

## Supplementary Information

### Oocyte formation by mitotically-active germ cells purified from ovaries of reproductive age women

Yvonne A. R. White, Dori C. Woods, Yasushi Takai, Osamu Ishihara, Hiroyuki Seki, and Jonathan L. Tilly

#### SUPPLEMENTARY METHODS (DETAILED PROTOCOLS)

**Animals** Young adult (2 months of age) female C57BL/6 mice and adult (3–4 months of age) male C57BL/6 mice were obtained from Charles River Laboratories, whereas young adult (1.5–3 months of age) NOD-SCID female mice were obtained from Jackson Laboratories. Transgenic (TgOG2) mice with germline-specific GFP expression<sup>1–3</sup> were obtained from J.R. Mann through K.J. MacLaughlin (University of Pennsylvania and Nationwide Children's Hospital), and were bred and genotyped as detailed previously<sup>4,5</sup>. All experimental protocols with animals described herein were reviewed and approved by the institutional care and use committee of Massachusetts General Hospital.

**Human samples** With written informed consent, ovaries were surgically removed from six female subjects between 22–33 ( $28.5 \pm 4.0$ ) years of age with gender identity disorder for sex reassignment at Saitama Medical Center. The outer cortical layer was carefully removed, vitrified and cryopreserved<sup>6</sup>. Briefly, 1 mm-thick cortical fragments were cut into 10 x 10 mm pieces, incubated in an equilibration solution containing 7.5% ethylene glycol and 7.5% dimethylsulfoxide (DMSO) at 26 C for 25 min, and then incubated in a vitrification solution containing 20% ethylene glycol, 20% DMSO and 0.5 M sucrose at 26 C for 15 min prior to submersion into liquid nitrogen. For our experimental analyses, cryopreserved ovarian tissue was thawed using the Cryotissue Thawing Kit (Kitazato Biopharma) and processed immediately for histology, xenografting or OSC isolation. All research experiments with human tissue

described herein were conducted without any subject identifiers under a human subjects protocol reviewed and approved by the institutional review board of Massachusetts General Hospital (protocol number 2008P001640).

**DDX4 antibodies** The antibody used previously to isolate mouse OSCs by immunomagnetic sorting<sup>7</sup> is a rabbit polyclonal against the last 25 amino acids of the COOH-terminus of human DDX4 (ab13840, Abcam). This region shares 96% overall homology with the corresponding region of mouse Ddx4. For comparative studies, we used a goat polyclonal antibody against the first 145 amino acids of the NH<sub>2</sub>-terminus of human DDX4 (AF2030, R & D Systems), which shares 91% overall homology with the corresponding region of mouse Ddx4.

**Immunomagnetic bead sorting** For each preparation of cells, ovaries from four mice were pooled and dissociated by mincing followed by a two-step enzymatic digestion involving a 15-min incubation with 800 U/ml collagenase [type IV; prepared in Hank's balanced salt solution minus calcium and magnesium (HBSS)] and a 10-min incubation with 0.05% trypsin-EDTA. Digestions were carried out in the presence of 1  $\mu\text{g ml}^{-1}$  DNase-I (Sigma-Aldrich) to minimize stickiness within the cell preparations, and trypsin was neutralized by addition of 10% fetal bovine serum (FBS; Hyclone). Ovarian dispersates were filtered through a 70- $\mu\text{m}$  nylon mesh and blocked in a solution composed of 1% fatty-acid free bovine serum albumin (BSA; Sigma-Aldrich) with either 1% normal goat serum (Millipore; for subsequent reactions using ab13840) or 1% normal donkey serum (Sigma-Aldrich; for subsequent reactions using AF2030) in HBSS for 20 min on ice. Cells were then reacted for 20 min on ice with a 1:10 dilution of primary antibody that recognizes either the COOH terminus (ab13840) or NH<sub>2</sub> terminus (AF2030) of Ddx4 and DDX4. Cells were washed two times in HBSS and incubated for 20 min on ice with a 1:10 dilution of either goat anti-rabbit IgG-conjugated microbeads (Miltenyi; ab13840 detection) or biotin-conjugated donkey anti-goat IgG (Santa Cruz Biotechnology; AF2030 detection) followed by incubation with streptavidin-conjugated microbeads (Miltenyi). After one additional wash, the cell preparations were loaded onto MACS columns and separated according to manufacturer's specifications (Miltenyi). For experiments to visualize potential antibody-bead interaction with oocytes, adult female mice were superovulated by a single intraperitoneal injection of pregnant mare serum gonadotropin (PMSG, 10 IU; Sigma-Aldrich) followed by a single intraperitoneal injection of human chorionic gonadotropin (hCG, 10 IU; Sigma-Aldrich) 46–48 h later. Oocytes were collected from oviducts 15–16 h after hCG injection, denuded of cumulus cells using hyaluronidase (Irvine Scientific) and washed with human tubal fluid (HTF;

Irvine Scientific) supplemented with 0.4% BSA. Ovarian cells, dispersed as described above, or isolated oocytes were then blocked and incubated with each of the primary antibodies against DDX4. After washing in HBSS, cells were reacted with species-appropriate secondary antibodies conjugated to 2.5- $\mu$ m Dynabeads (Invitrogen). Suspensions were placed into 1.5 ml Eppendorf tubes for separation using a Dynal MPC<sup>®</sup>-S Magnetic Particle Concentrator.

**Flow cytometry** For each experiment, ovarian tissue (mouse: four ovaries pooled; human: 10 x 10 x 1 mm thick, cortex only) was dissociated, blocked and reacted with primary antibody (ab13840 for detection of the COOH terminus of Ddx4 and DDX4, or AF2030 for detection of the NH<sub>2</sub> terminus of Ddx4 and DDX4) as described above. After washing with HBSS, cells were incubated with a 1:500 dilution of goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Invitrogen; ab13840 detection) or donkey anti-goat IgG conjugated to Alexa Fluor 488 (Invitrogen; AF2030 detection) for 20 min on ice, and washed with HBSS. Labeled cells were then filtered again (35- $\mu$ m pore diameter) and subjected to FACS using a BD Biosciences FACS Aria II cytometer (Harvard Stem Cell Institute), gated against negative (unstained and no primary antibody) controls. Propidium iodide was added to the cell suspension just prior to sorting for dead cell exclusion. Freshly-isolated Ddx4-positive (mouse) and DDX4-positive (human) viable cells were collected for gene expression profiling, assessment of teratoma formation capacity or *in-vitro* culture. For some experiments, cells were fixed in 2% neutral-buffered paraformaldehyde (PFA) and permeabilized with 0.1% Triton-X100 prior to reaction with primary antibody against the NH<sub>2</sub> terminus of DDX4 (AF2030) and detection by FACS after reaction with donkey anti-goat IgG conjugated to Alexa Fluor 488. For re-sort experiments, viable cells were reacted with DDX4-COOH antibody (ab13840) and sorted by FACS after reaction with a goat anti-rabbit IgG conjugated to allophycocyanin (APC) (Jackson ImmunoResearch). Resultant APC-positive (Ddx4-positive) viable cells were either left intact or fixed and permeabilized prior to incubation with DDX4-NH<sub>2</sub> antibody (AF2030), followed by incubation with donkey anti-goat IgG conjugated to Alexa Fluor 488 and analysis by FACS.

**Teratoma formation** Ovaries were collected from a total of 100 young adult female mice, dissociated and subjected to FACS for isolation of Ddx4-COOH-positive viable cells, as described above. Freshly-isolated mouse OSCs were injected subcutaneously near the rear haunch of NOD-SCID female mice (1 x 10<sup>5</sup> cells injected per mouse, *n* = 3 mice). As a control for assessment of tumorigenesis *in vivo*, mouse embryonic stem cells (mESC v6.5) were injected at the same site into a parallel cohort of age-matched female NOD-SCID mice (1 x 10<sup>5</sup>

cells injected per mouse,  $n = 3$  mice). Mice were monitored weekly for up to six months for evidence of tumor formation.

**OSC cultures** Mouse and human ovary-derived OSCs were cultured at 37 C in 5% CO<sub>2</sub>-95% air on a feeder layer of mitotically-inactivated immortalized mouse embryonic fibroblasts (MEFs), similar to conditions detailed previously<sup>7</sup>. Briefly, cells were cultured in MEM $\alpha$  (Invitrogen) supplemented with 10% FBS (Hyclone), 1 mM sodium pyruvate, 1 mM non-essential amino acids, 1x-concentrated penicillin-streptomycin-glutamine (Invitrogen), 0.1 mM  $\beta$ -mercaptoethanol (Sigma-Aldrich), 1x-concentrated N-2 supplement (R & D Systems), 10<sup>3</sup> units ml<sup>-1</sup> leukemia inhibitory factor (LIF; Millipore), 10 ng ml<sup>-1</sup> recombinant human epidermal growth factor (rhEGF; Invitrogen), 1 ng ml<sup>-1</sup> basic fibroblast growth factor (bFGF; Invitrogen), and 40 ng ml<sup>-1</sup> glial cell-derived neurotrophic factor (GDNF; R & D Systems). Cultures were refreshed by the addition of 40–80  $\mu$ l of new medium every other day, and cells were re-plated on fresh MEFs every two weeks. For passage, mouse cells were trypsinized and dispersed into single cell suspensions whereas human cells were trypsinized with gentle colony disruption to avoid single cell dispersion. To assess proliferation, MEF-free OSC cultures were treated with 10  $\mu$ M BrdU (Sigma-Aldrich) for 48 h prior to fixation in 2% PFA for dual immunofluorescence-based detection of BrdU incorporation (mitotically-active cells) and Ddx4 or DDX4 expression (germ cells), essentially as described<sup>7</sup>. No signal was detected if primary antibodies were omitted or replaced with an equivalent dilution of normal rabbit serum (data not shown).

**Gene expression** The presence of each indicated mRNA was assessed in total RNA samples by conventional RT-PCR using a SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) and Platinum *Taq* polymerase (Invitrogen). All products were sequenced to confirm identity. Sequences of forward and reverse primers used, along with GenBank accession numbers of the corresponding genes and amplified product sizes, are provided in **Supplementary Table S1** (mouse) and **Supplementary Table S2** (human).

**Immunoanalysis** For analysis of cultured OSCs, cells were washed with 1x-concentrated phosphate-buffered saline (PBS), fixed in 2% PFA for 45 min at 20 C, washed three times with PBS-T (PBS containing 0.01% Triton-X100) and incubated for 1 h at 20 C in blocking buffer (PBS containing 2% normal goat serum and 2% BSA). The cells were then incubated for 1 h at 20 C with a 1:100 dilution of one of the following primary antibodies: a biotinylated mouse monoclonal against PRDM1 (ab81961, Abcam), a rabbit polyclonal against Dppa3 (ab19878,

Abcam), a rabbit polyclonal against Ifitm3/IFITM3 (mouse: ab15592; human: ab74699; Abcam), a rabbit polyclonal against DMC1 (ab96613, Abcam) or a rabbit polyclonal against SYCP3 (NB300-232 Lot B1, NOVUS Biologicals). For detection of Prdm1/PRDM1, Dppa3/DPPA3 and Ifitm3/IFITM3, cultured OSCs were washed and incubated for 30 min at 20 °C with a 1:500 dilution of streptavidin-conjugated Alexa Fluor 488 (Invitrogen; Prdm1/PRDM1 detection) or goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Invitrogen; Dppa3/DPPA3 and Ifitm3/IFITM3 detection) in the presence of rhodamine-phalloidin (Invitrogen). For DMC1 and SYCP3 detection, cells were washed and incubated for 30 min at 20 °C with a 1:500 dilution of goat anti-rabbit IgG conjugated to Alexa Fluor 568 (Invitrogen). All cells were then washed, incubated with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich) and washed three additional times before imaging. No signal was detected in OSCs if primary antibody was omitted or replaced with an equivalent dilution of normal serum (data not shown). For those experiments designed to assess expression of meiotic recombination proteins (DMC1 and SYCP3) in human OSCs, human ovarian stromal cells were used as a negative control.

For assessment of oocytes generated *in vitro* by mouse and human OSCs, individual oocytes were collected from culture supernatants, washed, fixed with 2% PFA containing 0.5% BSA for 45 min at 37 °C, washed and blocked for 1 h at 20 °C in PBS containing 0.5% BSA and either 5% normal goat serum (Ddx4, DDX4 and LHX8 detection) or 1% normal donkey serum (Kit and KIT detection). After blocking, oocytes were incubated for 2 h at 20 °C with a 1:100 dilution (in PBS with 0.5% BSA) of one of the following primary antibodies: a rabbit polyclonal against DDX4 (ab13840, Abcam), a goat polyclonal against KIT (sc1494, Santa Cruz Biotechnology), a rabbit polyclonal against Lhx8 (ab41519, Abcam), or a rabbit polyclonal against Ybx2 (ab33164, Abcam). Cells were then washed and incubated with a 1:250 dilution of goat anti-rabbit IgG conjugated to Alexa Fluor 568 (Invitrogen; Ddx4, DDX4, Ybx2 and YBX2 detection) or Alexa Fluor 488 (LHX8 detection), or a 1:250 dilution of donkey anti-goat IgG conjugated to Alexa Fluor 488 (Kit and KIT detection). Cells were washed, incubated with DAPI and washed three additional times before imaging. No signal was detected if primary antibody was omitted or replaced with normal serum (data not shown).

For these latter experiments, detection of oocyte expression of Ddx4, DDX4, KIT, YBX2 and LHX8 in ovarian tissue sections served as a positive control for antibody specificity and reactivity. Mouse and human ovarian tissue was fixed in 4% PFA, paraffin-embedded and sectioned (6-µm) prior to high temperature antigen retrieval using 0.01 M sodium citrate buffer (pH 6.0). After cooling, sections were washed and blocked for 1 h at 20 °C using TNK buffer (0.1 M Tris-HCl, 0.55 M NaCl, 0.1 mM KCl, 0.5% BSA, and 0.1% Triton-X100 in PBS) containing

either 1% normal goat serum (for subsequent detection of Ddx4-COOH in mouse ovary, DDX4-COOH in human ovary, and YBX2 and LHX8 in human ovary) or 1% normal donkey serum (for subsequent detection of Ddx4-NH<sub>2</sub> in mouse ovary, DDX4-NH<sub>2</sub> in human ovary, and KIT in human ovary). Sections were then incubated with a 1:100 dilution of primary antibody (in TNK buffer with 1% normal serum) overnight at 4 C, washed in PBS, and incubated for 30 min at 20 C with a 1:500 dilution of goat anti-rabbit IgG conjugated to Alexa Fluor 568 (DDX4-COOH detection in human ovary), goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Ddx4-COOH detection in mouse ovary, and LHX8 detection in human ovary) or donkey anti-goat IgG conjugated to Alexa Fluor 488 (Ddx4-NH<sub>2</sub> detection in mouse ovary, DDX4-NH<sub>2</sub> detection in human ovary, and KIT detection in human ovary). For assessment of YBX2 expression in human ovary, primary antibody was detected using a goat anti-rabbit biotinylated IgG for 1 h at 20 C, followed by reaction with streptavidin-conjugated Alexa Fluor 568. After washing with PBS, sections were cover-slipped using Vectashield containing DAPI (Vector Labs). No signal was detected if primary antibody was omitted or replaced with normal serum (not shown). In addition to DDX4, KIT, YBX2 and LHX8, we also empirically tested antibodies against Nobox (ab41521, Abcam), Gdf9 (sc7407, Santa Cruz Biotechnology) and ZP3 (ab48895, Abcam) using human ovarian cortical tissue as a positive control for oocyte-specific expression. However, use of these antibodies over multiple dilutions and detection conditions showed either no reaction signal (GDF9 or ZP3) or inconsistent outcomes (NOBOX) with human ovarian tissue sections (data not shown), and thus were not pursued further.

**Ploidy analysis** Cultured mouse (48 h after passage) or human (72 h after passage) OSCs were collected by trypsinization, washed and re-suspended in ice-cold PBS, and counted with a hemocytometer. After fixation in ice-cold 70% ethanol for 1 h, cells were washed in ice-cold PBS and incubated with 0.2 mg ml<sup>-1</sup> RNase-A for 1 h at 37 C. Propidium iodide was then added (10 µg ml<sup>-1</sup> final), and ploidy status was determined using a BD Biosciences FACSaria II cytometer<sup>8</sup>. As a control somatic cell line, these experiments were repeated using human fetal kidney fibroblasts (KEK 293, Invitrogen).

**Generation of GFP-expressing OSCs** For cell tracking experiments, mouse and human OSCs were transduced using a retrovirus to obtain cells with stable expression of GFP (mouse, GFP-mOSCs; human, GFP-hOSCs). Briefly, 1 µg of *pBabe-Gfp* vector DNA (Addgene plasmid repository #10668) was transfected as per the manufacturer's protocol (Lipofectamine, Invitrogen) into the Platinum-A retroviral packaging cell line (Cell Biolabs). Viral supernatant was

collected 48 h after transfection. Transduction of OSCs was performed using fresh viral supernatant facilitated by the presence of polybrene ( $5 \mu\text{g ml}^{-1}$ ; Sigma-Aldrich). After 48 h, the virus was removed and replaced with fresh OSC culture medium. Mouse and human OSCs with expression of GFP were purified by FACS following an initial one week of expansion. Resultant GFP-expressing cells were then expanded for two additional weeks before a second round of FACS purification to obtain GFP-mOSCs for intraovarian transplantation experiments or GFP-hOSCs for human ovarian tissue re-aggregation and xenografting experiments.

**Intraovarian transplantation of GFP-mOSCs** To track the fate of OSCs in ovarian tissue, GFP-mOSCs ( $1 \times 10^4$ ) were injected directly into each ovary of recipient wild-type female C57BL/6 mice at 2 months of age. Between 7–8 months of age, the transplanted mice were subjected to an induced ovulation protocol using a single intraperitoneal injection of PMSG (10 IU) followed by hCG (10 IU) 46–48 h later. Ovulated cumulus-oocyte complexes were collected from the oviducts 15–16 h after hCG injection, transferred into HTF supplemented with 0.4% BSA, and assessed by direct fluorescence microscopy for GFP expression. For *in-vitro* fertilization (IVF), the cauda epididymides and vas deferens were removed from adult wild-type C57BL/6 male mice and placed into HTF medium supplemented with BSA. Sperm were obtained by gently squeezing the tissue with tweezers, capacitated for 1 h at 37 C, and mixed with cumulus-oocyte complexes ( $1\text{--}2 \times 10^6$  sperm  $\text{ml}^{-1}$  of HTF medium supplemented with BSA) for 4–5 h. Inseminated oocytes (fertilized and unfertilized) were then washed of sperm and transferred to 50  $\mu\text{l}$  drops of KSOM-AA medium (Irvine Scientific), and the drops were covered with mineral oil to support further preimplantation embryonic development. Light and fluorescence microscopic examination was performed every 24 h for a total of 144 h to monitor embryo development to the hatching blastocyst stage<sup>9</sup>. Ovarian tissue harvested at the time of ovulated oocyte collection was fixed and processed for immunohistochemical detection of GFP using a mouse monoclonal antibody against GFP (sc9996, Santa Cruz Biotechnology) along with the MOM™ kit (Vector Labs), as detailed previously<sup>4,5</sup>. Ovaries from non-transplanted wild-type female mice and from TgOG2 transgenic female mice served as negative and positive controls, respectively, for GFP detection.

**Human ovarian tissue re-aggregation** Human ovarian cortex was dissociated and washed as described above, and incubated with  $35 \mu\text{g ml}^{-1}$  phytohemagglutannin (PHA; Sigma-Aldrich) plus  $1 \times 10^5$  GFP-hOSCs for 10 min at 37 C. The cell mix was pelleted by centrifugation ( $10,000 \times g$  for 1 min at 20 C) to create the tissue aggregate, which was placed onto a Millicell 0.4- $\mu\text{m}$

culture plate insert (Millipore) contained in a 6-well culture dish with 1 ml of OSC culture medium. Aggregates were incubated at 37 C in 5% CO<sub>2</sub>-95% air, and live-cell GFP imaging was performed 24, 48 and 72 h later.

**Intraovarian OSC injection and xenografting** Forty human ovarian cortical tissue pieces (2 x 2 x 1 mm) were individually injected with approximately  $1.3 \times 10^3$  GFP-hOSCs using a 10- $\mu$ l NanoFil syringe with a 35-gauge beveled needle (World Precision Instruments). Recipient NOD-SCID female mice were anesthetized and a small incision was made along the dorsal flank for subcutaneous insertion of the human ovarian tissue (4 grafts per mouse), essentially as described<sup>10,11</sup>. Xenografts were removed 7 or 14 d after transplantation, fixed in 4% PFA, paraffin-embedded and serially sectioned (6  $\mu$ m) for immunohistochemical analysis using a mouse monoclonal antibody against GFP, essentially as described<sup>4,5</sup>. Briefly, high temperature antigen retrieval was first performed using 0.01 M sodium citrate buffer (pH 6.0). After cooling, sections were incubated for 10 min with 3% hydrogen peroxide in methanol to block endogenous peroxidase activity, washed and incubated in streptavidin-biotin pre-block solution as per the manufacturer's protocol (Vector Laboratories). Sections were then blocked for 1 h at 20 C using TNK buffer containing 1% normal goat serum and incubated overnight at 4 C with a 1:100 dilution of GFP antibody prepared in TNK buffer containing 1% normal goat serum. Sections were then washed, incubated with a 1:500 dilution of goat anti-mouse biotinylated secondary antibody for 30 min at 20 C, washed and reacted with Vectastain ABC reagents (Lab Vision) for 30 min at 20 C prior to detection of GFP-positive cells using diaminobenzidine (DAKO). Sections were lightly counterstained with hematoxylin to visualize cell and tissue architecture. Negative controls (complete immunohistochemical staining protocol on xenografted tissues that received vehicle injections) were always run in parallel and did not show a positive signal. To confirm and extend these observations, dual immunofluorescence-based detection of GFP and either YBX2 (diplotene stage oocyte-specific marker) or LHX8 (early stage oocyte transcription factor) in xenografted human ovarian tissues was performed with DAPI counterstaining, essentially as detailed under *Immunoanalysis*.

**Data presentation and analysis** All experiments were independently replicated at least three times. Quantitative data (means  $\pm$  s.e.m. of combined results) were analyzed by one-way analysis of variance (ANOVA) using Prism software, whereas qualitative images presented are representative of the outcomes obtained in the replicate experiments.

## REFERENCES FOR SUPPLEMENTARY METHODS

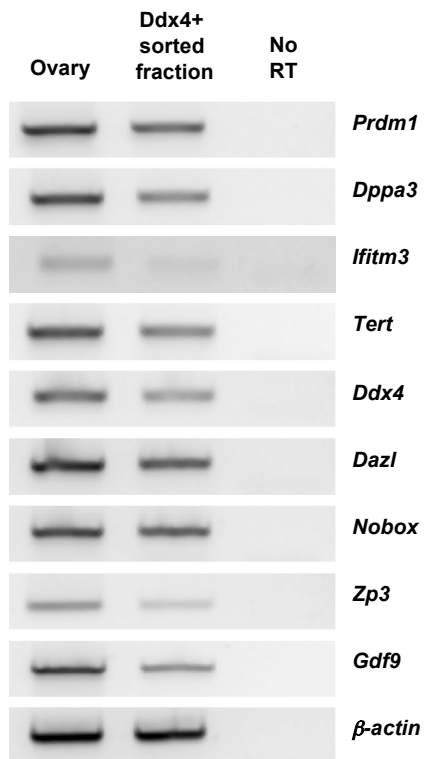
1. Yeom, Y.I. *et al.* Germline regulatory element of *Oct-4* specific for the totipotent cycle of embryonal cells. *Development* **122**, 881–894 (1996).
2. Yoshimizu, T. *et al.* Germline-specific expression of the *Oct-4*/green fluorescent protein (GFP) transgene in mice. *Dev. Growth Differ.* **41**, 675–684 (1999).
3. Szabo, P.E. *et al.* Allele specific expression of imprinted genes in mouse migratory primordial germ cells. *Mech. Dev.* **115**, 157–160 (2002).
4. Johnson, J. *et al.* Oocyte generation in adult mammalian ovaries by putative germ cells in bone marrow and peripheral blood. *Cell* **122**, 303–315 (2005).
5. Lee, H.-J. *et al.* Bone marrow transplantation generates immature oocytes and rescues long-term fertility in a preclinical mouse model of chemotherapy-induced premature ovarian failure. *J. Clin. Oncol.* **25**, 3198–3204 (2007).
6. Kagawa, N., Silber, S. & Kuwayama, M. Successful vitrification of bovine and human ovarian tissue. *Reprod. Biomed. Online* **18**, 568–577 (2009).
7. Zou, K. *et al.* Production of offspring from a germline stem cell line derived from neonatal ovaries. *Nat. Cell Biol.* **11**, 631–636 (2009).
8. West, F.D., Mumaw, J.L., Gallegos-Cardenas, A., Young, A. & Stice, S.L. Human haploid cells differentiated from meiotic competent clonal germ cell lines that originated from embryonic stem cells. *Stem Cells Dev.* **20**, 1079–1088 (2011).
9. Selesniemi, K., Lee, H.-J., Muhlhauser, A. & Tilly, J.L. Prevention of maternal aging-associated oocyte aneuploidy and meiotic spindle defects in mice by dietary and genetic strategies. *Proc. Natl. Acad. Sci. USA* **108**, 12319–12324 (2011).
10. Weissman, A. *et al.* Preliminary experience with subcutaneous human ovarian cortex transplantation in the NOD-SCID mouse. *Biol. Reprod.* **60**, 1462–1467 (1999).
11. Matikainen, T. *et al.* Aromatic hydrocarbon receptor-driven *Bax* gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals. *Nat. Genet.* **28**, 355–360 (2001).

**Supplementary Table S1.** Details for PCR primers used to analyze gene expression in total RNA samples prepared from mouse cells and tissues.

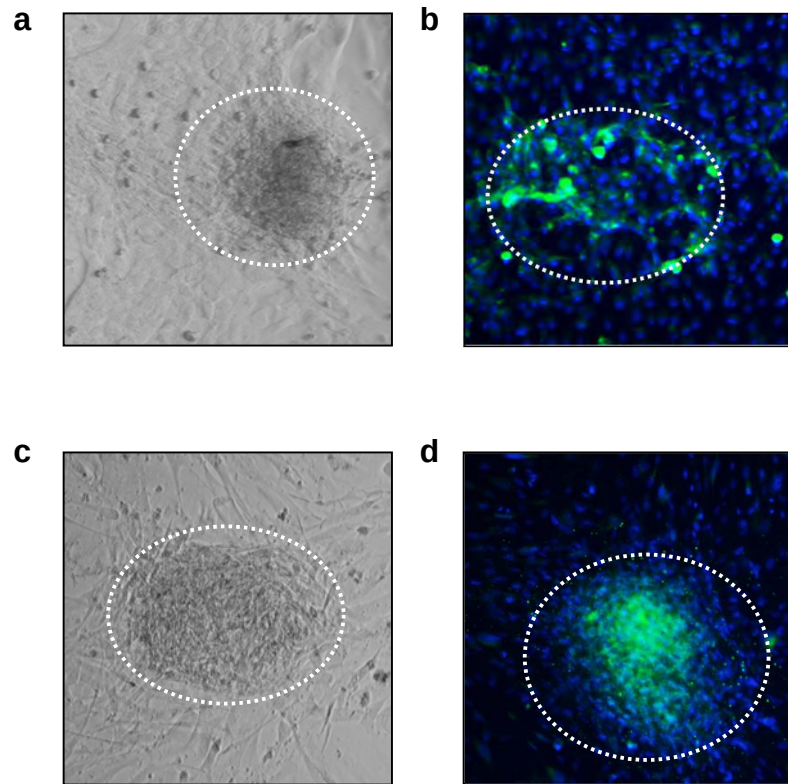
| <b>Gene</b>    | <b>Accession number</b> | <b>Primer sequences (5' to 3'; F, forward; R, reverse)</b>             | <b>Size (bp)</b> |
|----------------|-------------------------|------------------------------------------------------------------------|------------------|
| <i>Prdm1</i>   | NM_007548               | <b>F:</b> CGGAAAGCAACCCAAAGCAATAC<br><b>R:</b> CCTCGGAACCATAGGAAACATTC | 483              |
| <i>Dppa3</i>   | NM_139218               | <b>F:</b> CCCAATGAAGGACCCTGAAAC<br><b>R:</b> AATGGCTCACTGTCCCGTTCA     | 354              |
| <i>Ifitm3</i>  | NM_025378               | <b>F:</b> GTTATCACCATTGTTAGTGTCATC<br><b>R:</b> AATGAGTGTTACACCTGCGTG  | 151              |
| <i>Tert</i>    | NM_009354               | <b>F:</b> TGCCAATATGATCAGGCACTCG<br><b>R:</b> ACTGCGTATAGCACCTGTCACC   | 305              |
| <i>Ddx4</i>    | NM_001145885            | <b>F:</b> GGAAACCAGCAGCAAGTGAT<br><b>R:</b> TGGAGTCCTCATCCTCTGG        | 213              |
| <i>Dazl</i>    | NM_010021               | <b>F:</b> GTGTGTCGAAGGGCTATGGAT<br><b>R:</b> ACAGGCAGCTGATATCCAGTG     | 328              |
| <i>Ybx2</i>    | NM_016875               | <b>F:</b> CCTCCCCACTTTCCATAAT<br><b>R:</b> AATGGGTGGGGAAGAAAAAC        | 235              |
| <i>Sycp3</i>   | NM_011517               | <b>F:</b> AGCAGAGAGCTTGGTCGGG<br><b>R:</b> TCCGGTGAGCTGTCGCTGTC        | 100              |
| <i>Dmc1</i>    | NM_010059.2             | <b>F:</b> CTCACGCTTCCACAACAAGA<br><b>R:</b> TCTCGGGGCTGTCATAAATC       | 81               |
| <i>Nobox</i>   | NM_130869               | <b>F:</b> CCCTTCAGTCACAGTTTCCGT<br><b>R:</b> GTCTCTACTCTAGTGCCTTCG     | 379              |
| <i>Lhx8</i>    | NM_010713               | <b>F:</b> CGTCAGTCCCAACCATTCTT<br><b>R:</b> TTGTTGGTGAGCATCCATGT       | 157              |
| <i>Gdf9</i>    | NM_008110               | <b>F:</b> TGCCTCCTTCCCTCATCTTG<br><b>R:</b> CACTTCCCCCGCTCACACAG       | 709              |
| <i>Zp1</i>     | NM_009580               | <b>F:</b> GTCCGACTCCTGCAGAGAAC<br><b>R:</b> TGATGGTGAAGCGCTGATAG       | 208              |
| <i>Zp2</i>     | NM_011775               | <b>F:</b> AAGGTCTTGAGCAGGAACGA<br><b>R:</b> GGGTGGAAGTAGTGCGGTA        | 152              |
| <i>Zp3</i>     | NM_011776               | <b>F:</b> CCGAGCTGTGCAATTCCCAGA<br><b>R:</b> AACCTCTGAGCCAAGGGTGA      | 183              |
| <i>β-actin</i> | NM_007393               | <b>F:</b> GATGACGATATCGCTGCGCTG<br><b>R:</b> GTACGACCAGAGGCATACAGG     | 440              |

**Supplementary Table S2.** Details for PCR primers used to analyze gene expression in total RNA samples prepared from human cells and tissues.

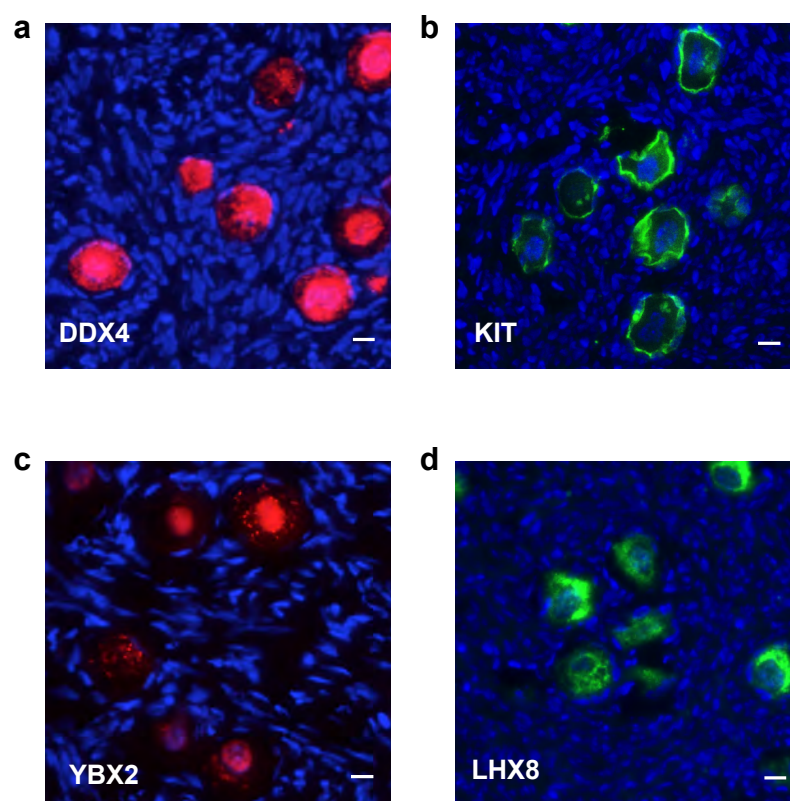
| <b>Gene</b>                     | <b>Accession number</b> | <b>Primer sequences (5' to 3'; F, forward; R, reverse)</b>                | <b>Size (bp)</b> |
|---------------------------------|-------------------------|---------------------------------------------------------------------------|------------------|
| <i>PRDM1</i>                    | NM_001198               | <b>F:</b> AAACATGACCGGCTACAAGACCCT<br><b>R:</b> GGCACACCTTGCATTGGTATGGTT  | 332              |
| <i>DPPA3</i>                    | NM_199286               | <b>F:</b> AGCAGTCCTCAGGGAAATCGAAGA<br><b>R:</b> TATGGCTGAAGTGGCTTGGTGTCT  | 276              |
| <i>IFITM3</i>                   | NM_021034               | <b>F:</b> ATGTCGTCTGGTCCCTGTTC<br><b>R:</b> GGGATGACGATGAGCAGAAT          | 205              |
| <i>TERT</i>                     | NM_198253               | <b>F:</b> AGACGGTGTGCACCAACATCTACA<br><b>R:</b> TGTCGAGTCAGCTTGAGCAGGAAT  | 271              |
| <i>DDX4</i>                     | NM_024415               | <b>F:</b> TTGTTGCTGTTGGACAAGTGGGTG<br><b>R:</b> GCAACAAGAACTGGGCACTTTCCA  | 283              |
| <i>DAZL</i>                     | NM_001190811            | <b>F:</b> TCGAACTGGTGTGTCCAAAGGCTA<br><b>R:</b> TAGGATTCATCGTGGTTGTGGGCT  | 260              |
| <i>YBX2</i>                     | NM_015982               | <b>F:</b> ACCCTACCCAGTACCCTGCT<br><b>R:</b> GCAAGAAAAGCAACCAGGAG          | 248              |
| <i>SYCP3</i>                    | NM_001177949            | <b>F:</b> TATGGTGTCTCTCCGAAAAA<br><b>R:</b> AACTCCAACCTCCTTCCAGCA         | 238              |
| <i>NOBOX</i>                    | NM_001080413            | <b>F:</b> ATAAACGCCGAGAGATTGCCCAGA<br><b>R:</b> AAGTCTGGTCAGAAAGTCAGCAGCA | 375              |
| <i>LHX8</i>                     | NM_001001933            | <b>F:</b> CAAGCACAATTTGCTCAGGA<br><b>R:</b> GGCACGTAGGCAGAATAAGC          | 230              |
| <i>GDF9</i>                     | NM_005260               | <b>F:</b> TCACCTCTACAACACTGTTCGGCT<br><b>R:</b> AAGGTTGAAGGAGGCTGGTCACAT  | 344              |
| <i>ZP1</i>                      | NM_207341               | <b>F:</b> CGCCATGTTCTCTGTCTCAA<br><b>R:</b> CGTTTGTTTACATCCCAGTG          | 219              |
| <i>ZP2</i>                      | NM_003460               | <b>F:</b> TCTTCTTCGCCCTTGTGACT<br><b>R:</b> CTCAGGGTGAGCTTTTCTGG          | 217              |
| <i>ZP3</i>                      | NM_001110354            | <b>F:</b> AGCAGGACCCAGATGAACTCAACA<br><b>R:</b> AAGCCCACTGCTCTACTTCATGGT  | 274              |
| <i><math>\beta</math>-actin</i> | NM_001101               | <b>F:</b> CATGTACGTTGCTATCCAGGC<br><b>R:</b> CTCCTTAATGTCACGCACGAT        | 250              |



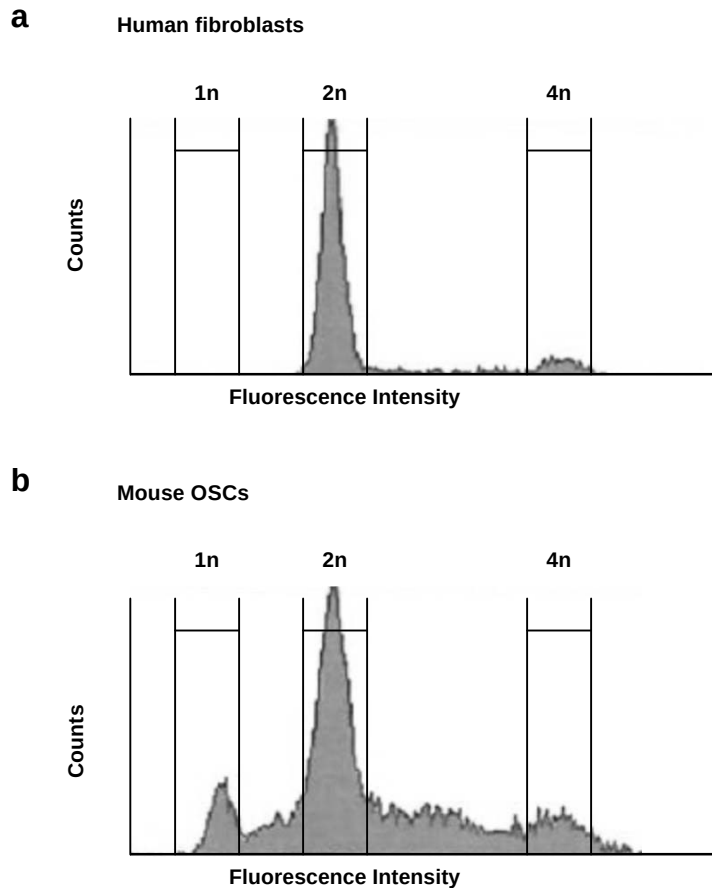
**Supplementary Fig. S1.** OSC fractions isolated from adult mouse ovaries by immunomagnetic bead sorting contain contaminating oocytes. Gene expression analysis of germline markers (*Prdm1*, *Dppa3*, *Ifitm3*, *Tert*, *Ddx4*, *Dazl*) and oocyte-specific markers (*Nobox*, *Zp3*, *Gdf9*) in young adult mouse ovaries (positive control) or the final cell fraction obtained following DDX4-COOH antibody-based immunomagnetic bead sorting of dispersed young adult mouse ovaries (No RT, PCR of sorted cell RNA sample without reverse transcription;  $\beta$ -actin, sample loading control).



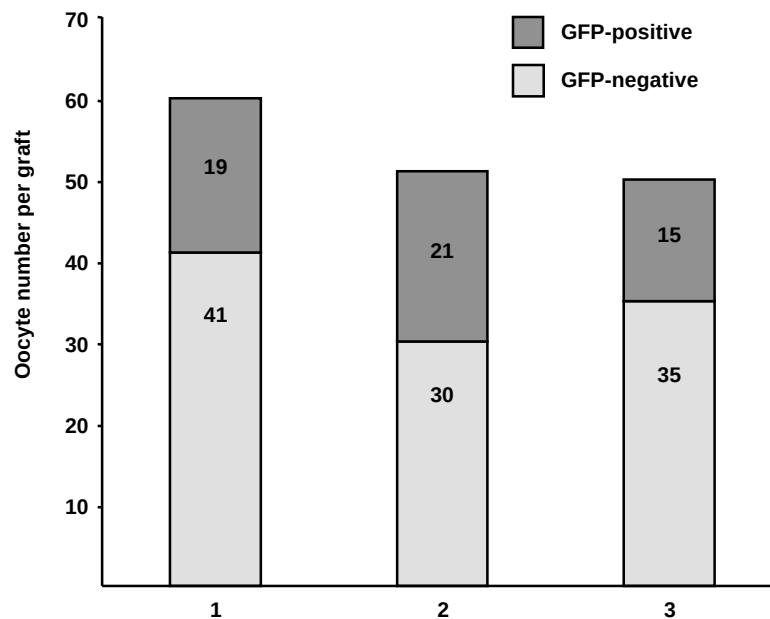
**Supplementary Fig. S2.** Germ cell colony formation by mouse and human OSCs *in vitro*. **a–d**, Bright field view (**a**, **c**) and immunofluorescence-based analysis of Ddx4/DDX4 expression (**b**, **d**; green with blue DAPI counterstain) in typical germ cell colonies formed by mouse (**a**, **b**) and human (**c**, **d**) OSCs after establishment on MEFs *in vitro* (typical colonies are highlighted by white dashed lines).



**Supplementary Fig. S3.** Detection of oocyte-specific markers in adult human ovaries. **a–d**, Immunofluorescence analysis DDX4 (**a**, red), KIT (**b**, green), YBX2 (**c**, red) and LHX8 (**d**, green) expression in oocytes in adult human ovarian cortical tissue (see also **Fig. 6h**). Sections were counterstained with DAPI (blue) for visualization of nuclei. Scale bars, 25- $\mu$ m.



**Supplementary Fig. S4.** Ploidy analysis of human fibroblasts and mouse OSCs in culture. **a, b**, Representative FACS-based assessment of ploidy status in cultures of actively-dividing human fetal kidney fibroblasts (**a**) and in mouse OSCs collected 48 h after passage (**b**). Haploid ( $1n$ ) cells were only detected in the germline cultures, consistent with results from analysis of human OSCs maintained *in vitro* (see **Fig. 5i**), whereas all cultures contained diploid ( $2n$ ) and tetraploid ( $4n$ ) populations of cells.



**Supplementary Fig. S5.** Morphometry-based assessment of oocyte formation in human ovarian xenografts following GFP-hOSC transplantation. Total number of primordial and primary follicles in three randomly selected human ovarian cortical tissue samples (labeled 1, 2 and 3), 7 d after injecting GFP-hOSCs and xenografting into NOD/SCID mice. Grafts contain both GFP-negative (host-derived) and GFP-positive (OSC-derived) oocytes (see **Fig. 6d–g** for examples).