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Comparative gene expression profiling of adult mouse ovary-derived oogonial stem cells supports a distinct cellular identity

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Abstract

Objective—Perform gene expression profiling of adult mouse ovary-derived oogonial stem cells (OSCs).

Design—Experimental animal study.

Setting—Research laboratory.

Animal(s)—Adult C57BL/6 female mice.

Intervention(s)—None.

Main outcome measure(s)—Gene expression profiles were compared between freshly isolated and cultured OSCs, as well as between OSCs and embryonic stem cells (ESCs), fetal primordial germ cells (PGCs) and spermatogonial stem cells (SSCs); OSC yield from ovaries versus meiotic gene activation during the estrous cycle was determined.

Result(s)—Freshly isolated OSCs, PGCs and SSCs exhibited distinct gene expression profiles. Cultured OSCs maintained their germline gene expression pattern, but gained expression of pluripotency markers found in PGCs and ESCs. Cultured OSCs also expressed the meiotic marker, *stimulated by retinoic acid gene 8 (Stra8)*. In vivo, OSC yield was higher from luteal versus follicular phase ovaries and this was inversely related to *Stra8* expression.

Conclusion(s)—Freshly isolated OSCs exhibit a germline gene expression profile that overlaps with, but is distinct from, that of PGCs and SSCs. After in vitro expansion, OSCs activate

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expression of pluripotency genes found in freshly isolated PGCs. In vivo, OSC numbers in the ovaries fluctuate during the estrous cycle, with the highest numbers noted during the luteal phase. This is followed by activation of *Stra8* expression during the follicular phase, which may signify a wave of neo-oogenesis to partially offset follicular loss through atresia and ovulation in the prior cycle.

Keywords

oogenesis; oocyte; germ cell; oogonial stem cell; *Stra8*

INTRODUCTION

Since the initial publication in 2004 challenging the decades-old belief that the ovaries of mammals lose replicative germ cells capable of generating new oocytes after birth (1), experimental evidence from many laboratories collectively supports a new paradigm in reproductive biology that accounts for the existence of female germline or oogonial stem cells (OSCs) in postnatal ovaries (reviewed in 2–6). In addition to the successful purification of OSCs by at least three laboratories using different techniques (7–10), transplantation studies analogous to those used for establishing the identity of spermatogonial stem cells (SSCs) in the testes of adult males have demonstrated that OSCs generate competent oocytes in adult ovaries that fertilize to produce viable embryos and offspring (7, 10–12). In addition, methods for the establishment and stable propagation of mouse and human OSCs in culture are now available (7–10, 13). These studies have shown that OSCs undergo spontaneous differentiation in vitro to produce what appear to be oocytes based on morphological criteria, gene expression profiling, meiotic activation and the ability to attract granulosa cells for folliculogenesis (8, 10, 13).

Central to this work is the development and validation of methods to purify OSCs from adult ovarian tissue (13). In 2009, Wu and colleagues reported an immunomagnetic sorting strategy for the isolation of mouse OSCs from dissociated ovarian tissue, relying on an externalized epitope of the germ cell protein, DEAD box polypeptide 4 (Ddx4; also commonly referred to as mouse vasa homolog or Mvh) (14, 15), which had not been identified previously (7, 16, 17). However, the magnetic sorting approach had limitations, as the OSC-containing cell fraction obtained was not homogeneous, and therefore analysis of freshly isolated OSCs could not be performed. This, combined with the relatively long culture period between isolation of the cells and their functional testing by transplantation strategies (7), prompted questions as to whether the cells isolated were truly innately germline at collection or had undergone some type of in vitro transformation event to a stem-like cell prior to transplantation.

To address this, we developed and reported a fluorescence-activated cell sorting (FACS)-based strategy, utilizing an antibody that targets the externalized epitope of Ddx4, for the purification of OSCs free of contaminating oocytes and other cells from ovaries of adult mice (10, 13). Importantly, the same technology was successfully applied to the isolation of OSCs from ovarian cortical tissue of reproductive age women (10, 13). A direct comparison of the immunomagnetic bead strategy reported by Wu and colleagues (7) and our FACS-

based approach (10) revealed that, while magnetic sorting results in a mixed population of cells including OSCs and oocytes, comparable fractions obtained by FACS are free of such contamination and can be used for characterization of freshly isolated OSCs. In this regard, OSCs analyzed immediately after FACS-based collection have a gene expression profile fully consistent with primitive germ cells during early specification, and are not the result of some random in-vitro transformation event as erroneously suggested by others (18). Along with the ability to isolate a pure population of OSCs from dissociated ovarian tissue, the use of FACS provides a quantitative assessment of OSC yield. In young adult mouse ovaries, the cells constitute $0.014\% \pm 0.002\%$ of the total ovarian cell population, and approximately $1.5\% \pm 0.2\%$ of the viable cell fraction sorted after dissociation (10).

We have also reported that ex-vivo expanded OSCs maintain a primitive germline gene expression profile that is uniform across essentially all cells in culture (10). In males, SSCs are unipotent, having the capability to self-renew and differentiate into spermatozoa (19). Following ex vivo culture, however, isolated SSCs acquire an embryonic stem cell (ESC)-like gene expression profile (20, 21). Whether OSCs propagated in culture possess this same capacity is not known. Accordingly, using our FACS-based methodology for OSC isolation (10, 13), herein we compared gene expression profiles between freshly isolated and cultured mouse OSCs, and between OSCs and other germline and pluripotent stem cell types. We also determined the relationship between OSC numbers and meiotic gene activation in ovaries in vivo during the estrous cycle, based on our observations from the profiling studies that OSCs cultured in vitro activate expression of the meiotic commitment gene, *stimulated by retinoic acid gene 8 (Stra8)* (22, 23), concomitant with oogenesis.

MATERIALS AND METHODS

Animals

Wild type C57BL/6 mice were obtained from Charles River Laboratories. To generate transgenic mice with *Stra8* promoter-driven GFP expression, a 1.4-kb fragment of the mouse *Stra8* promoter (−1400 to +11), previously shown to convey premeiotic germ cell-specific expression (24, 25), was amplified from mouse genomic DNA and cloned into the *XhoI/EcoRI* site of the *pEgfp-1* vector (BD Biosciences). The *pStra8-Gfp* vector was then sent to Genoway to generate *pStra8-Gfp* transgenic mice using their proprietary Quick Knock-in™ technology involving insertion of the transgene sequence into the neutral *Hprt* locus (www.genoway.com). This approach eliminates potential confounding effects of random integration of transgenes and variability in copy number commonly associated with conventional pronuclear injection. Mouse lines with germline transmission of the transgene were identified at Genoway, shipped to us and used to establish breeding colonies. All procedures were reviewed and approved by the institutional animal care and use committee of Massachusetts General Hospital.

Cell lines

Mouse ESCs (v6.5) were obtained from Novus Biologicals, expanded as undifferentiated cells in culture under standard conditions (26) and used at passage number 29.

Isolation of OSCs

Adult mouse ovary-derived OSCs were purified by FACS based on cell-surface expression of the C-terminus of Ddx4, and, for some experiments, established as actively dividing germ cell cultures without somatic feeder cells (10, 13). For studies of ex-vivo expanded OSCs, cells at passage number 23 were used.

Isolation of primordial germ cells (PGCs)

For each experimental replicate, 6–12 genital ridges were isolated from embryos at 11.5 days post-coitum (dpc), dissociated and processed by magnetic assisted cell sorting (MiniMACS; Miltenyi Biotec) using a mouse monoclonal antibody to stage specific embryonic antigen-1 (SSEA1; Abcam, ab16285) to isolate PGCs (27). The SSEA1-positive cell fraction enriched for PGCs was snap-frozen for gene expression analysis.

Isolation of SSCs

Spermatogonial stem cells were isolated from dissociated testes of 6-week-old male mice (28), and snap-frozen for gene expression analysis.

Gene expression analysis

Total RNA was extracted using the RNeasy Plus Micro or Mini kit (QIAGEN) and reverse-transcribed using the SuperScript VILO cDNA Synthesis Kit (Invitrogen), according to protocols supplied by the manufacturers. The absence or presence of each mRNA in each sample was assessed by conventional PCR using Platinum *Taq* polymerase (Invitrogen) and primer pairs specific for amplification of each gene (Supplementary Table 1). All products were sequenced to confirm identity. For quantitative analysis of *Stra8* mRNA levels, real-time PCR was performed using a Cepheid Smart Cycler II and primers specific for *Stra8* (FAM-labeled D-LUX™ Pre-designed Gene Expression Assays, MLUX3312362; Invitrogen), with levels normalized to β -actin mRNA levels (FAM-labeled certified LUX™ Primer Set for Mouse/Rat β -actin, 101M-01; Invitrogen) in each sample. Expression ratios were then calculated (29).

Immunofluorescence

Ovaries, testes and eviscerated e13.5 fetuses of *pStra8-Gfp* mice were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned for analysis using antibodies against *Stra8* (rabbit polyclonal ab49602, Abcam), *Ddx4* (rabbit polyclonal ab13840, Abcam) or GFP (mouse monoclonal sc-9996, Santa Cruz Biotechnology). For immunofluorescence, detection was performed using donkey anti-rabbit Alexa Fluor 546 or donkey anti-mouse Alexa Fluor 488 (Molecular Probes) as secondary antibody. Images were captured using a Nikon E800/BioRad Radiance 2000 confocal microscope or a Nikon ECLIPSE TE2000-S microscope.

Estrous cycle staging

Six-week-old female mice were housed in a controlled environment with food and water ad libitum. Daily vaginal smear cytology was used to monitor estrous cyclicity for each animal for 3–4 weeks prior to ovary collection at the specified stages (30). For each experimental

replicate, 2–4 ovaries per stage were prepared for OSC isolation as described above, and OSC yield per dissociated ovary was quantified using FlowJo software (version 9; Tree Star).

Data presentation and statistical analysis

All experiments were independently replicated a minimum of 3 times. Combined data from the replicates, expressed as the mean \pm standard error of the mean (SEM), were analyzed by a one-way analysis of variance followed by Student's *t*-test or Bonferroni post-hoc analysis using the Statistical Package for Social Sciences (SPSS, version 19) to identify significant differences ($P < 0.05$). Where appropriate, qualitative images representative of outcomes obtained in the replicate experiments are provided.

RESULTS

Gene expression profile comparisons

Expression of the germline markers *PR domain zinc finger protein 1* (*Prdm1*; also commonly referred to as *Blimp1*), *developmental pluripotency-associated protein 3* (*Dppa3*; also commonly referred to as *Stella*), *interferon-induced transmembrane protein 3* (*Ifitm3*; also commonly referred to as *Fragilis*), *Ddx4* and *deleted in azoospermia-like* (*Dazl*) were detected in freshly isolated OSCs, cultured OSCs, ESCs, PGCs and SSCs (Fig. 1A). Notably, of the cell types examined, only ESCs and cultured OSCs expressed detectable levels of *Stra8* mRNA (Fig. 1A). The pluripotency-associated transcripts *POU domain class 5 transcription factor 1* (*Pou5f1*; also known as *octamer binding transcription factor 4* or *Oct4*), *zinc finger protein 296* (*Zfp296*), dosage-sensitive sex reversal nuclear receptor subfamily 0 group B member 1 (*Nr0b1*; also commonly referred to as *Dax1*), undifferentiated embryonic cell transcription factor 1 (*Utf1*), *Nanog* and *SRY-box containing gene 2* (*Sox2*) were detectable in ESCs and PGCs, whereas SSCs expressed only *Pou5f1*, *Zfp296*, *Nr0b1* and *Utf1* (Fig. 1B). In striking comparison, of all the pluripotency-associated genes examined, only *Pou5f1* was detectable in freshly isolated OSCs; however, ex vivo culture of OSCs resulted in activation of all pluripotency-associated genes with the exception of *Nr0b1* (Fig. 1B). As a negative control, adult tail-snip fibroblasts did not show expression of any of the germline or pluripotency-associated genes tested (Fig. 1). A summary of the gene expression analyses, highlighting key comparative differences between the cell populations studied, is presented in Supplementary Table 2.

Meiotic gene activation

Expression of the meiotic commitment gene, *Stra8*, was detected only in cultured ESCs and cultured OSCs, which is in keeping with past work showing that both of these cell types can spontaneously generate oocytes in vitro (8, 10, 31). In turn, the absence of *Stra8* in freshly isolated OSCs attests to the undifferentiated (pre-meiotic) status of these germ cells prior to ex vivo expansion. To determine if the presence of externalized Ddx4 protein (extracellular-exposed Ddx4 or ecDdx4), which provides the means by which FACS-based isolation of OSCs is accomplished (10, 13), marks only undifferentiated OSCs, we analyzed expression of *Stra8* in the ecDdx4-positive and ecDdx4-negative cell fractions obtained from dissociated adult ovaries following FACS. Consistent with results shown earlier (Fig. 1A),

ecDdx4-positive cells (OSCs) did not express *Stra8* mRNA; however, *Stra8* mRNA was detected in the ecDdx4-negative cell fraction (Fig. 2A), which is composed of all other cells including differentiating germ cells as well as small oocytes that express and retain Ddx4 protein entirely in the cytoplasm (10).

Analysis of ecDdx4 in ovaries of *pStra8-Gfp* mice

To more clearly define the relationship between externalized Ddx4 expression and meiotic gene activation, we generated transgenic mice with GFP expression under control of the *Stra8* promoter (Fig. 2B). Consistent with the previously reported specificity of this promoter fragment for activation only in germ cells (24, 25), transgene expression in adult *pStra8-Gfp* mice was restricted to the gonads (Fig. 2B). Moreover, direct fluorescence imaging showed sex- and tissue-specific transgene expression during embryonic development paralleling that of the endogenous *Stra8* gene. Specifically, GFP was detected in female gonads at embryonic day 13.5 (e13.5) in an anterior to posterior pattern (Fig. 2C), mirroring that reported for endogenous *Stra8* expression associated with female germ cell meiotic commitment at this developmental stage (32). In contrast, GFP was not observed in any other organs of female embryos or in any tissues of male embryos including the testes (Fig. 2C). Dual immunofluorescence staining showed that most GFP co-localized with endogenous *Stra8* in e13.5 female gonads (Fig. 2D). A small number of cells were positive for only GFP or *Stra8* (Fig. 2D), which may be due to differential kinetics in transcription or protein translation from these two genes. As expected, an absence of *Stra8* promoter-driven GFP expression in embryonic testes (Fig. 2C) paralleled an absence of endogenous *Stra8* protein (Fig. 2D). However, GFP in male germ cells became detectable concomitant with initiation of meiosis in juvenile testes (Fig. 2E).

We also identified rare cells positive for *Stra8* promoter-driven GFP expression in ovaries of adult transgenic females (Fig. 2E). Dual immunofluorescence analysis indicated that these cells co-expressed the germ cell marker Ddx4, whereas adjacent oocytes contained within follicles of *pStra8-Gfp* mouse ovaries also expressed Ddx4 but lacked GFP (Fig. 2E). The absence of GFP in oocytes of this transgenic line is consistent with the absence of *Stra8* in mouse oocytes (33). Like endogenous *Stra8*-expressing cells in adult mouse ovaries (33, 34), these GFP-positive cells were extremely rare and located near the ovarian surface epithelium. Using FACS, we determined that the majority of cells positive for *Stra8* promoter-driven GFP expression were negative for externalized expression of Ddx4 (ecDdx4⁻; Fig. 2F); however, a small percentage of cells with externalized Ddx4 (ecDdx4⁺) were identified as also being positive for *Stra8* promoter-driven GFP expression (Fig. 2F).

OSC yield versus *Stra8* activation during the estrous cycle

Our observations that OSC differentiation (meiotic gene activation, viz. *Stra8* expression) is coupled to a loss of externalized Ddx4 protein (Fig. 2) prompted us to explore the physiological relationship between these events more thoroughly in vivo. Based on past studies, which have reported that the number of oocyte-containing primordial follicles in adult mouse ovaries fluctuates during the estrous cycle (30, 35), we next assessed OSC numbers versus *Stra8* activation in ovaries at different stage of the female reproductive cycle. Analysis of the ecDdx4-negative cell fraction by quantitative PCR throughout the

estrous cycle identified the highest level of *Stra8* expression during estrus, just prior to transition of the females from the follicular phase to the luteal phase of the ovarian cycle (Fig. 3). By comparison, the number of OSCs retrieved by FACS increased as females transitioned to the luteal phase (Fig. 4). Although no significant differences in OSC yield were detected after comparison of each of the four stages of the estrous cycle independently (Fig. 4A), stratification of the data into a comparison of the follicular (proestrus and estrus) versus luteal (metestrus and diestrus) phase revealed the presence of significantly more OSCs in luteal phase ovaries (Fig. 4B).

DISCUSSION

To our knowledge, comparative gene expression profiles between OSCs and other germ cell and stem cell lineages have not been previously reported, despite the fact that some scientists have questioned, without any supporting data, the identity of OSCs as being nothing more than misplaced or ‘laggard’ oogonia (viz. embryonic female PGCs) with no true biological function (36). As shown herein, the numerous and marked differences in gene expression patterns between embryonic PGCs and postnatal ovary-derived OSCs analyzed immediately after isolation provide strong evidence countering such a claim. In fact, in addition to confirming prior observations that freshly isolated OSCs express a primitive germline gene expression profile (7–10), our inclusion of several pluripotency markers in the present study actually demonstrates that PGCs and ESCs exhibit a much greater overlap in gene expression profiles than that of PGCs and OSCs. The most obvious difference is the absence, in OSCs, of the numerous ESC-like pluripotency genes expressed in PGCs. Interestingly, while freshly isolated OSCs do not innately express genes commonly associated with pluripotency, ex vivo expansion of the cells leads to acquisition of most of these factors such that cultured OSCs acquire a closer resemblance to PGCs in terms of gene expression patterns. It is important to point out that our experiments used OSCs at passage 23, which represents approximately 5–6 months of continuous culture. Future studies involving a passage-by-passage analysis of gene expression patterns in cultured OSCs, starting with their initial establishment in vitro (first passage at approximately 2 weeks after plating), will be of interest to determine exactly how much time is required for the gene expression profile of cultured OSCs to change to one more closely resembling that of PGCs. Irrespective, this observation aligns well with that reported for SSCs, which are unipotent in vivo (19) but acquire a multipotent ESC-like cell phenotype following extended culture (20, 21). These latter observations have generated interest in the potential use of SSCs as an alternative to ESCs for patient-specific stem cell therapies (37). Although more studies are needed to determine if cultured OSCs gain multipotency, the finding that OSCs activate genes associated with pluripotency following extended culture at least opens similar possibilities to those considered for multipotent adult germline stem cells derived from cultured SSCs.

Another important observation reported herein is the clear separation of two key processes in OSCs, namely externalized expression of *Ddx4* and meiotic commitment. As a point of note to avoid any confusion, we feel it is critical to refer to the viably sorted ovarian cell fractions with and without externalized *Ddx4* protein as ‘ec*Ddx4*-positive’ (extracellular *Ddx4* epitope exposed) and ‘ec*Ddx4*-negative’ (extracellular *Ddx4* epitope not exposed),

respectively. Since these cell fractions are sorted based on the immunological presence or absence of the C-terminus of Ddx4 protein on the cell surface, use of the more generic terms, Ddx4-positive and Ddx4-negative, may give the false impression that the latter fraction of cells is defined by the absence of *Ddx4* gene expression. This is not the case, a point exemplified most clearly by the fact that oocytes, which contain *Ddx4* mRNA as well as Ddx4 protein localized exclusively to the cytoplasm, do not exhibit cell surface expression of Ddx4 (10). Thus, small oocytes would be sorted into the ecDdx4-negative fraction using our FACS protocol (13).

It is currently unclear why OSCs, and not oocytes, exhibit cell surface expression of the C-terminus of Ddx4. One possibility we are now testing is that this difference in protein localization serves to sequester, and thereby silence, the RNA helicase function of Ddx4 in undifferentiated OSCs through membrane insertion of protein at the helicase domain. Once OSCs commit to an oocyte fate, the protein is completely drawn back into the cytoplasm to permit activation of RNA helicase function as a key step in germ cell differentiation. Our finding that mRNA for *Stra8*, which is one of the earliest known markers of germ cells exiting the mitotic cycle and entering the meiotic cycle (22, 23, 32), is present in germ cells that no longer exhibit externalized expression of Ddx4 [viz. *Stra8* mRNA found in only the ecDdx4-negative cell fraction; which remains positive for *Ddx4* mRNA (10) and thus still contains germ cells] is consistent with such a model. In turn, the absence of *Stra8* mRNA in freshly isolated OSCs, but its detection in pure cultures of OSCs that spontaneously generate oocytes in vitro (8, 10), supports that *Stra8*-expressing germ cells detected in the ecDdx4-negative ovarian cell fraction likely arise from OSCs undergoing differentiation in vivo. Our FACS-based analysis of ovaries of *pStra8-Gfp* transgenic mice extends this conclusion by revealing that transcriptional activation of the *Stra8* promoter (GFP expression) can be detected in ecDdx4-negative cells as well as in a very small population of cells that still exhibit externalized expression of Ddx4 protein. Since our ability to identify *Stra8* promoter activation probably precedes the point at which endogenous *Stra8* mRNA would accumulate to levels sufficiently high enough for PCR-based detection in differentiating germ cells, we believe the cells positive for both *Stra8* promoter-driven GFP expression and externalized Ddx4 protein represent a rare and transient population of OSC progenitors at the earliest stages of meiotic commitment just prior to complete cytoplasmic internalization of Ddx4.

Outcomes from our additional in vivo experiments agree with this model as well, in that we detected an inverse relationship between OSC numbers and meiotic gene activation in adult mouse ovaries during the estrous cycle. The highest yield of OSCs was obtained from luteal phase ovaries while *Stra8* expression peaked during estrus of the follicular phase. If the increase in OSC yield is indicative of proliferation, this finding may indicate that OSCs commit to a wave of mitosis during the luteal phase as a response to follicle loss from ovulation and atresia during the prior cycle. In turn, more OSCs would be available to activate *Stra8* and commit to meiosis during the next follicular phase as a means to at least partially rebuild the ovarian reserve. This sequence of events would help explain prior observations from two different labs that primordial follicle numbers in ovaries of young adult mice also fluctuate during the reproductive cycle, with the highest numbers detected during metestrus into diestrus (30, 35). Although additional work is needed to map the exact

relationship between OSC activity and oocyte numbers in vivo, the data presented add to a growing body of evidence that supports not only the presence of OSCs in postnatal mammalian ovaries but also the clear parallels and differences that exist in the genes and pathways that regulate the function of these cells compared to PGCs and SSCs. Studies aimed at identifying the hormones that control self-renewal versus meiotic activation in OSCs (38), as well as defining possible multipotential properties of OSCs after ex vivo expansion, are ongoing and are expected to reveal additional important features of these cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Capsule

Oogonial stem cells exhibited a gene expression profile partially overlapping with, but distinct from, other stem cells and germ cells, and showed altered numbers during the reproductive cycle inversely related to meiotic commitment.

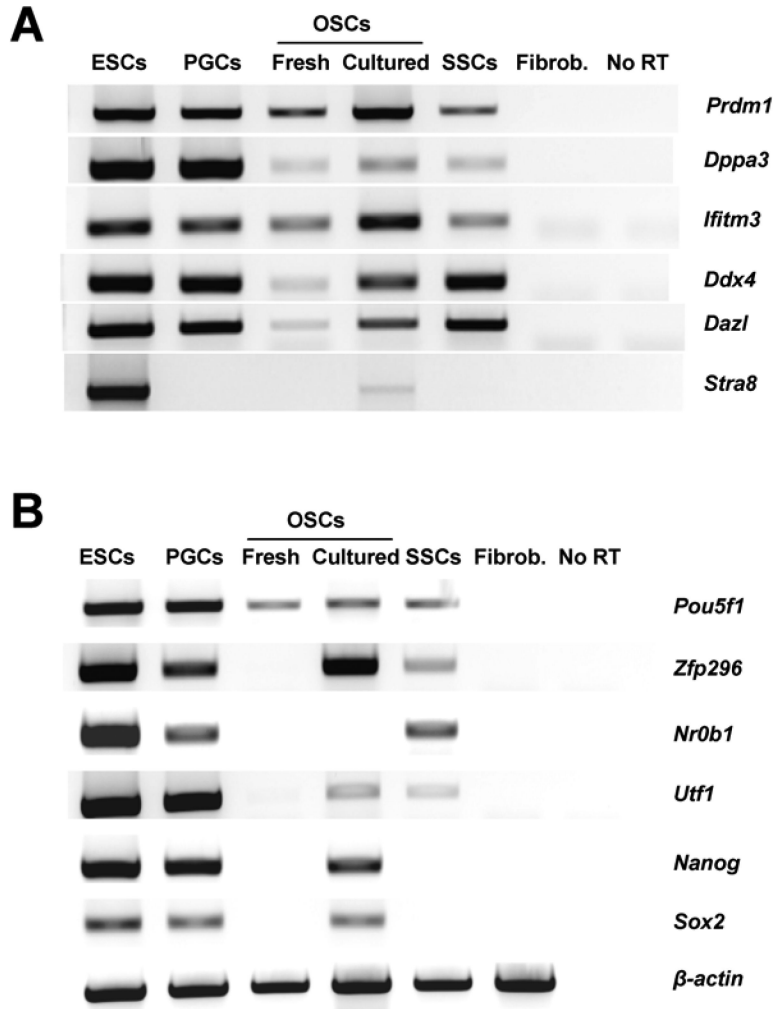


Figure 1. Comparative gene expression profiling analysis of germ cell and stem cell markers. (A, B) Representative expression pattern of genes associated with germline development and meiotic commitment (A) or pluripotency (B) in mouse ESCs, embryonic PGCs, adult ovary-derived OSCs (freshly isolated and cultured) and juvenile testis-derived SSCs. Adult tail-snip fibroblasts (Fibrob.) were used as a negative control for specificity of pluripotency and germline gene expression, whereas *β-actin* mRNA was used as a sample loading control (no RT, PCR analysis of RNA sample without reverse transcription used as a negative control for excluding genomic DNA contamination).

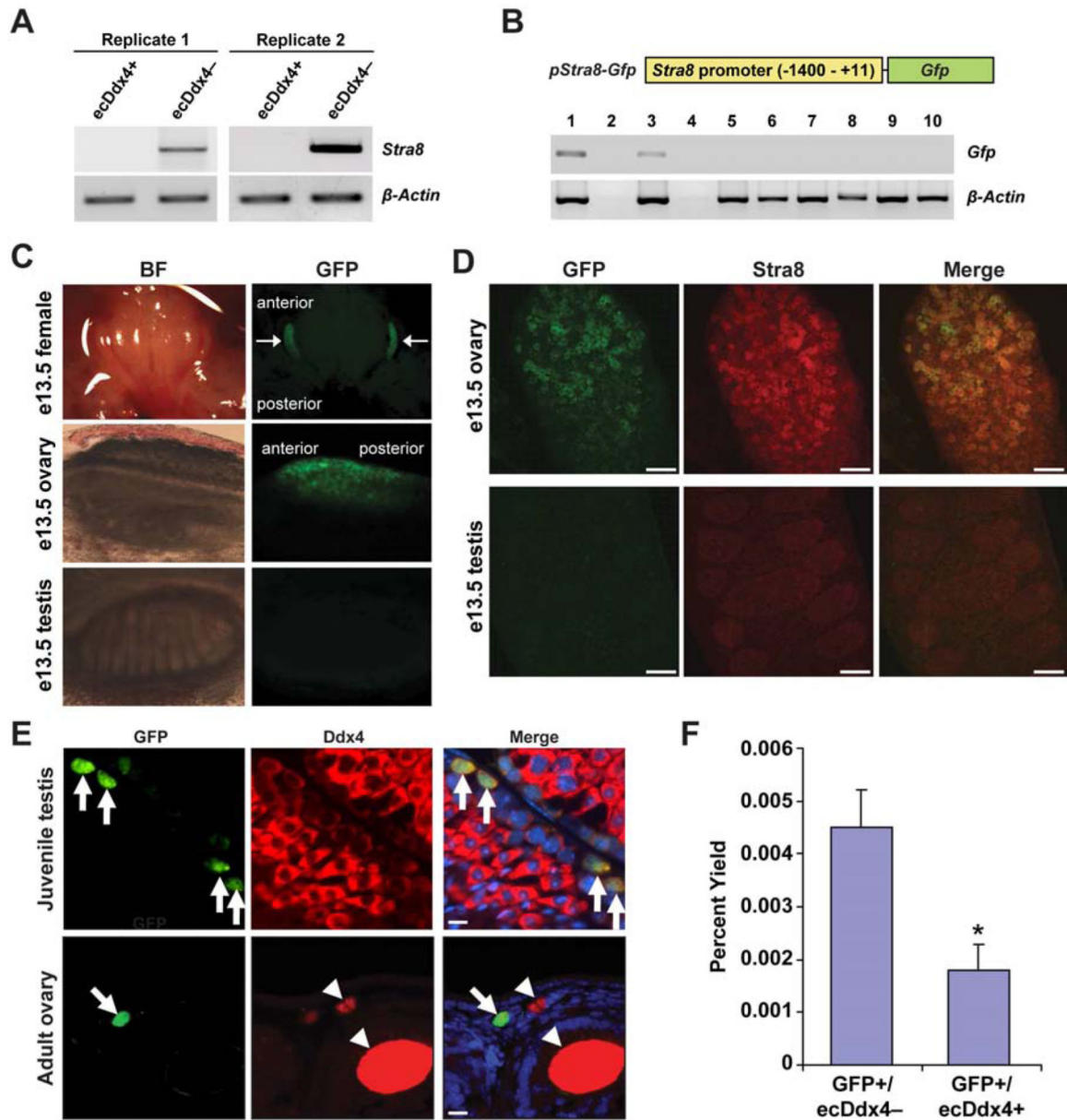


Figure 2. Relationship between externalized Ddx4 protein and meiotic gene activation in OSCs. (A) PCR analysis of *Stra8* expression in FACS-purified ovarian cell fractions with externalized Ddx4 protein (ecDdx4-positive or ecDdx4+) or without externalized Ddx4 protein (ecDdx4-negative or ecDdx4-). β -Actin, control gene for sample loading. (B) Schematic of the *Stra8* promoter (*pStra8*)-*Gfp* construct, and assessment of *Gfp* and β -actin mRNA levels in tissues from adult *pStra8-Gfp* transgenic mice: 1, testis; 2, PCR without reverse transcription of testicular RNA; 3, ovary; 4, PCR without reverse transcription of ovarian RNA; 5, heart; 6, kidney; 7, liver; 8, lung; 9, spleen; 10, brain. (C) Brightfield (BF, left panels) and direct fluorescence (GFP, right panels) imaging of female embryos (arrows highlight restricted GFP fluorescence in ovaries), female gonads and male gonads from *pStra8-Gfp* mice at embryonic day 13.5 (e13.5). (D) Dual immunofluorescence analysis of GFP and *Stra8*

ovaries and testes of *pStra8-Gfp* mice at e13.5, showing the presence of both proteins only in fetal ovaries. Scale bars, 10 μ m. (E) Dual immunofluorescence analysis of GFP (green) and Ddx4 (red) in juvenile testes of *pStra8-Gfp* mice after resumption of meiosis (upper panel; arrows indicate GFP-positive germ cells in a randomly selected tubule of a longitudinally sectioned testis) or in ovaries of 2-month-old (adult) *pStra8-Gfp* mice (lower panel, longitudinally sectioned ovary; arrow indicates a cell positive for both GFP and Ddx4, adjacent to GFP-negative/Ddx4-positive oocytes contained in follicles and highlighted by arrowheads). Merge also shows DAPI-counterstained nuclei (blue); scale bars, 10 μ m. (F) Yield of GFP-expressing cells (as a percent of total viable cells sorted) lacking externalized Ddx4 protein (GFP+/ecDdx4-), and of cells positive for both GFP and externalized Ddx4 (GFP+/ecDdx4+), from ovaries of adult *pStra8-Gfp* mice (mean \pm SEM, $n = 3$; *, $P < 0.05$).

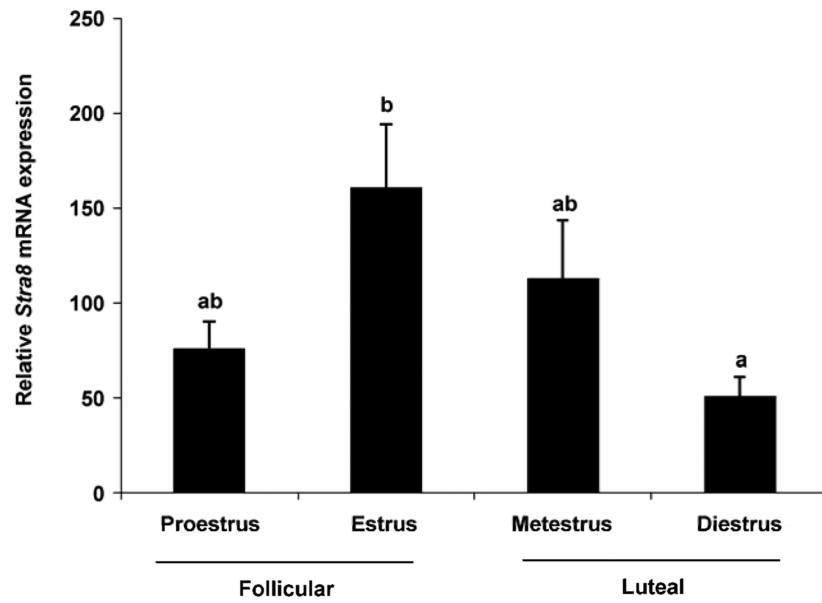


Figure 3. Ovarian *Stra8* expression patterns during the female reproductive cycle. Quantitative PCR analysis of *Stra8* mRNA levels (normalized to β -actin mRNA levels) in adult mouse ovaries at each indicated stage of the estrous cycle (mean \pm SEM, $n = 3$; different letters, $P < 0.05$).

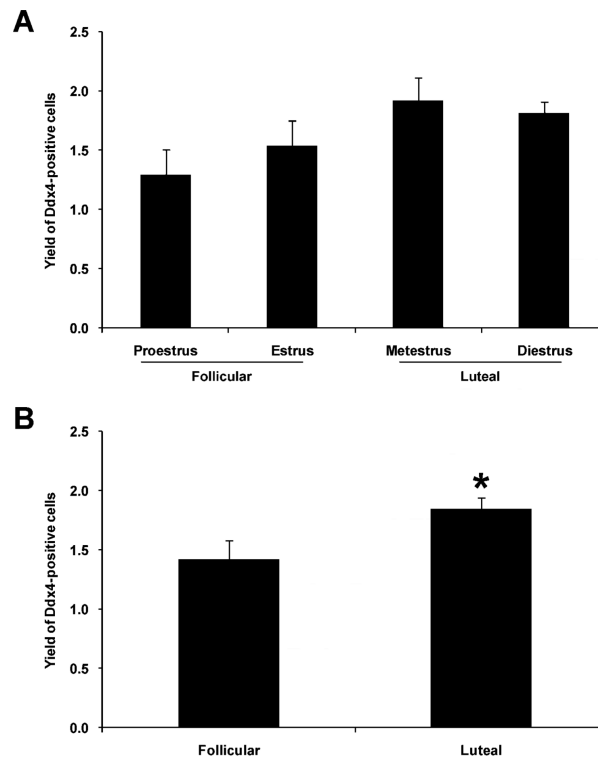


Figure 4.

Changes in OSC numbers in mouse ovaries relative to stage of the female reproductive cycle. (A, B) Yield of OSCs, as a percent of total viable cells sorted, from ovaries of adult female mice at each indicated stage of the estrous cycle (A) or following stratification of estrous cycle stages into the follicular and luteal phases of the ovarian cycle (B). The data shown represent the mean \pm SEM ($n = 3$; *, $P < 0.05$).