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COMMENTARY

Purification of germline stem cells from adult mammalian ovaries: a step closer towards control of the female biological clock?

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ABSTRACT: For decades it was believed that a non-renewable pool of oocyte-containing follicles is established in female mammals at birth. This cornerstone of reproductive biology was challenged 5 years ago by a study reporting on the presence of mitotically-active germ cells in juvenile and adult mouse ovaries. Additional findings presented in this study and others that followed further suggested that mammals retain the capacity to generate oocytes during adulthood; however, isolation of oocyte-producing germline stem cells (GSC) as unequivocal proof of their existence remained elusive. This piece of information now appears to have been provided by Ji Wu and colleagues. In addition to showing that proliferative germ cells resembling male spermatogonial stem cells can be purified from neonatal or adult mouse ovaries and maintained *in vitro* for months, transplantation studies demonstrated that these cells generate oocytes in ovaries of chemotherapy-sterilized recipients that fertilize and produce viable offspring. Although these findings do not establish that oogenesis occurs in adult females under physiological conditions, they strongly support the existence of GSC in adult mouse ovaries. If equivalent cells can be found in human ovaries, stem cell-based rejuvenation of the oocyte reserve in ovaries on the verge of failure may one day be realized.

Key words: germline stem cell / oogenesis / oocyte / ovary / menopause

Introduction

One of the most exciting, and controversial, developments in female reproductive biology over the past several years relates to an increasing body of evidence that the ovarian follicle pool may be replenished during adult life by a rare population of putative germline stem cells (GSC) (Johnson *et al.*, 2004; reviewed by Tilly *et al.*, 2009). Although the existence of GSC and the occurrence of oogenesis in the ovaries of adult flies (reviewed by Kirilly and Xie, 2007) and non-mammalian vertebrates (Pearl and Schoppe, 1921; Draper *et al.*, 2007) are well-accepted findings, claims that the ovaries of mammalian females retain a comparable population of oocyte-producing stem cells stand in stark contrast to more than five decades of traditional thinking. The dogma that female mammals are born with all of the oocytes they will ever possess has its foundations in a paper from Sir Solomon Zuckerman published in 1951 (Zuckerman, 1951), which overviews his reasons for arriving at this conclusion. Simply put, Zuckerman failed to find any experimental evidence available at that time that he felt was inconsistent with an earlier hypothesis (Waldeyer, 1870) that germ cell production in female mammals ceases prior to birth (reviewed by Zuckerman, 1971). This article and its principal conclusion profoundly affected the subsequent interpretation of experimental and clinical observations relating to ovarian development, function and failure for the next 50 years. Indeed, the entire premise of why age-related ovarian failure and menopause occur has its roots in the belief that mammalian females lack the ability to replenish their oocyte reserve, resulting in a progressive and irreversible decline in follicle numbers until the pool is exhausted at some point during adult life (Faddy *et al.*, 1992). The consequences of this are significant to consider, not only in the context of a loss of fertile potential but also in the broader picture of the diverse spectrum of age-related health problems that emerge in postmenopausal women linked to failure of their ovaries (Prior, 1998; Buckler, 2005). In turn, if it were possible to repopulate adult ovaries with new oocytes and follicles, the female biological clock would no longer be an unreachable target for clinical intervention.

Ovarian failure and quality of life in aging females

The existence of a close relationship between ovarian function and general health in females has been known for many years. In humans, ovarian failure at menopause has been causally associated with increased risks for the development of a long list of significant health complications, including osteoporosis, cardiovascular disease, recurrent depression and cognitive dysfunction (Prior, 1998; Buckler, 2005; Frey *et al*., 2008). In addition, other less physically debilitating problems, such as heat intolerance and hot flushes, also negatively impact on the quality of life in peri- and post-menopausal women (Santoro, 2008). That ovarian failure is directly tied to these events is borne out by follow-up studies of young girls and reproductive age women treated for cancer with cytotoxic drugs or radiation. Many of these treatments, whereas effective at killing cancer cells, also inadvertently accelerate depletion of immature ovarian follicles (reviewed by Tilly, 2001; see also Oktem and Oktay, 2007). In turn, a premature onset of infertility and menopause is frequently observed in these patients, along with many of the same health complications that occur in aging women after natural menopause (Molina *et al*., 2005; Oktay and Sönmezer, 2008; Schover, 2008; Wo and Viswanathan, 2009).

Evidence from laboratory animal studies agrees with these clinical data. Like that seen in humans (Richardson *et al*., 1987), female mice exhaust their follicle reserves long before death due to advanced age (Gosden *et al*., 1983). Subsequent to this ovarian failure, aged female mice exhibit an increased incidence of many health problems commonly associated with post-menopausal life in women, including obesity, declining muscle and bone strength and neurological defects (Perez *et al*., 2007). Thus, mice have been a model of choice to examine the consequences of age-related follicular depletion on overall health and even longevity. For example, aging female mice exhibit striking increases in life expectancy after receiving transplants of young adult ovary tissue (Cargill *et al*., 2003). In other studies, disruption of the gene encoding *Bax*, which is involved in promoting age-associated oocyte loss and follicle atresia (Perez *et al*., 1999), extends ovarian lifespan and reduces the incidence of bone and muscle loss, excess fat deposition, alopecia, cataracts, deafness, increased anxiety and selective attention deficit with age (Perez *et al*., 2007). Importantly, aged *Bax*-null females do not exhibit an increased incidence of cancer in any tissue, including the mammary glands and uterus which are highly responsive to steroids produced by the ovaries (Knudson *et al*., 2001; Perez *et al*., 2007). These findings collectively indicate that experimentally induced maintenance of ovarian function in aging females can be used to achieve significant improvements in health, well-being and life expectancy.

Evidence for the existence of GSC in mammalian females

In March of 2004, a study was published reporting the presence of germline cells, as deduced by morphological criteria and expression of an evolutionarily conserved germline-specific marker (mouse vasa homolog or MVH), in or proximal to the surface epithelium of juvenile and adult mouse ovaries that exhibited evidence of mitotic activity (i.e. incorporation of bromodeoxyuridine or BrdU, and chromatin changes consistent with cells in prometaphase and metaphase). The existence of these cells, designated as presumptive GSC, along with other supporting data were offered as evidence that the longstanding dogma that mammalian females lose the capacity to produce new oocytes at birth was incorrect (Johnson *et al*., 2004). Numerous commentaries followed with various assessments of both the data presented and the validity of this claim (reviewed by Tilly *et al*., 2009), which by most accounts were a shock to the field (Lemonick, 2004; Powell, 2007). A debate that started over a century ago (reviewed by Everett, 1945) and was long-since believed to be settled (Zuckerman, 1951), was now reopened for discussion. Although this debate has since been perceived as representing two clearly opposing viewpoints with no common ground (reviewed by Powell, 2007), the existence of GSC in mammalian ovaries is not necessarily inconsistent with the idea that females are born with all of the oocytes they will ever have. Indeed there is the possibility that both views can co-exist, with the formation of a fixed population of oocytes at birth that is normally not subject to renewal and the existence of GSC in adult ovaries that can only be activated under specific circumstances. Since it is impossible to prove beyond any doubt the absence of any given cell in a tissue, the debate cannot be fully resolved until the presence and function of GSC within adult ovaries can be unequivocally demonstrated.

In this regard, the initial report from Johnson *et al*. (2004) indicated that the cells they identified as putative GSC in juvenile and adult mouse ovaries were extremely rare, as one might expect of a stem cell population *in vivo*. Follow-up studies also suggested a possible extra-ovarian origin of female GSC in adult mice (Johnson *et al*., 2005a). This conclusion was based on observations of regulated germline marker expression in bone marrow- and peripheral blood-derived cells, as well as the formation of what appeared, at least by accepted morphological and gene expression characteristics, to be donor-derived immature oocytes in ovaries of chemotherapy-treated or genetically-sterile female recipients transplanted with bone marrow or blood cells from transgenic females with germline-specific expression of green fluorescent protein (GFP) (Johnson *et al*., 2005a). However, the interpretation and physiological significance of these findings, and their bearing on the validity of the dogma that the oocyte pool is fixed at birth, were quickly disputed (Telfer *et al*., 2005; see also reply by Johnson *et al*., 2005b). Subsequent investigations demonstrated that putative bone marrow- and blood-derived germ cells fail to generate developmentally competent oocytes after induced (Eggan *et al*., 2006) or spontaneous (Lee *et al*., 2007a) ovulations, although such outcomes do not contradict findings of donor-derived immature oocytes being formed in recipient ovaries following parabiosis (Tilly *et al*., 2009) or transplantation of marrow- or blood-derived cells (Johnson *et al*., 2005a; Lee *et al*., 2007a). It is also noteworthy here that bone marrow transplants reportedly sustain or restore the function of ovaries that are failing due to chemotherapy exposure (Lee *et al*., 2007a; Fu *et al*., 2008) or advancing age (Selesniemi *et al*., 2009), with all offspring derived from the host females.

In any case, the successful isolation and characterization of putative female GSC remained elusive, despite experimental results from a handful of laboratories offered as additional evidence against the idea that mammalian females lose the capacity to produce new

oocytes around the time of birth (reviewed by Tilly *et al*., 2009). Data presented in support of oocyte renewal during adulthood have ranged from morphometry-based results reporting the mathematical improbability of a non-renewable oocyte pool being established at birth in rodents (Johnson *et al*., 2004; Kerr *et al*., 2006; see also Allen, 1923)—consistent with historical assessments of oocyte dynamics in rhesus monkeys that reached the same conclusion (Vermande-Van Eck, 1956)—to outcomes of studies showing increased oocyte numbers in adult ovaries by either pharmacologic enhancement of basal oogenesis or oocyte regeneration after a pathological insult that initially depletes the resting follicle pool (Johnson *et al*., 2005a; Borovskaya *et al*., 2006). Genetic approaches have also been employed, leading to identification of the *Cables1* cell cycle-regulatory gene as a key regulator of post-natal oogenesis in adult mice (Lee *et al*., 2007b). Other studies identified the *Caspase-6* gene as a potential modulator of post-natal oogenesis (Skaznik-Wikiel *et al*., 2007). However, the interpretations and conclusions of these studies were based on the presumed existence of a cell type that had not yet been isolated, or at least demonstrated to have complete germline potential. Given this, and the fact that the case against oocyte regeneration during adulthood was represented by a body of work spanning more than 50 years (reviewed by Gosden *et al*., 2009), additional evidence supporting the existence of pre-meiotic germ cells capable of producing new oocytes in adult ovary tissue was deemed necessary by many scientists to understand and accept these claims (Powell, 2007; reviewed by Tilly *et al*., 2009).

Purification of mammalian female GSC

A key finding supporting claims that adult mouse ovaries retain the capacity for oogenesis came in April of 2009, with a report that the equivalent of male spermatogonial stem cells had been successfully isolated from neonatal and adult mouse ovaries (Zou *et al*., 2009). These cells, termed female germline stem cells (FGSC), were initially identified using the same criteria employed by Johnson *et al*. (2004)—namely, expression of MVH and BrdU incorporation. Immunomagnetic beads coupled to MVH antibody were then utilized to purify cells positive for MVH expression from enzymatically-dispersed neonatal or adult mouse ovaries, and these cells were subsequently placed in culture on mitotically-inactivated mouse embryonic fibroblasts. The medium used to achieve long-term and stable *in-vitro* propagation of the presumptive FGSC was similar in composition to that used for the *in-vitro* support of male GSC (often referred to as spermatogonial stem cells). As of the online publication date of this study, neonatal and adult ovary-derived FGSC had been successfully maintained and passaged for more than 20 and 10 months, respectively. Further, it was shown that cryopreserved and thawed FGSC could be re-established in culture (Zou *et al*., 2009).

Although these observations support the claim that mitotically-active germline cells were successfully isolated from post-natal mouse ovaries, the protocol employed by Zou *et al*. (2009) to accomplish this has already been viewed by some with skepticism since MVH is classically considered to be an intracellular protein in germ cells (Fujiwara *et al*., 1994; Toyooka *et al*., 2000). As such, the use of MVH antibodies coupled to magnetic beads to viably isolate these cells seems at odds with its spatial expression pattern. However, in reading the supplementary information provided by Zou *et al*. (2009), their computer-based bioinformatics analysis of MVH protein revealed the presence of two consensus membrane-spanning helix domains that had not been reported previously. Our assessment of MVH using the TMpred program employed by Zou and colleagues (http://www.ch.embnet.org/software/TMPRED_form.html) confirmed the presence of these two consensus transmembrane domain sequences. Further, our orientation analysis is fully compatible with the predicted extracellular C-terminal sequence of MVH being recognized on the outside of germ cells by the antibody used by Zou and colleagues to isolate FGSC (unpublished observations). It is important to emphasize, however, that these types of programs are only predictive in nature, and thus the possibility that MVH can exist in germ cells as a transmembrane protein remains to be experimentally proven.

Irrespective, perhaps the most striking and significant aspect of this study from Zou *et al*. (2009) was their observation that either neonatal or adult ovary-derived FGSC could reconstitute ovarian function in adult female mice rendered sterile by treatment with busulphan and cyclophosphamide. Using a retrovirus to convey expression of GFP in FGSC prior to transplantation, additional experiments showed that GFP-positive oocytes contained within follicles at all maturational stages were formed in the ovaries of chemo-ablated wild-type female mice transplanted with FGSC. Furthermore, mating trials performed with the transplanted females yielded offspring containing the GFP transgene in their genomic DNA, which was successfully carried over into a second generation of offspring. Parallel evaluations of chemotherapy-treated females not receiving the FGSC transplants revealed complete ovarian failure and infertility (Zou *et al*., 2009). Given that similar approaches have been employed to characterize GSC in males, the data presented in this new study offer a compelling argument for the existence of GSC in adult mammalian females which, at least under the experimental conditions described by Zou *et al*. (2009), are fully capable of generating oocytes that can fertilize and yield viable offspring.

Implications and limitations of these new results

As exciting as these new findings are, some caution needs to be exercised in evaluating the immediate significance of the work to our understanding of the *in-vivo* biology of ovarian function, as well as the ultimate relevance of this work to reproductive health in women. The successful purification and characterization of what appear to be *bona fide* female GSC from neonatal and adult ovary tissue in mice, although important, does not immediately equate to proof that these cells serve a contributory role in determining the size of the post-natal follicle pool or the timing of ovarian failure under normal physiological conditions. Indeed, it may be that the full germline potential of these cells only arises as a consequence of their long-term culture *in vitro* and that their oogenic activity is normally suppressed *in vivo*. Additional work will be needed to assess this, although the study of Zou *et al*. (2009) provides a critical springboard on which to launch such follow-up investigations. Furthermore, all of the work discussed has focused on the formation of new

oocytes; however, for oocytes to survive and function they need to interact with somatic (granulosa) cells. The location and characterization of a putative granulosa stem cell niche is still to be confirmed, and it may be that the number or activity of these cells restricts the function of GSC *in vivo*. Whatever the case, even if activity of these GSC *in vivo* is shown and it is accepted that these cells function to sustain the adult follicle pool by partially offsetting the high rate of follicle loss through atresia, these GSC still fail to maintain ovarian function with advancing age. Indeed, their existence and potential regenerative activity does not change the fact that natural menopause happens approximately halfway or so through a woman's chronological lifespan. What might change, however, is the thinking behind why the ovaries fail as a consequence of the aging process. If one draws parallels to that recently described for males, an intriguing story emerges that is now worthy of testing in females.

From two separate studies of male mice, it was reported that whereas age exerts a negative cell-intrinsic impact on the germline, quiescent spermatogonial stem cells capable of driving spermatogenesis do in fact persist in atrophied testes (Ryu *et al*., 2006; Zhang *et al*., 2006). Based on these observations, it has been proposed that an alteration in the function of somatic cells that support GSC activity is a key aspect of age-related gonadal failure. Such a conclusion aligns well with the outcomes of reciprocal transplantation studies in which atrophied testes of 12-month-old, but not 24-month-old, males were found to be permissive to a reconstitution of spermatogenesis by GSC collected from young donor animals (Zhang *et al*., 2006). If a similar situation exists in adult females, one could make the argument that age-related ovarian failure reflects impairment in somatic cell support of GSC activity. The net result would be a loss of input into the oocyte pool, thus allowing for atresia to rapidly deplete the remaining follicles in an unabated fashion. Accordingly, it would be of interest to test if FGSC can be retrieved from aged ovary tissue and whether such FGSC would retain their ability to reconstitute oogenesis if transplanted into a young host environment.

In parallel to continued investigations into the regulation and function of FGSC in mouse ovaries, efforts are needed to determine if a comparable population of cells exists in human ovaries. Earlier attempts to provide evidence of mitotic germ cells or meiotic entry in adult human ovarian tissue were reported as unsuccessful (Liu *et al*., 2007), although this work was questioned because of sensitivity issues with the assays employed to detect low abundance markers of germ cell renewal (Tilly and Johnson, 2007). Indeed, two subsequent studies have provided surprising insight into the presence of rare stem-like cells with germline characteristics in the ovarian surface epithelium of post-menopausal women. The first of these documented the isolation of these cells and their ability to spontaneously form oocytes or oocyte-like cells *in vitro* (Virant-Klun *et al*., 2008a). It was then reported that oocytes derived from these putative stem cells *in vitro* could undergo parthenogenetic activation to form blastocyst-like structures (Virant-Klun *et al*., 2008b). Although it remains unknown if the cells isolated from post-menopausal ovaries represent the human equivalent of the mouse FGSC identified by Zou *et al*. (2009), these studies nonetheless show that rare germline-like cells have until now gone undetected in both rodent and human ovaries. Further, it is noteworthy that the surface epithelial location of the stem-like cells in post-menopausal ovaries reported by Virant-Klun *et al*. (2008a, b) matches initial reports of the location of presumptive GSC (MVH–BrdU double-positive cells) in juvenile and young adult mouse ovaries (Johnson *et al*., 2004). A current challenge will be to isolate and characterize these cells from human ovaries, which may prove difficult because of the limited experimental tissue available for analysis. However, the recent development of a novel culture system that supports the growth of human ovarian cortical tissue *in vitro* (Telfer *at al*., 2008) may offer a valuable tool to identify putative GSC in human ovaries for further characterization.

In summary, this new study from Zou *et al*. (2009) has already garnered a substantial amount of interest from scientists in many disciplines (Normile, 2009). Although clearly supportive of and significantly extending earlier work claiming that mammalian GSC capable of supporting post-natal oogenesis exist, at least in the mouse (Johnson *et al*., 2004), there remain many unanswered questions. A few of these have been highlighted herein, with efforts to evaluate the physiological significance of these FGSC to adult ovarian function and failure requiring, at least in our view, some priority. It should also be noted that many stem cell experiments hailed as major breakthroughs have proven notoriously difficult to reproduce (reviewed by Check, 2007). Still, the results presented by Zou *et al*. (2009) represent an important advance in the fields of stem cell and reproductive biology, and several groups will now be poised to replicate and extend their findings. Time will tell if the reported isolation of female GSC from adult mouse ovaries is a reproducible observation, but in the meantime we should be open to its possibilities. Studies such as these move us a step closer to serious consideration of how stem cell-based regenerative medicine may one day become a safe and effective strategy to control the female biological clock and, as a consequence, the timing of age-related ovarian failure and menopause when it might be clinically desirable to do so.

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