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

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Abstract

Germline stem cells that produce oocytes *in vitro* and fertilization-competent eggs *in vivo* have been identified in and isolated from adult mouse ovaries. Here we describe and validate a FACS-based protocol that can be used with adult mouse ovaries and human ovarian cortical tissue to purify rare mitotically-active cells that exhibit a gene expression profile consistent with primitive germ cells. Once established *in vitro*, these cells can be expanded for months and spontaneously generate 35–50 μm oocytes, as determined by morphology, gene expression and attainment of haploid (1*n*) status. Injection of the human germline cells, engineered to stably express GFP, into human ovarian cortical biopsies leads to formation of follicles containing GFP-positive oocytes 1–2 weeks after xenotransplantation into immunodeficient female mice. Thus, ovaries of reproductive-age women, like adult mice, possess rare mitotically-active germ cells that can be propagated *in vitro* as well as generate oocytes *in vitro* and *in vivo*.

Since the early 1950s, management of ovarian insufficiency and failure, including infertility due to aging or insults, was restricted by the belief that the oocyte-containing pool of follicles set forth at birth is not amenable to renewal[1]. In 2004, studies with mice challenged the idea of a fixed ovarian reserve being endowed during the perinatal period[2]. Based on results from several approaches, it was concluded that ovaries of adult mice possess rare female germline or oogonial stem cells (OSCs) that generate oocytes analogous to spermatogonial stem cell (SSC) support of sperm production in adult testes[3]. Although this study was criticized by many in the field[4], OSCs were successfully isolated from neonatal and adult mouse ovaries several years later[5,6]. These cells stably proliferate *in vitro* for months[5,6] and spontaneously generate what appear to be immature oocytes in culture[6]. Transplantation studies showed that GFP-expressing OSCs injected into ovaries of chemotherapy-conditioned adult mice differentiate into mature eggs that are ovulated, fertilize and produce viable offspring[5]. These findings, along with other studies of mice[7–9],

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have opened the possibility of using OSCs as agents for transplantation and as targets for therapies designed to modulate ovarian function and fertility[4,10]. Additionally, identification of dormant OSCs in atrophic ovaries of aged mice, which resume *in-vivo* oogenesis when exposed to a young adult ovarian environment[11], indicates that ovarian aging may be reversible[10,12]. However, any consideration of clinical utility of these cells requires firm evidence that comparable oocyte-producing cells exist in ovaries of reproductive-age women.

As a first step towards this goal, we assessed the protocol recently reported to obtain OSCs from adult mouse ovaries[5]. This approach relies on immunological detection of a putative cell-surface variant of DEAD box polypeptide 4 (Ddx4; also commonly referred to as mouse vasa homolog or Mvh), an evolutionarily conserved germ cell-specific RNA helicase[13–15], coupled with cell sorting by use of magnetic beads. Two issues surfaced that we felt might preclude application of this protocol to isolate candidate OSCs from adult human ovaries. First, Ddx4 is widely considered to be a cytoplasmic protein, and thus its claimed detection on the outside of mouse OSCs is at odds with prior studies[10,16]. Second, even if a cell-surface variant of Ddx4 is expressed by mouse OSCs, immunomagnetic sorting is a relatively crude cell isolation approach that often results in enrichment of a desired cell type in fractions contaminated with non-targeted cells carried over during column washing and flushing steps. Magnetic bead sorting also does not distinguish between viable and damaged or dead cells, and does not allow simultaneous assessment of other cellular features such as yield, size or co-expression of additional markers. Our objectives were to initially test with mice whether or not OSCs possess an externally-exposed epitope of Ddx4, and if so to use this property for validation of a FACS-based protocol to purify viable OSCs from dispersed ovaries of adult mice. We then determined if this technology could also be used to purify an equivalent population of candidate OSCs from ovarian cortical tissue of healthy reproductive-age women.

RESULTS

Validation of a FACS-based protocol for OSC isolation

The Ddx4 antibody used previously to isolate mouse OSCs by immunomagnetic sorting is a rabbit polyclonal against the COOH-terminus of the protein[5]. We obtained this antibody along with a goat polyclonal antibody against the NH_2-terminus of Ddx4 for comparative studies. Immunofluorescence analysis of young adult (2-month-old) mouse ovaries using either antibody showed an identical pattern of Ddx4 expression that was restricted, as expected, to oocytes (Fig. 1a). We then used each antibody for immunomagnetic sorting of dispersed young adult mouse ovary tissue[5]. No cells were obtained in the bead fraction when the NH_2 antibody was used; however, 5–8 μm cells bound to the magnetic beads were observed when the COOH antibody was used (Fig. 1b). Analysis of cells isolated with the COOH antibody revealed a germline gene expression pattern consistent with that reported for mouse OSCs isolated previously using immunomagnetic sorting[5] (Supplementary Fig. S1). Although isolated oocytes assessed in parallel using the COOH antibody were always detected in the non-immunoreactive wash fraction (Fig. 1b), additional marker analysis of the Ddx4-positive viable cell fraction obtained by immunomagnetic sorting revealed several oocyte-specific mRNAs including *newborn ovary homeobox (Nobox), zona pellucida glycoprotein 3 (Zp3)* and *growth differentiation factor 9 (Gdf9)* (Supplementary Fig. S1). These findings indicate that while mouse oocytes do not show evidence of cell surface expression of Ddx4 when analyzed as individual entities (Fig. 1b), mouse oocytes are nonetheless a contaminating cell type following immunomagnetic sorting of OSCs from dispersed mouse ovary tissue. This outcome most likely reflects either a non-specific physical carry-over of small oocytes with OSCs during column washing and flushing or

reactivity of cytoplasmic Ddx4 in plasma membrane-compromised (damaged) oocytes with the COOH antibody.

We therefore next tested reactivity of each antibody with dispersed mouse ovarian cells by FACS. In agreement with the magnetic bead sorting results, viable Ddx4-positive cells were obtained only when the COOH antibody was used (Fig. 1c). However, if the ovarian cells were fixed and permeabilized prior to FACS, Ddx4-positive cells were obtained using the NH_2 antibody (Fig. 1c). Furthermore, if viable Ddx4-positive cells isolated by FACS using the COOH antibody were fixed, permeabilized and re-sorted, the same cell population was recognized by the NH_2 antibody (Fig. 1d). As a final means to confirm validity of this OSC isolation method, fractions of cells at each step of the protocol were assessed by gene expression analysis using markers for germ cells [*PR domain containing 1 with ZNF domain* or *Prdm1* (human gene ortholog, *PRDM1*; also commonly referred to as *BLIMP1*), *developmental pluripotency-associated 3* or *Dppa3* (human gene ortholog, *DPPA3*, also commonly referred to as *STELLA*), *interferon induced transmembrane protein 3* or *Ifitm3* (also commonly referred to as *Fragilis*; human gene ortholog, *IFITM3*), *telomerase reverse transcriptase* or *Tert* (human gene ortholog, *TERT*), *Ddx4* (human gene ortholog, *DDX4*), and *deleted in azoospermia-like* or *Dazl* (human gene ortholog, *DAZL*)] and for oocytes (*Nobox, Zp3*, and *Gdf9*). Adult mouse ovarian tissue was minced and enzymatically digested, passed through a 70-μm filter to remove large tissue clumps, and then passed through a 35-μm filter to obtain the final fraction of cells for FACS. Every fraction of cells through each step of the protocol, with the exception of the Ddx4-positive viable cell fraction obtained by FACS, expressed all germline and oocyte markers (Fig. 1f). While FACS-sorted Ddx4-positive cells expressed all germline markers, no oocyte markers were detected (Fig. 1f). Thus, unlike the oocyte contamination observed when OSCs were isolated by immunomagnetic sorting using the Ddx4-COOH antibody (Supplementary Fig. S1), use of this antibody with FACS provides a superior strategy to obtain OSCs free of oocytes.

FACS isolation of candidate OSCs from adult human ovaries

Using the COOH antibody with FACS, we consistently isolated viable DDX4-positive cells between 5–8 μm in diameter from human ovarian cortical tissue of all subjects analyzed, with a percent yield (1.7% ± 0.6% DDX4-positive versus total viable cells sorted; mean ± s.e.m., $n = 6$) that was comparable to the yield of OSCs from young adult mouse ovaries processed in parallel (1.5% ± 0.2% Ddx4-positive versus total viable cells sorted; mean ± s.e.m., $n = 15$). This percent yield is the incidence of these cells in the final pool of viable single cells sorted by FACS, which represents only a fraction of the total number of cells present in ovaries prior to processing. To estimate the incidence of OSCs per ovary, we used young adult mice to determine genomic DNA content per ovary (1,774.44 ± 426.15 μg; mean ± s.e.m., $n = 10$) and divided this value into genomic DNA content per fraction of viable cells sorted per ovary (16.41 ± 4.01 μg; mean ± s.e.m., $n = 10$). Assuming genomic DNA content per cell is equivalent, this allowed us to determine how much of the total ovarian cell pool was represented by the total viable sorted cell fraction obtained after processing. Using this correction factor, we estimated the incidence of OSCs per ovary to be 0.014% ± 0.002% [0.00926 × (1.5% ± 0.2%)]. With respect to OSC yield, this number varied across replicates but we consistently obtained between 250 to slightly over 1,000 viable Ddx4-positive cells from each young adult mouse ovary after FACS of dispersates initially prepared from a pool of 4 ovaries.

Analysis of freshly-isolated Ddx4/DDX4-positive cells from mouse and human ovaries (Fig. 2a,b) revealed a similar size and morphology (Fig. 2c,d) and a matched gene expression profile rich in markers for early germ cells[17–19] (*Prdm1/PRDM1, Dppa3/DPPA3, Ifitm3/IFITM3* and *Tert/TERT*; Fig. 2e). These findings agree with the morphology and gene expression profile of mouse OSCs reported earlier[5,6]. To further define characteristic

features of Ddx4-positive cells obtained from adult ovaries using FACS, we first performed an *in-vivo* teratoma formation assay. We felt this was important since a recent study has reported the isolation of *Oct3/4*-positive stem cells from adult mouse ovaries that possess the teratoma-forming capacity of embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs)[20]. As expected, 100% of NOD-SCID mice transplanted with 1×10^5 mouse ESCs used as a positive control ($n = 3$) developed teratomas within 3 weeks; however, no teratomas were observed in NOD-SCID mice transplanted in parallel with 1×10^5 Ddx4-positive cells freshly isolated from adult mouse ovaries ($n = 3$), even at 24 weeks post-transplant (Fig. 2f–h). Thus, while OSCs express numerous stem cell and primitive germ cell markers[5,6] (see also Figs. 1f and 2e), these cells are distinct from other types of pluripotent stem cells described to date.

We next assessed the ability FACS-purified mouse OSCs, engineered to express green fluorescent protein (GFP) through retroviral transduction after their establishment as actively-dividing germ cell-only cultures *in vitro* (see *In-vitro characterization of candidate human OSCs* below), to generate oocytes following transplantation into ovaries of adult female mice. To ensure the outcomes obtained were reflective of stable integration of the transplanted cells into the ovaries and also were not complicated by pre-transplantation induced damage to the gonads, we injected GFP-expressing mouse OSCs into ovaries of non-chemotherapy conditioned wild-type recipients at 2 months of age and animals were maintained for 5–6 months prior to analysis. Between 7–8 months of age, we induced the transplanted animals to ovulate with exogenous gonadotropins, after which their ovaries and any oocytes released into the oviducts were collected. Developing follicles containing GFP-positive oocytes were readily detected, along with host follicles containing GFP-negative oocytes, in ovaries of all females that received GFP-expressing mouse OSCs initially purified by FACS (Fig. 3a).

After oviductal flushing, complexes containing expanded cumulus cells surrounding centrally-located oocytes both lacking and expressing GFP were observed. Mixing of these complexes with sperm from wild-type males resulted in fertilization and development of preimplantation embryos, with those derived from fertilized GFP-positive eggs retaining GFP expression through the hatching blastocyst stage (Fig. 3b–d). From the five adult wild-type female mice transplanted with GFP-expressing OSCs 5–6 months earlier, a total of 31 cumulus-oocyte complexes were retrieved from the oviducts, 23 of which successfully fertilized to produce embryos. The presence of cumulus cells around each oocyte made it impossible for us to accurately determine the numbers of GFP-negative versus GFP-positive oocytes ovulated. However, evaluation of the 23 embryos produced following *in-vitro* fertilization (IVF) revealed that eight were GFP-positive, with all five mice tested releasing at least one egg at ovulation that fertilized to produce a GFP-positive embryo. These findings indicate that mouse OSCs purified by DDX4-COOH antibody-based FACS, like their previously reported counterparts isolated by immunomagnetic sorting[5], generate functional oocytes *in vivo*. However, our data also show that chemotherapy conditioning prior to transplantation is not, as previously reported[5], required for OSCs to engraft and generate functional oocytes in adult ovary tissue.

In-vitro characterization of candidate human OSCs

Using parameters described previously for *in-vitro* propagation of mouse OSCs[5], adult mouse and human ovary-derived Ddx4/DDX4-positive cells were placed into defined cultures with mitotically-inactive mouse embryonic fibroblasts (MEFs) as feeders. Freshly-isolated OSCs could be established as clonal cell lines, and the colony formation efficiency for human OSCs not seeded onto MEFs ranged from 0.18% to 0.40%. Accurate assessment of colony formation efficiency could not be performed using MEFs as initial feeders, the latter of which greatly facilitates establishment of mouse and human OSCs *in vitro*. After

10–12 weeks (mouse) or 4–8 weeks (human) in culture, actively-dividing germ cell colonies became readily apparent (Supplementary Fig. S2). Once established and proliferating, the cells could be re-established as germ cell-only cultures in the absence of MEFs without loss of proliferative potential. Dual analysis of Ddx4/DDX4 expression and bromodeoxyuridine (BrdU) incorporation in MEF-free cultures revealed large numbers of double-positive cells (Fig. 4a–d), confirming that adult mouse and human ovary-derived OSCs were actively dividing. At this stage, mouse cells required passage at confluence every 4–5 d with cultures split 1:6–1:8 (estimated doubling time of 14 h; Fig. 4e). The rate of mouse OSC proliferation was approximately 2–3 fold higher than that of human OSCs maintained in parallel, the latter of which required passage at confluence every 7 d with cultures split 1:3–1:4.

Cell surface expression of Ddx4 remained detectable on the surface of more than 95% of the cells after months of propagation (Fig. 4f). The remaining cells not detected by FACS using the COOH antibody were large (35–50 μm in diameter) spherical cells spontaneously produced by mouse and human OSCs during culture, which exhibited cytoplasmic expression of Ddx4/DDX4 and will be described in detail in the next section. Gene expression analysis of the cultured cells confirmed maintenance of early germline markers (Fig. 4g). Several oocyte-specific markers were also detected in these cultures, and the significance of these data will be discussed in the following section. To extend the mRNA analyses of *Prdm1/PRDM1, Dppa3/DPPA3* and *Ifitm3/IFITM3*, we performed immunofluorescence analysis of these three classic primitive germline markers[17,18]. All three proteins were easily and uniformly detected in mouse (Fig. 4h) and human (Fig. 4i) OSCs maintained *in vitro*. Notably, our detection of Ifitm3/IFITM3 in these cells agrees with a recent study reporting that this protein can also be used to isolate OSCs from mouse ovaries by immunomagnetic bead sorting[21].

In-vitro oogenic capacity of candidate human OSCs

Consistent with results from others[6], mouse OSCs cultured *in vitro* spontaneously generated large (35–50 μm in diameter) spherical cells that by morphology (Fig. 5a) and gene expression analysis (Fig. 5b,c) resembled oocytes. Peak levels of *in-vitro* oogenesis from mouse OSCs were observed within 24–48 h after each passage (Fig. 5d), followed by a progressive decline to nearly non-detectable levels each time the OSCs regained confluence. Parallel analysis of DDX4-positive cells isolated from adult human ovaries and maintained *in vitro* revealed that these cells, like mouse OSCs, also spontaneously generated oocytes as deduced from both morphological (Fig. 5f) and gene expression (Fig. 5c,g) analyses. The kinetics of *in-vitro* oogenesis from human OSCs differed slightly from mouse OSCs in that peak levels of oocyte formation were observed at 72 h after each passage (Fig. 5e). In addition to detection of many widely accepted oocyte markers [*Ddx4/DDX4, Kit/KIT, Nobox/NOBOX, LIM homeobox protein 8 (Lhx8/LHX8), Gdf9/GDF9,* and *zona pellucida glycoproteins 1–3 (Zp1/ZP1, Zp2/ZP2* and *Zp3/ZP3*)][22–26], mouse and human OSC-derived oocytes also expressed the diplotene oocyte stage-specific marker, *Y-box protein 2* (*Ybx2/YBX2;* also commonly referred to as *Msy2/MSY2* in both species, as well as *CONTRIN* in humans) (Fig. 5c), which is essential for meiotic progression and gametogenesis in both sexes[27,28]. Through empirical testing of commercially-available antibodies using adult human ovarian cortical tissue as a positive control, we identified four such antibodies against oocyte markers that specifically reacted with immature oocytes present in adult human ovaries (DDX4, KIT, YBX2 and LHX8) (Supplementary Fig. S3); all four of these proteins were also detected in oocytes generated by human OSCs *in vitro* (Fig. 5g).

The presence of mRNA encoding the meiotic marker YBX2 in oocytes newly formed from human OSCs *in vitro* prompted us to next explore the prospects of meiotic entry in these cultures. Immunofluorescence analysis of human OSCs 72 h after passage identified cells with punctate nuclear localization of the meiosis-specific DNA recombinase, dosage

suppressor of mck1 homolog (DMC1), and the meiotic recombination protein, synaptonemal complex protein 3 (SYCP3) (Fig. 5h). Both proteins are specific to germ cells and are necessary for meiotic recombination to proceed[29–31]. Furthermore, chromosomal DNA content analysis of human OSC cultures 72 h after passage revealed the presence of an expected diploid (2*n*) cell population; however, we also detected peaks corresponding to 4*n* and 1*n* cell populations, the latter being indicative of germ cells that had reached haploid status[32] (Fig. 5i). In actively-dividing cultures of human fibroblasts analyzed as controls in parallel, we detected only 2*n* and 4*n* populations of cells (Supplementary Fig. S4a). Haploid (1*n*) cells were also detected in mouse OSC cultures observed following FACS-based chromosomal analysis (Supplementary Fig. S4b).

Human OSCs generate oocytes in human ovaries *in vivo*

To confirm and extend the *in-vitro* observations of oogenesis from candidate human OSCs, in two final experiments DDX4-positive cells isolated from adult human ovaries were stably transduced with a GFP expression vector (GFP-hOSCs) to facilitate cell tracking. In the first experiment, approximately 1×10^5 GFP-hOSCs were re-aggregated with dispersed adult human ovarian cortical tissue. Numerous GFP-positive cells were observed, as expected, throughout the re-aggregated tissue (Fig. 6a). The aggregates were then placed in culture and assessed 24–72 h later by direct (live cell) GFP fluorescence. Within 24 h, several very large (≥ 50-μm) single cells had formed within the aggregates, many of which were enclosed by smaller GFP-negative cells in tightly compact structures resembling follicles; these structures remained detectable through 72 h (Fig. 6b,c). We interpreted these findings as evidence that GFP-expressing human OSCs spontaneously generated oocytes that became enclosed by somatic (pregranulosa/granulosa) cells present in the adult human ovarian dispersates.

To more clearly determine if this was the case, we next injected approximately 1.3×10^3 GFP-hOSCs into adult human ovarian cortical tissue biopsies (2 × 2 × 1 mm), which were then xenografted into NOD-SCID female mice ($n = 40$ grafts total). Grafts were collected 7 or 14 d later for assessment of GFP expression. All human ovary grafts contained easily discernible primordial and primary follicles with centrally-located GFP-negative oocytes. Inter-dispersed among and often adjacent to these follicles, which were presumably present in the tissue prior to GFP-hOSC injection, were other immature follicles containing GFP-positive oocytes (Fig. 6d,f). Serial section histomorphometric analysis of three randomly selected human ovarian tissue grafts injected with GFP-hOSCs revealed the presence of 15–21 GFP-positive oocytes per graft 7 d after transplanting into mice (Supplementary Fig. S5). As controls, GFP-positive oocytes were never detected in human ovarian cortical tissue prior to GFP-hOSC injection (Fig. 6e) or in xenografts that received mock injections (vehicle without GFP-hOSCs) prior to transplantation into NOD-SCID mice (Fig. 6g). Dual immunofluorescence-based detection of GFP along with either the diplotene stage oocyte-specific marker, YBX2, or the oocyte-specific transcription factor, LHX8, identified many dual-positive cells distributed throughout xenografts injected with GFP-hOSCs (Fig. 6h). As expected, no GFP-positive oocytes were detected in human ovarian tissue prior to GFP-hOSC injection or in xenografts that did not receive GFP-hOSC injections (not shown; see Fig. 6e,g); however, the GFP-negative oocytes present in the tissue were consistently positive for YBX2 and LHX8 (Fig. 6h and Supplementary Fig. S3).

DISCUSSION

In 2004, a major paradigm shift in reproductive biology was proposed from a study in mice challenging the longstanding belief that ovaries of female mammals lose the capacity for oocyte generation prior to birth[2]. Although this change in thinking was initially met with resistance from some members of the scientific community due to its sharp deviation from

traditional beliefs in the field[4], subsequent studies have since independently demonstrated that mouse OSCs can be isolated from adult ovaries for long-term propagation *in vitro*[5,6] and for generation of fertilization-competent eggs *in vivo* following intraovarian transplantation in chemotherapy-conditioned recipient female mice[5,21]. In addition, other work has reported that *de-novo* oocyte formation in adult mouse ovaries can be stimulated by small molecule inhibitors of histone deacetylases as well as by an as-yet unidentified factor(s) in the peripheral circulation of male mice[7–9]. However, the potential clinical relevance of these findings with mice has remained unclear due to a lack of definitive evidence that ovaries of reproductive-age women contain a comparable population of oocyte-producing germline cells that match the characteristic features of mouse OSCs.

As a first step towards accomplishing this, herein we describe in detail the conceptual and technical validation of a straightforward and highly repeatable FACS-based approach for the purification of viable OSCs from adult ovary tissue. The utility of an antibody directed against the COOH-terminus of Ddx4/DDX4 to isolate OSCs apparently reflects a differentiation-dependent switch in localization of this protein in female germ cells from the cell-surface (OSCs) to the cytoplasm (oocytes), with the latter being in accordance with traditional beliefs that Ddx4/DDX4 is a cytoplasmic protein in germ cells. The physiological significance of this change in localization remains to be determined; however, computer-based mapping of the Ddx4/DDX4 transmembrane spanning domain predicts that insertion of the COOH terminus of the protein across the cell membrane could potentially interfere with the RNA helicase domain[10,33]. Although additional work will be needed to test this, movement of proteins into and out of various cellular compartments is a post-translational mechanism commonly used by cells to regulate the activity of such proteins. In any case, we have incorporated this feature of Ddx4/DDX4 into a FACS-based protocol to demonstrate that a rare population of mitotically-active germ cells with a gene expression profile and growth characteristics remarkably similar to mouse OSCs can be reliably isolated from ovaries of healthy young women and propagated long-term *in vitro*.

Our successful purification of what appears to be, by all criteria tested, the human equivalent of mouse OSCs was facilitated by two main factors: nearly three years of work in our lab to test and refine the Ddx4/DDX4 antibody-based protocol for isolation of OSCs using FACS rather than immunomagnetic sorting as reported initially[5], and rare access to entire ovarian cortical tissue that had been vitrified and cryopreserved following removal of both ovaries from women in their twenties and early thirties (Supplementary Fig. S6a,b). Recent studies of mouse OSCs by others have demonstrated the ability of these cells to generate immature oocytes *in vitro*[6] (see also Fig. 5a–c) as well as oocytes that mature into developmentally-competent eggs *in vivo*[5] (see also Fig. 3). Regarding the latter, it is notable that prior studies of mammalian germline stem cell transplantation using SSCs or OSCs have been performed with chemotherapy-conditioned hosts as recipients[5,21,34,35], presumably to open niches for effective donor cell engraftment. Our observations show that for OSCs, adult females do not require chemotherapy conditioning prior to transplantation for these cells to effectively engraft in the ovaries and function long-term (≥ 5 months), at least as measured by their ability to generate oocytes that mature in follicles which yield fertilization-competent eggs following ovulation. Unfortunately, for ethical and legal reasons, as well as technical feasibility limitations related to a current lack of validated protocols for efficiently maturing human primordial follicles to the antral stage *in vitro* for isolation of metaphase II oocytes, our current endpoint analysis of the human equivalent cells could not be as comprehensive. Nonetheless, we have established a consistent and close parallelism between human ovary-derived DDX4-positive cells and mouse OSCs in terms of strategy of purification, yield from adult ovary tissue, morphology, primitive germline gene expression profile, *in-vitro* growth properties, mitotic activity, meiotic activity, and the ability to form oocytes in defined cultures *in vitro* and in injected ovary tissue *in vivo*.

Therefore, based on the multiple experimental lines of evidence reported herein we feel it is reasonable to conclude that these rare cells with cell-surface expression of DDX4 present in ovaries of reproductive-age women represent adult human OSCs. In addition to opening a new research field in human reproductive biology that was inconceivable less than ten years ago, clear evidence for the existence of these cells in women may offer new opportunities to expand on and enhance current fertility preservation strategies. For example, with assisted reproductive technologies involving cryopreservation of ovarian cortical tissue already in development for female cancer patients[36,37], isolation and expansion of OSCs from this tissue before or after cryopreservation might prove useful for new fertility applications. In fact, we have found that these cells can be consistently obtained from cryopreserved and thawed human ovarian tissue samples, and that the cells *per se* can be cryopreserved and thawed months later with minimal loss for successful establishment *in vitro* (Supplementary Fig. S6c). In addition, the availability of a detailed protocol for purification of these newly-discovered cells from human ovary tissue provides us and others with a much more physiologically relevant *in-vitro* model system to study human female germ cell development compared to ESC- or iPSC-derived germline cells currently used as models for human female gametogenesis[38–42].

METHODS

Animals

Wild-type C57BL/6 and NOD-SCID mice were from Charles River Laboratories and Jackson Laboratories, respectively. Transgenic mice with germline-specific GFP expression (TgOG2)[43–45] were obtained, bred and genotyped as described[7,46]. All animal protocols 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 years of age with Gender Identity Disorder for sex reassignment at Saitama Medical Center. The outer cortical layer was carefully removed, vitrified and cryopreserved[47]. For experiments, cryopreserved tissue was thawed using the Cryotissue Thawing Kit (Kitazato Biopharma). All experiments with human tissue were conducted without subject identifiers under a protocol reviewed and approved by the institutional review board of Massachusetts General Hospital (protocol number 2008P001640).

Ddx4/DDX4 antibodies

Polyclonal antibodies against the COOH- (ab13840; Abcam) or NH_2- (AF2030; R&D Systems) terminus of DDX4 were from Abcam or R&D Systems, respectively.

Immunomagnetic sorting

Dissociated ovarian cells or isolated oocytes were processed by immunomagnetic sorting using streptavidin-conjugated microbeads (Miltenyi) or secondary antibody-conjugated Dynabeads (Invitrogen), as described[5].

Flow cytometry

Antibody-labeled ovarian cell suspensions were subjected to FACS using a BD Biosciences FACSAria II cytometer (Harvard Stem Cell Institute), gated against negative controls (unstained cells and cell fractions processed without primary antibody).

Teratoma formation

Mouse ESCs (mESC v6.5) or freshly-isolated mouse OSCs were injected subcutaneously near the rear haunch of NOD/SCID female mice, which were then monitored weekly for up to 6 months for tumor formation.

OSC cultures

Mouse and human OSCs were established on mitotically-inactivated immortalized MEFs and propagated *in vitro*, essentially as described[5]. To assess proliferation, MEF-free OSC cultures were treated with 10 μM BrdU (Sigma-Aldrich) for 48 h for dual immunofluorescence-based detection of BrdU incorporation and Ddx4/DDX4 expression, essentially as described[5].

Gene expression

The presence of each indicated mRNA was assessed by conventional RT-PCR using primers detailed in Supplementary Table S1 (mouse) and Supplementary Table S2 (human).

Immunoanalyses

Fixed ovarian tissues, OSCs or oocytes were assessed by standard immunohistochemical or immunocytochemical methods using antibodies that recognize Ddx4/DDX4 (COOH terminus: ab13840, Abcam; NH_2 terminus: AF2030, R&D Systems), Kit/KIT (sc1494, Santa Cruz Biotechnology), Prdm1/PRDM1 (ab81961, Abcam), Dppa3/DPPA3 (ab19878; Abcam), Ifitm3/IFITM3 (mouse: ab15592, human: ab74699; Abcam), DMC1 (ab96613, Abcam), SYCP3 (ab85621, Abcam), YBX2 (ab33164, Abcam) or LHX8 (ab41519, Abcam).

Ploidy analysis

Chromosomal analysis of mouse and human OSCs was performed using a BD Biosciences FACSAria II cytometer, essentially as described[32]. Human fetal kidney fibroblasts (KEK 293, Invitrogen) were used as control somatic cells.

Generation of GFP-expressing OSCs

One μg of *pBabe-Gfp* vector DNA (Addgene plasmid repository #10668) was transfected (Lipofectamine, Invitrogen) into the Platinum-A retroviral packaging cell line (Cell Biolabs). Transduction of OSCs was performed using fresh viral supernatant in the presence of polybrene (Sigma-Aldrich). Mouse and human OSCs with expression of GFP were purified by FACS following an initial one week of propagation, and then expanded for two additional weeks before a second round of FACS purification to obtain cells used for experiments.

Transplantation of mouse OSCs

GFP-expressing mouse OSCs (1×10^4) were injected directly into each ovary of wild-type mice at 2 months of age. Between 7–8 months of age, transplanted mice were subjected to an induced ovulation protocol. Cumulus-oocyte complexes were collected from the oviducts and assessed by direct fluorescence microscopy for GFP expression. For *in-vitro* fertilization (IVF), cumulus-oocyte complexes were mixed wild-type sperm for 4–5 h, washed and transferred to KSOM-AA medium (Irvine Scientific). Light and fluorescence microscopic examination was performed every 24 h for a total of 144 h to monitor embryo development[48]. Ovarian tissue harvested at the time of oocyte collection was processed for immunohistochemical detection of GFP, as detailed[46].

Human ovarian tissue re-aggregation

Dissociated human ovarian cortex was incubated with 35 μg ml^{-1} phytohemaglutannin (PHA; Sigma) along with 1×10^5 GFP-expressing human OSCs for 10 min at 37 C. The cell mix was pelleted to create the tissue aggregate, which was placed onto a Millicell 0.4-μm culture plate insert (Millipore) in OSC culture medium. Aggregates were incubated at 37 C in 5% CO_2-95% air, and live-cell GFP imaging was performed 24, 48 and 72 h later.

Intraovarian OSC injection and xenografting

Human ovarian cortical tissue injected with approximately 1.3×10^3 GFP-expressing human OSCs were grafted into NOD-SCID female mice[49,50]. Xenografts were removed 7 or 14 d after transplantation and processed for immunohistochemical detection of GFP expression[46]. For dual marker analysis, we performed immunofluorescence-based detection of GFP and either YBX2 or LHX8 (see *Immunoanalysis*).

Data analysis

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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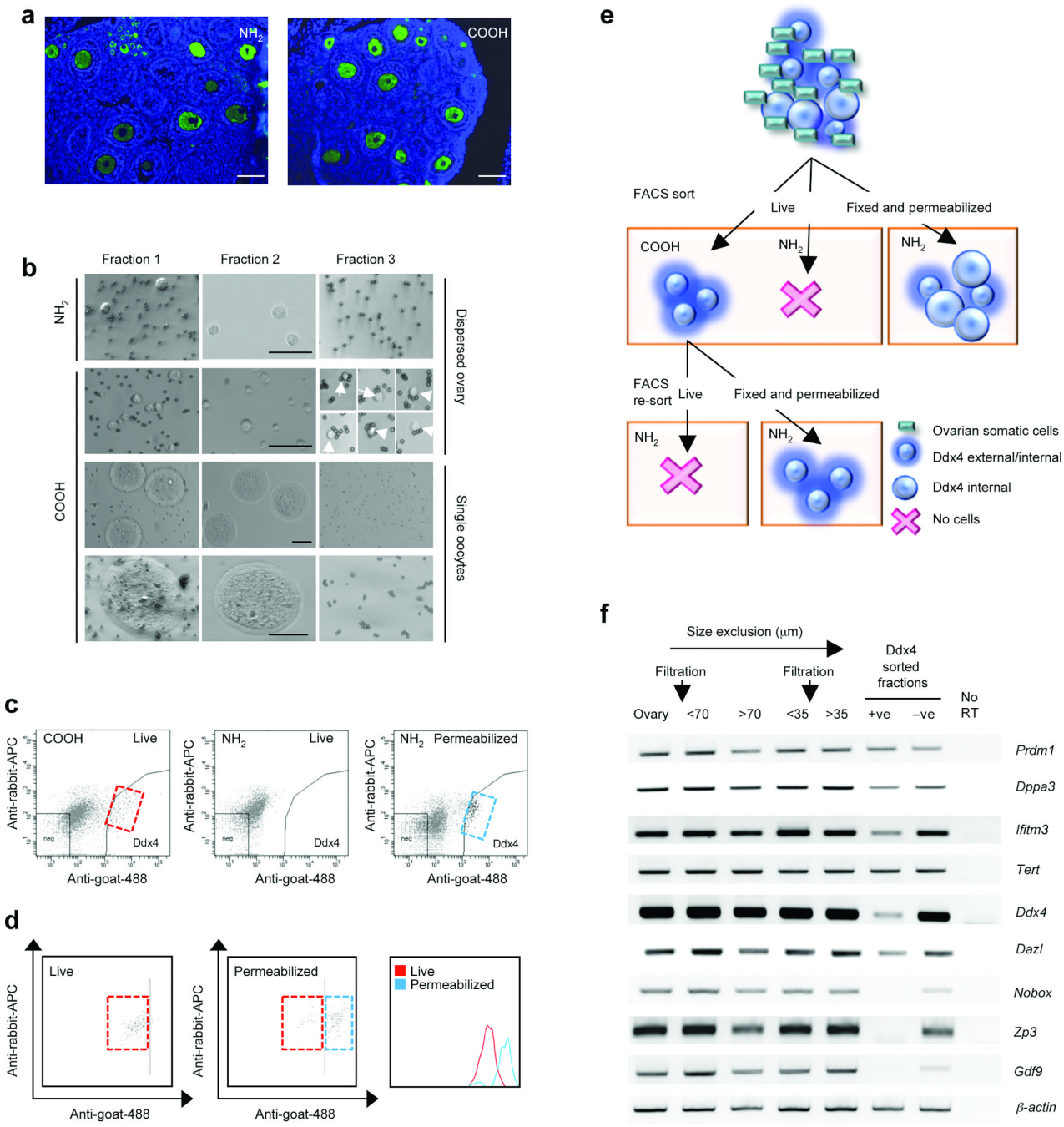

Fig. 1.

FACS-based protocol for OSC isolation. **a,** Immunofluorescence analysis of Ddx4 expression (green; blue DAPI counterstain) in adult mouse ovaries using antibodies against the NH_2 or COOH terminus (scale bars, 50-μm). **b,** Immunomagnetic sorting of dispersed mouse ovaries or isolated oocytes using antibodies against the NH_2 or COOH terminus of Ddx4. Fraction 1, cells plus beads before separation; Fraction 2, wash fraction (non-immunoreactive); Fraction 3, bead fraction (Ddx4-positive cells, highlighted by white arrows). **c,** FACS analysis of live or permeabilized cells from dispersed mouse ovaries using antibodies against the NH_2 or COOH terminus of Ddx4. Viable Ddx4-positive cells are only

detected with the COOH antibody (red dashed box) whereas permeabilization enables isolation of Ddx4-positive cells using the NH_2 antibody (blue dashed box). **d,** Permeabilization of viable Ddx4-positive cells obtained with the COOH antibody (red dashed box) enables re-isolation of the same cells by FACS using the NH_2 antibody (blue dashed box), as shown by shift in fluorescence (red to blue indicates shift from APC-positive live cells to 488-positive fixed and permeabilized cells). **e,** Schematic of FACS protocols employed to validate use of the Ddx4-COOH antibody for isolation of viable OSCs. **f,** Expression analysis of germline markers (*Prdm1, Dppa3, Ifitm3, Tert, Ddx4, Dazl*) and oocyte markers (*Nobox, Zp3, Gdf9*) in each cell fraction produced during the ovarian dispersion process to obtain cells for FACS-based isolation of OSCs (+ve, Ddx4-positive viable cell fraction after FACS; −ve, Ddx4-negative viable cell fraction after FACS; No RT, PCR of RNA sample without reverse transcription; *β-actin*, sample loading control).

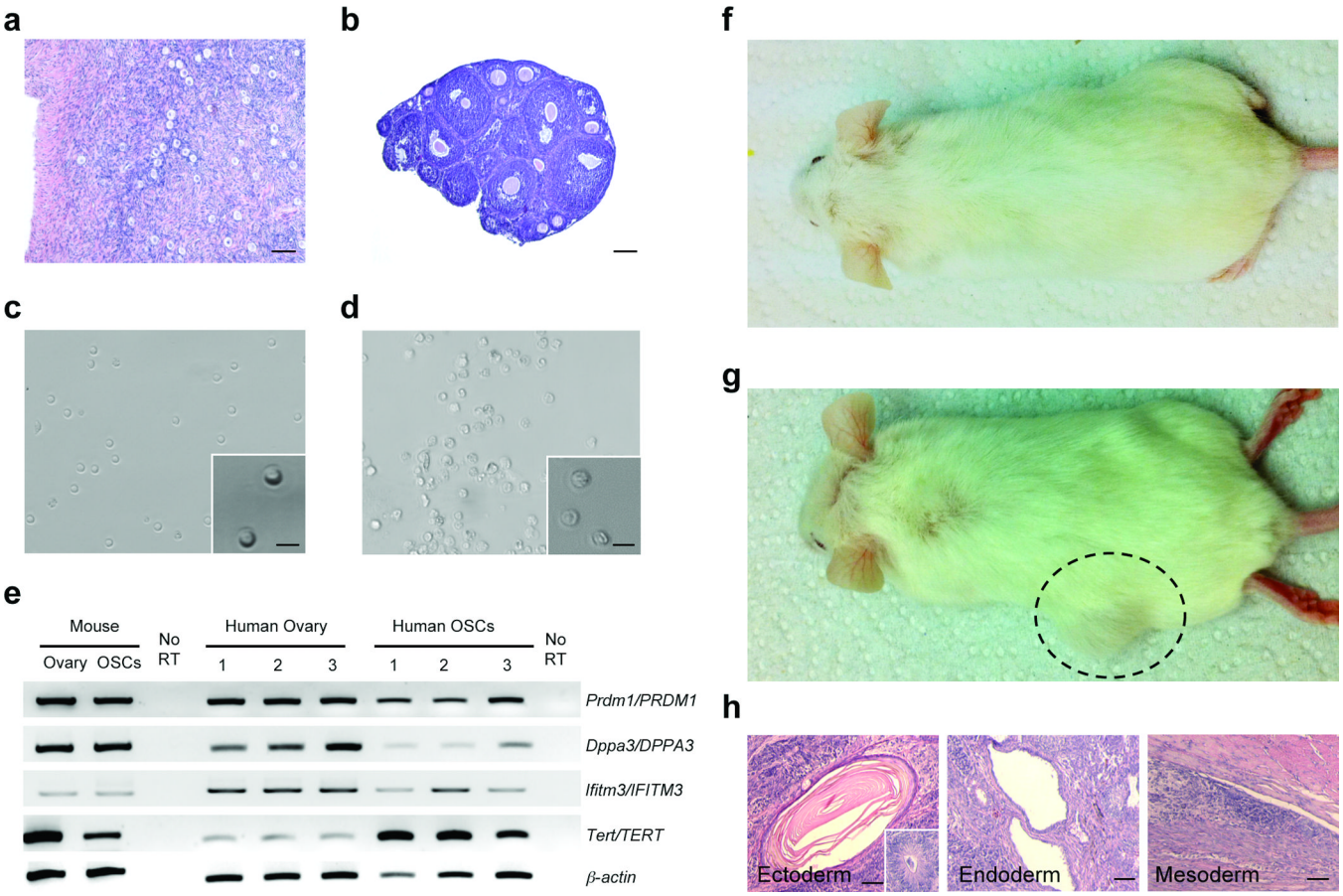

Fig. 2.

Isolation of OSCs from adult mouse and human ovaries. **a, b,** Representative histological appearance of adult ovarian tissue used for human (**a**) and mouse (**b**) OSC isolation. Scale bars, 100-μm. **c, d,** Morphology of viable cells isolated by FACS based on cell-surface expression of Ddx4/DDX4. Scale bars, 10-μm. **e,** Gene expression profile of starting ovarian material and freshly-isolated OSCs, showing assessment of three different subjects as examples for human ovarian tissue analysis (No RT: PCR of RNA sample without reverse transcription; *β-actin*, sample loading control). **f, g,** Teratoma formation assay showing an absence of tumors in NOD-SCID mice 24 weeks after receiving injections of freshly-isolated mouse OSCs (**f**) compared with development of tumors in mice 3 weeks after injection of an equivalent number of mouse ESCs (**g**; tumor highlighted by black-dashed oval). **h,** Examples of cells from all three germ layers in a representative ESC-derived teratoma are shown, with neural rosette highlighted in inset of left panel (scale bars, 100-μm).

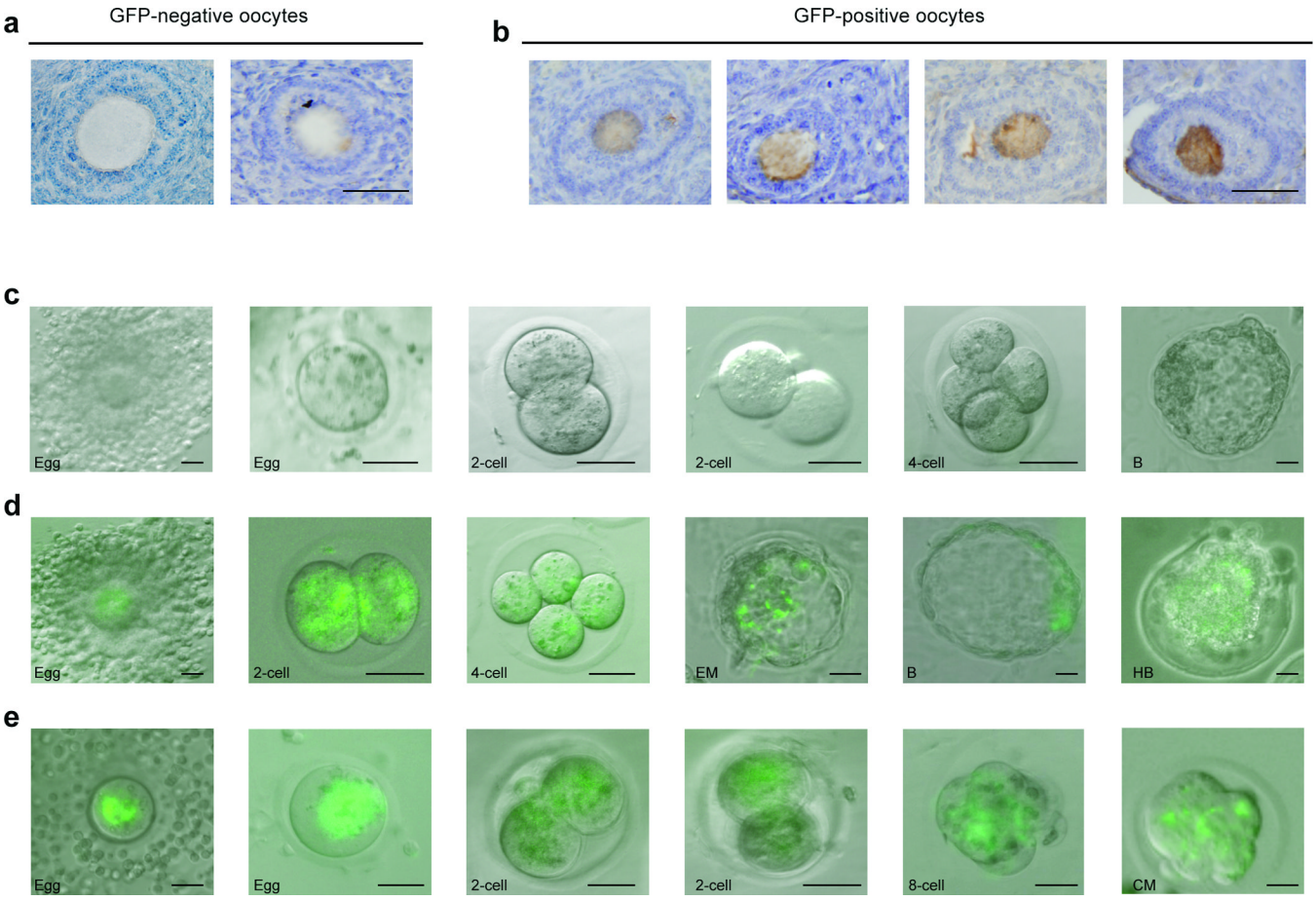

Fig. 3.

Mouse OSCs generate functional eggs after intraovarian transplantation. **a,** Examples of growing follicles containing GFP-negative and GFP-positive (brown; blue hematoxylin counterstain) oocytes in ovaries of wild-type mice injected with GFP-expressing OSCs 5–6 months earlier. **b,** Examples of ovulated GFP-negative eggs (in cumulus-oocyte complexes), and resultant embryos [2-cell, 4-cell, compact morula (CM) and early blastocyst (EB) stage embryos are shown as examples] generated by IVF, following induced ovulation of wild-type female mice that received intraovarian transplantation of GFP-expressing OSCs 5–6 months earlier. **c, d,** Examples of GFP-positive eggs (in cumulus-oocyte complexes) obtained from the oviducts following induced ovulation of wild-type female mice that received intraovarian transplantation of GFP-expressing OSCs 5–6 months earlier. These eggs were *in-vitro* fertilized using wild-type sperm, resulting in 2-cell embryos that progressed through preimplantation development [examples of GFP-positive embryos at the 2-cell, 4-cell, 8-cell, compacted morula (CM), expanded morula (EM), blastocyst (B) and hatching blastocyst (HB) stage are shown] to form hatching blastocysts 5–6 days after fertilization. Scale bars, 30-μm.

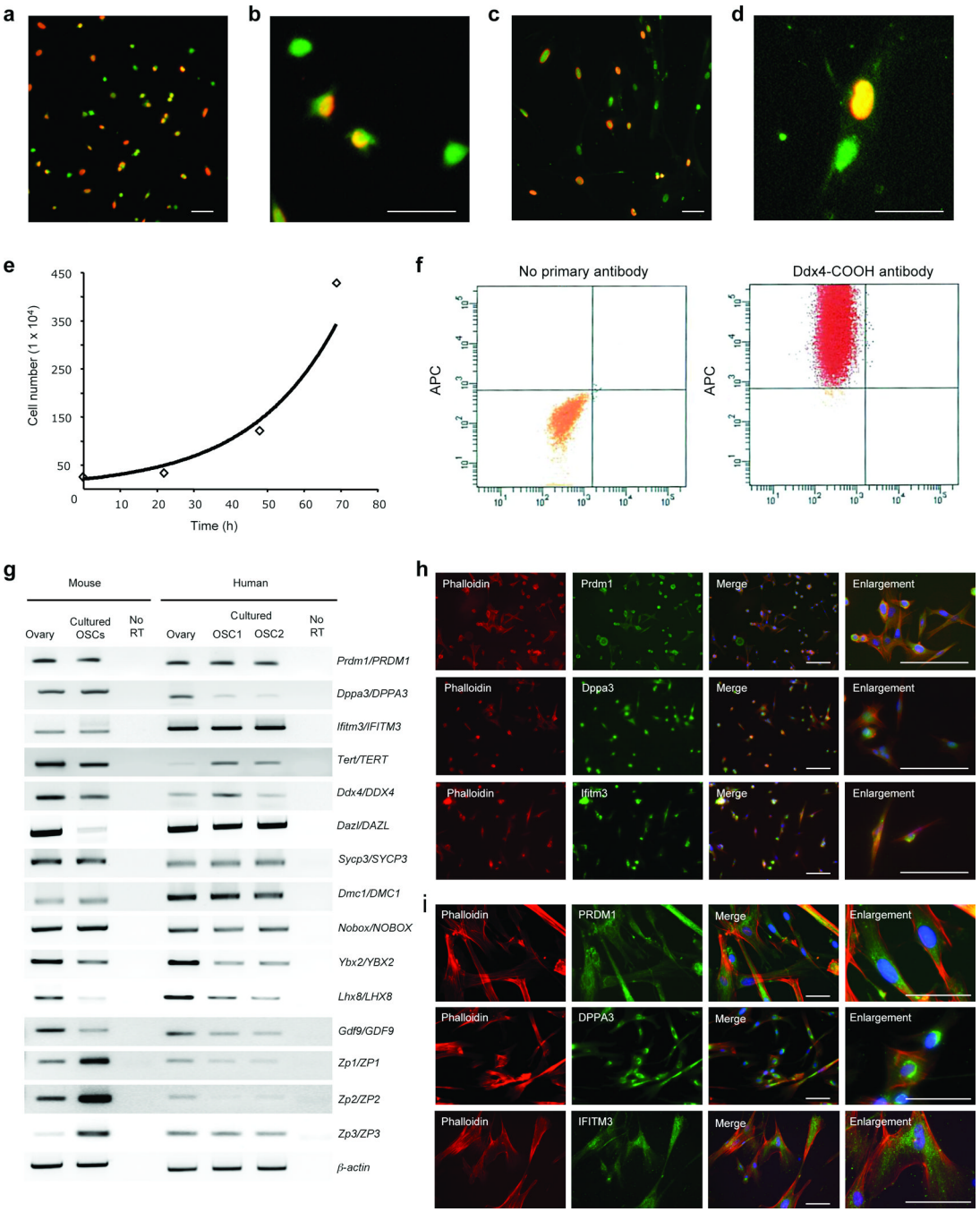

Fig. 4.

Evaluation of mouse and human ovary-derived OSCs in defined cultures. **a–d,** Assessment of OSC proliferation by dual detection of Ddx4/DDX4 expression (green) and BrdU incorporation (red) in mouse (**a, b**) and human (**c, d**) OSCs maintained in MEF-free cultures. Scale bars, 30-μm. **e,** Typical growth curve for MEF-free cultures of mouse OSCs after passage and seeding 2.5×10^4 cells in each well of 24-well culture plates. **f,** FACS analysis using the COOH antibody to detect cell-surface expression of Ddx4 in mouse OSCs after months of propagation (example shown, passage 45). **g,** Gene expression profile of starting ovarian material and cultured mouse and human OSCs after four or more months of

propagation *in vitro* (No RT, PCR of RNA sample without reverse transcription; *β-actin*, sample loading control). Two different human OSC lines (OSC1 and OSC2) established from ovaries of two different subjects are shown as examples. **h, i,** Immunofluorescence analysis of Prdm1/PDRM1, Dppa3/DPPA3 and Ifitm3/IFITM3 expression (green) in mouse (**h**) and human (**i**) OSCs in MEF-free cultures. Cells were counterstained with DAPI (blue) and rhodamine-phalloidin (red) to visualize nuclear DNA and cytoplasmic F-actin, respectively. Scale bars, 50-μm.

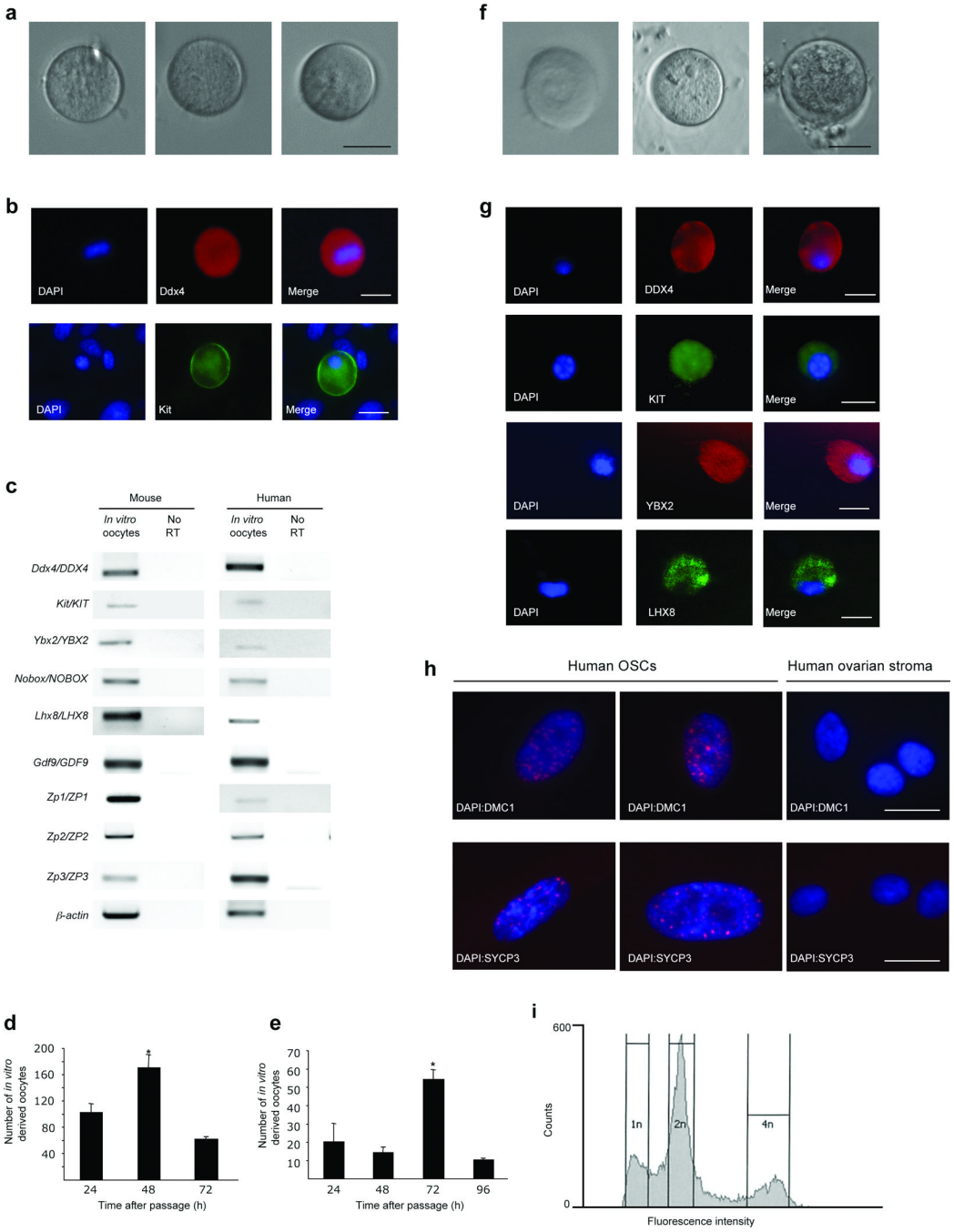

Fig. 5.

Spontaneous oocyte generation by cultured mouse and human OSCs. **a–c,** Oocytes formed by mouse OSCs in culture, as assessed by morphology (**a**), expression of oocyte marker proteins Ddx4 and Kit (**b**; note cytoplasmic localization of Ddx4 in oocytes), and presence of mRNAs encoding oocyte marker genes *Ddx4, Kit, Ybx2, Nobox, Lhx8, Gdf9, Zp1, Zp2* and *Zp3* (**c**; No RT: PCR of RNA sample without reverse transcription; *β-actin*, sample loading control). Scale bars, 25-μm. **d,** Number of oocytes formed by mouse OSCs after passage and seeding 2.5×10^4 cells in each culture well analyzed (values represent numbers generated over each 24-h block, not cumulative numbers; mean ± s.e.m., $n = 3$ independent

cultures; *, $P = 0.002$ versus other groups). **e–g,** *In-vitro* oogenesis from human OSCs, with examples of oocytes formed by human OSCs in culture (**f**, morphology; **g**, expression of oocyte marker proteins DDX4, KIT, YBX2 and LHX8) and numbers formed following passage and seeding of 2.5×10^4 cells in each culture well analyzed (**e**; mean ± s.e.m., $n = 3$ independent cultures; *, $P = 0.002$ versus other groups) shown. Expression of oocyte marker genes (*DDX4, KIT, YBX2, NOBOX, LHX8, GDF9, ZP1, ZP2, ZP3*) in human OSC-derived oocytes is shown in **c**, along with results for mouse OSC-derived oocytes. Scale bars, 25-μm. **h,** Immunofluorescence detection of the meiotic recombination markers, DMC1 and SYCP3 (red; blue DAPI counterstain), in nuclei of cultured human OSCs; human ovarian stromal cells served as a negative control. Scale bars, 15-μm. **i,** FACS-based ploidy analysis of cultured human OSCs 72 h after passage (see also Supplementary Fig. S4).

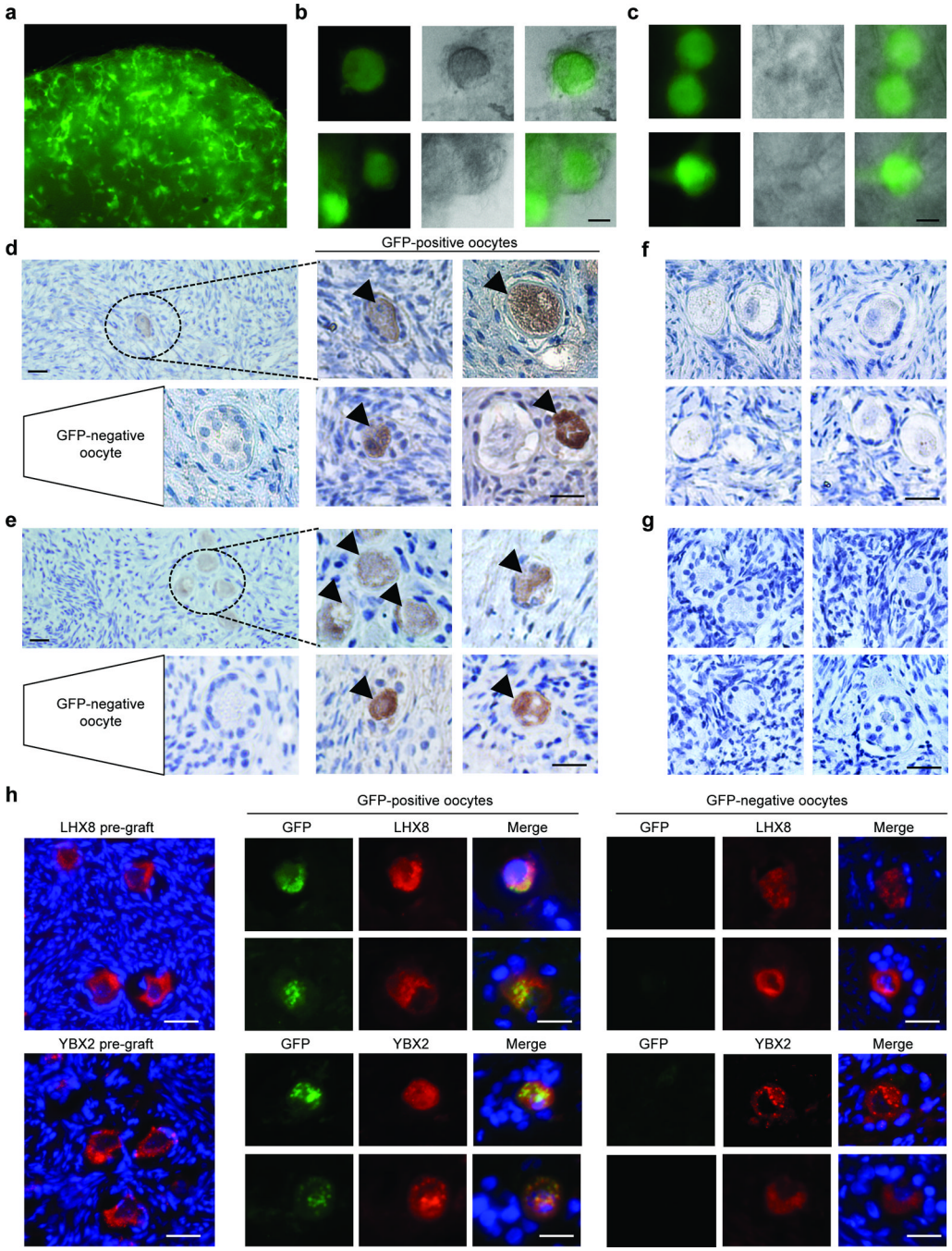

Fig. 6.

Human OSCs generate oocytes in human ovary tissue. **a–c,** Direct (live-cell) GFP fluorescence analysis of human ovarian cortical tissue following dispersion, re-aggregation with GFP-hOSCs (**a**) and *in-vitro* culture for 24–72 h (**b, c**), showing formation of large single GFP-positive cells surrounded by smaller GFP-negative cells in compact structures resembling follicles (**b, c**; scale bars, 50-μm). **d–g,** Immature follicles containing GFP-positive oocytes (brown, highlighted by black arrowheads, against a blue hematoxylin counterstain) in adult human ovarian cortical tissue injected with GFP-hOSCs and xenografted into NOD-SCID female mice (**d**, 1 week post-transplant; **f**, 2 weeks post-

transplant). Note comparable follicles with GFP-negative oocytes in the same grafts. As negative controls, all immature follicles in human ovarian cortical tissue prior to GFP-hOSC injection and xenografting (**e**) or that received vehicle injection (no GFP-hOSCs) prior to xenografting (**g**) contained GFP-negative oocytes after processing for GFP detection in parallel with the samples shown. Scale bars, 25-μm. **h,** Dual immunofluorescence analysis of GFP expression (green) and either the diplotene stage oocyte-specific marker YBX2 (red) or the oocyte transcription factor LHX8 (red) in xenografts receiving GFP-hOSC injections. Note that GFP was not detected in grafts prior to GFP-hOSC injection, whereas YBX2 and LHX8 were detected in all oocytes. Sections were counterstained with DAPI (blue) for visualization of nuclei. Scale bars, 25-μm.