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

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Objective: Perform gene expression profiling of adult mouse ovary-derived oogonial stem cells (OSCs).
Design: Experimental animal study.
Setting: Research laboratory.
Animal(s): Adult C57BL/6 female mice.
Intervention(s): None.
Main Outcome Measure(s): Gene expression profiles were compared between freshly isolated and cultured OSCs, as well as between OSCs and embryonic stem cells (ESCs), fetal primordial germ cells (PGCs), and spermatogonial stem cells (SSCs); OSC yield from ovaries versus meiotic gene activation during the estrous cycle was determined.
Result(s): Freshly isolated OSCs, PGCs, and SSCs exhibited distinct gene expression profiles. Cultured OSCs maintained their germline gene expression pattern but gained expression of pluripotency markers found in PGCs and ESCs. Cultured OSCs also expressed the meiotic marker, stimulated by retinoic acid gene 8 (*Stra8)*. In vivo, OSC yield was higher from luteal versus follicular phase ovaries, and this was inversely related to *Stra8* expression.
Conclusion(s): Freshly isolated OSCs exhibit a germline gene expression profile that overlaps with, but is distinct from, that of PGCs and SSCs. After in vitro expansion, OSCs activate expression of pluripotency genes found in freshly isolated PGCs. In vivo, OSC numbers in the ovaries fluctuate during the estrous cycle, with the highest numbers noted during the luteal phase. This is followed by activation of *Stra8* expression during the follicular phase, which may signify a wave of neo-oogenesis to partially offset follicular loss through atresia and ovulation in the prior cycle. (Fertil Steril® 2013;100:1451–8. ©2013 by American Society for Reproductive Medicine.)
Key Words: Oogenesis, oocyte, germ cell, oogonial stem cell, Stra8

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Since the initial publication in 2004 challenging the decades-old belief that the ovaries of mammals lose replicative germ cells capable of generating new oocytes after birth (1), experimental evidence from many laboratories collectively supports a new paradigm in reproductive biology that accounts for the existence of female germline or oogonial stem cells (OSCs) in postnatal ovaries (2–6). In addition to the successful purification of OSCs by at least three laboratories using different techniques (7–10), transplantation studies analogous to those used for establishing the identity of spermatogonial stem cells (SSCs) in the testes of male mice have demonstrated that OSCs generate competent oocytes in adult mouse ovaries that fertilize to produce viable embryos and offspring (7, 10–12). In addition, methods for the establishment and stable

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propagation of mouse and human OSCs in culture are now available (7–10, 13). These studies have shown that OSCs undergo spontaneous differentiation in vitro to produce what appear to be oocytes based on morphologic criteria, gene expression profiling, meiotic activation, and the ability to attract granulosa cells for folliculogenesis (8, 10, 13).

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

To address this, we developed and reported a fluorescence-activated cell sorting (FACS)–based strategy using an antibody that targets the externalized epitope of Ddx4 for the purification of OSCs free of contaminating oocytes and other cells from ovaries of adult mice (10, 13). Importantly, the same technology was successfully applied to the isolation of OSCs from ovarian cortical tissue of reproductive-age women (10, 13). A direct comparison of the immunomagnetic bead strategy reported by Wu and colleagues (7) and our FACS-based approach (10) revealed that, although magnetic sorting results in a mixed population of cells including OSCs and oocytes, comparable fractions obtained by FACS are free of such contamination and can be used for characterization of freshly isolated OSCs. In this regard, OSCs analyzed immediately after FACS-based collection have a gene expression profile fully consistent with primitive germ cells during early specification and are not the result of some random in vitro transformation event as erroneously suggested by others (18). Along with the ability to isolate a pure population of OSCs from dissociated ovarian tissue, the use of FACS provides a quantitative assessment of OSC yield. In young adult mouse ovaries, the cells constitute 0.014 ± 0.002% of the total ovarian cell population, and ~1.5 ± 0.2% of the viable cell fraction sorted after dissociation (10).

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

MATERIALS AND METHODS

Animals

Wild-type C57BL/6 mice were obtained from Charles River Laboratories. To generate transgenic mice with *Stra8* promoter–driven green fluorescent protein (GFP) expression, a 1.4-kb fragment of the mouse *Stra8* promoter (−1,400 to +11), previously shown to convey premeiotic germ cell–specific expression (24, 25), was amplified from mouse genomic DNA and cloned into the *XhoI*/*EcoRI* site of the *pEgfp-1* vector (BD Biosciences). The *pStra8-Gfp* vector was then sent to Genoway to generate *pStra8-Gfp* transgenic mice with the use of their proprietary Quick Knock-In technology involving insertion of the transgene sequence into the neutral *Hprt* locus (www.genoway.com). This approach eliminates potential confounding effects of random integration of transgenes and variability in copy number commonly associated with conventional pronuclear injection. Mouse lines with germline transmission of the transgene were identified at Genoway, shipped to us, and used to establish breeding colonies. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital.

Cell Lines

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

Isolation of OSCs

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

Isolation of Primordial Germ Cells (PGCs)

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

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