumulus granulosa cells (CCP) traversed the zona pellucida (ZP). The dashed arrow indicates a junction between the cytoplasmic processes of a granulosa cell and the *Stra8*-deficient oocyte-like cell. Scale bars, 2 μm.

germ cells should survive postnatally. However, if *Stra8* deficiency stringently blocked meiotic initiation and DSB formation, then germ cell survival in the double mutant should resemble that in the *Stra8* single mutant. We observed, as expected[29,30,33], that *Dmc1*-deficient ovaries at P30 contained no germ cells (**Fig. 3b**). Double-mutant (*Stra8*- and *Dmc1*-deficient) ovaries, however, contained oocyte-like cells similar to those of *Stra8*-deficient single-mutant ovaries (**Fig. 3a**,**c**). As the survival of *Stra8*-deficient oocyte-like cells did not require *Dmc1*, we conclude that these oocyte-like cells had not formed meiotic DSBs, reinforcing previous evidence that they had not entered meiotic prophase.

Follicle formation and ovulation

A critical function of wild-type oocytes is to actively organize somatic cells of the ovary into follicles, the hallmark of ovarian structure and function[34]. Like many wild-type oocytes, *Stra8*-deficient germ cells were enclosed in large follicles composed of multiple layers of cuboidal granulosa cells (**Fig. 1**), demonstrating their ability to drive folliculogenesis. By P30, large, preovulatory follicles were observed in 11 of 12 ovaries from 6 different *Stra8*-deficient mice (data not shown; see also **Figs. 3** and **4**).

To extend this observation, we examined *Stra8*-deficient ovaries for the expression of NOBOX, an oocyte-specific protein required for primary follicle formation[35]. We detected NOBOX protein in germ cells of all three *Stra8*-deficient and five control (wild-type or *Stra8*-heterozygous) ovaries examined by immunohistochemistry at P5 and P7 (**Fig. 4a**).

To confirm that proper granulosa cell differentiation had occurred in response to *Stra8*-deficient oocyte-like cells, we stained sections of wild-type and *Stra8*-deficient ovaries for FOXL2 protein, a key factor in granulosa cell differentiation and identity[36,37]. FOXL2 was expressed in the granulosa cells of both wild-type and *Stra8*-deficient ovaries at P10, when secondary follicles are present, and at P30, when large antral follicles are present. These findings confirm that *Stra8*-deficient oocyte-like cells support proper granulosa cell specification and differentiation (**Fig. 4b**,**c**).

We then asked whether oocyte-like cells in *Stra8*-deficient ovaries, together with surrounding somatic (cumulus) cells, could be ovulated. Because *Stra8*-deficient germ cells are lost before the mice reach sexual maturity, we could not assay natural ovulation. Instead, we hormonally stimulated wild-type and *Stra8*-deficient females at P20 to accelerate ovulation ('superovulation') and flushed their oviducts to identify any ovulated cumulus-oocyte complexes (COCs). We isolated ovulated COCs from wild-type and from all five *Stra8*-deficient females subjected to this regimen. In wild-type females, cumulus cells of the preovulatory follicle secrete hyaluronic acid, causing expansion of the cumulus cell mass (the 'cumulus oophorous') that surrounds the oocyte. This process depends on the oocyte, and competence to support cumulus oophorous expansion is acquired late in oogenesis[38]. Examination of wild-type and *Stra8*-deficient ovulated COCs showed that cumuli oophori were fully expanded in both groups (**Fig. 4d**). We conclude that *Stra8*-deficient oocyte-like cells can be ovulated with expanded cumuli oophori, implying that they can interact with the ovarian soma in a way that resembles that of wild-type oocytes.

Asymmetric division to produce polar bodies

In wild-type females, full-grown germinal vesicle–stage oocytes are arrested in diplotene of the first meiotic prophase. At ovulation, wild-type germinal vesicle–stage oocytes resume meiosis, breaking down the nuclear envelope, condensing their chromosomes and dividing asymmetrically to form a large secondary oocyte and a smaller polar body. Competence to undergo this division is acquired in late stages of oocyte growth and differentiation, making it a defining functional feature of a full-grown oocyte[39]. Two mutants that are defective in individual aspects of meiotic prophase (synapsis or recombination) have been reported to produce polar bodies[40,41], but it is not known whether blocking meiotic initiation (preventing the chromosomal program in its entirety) would preclude this asymmetric division. We therefore asked whether *Stra8*-deficient oocyte-like cells could produce polar bodies.

Full-grown wild-type oocytes can be induced to undergo maturation by removing them from follicles[42]. We extracted COCs from P22 wild-type and *Stra8*-deficient ovaries by follicle puncture and cultured them overnight. We then mechanically removed the cumulus cells and visually assayed polar body formation. Most *Stra8*-deficient cells underwent germinal vesicle breakdown (GVB), the first step on the path to polar body formation. Of the cells that underwent GVB,

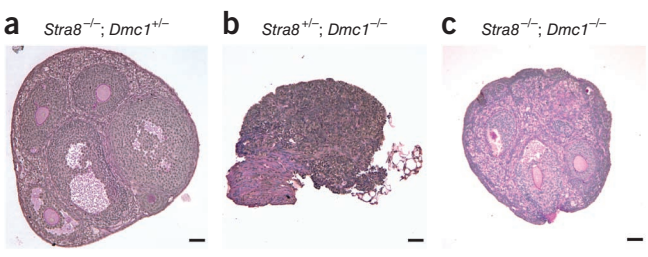

Figure 3 *Stra8*-deficient oocyte-like cells grow and differentiate without meiotic prophase. (**a**–**c**) Sections from P30 *Stra8*-deficient (**a**), *Dmc1*-deficient (**b**) and *Stra8* and *Dmc1* double-deficient (**c**) ovaries stained with PAS and hematoxylin. We observed oocyte-like cells in 16 of 17 *Stra8*-deficient ovaries, in 0 of 9 *Dmc1*-deficient ovaries and in all 3 double-mutant ovaries examined. *Dmc1*-deficient ovaries in **b** were small and dense, lacking germ cells or follicles. Scale bars, 50 μm.

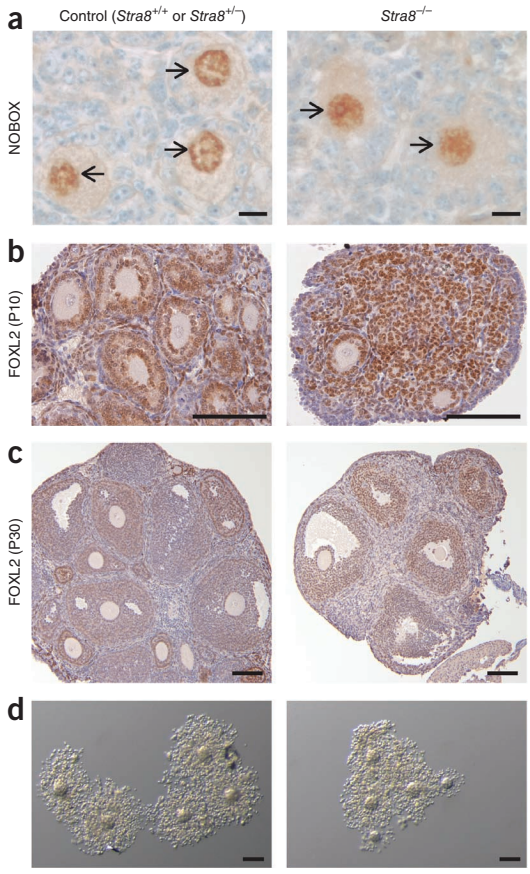

Figure 4 *Stra8*-deficient oocyte-like cells organize ovulation-competent follicles. (**a**) Immunohistochemical staining of sections of P5 control and *Stra8*-deficient ovaries ($n = 3$) for folliculogenesis-essential protein NOBOX; sections are counterstained with hematoxylin. Arrows indicate representative germ cells. (**b**,**c**) Immunohistochemical staining of P10 (**b**) and P30 (**c**) control and *Stra8*-deficient ovary sections for granulosa cell marker FOXL2. (**d**) Representative cumulus-oocyte complexes isolated from wild-type and *Stra8*-deficient superovulated mice ($n = 5$). Scale bars, 10 μm in **a** and 100 μm in **b**–**d**.

28 of 35 wild-type oocytes and 11 of 42 *Stra8*-deficient oocyte-like cells formed polar bodies (**Fig. 5**), showing that *Stra8*-deficient cells can undergo maturation and an asymmetric division. (We also observed polar body formation *in vivo*, after superovulation (**Supplementary Fig. 5**), corroborating our findings *in vitro*.)

The failure of many *Stra8*-deficient oocyte-like cells to produce polar bodies was likely due to a failure of spindle assembly in the absence of paired homologous chromosomes. Of 31 *Stra8*-deficient cells that underwent GVB but did not produce polar bodies, 7 fragmented, and the other 24 remained arrested without a visible germinal vesicle or polar body. The latter group of cells contained aberrant spindles reminiscent of those observed in *Mlh1*-deficient oocytes, which do not maintain homologous chromosome pairing (**Supplementary Fig. 6**)[40].

Premeiotic chromosome replication has not occurred

We reported previously that fetal germ cells in *Stra8*-deficient females do not undergo premeiotic chromosome replication[11]. Accordingly, we tested whether the *Stra8*-deficient oocyte-like cells had replicated their chromosomes. First, we compared diamidino-2-phenylindole (DAPI) intensity in germinal vesicle–stage wild-type oocytes and *Stra8*-deficient oocyte-like cells. Using this crude approach, we found that wild-type oocytes, with replicated chromosomes, contained significantly more DNA than *Stra8*-deficient oocyte-like cells (**Supplementary Fig. 7**).

To validate this preliminary observation, we examined the configurations of chromosomes in *Stra8*-deficient oocyte-like cells that had matured to produce polar bodies. Haploid mouse gametes have 20 chromosomes, and diploid cells have 40 chromosomes. In wild-type females, postnatal germinal vesicle–stage oocytes have 40 cohesed pairs of sister chromatids—80 chromatids in all—as a consequence of premeiotic chromosome replication having occurred during fetal development. (The number of chromatids is subsequently halved, during the first meiotic division, and halved again, during the second meiotic division.)

After the first division, chromatids (more precisely, their centromeres—one per chromatid) can be visualized and counted in the oocyte and polar body. We stained *in vitro*–matured cells with DAPI and antibodies to tubulin and centromere (ACA) to visualize, respectively, DNA, the spindle and centromeres. We mounted stained cells on slides in a manner that preserved three-dimensional structure, and we collected *z* stacks of images through the entire volume. We then counted centromeres and visualized the configurations of sister chromatids in both the oocyte (or oocyte-like cell) and its adjoining polar body.

As expected, in each of 16 wild-type samples, we observed a total of 80 chromatids: 20 pairs of cohesed chromatids in the matured oocyte and another 20 pairs of cohesed chromatids in the polar body (**Fig. 6a**,**b**). By contrast, in each of 11 *Stra8*-deficient samples, we observed a total of 40 uncohesed chromatids distributed unevenly between the oocyte-like cell and the polar body (**Fig. 6a**,**b**). Examination at high magnification confirmed that, whereas wild-type oocytes contained cohesed sister chromatids, *Stra8*-deficient oocyte-like cells contained single chromatids (**Fig. 6a**,**b**, insets). The only explanation for this chromosomal configuration is that, before maturation and polar body formation, these *Stra8*-deficient oocyte-like cells had 40 uncohesed chromatids—as would be found in a premeiotic germ cell that had not replicated its chromosomes. In the absence of premeiotic replication and meiotic prophase, bipolar spindle attachment of homologous chromosomes would not have been possible. This evidently led to chaotic chromosome segregation, with one, both or neither member of each homologous chromosome pair being apportioned to the polar body (**Fig. 6c**).

Taken together, these results argue strongly that *Stra8*-deficient germ cells completed oocyte-like differentiation and maturation without having undergone premeiotic chromosome replication and without having entered meiotic prophase.

Stra8-deficient cells yield two-cell embryos upon fertilization

Because *Stra8*-deficient oocyte-like cells have only 40 chromatids (compared to 80 in wild-type oocytes), which they segregate in an

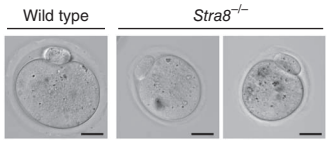

Figure 5 *Stra8*-deficient oocyte-like cells divide asymmetrically upon maturation. Representative differential interference contrast (DIC) photomicrographs of *in vitro*–matured wild-type oocytes and *Stra8*-deficient oocyte-like cells. Twenty-eight of 35 wild-type oocytes and 11 of 42 *Stra8*-deficient oocyte-like cells that underwent GVB formed polar bodies. Scale bars, 20 μm. For *in vivo* polar body formation, see also **Supplementary Figure 5**.

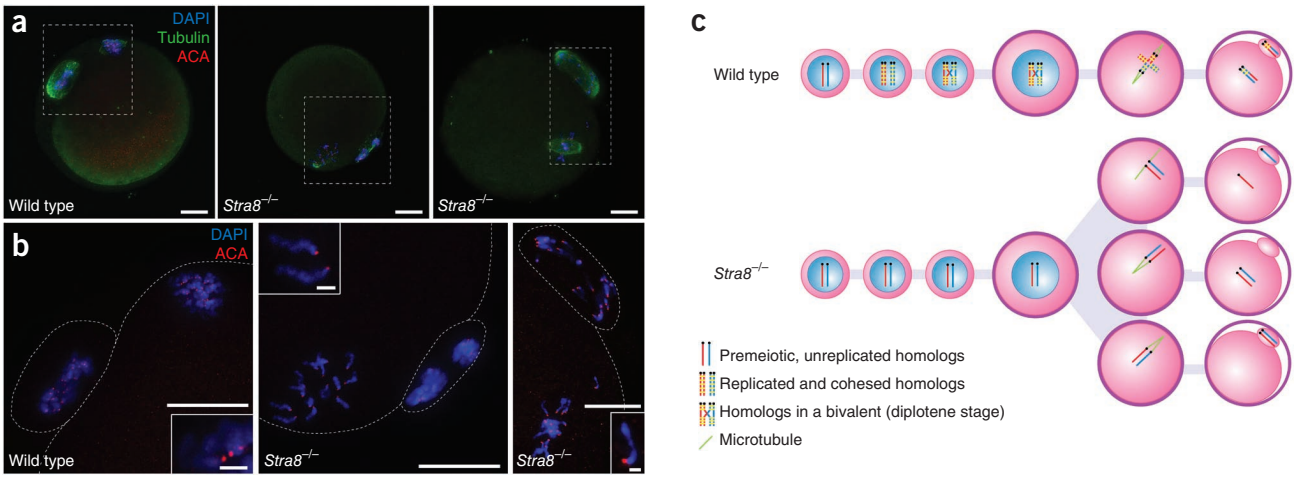

Figure 6 Premeiotic chromosome replication is dispensable for oocyte differentiation. (**a**) Deconvolved, projected *z* stacks of images of a wild-type oocyte and *Stra8*-deficient oocyte-like cells that were matured *in vitro*. Cells were immunofluorescently labeled with antibodies to tubulin and centromeres (ACA). Chromosomes were stained with DAPI. (**b**) High-magnification views of boxed areas in **a**, with the tubulin channel deleted. In each image, the polar body and oocyte or oocyte-like cell are outlined with a dashed line. Insets provide ultra-high-magnification views of representative chromatids. Wild-type oocytes ($n = 16$) invariably displayed pairs of sister chromatids (as a result of chromosome replication), whereas mutants ($n = 11$) displayed unreplicated single chromatids. The brightness and contrast of channels were adjusted independently. Scale bars, 20 μm (2 μm in insets). (**c**) Schematic interpretation of data presented in **a**,**b**. Top, wild-type oocytes progress through meiotic prophase, with homologous chromosomes in bivalents. During growth and differentiation, chromosomes remain in bivalents. Upon maturation, homologous chromosomes segregate in an orderly fashion, one to the main cell and the other to the polar body. Bottom, by contrast, during growth and differentiation of *Stra8*-deficient oocyte-like cells, chromosomes remain in an unreplicated, premeiotic configuration. Upon maturation of *Stra8*-deficient oocyte-like cells, the univalent homologous chromosomes segregate chaotically, with one, both or neither going to the polar body.

apparently haphazard manner, the probability of achieving a euploid egg that could yield a live-born pup is remote. Nevertheless, we wondered whether these oocyte-like cells could undergo fertilization and support early events of embryogenesis. To address this question, we performed *in vitro* fertilization (IVF) experiments using control (wild-type or *Stra8*-heterozygous) oocytes and *Stra8*-deficient oocyte-like cells harvested by superovulation of juvenile females (**Fig. 7**). Six hours after IVF, we observed that control oocytes and *Stra8*-deficient oocyte-like cells had extruded a second polar body and contained two pronuclei, indicating successful fertilization. At 22 h, 52 of 68 control oocytes and 7 of 39 *Stra8*-deficent oocyte-like cells had progressed to become 2-cell embryos. At 48 h, control two-cell embryos had progressed to the four-cell stage, but all *Stra8*-deficent embryos remained arrested at the two-cell stage. These results show that meiotic initiation and prophase are not required for the formation of fertilization-competent egg-like cells or for the subsequent division that gives rise to two-cell embryos.

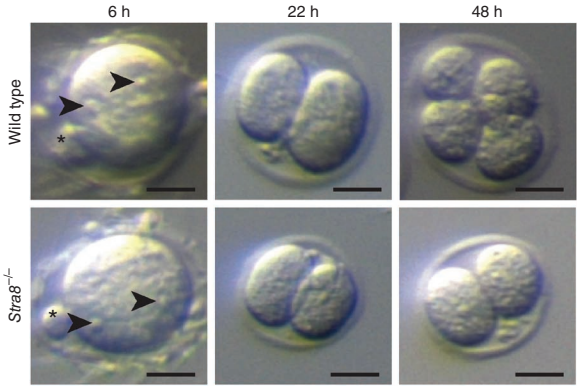

Figure 7 *Stra8*-deficient oocyte-like cells cleave to yield two-cell embryos upon fertilization. Wild-type oocytes and *Stra8*-deficient oocyte-like cells at 6, 22 and 48 h after IVF. At 22 h, 52 of 68 control oocytes and 7 of 39 *Stra8*-deficent oocyte-like cells had progressed to become 2-cell embryos. Arrowheads indicate paternal and maternal pronuclei. Asterisks indicate second polar bodies. Scale bars, 20 μm.

DISCUSSION

We investigated the functional relationship between two components of oogenesis: meiosis and cellular growth and differentiation. Using *in vivo* genetic analysis, we demonstrated that growth and differentiation are dissociable from the chromosomal events of meiosis. Although meiosis is an essential part of oogenesis and is absolutely required for orderly chromosomal segregation, we found that fertilization-competent oocyte-like cells can develop in its absence (**Fig. 8**).

We previously reported that *Stra8*-deficient ovarian germ cells do not initiate meiosis during fetal development[11]. Here we confirmed our previous findings on an inbred genetic background. We extended these observations by directly examining the chromosomes of postnatal *Stra8*-deficient germ cells during the first wave of oogenesis. We found that the chromosomal content and configuration of *Stra8*-deficient germ cells remained identical to that of premeiotic germ cells that had not replicated their chromosomes—even many weeks after wild-type germ cells would have initiated meiosis. This finding highlights a fundamental difference between the *Stra8*-deficient phenotype and the meiotic phenotypes of mice deficient for *Spo11*, *Dmc1*, *Rec8*, *Sycp1*, *Sycp3* and *Mlh1*, among others. These meiotic mutants enter prophase of meiosis I, and their defects are limited to a subset of meiotic chromosomal processes[29,30,32,41,43–45]. In contrast, the *Stra8* mutant does not undergo any of the chromosomal events associated with prophase of meiosis I and therefore is non-meiotic. The *Stra8* mutant represents a unique and powerful tool with which to study ovarian germ cell differentiation in the absence of meiosis.

After our original report that *Stra8* is required for meiotic initiation in the fetal ovary, other investigators inferred, given prevailing models,

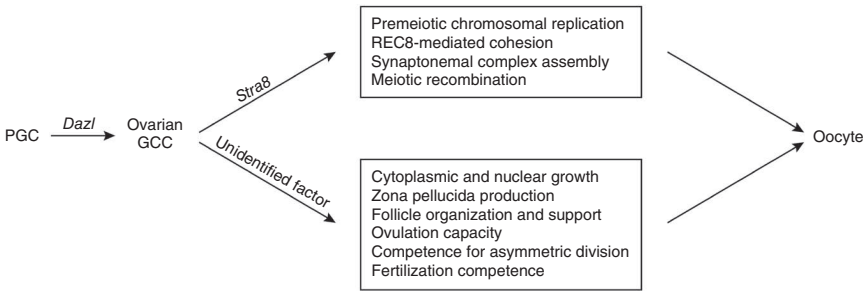

Figure 8 A proposed model for the initiation of both meiosis (top) and oocyte growth and differentiation (bottom) in the mouse ovary. The GCC, which derives from a PGC, embarks on meiosis through the action of the meiotic initiation factor *Stra8*, and it embarks on the *Stra8*-independent program of growth and differentiation through the action of one or more factors yet to be identified. The two programs thus set in motion constitute oogenesis.

that *Stra8* must also be required for oocyte differentiation[7,9,10,46,47]. To the contrary, we report that *Stra8* function and, hence, meiotic initiation and prophase are not required for oocyte differentiation, as judged on the basis of morphological criteria, molecular markers and functional assays. In the absence of meiosis, *Stra8*-deficient ovarian germ cells can develop a large cytoplasm, synthesize zonae pellucidae, organize surrounding somatic cells into follicles, acquire competence for fertilization and the first cleavage division, undergo asymmetric cell divisions to form polar bodies and be ovulated in response to hormonal stimulation. We conclude that oogenesis in mice is a genetically dissociable union of two concurrent processes—meiosis and differentiation—and that meiotic initiation and prophase are not prerequisite to oocyte differentiation (**Fig. 8**). We postulate the existence of a *Stra8*-independent pathway, yet to be identified, that regulates oocyte growth and differentiation (**Fig. 8**). More broadly, our findings open to study the independent contributions of meiosis and oocyte differentiation to the making of a functional egg.

Our findings should also motivate a re-evaluation of hypotheses concerning mouse germ cell sex determination, that is, how a primordial germ cell chooses between becoming an egg or a sperm. For decades, experimentalists have interpreted the presence or absence of fetal meiotic prophase as a proxy for commitment to, respectively, oogenesis or spermatogenesis[1,4–10]. In contrast, our finding of oocyte-like growth and development in *Stra8*-deficient germ cells clearly shows that the absence of meiotic prophase is not, by itself, evidence that fetal germ cells have adopted a spermatogenic fate. Conversely, entry into meiotic prophase should no longer be taken by itself as evidence that experimentally manipulated fetal germ cells have adopted an oogenic fate. For these reasons, many published conclusions regarding the timing and control of mouse germ cell sex determination must be reconsidered, with new phenotypic end points and assays adopted.

Our work also suggests additional opportunities for future study. Because the occurrence of oocyte growth and differentiation during meiotic prophase arrest is broadly conserved[13,14], we wonder whether oocyte differentiation is genetically dissociable from meiosis in other vertebrate and invertebrate animals. A gene orthologous to mouse *Stra8* has been identified in the *Xenopus tropicalis* genome (XM_002941430), but its roles, if any, in meiotic initiation and oogenesis remain unknown. It should also be possible to address this question in other animals once the genes required for meiotic initiation are identified.

Indeed, it has been reported that sporulation is dissociable from meiosis in yeast and that gametogenesis is dissociable from the meiotic pattern of chromosome segregation in *Arabidopsis thaliana*[48,49]. Most recently, the sperm-oocyte decision has been decoupled from the mitosis-meiosis decision in *Caenorhabditis elegans*[50]. Going forward, it will be of great interest to explore similarities and differences among these diverse eukaryotic systems with respect to the separability of meiosis and gamete differentiation.

The question remains as to how the non-meiotic 2C diploid genome in *Stra8*-deficient ovarian germ cells drives oogenic growth and differentiation in a manner that so closely resembles that of the 4C diploid genome in wild-type oocyte arrested during meiotic prophase. This question might be addressed in the future by assessing the epigenetic and transcriptional status of the *Stra8*-deficient oocyte-like cells, recognizing that it will be challenging to obtain those cells in substantial numbers.

After IVF of *Stra8*-deficient egg-like cells, we observed seven embryos that progressed to but not beyond the two-cell stage. A full understanding of this arrest at the two-cell stage—a time when activation of the zygotic genome occurs in wild-type embryos[51]—will require further investigation. The arrest may be due to activation of a grossly aberrant zygotic genome, with the non-meiotic *Stra8*-deficient egg-like cells having contributed chaotic samplings of chromosomes to their respective embryos. Indeed, one would expect these embryos to be monosomic for several autosomes (or, in some cases, trisomic) and about one-quarter to have no X chromosome. The question arises of whether the cytoplasm of a non-meiotic, *Stra8*-deficient oocyte-like cell would be capable of supporting embryogenesis beyond the two-cell stage if presented with a normal complement of chromosomes. This possibility might be tested by nuclear transplantation, with the results delineating the limits of oogenic differentiation in the absence of meiosis.

As described here and in our previous report[11], the absence of *Stra8* function accelerates the germ cell loss that is a prominent feature of the wild-type ovary; here, as in the wild-type ovary, we do not know the cause(s) of this germ cell death. It is probably not due to the checkpoints usually associated with progression through meiotic prophase, as DSBs and asynapsed axial elements, recognized by DNA damage or asynapsis checkpoints, respectively[33,52], are absent in the *Stra8*-deficient oocyte-like cells.

Our findings may have practical implications, for infertility and for *in vitro* oogenesis. Oocyte-like differentiation without meiosis may explain some cases of infertility, especially where women cannot achieve or sustain pregnancy, despite the presence of cells that histologically resemble oocytes. Similarly, some mouse or human oocytes in recent reports derived from cells grown in culture[16–22] may actually be demonstrating oocyte-like differentiation without meiosis[23]. Our observations show that claims of successful oogenesis *in vitro* cannot rest solely on evidence of oocyte-like differentiation, as oocyte-like morphology and functionality can arise in the absence of meiotic initiation and meiotic prophase. Therefore, meiotic initiation and progression must be documented directly. Recently, successful oogenesis from ES and iPS cells was achieved in mice by culturing induced PGC-like cells within gonadal aggregates and then transplanting them into the ovary of an adult female[53]. Understanding the functional interdependencies, if any, of meiosis and oocyte differentiation may help to move this technology entirely *in vitro*.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

G.A.D., A.E.B., J.J.E. and D.C.P. designed the experiments. G.A.D. and J.J.E. performed the experiments. G.A.D. and D.C.P. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. *Stra8*-deficient mice were generated and backcrossed as previously described[11,12]. All *Stra8* mice used were backcrossed to C57BL/6NtacfBR mice for at least 18 generations. *Dmc1*-deficient mice[28], which had been backcrossed to C57BL/6J mice for at least ten generations, were purchased from Jackson Laboratory. *Stra8*-deficient females were generated by mating heterozygotes. *Dmc1*-deficient females and *Stra8* and *Dmc1* double-deficient females were generated by mating *Stra8* and *Dmc1* double heterozygotes. All experiments involving mice were approved by the Committee on Animal Care at the Massachusetts Institute of Technology.

Histology. Dissected ovaries were fixed in Bouin's solution (Polysciences) overnight at 4 °C, embedded in paraffin and sectioned. Slides were stained with PAS reagent (Sigma) according to the manufacturer's protocol and counterstained with Mayer's modified hematoxylin (Invitrogen). For every genotype, at least four samples were analyzed.

Electron microscopy. The ovaries of P21 mice were fixed in 85% Karnovsky's fixative and trimmed in the fixative to individual antral follicles. Samples were prepared for electron microscopy using standard procedures.

Immunocytochemistry. Ovarian germ cell spreads were prepared according to a published protocol[54]. Cells were permeabilized with 0.5% Triton X-100 for 5 min and blocked with 1% BSA for 1 h. Rabbit antibody to SYCP3 (Abcam, ab15092) was used at a 1:1,000 dilution. Rabbit antibody to REC8 (courtesy of C. Heyting) was used at a 1:250 dilution.

Immunohistochemistry. Dissected ovaries were fixed in Bouin's solution or 4% paraformaldehyde overnight at 4 °C, embedded in paraffin and sectioned. Slides were dewaxed in xylenes, rehydrated through an ethanol gradient and boiled in sodium citrate buffer (pH 6.0) for 15 min. For colorimetric immunohistochemistry, slides were pretreated for 10 min with 0.3% H_2O_2. Mouse monoclonal antibody to γH2A.X (clone JBW301; Millipore, 05-636) was used at a 1:100 dilution. Goat antibody to hVASA (R&D Systems, AF2030) was used at a 1:250 dilution. Rat monoclonal antibody to ZP2 (clone IE-3; Santa Cruz Biotechnology, sc-32752) was used at 1:100 dilution. Rabbit antibody to NOBOX (Abcam, ab41612) was used at a 1:1,000 dilution. Goat antibody to FOXL2 (Abcam, ab5096) was used at 1:500 dilution.

Superovulation and isolation of ovulated COCs. Female mice were stimulated with 5 IU of pregnant mare serum gonadotropin (PMSG; Sigma) at P20. Forty-four to 48 h after PMSG injection, mice were stimulated with 5 IU of human chorionic gonadotropin (hCG; Sigma). Fifteen to 16 h after the second injection, mice were sacrificed, and their oviducts were dissected away from the uterus and ovaries. Oocytes were released by slicing open the oviducts and allowing the COCs to spill into a drop of KSOM (Potassium Simplex Optimized Medium; Millipore) under oil. Cumulus cells were removed by treating COCs with hyaluronidase (Sigma) for 5 min and washing twice in KSOM.

***In vitro* maturation of oocytes.** Female mice were stimulated with 5 IU of PMSG at P20. Forty-four to 48 h after injection, mice were sacrificed, and ovaries were dissected. COCs were isolated by follicle puncture and were cultured overnight in MEM-α (minimum essential medium α; Invitrogen) supplemented with 0.3% BSA. The next day, cumulus cells were removed mechanically, and polar body formation was assessed.

DAPI intensity integration. Germinal vesicle–stage oocytes were isolated by follicle puncture, mechanically denuded and spread by drying down on slides wetted with 1% paraformaldehyde. Slides were imaged using constant exposure time across all samples. DAPI intensity was quantified using CellProfiler software.

Whole-oocyte immunocytochemistry. *In vitro*–matured oocytes were fixed in 4% paraformaldehyde for 45 min, permeabilized with 0.1% Triton X-100 and blocked with 10% donkey serum. Monoclonal rat antibody to tubulin (clone YL/2; Abcam, ab6160) was used at a 1:250 dilution. Human antibody to centromere (ACA, Antibodies, 15-235) was used at a 1:100 dilution.

Microscopy. Images were acquired using a DeltaVision deconvolution microscope (Applied Precision). For oocytes and oocyte-like cells, *z* stacks were collected at 0.5-μm spacing. All images within the stacks were deconvolved using DeltaVision softWoRx software. *z*-stack projections were generated using the maximum-intensity method in ImageJ. ACA signals were counted on projected images. Individual images from the *z* stacks were used to resolve centromeres that were superimposed in the *z* plane.

IVF. Superovulated COCs were collected as described and maintained in MEM-α supplemented with 5% FBS to prevent zona pellucida hardening. To collect sperm, epididymi were dissected from adult males and cut to release sperm in serum-free MEM-α supplemented with 0.3% BSA (Sigma). COCs were washed out of serum into MEM-α supplemented with 0.3% BSA and added to sperm, which had been diluted 1:90 in MEM-α supplemented with 0.3% BSA under oil. Fertilization dishes were incubated for 6 h before washing away sperm and were then incubated overnight. Two-cell embryos were transferred to KSOM for further culture.

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