Workflow optimization for identification of female germline or oogonial stem cells in human ovarian cortex using single-cell RNA sequence analysis

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Running Head: Analysis of human ovaries by scRNA-seq

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Significance Statement The recent discovery of a rare stem cell population in the ovaries of women that is capable of supporting the production of new eggs cells or oocytes has the potential to significantly change the current landscape for the clinical management of female infertility as well as the hormonal imbalance resulting from ovarian failure at menopause. This study further documents the existence of these rare cells in the ovaries of women, the genetic profile of these cells, and the occurrence of the earliest steps of the differentiation of these cells into new oocytes in the ovaries of women under normal physiological conditions.

Data Availability Statement The data underlying this article are available in the article and in its online supporting information.

ABSTRACT

In 2004, the identification of female germline or oogonial stem cells (OSCs) that can support postnatal oogenesis in ovaries of adult mice sparked a major paradigm shift in reproductive biology. Although these findings have been independently verified, and further extended to include identification of OSCs in adult ovaries of many species ranging from pigs and cows to non-human primates and humans, a recent study rooted in single-cell RNA sequence analysis (scRNA-seq) of adult human ovarian cortical tissue claimed that OSCs do not exist, and that other groups working with OSCs following isolation by magnetic-assisted or fluorescence-activated cell sorting have mistaken perivascular cells (PVCs) for germ cells. Here we report that rare germ lineage cells with a gene expression profile matched to OSCs but distinct from that of other cells, including oocytes and PVCs, can be identified in adult human ovarian

cortical tissue by scRNA-seq after optimization of analytical workflow parameters. Deeper cell-by-cell expression profiling also uncovered evidence of germ cells undergoing meiosis-I in adult human ovaries. Lastly, we show that, if not properly controlled for, PVCs can be inadvertently isolated during flow cytometry protocols designed to sort OSCs because of inherently high cellular autofluorescence. However, human PVCs and human germ cells segregate into distinct clusters following scRNA-seq due to non-overlapping gene expression profiles, which would preclude the mistaken identification and use of PVCs as OSCs during functional characterization studies.

INTRODUCTION

A central underpinning of reproductive biology has held that oocyte generation in ovaries of female mammals is restricted to the embryonic period [1]. This thinking deviates markedly from spermatogenesis in males throughout adult life, which involves meiotic differentiation of male germline or spermatogonial stem cells (SSCs) in the testes [2]. However, the longstanding paradigm of a non-renewing oocyte pool was challenged by a study with mice in 2004, which reported the existence of female germline or oogonial stem cells (OSCs) and the continuation of oocyte production in adult mouse ovaries [3]. While this study sparked significant debate [4, 5], more than 80 corroborating studies now support the existence of OSCs and/or active oogenesis in adulthood across species [6], including humans [7–15] (Supporting Information Tables 1–3). The discovery of OSCs, which brings the biology of male and female gametogenesis in mammals more closely in line with one another and with that of non-vertebrate species [16], has significant ramifications for development of in-vitro models to investigate human oogenesis as well as of new technologies to combat ovarian failure and female infertility caused by aging or insults [17–19].

A major breakthrough in the study of OSCs came in 2009, with the first report that the cells could be retrieved as a distinct population from mouse ovaries using DEAD-box helicase 4 (DDX4)

antibody-based sorting [20]. Through extensive in-vitro characterization and in-vivo transplantation studies, the germline identity of the cells was established, as was the functional identity of the cells as bona fide precursors to oocytes that can be fertilized to produce viable offspring [20]. More than 60 other publications have since isolated OSCs from ovaries of mice, rats, pigs, cows, baboons and humans [6] (Supporting Information Tables 1 and 2). Moreover, inducible genetic lineage tracing studies with mice have fate-mapped new oocytes produced during adulthood to generation of healthy offspring in natural mating trials, thus establishing the physiological importance of OSC-supported oogenesis to adult ovarian function and female fertility [21]. A second major breakthrough came in 2012 with the successful purification of OSCs from adult human ovarian cortical tissue [7, 8], the findings of which have since been independently verified and extended by many other groups [9, 11, 12, 14, 15]. Human OSCs express a profile of genes characteristic of primitive germ cells, and these cells differentiate through meiosis into oocyte-like cells in vitro and into oocytes that are enclosed within newly-formed follicles after transplantation into human ovarian cortical tissue [7–15, 17]. Human OSCs isolated by fluorescence-activated cell sorting (FACS) with monoclonal antibodies against DDX4 have also been used in approved clinical studies [17, 22–24].

Discordant with this large body of work, a recent study concluded from single-cell RNA sequence analysis (scRNA-seq) that human OSCs do not exist [25]. These authors identified only six clusters (viz. six cell types) in adult human ovarian cortical tissue biopsies using scRNA-seq: stromal cells, perivascular cells (PVCs), endothelial cells, granulosa cells, immune cells and oocytes. That non-oocyte germ lineage cells were apparently missing from their analysis was subsequently put forth as evidence of OSCs being absent from adult human ovaries. However, the expression and clustering analysis reported in this study was performed with an early version of Cell Ranger software (2.1.1 or v2), which has widely known limitations in its ability to detect low-expression cells. An improved version of Cell Ranger software (3.0.2 or v3), which was available and actually used by the authors in the same study for analysis of

human ovarian cells after flow cytometric sorting, increases the sensitivity for cell calling by approximately one log-order over that using Cell Ranger v2 [26] (https://www.10xgenomics.com). While the preprocessed data obtained from Cell Ranger v2 and v3 are fairly consistent, the ability of Cell Ranger v3 to detect more cells, especially those with low abundance transcripts, can change batch-effect corrections and thus the accuracy of the output data analysis [26]. Another issue which can affect the resolution of scRNA-seq is the human reference genome used for read alignments, with HG38 preferred over HG19 for optimal depth of analysis [27]. All of this is highly relevant because OSCs, like other stem cell types, are very rare, with a reported frequency of ~0.014% in adult ovaries [7]. If one is seeking to identify as many cells, and as many cell types, as possible in a highly heterogenous cell sample using scRNA-seq, decisions about which analytical approaches will be used become critically important to consider prior to performing the experiments [26, 27].

In this same study, Wagner et al. [25] also employed DDX4 antibody-based FACS coupled with scRNA-seq to claim that OSCs isolated and studied by many others for over a decade using the same cell sorting strategy [7–13, 20, 22, 23, 28; see also 14, 15] (Supporting Information Table 1) are actually PVCs lacking any germ lineage characteristics. Given past debate over whether mammalian OSCs exist [4–6], and with our labs being directly involved in studies of OSCs for nearly two decades [3, 7, 8, 10, 11, 13, 19, 29, 30], we felt it was important to experimentally assess the conclusions reached by Wagner et al. [25] in an effort to reconcile this recent report with the opposite conclusions reached in over 60 other published studies that have isolated and characterized OSCs since 2009 [6] (Supporting Information Tables 1 and 2). In parallel, we evaluated the possibility that a technology like scRNA-seq could provide further evidence of not just the existence of human OSCs but also of primitive germ cells committing to, or progressing through, the early stages of meiotic differentiation into oocytes in adult human ovarian tissue under normal physiological conditions in vivo. Such an outcome, which has not yet been demonstrated in humans, would be consistent with recent genetic tracing studies in adult female mice

showing that active meiotic entry and oogenesis occur naturally in adult ovaries during reproductive life [21, 31, 32], and that oocytes formed in the ovaries during adulthood contribute directly to the pool of eggs used for natural species propagation [21].

MATERIALS AND METHODS

Animals

Freshly-collected ovarian tissues from adult heifers (*Bos taurus*) were obtained from Blood Farms (Groton, MA) and processed immediately for cortex isolation and cryopreservation until use.

Human Subjects

All research with human tissues was approved by the institutional review boards of Northeastern University (IRB#14-03-22), University of Edinburgh (LREC 16/SS/0144), and Saitama Medical University (630-III). Informed consent was obtained from all participants, and all tissue samples were de-identified prior to use. A total of 7 ovarian cortical tissue samples from two caesarean section patients (CSP) and one gender reassignment patient (GRP) between 26–34 years of age [7, 8, 11] were used.

Adult Human Unsorted Ovarian Cortical Cell scRNA-seq Data and Code Availability

The scRNA-seq data referenced in this study were originally generated by Wagner et al. [25] from adult human ovarian cortical biopsies of four subjects (CSP, n = 3; GRP, n = 1). The 10x Genomics dataset of Wagner et al. [25] for adult human unsorted ovarian cortical cells was deposited by these authors to, and accessed by us through, the European Molecular Biology Laboratory's European Bioinformatics Institute (EMBL-EBI) under the accession code E-MTAb−8381. Analyses of scRNA-seq data were completed using the lines of code for adult human unsorted ovarian cortical cells deposited by Wagner

et al. [25] on GitHub (https://github.com/wagmag/SingleCellOvary). For Cell Ranger v6 analysis, additional lines of code were run in parallel to select proper quality control metrics as well as to determine the parameters for dimensionality reduction that best represented the data. The code used with the Cell Ranger v6 analysis is available on GitHub (https://github.com/hanrico/Ovarian-scRNA-seq).

Clustering and Analysis of scRNA-seq Data

In the Wagner et al. [25] study, output files for their adult human unsorted ovarian cortical cell samples were converted using Cell Ranger v2. We first re-analyzed their raw fastq files using the same version of Cell Ranger and the same human genome assembly (HG19), along with Seurat version 3.0.0 (v3) and the lines of code for unsorted human ovarian cortical cells deposited by Wagner et al. [25]. We then repeated this analysis using Cell Ranger version v3, since this newer software version was also available to the authors at the time of their study. In fact, Wagner et al. [25] elected to use Cell Ranger v3 for their analysis of sorted ovarian cells, but for unclear reasons they chose Cell Ranger v2 for their unsorted ovarian cell analysis. Finally, the same dataset was analyzed using current versions of Cell Ranger (version 6.0.1 or v6) and Seurat (version 4.0.4 or v4), along with HG38 as the human reference genome (Supporting Information Table 4). After completing the Seurat analysis of the Cell Ranger v6 output data, we then replaced Seurat with Loupe Browser (version 5.1.0 or v5; 10x Genomics) for deeper expression analysis of single cells, using the same filtering and data visualization parameters utilized with Seurat (see Supporting Information Method 1 for additional details).

Expression of *PRDM1* (PR domain containing 1 with ZNF domain), *DPPA3* (developmental pluripotency-associated 3), *IFITM3* (interferon-induced transmembrane protein 3), *TUBB8* (tubulin beta 8 class VIII) and *DDX4* was used to identify primitive germ cells, noting that Wagner et al. [25] used a more limited profile of only *PRDM1*, *DPPA3* and *DAZL* (deleted in azoospermia like). Analysis of *FIGLA* (factor in the germline alpha), *OOSP2* (oocyte secreted protein 2), *GDF9* (growth differentiation factor 9)

and *ZP3* (zona pellucida glycoprotein 3) was used to identify oocytes, as reported by Wagner et al. [25]. However, we also analyzed expression of *ZP1*, *ZP2* and *NOBOX* (newborn ovary homeobox) as oocyte markers. Expression of *SYCP3* (synaptonemal complex protein 3), *STAG3* (stromal antigen 3), *SMC1α* (structural maintenance of chromosomes 1 alpha), *SMC3* (structural maintenance of chromsomes 3) and *STRA8* (stimulated by retinoic acid gene 8) was used to identify germ cells in the first meiotic division. Expression of *RGS5* (regulator of G-protein signaling 5), *MCAM* (melanoma cell adhesion molecule), *MYH11* (myosin heavy chain 11), *RERGL* (Ras-related and estrogen-regulated growth inhibitor-like) and *TAGLN* (transgelin) was used to identify PVCs, as reported by Wagner et al. [25]. For ease of referral, a listing and brief overview of each gene analyzed is provided in Supporting Information Table 5.

Flow Cytometry

Ovarian cortical tissue from adult heifers or reproductive-age women was cryopreserved, thawed, and dissociated into single-cell suspensions for flow cytometry using a BD FACSAria™ III, as described previously [8, 33] (see Supporting Information Method 2 for more details). Primary antibodies against SMA (ab5694, 1:50; Abcam, Cambridge, MA) or CD31 (MA3100, 1:50; Invitrogen–ThermoFisher Scientific, Waltham, MA), each directly conjugated to APC (Abcam, ab201807), were used for determination of the total percentage of autofluorescent events that were positive for expression of either PVC marker. Flow cytometry data were acquired using BD FACSDiva 8.0.1 software and analyzed by FlowJo 10.7 software.

Data Analysis

For scRNA-seq, data analysis was performed using the human unsorted ovarian cortical cell dataset of Wagner et al. [25]. For FACS, the data represent the mean ± SE of combined results; n = 4 (CD31) or 7 (SMA), and n = 5 (SMA) or 6 (CD31), for bovine and human ovarian tissue sample analysis, respectively.

RESULTS

Analysis of Unsorted Cells Isolated from Human Ovarian Cortical Tissue Biopsies

We first used the reported frequency of OSCs in adult ovarian tissue (0.014%; [7]) with software for estimating the number of cells required to detect a given cell type with scRNA-seq (www.satijalab.org/howmanycells). Assuming six general cell types based on the clusters reported by Wagner et al. [25], we determined that 84,550 viable ovarian cells would be needed for detection of at least five OSCs at 95% confidence (Supporting Information Fig. 1). Any number less than five cells failed to produce a reliable assessment of input cell number required. Notably, the 12,160 cells analyzed by Wagner et al. [25] was 14.4% of the minimal cell input number needed for detection of five OSCs at 95% confidence under these modeling parameters (see Supporting Information Method 3 for additional details). To then assess if use of Cell Ranger v2 versus v3 would have altered the outcomes reported by Wagner et al. [25], we reanalyzed their adult human unsorted ovarian cortical cell dataset using Cell Ranger v2 and v3 with their published code, HG19 and Seurat v3. With Cell Ranger v2, we identified the six clusters reported by these authors from 12,020 total cells called after preprocessing and filtering the Cell Ranger output data (Fig. 1A). When we switched to Cell Ranger v3, we identified the same six clusters plus two additional clusters that emerged from more than double the number (27,376) total cells called (Fig. 1B, C). Through gene ontology (GO) analysis, the two additional clusters were enriched for genes associated with stromal and immune cells. Wagner et al. [25] reported that one of the six clusters they identified, which was comprised of 18 total cells, exhibited higher expression levels of four markers commonly associated with oocytes (*FIGLA*, *OOSP2*, *GDF9* and *ZP3*) relative to expression levels of these genes in the other five clusters. Our analysis using Cell Ranger v2 similarly identified 18 cells that constituted a single cluster enriched for expression of these four genes (Supporting Information Fig.

2; Supporting Information Table 6). However, when analyzed using Cell Ranger v3, this cluster increased from 18 to 62 cells (Supporting Information Table 6). Deeper analysis of this cluster identified two cells, not detected with Cell Ranger v2, that were positively associated with genes used by Wagner et al. [25] to identify OSCs (*PRDM1*, *DPPA3* and *DAZL*) but had an oocyte gene expression score of zero (Fig. 1D).

Workflow Adjustments to Optimize scRNA-seq of Human Ovarian Cortical Cells

Our identification of at least two potential non-oocyte germ cells using Cell Ranger v3 that were missed when Cell Ranger v2 was employed prompted us to ask if further optimization of the scRNA-seq workflow could provide additional insights into whether evidence of OSCs in this dataset could be uncovered. To do this, we analyzed the same dataset using current versions of Cell Ranger software (v6) and Seurat (v4), along with HG38 and with the number of principal components, dimensions and resolution set at 30, 1:18 and 0.1, respectively. Using this updated workflow, UMAP analysis identified nine clusters from a total of 27,710 cells called (Fig. 1E; Supporting Information Table 6). Using GO analysis, we identified the clusters as likely representing stromal cells (two separate clusters), PVCs, endothelial cells (two separate clusters), granulosa cells, immune cells (two separate clusters), and germ cells/oocytes. We then compared outcomes obtained using Seurat v4 versus Loupe Browser v5 for downstream analysis of the preprocessed Cell Ranger v6 data, since Loupe Browser is more user-friendly and does not require specific lines of code, like Seurat, for interactive visualization of the results. Both Seurat and Loupe Browser identified the same germ cell/oocyte cluster based on similarity in overall expression patterns. However, cell-by-cell analysis with Loupe Browser revealed that only 10 of 62 total cells in this cluster co-expressed all four marker genes associated with oocytes (*FIGLA*, *OOSP2*, *GDF9* and *ZP3*) (Fig. 1F). However, since premeiotic and postmeiotic germ cells are known to express a common suite of genes that define the germ lineage, we suspected that these cells were likely clustered based on germline, rather than on oocyte-associated, gene expression patterns.

Analysis of Human Unsorted Ovarian Cortical Cells for Evidence of Oocytes

Using our optimized analytical pipeline, 10 cells were identified in the germ cell/oocyte cluster as oocytes based on co-expression *FIGLA*, *OOSP2*, *GDF9* and *ZP3* in each cell (Fig. 1F). While *FIGLA* is often referred to as an oocyte-specific gene [34–36], *FIGLA* encodes a transcription factor that functions at various stages of oogenesis, including the regulation of key genes needed for meiosis-I progression in premeiotic germ cells [37]. We therefore performed a gene-by-gene analysis of the 62 cells in this cluster using Loupe Browser. We identified a total of 40 *FIGLA*-expressing cells, of which only 21 co-expressed *OOSP2*, 13 co-expressed *GDF9*, 37 co-expressed *ZP3*, and 12 co-expressed both *GDF9* and *ZP3* (Fig. 2). Likewise, when the other oocyte markers were analyzed individually, we identified 25 *OOSP2*-expressing cells, 14 *GDF9*-expressing cells and 42 *ZP3*-expressing cells in this cluster (Fig. 2). The discordance in numbers of cells expressing each gene individually or in pairs versus combined as a four-gene panel may be due to differences in timing of expression of the various genes relative to oocyte maturational stage, expression in cells other than oocytes, and/or degradation of mRNA transcripts during sample processing that led to the expression of a given gene in a given cell falling below the detection threshold (see **DISCUSSION** and Supporting Information Discussion 1 for additional details).

While DDX4 is widely accepted as being expressed in all oocytes in vivo [7, 38–40], only 12 of the 62 cells in this cluster expressed *DDX4* (Fig. 3) Moreover, only 5 of the *FIGLA*/*OOSP2*/*GDF9*/*ZP3*-expressing cells co-expressed *DDX4* (Fig. 3). Pairwise gene analysis identified 12 *DDX4*/*FIGLA*-expressing cells, 8 *DDX4*/*OOSP2*-expressing cells, 6 *DDX4*/*GDF9*-expressing cells and 10 *DDX4*/*ZP3*-expressing cells (Fig. 3). Thus, out of the four marker genes utilized by Wagner et al. [25] to identify oocytes, the only gene co-expressed in all *DDX4*-expressing cells in this cluster was *FIGLA*, which is expressed in both pre- and postmeiotic germ cells [37]. A parallel analysis of *NOBOX*, which in mouse and human ovaries is robustly expressed in oocytes throughout development from the primordial follicle to metaphase-II egg

stage [41, 42], failed to identify a single *NOBOX*-expressing cell in the germ cell/oocyte cluster (Fig. 3). Likewise, there were no *ZP1*-expressing cells, and only two *ZP2*-expressing cells, identified in this cluster of cells containing candidate oocytes (Fig. 3). Of the two *ZP2*-expressing cells, only one co-expressed the four-gene marker panel used by Wagner et al. [25] to identify oocytes, whereas the other co-expressed *FIGLA*, *OOSP2* and *ZP3*, but not *GDF9* (data not shown).

Analysis of Human Unsorted Ovarian Cortical Cells for Evidence of Non-oocyte Germ Cells

We next moved to analysis of genes known to be expressed in primitive germ cells (see Supporting Information Table 5 for more details). We identified one cell in the germ cell/oocyte cluster with expression of all five germline genes analyzed (*PRDM1*, *DPPA3*, *IFITM3*, *TUBB8* and *DDX4*), and two additional cells with expression of *DPPA3*, *IFITM3*, *TUBB8* and *DDX4* but lacking detectable *PRDM1* (Fig. 4). All three cells localized to the same cluster of 62 cells which contained the 10 *FIGLA*/*OOSP2*/*GDF9*/*ZP3*-expressing cells. However, these three cells were clearly distinct from the ten cells classified as oocytes (Fig. 4). In the two non-oocyte germ cells lacking *PRDM1* expression, we detected expression of *SYCP3* (Table 1), which is required for progression of germ cells through the early stages of the first meiotic cell division [43, 44]. This observation prompted us to explore additional genes involved in the early stages of meiosis-I. From this, we found that both *SYCP3*-expressing germ cells co-expressed *STAG3* and *SMC3*, and one of the *SYCP3*/*STAG3*/*SMC3*-expressing germ cells also co-expressed *SMC1α* (Table 1). Notably, the proteins encoded by *STAG3*, *SMC1α* and *SMC3* are all meiosis-specific cohesin complex components involved in formation of axial elements and cohesion of sister chromatids during meiotic prophase-I [45–47]. We also identified two *SYCP3*/*STAG3*/*SMC3*-expressing cells in this cluster with co-expression of *STRA8* (Supporting Information Fig. 3), the latter of which is considered a critical early gene for meiosis-I progression in germ cells of both sexes [49].

It is worth noting that *FIGLA* was detected in all three *DPPA3*/*IFITM3*/*TUBB8*/*DDX4*-expressing cells (Table I). However, since these cells were distinct from the *FIGLA*/*OOSP2*/*GDF9*/*ZP3*-expressing cells (viz. candidate oocytes), the presence of *FIGLA*, which is not oocyte-specific [37], is still aligned with these three cells being classified as non-oocyte germ cells. Expression of *OOSP2* was detected in the two *DPPA3*/*IFITM3*/*TUBB8*/*DDX4*-expressing cells, but not in the single *PRDM1*/*DPPA3*/*IFITM3*/*TUBB8*/*DDX4*-expressing cell (Table 1). While *OOSP* gene family members were first identified as encoding oocyte-enriched proteins in the mouse ovary [50, 51], lineage specificity of *OOSP2* in human ovaries has not been evaluated to date, and transcriptomic expression of the gene in humans is not restricted to oocytes [52].

Continuing with our analysis, *GDF9* was not detected in any of the *DPPA3*/*IFITM3*/*TUBB8*/*DDX4*-expressing cells found in this cluster, whereas *ZP3* was detected in all three cells (Table 1). However, *ZP3* expression was far more ubiquitous than expected, in that a total of 567 cells with *ZP3* expression were identified across the population of 27,710 cells called in this dataset (Supporting Information Fig. 4). Strikingly, 525 of these *ZP3*-expressing cells were localized outside of the germ cell/oocyte cluster (see Supporting Information Results 1 for additional details). This widespread detection of *ZP3* expression across clusters representing different lineages, most of which are somatic, is consistent with the reported low-level expression of this gene in diverse cell types and tissues in humans [52]. To further assess the promiscuous nature of *ZP3* expression outside of oocytes, we analyzed a different scRNA-seq dataset derived from adult human ovarian medullary tissue [53]. We did not identify a single cell with co-expression of the four-gene marker profile used by Wagner et al. [25] to identify oocytes; however, parallel analysis of this dataset identified 673 cells expressing *ZP3*, again distributed randomly across the various clusters (data not shown).

Finally, given that Wagner et al. [25] used *DAZL* as one of their three genes for OSC screening, we identified twenty *DAZL*-expressing cells in the entire dataset of 27,710 cells called using Cell Ranger

v6, ten of which were localized to the germ cell/oocyte cluster. Of these ten cells, five co-expressed the four-gene marker panel used by Wagner et al. [25] to identify oocytes (Supporting Information Fig. 5), consistent with past studies establishing expression and function of *DAZL* in both pre- and postmeiotic germ cells [54, 55]. Breaking the oocyte marker panel down further, we identified eight *DAZL*/*FIGLA*-expressing cells, eight *DAZL*/*OOSP2*-expressing cells, eight *DAZL*/*FIGLA*/*OOSP2*-expressing cells, five *DAZL*/*GDF9*-expressing cells, five *DAZL*/*ZP3*-expressing cells, and five *DAZL*/*GDF9*/*ZP3*-expressing cells in the germ cell/oocyte cluster (data now shown).

Analysis of Cells Sorted from Human Ovarian Cortical Tissue Biopsies using Flow Cytometry

In the Wagner et al. [25] study, the authors also reported that DDX4 antibody-based FACS, a method used by many others to specifically sort OSCs across species since 2009 [7–13, 20, 22, 23, 28–30] (Supporting Information Table 1), led to the isolation of PVCs and not OSCs. While initially puzzling, we noted that their flow cytometry was performed using AF594 detected with a 561-nm laser in the red channel, which is widely known to detect autofluorescence as a “positive” event during FACS. In evaluating the antibody validation and gating strategies shown in the supplementary data section of their study, we observed that the area above the cutoff designating the negative versus positive fractions in their negative control sample lacking antibody contained positive events, which represent autofluorescence. With this information in mind, PVCs are known to express autofluorescent biomolecules, such as collagen and elastin, which produce widely known artifacts in flow cytometry [56, 57]. We therefore sorted dispersed ovarian cortical tissue with a 561-nm laser in the red channel following the parameters published by Wagner et al. [25]. Using adult bovine ovarian cortical tissue for validation, a distinct population of autofluorescent events was obtained (Fig. 5). Three-quarters of these cells were positive for SMA or CD31, which respectively mark the two cell types that comprise PVCs: vascular smooth muscle cells and pericytes. A distinct population of autofluorescent events was similarly

detected in dispersed ovarian cortical tissue of reproductive-age women. Likewise, almost two-thirds of these events were identified as being SMA- or CD31-positive (Fig. 5). Moreover, these autofluorescent events were detectable in dispersed cell preparations from human ovarian cortical tissue irrespective of whether the samples were gated versus FSC-A or SSC-A, or if PE-Texas Red-A was plotted against a different laser (APC-A) (Supporting Information Fig. 6).

Germ Cells and PVCs Segregate into Distinct Clusters

We then dug deeper into the conclusion of Wagner et al. [25] that we and others have mistakenly worked with PVCs in studies that have explored the characteristic germline properties, and potential clinical utility, of human OSCs [7–15, 17, 22, 23] (Supporting Information Tables 1 and 2). Using the optimized scRNA-seq workflow described above, we identified a cluster comprised of 3,310 total cells, 479 of which had the five-gene gene expression profile utilized by Wagner et al. [25] to identify PVCs (*RGS5*, *MCAM*, *MYH11*, *RERGL* and *TAGLN*) (Fig. 6A). None of the cells comprising this cluster co-expressed the gene panel which identified the three non-oocyte germ cells (*DPPA3*, *IFITM3*, *TUBB8* and *DDX4*, without or with *PRDM1*; data not shown), and only 3 of the 3,310 total cells in this cluster expressed *DDX4* (Fig. 6B). Moreover, none of the 479 cells identified as PVCs using the five-gene profile of Wagner et al. [25] co-expressed *DDX4* (Fig. 6B). Likewise, none of the three non-oocyte germ cells expressed the five-gene profile used by Wagner et al. [25] to cluster PVCs (Table 1), which led to the expected segregation of these two cell types into distinct and non-overlapping clusters (Fig. 6C). In fact, of the five genes used by Wagner et al. [25] to cluster PVCs, *TAGLN* was the only gene identified through individual gene-by-gene analysis to be co-expressed in the three *DPPA3*/*IFITM3*/*TUBB8*/*DDX4*-expressing germ cells (Table 1). However, *TAGLN* expression is not specific to any single type, and thus its utility as a lineage marker must be viewed in context with other genes as a profile associated with a given cell type. Supporting this statement, we found that 16,291 cells of the 27,710 total cells called

expressed *TAGLN* (Fig. 6C). This included 23 cells in the 62-cell germ cell/oocyte cluster, four of which co-expressed the four-gene profile used by Wagner et al. [25] to identify oocytes (Fig. 6C).

DISCUSSION

While scRNA-seq is useful as a tool to gain insights into cell lineage heterogeneity within a sample [58], a major caveat of this approach is that its failure to detect gene expression-based evidence of a given cell type after clustering analysis does not, by default, equate to that cell type being absent in the sample analyzed. This is especially apropos in attempts to identify either very rare cells or low-expression cells in a dispersed cell preparation that is heterogenous in nature, highlighting the challenges associated with detection of stem cells in tissues by scRNA-seq [59]. The analytical pipeline employed also has a significant impact on the depth and accuracy of the data obtained, especially if the objective is to produce a comprehensive snapshot of as many cells, and as many cell types, as possible in the sample analyzed. In the Wagner et al. [25] study, their attempts to identify OSCs in a pool of 12,160 total cells called, given the extreme rarity of OSCs in adult ovaries [7], would be difficult even under the best of conditions (see Supporting Information Discussion 1 for additional details). Our rigorous reassessment of their unsorted cell dataset following empirical testing of numerous variables that affect the depth, resolution, and accuracy of scRNA-seq highlight how multiple decisions made by these authors for their analysis of unsorted cells actually minimized, rather than optimized, the probability of detecting rare or low-expression cell types such as OSCs. In fact, several other ovarian stem cell types were also missed by Wagner et al. [25], including pluripotent embryonic stem cell (ESC)-like cells, mesenchymal stem cells (MSCs), and very small embryonic-like stem cells (VSELs) (Supporting Information Table 7; see also Supporting Information Discussion 2), the latter of which have been reported to support postnatal oogenesis in mammalian ovaries [32].

These challenges were further complicated by the fact that Wagner et al. [25] restricted their efforts to find evidence of OSCs in their entire dataset of 12,180 cells to only 15 cells that were manually selected by these authors based on the required presence of *DDX4* mRNA to establish the only "cluster" of cells that could contain OSCs. The a priori assumption that all candidate OSCs must have detectable *DDX4* expression using scRNA-seq is fraught with interpretational problems. For example, we found that only 5 of the 10 cells identified as oocytes using the four-gene profile reported by Wagner et al. [25] co-expressed *DDX4*. Based on their reasoning, such an approach would have removed the remaining five *FIGLA*/*OOSP2*/*GDF9*/*ZP3*-expressing cells lacking detectable *DDX4* mRNA from further consideration as oocytes. Likewise, we could not identify a single *NOBOX*-expressing cell in the germ cell/oocyte cluster, even though *NOBOX* is highly expressed in oocytes at all developmental stages [41, 42]. If one evaluated this dataset for evidence of oocytes based solely on *NOBOX* expression, or manually created a "cluster" of *NOBOX*-expressing cells as the sole cell population in which any potential oocytes would be found, the reasoning of Wagner et al. [25] with OSCs would lead to the erroneous conclusion that oocytes do not exist in adult human ovarian cortex. At the other end of the spectrum, our evaluation of *ZP3*, which is widely used as an "oocyte-specific" marker [35], revealed low-level but widespread expression of this gene across all cell clusters, most of which are somatic in origin. Thus, scRNA-seq workflow design, and any conclusions drawn, based on the expression of a single gene being detected or not in a cell of interest lack scientific rigor or confidence.

However, optimization of the scRNA-seq workflow pipeline using a version of Cell Ranger (v3), which was available to, and used by, Wagner et al. at the time of their study [25] but was not used for their unsorted cell analysis, allowed us to identify rare cells in their adult human unsorted ovarian cortical cell dataset with a gene expression profile that closely aligns with that of primitive germ cells, such as embryonic PGCs [60–63] and adult ovary-derived OSCs [6, 7–9, 11, 12, 20]. Further analysis showed that two of these non-oocyte germ cells expressed multiple markers of meiosis-I commitment

and progression. These observations, which offer evidence of ongoing de-novo oogenesis in adult human ovaries under normal physiological conditions, are consistent with prior studies with mice which have demonstrated that resident germ cells routinely undergo meiosis in adult ovaries [21, 31, 32]. Interpretational caution must still be exercised here, however, since gene expression profiling does not offer definitive evidence of the existence, or not, of human OSCs or of active meiotic entry. In this regard, all scientific studies of isolated human OSCs published to date have characterized the cells, following isolation, by gene expression profiling along with various downstream functional tests of meiotic cell division capability and/or oocyte-forming potential [7–15]. Like other gene expression-based studies, the inability of scRNA-seq as a standalone approach to offer any type of functional verification of suspected lineage identity is another caveat of the use this type of "big-data" technology and the interpretations drawn from it [64].

Moving on to the FACS analysis of cells identified by Wagner et al. [25] as DDX4-positive (+) or DDX4-negative (–), the sorting strategy for OSC isolation relies on detection of an externalized region of the C-terminus of DDX4 exposed on the outside of viable cells and not simply DDX4 expression [7, 8, 20, 28]. Dual-antigen single-protein sorting studies conducted almost 10 years ago showed that OSCs could be sorted as viable cells using a C-terminal, but not N-terminal, DDX4 antibodies, noting that both antibodies recognize DDX4 in oocytes in fixed ovarian sections [7]. In turn, the viable cells sorted with the C-terminal antibody show a near-complete population shift by FACS when the same cells are permeabilized and then analyzed with an N-terminal DDX4 antibody [7], verifying the specificity of the sorting protocol for detection of externalized DDX4. It has also been shown that proper tissue dispersion is a crucial step to achieve viable cell isolation and to release OSCs as single cells [8, 20, 28]. Human ovary tissue is particularly fibrous and difficult to disaggregate, and thus extreme care must be exercised during disaggregation to maintain cell viability [8, 11, 20, 28]. If this is not done, the possibility of non-specific antibody binding is markedly increased, which may explain why the yield of "DDX4+" events

obtained by Wagner et al. [25] with the Abcam DDX4 antibody was 3.0–6.5-fold higher than the yield of human OSCs reportedly previously using the same sorting approach with the same antibody [7].

Putting potential technical issues aside, it is important to emphasize that no other study which has used DDX4 antibody-based sorting to isolate OSCs, an approach first reported over 10 years ago [20] with more than 30 corroborating studies since then (Supporting Information Table 1), has retrieved PVCs. In addition, DDX4 antibodies have been used to sort PGC-like cells from cultures of human embryonic stem cells and induced pluripotent stem cells [65, 66], indicating that the utility of this approach to specifically isolate primitive germ cells is not unique to OSCs. Notably, only PGC-like cells were obtained after DDX4 antibody-based sorting in these two studies [65, 66], even though PVCs also arise in cultures of differentiating human pluripotent stem cells [67]. This discordance in what Wagner et al. [25] report regarding their isolation of PVCs instead of OSCs by this approach also extends to their own previously published findings, in which identical claims were made that human OSCs do not exist and that the sorting strategy for OSC isolation using DDX4 antibodies does not work [68]. While those claims were experimentally disputed [69], in this earlier study the authors similarly performed scRNA-seq on “DDX4+” cells obtained from human ovarian cortical biopsies. Their analysis in that prior study did not, however, identify PVCs as the primary cell type retrieved by FACS. Instead, out of a randomly selected population of 41 “DDX4+” cells, their gene expression associations identified a mixed population of very diverse cell types [68]. The inconsistent outcomes reported by these authors when using DDX4 antibodies for cell sorting in their own studies [25, 68] may help explain why their findings diverge widely from what many others have consistently reported using the same cell sorting strategy since 2009 (Supporting Information Table 1) (see also Supporting Information Discussion 1).

With this information as a preface, we designed experiments to determine how PVCs could be erroneously isolated as cells perceived to be antibody-positive using flow cytometry. Our data presented herein offer at least a reasonable explanation for this outcome, which accounts for the inherent

autofluorescence of PVCs being detected as a false-positive signal during FACS. This would lead to an artifactual enrichment of these cells rather than true antibody-positive cells. In turn, our analysis of PVCs and non-oocyte germ cells in the Wagner et al. [25] dataset demonstrated that these two cell types, not surprisingly, cluster separately and exhibit no overlap in gene expression profiles associated with each cell type. Thus, even if PVCs were isolated by DDX4 antibody-based FACS for reasons unrelated to endogenous autofluorescence, any downstream analysis of these cells would generate data that differ considerably from the published results from many other groups that have successfully sorted human OSCs for characterization of their germline identity and oocyte-forming properties [7–15].

CONCLUSIONS

Since the initial report on OSCs almost two decades ago [3], over 80 primary research studies have been published supporting the existence of OSCs and/or postnatal oogenesis in mammals [6] (Supporting Information Tables 1–3). More than 30 of these have sorted OSCs from ovaries with polyclonal or monoclonal antibodies directed against the C-terminus of DDX4 for in-depth characterization (Supporting Information Table 1). In this same time frame, only 10 primary research papers have been published disputing the existence of OSCs and/or the occurrence of postnatal oogenesis in mammals (Supporting Information Tables 1 and 3), and only 4 of these studies claimed that DDX4 antibody-based sorting fails to isolate OSCs (Supporting Information Table 1; see also Supporting Information Discussion 3). With respect to human OSCs, at least six different groups have established, and independently corroborated, that extracellular DDX4-positive cells sorted from adult human ovarian cortex express primitive germ lineage (but not oocyte) markers, can be expanded in number in culture, activate meiosis, and generate oocyte-like cells in vitro and oocytes in ovarian tissue [7–15]. Aside from the fact that these outcomes are fully consistent with a large body of work on OSCs in other species [6, 18, 70], none of these endpoints are features of PVCs.

In consideration of this, along with the experimental evidence presented herein, a more reasonable conclusion from the Wagner et al. [25] study is that the scRNA-seq workflow employed by the authors was not designed appropriately to identify candidate OSCs, or in fact any stem cell type, in their sample. When the analytical workflow was optimized and applied to all cells of their sample equally, we uncovered evidence in their dataset of the existence of both OSCs and primitive germ cells entering meiosis-I. Likewise, a more reasonable conclusion from their flow cytometry work is that these authors have had recurrent technical difficulties with FACS over the years [25, 68] in achieving what more than 30 other studies have already reported with respect to the sorting of OSCs from adult ovarian tissue for in-vitro and in-vivo characterization (Supporting Information Table 1). This alternative conclusion would also remove the erroneous inference made by Wagner et al. [25] that numerous other groups have mistakenly worked with PVCs, and not germ cells, in the many reports of OSCs [6] (Supporting InformationTable 1) or PGC-like cells [64, 65] published to date using DDX4 antibody-based sorting to isolate primitive germ lineage cells.

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AUTHOR CONTRIBUTIONS

H.A., Z.F. and D.C.W. performed the analysis of the scRNA-seq dataset; H.A. performed the flow cytometric analysis; T.B. assisted with the experiments and performed analysis of published studies of OSCs, postnatal oogenesis, and other ovarian stem cell types. R.A.A., E.E.T. Y.T., O.I. and H.S. collected and cryopreserved human ovarian cortical tissue for analysis; D.C.W. and J.L.T. directed the experiments

and J.L.T. wrote the manuscript; all authors approved the results and the final manuscript for submission.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

H.A., Z.F., T.B., Y.T., O.I., H.S., R.A.A. and E.E.T. declare no potential conflicts of interests. D.C.W. declares interest in intellectual property described in U.S. Patent 8,642,329, U.S. Patent 8,647,869, U.S. Patent 9,150,830 and U.S. Patent 10,525,086. J.L.T. declares interest in intellectual property described in U.S. Patent 7,195,775, U.S. Patent 7,850,984, U.S. Patent 7,955,846, U.S. Patent 8,642,329, U.S. Patent 8,647,869, U.S. Patent 8,652,840, U.S. Patent 9,150,830, U.S. Patent 9,267,111, U.S. Patent 9,845,482 and U.S. Patent 10,525,086.

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FIGURE LEGENDS

Figure 1. Clustering and analysis of unsorted adult human ovarian cortical cells following scRNA-seq. **(A):** Identification of a total of 12,020 cells that formed 6 clusters following analysis of the dataset using Cell Ranger v2. **(B):** Identification of a total of 27,376 cells that formed 8 clusters following reassessment of the same dataset with Cell Ranger v3. **(C):** Cluster dendrogram depicting the lineage relationships between the 8 clusters identified using Cell Ranger v3. **(D):** Scatterplot analysis of the 62-cell germ cell/oocyte cluster identified using Cell Ranger v3, showing OSC gene expression scores plotted against oocyte gene expression scores. Two cells with very high OSC gene expression scores and an oocyte gene expression score of zero are highlighted by black arrows. **(E):** Identification of a total of 27,710 cells that formed 9 clusters following reassessment of the dataset with Cell Ranger v6. **(F):** Loupe Browser analysis of the Cell Ranger v6 output data, with the germ cell/oocyte cluster highlighted by the expanded box. Of the 62 cells in this cluster (each cell is depicted as an individual dot), 10 cells were identified as positive for co-expression of *FIGLA*, *OOSP2*, *GDF9* and *ZP3* (purple dots; examples are highlighted by black arrows) whereas 52 cells did not show co-expression of this four-gene oocyte marker panel (light-gray dots; examples are highlighted by open arrowheads).

Figure 2. Cell-by-cell analysis of oocyte-associated markers in the germ cell/oocyte cluster using Cell Ranger v6 followed by Loupe Browser. Cells identified as positive for expression of the indicated gene(s) is/are colorized purple (examples are highlighted by black arrows in the uppermost panel) whereas cells negative for expression of the indicated gene(s) is/are light-gray (examples are highlighted by white arrowheads in the uppermost panel).

Figure 3. Further analysis of the germ cell/oocyte cluster for expression of *DDX4* and oocyte markers on a cell-by-cell basis using Loupe Browser. Cells identified as positive for expression of the indicated gene(s) is/are colorized purple whereas cells negative for expression of the indicated gene(s) is/are light-gray. No *ZP1*-expressing cells were identified in this cluster (data not shown).

Figure 4. Loupe Browser analysis of the germ cell/oocyte cluster for expression of genes highly enriched in primitive germ cells. Cells identified as positive for expression of the indicated gene(s) is/are colorized (see legends in each panel) whereas cells negative for expression of the indicated gene(s) is/are light-gray.

Figure 5. Flow cytometric detection, isolation, and characterization of autofluorescent events in adult cow and human ovarian cortical tissue. **(A–D):** Representative gating strategy for doublet discrimination (forward-scatter or FSC-A: B; side-scatter or SSC-A: C) and for dead cell exclusion using 4',6-diamidino-2-phenylindole (DAPI) labeling (D). **(E, F):** Comparison of autofluorescent events detected in the APC-A far-red channel (640-nm laser; E) versus the PE-Texas red-A channel (561-nm laser; F). **(G–K):** Autofluorescent events detected in the PE-Texas red-A channel were collected, fixed and permeabilized (G and H), and then incubated with APC-conjugated primary antibodies against SMA (Abcam ab5694) or CD31 (Invitrogen MA3100) (I and J) for determination of the total percentage of autofluorescent events that were positive for expression of either PVC marker in bovine and human ovarian cortical tissue samples (K). Data shown in (K) are the mean $\pm$ SE; n = 4 (CD31) or 7 (SMA), and n = 5 (SMA) or 6 (CD31), for bovine and human sample analysis, respectively.

Figure 6. Analysis of PVC markers in adult human unsorted ovarian cortical cells using Loupe Browser. **(A):** Identification of cells with co-expression of *RGS5*, *MCAM*, *MYH11*, *RERGL* and *TAGLN* (green dots) in

a larger population of cells clustered together as PVCs based on overall similarities in gene expression. **(B):** Assessment of *DDX4* expression (black dots) in the PVC cluster, highlighting the absence of *DDX4* in the subpopulation of cells that co-express *RGS5*, *MCAM*, *MYH11*, *RERGL* and *TAGLN* (green dots). **(C):** Analysis of *TAGLN* expression (purple dots) across all cells called in the adult human unsorted ovarian cortical scRNA-seq dataset, with an expanded view of the germ cell/oocyte cluster shown (cells lacking expression of *TAGLN* are shown as light gray). Note that the cluster highlighted by the asterisk in (C) is the PVC cluster shown in (A) and (B). Light-gray dots: cells negative for expression of the indicated gene(s). See also related data shown in Table 1.

Table 1. Gene expression profiling analysis of non-oocyte germ cells identified in adult human ovarian cortical tissue. Loupe Browser analysis of the three non-oocyte germ lineage cells for expression of oocyte marker genes (tan-shaded boxes), genes associated with meiosis-I activation and progression (green-shaded boxes), and PVC marker genes (blue-shaded boxes) (–, expression not detected; +, expression detected).

	DDX4/IFITIM3/DPPA3/ TUBB8 Cell 1	*DDX4/IFITIM3/DPPA3/ TUBB8* Cell 2	*DDX4/IFITIM3/DPPA3/ TUBB8/PRDM1*
FIGLA/OOSP2/ GDF9/ZP3	–	–	–
FIGLA	+	+	+
OOSP2	+	+	–
GDF9	–	–	–
ZP3	+	+	+
SYCP3	+	+	–
STAG3	+	+	–
SMC1α	+	–	–
SMC3	+	–	–
RGS5/MCAM/ MYH11/RERGL/ TAGLN	–	–	–
RGS5	–	–	–
MCAM	–	–	–
MYH11	–	–	–
RERGL	–	–	–
TAGLN	+	+	+

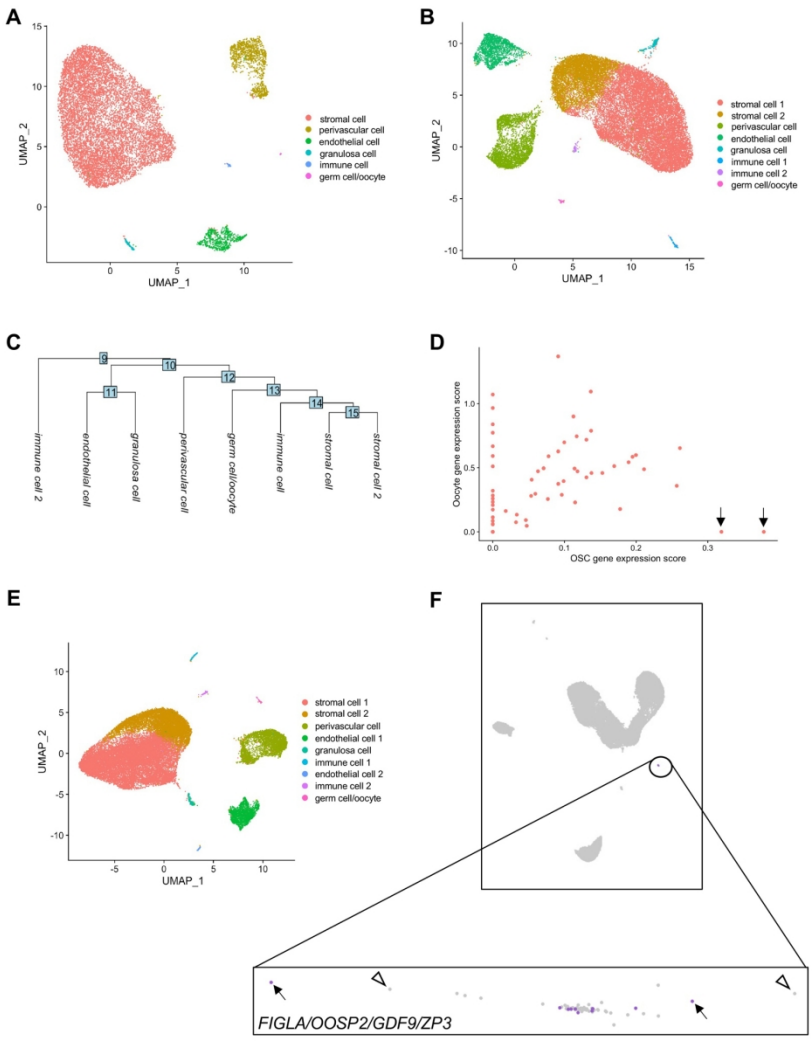
A
UMAP_2
UMAP_1
stromal cell
perivascular cell
endothelial cell
granulosa cell
immune cell
germ cell/oocyte
B
UMAP_2
UMAP_1
stromal cell 1
stromal cell 2
perivascular cell
endothelial cell
granulosa cell
immune cell 1
immune cell 2
germ cell/oocyte
C
9
10
11
12
13
14
15
immune cell 2
endothelial cell
granulosa cell
perivascular cell
germ cell/oocyte
immune cell
stromal cell
stromal cell 2
D
OSC gene expression score
OSC gene expression score
E
UMAP_2
UMAP_1
stromal cell 1
stromal cell 2
perivascular cell
endothelial cell 1
granulosa cell
immune cell 1
endothelial cell 2
immune cell 2
germ cell/oocyte
F
FIGLA/OOSP2/GDF9/ZP3

Figure 1


FIGLA
FIGLA/OOSP2
FIGLA/GDF9
FIGLA/ZP3
GDF9/ZP3
OOSP2
GDF9
ZP3

Figure 2

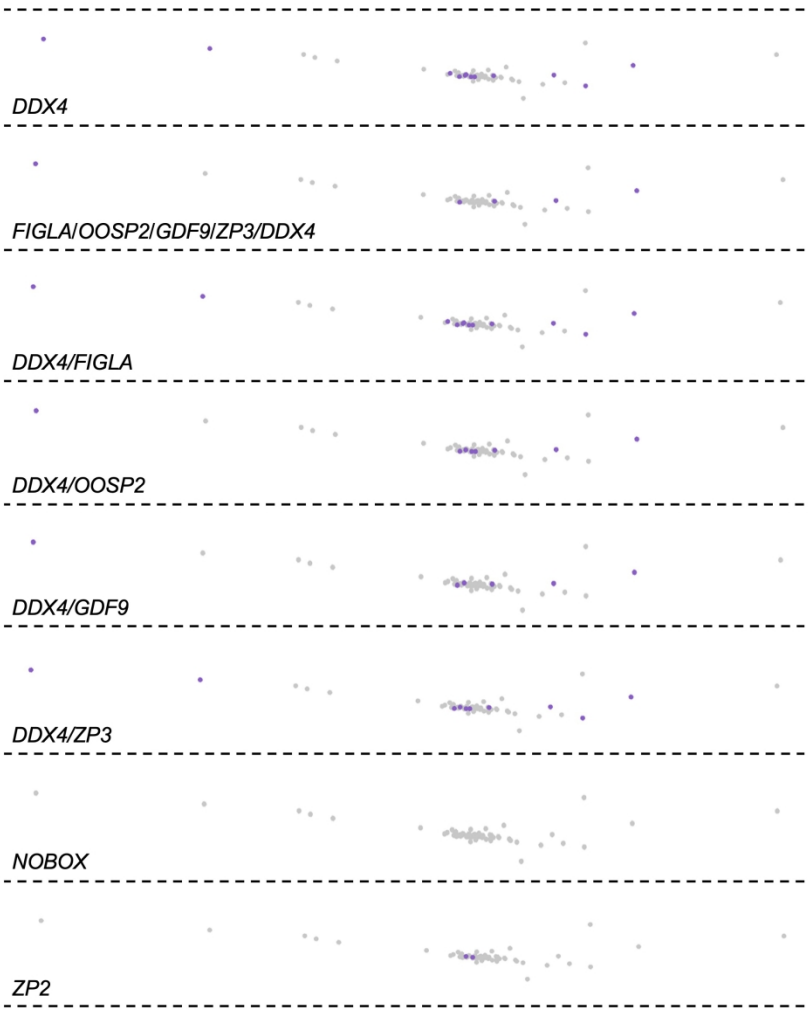
DDX4
FIGLA/OOSP2/GDF9/ZP3/DDX4
DDX4/FIGLA
DDX4/OOSP2
DDX4/GDF9
DDX4/ZP3
NOBOX
ZP2

Figure 3

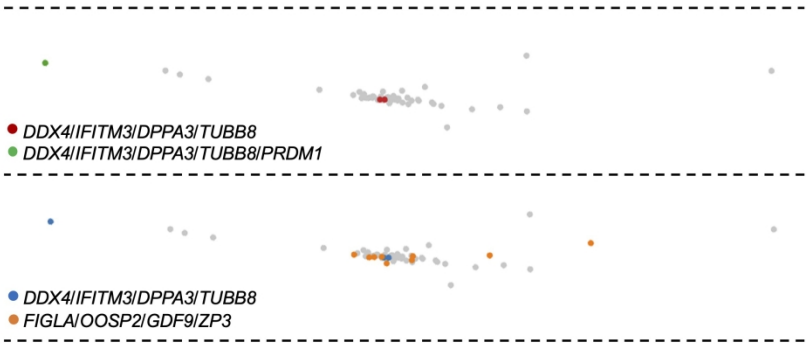
DDX4/IFITM3/DPPA3/TUBB8
DDX4/IFITM3/DPPA3/TUBB8/PRDM1
DDX4/IFITM3/DPPA3/TUBB8
FIGLA/OOSP2/GDF9/ZP3

Figure 4

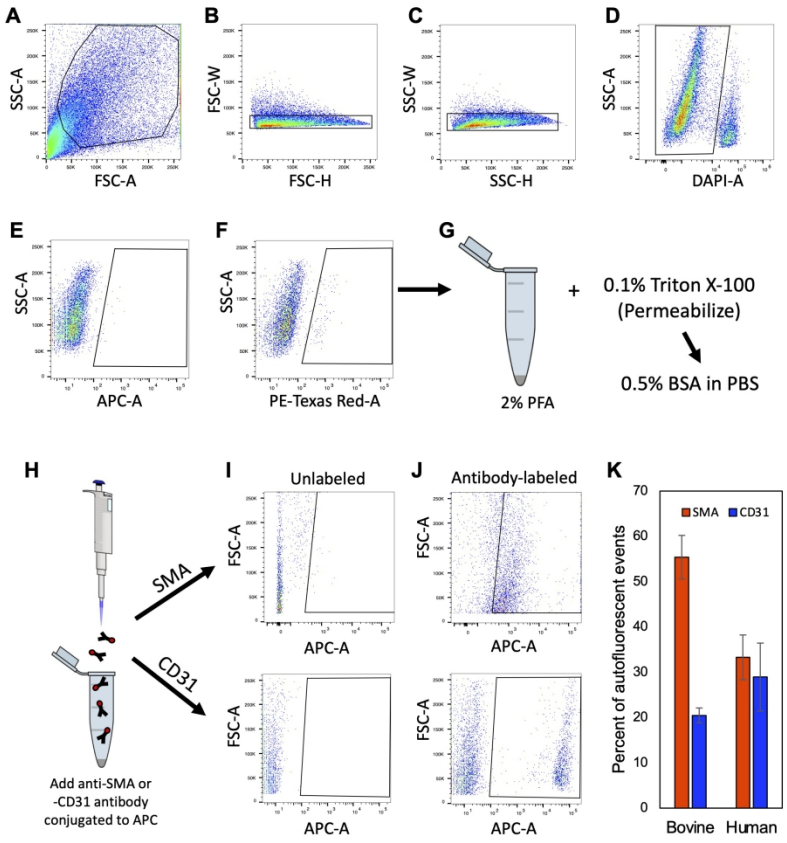
A
SSC-A
FSC-A
B
FSC-W
FSC-H
C
SSC-W
SSC-H
D
SSC-A
DAPI-A
E
SSC-A
APC-A
F
SSC-A
PE-Texas Red-A
G
2% PFA
+
0.1% Triton X-100
(Permeabilize)
0.5% BSA in PBS
H
SMA
CD31
Add anti-SMA or
-CD31 antibody
conjugated to APC
I
Unlabeled
FSC-A
APC-A
J
Antibody-labeled
FSC-A
APC-A
K
SMA
CD31
Percent of autofluorescent events
70
60
50
40
30
20
10
0
Bovine
Human

Figure 5

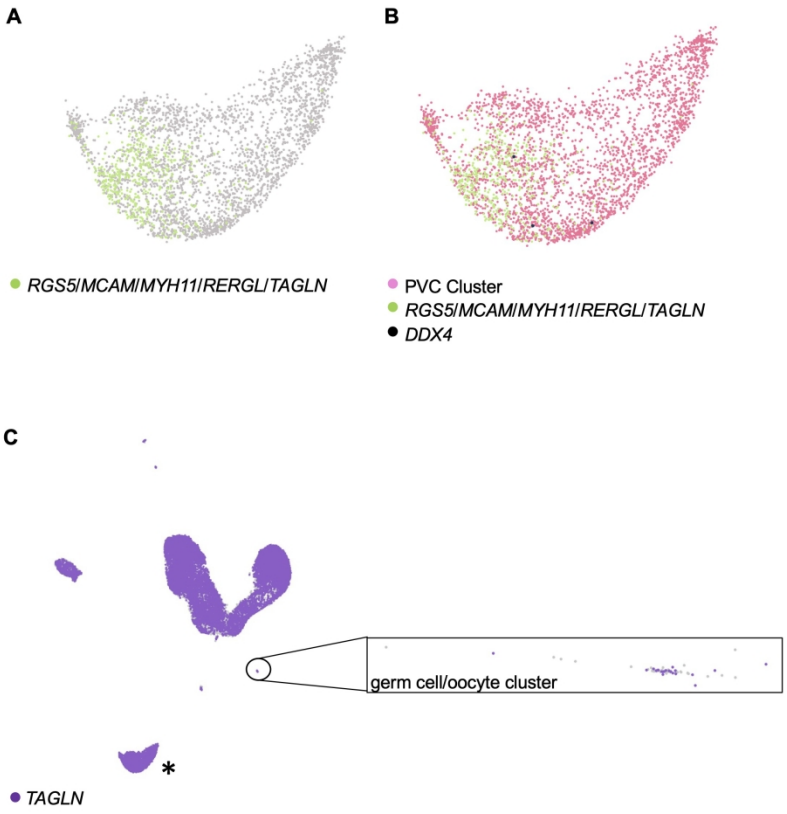
A
B
RGS5/MCAM/MYH11/RERGL/TAGLN
PVC Cluster
RGS5/MCAM/MYH11/RERGL/TAGLN
DDX4
C
germ cell/oocyte cluster
*
TAGLN

Figure 6

SUPPORTING INFORMATION

Workflow optimization for identification of female germline or oogonial stem cells in human ovarian cortex using single-cell RNA sequence analysis

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Supporting Information:

SUPPORTING INFORMATION MATERIALS AND METHODS

Method 1: Clustering and Analysis of scRNA-seq Data

After the initial Cell Ranger v6 filtration (see **MATERIALS AND METHODS** of the main text for additional details), the data for human unsorted ovarian cortical cells deposited by Wagner et al. (2020) were manually filtered further in Seurat for quality control. Genes found to be expressed in a minimum of three cells were retained for further analysis, and cells expressing between 200 to 5,000 genes, with no more than 20% of these being mitochondrial genes, were included in the downstream analyses. These parameters were selected after visualization of violin plots, as well as of scatter plots depicting mitochondrial gene percentage, 'nFeature-RNA' and 'nCount-RNA', in Seurat. After filtration, the cell pool consisted of 27,710 total cells (Supporting Information Table 6). Integration of the two data sets, derived from three caesarean section patient (CSP) samples (17,430 of the 27,710 total cells called) and one gender reassignment patient (GRP) sample (10,280 of the 27,710 total cells called), was completed using the CCA integration tool in Seurat. The preprocessed data were merged and regressed for batch effects. As reported by Wagner et al. (2020), the datasets were column-normalized and log-transformed prior to principal component analysis. For analysis of the data generated by Cell Ranger v6, elbow plots were generated in Seurat to determine the ideal number of principal components to include in downstream Uniform Manifold Approximation and Projection (UMAP) dimension reduction analysis. The elbow plots, as well as the calculated value for cumulative percent of variation, led us to utilize the first 18 principal components in our UMAP projection.

When Seurat was replaced with Loupe Browser to analyze the preprocessed data derived from Cell Ranger v6, distribution and clustering of the 27,710 total cells called was comparable across the two analytical platforms. However, we noted that the germ cell/oocyte cluster was reduced from 65 total cells using Seurat to 62 total cells using Loupe Browser; the three cells clustered by Seurat as germ cells/oocytes that were not included in the germ cell/oocyte cluster by Loupe Browser likely represented oocyte-granulosa cell doublets (see Supporting Information Results 1 for more details), which were excluded by Loupe Browser, but not Seurat, from this cluster. For Loupe Browser analysis, the following parameters were used:

- minimum and maximum thresholds for unique molecular identifier (UMI) counts: 339 and 160,107, respectively
- minimum and maximum thresholds for features expressed: 200 and 5,000, respectively
- maximum mitochondrial UMI counts: 20%
- number of principal components: 18
- minimum distance: 0.1
- number of neighbors: 59

Method 2: Flow Cytometric Analysis

For fluorescence-activated cell sorting (FACS), a combined approach of doublet discrimination using forward-scatter (FSC-A) versus side-scatter (SSC-A) parameters of pulse-height, pulse-width, and pulse-area, as well as dead cell exclusion using 4',6-diamidino-2-phenylindole (DAPI) labeling, was applied. Comparison of autofluorescent events detected in the APC-A far-red channel (640-nm laser) versus the

PE-Texas red-A channel (561-nm laser) was completed by gating the FACS plots manually using the same gating strategy across all samples. To identify whether autofluorescent cells were PVCs, positive events detected in the PE-Texas red-A channel were sorted directly into 2% paraformaldehyde for fixation, permeabilized with 0.1% Triton X-100 on ice for 10 minutes, and then centrifuged to obtain cell pellets. The pellets were resuspended in cell permeabilization and blocking buffer (0.3% Triton X-100 and 0.5% bovine serum albumin in 1X-concentrated phosphate-buffered saline) and incubated overnight at 4 C. Samples were then separated into three tubes to allow for fluorescence-gating controls before addition of primary antibodies (see **MATERIALS AND METHODS** of the main text for additional details).

Method 3: Estimating Required Input Cell Number to Detect OSCs

The identification of three non-oocyte germ cells in this dataset was initially unexpected given the estimate that 85,440 total cells would be needed to identify five OSCs, based on their rarity, at 95% confidence (see **RESULTS** of the main text for additional details; see also Supporting Information Fig. 1). However, only one of these three cells could be viewed as a candidate OSC since the other two cells exhibited a meiotic gene expression profile consistent with early germ cell differentiation associated with oogenesis. It should be emphasized that OSC frequency in ovaries in situ (0.014%) (White et al., 2012) will differ from their frequency in a single-cell suspension following enzymatic tissue disaggregation, filtration, and processing for scRNA-seq or FACS, which will also differ from the yield of OSCs, calculated as a percent of the total viable cells sorted, following FACS (1.7%) (White et al., 2012). In other words, each step of processing and analysis alters cell ratios, which explains the variation in OSC frequency at the various steps of sample processing. Therefore, modeling analysis in this particular case is extremely difficult to perform since the actual frequency of OSCs in a single-cell preparation submitted for scRNA-seq is unknown.

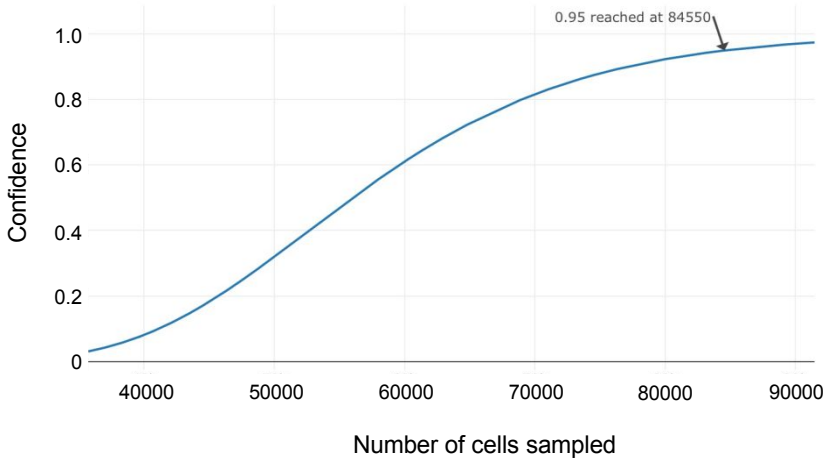
SUPPORTING INFORMATION RESULTS

Results 1: *ZP3* Expression in the Granulosa Cell Cluster

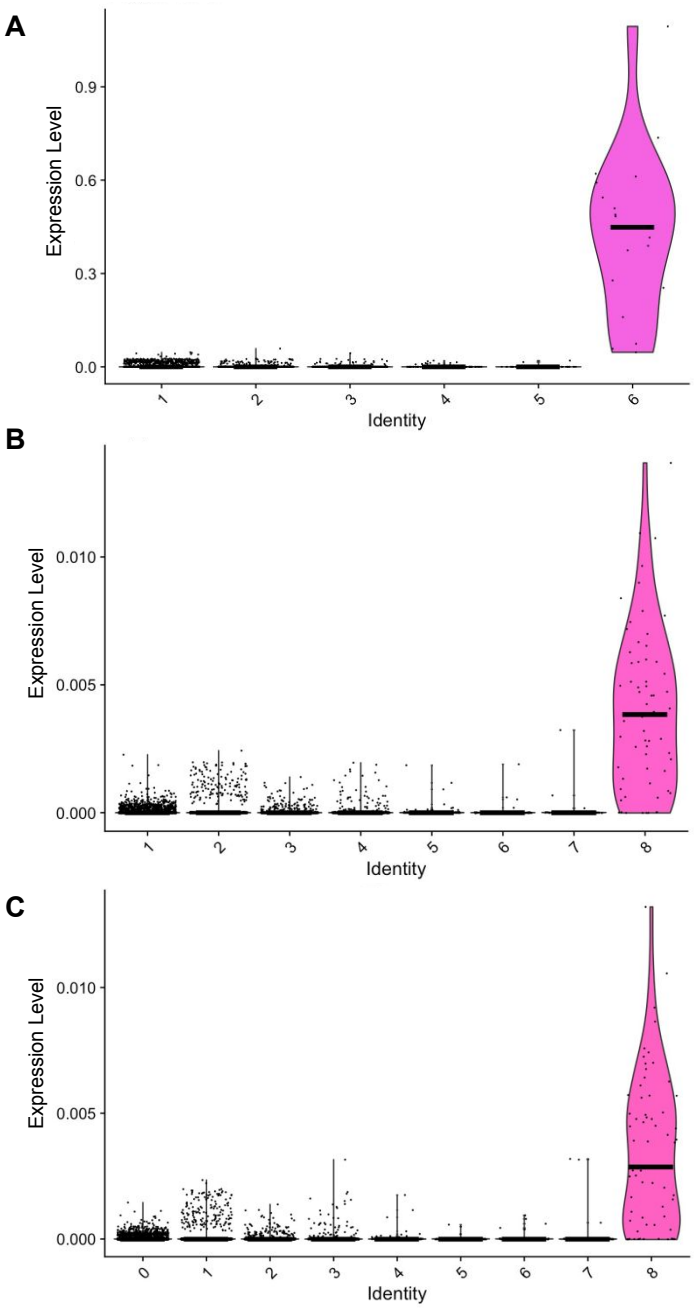
Following analysis with Cell Ranger v6 and Loupe Browser, we identified seven *ZP3*-expressing cells in the granulosa cell cluster (data not shown). Two of these *ZP3*-expressing cells also expressed *FIGLA*, whereas one co-expressed *GDF9*. Even more surprisingly, of the seven *ZP3*-expressing cells in the granulosa cell cluster, five co-expressed *FOXL2* (forkhead box L2), six co-expressed *AMHR2* (anti-Müllerian hormone receptor type 2), and four co-expressed both *FOXL2* and *AMHR2* (data not shown). The finding that two widely-used markers of granulosa cells (*FOXL2* and *AMHR2*) were co-expressed with *ZP3* indicates that either granulosa cell-oocyte doublets exist in their reported single-cell dataset or that expression of *ZP3*, as a standalone gene, is not specific to oocytes (see also **RESULTS** and **DISCUSSION** of the main text for additional details on *ZP3* expression analysis).

Figures 1–6 and Tables 1–7

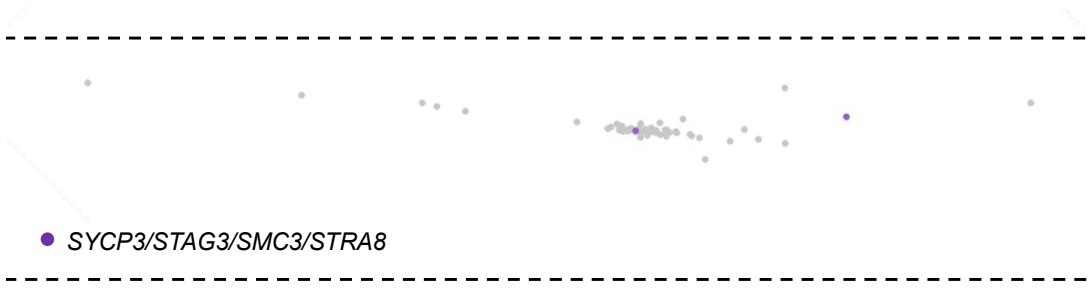
These Supporting Information display items are provided, starting on the next page, in sequence below.



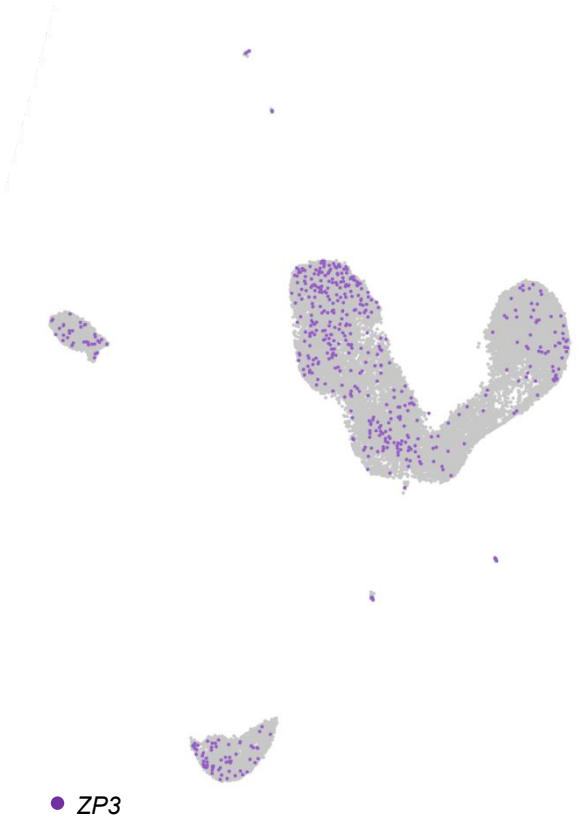
Supporting Information Figure 1. Estimated number of cells needed to detect, at 95% confidence, at least five OSCs in a dispersed human ovarian cortical cell preparation consisting of six cell types using scRNA-seq.



Supporting Information Figure 2. Violin plots showing the expression scores of genes associated with identification of oocytes (*FIGLA*, *OOSP2*, *GDF9* and *ZP3*) using Cell Ranger v2 (**A**), v3 (**B**) or v6 (**C**). Median gene expression is depicted by horizontal lines in each violin plot.

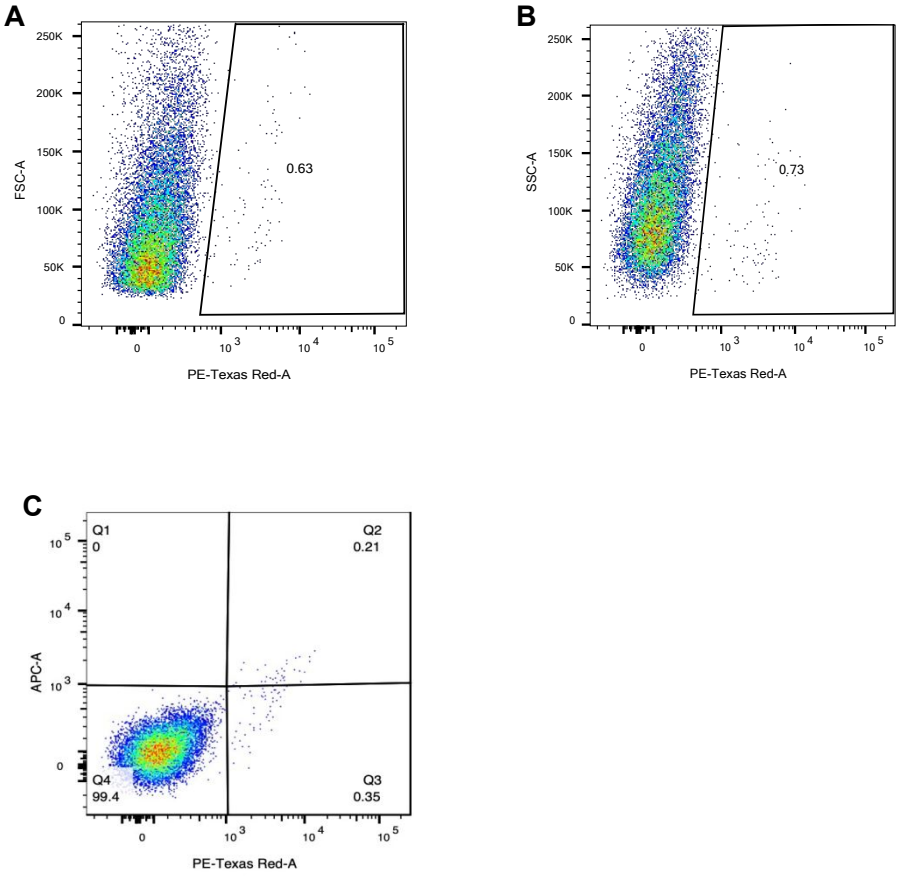


Supporting Information Figure 3. Loupe Browser analysis of expression of meiosis-I genes (*SYCP3*, *STAG3*, *SMC3* and *STRA8*) in the 62-cell germ cell/oocyte cluster identified in the adult human unsorted ovarian cortical cell dataset of Wagner et al. (2020). The two purple dots are cells identified as positive for co-expression of the indicated genes whereas all remaining light-gray dots are cells lacking detectable levels of expression of the indicated genes.



Supporting Information Figure 4. Loupe Browser analysis of *ZP3* expression across the entire adult human unsorted ovarian cortical cell dataset of Wagner et al. (2020), highlighting its promiscuous expression in all clusters (cell types). The purple dots are cells identified as positive for *ZP3* expression whereas light-gray dots are cells lacking detectable levels of *ZP3* expression.

Supporting Information Figure 5. Loupe Browser analysis of the 62-cell germ cell/oocyte cluster identified in the adult human unsorted ovarian cortical cell dataset of Wagner et al. (2020) for *DAZL* expression alone (upper panel, purple dots) or *DAZL* co-expressed with the four genes used by Wagner et al. (2020) to identify oocytes (lower panel, blue dots). Light-gray dots: negative for expression of the indicated gene(s).



Supporting Information Figure 6. Detection of autofluorescent events in DAPI-negative cells sorted from dispersed human ovarian cortical tissue independent of whether the samples were gated against FSC-A or SSC-A, or if PE-Texas red-A (AF594, 561-nm) was plotted against another laser (APC, 640-nm).

Supporting Information Table 1. Published studies that have used C-terminal DDX4 antibodies with magnetic-assisted cell sorting (MACS) or fluorescence-activated cell sorting (FACS) to isolate oogonial stem cells (OSCs). Green-shaded boxes: successful OSC isolation; yellow-shaded boxes: successful OSC isolation with questions about antibody-specificity; red-shaded boxes: unsuccessful OSC isolation.

Species	Method	Reference
Mouse	MACS	Zou et al. 2009 (Nat Cell Biol 11: 631–636)
Mouse	MACS	Zhang et al. 2011 (J Mol Cell Biol 3: 132–141)
Mouse, Human	MACS/FACS	White et al. 2012 (Nat Med 18: 413–421)
Mouse	FACS	Imudia et al. 2013 (Fertil Steril 100: 1451–1458)
Mouse	FACS	Park et al. 2013 (Fertil Steril 100: 1468–1475)
Mouse, Human	FACS	Woods and Tilly 2013a (Nat Protoc 8: 966–988)
Baboon	FACS	Woods and Tilly 2015a (Nat Med 21: 1118–1121)
Mouse	FACS	Park and Tilly 2015 (Mol Hum Reprod 21: 58–65)
Human	FACS	Fakih et al. 2015 (JFIV Reprod Med Genet 3: 154)
Human	FACS	Oktay et al. 2015 (Reprod Sci 12: 1612–1617)
Mouse	MACS	Khosravi-Farsani et al. 2015 (Cell J 16: 406–415)
Mouse	FACS	Navaroli et al. 2016 (Methods Mol Biol 1457: 253–268)
Mouse, Human	MACS	Ding et al. 2016 (Sci Rep 6: 28218)
Mouse	MACS	Wu et al. 2017 (Mol Ther 25: 1408–1419)
Mouse	FACS	Wang et al. 2017 (Sci Rep 7: 10011)
Mouse	MACS	Ye et al. 2017 (Cell Physiol Biochem 41: 1051–1062)
Porcine	FACS	Tasi et al. 2017 (Oncotarget 8: 63484–63505)
Mouse	MACS	Liu et al. 2017 (J Biol Chem 292: 16003–16013)
Mouse	MACS	Yazdekhasti et al. 2017 (Cell Reprogram 19: 132–144)
Human	FACS	Clarkson et al. 2018 (Sci Rep 8: 6953)
Human	MACS	Silvestris et al. 2018 (Hum Reprod 33: 464–473)
Human	FACS	Bothun et al. 2018 (Stem Cells Dev 27: 723–735)
Mouse, Human	FACS	MacDonald et al. 2019 (Fertil Steril 111: 794–805)
Mouse	MACS	Ma et al. 2019 (DNA Res 26: 105–117)
Mouse	MACS	Zou et al. 2019 (Cell Prolif 52: e12530)
Mouse	FACS	Satirapod et al. 2020 (Aging (Albany NY) 12: 7313–7333)
Mouse	MACS	Zhao et al. 2021a (Mol Ther Nucleic Acids 23: 431–439)
Mouse	MACS	Huang et al. 2021 (Reprod Biol Endocrinol 19: 14)
Mouse	MACS	Yuan et al. 2021 (Cell Biosci 11: 107)
Mouse	MACS	Li et al. 2021a (Front Cell Dev Biol 9: 640402)
Mouse	FACS	MacDonald et al. 2021 (Stem Cells Dev 30: 749–757)
Human	MACS	Ariyath et al. 2021 (Cells Tissues Organs doi:10.1159/000519087)
Human	MACS	Sequeira et al. 2021 (Cell Tissue Res 386: 145–156)

Mouse	MACS	Li et al. 2021b (Biomaterials doi:10.1016/j.biomaterials.2021.121213)
Monkey		Li et al., 2021c (Stem Cell Rev Rep doi:10.1007/s12015-021-10278-9)
Mouse	MACS	Ma et al. 2021 (J Int Med Res 49: 3000605211029461)
Mouse	MACS	Tian et al. 2022 (Cell Mol Life Sci 79: 22)
Mouse, Monkey, Human	FACS	Hernandez et al. 2015 (Nat Med 21: 1114–1116)
Monkey	FACS	Yuan et al. 2013 (Stem Cells 31: 2538–2550)
Mouse, Human	FACS	Zhang et al. 2015 (Nat Med 21: 1116–1118)*†
Mouse	FACS	Zarate-Garcia et al. 2016 (Sci Rep 6: 27991)
Human	FACS	Wagner et al. 2020 (Nat Commun 11: 1147)*

*Same research group contributors.
†See rebuttal in Woods and Tilly (2015b).

Supporting Information Table 2. Published strategies other than DDX4 antibody-based sorting that have been successfully used to isolate mammalian oogonial stem cells for analysis. EGFP, enhanced green fluorescent protein; EYFP, enhanced yellow fluorescent protein; FACS, fluorescence-activated cell sorting; IFITM3, interferon-induced transmembrane protein 3; MACS, magnetic-assisted cell sorting; SSEA4, stage-specific embryonic antigen 4.

Species	Method	Reference
Mouse	EGFP-based selection	Pacchiarotti et al. 2010 (Differentiation 79: 159–170)
Mouse	IFITM3 MACS	Zou et al. 2011 (Stem Cells Dev 20: 2197–2204)
Mouse	Selection in culture	Hu et al. 2012 (Cell Prolif 45: 287–298)
Porcine	Selection in culture	Bai et al. 2013 (Cell Prolif 46: 516–528)
Mouse	IFITM3 MACS	Wang et al. 2014 (J Mol Cell Biol 6: 164–171)
Rat	IFITM3 MACS	Zhou et al. 2014 (Mol Hum Reprod 20: 271–281)
Mouse	IFITM3 MACS	Xie et al. 2014 (Sci Rep 4: 5580)
Mouse	IFITM3 MACS	Xiong et al. 2015 (PLoS One 10: e0139824)
Mouse	Selection in culture	Parvari et al. 2015 (Arch Med Sci 11: 670–678)
Mouse	Selection in culture	Parvari et al. 2016 (Cell Reprogram 18: 419–428)
Mouse	EGFP FACS	Zhang and Wu 2016 (Mol Hum Reprod 22: 457–464)
Mouse	IFITM3 MACS	Lu et al. 2016 (Stem Cells Int 2016: 2749461)
Mouse	IFITM3 MACS	Zhang et al. 2016 (Genome Biol 17: 162)
Mouse	EYFP-based selection	Guo et al. 2016 (Mol Hum Reprod 22: 316–328)
Mouse	IFITM3 FACS	Navaroli et al. 2016 (Methods Mol Biol 1457: 253–268)*
Mouse	EGFP FACS	Li et al. 2017a (Oncotarget 8: 26573–26590)
Mouse	EGFP FACS	Li et al. 2017b (Sci Rep 7: 3729)
Bovine	Differential migration	de Souza et al. 2017 (Reprod Domest Anim 52: 243–250)
Mouse	IFITM3 MACS	Wang et al. 2018 (Gene 653: 14–21)
Porcine	IFITM3 MACS	Hou et al. 2018 (Cell Transplant 27: 1195–1202)
Mouse	Differential adhesion	Wu et al. 2018 (Cell Physiol Biochem 46: 2114–2126)
Mouse	IFITM3 MACS	Gu et al. 2018 (Mol Omics 14: 95–102)
Mouse	EGFP-based selection	Yang et al. 2018 (Cytotechnology 70: 843–854)
Mouse	IFITM3 MACS	Zhang et al. 2018 (Cell Mol Life Sci 75: 1241–1253)
Mouse	IFITM3 MACS	Zhu et al. 2018 (Arch Toxicol 92: 1581–1591)
Mouse	Selection in culture	Jiang et al. 2019a (Int J Mol Sci 20: 3605)
Mouse	Selection in culture	Jiang et al. 2019b (Stem Cell Res Ther 10: 198)
Mouse	EGFP FACS	Li et al. 2019a (Mol Ther Nucleic Acids 17: 436–447)
Mouse	EGFP FACS	Li et al. 2019b (Cells 8: 606)

Mouse	EGFP FACS	Li et al. 2019c (Sci Data 6: 8)
Porcine	SSEA4 MACS	Nguyen et al. 2019 (J Reprod Dev 65: 423–432)
Mouse	IFITM3 MACS	Wu et al. 2019 (Aging (Albany NY) 11: 1030–1044)
Mouse	IFITM3 MACS	Zhang et al. 2019 (Stem Cells 37: 1095–1107)
Mouse	IFITM3 MACS	Zhao et al. 2021b (Stem Cell Rev Rep 17: 938–951)
Mouse	EGFP FACS	Chen et al. 2021 (Gene 766: 145150)
Mouse	Differential adhesion	Saber et al. 2021 (Toxicol In Vitro 70: 105032)
Porcine	SSEA4 MACS	Pennarossa et al. 2021 (Methods Mol Biol 2273: 139–149)
Human	DDX4/IFITM3 MACS	Sequeira et al. 2021 (Cell Tissue Res 386: 145–156)*

*Also cited in Supporting Information Table 1.

Supporting Information Table 3. Contemporary studies published since 2004 supporting or refuting the existence of oogonial stem cells (OSCs) and/or postnatal oogenesis in mammals. Green-shaded boxes: studies that support OSCs and/or postnatal oogenesis; red-shaded boxes: studies that refute OSCs and/or postnatal oogenesis. These publications do not include any of those already listed in Supporting Information Tables 1 and 2.

Species	Reference
Mouse	Johnson et al. 2004 (Nature 428: 145–150)
Mouse	Johnson et al. 2005 (Cell 122: 303–315)
Mouse	Lee et al. 2007a (Cell Cycle 6: 2678–2684)
Mouse	Lee et al. 2007b (J Clin Oncol 25: 3198–3204)
Mouse	Niikura et al. 2009 (Aging (Albany NY) 1: 971–978)
Mouse	Wang and Tilly 2010 (Cell Cycle 9: 339–349)
Mouse	Joo et al. 2014 (Reprod Biol Endocrinol 12: 113)
Mouse	Lai et al. 2015 (J Transl Med 13: 155)
Mouse	Li et al. 2015 (Cell Physiol Biochem 36: 1712–1724)
Mouse	Pan et al. 2015 (Cell Physiol Biochem 37: 2311–2322)
Mouse	Sharma and Bhartiya 2021 (Stem Cell Rev Rep 17: 1695-1711)
Mouse	Bristol-Gould et al. 2006 (Dev Biol 298: 149–154)
Mouse	Liu et al. 2007 (Dev Biol 306:112–120)*¶
Mouse	Veitia et al. 2007 (Stem Cells 25: 1334–1335)
Mouse	Zhang et al. 2012 (Proc Natl Acad Sci USA 109: 12580–12585)†
Mouse	Lei et al. 2013 (Proc Natl Acad Sci USA 110: 8585–8590)
Mouse	Zhang et al. 2014 (Proc Natl Acad Sci USA 111: 17983–17988)†‡

*Same senior/corresponding authors for Yuan et al. (Stem Cells 2013 31: 2538–2550) in Supporting Information Table 1.

¶See rebuttal in Tilly and Johnson (2007). See also Tilly et al. (2009), Woods and Tilly (2013b), Woods et al. (2013), Woods and Tilly (2015b), Akahori et al. (2019), and Martin et al. (2019) for additional points of discussion on OSCs and postnatal oogenesis.

†Same corresponding author for Zhang et al. (Nat Med 2015 21: 1116–1118) in Supporting Information Table 1.

‡Same co-corresponding author for Zhang et al. (Nat Med 2015 21: 1116–1118), and same contributing author for Wagner et al. (Nat Commun 2020 11: 1147), in Supporting Information Table 1.

Supporting Information Table 4. Comparison of output data obtained using three different versions of Cell Ranger software to analyze the human unsorted ovarian cortical cell scRNA-seq dataset of Wagner et al. (2020). GRP, gender reassignment patient (n = 1); CSP, caesarean section patients (n = 3).

	GRP	CSP	GRP	CSP	GRP	CSP	GRP	CSP	GRP	CSP
Cell Ranger version	v2.1.1		v3.0.2		v6.0.1		v3.0.2		v6.0.1	
Human genome assembly	HG19		HG19		HG19		HG38		HG38	
Estimated cell number	5725	6315	10167	17310	10165	17401	10329	17600	10387	17622
Fraction reads in cells (%)	69.3	55.4	81.7	79.5	81.6	79.7	82.0	79.9	82.2	79.9
Mean reads per cell	78871	66806	44412	24372	44421	24245	43715	23970	43472	23941
Genes per cell*	1517	1494	1183	932	1179	929	1213	955	1205	952
Total genes detected	22351	22831	22724	23547	22719	23526	25284	26368	25306	26347
Median UMI counts per cell	5729	5401	4127	2648	4116	2639	4210	2694	4175	2680

*Due to their use of Cell Ranger v2 for analysis of human unsorted ovarian cortical cells, Wagner et al. (2020) report their results as mean genes per cell, but all subsequent versions of Cell Ranger (version 3 and above) only report median genes per cell. Thus, values for this endpoint using v2.1.1 are displayed as mean genes per cell whereas all remaining values for this endpoint (v.3.0.2 and v.6.0.1) are displayed as median genes per cell.

Note: the following alert was displayed for both GRP and CSP samples when run in Cell Ranger v2, as performed by Wagner et al. (2020), but not in any of the subsequent versions of Cell Ranger software (v3 and above): "The analysis detected some issues. Low Fraction Reads in Cells. Value 61.3% [GRP, 69.3%; CSP, 55.4%]. Ideal= >70%. Application performance may be affected. Many of the reads were not assigned to cell-associated barcodes. This could be caused by high levels of ambient RNA or by a significant population of cells with a low RNA content, which the algorithm did not call as cells. The latter case can be addressed by inspecting the data to determine the appropriate cell count and using --force-cells." This automatic alert to a significant issue with analysis of the unsorted ovarian cortical cell dataset using Cell Ranger v2 would have been easily remedied by using Cell Ranger v3. However, Wagner et al. (2020) elected to continue with Cell Ranger v2 for their *unsorted* cell analysis while switching to Cell Ranger v3 for their *flow cytometry-sorted* cell analysis.

Supporting Information Table 5. Overview of the genes utilized to identify the indicated cell type(s) or biological process commonly associated with each gene, along with the reported principal role(s) of the protein encoded by each gene. Refer to the main text for relevant references; also see Fagerberg et al., 2014. Multi-gene panels are needed to perform each cell type or process association with confidence

Cell Type or Process	Gene	Principal Role(s) of Encoded Protein
Germ cells		
	PRDM1	Epigenetic modulator commonly used to identify primitive (premeiotic) germ cells
	DPPA3	Epigenetic modulator commonly used to identify primitive (premeiotic) germ cells
	IFITM3	Transmembrane protein implicated in the homing of embryonic (premeiotic) germ cells during migration
	*TUBB8**	Beta-tubulin isotype implicated in oocyte maturation; also expressed in premeiotic (primordial) germ cells but function unknown
	*DDX4**	Putative RNA helicase commonly used to identify pre- and post-meiotic germ cells
	*DAZL**	RNA binding-protein expressed in both pre- and post-meiotic germ cells
Oocytes		
	FIGLA	Transcription factor commonly used to identify oocytes, but expressed in both pre-and post-meiotic germ cells
	OOSP2	Oocyte-enriched protein identified in mouse ovaries; lineage specificity in human ovaries is unknown, but transcriptomic expression of the gene in humans is not restricted to oocytes
	GDF9	Transforming growth factor (TGF)-β family member expressed at high levels in oocytes
	ZP1	Glycoprotein component of the oocyte zona pellucida that ensures structural integrity
	ZP2	Glycoprotein component of the oocyte zona pellucida that may act as a secondary sperm receptor along with ZP3
	ZP3	Glycoprotein component of the oocyte zona pellucida involved in sperm binding during fertilization; exhibits promiscuous low-level expression in many other cell types
	NOBOX	Transcription factor expressed in oocytes at all stages of development

Meiosis		
	SYCP3	Core component of the synaptonemal complex; involved in recombination and segregation of chromosomes in germ cells during meiosis-I
	STAG3	Meiosis-specific component of the cohesin complex which is required for cohesion of sister chromatids in germ cells after DNA replication
	SMC1a	Central component of the cohesin complex which forms during meiosis-I in germ cells
	SMC3	A central component of the cohesin complex which heterodimerizes with SMC1A during meiosis-I in germ cells
	STRA8	Drives meiotic commitment in germ cells; required for premeiotic DNA replication and progression through meiotic prophase
Perivascular Cells (PVCs)		
	RGS5	Regulator of G-protein signaling; GTPase inhibitor
	MCAM	Cell surface glycoprotein that acts as an adhesion and cohesion molecule in vascular endothelial cells
	MYH11	Smooth muscle myosin involved in cytoskeletal motor activity
	RERGL	Modulator of G protein signaling
	TAGLN	Actin-binding protein involved in calcium-dependent smooth muscle contraction

*****These genes are also expressed in oocytes, and thus each gene, when used alone, should be viewed as a general germ lineage marker.

Supporting Information Table 6. Number of cells called in the clusters identified in the human unsorted ovarian cortical cell dataset of Wagner et al. (2020) under the various analytical conditions (GRP: gender reassignment patient, n = 1; CSP: caesarean section patients, n = 3). The oocyte/germ cell cluster is highlighted by gray shading in each analysis.

Cell Ranger v2, Seurat v3, HG19

Cluster	Number of GRP Cells	Number of CSP Cells	GRP + CSP Cells Identified
1	5127	4872	9999
2	421	770	1191
3	142	485	627
4	5	132	137
5	19	29	48
6	4	14	18
			Total cells identified: 12020

Cell Ranger v3, Seurat v3, HG19

	Number of GRP Cells	Number of CSP Cells	GRP + CSP Cells Identified
1	7032	8414	15446
2	1876	4178	6054
3	739	2633	3372
4	387	1593	1980
5	13	192	205
6	35	127	162
7	35	60	95
8	14	48	62
			Total cells identified: 27376

Cell Ranger v6, Seurat v4, HG38

	Number of GRP Cells	Number of CSP Cells	GRP + CSP Cells Identified
1	6885	8213	15098
2	2196	4604	6800
3	729	2624	3353
4	355	1492	1847
5	13	185	198
6	35	124	159
7	16	88	104
8	33	53	86
9	18	47	65
			Total cells identified: 27710

Supporting Information Table 7. Stem cell types other than OSCs that have been identified in adult mammalian ovaries. ESC, embryonic stem cell; MSC, mesenchymal stem cell; VSEL, very small embryonic-like stem cell. See also Supporting Information Discussion 2 for more details.

Cell Type	Species	Reference
ESC-like*	Mouse	Szotek et al. 2008 (Proc Natl Acad Sci USA 105: 12469–12473)
ESC-like*	Mouse	Gong et al. 2010 (Fertil Steril 93: 2594–2601)
VSEL	Human	Virant-Klun et al. 2008 (Differentiation 76: 843–856)
VSEL	Human	Virant-Klun et al. 2009 (Stem Cells Dev 18: 137–149)
VSEL	Human	Virant-Klun et al. 2011 (J Biomed Biotechnol 2011: 381928)
VSEL	Rabbit, Sheep, Monkey, Human	Parte et al. 2011 (Stem Cells Dev 20: 1451–1464)
VSEL	Human	Virant-Klun et al. 2013a (Biomed Res Int 2013: 690415)
VSEL	Human	Virant-Klun et al. 2013b (Biomed Res Int 2013: 861460)
VSEL	Human	Virant-Klun et al. 2013c (J Ovarian Res 6: 24)
VSEL	Mouse, Sheep	Bhartiya et al. 2013 (J Ovarian Res 6: 65)
VSEL	Sheep	Patel et al. 2013 (J Ovarian Res 6: 52)
VSEL	Marmoset, Human	Parte et al. 2013 (J Ovarian Res 6: 20)
VSEL	Human	Stimpfel et al. 2013 (Cell Tissue Res 354: 593–607)
VSEL	Sheep, Human	Parte et al. 2014 (J Ovarian Res 7: 25)
VSEL	Porcine	Bui et al. 2014 (Development 141: 2235–2244)
VSEL	Sheep	Bhartiya 2015 (J Ovarian Res 8: 70)
VSEL	Mouse, Sheep, Rabbit, Monkey, Human	Parte et al. 2015 (Methods Mol Biol 1235: 203–229)
VSEL	Mouse	Sriraman et al. 2015 (Reprod Sci 22: 884–903)
VSEL	Mouse	Esmaeilian et al. 2017 (Zygote 25: 358–375)
VSEL	Human	Virant-Klun 2018 (Stem Cell Rev Rep 14: 715–721)
VSEL	Sheep	Patel et al. 2018 (J Ovarian Res 11: 3)
VSEL	Mouse	Sharma and Bhartiya, 2021 (Stem Cell Rev Rep 17: 1695-1711)
MSC	Human	Kossowka-Tomaszczuk et al. 2009 (Stem Cells 27: 210–219)
MSC	Porcine	Song et al. 2011 (Stem Cells Dev 20: 1359–1370)
MSC	Human	Stimpfel et al. 2013 (Cell Tissue Res 354: 593–607)
MSC	Human	Stimpfel et al. 2014 (J Assist Reprod Genet 31: 959–974)
MSC	Human	Riva et al. 2014 (Reprod Biomed Online 29: 457–469)

MSC	Human	Dzafic et al. 2014 (Biomed Res Int 2014: 508216)
MSC	Bovine	Lange-Consiglio et al. 2016 (Cell Reprogram 18: 116–126)
MSC	Mouse	Lee et al. 2016 (J Ovarian Res 9: 24)
MSC	Canine	Trindale et al. 2017 (Cell Prolif 50: e12391)
MSC	Canine	Hill et al. 2018 (J Vis Exp 142)
MSC	Human	Virant-Klun et al. 2019 (Stem Cell Rev Rep 15: 543–557)
MSC	Mouse	Zolbin et al. 2020 (In Vitro Cell Dev Biol Anim 56: 59–66)
MSC	Human	Rungsiwiwut et al. 2021 (Hum Cell 34: 300–309)

*****The stem cells reported in these studies are likely VSELs but were not identified as such.

SUPPORTING INFORMATION DISCUSSION

Discussion 1: Significant Cell Damage and Loss in the Wagner et al. scRNA-seq Workflow

A major factor that affects the interpretational quality of the data obtained by scRNA-seq is the initial quality of the sample analyzed. To this end, we note that Wagner et al. (2020) identified a total of only 18 oocytes in their dataset following Cell Ranger v2 analysis, which is alarmingly low compared to the total number of oocytes that would be expected in a dataset derived from four human ovarian cortical tissue biopsies as starting material. If one evaluates the representative histological section of an ovarian biopsy shown in the first figure of the Wagner et al. (2020) study, more than 200 oocytes are visible in the cortex of a single section, which is far less than the total number of oocytes present in an entire cortical biopsy. With four cortical biopsies used as starting material for their unsorted ovarian cell analysis, thousands of oocytes should be present. Even with the optimized analytical workflow reported herein, our analysis of their dataset using the four-gene panel selected by Wagner et al. (2020) for defining the oocyte cluster did not improve this outcome in terms of total number of oocytes identified. It is therefore not surprising that OSCs, as well as other rare stem cell types not acknowledged or discussed by Wagner et al. (2020) but which are known to be present in human ovarian cortex (Supporting Information Table 7), went undetected in their study (see also Supporting Information Discussion 2).

Digging deeper into the issue of extensive cell damage and loss in the Wagner et al. (2020) study, the authors state for their flow cytometry studies that after "*quality control and filtration, there were 5479 Ab+ and 6690 Ab− cells available for* [scRNA-seq] *analysis*". This statement raises two key questions. Why did the authors only obtain a total of 12,169 cells from their starting material, which contains hundreds of thousands, if not millions, of cells? How did the large indistinct smear of "DDX4+" events depicted in their FACS analysis shown in their supplementary data represent only 5,479 viable cells? The answer to both questions is again, most likely, a very high level of cell damage and death during their sample processing, which would be consistent with the massive cell loss in their analytical pipeline reflected by actual versus expected numbers of oocytes detected in their unsorted ovarian cell dataset discussed above. In turn, non-specific antibody binding is a well-established artifact of samples containing damaged and dead cells when analyzed by FACS, which produces a smear of events representing "positive" and "negative" cells [as shown by Wagner et al. (2020) in the supplementary data section of their published study] rather than a distinct population shift of true antibody-positive cells. When considered with the issue of cellular autofluorescence associated with PVCs documented in the main text (see **RESULTS**), along with the recurrent difficulty these authors have had in obtaining consistent results in their own studies using FACS with human ovarian cortical tissues [compare Zhang et al. (2015) and Wagner et al. (2020)], we believe that these observations collectively offer several reasonable explanations for how Wagner et al. (2020) isolated a cell fraction enriched for PVCs, but also containing several other random somatic cell types, following DDX4 antibody-based sorting of dispersed ovarian cortical tissues instead of OSCs.

Discussion 2: Ovarian Stem Cells Other than OSCs also Missed by Wagner et al. (2020)

Considering that adult mammalian ovaries harbor a variety of rare stem cell types in addition to OSCs (Supporting Information Table 7), it is noteworthy that Wagner et al. (2020) failed to identify *any* stem cell type in their adult human ovarian cortical cell dataset by scRNA-seq. Other than OSCs, very small

embryonic-like stem cells (VSELs) have probably been the most well-studied type of stem cell in mammalian ovaries (see Supporting Information Table 7 for references). It has been postulated that VSELs represent primitive multi-potent stem cells which, in adult ovaries, give rise to OSCs as a more lineage-committed stem cell type (Parte et al., 2011; Bhartiya, 2015; Bhartiya et al., 2018). However, experimental evidence unequivocally demonstrating that OSCs are direct descendants of VSELs is lacking (see Martin et al., 2019 for a detailed overview), even though ovarian VSELs have been a subject of investigation for nearly 15 years (Virant-Klun et al., 2008). For example, there are no studies showing that purified VSELs expressing a fluorescent gene reporter for cell fate tracking can differentiate directly into OSCs following intraovarian transplantation in vivo or following extended culture in vitro. Moreover, purified VSELs transplanted into ovaries of recipient females have not been shown to generate eggs, embryos or offspring that can be traced back to the transplanted cell fraction. By comparison, these outcomes have been repeatedly shown for more than a decade using purified OSCs as the donor cells (Zou et al., 2009; Zhang et al., 2011; Zhou et al., 2014; Xiong et al., 2015; Zhang et al., 2016; Wang et al., 2017; Wu et al., 2017), as well as by very recent ovarian tissue reconstitution studies with OSCs in vitro (Li et al., 2021b).

As mentioned in the main text (see **DISCUSSION**), a very recent study based on immunofluorescence analysis of various markers associated with germ cells, mitosis and meiosis concluded that that green fluorescent protein-expressing VSELs transplanted into ovaries of wild type female mice could subsequently be traced to multi-cellular structures referred to by the authors as newly formed germ cell nests (Sharma and Bhartiya, 2021). However, the protocol employed in this study for the isolation of VSELs that were then used for transplantation relied solely on detection of stage-specific embryonic antigen-1 (SSEA1), which is a generic marker for the detection and sorting of diverse stem cell types, including OSCs. This is not an insignificant issue, and thus the sorting of VSELs as a pure population requires the use of multiple antibodies to both include and exclude cells expressing a spectrum of different externalized epitopes (Kucia et al., 2006; Ratajczak et al., 2013; Havens et al., 2014; Kim et al., 2014). It is also important to emphasize that VSELs do not express the extracellular C-terminal epitope of DDX4 to enable antibody recognition on viable cells (Martin et al., 2019). Consequently, the DDX4 antibody-based sorting protocols commonly used for more than a decade to isolate viable OSCs for downstream analysis (Zou et al., 2009; White et al., 2012; Woods and Tilly, 2013a; Navaroli et al., 2016; see also Supporting Information Table 1) do not retrieve VSELs in parallel. Hence, all studies of OSCs published to date, which initially sorted the cells using DDX4 antibodies, reflect the characteristic features of OSCs and not a mixed OSC/VSEL preparation or a pool of differentiating VSELs. For these reasons, VSELs and OSCs are considered distinct types of ovarian stem cells.

Others have expressed similar reservations about a functional lineage connection between VSELs and OSCs. For example, De Felici and Barrios (2013), in a detailed review of the potential origins of mammalian OSCs, stated that "*a demonstration of their [VSELs] ability to commit to the germ cell fate is, to the best of our knowledge, totally lacking. Lineage tracing experiments could shed light into the origin and differentiation potential of the VSEL stem cells, although this could be difficult to implement if these cells have no unique gene to distinguish their identity*." The latter issue raised by De Felici and Barrios is also very relevant to the specific identification of VSELs, which share tremendous overlap in gene expression patterns with other primitive or multipotent stem cells, in a heterogenous cell preparation through mRNA-based technologies like scRNA-seq. Differences in cell size and morphology, as well as intracellular localization of proteins like Octamer-binding transcription factor 4 (OCT-4, encoded by the

POU5F1 gene, with OCT-4 being nuclear in VSELs and cytoplasmic in OSCs), have often been used to distinguish these two stem cell types in prior studies (see, for example, Parte et al., 2011). However, such endpoints are not useful with technologies reliant solely on gene transcription (viz. gene promoters for construction of cell type-specific transgenes) or mRNA transcript content (viz. scRNA-seq). Hence, the current absence of a distinct gene expression signature for VSELs creates significant challenges for lineage tracing, as pointed out by De Felici and Barrios (2013), and also for detection of the cells in a mixed cell preparation by scRNA-seq. Future studies in which bulk RNA-seq is performed on ovarian VSELs purified by multi-parametric FACS (Kucia et al., 2006; Ratajczak et al., 2013; Havens et al., 2014; Kim et al., 2014) may prove especially helpful in this regard for those who study this specific stem cell type.

Finally, since Wagner et al. (2020) focused their conclusions on disputing solely the existence of OSCs in adult human ovaries (NB: these authors did not acknowledge or discuss any other stem cell type), we in turn focused our experiments reported herein on specifically OSCs and not on all stem cell types in adult ovaries. However, it is highly likely that the failure of Wagner et al. (2020) to identify any type of stem cell in their study is due to the same analytical workflow limitations demonstrated herein which precluded their identification of candidate OSCs and rare germ cells in meiosis-I. It is worth mentioning that for VSELs, understanding the basis of failed detection of this cell type by Wagner et al. (2020) may be less complicated than for OSCs since these cells, unlike OSCs and MSCs, are localized exclusively to the outermost layer of cells on the surface of the female gonad termed the ovarian surface epithelium (Virant-Klun et al., 2008; Parte et al., 2011). In most studies published on VSELs over the years (see Supporting Information Table 7 for example references), the cells were obtained as part of a heterogenous cell preparation retrieved by gently “scraping” or “brushing” the outer surface of ovarian tissue or by simply removing and culturing the ovarian surface epithelium ex vivo. In our experience working with cryopreserved human ovarian cortex to study human OSC biology and function for more than a decade (White et al., 2012; Bothun et al., 2018; MacDonald et al., 2019), we have routinely observed that the ovarian surface epithelium is loosely adhered to the underlying cortical region and is frequently lost during tissue preparation and handling associated with cortical strip cryopreservation. Therefore, the inability of the scRNA-seq workflow reported by Wagner et al. (2020) to find evidence of VSELs in cryopreserved human ovarian cortical tissue may be due to inadvertent loss of the ovarian surface epithelium, and thus the VSELs contained within it, from their tissue samples prior to any downstream analysis.

Discussion 3: Contemporary Publications Supporting OSCs Far Outweigh Those Refuting OSCs

As overviewed in the main text (see **DISCUSSION**), over 80 primary research papers have been published since the initial report of OSCs in mouse ovaries almost 20 years ago (Johnson et al., 2004) that support the existence of female germline stem cells and/or the occurrence of active oogenesis in adult ovaries of numerous mammalian species, including humans [reviewed in Martin et al. (2019); see also Supporting Information Tables 1–3]. By comparison, over this same time span only 10 primary research papers have been published that question the existence of OSCs and/or postnatal oogenesis in mammals. Notably, all ten of these latter studies are rooted in negative data and “absence of evidence is evidence of absence” based arguments (Supporting Information Tables 1–3) [see also discussion points made in Tilly and Johnson (2007), Tilly et al. (2009), and Woods et al. (2013)]. Despite this more than 8-fold difference in contemporary publications supporting versus refuting mammalian OSCs and postnatal oogenesis, Hainaut and Clarke recently claimed in a very recent review of mammalian germ cells that “*the weight of evidence*

strongly supports the traditional interpretation that germ-line stem cells do not exist post-natally in female mammals" (Hainaut and Clarke, 2021). If one considers the actual weight of evidence based on primary data in published research papers since 2004 (Supporting Information Tables 1–3), and the fact that numerous groups have now independently isolated mitotically-active germ lineage cells from adult mammalian ovaries that differentiate through meiosis to generate oocytes in vitro and in vivo [recently reviewed in Martin et al. (2019) and Akahori et al. (2019)], such an opinion, which at this point unnecessarily fuels continued confusion and debate over cells that undeniably exist across mammalian species including humans, is simply inaccurate and scientifically unfounded.

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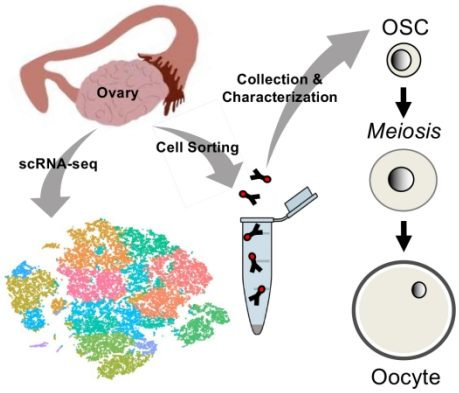

Single-cell RNA sequence analysis (scRNA-seq) of adult human ovarian cortex reveals transcriptional profiles consistent with the presence of oogonial stem cells (OSCs) and active meiotic cell division. These findings reinforce and extend a growing body of work collectively demonstrating the existence and functional properties of OSCs, as well as the differentiation of these stem cells through meiosis to form new oocytes.