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Isolation, characterization and propagation of mitotically active germ cells from adult mouse and human ovaries

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Abstract

Accruing evidence indicates that production of new oocytes (oogenesis) and their enclosure by somatic cells (folliculogenesis) are processes not limited to the perinatal period in mammals. Endpoints ranging from oocyte counts to genetic lineage tracing and transplantation experiments support a paradigm shift in reproductive biology involving active renewal of oocyte-containing follicles during postnatal life. The recent purification of mitotically active oocyte progenitor cells, termed female germline stem cells (fGSCs) or oogonial stem cells (OSCs), from mouse and human ovaries opens up new avenues for research into the biology and clinical utility of these cells. Here we detail methods for the isolation of mouse and human OSCs from adult ovarian tissue, cultivation of the cells after purification, and characterization of the cells before and after *ex vivo* expansion. The latter methods include analysis of germ cell-specific markers and *in vitro* oogenesis, as well as the use of intraovarian transplantation to test the oocyte-forming potential of OSCs *in vivo*.

INTRODUCTION

The recent identification and isolation of OSCs (also referred to as fGSCs) from mouse and human ovaries has sparked both enthusiasm and controversy in the fields of reproductive biology, stem cell biology and clinical reproductive medicine^{1–6}. Although multiple lines of compelling evidence now exist that support the concept of postnatal oogenesis and follicle renewal in mammals, these new findings are at odds with what has become dogmatic thinking in the field for more than 5 decades (ref. 7). To this day, age-related depletion of a ‘fixed ovarian reserve’ of oocytes endowed at birth remains a popular, if not convenient, means to explain why mammalian females undergo reproductive senescence approximately halfway through their chronological lifespan. However, after the initial challenge to this decades-old dogma¹, work from many laboratories now strongly supports that the ovaries of mammals generate new oocytes and follicles during the reproductive period^{1–6,8–12}.

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Circumstantial evidence for the existence of OSCs

The field of OSC biology actually arose serendipitously from oocyte-counting experiments in mice, which were not originally designed to test whether oogenesis ceases before birth. Simply by asking whether the net decline in the number of healthy oocyte-containing follicles during postnatal life was paralleled by the detection of an equivalent number of dying oocytes over the same time period, a mathematical dilemma was uncovered that could not be solved under the constraints of the prevailing dogma. The incidence of oocyte loss through follicle atresia, assessed directly by scoring degenerative oocytes in serial ovarian sections, far exceeded the net decline in the ovarian reserve of oocytes in female mice as they transitioned from juvenile to adult life¹. It was therefore logically concluded that this surprisingly high rate of oocyte loss during postnatal life must be partially offset by routine addition of new oocyte-containing follicles for ovarian function to persist as long as it does. Mathematical models were then used to estimate that mouse ovaries are replenished with ~77 new follicles per d, at least early in reproductive life¹. This suggested that the oocyte progenitor cells responsible for oogenesis are either very rare or slow to proliferate. Either way, this model represents a striking departure from the thousands of spermatogonial stem cells (SSCs) and spermatogenic progenitor cells present in the testes of adult male mice that produce millions of spermatozoa daily^{13–15}.

The presumed rarity of OSCs is in keeping with data showing that the germ cell-specific meiosis-commitment gene, *Stra8* (stimulated by retinoic acid gene 8) (refs. 16,17), which is abundantly expressed in adult testes and in embryonic ovaries during the period of oogenesis^{18,19}, is rare but not absent in ovaries of reproductive-age mice^{8,20}. Although the presence of these *Stra8*-expressing cells within adult ovaries is highly suggestive of ongoing *de novo* oocyte formation, recent genetic evidence further substantiates the occurrence of postnatal oogenesis and follicle renewal by demonstrating that the number of traceable mitotic divisions in oocytes of aged mice exceeds those in younger counterparts²¹. As oocytes themselves do not divide, the mitotic ‘depth’ of these cells reflects the number of mitoses that the progenitor germ cells underwent before production of a given oocyte at the time of analysis²². Coupled with the findings that unilateral ovariectomy in female mice at 1 month of age accelerates the mitotic depth in oocytes of the remaining ovary 3 months later, the simplest explanation for these findings is that the follicle pool is partly maintained during reproductive life by a relatively rare population of premeiotic germ cells capable of generating new oocytes that form follicles^{21,22}.

Purification of OSCs as proof of their existence

Over the past few years, several laboratories have independently reported the isolation of mammalian OSCs and their subsequent propagation in defined cultures^{2,3,9,23}. As expected of a rare pool of adult stem cells, the number of OSCs within the ovary is very low, representing $\sim 0.014 \pm 0.002\%$ of the total ovarian cell population in adult female mice³. Cultivation of OSCs *in vitro* results in stable expansion of these premeiotic germ cells, as well as the spontaneous formation of immature oocytes^{3,9}. Furthermore, transplantation of *ex vivo*-expanded mouse or human OSCs expressing GFP into adult ovarian tissue leads to the formation of new oocytes, as determined by the detection of GFP-positive oocytes contained within follicles composed of host (wild-type, non-GFP) somatic granulosa

cells^{2,3}. In mice, these oocytes are ovulated to produce mature, fertilizable eggs and viable offspring^{2,3,24}. Although this complete developmental potential has yet to be shown using the equivalent human cells, human OSCs injected directly into adult human ovarian cortical tissue pieces transplanted into immunocompromised mice generate oocytes and participate in follicle formation in an *in vivo* environment³. These findings not only demonstrate the ability of adult human ovarian tissue to support folliculogenesis, but also they provide the first evidence that the ovarian follicle pool in women may, similar to that in mice, be amenable to renewal. Moving forward, the study of oogenesis in mammals, as well as the development of therapeutic strategies for the treatment of female infertility, should now take into account OSCs as natural precursor cells of oocytes^{5,6,25}.

Although multiple methods have been reported for the successful propagation of OSCs *in vitro*, the relative purity of the initial population of cells retrieved from the ovaries varies on the basis of the isolation strategy used^{2,3,9,11,23}. Antibody-based live cell-sorting strategies targeting an extracellular domain of the evolutionarily conserved germ cell marker, DEAD box polypeptide 4 (Ddx4) (refs. 26–28), yield viable premeiotic germ cells from adult mouse ovaries that stably proliferate *ex vivo* and ultimately give rise to fertilizable oocytes *in vivo*^{2,3}. In addition to the Ddx4 antibody-based approach for OSC purification², which has been rigorously and independently validated³, antibodies targeting an extracellular domain of the primitive germ cell marker interferon-induced transmembrane protein 3 (Ifitm3; refs. 29,30) can also be used to isolate mouse OSCs¹¹. We have confirmed the utility of this approach to isolate OSCs from adult mouse ovaries (D.C.W. and J.L.T., unpublished observations), which is important for reasons other than independent verification. Some scientists have questioned the use of antibodies against the C terminus of Ddx4 for isolating OSCs because the protein has historically been viewed, but never proven, to be a cytoplasmic protein in all germ cells^{26–28}; however, there is a clear precedent for externalized expression of Ifitm3 in primitive germ cells^{31,32}. Thus, any doubts regarding the validity of Ddx4 antibody-based OSC sorting are scientifically unsubstantiated simply on the basis of the fact that the same population of premeiotic germ cells can be isolated from postnatal mouse ovaries by using antibodies against either Ddx4 or Ifitm3 in cell-sorting protocols^{2,3,11}. Because the utility of IFITM3 antibodies for purifying OSCs has not yet, at least to our knowledge, been tested with human ovarian tissue, the protocol detailed here focuses specifically on the use of Ddx4 (mouse; DDX4 in humans) as the target protein. Nonetheless, given the strong evolutionary conservation of mammalian germ cell development, it stands to reason that antibodies targeting an extracellular portion of IFITM3 would probably be successful for the immunological isolation of OSCs from human ovaries.

Although initial characterization of the *Ddx4* gene in mice noted intracellular localization of the protein in oocytes by immunodetection²⁶, whether or not the protein contains consensus transmembrane spanning or extracellular domains was not evaluated. Several years later, Wu and colleagues² performed a bioinformatic analysis of the Ddx4 protein sequence, resulting in the identification of a putative extracellular region at the C terminus of the protein. In addition to confirming this prediction³³, we have also compared the externalized Ddx4 amino acid sequence with other proteins across databases to determine whether this sequence is unique or whether other proteins containing a similar sequence might be expressed on the surface of OSCs. We found very little sequence homology to other

proteins, known or predicted, which contain a cell-surface domain that would be recognized by the Ddx4 antibody used for sorting. The closest match of common sequence homology we found was between the Ddx4 target sequence and a small region of ATP-binding cassette subfamily C member 12 (Abcc12). However, the amino acid sequence in Abcc12 that shares any homology to the externalized domain of Ddx4 is restricted to an intracellular portion of the protein³⁴. Thus, it is highly unlikely that the C-terminal Ddx4 antibody used by others and us to isolate OSCs cross-reacts with Abcc12 in living (non-fixed, non-permeabilized) cells.

In order to confirm the suitability of antibodies directed against the extracellular portion of Ddx4 for live-cell sorting, Wu and colleagues² also performed a visualization experiment using a Ddx4 antibody targeting the exposed C terminus, followed by a secondary antibody conjugated to large beads. This enabled visualization of the antibody-binding sites on the surface of Ddx4-positive cells. We repeated this visualization experiment essentially as described using dissociated mouse ovaries as starting material and found that antibodies against the C terminus, but not the N terminus, of Ddx4 bind to the surface of OSCs and not to isolated oocytes³. Nonetheless, because nonspecific binding of antibodies can be a problem in these types of immunolabeling experiments, further study was required to ensure that the antibody binding was specific to Ddx4.

To this end, we extensively validated the specificity of a C-terminal Ddx4 antibody for the purification of OSCs through FACS. By using two antibodies targeting Ddx4, one directed against the extracellular C-terminal portion and another targeting the cytoplasmic N terminus, we demonstrated that the cells to which the C-terminal antibody bound were Ddx4-positive and not a by-product of nonspecific binding³. The cell fraction obtained by the C-terminal Ddx4 antibody-based FACS approach yields a uniform population of cells that are less than 10 μm in diameter and positive for expression of Ddx4 along with many well-documented primitive germ cell markers, including PR domain containing 1 with ZNF domain (*Prdm1*), developmental pluripotency-associated 3 (*Dppa3*), *Ifitm3* and telomerase reverse transcriptase (*Tert*, catalytic subunit of telomerase)³. Notably, once the OSCs are plated, many of the cells quickly expand in size (up to 20 μm in diameter), but they continue to actively proliferate for months while retaining a germline gene expression profile³.

Non-immunological-based methods to isolate OSCs

Alternative strategies aimed at obtaining OSCs from postnatal mouse ovaries have also been reported. However, unlike the antibody-based strategies discussed above, other methods described to date have inherent pitfalls that may confound endpoint analysis and make data interpretation difficult if not impossible. For example, the use of ovarian tissue from transgenic POU domain class 5 transcription factor 1 (*Pou5f1*) promoter-driven GFP reporter mice (Jackson Laboratory strain B6;CBA-Tg(Pou5f1-EGFP)2Mnn/J, also commonly referred to as TgOG2 or PE-*Oct4-Gfp* transgenic mice) for FACS-based isolation of OSCs from postnatal ovaries has been described⁹. Although the cells obtained have all of the general properties of OSCs reported by us and others using Ddx4 antibody-based sorting, including the ability of the cultured cells to form oocytes *in vitro*⁹, we believe

the use of TgOG2 mice for sorting of OSCs from ovarian tissue is not optimal for several reasons.

The most important of these is that expression of the endogenous *Pou5f1* gene in germ cells fluctuates widely both *in vivo* and *in vitro*. During fetal ovarian development, *Pou5f1* expression is maintained in primordial germ cells (PGCs) until the onset of meiotic entry (oocyte formation) and then downregulated, only to be activated once again after the differentiating germ cells have reached prophase of the first meiotic division^{35–38}. However, female germ cells continue to express *Ddx4* during this entire time, even when *Pou5f1* expression is essentially shut down, thereby highlighting a disconnect between the expression patterns of these two genes in developing germ cells. As one might expect, multiple populations of PE-*Pou5f1*-GFP-expressing cells are actually observed in postnatal ovaries of TgOG2 mice. In addition to GFP-positive tetraploid cells (presumably small oocytes), a distinct population of much smaller GFP-positive diploid cells is detectable⁹. Of particular note, although GFP-positive cells obtained from TgOG2 mouse ovaries by FACS express germ cell markers as expected, many germ cell markers including *Ddx4* are also readily detected in the cell fraction identified as negative for PE-*Pou5f1* driven GFP expression⁹.

Given this information, we performed FACS analysis on dissociated TgOG2 mouse ovaries labeled with C-terminal *Ddx4* antibody. We observed both GFP-positive and *Ddx4*-positive cell populations; however, we detected few, if any, cells that expressed both GFP and cell-surface *Ddx4*. Moreover, when transplanted into the ovaries of recipient wild-type mice, the isolated GFP-negative/*Ddx4*-positive cells generated GFP-positive oocytes (D.C.W. and J.L.T., unpublished observations)⁶, confirming both the identity of the isolated cells as oocyte progenitor cells and faithfulness of the transgene to be activated once oocytes are formed. Thus, although PE-*Pou5f1*-GFP-positive ovarian cells appear to have at least some of the basic characteristics of OSCs when cultured *in vitro*, these cells probably represent a different developmental stage of germ cells compared with OSCs purified by antibody-based sorting. In this regard, it is notable that PE-*Pou5f1*-GFP-positive cells isolated from TgOG2 ovaries lose expression of GFP shortly after initiation of *ex vivo* culture, while maintaining expression of germline and stem cell markers⁹. Whether this is due to transient expression of the PE-*Pou5f1*-GFP transgene or perhaps to contamination with small oocytes that die off shortly after culture initiation is not known. However, because oocytes and OSCs exhibit a partially overlapping germline gene expression profile, any claims to study OSCs isolated on the basis of transgenic reporters must be certain that the cells under investigation are indeed OSCs and not immature oocytes.

A clearly illustrative example of this type of interpretational ambiguity recently arose in a study from Liu and colleagues³⁹ with a different transgenic reporter mouse line. In their attempt to find evidence of proliferative *Ddx4*-expressing cells in postnatal mouse ovaries, gonads were obtained from female offspring of *Ddx4-Cre* transgenic mice crossed with *Rosa26^{trbw/+}* mice, which leads to a permanent genetic ‘switch’ in fluorescence from GFP (nonrecombined) to red, yellow or cyan fluorescent protein in any cell that has at some point transcriptionally activated the *Ddx4* gene (recombined). Unfortunately, because both types of germ cells—premeiotic and postmeiotic—arise from more primitive germ cells that have

already activated *Ddx4* expression, the *Ddx4*-Cre recombinase-based switch in fluorescence is not specific for the identification of any one type of germ cell. In other words, OSCs and oocytes have an identical fluorescence pattern, which makes data interpretation in this type of study impossible unless additional steps are taken to unequivocally confirm the identity of the cells being evaluated. As a case in point, after crossing *Ddx4*-Cre transgenic mice with *Rosa26^{bw/+}* mice to obtain female offspring for analysis, Liu and colleagues³⁹ placed dispersed cell preparations obtained from ovaries of offspring at postnatal day 8 into culture. Over a subsequent 72-h period, recombined (red fluorescent, *Ddx4*-expressing) cells failed to proliferate, which prompted the conclusion that, contrary to many reports from us and others^{1–3,9,11,23}, mitotically active germ cells do not exist in postnatal mouse ovaries³⁹. However, no effort was made by these investigators to confirm the identity of the recombined cells on which this conclusion was based. In fact, the relatively large size of the red fluorescent cells shown³⁹, coupled with their inability to proliferate, suggests that the cells studied were actually small oocytes and not OSCs. Unfortunately, the interpretational problems with this experiment and its conclusions were only worsened by the absence of even a simple PCR-based gene expression profile showing the presence of markers of germ cells but not oocytes in the red fluorescent cells claimed as evidence against the existence of OSCs. These limitations, along with a lack of any experimental confirmation that the *Ddx4* promoter fragment used to drive Cre expression is not ‘leaky’ (i.e., activated *only* in germ cells), combine to make this study and its interpretations far from conclusive.

Finally, crudely dispersed ovarian cell preparations and surface epithelial cell ‘scrapings’ have also been reported as a means to study fGSCs *in vitro*^{23,40–42}. It is important to note, however, that these methods invariably yield a heterogeneous cell population. Approaches such as these that do not target a specific cell type are therefore inherently problematic, as ovaries contain multiple stem cell populations^{43–45}, none of which have been shown to possess the *in vivo* oocyte-forming capacity of OSCs. In addition, although germ cells derived from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) differentiated in culture have been reported to form developmentally competent eggs after being recombined with fetal ovarian tissue and transplanted adjacent to adult ovarian tissue *in vivo*⁴⁶, the differences between OSCs and germ cells derived from ESCs or iPSCs are many and substantial. Aside from the fact that OSCs are natural precursor cells for oocyte formation, and they accordingly perform this task without issue in adult ovarian tissue^{2,3}, ESC- and iPSC-derived germ cells require instructive cues from embryonic ovarian tissue to generate functional oocytes. In addition, oocyte formation after transplantation of ESC- or iPSC-derived germ cells recombined with fetal ovaries occurs in a single synchronous wave⁴⁶. This outcome mirrors that observed with transplanted embryonic PGCs but differs from the sustained oogenesis achieved with OSCs after transplantation into adult ovaries^{2,3}.

Summation

With this background information in mind, the following sections detail methods for *Ddx4* antibody-based sorting of OSCs from adult human and mouse ovarian tissues, and for studying the properties and functions of purified OSCs *in vitro* and *in vivo*. A comprehensive schematic overview of the major protocol steps is shown in Figure 1, whereas a detailed description of potential pitfalls and a guide for protocol troubleshooting

are provided in Table 1. It also should be mentioned that, although the protocols described herein are targeted at the purification and study of adult mammalian ovary-derived OSCs, we have tested and validated the C-terminal Ddx4 antibody-based FACS protocol for the purification of PGC-like cells from cultures of mouse ESCs after spontaneous differentiation⁴⁷ (D.C.W. and J.L.T., unpublished observations). Hence, the methods described here should be easily adaptable for those who use ESCs or iPSCs as a source of stem cells for germ cell derivation. Finally, our referral to the mitotically active germ cells that support oogenesis in adult ovaries as OSCs is intended to simply parallel the term SSCs, which is frequently used to describe the analogous population of mitotically active germ cells present in the adult testes that are responsible for spermatogenesis.

Experimental design

Isolation of OSCs from mouse ovaries or human ovarian cortex—Although OSCs can be isolated from ovaries of mice throughout the reproductive lifespan⁴⁸, for initial validation of the protocol, we recommend using ovaries from mice between 6 and 8 weeks of age, as the ovarian tissue is more easily dispersed with collagenase. For the isolation of human OSCs, we have used freshly collected, vitrified-warmed and slow-frozen-thawed ovarian cortical tissue pieces with equal success. In addition, we have successfully isolated OSCs from ovarian cortical tissue of women in their 20s, 30s, 40s and 50s (ref. 3; D.C.W. and J.L.T., unpublished observations).

Dissociation of ovarian tissue—Both mouse and human tissues are dispersed into single-cell suspension using mechanical dissociation with collagenase. It should be noted that the enzymatic activity of collagenase varies considerably across suppliers and even across lots from a single supplier. As such, the dilution of collagenase needs to be carefully considered and adjusted as needed to reflect differences in units of enzymatic activity per mg of protein. Efficient retrieval and optimal viability of OSCs are both markedly affected by the amount of collagenase activity and length of exposure time of the ovarian tissue to collagenase for cell dispersion (Table 1).

Isolation of OSCs by FACS or MACS—Both FACS and MACS have been described as methods for the isolation of OSCs from adult ovary tissue^{2,3,11}. Although both methods use antibody-based detection of externally exposed protein epitopes on OSCs, in our experience, the cell population obtained by FACS (versus MACS) shows much greater viability and purity, with the latter demonstrated by a lack of oocyte markers in the final OSC fraction collected³. In addition, dead-cell exclusion and cell size-based inclusion can be performed simultaneously using FACS, further enhancing the viability and purity of the cell population obtained. Both FACS- and MACS-based isolation methods rely on the use of a C-terminal Ddx4 antibody to detect expression of this protein on the cell surface of OSCs. Although Ddx4 is expressed and detectable in both OSCs and oocytes in adult ovaries, OSCs express the C terminus of the protein on the cell surface, whereas Ddx4 is entirely cytoplasmic in oocytes³.

Alternative approaches and potential pitfalls—In addition to antibody-based isolation methods for the purification of OSCs from dissociated ovarian tissue^{2,3,11}, OSCs

have reportedly been isolated from ovaries using transgenic reporter mice⁹, as well as by crudely dispersing ovarian cell preparations, after which OSCs are selected by differential adhesion²³. However, because of considerable pitfalls associated with interpretation of data from the study of cells obtained by these and other approaches (see INTRODUCTION, Non-immunological-based methods to isolate OSCs), it is highly recommended that antibody-based sorting methods be used to purify OSCs.

Analysis of freshly isolated OSCs—Verification of the successful isolation of OSCs from ovarian tissue can be accomplished by simple PCR-based expression analysis of a panel of four genes that are widely accepted as primitive germ cell markers: *Prdm1*, *Dppa3*, *Ifitm3* and *Tert*. If desired, a defined homogenous pool of somatic cells, such as fibroblasts, can be used as a negative control for sample comparison; however, fibroblasts from adult tissue (such as tail-tips) should be used rather than fibroblasts from an embryonic or fetal source. The presence of contaminating oocytes can simultaneously be ruled out by the assessment of several oocyte-specific markers not expressed in OSCs, including newborn ovary homeobox (*Nobox*), growth differentiation factor 9 (*Gdf9*) and zona pellucida glycoproteins 1–3 (*Zp1–Zp3*) (ref. 3). It should be noted that of all of the OSC purification strategies reported to date, only the antibody-based FACS approach yields a purified population of homogenous cells free from contaminating oocytes and non-germline cell lineages. Notably, methods that do not exclude oocytes on the basis of the use of transgenic germline reporters that are not specific for only OSCs, or in the case of MACS oocytes that are dead or damaged (i.e., membrane compromised), can yield results that are ambiguous or impossible to accurately interpret (see INTRODUCTION, Non-immunological-based methods to isolate OSCs).

Establishment and propagation of OSCs in culture—As is the case with cultured SSCs, mouse and human OSCs require the presence of FBS and a cocktail of growth factors in the culture medium for viability and sustained growth. Serum is undefined, with substantial lot-to-lot variability. In our experience, FBS lots intended for use with ESCs provide adequate support for mouse and human OSCs maintained *in vitro*. Nonetheless, it may be beneficial to batch-test FBS lots for optimal performance and outcomes. This can be easily done using ESC cultures, with serum lots that successfully maintain ESCs in an undifferentiated state identified as suitable for use with OSC cultures. Furthermore, although an underlying monolayer of somatic feeder cells is not absolutely required for the successful establishment of mouse or human OSCs *in vitro*, the initial rate of proliferation is greatly enhanced using a coculture system in which freshly isolated OSCs are seeded onto mitotically inactivated mouse embryonic fibroblasts (MEFs). In our experience, optimal outcomes are achieved if MEFs are replaced every 2 weeks, and, during the initial establishment of the OSC cultures, the cells are passaged onto new MEFs every 10–12 d.

Analysis of *in vitro*-derived oocytes—After establishment in culture, a subset of mouse and human OSCs spontaneously initiate a differentiation program and form immature oocytes immediately after each passage of the cells that persists for up to 3 d (Fig. 2). The OSC-derived oocytes are non-adherent (released into the culture medium) and are easily distinguished from the cells in culture on the basis of their size and morphology. Oocytes

can be removed from the culture medium by selective aspiration and analyzed for expression of oocyte-specific markers by PCR or immunofluorescence-based cytochemistry (Fig. 2; ref. 3). In our experience, the number of oocytes produced each day of the 3 d after passage is consistent from passage to passage, and it can be used as a quantitative assessment of the rate of oocyte production from OSCs maintained under different experimental conditions *in vitro*⁴⁹.

Transplantation of human OSCs into human ovary tissue and xenografting—

For transplantation and fate-mapping experiments, OSCs can be readily transformed to express a fluorescence reporter, such as GFP, using standard viral transduction protocols. The reagents used to produce retrovirus are included in the Reagents section. The use of retroviruses has a distinct advantage for transduction of OSCs cocultured with MEFs, as retroviruses generally target only proliferating cells. As the feeder cells have been mitotically inactivated, the virus does not infect these cells. Once the protocol is performed, resultant GFP-expressing OSCs can be purified by FACS and used directly for injection or subsequently propagated without the loss of GFP expression. After stable integration of GFP, expanded human OSCs can be directly injected into small pieces (in general, pieces 2 mm long $\times$ 2 mm wide $\times$ 1 mm thick work well) of outer cortex obtained from ovaries of reproductive age women. The injected cortical samples can then be transplanted subcutaneously into immunocompromised mice. Successful xenografting occurs most frequently when the tissue is sutured in close proximity to the cutaneous vasculature. Retrieval of the grafts at 1–2 weeks after transplantation is sufficient for the subsequent immunodetection of GFP-positive human oocytes contained within histologically normal follicles comprising GFP-negative granulosa cells (Fig. 3; ref. 3).

Intraovarian transplantation of mouse OSCs into syngeneic recipient mice—

Using microdissection scissors to open the ovarian bursa surrounding each ovary of the recipient wild-type female mice, GFP-expressing mouse OSCs can be directly microinjected into the exposed ovarian tissue at several sites. Similar to what is observed after the delivery of GFP-expressing human OSCs into human ovarian cortical tissue, GFP-positive mouse oocytes are detectable in the recipient ovaries, enclosed within wild-type somatic cells as follicles, within 1–2 weeks (Fig. 3). These chimeric follicles progress through all stages of maturation, and they are responsive to gonadotrophic hormone stimulation, resulting in ovulation of the enclosed GFP-expressing oocytes (Fig. 4). These oocytes can be retrieved from the oviducts and embryonic development can be monitored after *in vitro* fertilization (IVF) using wild-type spermatozoa (Box 1 and Fig. 4; ref. 3). In parallel, mating trials can be conducted by housing wild-type female mice after intraovarian injection of GFP-expressing OSCs with wild-type male mice of proven fertility. Conventional PCR-based genotyping is then performed to determine whether the oocyte fertilized *in vivo* to produce a given offspring was derived from the recipient (wild-type) or from the transplanted OSCs (GFP-positive). For these types of functional tests, OSCs isolated from ovaries of wild-type female mice and transduced with GFP, or OSCs isolated from ovaries of transgenic female mice with documented expression of GFP in germ cells (for example, TgOG2), can be used to track cell fate after transplantation into wild-type recipients. It should be noted that, although a prior report of offspring production from transplanted mouse OSCs used

chemotherapy-conditioned female mice as recipients², we have found that mouse OSCs successfully engraft and produce functional oocytes after transplantation into ovaries of female mice without the need for any prior conditioning protocol^{3,24}.

MATERIALS

REAGENTS

- Avertin (or comparable anesthetic)
- Basic fibroblast growth factor, human recombinant (bFGF; Invitrogen, cat. no. 13256-029)
- BSA, fatty acid free (Sigma-Aldrich, cat. no. 85041C)
- Collagenase type IV (Worthington Biochemical Corporation, cat. no. LS004188)
▲ CRITICAL We have found that there is a substantial variability in the enzymatic activity of collagenase from different suppliers, and thus we recommend this specific supplier; we have also observed lot-to-lot variability in collagenase activity from this supplier, and we recommend batch-testing each lot empirically before use.
- Cryotissue thawing kit (Kitazato, cat. no. VT302-CT), optional (Step 1A)
- DAPI (Sigma-Aldrich, cat. no. 9542) **! CAUTION** This reagent is a biohazard; adequate safety precautions should be taken when handling as per the supplier's specifications.
- DMSO (Sigma-Aldrich, cat. no. D2650), optional (Step 18A)
! CAUTION This reagent is a biohazard; adequate safety precautions should be taken when handling as per the supplier's specifications.
- DNase I, recombinant grade I (Roche Applied Science, cat. no. 04536282001)
- Donkey anti-goat IgG antibody conjugated to Alexa Fluor 488 (Invitrogen, cat. no. A-11055)
- DMEM, 4.5 g per liter glucose (Invitrogen cat. no. 21969-035)
- Dulbecco's PBS, 1×-concentrated (calcium-free and magnesium-free; Invitrogen, cat. no. 14190)
- EDTA, 500 mM (Millipore, cat. no. 324504-500ML)
- Ethanol solution, 70% (vol/vol), sterile (Decon Laboratories, cat. no. 8116)
- Epidermal growth factor, human recombinant (EGF; Invitrogen, cat. no. PHG0311)
- FBS (ESC grade for optimal results; Invitrogen, cat. no. 26140-111)
- Gelatin solution, 0.1% (wt/vol) (Millipore, cat. no. ES-006-B)
- Glial-derived neurotrophic factor, human recombinant (GDNF; R&D Systems, cat. no. 212-GD-010)

- Goat anti-KIT antibody (Santa Cruz Biotechnology, cat. no. sc1494)
- Goat anti-rabbit IgG antibody conjugated to Alexa Fluor 488 (Invitrogen, cat. no. A-11008)
- Goat anti-rabbit IgG antibody conjugated to Alexa Fluor 568 (Invitrogen, cat. no. A-11011)
- Goat anti-rabbit IgG antibody conjugated to allophycocyanin (APC; Jackson ImmunoResearch, cat. no. 111-136-144), optional (Step 17A)
- Goat anti-rabbit IgG antibody-conjugated microbeads (Miltenyi Biotec, cat. no. 130-048-602), optional (Step 17B)
- HBSS (calcium-free, magnesium-free and phenol red-free; Gibco, cat. no. 14175-095)
- Human chorionic gonadotrophin (hCG; Sigma-Aldrich, cat. no. CG10-1VL), optional (Step 30C; Box 1)
- Human tubal fluid (HTF; Irvine Scientific, cat. no. 90125) supplemented with 0.4% (wt/vol) BSA (Reagent Setup), optional (Step 30; Box 1)
- Hyaluronidase solution (Irvine Scientific, cat. no. 90101), optional (Step 30C; Box 1)
- KSOM-AA medium (Millipore, cat. no. MR106), optional (Step 30C; Box 1)
- Leukemia inhibitory factor, human recombinant (LIF; ESGRO; Millipore, cat. no. ESG1106)
- Lipofectamine 2000 (Invitrogen, cat. no. 11668-027), optional (Step 30B or 30C)
- M199 culture medium (Sigma-Aldrich, cat. no. m4530), optional (Steps 1B and 30B)
- Mouse embryonic fibroblasts, irradiated, mitotically inactivated (MEFs; GlobalStem, cat. no. GSC-6201G)
- MEM- α (GlutaMax; Invitrogen, cat. no. 32561-102)
- β -Mercaptoethanol (β -ME; Pierce, cat. no. 35602) ! **CAUTION** This reagent is biohazardous; adequate safety precautions should be taken when handling as per the supplier's specifications.
- Mineral oil (Sigma-Aldrich, cat. no. M8410), optional (Step 30C; Box 1)
- Mouse anti-PRDM1 antibody (Abcam, cat. no. ab81961)
- Mouse monoclonal anti-GFP antibody (Santa Cruz Biotechnology, cat. no. sc9996), optional (Step 30B or 30C)
- Mouse on mouse (MOM) antibody kit (Vector Labs, cat. no. BMK-2202), optional (Step 30B or 30C)
- N-2 MAX media supplement (R&D Systems, cat. no. AR009)

- Non-essential amino acids (NEAA; Invitrogen, cat. no. 11140-050)
- Normal donkey serum (Sigma-Aldrich, cat. no. D9663)
- Normal goat serum (Millipore, cat. no. S26-Liter)
- Nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice (6-week-old females; Charles River Laboratories or equivalent), optional (Step 30B) ! **CAUTION** Experiments involving mice must conform to the policies, rules and regulations of the user's institutional, local and/or national oversight committee(s) for the care and use of laboratory research animals.
- Paraformaldehyde (formaldehyde, methanol-free), 16% (wt/vol) (PFA; Pierce, cat. no. 28906) ! **CAUTION** This reagent is biohazardous; adequate safety precautions should be taken when handling as per the supplier's specifications.
- *pBabe-Gfp* plasmid (Addgene plasmid repository, cat. no. 10668), optional (Step 30B or 30C)
- Penicillin-streptomycin-glutamine solution (PSG; Invitrogen, cat. no.10378-016)
- Platinum Taq polymerase (Invitrogen, cat. no. 10966-018)
- Platinum-A retroviral packaging cell line (Cell Biolabs, cat. no. RV-102), optional (Step 30B or 30C)
- Pregnant mare serum gonadotrophin (PMSG; Sigma-Aldrich, cat. no. G4877-1000 IU), optional (Step 30C; Box 1)
- Polybrene (Millipore, cat. no. TR-1003-G), optional (Step 30B or C)
- Rabbit anti-DDX4 antibody (Abcam, cat. no. ab13840) ▲ **CRITICAL** We have observed substantial lot-to-lot variation in DDX4 antibody concentration from this supplier, and therefore we recommend empirical testing of each lot before experimental use. We have tested and validated the following antibody lots from Abcam for use in FACS for successfully isolating OSCs: 957695, 960162, GR7257-1, GR29711-1 and GR61610-1; lot number GR37657-1 was of low concentration and should be avoided.
- Rabbit anti-Ifitm3/IFITM3 antibody (Abcam; mouse cat. no. ab15592; human cat. no. ab109429)
- Rabbit anti-Dppa3 antibody (Abcam, cat. no. ab19878)
- Rabbit anti-GFP antibody (Cell Signaling, cat. no. 2555), optional (Step 30B or 30C)
- Rabbit anti-LHX8 antibody (Abcam, cat. no. ab41519)
- Rabbit anti-YBX2 antibody (Abcam, cat. no. ab33164)
- Rhodamine-phalloidin (Invitrogen, cat. no. R415)
- Sodium pyruvate (Invitrogen, cat. no. 11360-070)
- Streptavidin-conjugated Alexa Fluor 488 (Invitrogen, cat. no. S-11223)

- SuperScript VILO cDNA synthesis kit (Invitrogen, cat. no. 11754-050)
- Triton X-100 (Promega, cat. no. H5142)
- Trypsin (0.05% wt/vol)-EDTA (Invitrogen, cat. no. 25300-054)
- Young adult (6–8 weeks of age) female C57BL/6 mice (Charles River Laboratories or equivalent), optional (Step 2A) ! **CAUTION** Experiments involving mice must conform to the policies, rules and regulations of the user's institutional, local and/or national oversight committee(s) for the care and use of laboratory research animals.
- Young adult (6–8 weeks of age) female B6;CBA-Tg(*Pou5f1-EGFP*)2 Mnn/J (TgOG2 or PE-*Oct4-Gfp*) transgenic mice (Jackson Laboratories, stock no. 004654), optional (Step 30C) ! **CAUTION** Experiments involving mice must conform to the policies, rules and regulations of the user's institutional, local and/or national oversight committee(s) for the care and use of laboratory research animals.
- Young adult (3–4 months of age) male C57BL/6 mice (Charles River Laboratories or equivalent), optional (Step 30; Box 1) ! **CAUTION** Experiments involving mice must conform to the policies, rules and regulations of the user's institutional, local and/or national oversight committee(s) for the care and use of laboratory research animals.

EQUIPMENT

- Analytical balance (Mettler Toledo, model AB204-S or similar)
- Biosafety cabinet (Thermo Scientific, 1300 series or similar)
- C-tubes (Mitenyi Biotec, cat. no. 130-093-237), optional (Step 2B)
- Cell strainers/filters, 100- μ m pore size (Partec Celltrics, cat. no. 04-004-2328)
- Centrifuge (Eppendorf, model 5810R or similar)
- Conical centrifuge tubes, polypropylene, 15 ml (17 $\times$ 120 mm; BD Falcon, cat. no. 352097)
- Cryogenic handling gloves (Thermo Scientific, cat. no. 189573), optional (Steps 1 and 18A)
- Cryogenic unit for liquid nitrogen storage (MVE Cryogenics, TEC2000 or similar), optional (Steps 1 and 18A)
- Cryogenic storage vials, 2 ml (Corning, cat. no. 430488 or equivalent), optional (Step 18A)
- Disposables for use in conventional cell culture (culture dishes, multiwell plates, filter units, syringe filters, pipettes, pipette tips and so on)
- Ethanol-resistant pen (Phoenix Research Products, cat. no. MP-1B or similar)
- Eye protection (according to institutional guidelines)

- Fine forceps no. 5 (Fine Science Tools, cat. no. 11254-20)
- Fine scissors (Fine Science Tools, cat. no. 14090-09)
- FACS; Becton Dickinson, model Aria II with software or similar), optional (Step 17A)
- Fluorescent microscope with appropriate lasers and objectives (Nikon Eclipse TE2000-S or equivalent)
- Gel electrophoresis equipment (CBS Scientific, cat. no. GCMGU-102T)
- GentleMACS tissue dissociator (Miltenyi Biotec, cat. no. 130-093-235), optional (Step 2B)
- Glass tissue culture dishes (Electron Microscopy Sciences, cat. no. 70648-14)
- Heated water bath (Thermo Scientific, cat. no. 2827 or similar)
- Image analysis system (UVP GelDocIt-300 or equivalent)
- Isopropanol cell-freezing container, Mr. Frosty (Nalgene, cat. no. 5100-0001), optional (Step 18A)
- Laboratory shaker (Lab-line orbital environmental shaker, model 3527 or similar)
- Laminar flow hood (Forma Scientific, model 1828 or similar)
- Light microscope
- MACS cell separation columns (Miltenyi Biotec, cat. no. 130-042-201), optional (Step 17B)
- Microcentrifuge (Eppendorf, model 5430 or similar)
- Micropipette tips for Pneumatic Pico Pump, glass (World Precision Instruments, cat. no. TIPMIX05-10), optional (Step 30C)
- MiniMACS separator (Miltenyi Biotec, cat. no. 130-090-312), optional (Step 17B)
- Multistand (Miltenyi Biotec, cat. no. 130-042-303), optional (Step 17B)
- NanoFil needles, 35-gauge beveled (World Precision Instruments, cat. no. NF35BV-2), optional (Step 30B)
- NanoFil syringes, 10- μ l (World Precision Instruments, cat. no. NANOFIL), optional (Step 30B)
- Orbital shaker (Thermo Scientific MaxQ 4000 or equivalent)
- PCR machine (Applied Biosystems GeneAmp 9700 or equivalent)
- Pipet-aid (Drummond, cat. no. 4-000-110 or similar)
- Pneumatic PicoPump (World Precision Instruments, cat. no. SYS-PV820 or similar), optional (Step 30C)

- Refrigerated centrifuge (Eppendorf, model 5810R or similar)
- Round-bottom test tubes with 35- μ m cell strainer cap, 5 ml, polystyrene (BD Biosciences, cat. no. 352235)
- Scalpel blades (Complete Medical Supplies, cat. no. 19135G)
- Serological pipettes, 5 ml, borosilicate glass, disposable, individually wrapped and sterile (Fisher Scientific, cat. no. 13-678-27E)
- Serological pipettes, 10 ml, borosilicate glass, disposable, individually wrapped and sterile (Fisher Scientific, cat. no. 13-678-27F)
- Tissue culture incubator set to 37 °C, gassed with 5% CO₂–95% air under humidified conditions
- Vortex (Scientific Industries, model Vortex Genie 2, cat. no. G-560 or similar)

REAGENT SETUP

Slow-frozen human cortical tissue (optional; can use fresh)—There are many methods reported to slow-freeze ovarian tissue for long-term storage in liquid nitrogen. In our laboratory, we perform a very simple procedure, which entails the following steps: if working with human ovarian cortex, cut tissue into small pieces (3 mm long $\times$ 3 mm wide $\times$ 1 mm thick or smaller); if you are working with mouse ovaries, simply clean away non-ovarian tissue and freeze entire ovaries intact. Place 1 ml of freezing medium (FBS containing 10% (vol/vol) DMSO, precooled to 4 °C) into a 2-ml cryovial. Place up to five pieces of human ovarian tissue or five mouse ovaries into the cryovial. Close the vial and place the tube into an isopropanol cell-freezing device (Mr. Frosty). Transfer it to a –80 °C freezer and incubate overnight. The next day, transfer the cryovial to a liquid nitrogen storage container.

! CAUTION Informed consent must be obtained from human subjects who donate ovarian tissue for research purposes, and proposed experiments must conform to all required institutional, local and/or national policies and regulations for human subjects research.

Antibody blocking/dilution solution—Dissolve 200 mg of BSA in 9.8 ml of HBSS, and then add 200 μ l of normal goat serum. Sterile-filter the solution through a 0.22- μ m filtration device and place it on ice until use. The solution should be prepared fresh each time.

bFGF (human recombinant)—Prepare a 10 μ g ml^{–1} stock solution by adding 1 ml of sterile PBS containing 0.1% (wt/vol) BSA to a 10- μ g vial of bFGF. Working aliquots (50 μ l) can be prepared and stored at –20 °C for up to 6 months.

Collagenase/DNase I solution—Dissolve lyophilized collagenase type IV according to the units of enzymatic activity, which is specific for each lot, in HBSS. For mechanical dissociation of human ovarian cortex using the GentleMACS dissociator, dissolve collagenase to a final concentration of 400 U ml^{–1}. For manual dissociation of mouse ovarian tissue, dissolve collagenase to a final concentration of 800 U ml^{–1}. Prepare a 1 mg

ml⁻¹ stock solution of DNase I, divide it into aliquots and store them at -20 °C. Before use, add DNase I solution to collagenase solution at a 1:1,000 (vol:vol) final ratio. Filter-sterilize the solution through a 0.22-μm filtration device and place it in a 37 °C water bath until use. This solution should be freshly prepared each time.

DAPI stock solution—Dissolve 10 mg of DAPI in 2 ml of sterile molecular biology-grade water. This solution can be stored at 4 °C for up to 6 months.

EGF (human recombinant)—Prepare a 100 μg ml⁻¹ stock solution by adding 1 ml of sterile PBS to a 100-μg vial of EGF. Working aliquots (50 μl) can be prepared and stored at -20 °C for up to 6 months.

FACS buffer—Add 50 μl of FBS to 50 ml of cold HBSS. Place the buffer on ice. This buffer should be freshly prepared each time.

GDNF (human recombinant)—Prepare a 10 μg ml⁻¹ stock solution by adding 1 ml of sterile PBS containing 0.1% (wt/vol) BSA to a 10-mg vial of GDNF. Working aliquots (40 μl) can be prepared and stored at -20 °C for up to 6 months.

hCG—Reconstitute a 10,000-IU vial with 10 ml of sterile PBS. Divide 50-μl aliquots into individual vials, and then add an additional 200 μl of sterile PBS to each vial for a stock concentration of 200 IU ml⁻¹. Each aliquot can be stored at -20 °C for up to 3 months. Immediately before use, dilute a single stock vial with 750 μl of sterile PBS, for a final working concentration of 50 IU ml⁻¹ (injection volume per mouse is 100 μl, to deliver 5 IU total).

MACS buffer—Dissolve 50 mg of BSA into 9.96 ml of PBS, and then add 40 μl of 500 mM EDTA solution; place on ice until use. This buffer should be freshly prepared each time.

OSC culture medium—OSC culture medium is MEM-α (GlutaMAX) supplemented with 10% (vol/vol) FBS, 1 mM sodium pyruvate, 1 mM NEAA, 1×-concentrated PSG, 0.1 mM β-ME, 1×-concentrated N-2 supplement, 10³ units per ml LIF, 10 ng ml⁻¹ EGF, 1 ng ml⁻¹ bFGF and 40 ng ml⁻¹ GDNF. Filter-sterilize the solution through a 0.22-μm filtration device. Store the solution at 4 °C for up to 14 d.

OSC freezing (cryopreservation) medium—Freezing medium is DMEM containing 20% (vol/vol) FBS and 10% (vol/vol) DMSO. Store the medium at 4 °C for up to 14 d.

PFA—For a 4% solution of PFA, bring 10 ml of 16% PFA stock solution to 40 ml in PBS. The solution can be stored at 4 °C for up to 2 weeks. For immunocytochemical analysis, the 4% solution can be further diluted with PBS to make a 2% PFA solution.

PMSG—Reconstitute a 1,000-IU vial with 5 ml of sterile PBS for a stock solution of 200 IU ml⁻¹. Divide 250-μl aliquots of the solution into individual vials and freeze them at -20 °C for up to 3 months. Immediately before use, dilute the stock vial with 750 μl of

sterile PBS for a final working concentration of 50 IU ml⁻¹ (injection volume per mouse is 100 µl, to deliver 5 IU total).

Primary antibody dilution for either FACS or MACS—Prepare 100 µl of primary antibody solution by adding 10 µl of rabbit anti-DDX4 antibody to 90 µl of antibody blocking/dilution solution. Place the solution on ice until use. The solution should be freshly prepared each time.

Secondary antibody dilution for FACS only—Prepare 500 µl of secondary antibody solution by adding 2 µl of secondary antibody (goat anti-rabbit IgG antibody conjugated to APC) to 498 µl of antibody blocking/dilution solution. The solution should be prepared fresh each time.

Secondary antibody dilution for MACS only—Prepare 100 µl of secondary antibody solution by adding 10 µl of goat anti-rabbit IgG antibody-conjugated microbeads to 90 µl of antibody blocking/dilution solution. The solution should be freshly prepared each time.

EQUIPMENT SETUP

FACS—For our studies of OSCs, we have used a FACSAria II benchtop flow cytometer running DiVa 6 software and equipped with 405-, 488- and 633-nm lasers. Unlabeled and secondary antibody only-labeled dissociated ovarian tissue samples are used each time as controls to gate against background autofluorescence and to rule out non-specific binding of the secondary antibody.

Gelatin-coated dishes—Prepare gelatin-coated tissue culture dishes by adding 0.1% (wt/vol) gelatin solution so as to completely cover the bottom of each dish. Incubate the dishes for 1–4 h at 37 °C. Remove the gelatin solution before use with cells.

MEF feeder cell plates—MEF culture medium is DMEM supplemented with 10% (vol/vol) FBS and 1×-concentrated PSG solution. Thaw a frozen vial of irradiated MEFs and resuspend with MEF culture medium at a density of 1 × 10⁵ cells ml⁻¹. Seed 0.5 ml of the MEF cell suspension into each well of a 24-well gelatin-coated tissue culture dish. Culture the plates in a humidified chamber at 37 °C under 5% CO₂–95% air.

PROCEDURE

! CAUTION We have found that the use of trypsin during any stage of the ovarian tissue dissociation procedure is unnecessary and should be avoided entirely to minimize damage to externally exposed protein epitopes on viable cells that may preclude successful binding of the C-terminal Ddx4 antibody to its target on OSCs for effective cell sorting. Although the use of very low concentrations or, more preferably, complete avoidance of trypsin in studies that rely on the use of cell-surface antigens, is generally well known in the field, there are recent published examples of trypsin-based (with no collagenase) ovarian tissue dispersion being used to generate cell suspensions for analysis of female germ cells³⁹. Such approaches would generate cell suspensions that are not amenable to successful Ddx4 antibody-based sorting of OSCs from dispersed ovaries.

▲ **CRITICAL** Before beginning the PROCEDURE, place 50 ml of HBSS on ice to chill (cold HBSS) and 50 ml of HBSS in a 37 °C water bath to prewarm (warm HBSS).

Cryopreserved ovarian tissue preparation

- 1| If you are working with vitrified or slow-frozen tissue, follow option A (warming of vitrified ovarian tissue) or B (thawing of slow-frozen ovarian tissue) before proceeding with Step 2. If you are using fresh tissue, proceed directly to Step 2. We warm vitrified tissue via an osmotic gradient using a cryotissue thawing kit according to the details provided by the manufacturer (Kitazato). We have used freshly collected, vitrified-warmed and slow frozen–thawed human ovarian cortical tissue pieces to obtain OSCs with equal success.

? TROUBLESHOOTING

(A) Warming of vitrified ovarian tissue ● TIMING ~20 min

- i. Remove the vitrified tissue sample from liquid nitrogen storage.
- ii. Place the cryovial containing the sample into a Dewar container into which liquid nitrogen has been added.

! **CAUTION** Take adequate safety precautions when working with liquid nitrogen.
- iii. Warm the thawing solution contained in a 50-ml tube provided in the kit to 37 °C in a water bath; water temperature should be verified and recorded using a calibrated thermometer.
- iv. Bring the diluent solution, washing solution 1 and washing solution 2 provided in separate tubes in the kit to 20–21 °C (room temperature); all three solutions should be at room temperature for ~1 h before use.
- v. Place all items (tubes containing thawing, diluent and washing solutions) in a sterile hood; all items placed in the hood should be sprayed with 70% (vol/vol) ethanol and wiped down. Quickly remove the cryovial from liquid nitrogen and remove the cap from the cryovial.
- vi. Remove the cryoapparatus from the cryovial and quickly place into the prewarmed thawing solution.
- vii. The tissue will fall off the cryoapparatus and sink to the bottom of the tube; leave the tissue in the thawing solution for 1 min after immersion.
- viii. Pour the contents of the tube (thawing solution and sample) into an appropriately labeled 150-mm × 20-mm cell culture dish (e.g., Nunc, cat. no. 168381).
- ix. Pour the contents of the diluent solution into an appropriately labeled 100-mm × 20-mm cell culture dish (e.g., Falcon, cat. no. 353003).
- x. By using a new pair of sterile forceps, transfer the tissue from the thawing solution dish to the diluent solution dish and let it sit for 3 min.

- xi.** Pour the full containers of washing solution 1 and washing solution 2 into separate appropriately labeled 100-mm × 20-mm cell culture dishes. This can be done while the tissue is in the diluent solution.
- xii.** Transfer the tissue from the diluent solution dish to the washing solution 1 dish and let it sit for 5 min.
- xiii.** Transfer the tissue from the washing solution 1 dish to the washing solution 2 dish and let it sit for 5 min.
- xiv.** Remove the tissue from the washing solution 2 dish and proceed with Step 2.

(B) Thawing of slow-frozen ovarian tissue ● TIMING ~35 min

- i.** By using a 5-ml glass serological pipette, add 1.5 ml of thaw medium (HEPES-buffered M199 medium containing 10% (vol/vol) FBS) to each well of a six-well tissue culture dish. Label the wells no. 1, no. 2, no. 3, no. 4, no. 5 and no. 6.
- ii.** Leave the dish for 15 min or until medium has reached room temperature.
- iii.** Remove the frozen cryovial containing slow-frozen ovarian tissue from liquid nitrogen tank.
! CAUTION Take adequate safety precautions when working with liquid nitrogen.
- iv.** Warm the cryovial by rubbing between hands until freezing medium has thawed (~2–3 min).
- v.** By using a pair of sterile forceps, remove the pieces of ovarian tissue from the cryovial and place into well no. 1 of the six-well plate containing the thaw medium (prepared above). Keep the sterile forceps for further use in sequentially transferring the pieces of tissue from one well to another as described below.
- vi.** Gently rotate the dish by rocking back and forth to quickly rinse the tissue pieces (5–10 s).
- vii.** Remove the tissue pieces from well no. 1 and place them into well no. 2, and then briefly rinse as described above; repeat this procedure through well no. 3 until the tissue has been placed into well no. 4.
- viii.** Incubate the tissue in well no. 4 for 5 min, periodically rotating the plate to rinse the tissue pieces. Transfer the tissue to well no. 5 and repeat the 5-min incubation with periodic rotation (rinsing).
- ix.** Transfer the tissue to well no. 6 and repeat the 5-min incubation with periodic rotation (rinsing).
- x.** Remove the tissue from well no. 6, and proceed with Step 2.

Dissociation of ovarian tissue

- 2| If you are working with mouse tissue, follow option A (dissociation of mouse ovarian tissue). If you are working with human tissue, follow option B (dissociation of human ovarian cortex).

(A) Dissociation of mouse ovarian tissue ● TIMING ~70 min

- i. Just before ovarian tissue collection, prewarm an orbital shaker to 37 °C. Carefully dissect the ovaries from female mice, taking care to remove the attached fat pad, bursa and oviduct from each ovary.
- ▲ **CRITICAL STEP** Tissue dispersion into single-cell suspension is easier using ovaries from younger adult animals (6–8 weeks of age), which may help to improve overall OSC yield. In our experience, optimal OSC retrieval is obtained using four ovaries pooled as a single sample, and retrieval efficiency is not improved further with additional ovaries added to the sample.

? TROUBLESHOOTING

- ii. By using a scalpel blade or mincing scissors, mince the ovaries into slurry in 0.5 ml of collagenase/DNase I solution (Reagent Setup) in a glass tissue culture dish.

? TROUBLESHOOTING

- iii. By using a 5-ml glass serological pipette, rinse the slurry to the bottom of the dish with 2.5 ml of collagenase/DNase I solution and collect the slurry by placing the entire 3 ml of solution into a 15-ml conical tube.

? TROUBLESHOOTING

- iv. Incubate the tube in a prewarmed (37 °C) orbital shaker for 15 min at 250 r.p.m.
- v. Remove the tube from the orbital shaker and manually disperse with gentle pipetting using a 5-ml glass serological pipette.

? TROUBLESHOOTING

- vi. Incubate the tube in the orbital shaker at 37 °C for an additional 15 min at 250 r.p.m.

- vii. Remove the tube from the orbital shaker and manually disperse with gentle pipetting until no visible pieces of ovary are present.

? TROUBLESHOOTING

- viii. Filter the cell suspension through a 100-µm nylon mesh cell strainer, collecting the filtrate into a new 15-ml conical tube.

? TROUBLESHOOTING

- ix. Add 10 ml of warm HBSS to the conical tube containing the strained cell suspension (from Step 8), and centrifuge the tube at 300g for 5 min at room temperature, with the centrifuge brake turned off (brake rate set to 0; this is very important so as to minimize disruption or dislodging of the cell pellet).

? TROUBLESHOOTING

- x. After centrifugation, carefully decant the liquid and save the cell pellet. Take care to remove as much of the supernatant as possible without disturbing the cell pellet (this can be done by gently touching the opening of the inverted 15-ml conical tube on sterile gauze). Proceed with Step 3.

▲ **CRITICAL STEP** Aspiration of the liquid, rather than gentle decanting, is not recommended as the vacuum suction created by aspiration can dislodge or disrupt the cell pellet and result in cell loss.

(B) Dissociation of human ovarian cortex ● TIMING ~55 min

- i. Tissue size should be 2 mm long × 2 mm wide × 1 mm thick. Cut tissue pieces to size with a sterile scalpel, if necessary. Transfer the tissue sample to a sterile C-tube. Multiple tissue pieces can be placed into the same tube.

▲ **CRITICAL STEP** Increasing the amount of tissue digested can improve total cell yield; however, OSCs can be successfully isolated from a single 2 mm × 2 mm × 1 mm piece of adult human ovarian cortical tissue.

- ii. Add 3 ml of prewarmed (37 °C) collagenase/DNase I solution to the C-tube containing the tissue. Tightly close the C-tube with the screw cap provided, invert the tube and place it into the GentleMACS tissue dissociator. When placing the tube into the dissociator, ensure that the tissue is in the collagenase/DNase I solution and not stuck on the side of the tube.

? TROUBLESHOOTING

- iii. Run the manufacturer's program '1-H_tumor_01.01' on the GentleMACS dissociator.

▲ **CRITICAL STEP** Inspect the sample in the tube following completion of the run. In the event that tissue fragments become trapped in the C-tube dispersion mechanism, open the tube in a sterile hood and place the tissue back into the collagenase/DNase I solution using sterile forceps.

- iv. Place the C-tube in an orbital shaker at 37 °C and incubate it for 20 min at 250 r.p.m.

- v. Remove the C-tube from the orbital shaker and place it, inverted, onto the GentleMACS dissociator. Run the manufacturer's program '1-H_tumor_02.01'.

▲ **CRITICAL STEP** Inspect the sample in the tube after completion of the run. In the event that tissue fragments become trapped in the C-tube dispersion mechanism, open the tube in a sterile hood and place the tissue back into the collagenase/DNase I solution using sterile forceps.

- vi. Place the C-tube in an orbital shaker set at 37 °C and incubate it for 20 min at 250 r.p.m.

- vii. Remove the C-tube from the orbital shaker and place it, inverted, onto the GentleMACS dissociator. Run the manufacturer's program '1-H_tumor_03.01'.

▲ CRITICAL STEP Inspect the sample in the tube after completion of the run. In the event that tissue fragments become trapped in the C-tube dispersion mechanism, open the tube in a sterile hood and place the tissue back into the collagenase/DNase I solution using sterile forceps.

- viii. Inspect the tissue. By using a 5-ml glass serological pipette, gently pipette up and down until the majority of the tissue has been dispersed.

? TROUBLESHOOTING

- ix. Filter the solution including the ovarian remnants through a 100- μ m cell strainer into a 15-ml conical tube. Keep the strainer for use in Step 2B(xi) below.

▲ CRITICAL STEP We recommend using 15-ml conical tubes rather than round-bottom tubes for effective pelleting of cells by centrifugation.

- x. Rinse the C-tube by adding 10 ml of warm HBSS. Tightly cap the tube and invert it to rinse. Remove the cap.
- xi. Collect the HBSS with a 10-ml glass serological pipette and use the collected HBSS to rinse the strainer from Step 2B(ix) into the 15-ml conical tube containing the strained cell suspension from Step 2B(ix).
- xii. Centrifuge the strained ovarian cell suspension (in the 15-ml conical tube) at 300g for 5 min at room temperature with the brake on the centrifuge turned off (brake rate set to 0).

? TROUBLESHOOTING

- xiii. After centrifugation, carefully decant the liquid and save the cell pellet. Take care to remove as much of the supernatant as possible without disturbing the cell pellet (this can be done by gently touching the opening of the inverted tube on sterile gauze). Proceed with Step 3.

▲ CRITICAL STEP We do not recommend aspiration of the liquid, rather than gentle decanting, as the vacuum suction created by aspiration can dislodge or disrupt the cell pellet and result in cell loss.

- 3| By using a sterile 5-ml glass serological pipette, gently resuspend the cell pellet in 4 ml of warm HBSS. Bring the volume to 10 ml with warm HBSS.
- 4| Centrifuge the cell suspension at 300g for 5 min at room temperature with the brake on the centrifuge turned off (brake rate set to 0).

? TROUBLESHOOTING

- 5| After centrifugation, carefully decant the liquid and save the cell pellet. Take care to remove as much of the supernatant as possible without disturbing the cell pellet (this can be done by gently touching the opening of the inverted vial on sterile gauze).

▲ CRITICAL STEP All prior steps are performed at 37 °C or room temperature, as indicated, for optimal enzymatic digestion and centrifugation. Subsequent steps are performed at 4 °C.

? TROUBLESHOOTING

Preparation of cells for antibody labeling ● TIMING 90 min

- 6| Gently resuspend the cell pellet obtained in Step 5 in 500 µl of cold antibody blocking/dilution solution. Label this tube as ‘sample’ and place it on ice for 20 min.

! CAUTION All incubations on ice should be performed using fresh (non-melted) ice to minimize cell damage (all tubes containing cells should be in the ice phase and not exposed to the ice-melted ice water interface).

? TROUBLESHOOTING

- 7| Set the centrifuge to 4 °C. Perform this step when the tube of cells is first placed on ice in Step 6 to give ample time for the centrifuge to cool to 4 °C. The brake speed should be set to 9 or maximum.
- 8| Label a 15-ml conical tube as ‘negative control’, and label another 15-ml conical tube as ‘secondary antibody only’.
- 9| Add 100 µl of cell suspension from the sample tube (Step 6) to the negative control and secondary antibody only tubes. Place the tubes on ice.
- 10| Bring the remaining 300 µl of cell suspension in the sample tube to 10 ml with cold HBSS. Centrifuge the suspension at 300g for 5 min at 4 °C.

? TROUBLESHOOTING

- 11| Remove the sample tube from the centrifuge and discard the supernatant, being careful not to disturb or dislodge the cell pellet.

▲ CRITICAL STEP Ensure that as much supernatant as possible is removed as any residual supernatant volume will dilute the final primary antibody concentration at the next step; we recommend that the sample tube be gently inverted over sterile gauze to blot, and then any residual supernatant volume should be carefully collected away from the cell pellet with a small pipette tip.

? TROUBLESHOOTING

- 12| Gently resuspend the cell pellet in 100 µL of prepared primary antibody solution (Reagent Setup).

? TROUBLESHOOTING

- 13| Place the suspension on ice and incubate it for 20 min.
- 14| Bring the resuspended cell sample mixed with primary antibody in the ‘Sample’ tube to a total of 10 ml with cold HBSS. Centrifuge the mixture at 300g for 5 min at 4 °C.

? TROUBLESHOOTING

- 15| Remove the sample tube from the centrifuge and carefully discard supernatant. Depending on the amount of initial starting material (ovarian tissue), a cell pellet may or may not be readily visible with the naked eye at this time.
- 16| Gently resuspend the sample tube cell pellet in 10 ml of cold HBSS. Centrifuge the suspension at 300g for 5 min at 4 °C. At this time, centrifuge the cells in the secondary antibody only tube (from Step 9). Discard the supernatants.

? TROUBLESHOOTING**Isolation or enrichment of OSCs**

- 17| If you are using FACS to isolate OSCs, follow option A. If you are using MACS to enrich for OSCs, follow option B.

▲ CRITICAL STEP We highly recommend the use of FACS instead of MACS for isolation of OSCs, as we have found that OSC preparations obtained by MACS are often contaminated with small oocytes³. In addition, FACS offers the user the advantages of damaged or dead cell exclusion and homogenous cell-size selection. If, however, the user does not have access to FACS facilities, MACS can be used to obtain OSCs, although subsequent use and analysis of the cells should take into account the possibility of oocyte contamination.

(A) Isolation of OSCs via FACS ● TIMING ~1.5–2 h

- i. Gently resuspend cell pellet in the sample tube, as well as the cell pellet in the secondary antibody only tube (from Step 16), in 250 µl of prepared secondary antibody solution for FACS (Reagent Setup).

▲ CRITICAL STEP Depending on the FACS equipment available, the laser setup or even the experimental design, it may be necessary to use a fluorophore other than APC. Alternative methods for fluorescent labeling, such as the use of a different fluorophore or direct conjugation of the primary antibody, should be tested empirically.

? TROUBLESHOOTING

- ii. Place the cell suspension on ice and incubate it for 20 min.
- iii. Bring the volume in the sample tube and the secondary antibody only tube to a total of 10 ml with cold HBSS. Centrifuge the cell suspension at 300g for 5 min at 4 °C. Discard the supernatants.

? TROUBLESHOOTING

- iv. Gently resuspend the cell pellets in the sample tube and the secondary antibody only tube in 10 ml of cold HBSS. At this time, bring the volume of the negative control tube (from Step 9) to 10 ml with cold HBSS.
- v. Centrifuge all three tubes at 300g for 5 min at 4 °C. Discard the supernatants.

? TROUBLESHOOTING

- vi. Gently resuspend each cell pellet (sample tube, secondary antibody only tube and negative control tube) in 0.5 ml of FACS buffer (Reagent Setup). Place the samples on ice until FACS analysis.

▲ **CRITICAL STEP** FACS should be initiated within 30 min of completing this step. Sorting gates for Ddx4-positive and Ddx4-negative cells should be determined each time on the basis of an initial screening analysis of the negative control sample, the secondary antibody only sample and the actual experimental sample (with and without the addition of DAPI solution for assessment of viability), and they will vary on the basis of the type of FACS equipment and software used.

? TROUBLESHOOTING**(B) Enrichment of OSCs by MACS ● TIMING ~30 min**

- i. Gently resuspend the cell pellet in the sample tube, as well as the cell pellet in the secondary antibody only tube (from Step 16), in 250 µl of prepared secondary antibody solution for MACS (see Secondary antibody dilution for MACS only under Reagent Setup).

? TROUBLESHOOTING

- ii. Place the cell suspension on ice and incubate for 20 min.
- iii. Bring the volume in the sample tube and the secondary antibody only tube to 10 ml with cold HBSS. Centrifuge the cell suspension at 300g for 5 min at 4 °C. Discard the supernatants.
- iv. Gently resuspend the cell pellets in the sample tube and the secondary antibody only tube in 10 ml of cold HBSS. At this time, also bring the volume of the negative control tube (from Step 9) to 10 ml with cold HBSS.
- v. Centrifuge all the three tubes at 300g for 5 min at 4 °C. Discard the supernatants.
- vi. Gently resuspend each cell pellet (sample tube, secondary antibody only tube and negative control tube) in 0.5 ml of MACS buffer (Reagent Setup). Place the samples on ice until MACS analysis.
- vii. Prepare and run each sample through separate MACS columns according to the instructions provided by the manufacturer (Miltenyi Biotec).

▲ **CRITICAL STEP** MACS has been used to successfully enrich OSCs from mouse ovaries^{2,3}; however, because of evidence of contamination of the resultant OSC preparation obtained by MACS with dead or damaged oocytes³, any analyses performed on freshly isolated OSCs isolated with MACS should take this caveat into account.

OSC culture

- 18| Purified OSCs obtained directly from FACS or MACS, or from *ex vivo*-expanded OSC cultures after trypsinization, can be cryopreserved (slow frozen) and thawed for later use. If you wish to do this, follow option A; otherwise, follow option B to plate the cells for *ex vivo* expansion (Fig. 1) followed by further analysis (e.g., gene expression).

(A) Cryopreservation and thawing of freshly isolated and *ex vivo*-expanded OSCs

- i. Centrifuge the OSCs for 5 min at 200*g* at room temperature to obtain a cell pellet.
- ii. Carefully discard the liquid portion, taking care not to disturb the cell pellet.
- iii. Gently resuspend the cell pellet in 1 ml of cold OSC culture medium supplemented with 10% (vol/vol) DMSO.
- iv. Transfer the 1-ml volume to an appropriately labeled 2-ml cryovial. Close the vial and place the tube into an isopropanol cell freezing device (e.g., Mr. Frosty).
- v. Transfer to the device to a -80 °C freezer and incubate it overnight.
- vi. The next day, transfer the cryovial to a liquid nitrogen storage container. !
CAUTION Adequate safety precautions should be taken when working with liquid nitrogen.

■ **PAUSE POINT** The cells can be kept in liquid nitrogen storage for months, or longer if necessary. Viability of the cells, once thawed, varies and depends in large part on whether freshly isolated or *ex vivo*-expanded cells are frozen for long-term storage. Freshly isolated cells have very little cytoplasm compared with *ex vivo*-expanded cells; thus, viability after thawing is markedly higher using freshly isolated cells (~90%) compared with *ex vivo*-expanded cells (~50%).

- vii. To retrieve the cells, remove the cryovial from liquid nitrogen storage and place it in a 37 °C water bath until only a small ice crystal remains.
- viii. Add 5 ml of prewarmed (37 °C) OSC culture medium to a 15-ml conical vial.
- ix. Pipette the thawed cells from the cryovial and slowly add them dropwise into the OSC culture medium in the 15-ml conical tube while gently agitating the tube to distribute the cells.
- x. Centrifuge the cells for 5 min at 200*g* at room temperature. Discard the supernatant containing DMSO, taking care not to disturb the cell pellet.
- xi. By using a 5-ml glass serological pipette, resuspend the thawed cell pellet in 2 ml of freshly prepared OSC culture medium (Reagent Setup). Transfer into one or more wells of a 24-well culture plate, depending on cell number (maximum of 1×10^5 cells per well); we do not recommend seeding the cells into culture plates containing fewer than 12 wells. Proceed with Step 19.

▲ CRITICAL STEP In our experience, successful establishment of OSC cultures is greatly facilitated by seeding the cells onto an MEF feeder cell layer; however, cryopreserved stocks of previously established OSC lines can be easily re-established after thawing without the use of MEFs.

? TROUBLESHOOTING

(B) Establishment and propagation of freshly isolated OSCs *in vitro*

- i. Centrifuge the original collection tube (1.5-ml Eppendorf) containing the Ddx4-positive cell fraction obtained from FACS (Step 17A(vi)) or MACS (Step 17B(vi)) at 300*g* for 5 min at room temperature. Discard the supernatant.
- ii. Gently resuspend the cell pellet in 1 ml of freshly prepared OSC culture medium (Reagent Setup).
- iii. Transfer the cells and the medium into a single well of a 24-well culture plate. Proceed with Step 19.

▲ CRITICAL STEP To establish and expand freshly isolated OSCs on MEFs, remove the MEF culture medium from a previously seeded MEF feeder cell plate (Equipment Setup) and place the resuspended OSCs into the well containing the MEFs.

? TROUBLESHOOTING

- 19| Place the cells into a humidified tissue culture incubator at 37 °C, maintained under 5% CO₂–95% air. Freshly isolated OSCs will take ~5–7 d to establish ('plate down') and begin colony formation, although actively dividing germ cell colonies (Fig. 5) may not become apparent to the naked eye for 2 weeks or more. During this time, the culture medium should be refreshed by adding several drops of fresh OSC culture medium to the wells every other day.

▲ CRITICAL STEP Do not attempt to remove culture medium or passage the cells during this initial growth phase.

- 20| Allow cells to form visible colonies (human OSCs) or reach 90% confluence (mouse OSCs) before passaging. Human OSC cultures generated from freshly isolated cells are ready to pass when they have established 'proliferative centers' (clusters of cells). The cells are generally elongated in appearance, and they may vary in size, possibly related to their individual state of differentiation. Allowing the colony size to become too large will result in increased cell death.

? TROUBLESHOOTING

- 21| For passage, remove the spent culture medium and place it into a 15-ml conical tube labeled with the name of the OSCs (for example, mouse or human and sort date) and the new passage number (the old passage number plus 1). Set it aside.

▲ CRITICAL STEP Save the remaining spent culture medium and use it to neutralize trypsin after passage (Step 23); moreover, nonadherent OSCs will be present in the spent culture medium and thus it should not be discarded.

? TROUBLESHOOTING

- 22| Gently passage by adding 0.1 ml of 0.05% (wt/vol) trypsin-EDTA solution to the well containing the cells. Human OSCs should be passaged in clusters (do not attempt to disrupt human OSCs into a single-cell suspension), whereas mouse OSCs should be passaged as a single-cell suspension.

! CAUTION Watch this step carefully so as to not ‘overtrypsinize’ the cells (especially human OSCs); also, do not resuspend human OSCs into a single-cell suspension for passage.

? TROUBLESHOOTING

■ **PAUSE POINT** Although the use of trypsin for the initial dissociation of ovarian tissue to obtain cell suspensions for OSC sorting is unnecessary and strongly discouraged in order to minimize damage to the cell surface exposed protein epitopes required for successful antibody-based FACS or MACS, once OSCs have been purified and established in culture, a brief exposure to very low amounts of trypsin (0.05%, wt/vol) for the purpose of cell passage is fine.

- 23| To neutralize the trypsin, tilt the plate to a 45° angle and add spent medium (from Step 21) to the top of the well. Rinse all contents of the well to the bottom.

? TROUBLESHOOTING

- 24| Collect and return everything (cells and medium) to the 15-ml conical vial containing the spent culture medium. Centrifuge the vial at 200g for 5 min at room temperature.

? TROUBLESHOOTING

- 25| Remove the vial from the centrifuge. In a sterile hood, aspirate or decant the spent medium and supernatant from the vial and discard, taking care not to disturb the cell pellet.

? TROUBLESHOOTING

- 26| By using a 5-ml glass serological pipette, add 2 ml of fresh, prewarmed (37 °C) OSC culture medium to the 15-ml conical tube.

? TROUBLESHOOTING

- 27| Gently resuspend the cell pellet by holding the 15-ml tube at a 20° angle and gently running the cell suspension down the side of the tube. For human OSCs, pipette up and down approximately five times. Small clusters of cells should remain visible to the naked eye. For mouse OSCs, pipette up and down approximately ten times. No clusters of cells should be visible to the naked eye.
- 28| For mouse OSCs, seed cells at a density of 2.5×10^4 cells into single wells of a 24-well plate (total volume of the medium plus cells in each well should be 1 ml). For human OSCs, we highly recommend passaging the cells in a ratio of

1:2 or 1:3 for optimal propagation. Do not seed human OSCs sparingly, and do not resuspend the cells into a single-cell suspension for passage.

- 29| Continue to refresh the culture medium every other day and passage the cells routinely as necessary. We recommend that aliquots of cells at later passages (once the cultures are established) be periodically cryopreserved and stored, with clear labeling indicating the type of cell (human or mouse), estimated number of cells, passage number and passage date. If desired or needed, these stocks can be thawed to re-establish new cultures at a later time. If the user wishes to cryopreserve cells, follow Step 18A.

? TROUBLESHOOTING

- 30| Perform the desired endpoint analysis on the cells obtained at Step 29, which can include the following types of studies. In established OSC cultures (mouse and human), spontaneous oocyte formation (oogenesis) occurs within 1–3 d after each passage (Fig. 2). At the seeding densities recommended above, we routinely observe the formation of ~100–300 oocytes in each well³. The process of spontaneous oogenesis subsides as the cells reach maximum density again. The oocytes formed are released into the medium, and they can be easily distinguished from OSCs on the basis of size and morphology³. If *in vitro* oogenesis is desired as an endpoint of study, the user should follow option A below. In addition to analysis of oocyte formation *in vitro*, the fate of OSCs carrying a traceable marker gene (for example, *GFP*) after injection into ovarian tissue can also be studied using transplantation models reported recently³. To perform such studies using human OSCs, follow option B below; if you are using mouse OSCs, follow option C below. For monitoring the fate of human OSCs injected into adult human ovarian cortical tissue, the OSCs need to be engineered to carry a traceable marker gene that will be expressed in oocytes formed from these cells. For our experiments, we use retroviral transduction as a means to introduce stable expression of GFP in human OSCs before injection³. Briefly, transfect 1 µg of *pBabe-Gfp* vector DNA into the Platinum-A retroviral packaging cell line using Lipofectamine according to the manufacturer's guidelines. Seventy-two hours later, transduce actively dividing OSC cultures using fresh viral supernatant in the presence of 10 µg ml⁻¹ polybrene. After 1 week of propagation, purify the GFP-expressing cells by FACS. Expand the purified cells for an additional 1–2 weeks, re-sort by FACS and repeat expansion of the GFP-expressing cells for 1 additional week before the experiment.

▲ CRITICAL STEP To monitor the fate of mouse OSCs injected into wild-type adult mouse ovaries, the OSCs must carry a traceable marker gene that will be expressed in oocytes formed from these cells. We have used two different approaches with equal success. The first uses OSCs purified from ovaries of adult female TgOG2 (PE-*Oct4-Gfp*) transgenic mice, which generate oocytes with GFP expression after introduction into the wild-type adult mouse ovaries⁶ (D.C.W. and J.L.T., unpublished observations). If such mice are not available to the user, retroviral transduction can be used as a means to introduce stable

expression of GFP in wild-type mouse OSCs before injection³. This is performed as described above for transduction of human OSCs.

(A) *In vitro* oogenesis in OSC cultures

- i. After establishing OSCs in culture, prepare a cell suspension of 5×10^4 cells ml^{-1} in 1.5 ml of OSC culture medium (7.5×10^4 cells total).
- ii. In a 24-well tissue culture dish, add 500 μl of the prepared cell suspension to each of the three wells, for a seeding density of 2.5×10^4 cells in each well.
- iii. At 24 h after seeding, remove 100 μl of the culture medium from each well and transfer it to a new well.
- iv. Under light microscopy, count the number of oocytes in the 100- μl of transferred medium. Oocytes are easily distinguishable from other cells and debris in the dish on the basis of their size (greater than 35- μm in diameter) and morphology (round or ovoid)³. Multiply this number by 5 to correct for the total amount of medium in the original culture well. Calculate the mean and standard error of the counts across the three replicates.
- v. Change the medium on the OSCs to remove all the remaining floating cells and oocytes, and bring the volume back to 500 μl in each well using fresh prewarmed OSC culture medium.
- vi. Repeat the counting procedure and medium change every 24 h. If desired, oocytes collected from the culture supernatants can be processed for a number of endpoint analyses, including PCR-based screens of gene expression (for germ cell and oocyte markers) and single-cell immunofluorescence-based assessment of oocyte-specific proteins (Fig. 2; ref. 3).

(B) Injection of human OSCs into human ovarian cortical tissue and xenografting

- i. Prepare human OSCs for injection by centrifuging (200g for 5 min at room temperature) and resuspending the FACS-purified GFP-expressing cells in sterile PBS at a concentration of 1×10^6 cells per ml. If desired, trypan blue (0.04% wt/vol final concentration) can be added at this step to facilitate visualization of the injection.

▲ CRITICAL STEP We recommend that a minimum of 500 cells (0.5 μl of cell suspension) be injected per piece of human ovarian cortical tissue (2 mm long $\times$ 2 mm wide $\times$ 1 mm thick).
- ii. Load the cell suspension into a 10- μl NanoFil syringe using the supplied loading needle.
- iii. Remove the loading needle and replace it with a 35-gauge beveled NanoFil needle.

- iv. Prepare freshly collected or cryopreserved-thawed human ovarian cortical tissue by cutting into strips 2 mm long $\times$ 2 mm wide $\times$ 1 mm thick; place the tissue strips into a culture dish containing M199 medium.
- v. By using sterile forceps, hold the tissue in place and carefully insert the injection needle.
- vi. Inject the cell suspension directly into the tissue. Repeat with multiple injection sites if possible, as tissue may not be uniform.
- vii. Anesthetize a recipient female immunocompromised (NOD/SCID) mouse according to your institutional guidelines. We use 2% (wt/vol) Avertin prepared in sterile PBS (0.03 ml per g of body weight) injected into the peritoneal cavity in a total volume of 0.1 ml. If desired, prophylactic analgesics (such as buprenorphine; 0.05–0.1 mg kg⁻¹, s.c. in a 20- μ l volume) can be provided to mice 30 min before surgery and for 72 h after surgery (and longer as may be needed after consultation with institutional veterinary staff).
- viii. Prepare the incision site by removing fur and cutting the skin along the dorsal midline (~20 mm).
- ix. Directly suture the OSC-injected human ovarian cortical tissue in close proximity to the cutaneous vasculature under the skin flap.
- x. Close the incision site with sutures and allow the mouse to recover according to your institutional guidelines. We place the mice in clean cages on warming trays set to 37 °C. Once the mice are fully awake (~30 min), they are monitored for an additional 30 min afterward to ensure that each animal regains alertness and the ability to move freely and easily (for example, to reach food and water), and that animals show minimal discomfort resulting from the surgery. After the mice are transferred back to housing rooms, they are assessed at least twice daily for the first 3 d after surgery, once daily afterward for 4 d and (depending on the experimental outcome) every 3 d after the first week of surgery for signs of complications, discomfort or infections related to the surgery.
- xi. To collect and analyze the grafts, euthanize the mouse according to your institutional guidelines (we use i.p. injection of Avertin (200 mg kg⁻¹), followed by decapitation) and retrieve the graft(s) from the mouse. Take care to remove any remaining suture material from the graft.
- xii. Once they have been collected, process the tissue grafts for desired endpoint analyses such as histological surveys, immunohistochemical detection of GFP in oocytes contained in follicles (if you are using GFP-expressing OSCs as the injected cells) and dual-immunofluorescence detection of GFP (if you are using GFP-expressing OSCs as the injected cells) along with oocyte-specific markers to identify dual-positive cells³.

(C) Intraovarian injection of mouse OSCs

- i. Prepare mouse OSCs for injection by centrifuging (200g for 5 min at room temperature) and resuspending the FACS-purified GFP-expressing cells in sterile PBS at a concentration of 1×10^4 cells μl^{-1} . If desired, trypan blue (0.04% (wt/vol) final concentration) can be added at this step to facilitate visualization of the injection.
▲ CRITICAL STEP We recommend that each ovary receives a minimum of 5×10^4 cells (5 μl of the cell suspension, with a single injection site preferred to minimize the chance for hemorrhage).
- ii. For injection, load the cell suspension into a glass micropipette tip. Attach the micropipette to the nozzle supplied with the Pneumatic PicoPump.
▲ CRITICAL STEP The precision of the Pneumatic PicoPump allows for optimum control and delivery of the cells to be injected; however, if a PicoPump or a similar device is not available, intraovarian injection can be done manually.
- iii. Anesthetize the recipient female mouse as per the user's institutional guidelines. We use 2% (wt/vol) Avertin prepared in sterile PBS (0.03 ml per g of body weight) injected into the peritoneal cavity in a total volume of 0.1 ml. If desired, prophylactic analgesics (such as buprenorphine; 0.05–0.1 mg kg^{-1} , s.c. in a 20- μl volume) can be provided to mice 30 min before surgery and for 72 h after surgery (and longer as may be needed following consultation with institutional veterinary staff).
- iv. Prepare surgical sites above the haunches of the mouse by removing hair and making a 10-mm incision through the skin and muscle, exposing the fat pad attached to the ovary.
- v. By using sterile forceps to grab the fat pad, gently remove the ovary (attached to oviduct and uterine horn) from the body cavity.
- vi. By using fine-tipped sterile forceps, open and remove the ovarian bursa, exposing the ovary.
- vii. While holding the ovary with forceps, insert the micropipette tip into the ovary and inject the cell suspension into the ovary using the foot pedal supplied with the PicoPump.
- viii. Once the entire volume has been injected, allow the ovary to settle back into the body cavity and close incision site with sutures.
- ix. Allow the mouse to recover as your institutional animal care guidelines. We place the mice in clean cages on warming trays set to 37 °C. Once the mice are fully awake (~30 min), they are monitored for an additional 30 min afterward in order to ensure that each animal regains alertness, regains the ability to move freely and easily (for example, reach food and water) and shows minimal discomfort resulting from the surgery. After the mice are transferred back to housing rooms, they are assessed at least twice daily for the first 3 d after

surgery, once daily afterward for 4 d and (depending on the experimental outcome) every 3 d after the first week of surgery for signs of complications, discomfort or infections related to the surgery.

- x. After intraovarian transplantation of OSCs, the recipient mice can be used for analysis of OSC-derived oocyte formation by immunohistochemical detection of GFP in oocytes contained in follicles³, or IVF-based studies of OSC-derived oocytes (Box 1; ref. 3); alternatively, they can be placed in mating trials for evaluation of germline transmission of the OSC reporter gene to offspring^{2,12,24}.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

● TIMING

Step 1A, warming of vitrified ovarian tissue, including setup: ~20 min

Step 1B, thawing of slow-frozen ovarian tissue, including setup: ~35 min

Steps 2A–5, dissociation of mouse ovarian tissue: ~70 min

Steps 2B–5, dissociation of human ovarian cortex: ~55 min

Steps 6–16, preparation of cells (antibody labeling) for FACS or MACS: ~90 min

Step 17A, purification of OSCs by FACS: ~1.5–2 h

Step 17B, enrichment of OSCs by MACS: ~30 min

Steps 18–20, OSC culture: variable (Once OSCs are purified by FACS or enriched by MACS, establishment of OSC colonies will take ~2 weeks before the cells can be passaged. Establishment of robustly dividing OSC cultures will take ~2 months following initial seeding, with routine passage and maintenance required throughout this period.)

Steps 21–30, OSC passage: 15 min

Box 1, IVF of OSC-derived eggs after superovulation: 5 d

ANTICIPATED RESULTS

By using the FACS-based isolation approach described herein, both mouse and human OSCs can be isolated from adult ovarian tissue for study (Fig. 1). The anticipated total yield of cells will vary depending on the amount of sample input, effectiveness of tissue dispersion, level of antibody binding and variables commonly associated with FACS analysis (equipment and software type, gating parameters and so on). In our experience, the yield of OSCs out of the total number of viable cells sorted is routinely $1.7 \pm 0.6\%$ (mean $\pm$ s.e.m.) for adult human ovarian cortical tissue samples and $1.5 \pm 0.2\%$ (mean $\pm$ s.e.m.) for adult mouse ovarian samples³. The yield of OSCs from mouse ovaries may vary from strain to strain, and, for both mouse and human ovary studies, we have observed that the yield of

OSCs varies depending on the age of the female mouse at the time of ovary collection⁴⁸ (D.C.W. and J.L.T., unpublished observations). Nonetheless, although our previously published work shows that OSCs can be reliably isolated from ovarian cortical tissue of women in their 20s and 30s (ref. 3), we have since then validated the use of the FACS-based protocol described here to successfully purify OSCs from ovarian cortical tissue of women in their 40s and 50s as well (D.C.W. and J.L.T., unpublished observations).

After isolation, the identity of freshly isolated OSCs can be verified by monitoring expression of well-accepted germline markers, such as *Prdm1*, *Ifitm3*, *Dppa3*, *Tert* and *Ddx4*, while simultaneously confirming the absence of contaminating oocytes through the analysis of markers specific to this stage of advanced female germ cell development (for example, *Nobox*, *Gdf9* and *Zp1–3*) (ref. 3). Notably, this primitive germline gene expression profile should be maintained after establishment of OSCs *in vitro*. In addition, the cultured cells should remain relatively uniform in population, which can be easily assessed by immunocytochemical analysis of Prdm1, Ifitm3 and Dppa3 protein expression (Fig. 6; ref. 3). Both mouse and human OSCs can be cultured in the absence or presence of MEF feeder cells; however, initial establishment in culture and rate of proliferation is greatly enhanced by the use of MEFs. After several passages on MEFs, the cells will become more firmly established in culture. At this stage, the OSCs can be removed from MEFs and maintained as pure germ cell cultures³. In our laboratory, we have continually propagated human OSCs for well over 4 months, whereas mouse OSCs have been maintained for more than 18 months, without loss of proliferative potential, germ cell identity or oogenic activity. With regard to the latter, oocytes formed *in vitro* from OSCs can be detected as large (35–50 μ m) ovoid cells that detach from the plates and float in the culture medium (Fig. 2). These oocytes can be counted for a quantitative assessment of oogenesis under different experimental conditions⁴⁹, collected and pooled for PCR analysis of oocyte-specific gene expression or collected and individually analyzed by fluorescence-based immunocytochemical procedures for expression of oocyte-specific proteins such as Ybx2, Lhx8 and Kit. All of these approaches have been tested using both mouse and human OSCs maintained *in vitro*³.

An important validation step for the assessment of OSC identity and function is transplantation of the purified cells back into an ovarian environment in order to monitor the formation of new oocytes (Fig. 3) and, in the case of mouse OSCs, the ability of OSC-derived oocytes to mature into fully functional (fertilization competent) eggs^{2,3,12,24}. By analogy, intragonadal transplantation remains the litmus test for testing male GSC identity in mammals after nearly 20 years since the delivery of isolated mouse SSCs back into testes of recipient male mice was first attempted^{50,51}. Notably, the first successful SSC transplantation in non-human primates has recently been published⁵², confirming that the spermatogenic properties of isolated mouse SSCs are conserved in primates, as appears to be the case with OSCs isolated from mouse or human ovaries³. It should be emphasized that most GSC transplantation studies to date have used *ex vivo*-expanded SSCs or OSCs, which allow for the generation of sufficient numbers of cells (especially in the case of OSCs, which are limited in number in adult ovaries) to perform the experiments. However, transplantation of freshly isolated cells can be accomplished by cryopreserving OSCs isolated from successive FACS runs and then pooling the samples after thawing. Although this requires a

much longer time frame for experimental execution as the needed stockpile of freshly cells is accumulated, it is important to document that the ability of transplanted OSCs to produce functional eggs *in vivo* is not somehow related to *in vitro* expansion of the cells before testing⁶.

The fate of transplanted OSCs can be monitored using ‘traceable’ OSCs, such as those expressing GFP either through retroviral transduction of wild-type OSCs or purification of OSCs from ovaries of transgenic mice with documented germ cell-specific reporter gene expression. Mouse OSCs expressing GFP can be transplanted into the ovaries of young adult wild-type syngeneic recipients, and the subsequent formation of GFP-positive oocytes contained within follicles composed of wild-type (host-derived) somatic granulosa cells can be detected by conventional immunohistochemical protocols (Fig. 3; refs. 2,3). Moreover, after superovulation with exogenous gonadotrophins and retrieval of eggs from the oviducts, fertilization competency and embryonic developmental potential of OSC-derived (GFP-expressing) oocytes can be performed after IVF (Box 1 and Fig. 4; ref. 3). Alternatively, female mice transplanted with OSCs can be monitored in natural mating trials for giving birth to offspring carrying the traceable gene of interest^{2,12,24} (J.L.T. laboratory, unpublished observations). The ability of human OSCs to generate new oocytes that then participate in follicle formation in adult human ovary tissue can be determined by injecting human ovarian cortical tissue with human GFP-expressing OSCs. Within 1–2 weeks of s.c. grafting of the injected tissue into immunocompromised (NOD/SCID) female mice, GFP-positive human oocytes can be found localized within primordial and primary follicles throughout the tissue (Fig. 3; ref. 3). To verify that the GFP-positive cells within these follicles are oocytes, dual-immunofluorescence detection of GFP with oocyte-specific gene products, such as the meiotic diplotene-stage germ cell marker Y-box binding protein 2 (YBX2) (refs. 53,54) or the transcriptional regulator LIM homeobox 8 (LHX8) (ref. 55), can be used to reveal dual-positive oocytes³. Finally, injection of human GFP-expressing OSCs into human ovarian cortical tissue placed in multistep culture systems, which have been shown to support formation and maturational development of follicles entirely *ex vivo*^{56,57}, provides a means to study human oogenesis and folliculogenesis under defined conditions without the need for a ‘foreign’ (mouse) host⁵.

Finally, we would like to note that use of the terms ‘Ddx4-positive’ and ‘Ddx4-negative’ when referring to the fractions of cells obtained following FACS-based immunological detection of the C-terminus of Ddx4 protein in dispersed ovarian cells is not intended to indicate the absence of *Ddx4* gene expression in all cells that lack externalized Ddx4 protein. As a case in point, small oocytes express the *Ddx4* gene and contain Ddx4 protein, but the protein is completely cytoplasmic. Accordingly, and unlike OSCs, viable (non-fixed, non-permeabilized) oocytes show no interaction with antibodies against the C-terminus of Ddx4 (ref. 3). While oocytes would be considered ‘Ddx4-negative’ in the context of the antibody-based OSC sorting protocols described herein, oocytes are nonetheless ‘Ddx4-positive’ in the general context of *Ddx4* gene expression. We therefore propose that viable cells identified as reactive with the C-terminal Ddx4 antibody (i.e., OSCs) should be referred to as ‘ecDdx4-positive’ (for ‘extracellular Ddx4-positive’), and the remaining cells lacking externalized expression of Ddx4 protein, which can include small oocytes, should be referred to as ‘ecDdx4-negative’. Use of this terminology will help minimize confusion in

future studies of germ cells at different stage of development, all of which express the *Ddx4* gene but differ in their ability to be separated and identified on the basis of externalized Ddx4 protein.

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Box 1**IVF of OSC-derived eggs after superovulation**

This is a 5-d procedure that is performed after transplantation of mouse OSCs bearing a traceable marker gene into ovaries of adult wild-type female recipient mice (Step 30C); we recommend that the injected cells be allowed to engraft and differentiate for at least 4 weeks before performing this procedure.

1. Begin by injecting 5 IU of PMSG solution (100 μ l of prepared stock; depending on age and weight of the females, this dose can be increased to 10 IU) using an insulin syringe into the peritoneal cavities of female mice that have received intraovarian OSC transplants.
2. Approximately 46 h later, inject 5 IU of hCG solution (100 μ l of prepared stock; depending on age and weight of the females, this dose can be increased to 10 IU) into the peritoneal cavities of the same mice.
3. Add 1 ml of HTF medium to a center-well organ culture dish to be used for sperm collection (label this as 'sperm collection dish').
4. For each female mouse, add 500 μ l of HTF medium to a center-well organ culture dish to be used for IVF (label this as 'IVF dish').
5. For each female mouse, place five separate 40- μ l droplets of HTF medium into a 60-mm culture dish to be used for washes; gently cover each HTF droplet in mineral oil (label this as 'wash dish'), ensuring that the droplets remain separate.
6. For each female mouse, place five separate 40- μ l droplets of KSOM-AA medium into a 60-mm culture dish to be used for culture; gently cover each KSOM-AA droplet medium with mineral oil (label this as 'Culture Dish'), ensuring that the droplets remain separate.
7. Place the labeled dishes prepared in steps 3–6 in a humidified tissue culture incubator at 37 °C under 5% CO₂–95% air.
8. At 13 h after hCG injection, euthanize an adult male mouse according to your institutional guidelines (we use i.p. injection of Avertin (200 mg kg⁻¹), followed by decapitation).
9. Dissect out the cauda epididymis and vas deferens, taking care to remove any adherent fat, and place the tissue into the sperm collection dish.
10. Use an insulin syringe needle to cut open the cauda epididymis.
11. By using sterile forceps, gently squeeze the spermatozoa from the vas deferens through the opened cauda epididymis; remove tissue and discard.
12. To capacitate the extruded spermatozoa, place the sperm collection dish containing the spermatozoa in a humidified tissue culture incubator at 37 °C under 5% CO₂–95% air for 1 h.

13. After 1 h, add ~10 μ l of capacitated spermatozoa to the HTF medium in the IVF dish and return the dish to incubator.
14. At this time, prepare a 60-mm culture dish containing 1 ml of HTF medium and label it as 'ovary/oviduct holding dish'; at this time, for each female mouse label a separate empty dish as 'COC dish' for collection of ovulated COCs from the oviducts.
15. At 14 h after hCG injection (once the sperm capacitation step is completed), euthanize the transplanted female mice according to the user's institutional guidelines (we use i.p. injection of Avertin (200 mg kg⁻¹), followed by decapitation).
16. Quickly remove the ovaries and attached oviducts from the body cavity of each mouse and place them into the ovary/oviduct holding dish containing HTF medium.
17. Transfer one ovary and attached oviduct to an empty COC dish; use an insulin syringe needle to open the ampulla (bulge) of the oviduct to release the superovulated COCs. Repeat the procedure with oviducts from each ovary in a separate dish.

▲ CRITICAL STEP To facilitate the collection of the COCs released from the oviducts, this step should be performed in an empty dish (no medium); the medium associated with the tissue when transferred from the ovary/oviduct holding dish to the COC dish is of sufficient volume to perform this step and maximize COC retrieval.
18. Identify and transfer the COCs from the COC dish to 500 μ l of HTF medium in the IVF dish (previously prepared in step 4) containing the capacitated sperm (previously prepared in step 3).
19. Incubate the IVF dish at 37 °C in a humidified tissue culture incubator under 5% CO₂–95% air for 4–6 h.
20. After IVF, wash the oocytes/zygotes by successive transfer through the five droplets of the HTF medium in the wash dish.
21. After the final wash, transfer the oocytes/zygotes to the culture dish containing the droplets of KSOM-AA medium (up to 20 oocytes/zygotes per droplet) and incubate the dish overnight at 37 °C in a humidified tissue culture incubator under 5% CO₂–95% air.
22. The next morning, evaluate each drop for the presence of two-cell stage embryos, which can be used as a measure of fertilization success. Embryo culture can then be continued to later stages of preimplantation development (up to hatching blastocysts), as desired.

Figure 1. Schematic overview of the OSC isolation protocol. Depicted are key steps in the FACS-based protocol for the purification of OSCs from adult mouse or human ovarian tissue, and the main steps involved in the subsequent characterization and expansion of the cells once isolated.

Figure 2. Oogenesis in OSC cultures. **(a,b)** Evaluation of oocytes formed *in vitro* in cultures of mouse **(a)** and human **(b)** OSCs isolated from adult ovary tissue, as shown by morphology (bright-field, top images; three different oocytes shown for each), immunofluorescence-based detection of the oocyte markers Ddx4/DDX4 and Kit/KIT (middle images), and kinetics of production each day after seeding 2.5×10^4 OSCs per well (bottom images). Scale bars, 25 μ m. Error bars are means $\pm$ s.e.m. **(c)** After collection of oocytes from OSC culture supernatants, PCR-based detection of nine distinct and well-accepted oocyte marker genes (*Ddx4/DDX4*, *Kit/KIT*, *Ybx2/YBX2*, *Nobox/NOBOX*, *Lhx8/LHX8*, *Gdf9/GDF9*, *Zp1/ZP1*, *Zp2/ZP2* and *Zp3/ZP3*) further confirms their cellular identity (β -actin, housekeeping gene). See White et al.³.

Figure 3.

In vivo differentiation of OSCs into oocytes. (**a,b**) Examples of follicles containing GFP-negative (host ovary-derived) and GFP-positive (OSC-derived; brown immunostain against blue hematoxylin counterstain) oocytes in human ovarian cortical tissue injected with human GFP-expressing OSCs and then grafted s.c. into immunocompromised mice (**a**; scale bar, 25 μ m) or in ovaries of wild-type mice injected with mouse GFP-expressing OSCs (**b**; scale bar, 30 μ m). Reproduced from ref. 3.

Figure 4.

Testing the functionality of OSC-derived oocytes. Following exogenous gonadotrophin stimulation, follicles containing oocytes generated by GFP-expressing mouse OSCs transplanted into ovaries of wild-type female recipients undergo ovulatory release of cumulus cell-enclosed GFP-positive eggs (cumulus-oocyte complexes or COCs; adjacent panel shows eggs denuded of cumulus cells). Functionality of these eggs can be tested by IVF and then monitoring the progression of preimplantation embryonic development through the blastocyst (**B**) stage. Paired examples of host-derived (wild-type) and OSC-derived (GFP-positive) oocytes and embryos are provided for comparison. Reproduced from ref. 3. Scale bars, 30 μ m.

Figure 5.

Morphology of freshly isolated and *ex vivo*-expanded OSCs. **(a)** After human ovarian tissue dissociation and C-terminal DDX4 antibody-based FACS, a uniform population of small, round cells less than 10- μm in diameter (DDX4-positive) is obtained, whereas the DDX4-negative fraction comprises a mixed population of much larger cells. Ovarian stromal cells 1 d after plating are shown for size comparison. All images shown are at the same magnification. Scale bars, 30 μm . **(b)** After establishment of OSCs *in vitro*, actively dividing germ cell colonies begin to develop (typical colonies of human and mouse OSCs are shown) Scale bars, 30 μm . **(c)** Schematic depiction of the rapid changes in OSC size and morphology once freshly isolated OSCs (<10 μm) are established in culture (~20 μm). The size of mouse OSCs purified using FACS and then established *in vitro* is consistent with a previous report on the size of mouse OSCs cultured for as little as 24 h after MACS-based isolation².

Figure 6. Expanded OSCs maintain a primitive germline gene expression profile. (a,b) Immunocytochemical detection (green) of Dppa3/DPPA3, Ifitm3/IFITM3 and Prdm1/PRDM1 in mouse (a) and human (b) OSCs after months of *ex vivo* propagation. See White *et al.*³ for additional examples. Scale bars, 10 μ m.

TABLE 1

Troubleshooting table.

Step	Problem	Solution
<i>Ovarian tissue dispersion</i>		
1	Poor cell viability	Evaluate the viability of the tissue source; if you are using frozen or vitrified tissue, ensure that the tissue is free of freezing-related damage
2A(i), 2B(ii)		Worthington type IV collagenase should be used
2A(ii, iii), 2B(ii)		Reduce the collagenase enzyme concentration by 5% decrements until a point just before optimal tissue dissociation is lost, so as to maintain adequate tissue dispersion while reducing cell damage
2A(iii, v, vii), 2B(viii)		Use 5-ml glass (not plastic) serological pipettes during manual tissue dispersion to reduce cell damage
2A(v, vii) and 2B(viii)		Decrease the frequency or vigor of manual dissociation (repeated pipetting with a 5-ml glass serological pipette)
10, 14, 16, 17A(iii, v)		Reduce centrifugation times from 5 min to 3 min
2		Work as quickly as possible through the entire tissue digestion protocol
2A, 2B, 17		Minimize temperature variations during key steps; maintain samples at 37 °C during enzymatic dissociation and at 4 °C during antibody labeling steps; avoid submersion of samples into “wet” ice (ice-melted ice water interface) in ice containers, as this will cause extensive damage to cells
2	Poor tissue dissociation	Avoid use of trypsin for tissue dissociation
2A(i), 2B(ii)		Increase the collagenase enzyme concentration in 5% increments until desired degree of dispersion is achieved; use freshly prepared collagenase/DNase I solution for each dissociation
		Perform collagenase digestions steps at 37 °C for optimal enzymatic activity
2A(v, vii), 2B(viii)		Increase the frequency or vigor of manual dissociation by repeated pipetting with 5-ml glass serological pipettes
2B		If you are using manual dispersion throughout (as opposed to manual and mechanical, i.e., GentleMACS), mince the tissue into a slurry using fine scissors with a curved blade; this process may take 5–7 min of continuous/repeated mincing
2A(i), 2B(ii)		Avoid freeze-thaw cycles of all dissociation enzymes to maintain expected activity
	Clumping	Reduce the collagenase enzyme concentration by 5% decrements to reduce cell damage while maintaining tissue dispersion (genomic DNA released from damaged cells can increase clumping)
		Try a freshly prepared aliquot of DNase I solution
		Avoid freeze-thaw cycles of all dissociation enzymes to maintain expected activity
2A(v, vii) and 2B(viii)		Use 5-ml glass (not plastic) serological pipettes during manual tissue dispersion to reduce cell damage (genomic DNA released from damaged cells can increase clumping)
2A(x), 2B(xiii), and 5, 11, 15 and 16	Cell pellet small or absent after digestion	Gently decant supernatants after centrifugation; aspiration should be avoided to minimize dislodging/disruption of the cell pellet and cell loss
2A(ix), 2B(xii), 4, 10, 14, 16, 17A(iii, v), 17B(iii, v) and 18B(i)		For ineffective pelleting, increase centrifuge speed to 600g and use 15-ml conical tubes, as these most effectively pellet cells (avoid use of round-bottom tubes for centrifugation)
<i>Antibody labeling and FACS</i>		
2A(ix), 2B(xii), 4, 10, 14 and 16, 17A(iii, v), 17B(iii, v), 18B(i)	Low yield of Ddx4/DDX4-positive cells	Increase centrifugation from 300g to 600g to improve pelleting of the cells; perform all centrifugation steps using 15-ml conical vials to improve cell pelleting (do not use round-bottom tubes); when removing supernatants after centrifugation, do not use suction, as this can dislodge/disrupt the pellet and result in cell loss

Step	Problem	Solution
11, 12		When discarding supernatant before adding primary antibody solution, make sure <100 µl of supernatant remains, as this additional volume will dilute the primary antibody; adjust the amount of primary antibody (for a final concentration of 1:10–1:20) accordingly
12		Primary antibody concentration varies by lot number; try a new lot of primary antibody
Step 2A(i), 2B(ii)		Avoid the use of trypsin, which can damage or remove externally exposed protein epitopes on cells required for adequate primary antibody binding
2A(i), 2B(ii)	Nonspecific antibody binding	Avoid the use of trypsin, as this can increase nonspecific binding and isotype control antibody binding
6		Increase the antibody blocking time from 20 min to 40 min
12, 17A(i), 17B(i)		Reduce primary or secondary antibody concentration
17A(vi)	Low viability of cells after FACS collection	If cell viability before and during FACS is high but reduced after sorting, change the collection method; decrease surface tension by increasing the amount of medium in the collection tube into which the cells are collected; do not collect cells into plates, as cells will lyse on impact. We recommend sorting into 1.5-ml Eppendorf tubes containing 1 ml of medium and located as close to the emitter as possible
		The final cell fraction prepared for FACS will contain cells of multiple sizes as well as debris from the tissue digestion; an experienced FACS technician should be consulted to distinguish between cell fragments and viable small cells (the desired cell population is very small, 10 µm or less in diameter) and include a viability dye, such as DAPI or propidium iodide
17A(vi), FACS		Avoid bubbles in the FACS collection tube, as this increases surface tension, which can lyse cells being collected
17		During the antibody blocking and labeling steps, which are performed on ice, “wet” ice (melted ice water with ice) has a much lower temperature than fresh ice and is lethal to cells; when performing antibody blocking and labeling steps, use fresh ice and ensure that all tubes are maintained only in the upper ice phase
<i>OSC culture</i>		
18A(xi), 18B(iii)	Onset to proliferation is slow	Increase the initial plating density of OSCs by adding more OSCs per well of a 24-well culture dish, or by plating cells in one well of a 48-well culture dish
18B(iii)		Initially seed freshly collected OSCs onto a MEF feeder cell layer
18A(xi), 18B(iii)	Cells cease proliferation	If cells stop proliferating in the absence of MEFs, re-establish the cells on MEFs until proliferation resumes
		If maintaining OSCs on MEF feeder cells, passage the cells onto newly prepared MEFs to maximize feeder cell activity
20		If mouse OSCs are allowed to exceed 90–95% confluence, viability can be compromised and cells may cease proliferation
21		Make sure to collect spent medium at each passage and use it to neutralize trypsin; this also ensures that any nonadherent OSCs present in the spent medium are included at each passage
22		If human OSCs are passed into single-cell suspension, proliferation will cease and pass as cell clusters
		If human OSCs are seeded too sparingly, proliferation will cease; seed more cells per well, reduce passage frequency or reduce extent of passage (for example, from 1:3 or 1:4 to 1:2)
18A(xi), 21, 23, 24, 29		Use of 5-ml glass (not plastic) serological pipettes during OSC maintenance improves cell viability
25, 26		Cryopreserve cells at early passages and return to use of early passage cells if necessary