

Use of *DEAD*-box polypeptide-4 (*Ddx4*) gene promoter-driven fluorescent reporter mice to identify mitotically active germ cells in post-natal mouse ovaries

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Submitted on April 24, 2014; resubmitted on June 25, 2014; accepted on July 11, 2014

ABSTRACT: Several laboratories have independently isolated mitotically active germ cells, termed female germline stem cells or oogonial stem cells (OSCs), from adult mouse ovaries. However, a recent study using *Ddx4-Cre;Rosa26* reporter mice concluded that such germ cells do not exist. Given the disparity in conclusions drawn in this study compared with others, we felt it was important to re-assess the utility of *Ddx4-Cre;Rosa26* reporter mice for identification of OSCs in adult mouse ovaries. Transgenic *Ddx4-Cre* mice were crossed with *Rosa26^{tdTm/tdTm}* mice to drive restricted *tomato red (tdTm)* gene expression in cells in which the *Ddx4* gene promoter has been activated. Crude dispersion of ovaries from recombined offspring generated cell fractions containing tdTm-positive immature oocytes, which are incapable of proliferation and thus probably represent the uncharacterized reporter-positive ovarian cells identified in the paper Zhang *et al.* (2012) as being mitotically inactive. Dispersed ovaries further subjected to fluorescence-activated cell sorting yielded a large population of non-germline tdTm-positive cells, indicative of promoter ‘leakiness’ in the *Ddx4-Cre* mouse line. Nonetheless, a small percentage of these tdTm-positive cells exhibited externalized (extracellular, ec) expression of *Ddx4* protein (ec*Ddx4*-positive), expressed markers of primitive germ cells but not of oocytes, and actively proliferated in culture, all of which are characteristic features of OSCs. Thus, crude dispersion of ovaries collected from *Ddx4* gene promoter-driven reporter mice is not, by itself, a reliable approach to identify OSCs, whereas the same ovarian dispersates further subjected to cell sorting strategies yield purified OSCs that can be expanded in culture.

Key words: *Ddx4* / germline stem cell / oogonial stem cell / oogenesis / oocyte

Introduction

A decade ago, several initial lines of evidence using mice as a model were published, which challenged the longstanding paradigm in reproductive biology that female mammals lose the capacity for germ cell renewal around the time of birth (Johnson *et al.*, 2004). Subsequently, many independent studies have corroborated and extended these early findings (reviewed in Woods and Tilly, 2013a, b). Some of these studies reported the successful isolation of mitotically active germ cells from adult mouse, rat, cow, monkey or human ovaries using magnetic-assisted cell sorting (MACS) or fluorescence-activated cell sorting (FACS) approaches (Zou *et al.*, 2009, 2011; Pacchiarotti *et al.*, 2010; Zhang *et al.*, 2011; White *et al.*, 2012; Anderson *et al.*, 2013; Woods and Tilly, 2013a; Dunlop *et al.*, 2014; Wolff *et al.*, 2014; Zhou *et al.*, 2014). Additionally, intragonadal transplantation studies, essentially identical to those used for 20 years to establish the identity of spermatogonial

stem cells in mammalian testes (Brinster and Zimmermann, 1994; Brinster and Avarbock, 1994; Hermann *et al.*, 2012), have shown that these adult ovary-derived germ cells, termed female germline stem cells or oogonial stem cells (OSCs), differentiate into fully functional eggs that ovulate and fertilize to produce viable embryos and offspring (Zou *et al.*, 2009; Zhang *et al.*, 2011; White *et al.*, 2012; Woods and Tilly, 2013a; Zhou *et al.*, 2014). Parallel *in vitro* studies of mammalian OSCs have established several characteristic features of these cells, including stable long-term proliferative potential (Zou *et al.*, 2009, 2011; Pacchiarotti *et al.*, 2010; Zhang *et al.*, 2011; White *et al.*, 2012; Anderson *et al.*, 2013; Woods and Tilly, 2013a; Wolff *et al.*, 2014; Zhou *et al.*, 2014), inherent gene expression profile differences between embryonic primordial germ cells (PGCs) and OSCs (Imudia *et al.*, 2013), and responsiveness of OSCs to endogenous cues that activate meiotic gene expression and the subsequent formation of immature oocytes (Park *et al.*, 2013a; Woods and Tilly, 2013a).

However, the emerging field of mammalian OSC biology is not without controversy (Tilly *et al.*, 2009; Woods *et al.*, 2013), as would be expected of any major paradigm shift, and this has been refueled in part by a recent study from Liu and colleagues (Zhang *et al.*, 2012). By evaluating the ovaries of a genetic mouse model in which expression of a fluorescent reporter gene is driven by the promoter of the germ cell-specific gene, *Ddx4* (DEAD-box polypeptide 4; also referred to as mouse vasa homolog or *Mvh*), Zhang *et al.* (2012) claimed their findings refute a large body of work regarding the existence of mitotically active germ cells in post-natal mammalian ovaries (Johnson *et al.*, 2004; Zou *et al.*, 2009, 2011; Pacchiarotti *et al.*, 2010; Zhang *et al.*, 2011; White *et al.*, 2012; Anderson *et al.*, 2013; Imudia *et al.*, 2013; Park *et al.*, 2013a; Woods and Tilly, 2013a, b; Dunlop *et al.*, 2014; Wolff *et al.*, 2014; Zhou *et al.*, 2014). That study, which received extensive attention (Hamzelou, 2012; Underwood, 2012; Yong, 2012), used a genetic approach reliant on the generation of recombined offspring derived from crossing two transgenic mouse lines: *Ddx4-Cre* and *Rosa26^{tbw/tbw}*. The resultant Cre-mediated recombination event at the *Rosa26^{tbw/+}* locus triggers an irreversible switch from ubiquitous green fluorescent protein expression in all cells of the parental reporter mouse line to restricted expression of red fluorescent protein (RFP), orange fluorescent protein (OFP) or cyan fluorescent protein (CFP) in any cells that have at any point in development activated the *Ddx4* gene promoter. Assuming the fragment of the *Ddx4* gene promoter used to drive *Cre* expression in this transgenic mouse line is, like the endogenous *Ddx4* gene, germ cell-specific (Fujiwara *et al.*, 1994), recombined offspring should exhibit restricted RFP, OFP or CFP expression in all cells of the germ lineage.

Liu and colleagues presented four sets of experimental outcomes in their study using these mice (Zhang *et al.*, 2012); however, three of the four data sets were concerned with either transplantation of transgenic fetal ovary-derived cells into wild-type adult ovaries or *ex vivo* study of post-natal ovary-derived cells that were identified as *Ddx4*-negative in recombined offspring. These experiments do not address the question of whether OSCs, which clearly express *Ddx4* (Zou *et al.*, 2009, 2011; Pacchiarotti *et al.*, 2010; White *et al.*, 2012), exist in adult ovaries. The main case tendered by Zhang *et al.* (2012) against the existence of OSCs in mice centers on their data which show only fluorescent images of RFP-expressing (*Ddx4-Cre* recombined) cells in 24-, 48- and 72-h cultures of cell fractions obtained by crude dispersion of post-natal day 8 ovaries from recombined offspring. Assessment of the RFP-expressing cells did not reveal evidence of active mitosis over the first 3 days of culture. It was therefore concluded that the germ cells identified in post-natal ovaries as having activated the *Ddx4* gene promoter do not proliferate, and consequently conclusions drawn from prior studies that have purified mitotically active germ cells from post-natal mouse ovaries are incorrect (Zhang *et al.*, 2012).

Unfortunately, the identity of the recombined cells present in the crudely dispersed ovarian cell fractions that were studied in short-term cultures were not discerned. Based on the relatively large size of the RFP-expressing cells shown in Fig. 2 of their paper (Zhang *et al.*, 2012), it is possible that these cells were immature oocytes, which express the *Ddx4* gene and thus would be RFP-positive, but are no longer capable of mitosis. Furthermore, the authors did not reproduce the experiments they claim to invalidate, by simply enriching for or purifying OSCs from ovaries of their recombined mice as others have done (Zou *et al.*, 2009, 2011; Pacchiarotti *et al.*, 2010; White *et al.*, 2012). Detailed methods for mouse OSC purification are available and fully validated,

and would have removed the possibility of interpretational ambiguity resulting from oocyte contamination in any end-point analysis directed at detection or analysis of premeiotic germ cells (reviewed in Woods and Tilly, 2013a).

Given these questions, and the discordance in the conclusions drawn by Zhang *et al.* (2012) compared with those of other laboratories (Johnson *et al.*, 2004; Zou *et al.*, 2009, 2011; Pacchiarotti *et al.*, 2010; Zhang *et al.*, 2011; White *et al.*, 2012; Anderson *et al.*, 2013; Imudia *et al.*, 2013; Park *et al.*, 2013a; Woods and Tilly, 2013a, b; Dunlop *et al.*, 2014; Wolff *et al.*, 2014; Zhou *et al.*, 2014), we felt it was imperative to more comprehensively examine the utility of the *Ddx4-Cre* mouse line employed by these authors for the detection of OSCs in ovaries of fluorescent reporter mice. Here, we show that the methods used by Liu and colleagues lacked certain key control experiments, leading to an erroneous conclusion that mitotically active germ cells do not exist in post-natal mouse ovaries. We further show that by employing available sorting techniques to purify OSCs (Zou *et al.*, 2009; White *et al.*, 2012; Woods and Tilly, 2013a), ovaries of genetically recombined offspring resulting from crosses of *Ddx4-Cre* mice with *Rosa26* reporter mice do contain mitotically active germ cells that are RFP-positive.

Materials and Methods

Animals

Rosa26^{tdTm/tdTm} female mice (JAX stock 007914) and *Ddx4-Cre* male mice (JAX stock 006954) were obtained from the Jackson Laboratory (Bar Harbor, Maine, USA). The *Rosa26^{tdTm/tdTm}* mice harbor a loxP-flanked STOP-cassette preventing transcription of the downstream CAG promoter-driven RFP variant, tdTomato (tdTm). In recombined (*Ddx4-Cre;Rosa26^{tdTm/+}*) mice, *Ddx4* promoter-driven expression of *Cre* recombinase excises the STOP-cassette to permanently activate expression of the *tdTm* gene, which should be restricted to *Ddx4*-expressing germ lineage cells. All procedures reported herein were reviewed and approved by the appropriate institutional animal care and use committee.

Preparation of crude ovarian cell fractions

Ovaries from post-natal Day 8 *Ddx4-Cre;Rosa26^{tdTm/+}* female mice were collected, minced, dissociated with trypsin, and then filtered through a 40- μ m-pore cell strainer to remove large oocytes, debris and cell aggregates, exactly as described previously (Zhang *et al.*, 2012). The resultant cell fractions were analyzed for the presence of immature oocytes by evaluating expression of oocyte-specific genes (see 'Gene Expression Analysis').

Isolation and culture of OSCs

Ovaries from 2-month-old *Rosa26^{tdTm/+}* and *Ddx4-Cre;Rosa26^{tdTm/+}* female mice were processed for FACS-based isolation of viable *ecDdx4*-positive cells (*viz.* OSCs), as described (White *et al.*, 2012; Woods and Tilly, 2013a). Sorted OSCs were then established as pure germ cell cultures without somatic feeder cells (White *et al.*, 2012; Woods and Tilly, 2013a). Fluorescence images of whole ovaries before dispersion, and of purified OSC colonies formed after 1 or 2 weeks of culture *in vitro*, were taken and analyzed with Spot Advanced Software (Diagnostic Instruments, Sterling Heights, MI, USA).

Cell proliferation analysis

Purified tdTm-expressing cells were seeded onto glass coverslips coated with poly-L-lysine, and then cultured with 10 μ M BrdU (Sigma-Aldrich, St. Louis, MO, USA) for 48 h. The cells were then gently rinsed with 1 \times -concentrated

phosphate-buffered saline prior to fixation in 1% paraformaldehyde for dual immunofluorescence-based detection of bromodeoxyuridine (BrdU) incorporation (mitotically active cells) and RFP expression, as described (White et al., 2012).

Gene expression analysis

Total RNA was isolated from ovaries (intact or crude dispersates) and testes using TRIzol reagent (Invitrogen—Life Technologies, Grand Island, New York, USA), and from freshly isolated ecDdx4-positive cells (OSCs) using the RNeasy Plus Micro kit (Qiagen, Valencia, CA, USA). Each pool of RNA was then quantified and assessed by reverse transcription–polymerase chain reaction (RT–PCR) using Platinum Taq polymerase (Invitrogen—Life Technologies) for the presence of germ cell-specific and oocyte-specific mRNAs. Sequences of the forward and reverse primers used, along with GenBank accession numbers of the corresponding genes, are as reported previously (White et al., 2012).

Data presentation and analysis

All experiments were independently replicated at least three times using different mice or cell preparations in each experimental replicate. Where appropriate, qualitative images representative of outcomes obtained in the replicate experiments are provided.

Results

Crudely dispersed *Ddx4-Cre;Rosa26^{tdTm/+}* ovarian cell fractions contain oocytes

We first replicated the experiment presented by Zhang et al. (2012) in Fig. 2 of their paper using the same *Ddx4-Cre* transgenic mouse line crossed with transgenic mice carrying the *Rosa26^{tdTm/tdTm}* reporter locus as a target for Cre-mediated recombination to drive restricted *tdTm* fluorescence gene expression in only those cells that have activated the *Ddx4* promoter. Ovaries from recombined offspring were collected on post-natal Day 8 and subsequently processed through the trypsin-based dispersion procedure detailed in their paper (Zhang et al., 2012). Prior to dispersion, we observed a generalized and widespread pattern of *tdTm* fluorescence in recombined ovaries (Fig. 1A), which would not be expected of a transgenic reporter designed to mimic endogenous *Ddx4* expression and thus be specific for germ cells. This finding led us to speculate that the *Ddx4-Cre* mouse line is prone to ‘leaky expression’ in non-germline cells, which was confirmed in subsequent FACS experiments (see next section below). Dispersion of ovaries by trypsin, mechanical disruption and filtration through a 40- μ m cell-strainer, exactly as reported by Zhang et al. (2012), produced crude cell fractions containing uncharacterized debris and cell aggregates (Fig. 1B). Not surprisingly, we found that these fractions also contained immature oocytes, as determined by PCR analysis of a panel of oocyte-specific markers (Fig. 1C).

Identification of OSCs in *Ddx4-Cre; Rosa26^{tdTm/+}* ovaries by FACS

Based on the issues encountered thus far, we elected to refine the strategy for identification of premeiotic germ cells in recombined ovaries by incorporating a recently detailed FACS-based method for the purification of OSCs (White et al., 2012; Woods and Tilly, 2013a). This approach takes advantage of the fact that the C-terminus of the *Ddx4* protein is exposed on the outer plasma membrane of OSCs, but

becomes localized entirely in the cytoplasm upon differentiation of the cells into oocytes (Zou et al., 2009; White et al., 2012; Imudia et al., 2013; Woods and Tilly, 2013a). Accordingly, antibodies that target the C-terminus of *Ddx4* can be used with FACS to obtain from dispersed ovaries a purified fraction of viable OSCs, which are extracellular *Ddx4* protein (ecDdx4)-positive; these cells are completely free of contaminating oocytes, which are ecDdx4-negative but still positive for *Ddx4* gene expression (White et al., 2012; Woods and Tilly, 2013a). The other significant modification we made was to move away from trypsin-based digestion of ovarian tissue used by Liu and colleagues (Zhang et al., 2012), since this approach would have a very high likelihood of stripping away the *Ddx4* antigen exposed on the plasma membrane of OSCs and consequently prevent antibody (FACS or MACS)-based collection of the cells (Woods and Tilly, 2013a).

Similar to ovaries harvested from recombined mice on post-natal Day 8 (Fig. 1A), ovaries collected from *Ddx4-Cre;Rosa26^{tdTm/+}* mice during adulthood (post-natal Day 60) also exhibited a generalized and widespread pattern of *tdTm* fluorescence (Fig. 2A). After dissociation using a collagenase-based protocol (White et al., 2012; Woods and Tilly, 2013a), we analyzed the resultant ovarian cell fraction by FACS for *tdTm* expression, APC-coupled ecDdx4 protein expression, or the two end-points together. As anticipated, ovaries of non-recombined (*Rosa26^{tdTm/+}*) female offspring derived from crosses of *Ddx4-Cre* and *Rosa26^{tdTm/tdTm}* mice did not contain any cells positive for *tdTm* expression (Fig. 2B and F); however, a small population of ecDdx4-positive cells (1.53% of the total viable cells sorted) was readily detectable in dispersed *Rosa26^{tdTm/+}* ovaries (Fig. 2C and F). Also as expected based on the widespread *tdTm* fluorescence in recombined (*Ddx4-Cre;Rosa26^{tdTm/+}*) ovaries at collection (Figs 1A and 2A), more than 60% of the viable cells sorted from these ovaries were *tdTm*-positive (Fig. 2D and F). In addition, of the viable cells comprising the *tdTm*-positive fraction, only 3.15% were dual-positive for both *tdTm* and ecDdx4 expression (Fig. 2E and F). This ecDdx4-positive population represented 1.83% of the total viable cells sorted from dispersed *Ddx4-Cre;Rosa26^{tdTm/+}* ovaries (Fig. 2F). The yield of ecDdx4-positive cells from ovaries of 2-month-old *Rosa26^{tdTm/+}* mice (non-recombined) and *Ddx4-Cre; Rosa26^{tdTm/+}* mice (recombined) is fully consistent with data from past studies of OSC yield from adult mouse ovaries (White et al., 2012; Imudia et al., 2013; Woods and Tilly, 2013a).

Cells positive for both *tdTm* and ecDdx4 are mitotically active germ cells

The purified *tdTm*/ecDdx4 dual-positive cell fraction obtained by FACS from dissociated *Ddx4-Cre;Rosa26^{tdTm/+}* ovaries expressed the primitive germ cell markers, *Prdm1* (*PR domain containing 1 with ZNF domain*; also referred to as *Blimp1*), *Dppa3* (*developmental pluripotency-associated 3*; also referred to as *Stella*), *Ifitm3* (*interferon-induced transmembrane protein 3*; also referred to as *Fragilis*), *Tert* (*telomerase reverse transcriptase*) and *Dazl* (*deleted in azoospermia-like*), but did not express any of a panel of widely accepted oocyte-specific markers [*Nobox* (*newborn ovary homeobox*), *Zp3* (*zona pellucida glycoprotein 3*), *Gdf9* (*growth differentiation factor 9*)] (Fig. 3A). Levels of *Ddx4* mRNA in freshly isolated *tdTm*/ecDdx4 dual-positive cells were low, but could be detected with increased PCR cycle number. In addition, plating of *tdTm*/ecDdx4 dual-positive cells in culture resulted in the formation of visibly fluorescent germ cell colonies within 1 week, which rapidly expanded

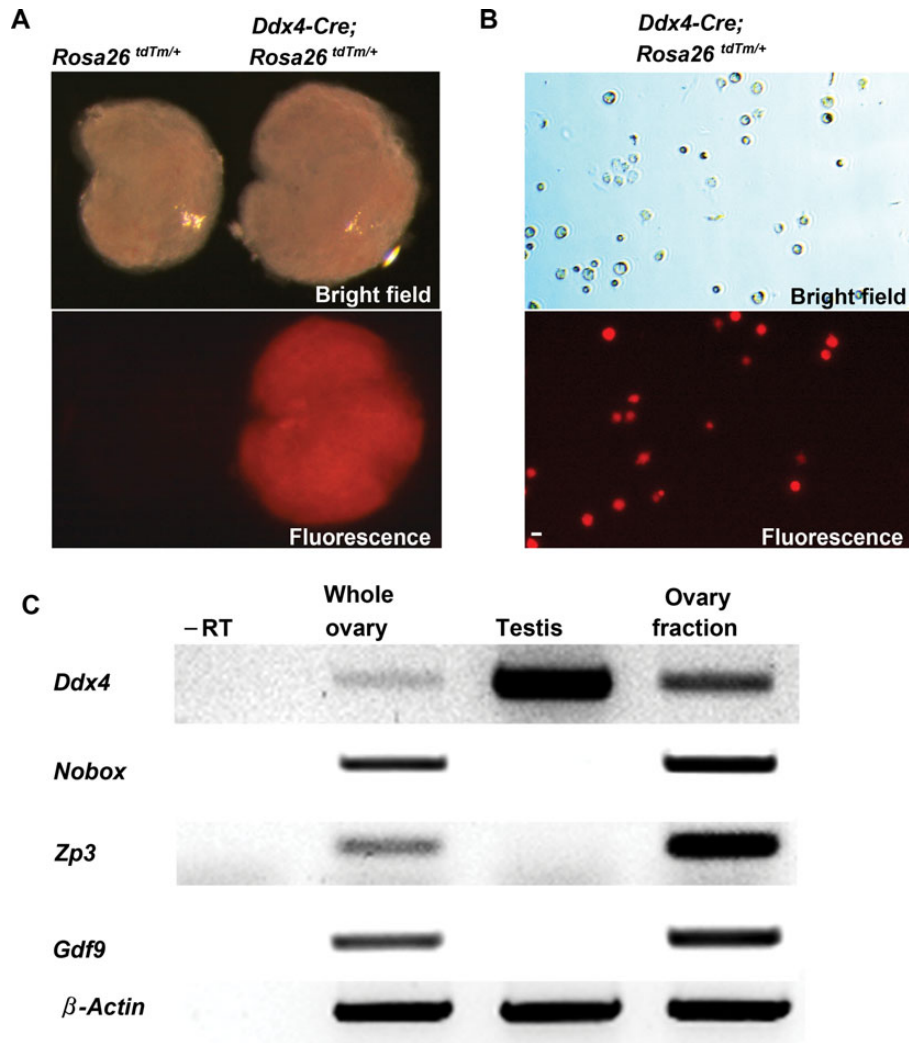


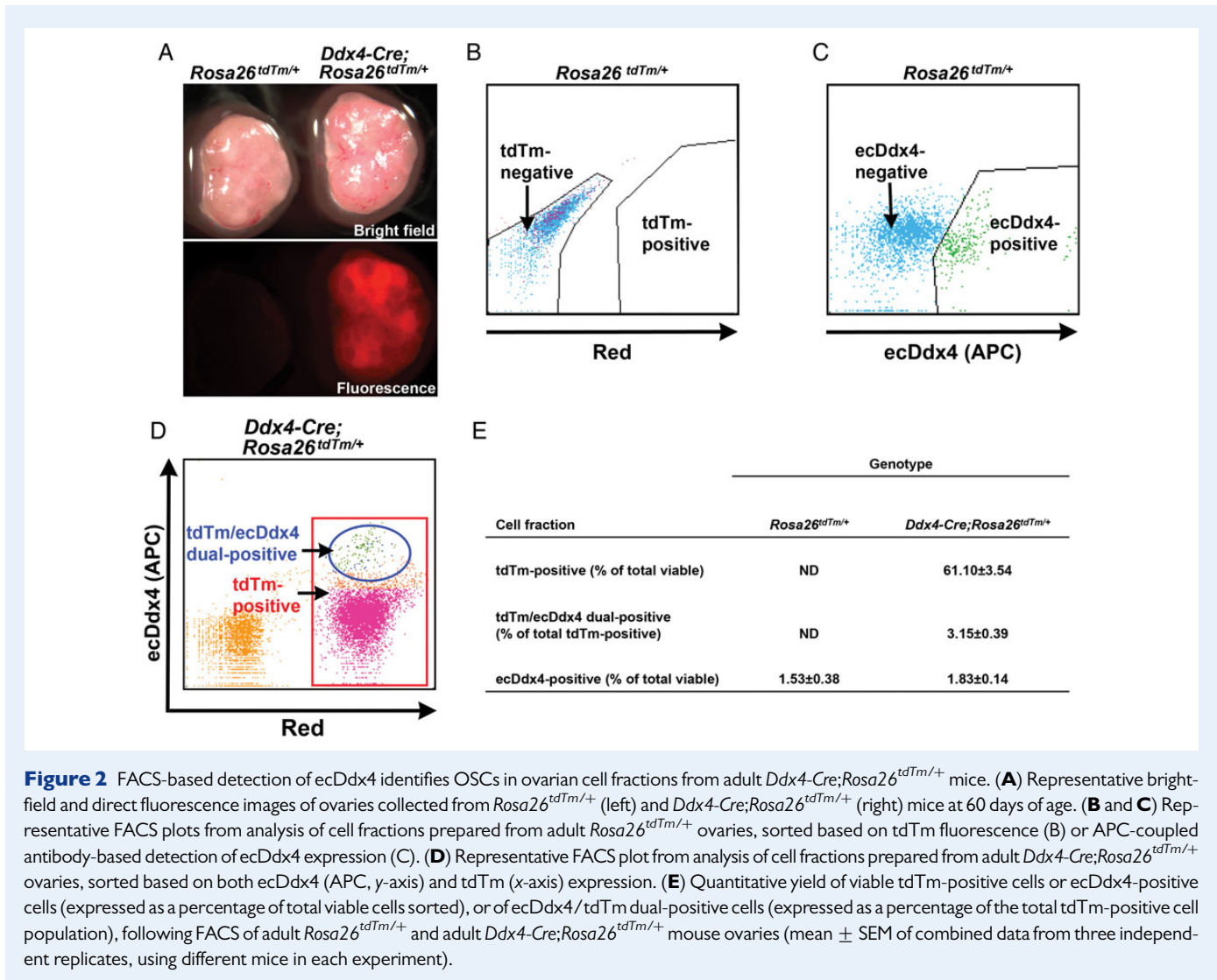
Figure 1 Assessment of *Ddx4-Cre;Rosa26^{tdTm/+}* mouse ovaries reveals both reporter 'leakiness' and oocyte contamination in crudely dispersed cell fractions. **(A)** Representative bright-field and direct fluorescence images of ovaries collected from *Rosa26^{tdTm/+}* (non-recombined offspring, reporter not activated; left panels) and *Ddx4-Cre;Rosa26^{tdTm/+}* (recombined offspring, reporter activated; right panels) mice at post-natal Day 8. **(B)** Representative bright-field and direct fluorescence images of crudely dissociated cell fractions prepared from *Ddx4-Cre;Rosa26^{tdTm/+}* ovaries, exactly as described (Zhang *et al.*, 2012). Scale bar, 50- μ m. **(C)** Expression analysis of *Ddx4* (germ cell marker), oocyte-specific genes (*Nobox*, *Gdf9*, *Zp3*) and β -actin (house-keeping gene) in post-natal Day 8 whole ovary, adult testis and the crudely dissociated post-natal Day 8 ovary cell fractions shown in (B) (-RT, PCR analysis of ovary cell fraction RNA sample processed without reverse transcription to rule out the possibility of target gene amplification from genomic DNA contamination).

in numbers with continued culture (Fig. 3B). Dual immunostaining analysis of RFP expression and BrdU incorporation confirmed that the vast majority of tdTm-positive germ cells plated in culture were actively dividing (Fig. 3C and D).

Discussion

Over the last decade, an increasing number of academic (Johnson *et al.*, 2004; Zou *et al.*, 2009, 2011; Zhang *et al.*, 2011; White *et al.*, 2012; Anderson *et al.*, 2013; Imudia *et al.*, 2013; Park *et al.*, 2013a, b; Woods and Tilly, 2013a, b; Dunlop *et al.*, 2014; Zhou *et al.*, 2014), industry (Pacchiarotti *et al.*, 2010) and government (e.g. National Institutes of Health; Wolff *et al.*, 2014) laboratories have produced experimental

evidence questioning the decades-old belief that female mammals are incapable of post-natal oocyte renewal (Zuckerman, 1951). However, this line of study remains a source of contention in the field of reproductive biology (Tilly *et al.*, 2009; Hamzelou, 2012; Underwood, 2012; Yong, 2012; Gura, 2012; Woods *et al.*, 2013), despite the fact that the female equivalent of spermatogonial stem cells in males have not only been purified from ovaries of adult mice, rats, cows, monkeys and humans (Zou *et al.*, 2009, 2011; Pacchiarotti *et al.*, 2010; Zhang *et al.*, 2011; White *et al.*, 2012; Anderson *et al.*, 2013; Dunlop *et al.*, 2014; Wolff *et al.*, 2014; Zhou *et al.*, 2014), but also shown by intraovarian transplantation studies in mice and rats to possess the capacity to differentiate into fully functional eggs (Zou *et al.*, 2009; Zhang *et al.*, 2011; White *et al.*, 2012; Park *et al.*, 2013b; Zhou *et al.*, 2014). Countering



these reports, Liu and colleagues recently claimed that their experiments using *Ddx4-Cre;Rosa26^{fbw/+}* reporter mice provide strong genetic proof that post-natal mouse ovaries do not contain mitotically active germ cells (Zhang et al., 2012).

The first data set offered by these authors, shown in Fig. 1 of their paper (Zhang et al., 2012), assessed the fate of crudely dispersed *Rosa26^{fbw/+}* (viz. non-recombined) fetal ovary-derived cell fractions transplanted into ovaries of post-natal wild-type mice. The rationale for this experiment is not clear; the oocyte-forming capacity of embryonic PGCs in adult ovaries have little relevance to the question of whether adult ovaries contain mitotically active germ cells. Recent studies have shown PGCs have a gene expression profile more closely matched to embryonic stem cells rather than adult ovary-derived OSCs (Imudia et al., 2013), indicating that the two germ cell populations are quite distinct. Furthermore, it is already well established that PGCs require developmentally matched ovarian somatic cells to generate follicles (Lei et al., 2006). With respect to this latter point, Liu and colleagues injected only dispersed fetal ovary fractions into adult ovaries rather than separate injections of PGCs or embryonic ovarian somatic cells, which would be feasible with their genetic recombination model. With both fetal cell

lineages present in post-natal ovaries after injection, it is expected that all new follicles formed would contain donor-derived oocytes surrounded by donor-derived somatic cells, as these embryonic-derived cells are natural and prerequisite partners for folliculogenesis (Lei et al., 2006; Hayashi et al., 2012).

The second experimental data set from Zhang et al. (2012), involves a direct analysis of post-natal ovaries for evidence of mitotically active germ cells. However, three issues uncovered in our study weaken clear interpretation of the experimental data presented by these authors. First, in our hands Cre-mediated recombination in *Ddx4-Cre;Rosa26* fluorescent reporter mice is 'leaky' and not specific for germ cells. Although the commercial source of *Ddx4-Cre* mice used in our study and that of Liu and colleagues (Zhang et al., 2012) is the same, it is possible that variation exists in the specificity of Cre expression driven by the *Ddx4* promoter amongst different mice in this particular transgenic line (Gallardo et al., 2007). Even if this were the case, a second issue in their study design is that the crudely dispersed ovarian cell fractions were contaminated with immature oocytes, which would be genetically recombined but incapable of proliferation. Hence, the mitotically inactive RFP-expressing cells studied by these authors are in all likelihood immature oocytes. The third issue is

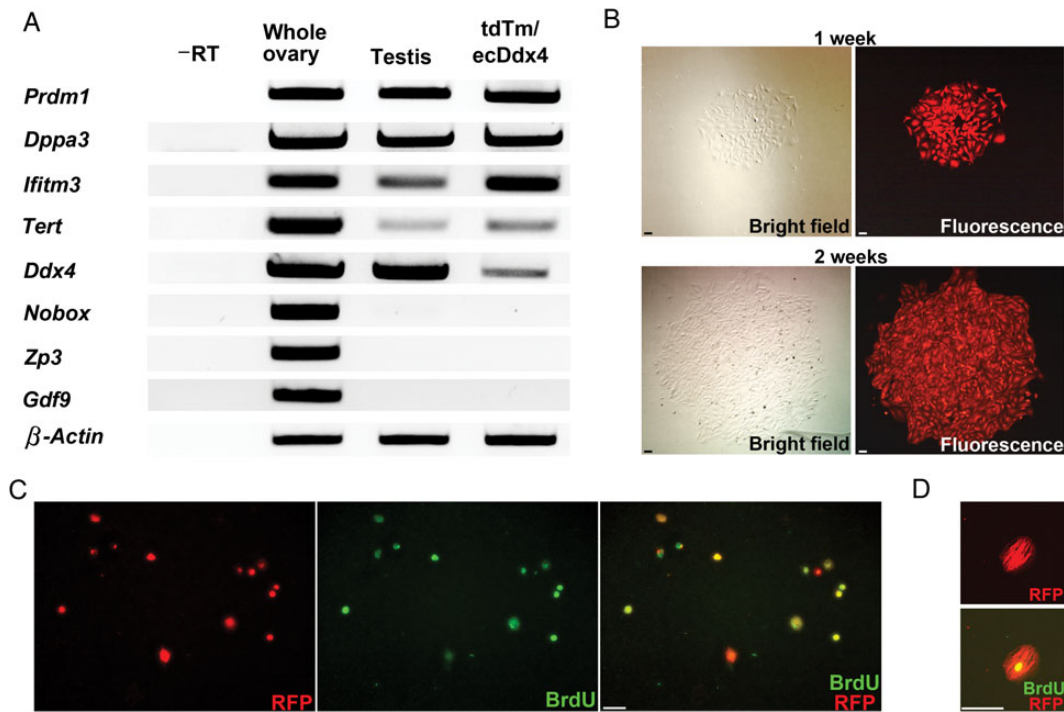


Figure 3 Ovaries of adult *Ddx4-Cre;Rosa26^{tdTm/+}* reporter mice contain mitotically active germ cells. **(A)** Representative expression analysis of primitive germline markers (*Prdm1*, *Dppa3*, *Ifitm3*, *Tert*, *Ddx4*) and oocyte markers (*Nobox*, *Zp3*, *Gdf9*) in adult whole ovary, adult testis and ecDdx4/tdTm dual-positive cells isolated by FACS from ovaries of adult *Ddx4-Cre;Rosa26^{tdTm/+}* mice (-RT, PCR analysis of ecDdx4/tdTm dual-positive cell RNA sample processed without reverse transcription to rule out the possibility of target gene amplification from genomic DNA contamination; β -actin, internal sample loading control). **(B)** Time sequence analysis of a typical germ cell colony formed after plating ecDdx4/tdTm dual-positive cells isolated by FACS from adult *Ddx4-Cre;Rosa26^{tdTm/+}* ovaries, which doubles in size between Week 1 (upper panels) and Week 2 (lower panels) of culture. Scale bars, 50- μ m. **(C and D)** Assessment of proliferation by dual detection of RFP expression (red) and BrdU incorporation (green) in cultures of ecDdx4/tdTm dual-positive cells isolated by FACS from adult *Ddx4-Cre;Rosa26^{tdTm/+}* ovaries and cultured without a somatic feeder cell layer. Scale bars: C, 50- μ m; D, 20- μ m.

that Zhang *et al.* (2012) did not separate OSCs away from oocytes (and other cell types if there is promoter leakiness), using well-validated protocols that have been successfully employed with ovaries from a variety of mammalian species (Zou *et al.*, 2009, 2011; White *et al.*, 2012; Anderson *et al.*, 2013; Woods and Tilly, 2013a; Dunlop *et al.*, 2014; Wolff *et al.*, 2014; Zhou *et al.*, 2014). After publication of the Zhang *et al.* (2012) study, the senior author of this report subsequently clarified that his laboratory was unable to repeat the technique of Ddx4 antibody-based sorting, thus necessitating use of an alternative strategy to track potential germ cells in post-natal mouse ovaries (Gura, 2012). Since Liu and colleagues used trypsin to disperse ovaries for analysis (Zhang *et al.*, 2012), it is not surprising that subsequent isolation of cells based on detection of a cell surface protein epitope (*viz.* extracellular Ddx4) would prove problematic (Woods and Tilly, 2013a). We found that replacing trypsin with collagenase and further processing the ovarian dispersates by FACS using a C-terminal Ddx4 antibody (White *et al.*, 2012; Woods and Tilly, 2013a), RFP-expressing OSCs were readily detectable in adult ovaries of *Ddx4-Cre;Rosa26* fluorescent reporter mice. Further, these cells, as reported previously by others and us (Zou *et al.*, 2009, 2011; Zhang *et al.*, 2011; White *et al.*, 2012; Anderson *et al.*, 2013; Woods and Tilly, 2013a; Wolff *et al.*, 2014; Zhou *et al.*, 2014), can be easily established in culture for propagation *in vitro*.

The third and fourth data sets tendered by Liu and colleagues, shown in Fig. 3 and Figure 4 of their paper (Zhang *et al.*, 2012), are devoted to *in vitro* analyses of unidentified post-natal ovarian cells that did not undergo recombination in their *Ddx4-Cre;Rosa26^{tbw/+}* offspring. The absence of recombination at the *Rosa26^{tbw/+}* locus indicates that the cells under analysis for these final sets of experiments had never activated the *Ddx4* gene promoter, leaky or otherwise. Accordingly, the gene expression and growth characteristics of these non-germline cells have no bearing on questions about OSCs or the oocytes that OSCs generate, all which are positive for *Ddx4* expression at the mRNA and protein levels (Zou *et al.*, 2009, 2011; Pacchiarotti *et al.*, 2010; White *et al.*, 2012; Woods and Tilly, 2013a).

It is worthwhile noting that a second genetic mouse study was published in 2013 in the same journal as the Zhang *et al.* (2012) paper, which also concluded that post-natal mouse ovaries lack mitotically active germ cells capable of *de novo* oogenesis (Lei and Spradling, 2013). With our re-assessment of the Zhang *et al.* (2012) study completed, we have now turned our attention to a similar re-assessment of the experimental designs and results reported by Lei and Spradling (2013). While these new experiments will take some time to complete, several key issues are already apparent with the elliptical arguments and data sets tendered by these authors in disputing the existence of OSCs in

adult mouse ovaries (Woods and Tilly, 2013a, b). Most notably, the genetic approach of labeling all cell types at low frequency does not provide a 'sensitive lineage-labeling system' to 'directly identify germline stem cells' (Lei and Spradling, 2013) since this non-targeted approach makes it impossible to fate-map if a given oocyte arises from the subsequent differentiation of a labeled OSC. This report also did not enrich for or purify OSCs from ovaries of their mice prior to concluding from indirect experiments that such germ cells do not exist (Lei and Spradling, 2013). As we demonstrate here, if genetic reporter mouse lines are coupled with OSC sorting strategies (Woods and Tilly, 2013a), direct evidence for the existence of mitotically active germ cells, which are fully capable of differentiating into functional eggs following intraovarian transplantation *in vivo* (Zou *et al.*, 2009; Zhang *et al.*, 2011; White *et al.*, 2012; Park *et al.*, 2013b; Zhou *et al.*, 2014), continues to emerge.

Acknowledgements

This study was initiated while the authors were at Massachusetts General Hospital and Harvard Medical School, and completed at Northeastern University.

Authors' roles

E.S.P. and J.L.T. designed the experiments. E.S.P. conducted the experiments. E.S.P. and J.L.T. analyzed the results, wrote the manuscript and approved the final manuscript for submission.

Funding

This study was supported by the U.S. National Institutes of Health (National Institute on Aging Method to Extend Research in Time Award R37-AG012279); and, the Glenn Foundation for Medical Research.

Conflict of interest

E.S.P. has nothing to disclose; J.L.T. discloses interest in intellectual property described in United States Patent 7195775, United States Patent 7850984, United States Patent 7955846 and United States Patent 8642329, and is a scientific co-founder of OvaScience, Inc. (Cambridge, MA, USA).

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