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Oogonial stem cells as a model to study age-associated infertility in women

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Abstract

Fertility is the first biological process to break down during aging, thereby making it a useful tool to understand fundamental processes of aging. Reproductive aging in females is associated with a loss of ovarian function characterised by a reduction in the number and quality of oocytes. The central dogma, namely that females are born with a fixed pool of oocytes that progressively decline with increasing maternal age, has been challenged by evidence supporting postnatal oogenesis in mammals. Reports demonstrating formation of new oocytes from newly discovered germline stem cells, referred to as oogonial stem cells (OSCs), has opened new avenues for treatment of female infertility. In this review we discuss why the OSCs possibly lose their regenerative potential over time, and focus specifically on the aging process in germline stem cells as a possible mechanism for understanding female age-related infertility and how we can slow or delay ovarian aging.

Additional keywords

germline stem cells; meiosis; oocyte; postnatal oogenesis

Introduction

Reproductive aging is characterised by a progressive decline in fertility and fecundity with advancing age (te Velde and Pearson 2002). In women, fertility decreases drastically beyond 37 years of age (The American College of Obstetricians and Gynecologists Committee on Gynecologic Practice and The Practice Committee of the American Society for Reproductive Medicine 2014) and is associated with reduced ovarian function. This decline is believed to contribute to the development of health complications, including osteoporosis, cardiovascular disease, cancer and urinary problems (Pérez-López *et al.* 2009). Combined with increasing numbers of women delaying childbirth, immense efforts are being made to diagnose and counteract age-linked female infertility.

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Although it is known that age is the single most important determinant of female fertility (Balasch 2010), recent work has shown that the decline in fertility with age is far more malleable than originally thought. Studies in lower organisms such as *Drosophila* have demonstrated that, with age, there is a gradual decline in the rate of germline stem cell division accompanied by reduced egg production and increased incidence of cell death of developing eggs, especially in oldest females (Zhao *et al.* 2008). In mammals, the female reproductive axis is the first to fail with advancing age and is associated with changes in ovarian function (te Velde and Pearson 2002; Selesniemi *et al.* 2009). The primary functional unit of the mammalian ovary is the follicle, consisting of an oocyte and supporting somatic cells that function in the growth and development of the oocyte (Channing *et al.* 1980). Each oocyte, enclosed within a primordial follicle, is arrested in the diplotene stage of meiotic prophase I (Borum 1961). The pool of primordial follicles laid down at birth represents the total population of follicles existing in the ovary throughout the reproductive lifespan of a female (Kezele *et al.* 2002). In mammals, several of the oocytes die during development or shortly after birth, and those remaining continue to decline with age (De Felici *et al.* 2005). This eventual exhaustion of the ovarian follicular reserve culminates in a complete cessation of normal ovarian function, driving menopause and a decline in female physiology (Richardson *et al.* 1987).

A central dogma in the field of reproductive biology is that ovaries of mammalian females are endowed with a finite non-renewable pool of oocytes at the time of birth (Borum 1961). The foundation for this notion is the supposed absence of mitotically active germ cells in postnatal mammalian ovaries (Pepling 2006). In contrast, females of non-mammalian species, including flies and fish, retain germline stem cells and continue producing new oocytes well into adult life (Kirilly and Xie 2007; Nakamura *et al.* 2010). Germline stem cells are a unique stem cell population involved in reproduction and transmitting genetic information (Lehmann 2012). In males, spermatogenesis continues throughout adult life due to the presence of spermatogonial stem cells (Valli *et al.* 2014). However, recent studies may have disproved the theory of fixed ovarian reserve in adult females by providing evidence for the presence of germline stem cells in the postnatal mammalian ovary that are capable of undergoing differentiation into oocytes both *in vitro* and *in vivo* (Johnson *et al.* 2004; White *et al.* 2012). Nonetheless, this field is still in its infancy and lacks a complete understanding of how these putative germline stem cells and fertility respond during aging. Given that oocyte numbers decline with age, it is possible that menopause in females is not a result of depletion of the ovarian reserve, but rather a result of aging of germline stem cells (Dunlop *et al.* 2014). In this review we discuss the connection between stem cells, fertility and aging.

Postnatal oogenesis, germline stem cells and fertility

In mammals, oogenesis is believed to be primarily prenatal. During development in females, precursor germ cells known as primordial germ cells arise extra-embryonically and migrate to their final destination in the gonads, where they enter meiosis and differentiate into oocytes, thereby ending their stem cell potential (Bowles and Koopman 2007). The possibility of postnatal oogenesis in the mammalian ovary was a topic of debate for a long time (Greenfeld and Flaws 2004). In 2004, multiple lines of evidence were provided, for the first time, for the existence of germline stem cells in adult mouse ovaries capable of

generating oocytes to form new follicles (Johnson *et al.* 2004). A small population of mitotically active germ cells was detected in the surface epithelium of adult ovaries, which, upon isolation, differentiated to produce oocyte-like cells in culture (Johnson *et al.* 2004). By calculating the number of follicles undergoing atresia, it was concluded that postnatal production of follicles to the existing ovarian reserve was necessary to reach the expected reproductive lifespan in mice (Johnson *et al.* 2004). Thus, the demonstration of the existence of putative postnatal germline stem cells changed the traditional thinking that mammalian oogenesis is prenatal.

Following the work of Johnson *et al.* (2004), several independent laboratories demonstrated evidence for the existence of postnatal oogenesis, each having isolated germline stem cells from neonatal and adult mouse ovaries using different isolation techniques (Zou *et al.* 2009; Pacchiarotti *et al.* 2010; Zhang *et al.* 2011; White *et al.* 2012). The study by White *et al.* (2012) further went on to isolate these germline stem cells, henceforth referred to as oogonial stem cells (OSCs), from adult human ovaries. The OSCs were isolated using a ATP-dependent DEAD-box RNA helicase (DDX4) antibody-based immunomagnetic sort or fluorescence-activated cell sorting (FACS; Zou *et al.* 2009; White *et al.* 2012). These OSCs constitute a very small subset, approximately 0.01%, of the total ovarian cell population. Freshly isolated mouse and human OSCs express germline markers, namely PR domain zinc finger protein 1 (*Prdm1*), developmental pluripotency associated 3 (*Dppa3*), interferon-induced transmembrane protein 3 (*Ifitm3*), telomerase reverse transcriptase (*Tert*), *Ddx4* and deleted in azoospermia-like gene family (*Dazl*), possess pluripotency markers octamer-binding transcription factor 4 (*Oct4*) and stage-specific mouse embryonic antigen (*Ssea1*) and have a moderate level of telomerase activity (White *et al.* 2012; Woods and Tilly 2013). The OSCs maintain their germline profile even when cultured for a long time *in vitro*. In culture, a subset of these OSCs undergoes spontaneous meiosis to generate oocyte-like cells that express classic oocyte markers, including *Kit*, newborn ovary homeobox (*Nobox*), Y-box-binding protein 2 (*Ybx2*), LIM homeobox 8 (*Lhx8*) and growth differentiation factor 9 (*Gdf9*), as well as zona pellucida glycoprotein (*Zp1*, *Zp2* and *Zp3*) (White *et al.* 2012). In transplantation studies, mouse OSCs with stable green fluorescent protein (GFP) expression have been shown to differentiate into follicle-enclosed GFP-positive oocytes that, upon ovulation, are released as mature MII eggs. These eggs are fertilisation competent and can produce viable offspring (Zou *et al.* 2009; White *et al.* 2012). Like mouse OSCs, human GFP-tagged OSCs also undergo neo-oogenesis, producing large, ovoid, GFP-positive oocytes in adult human ovarian cortical tissue *in vivo* (White *et al.* 2012).

Germline stem cell aging

Aging is characterised by a progressive decline of tissue and organ function. The human body is composed of a variety of adult stem cells that are capable of replenishing somatic cells and tissues as they get damaged, diseased or die to maintain tissue homeostasis (Jones and Rando 2011). However, during aging, stem cells lose their regenerative and proliferative potential and this loss of stem cell function over time is considered a major cause of age-related organ deterioration and disease (Jones and Rando 2011; Oh *et al.* 2014). To date, the mechanisms of such stem cell aging are poorly understood. Comprehending the molecular pathways that regulate stem cell survival, self-renewal, proliferation and commitment to

specific differentiated cell lineages and age-dependent stem cell quiescence is critical in determining age-associated stem cell dysfunction. Furthermore, such knowledge will be essential for the development of therapeutic interventions that can delay age-related degenerative processes to enhance repair and maintain healthy function in aging tissues.

In the context of regenerative medicine and treating age-related diseases, adult somatic stem cells and embryonic stem cells have attracted much attention. However, germline stem cells have been largely ignored. This was mainly because these cells were not even known to exist. Not only have these cells now been identified, but they also populate atrophic ovaries of reproductively aged mice despite complete depletion of oocytes (Niikura *et al.* 2009). This suggests that premeiotic germ cells or OSCs, although present in ovaries of aged mice, exist in a dormant state and are apparently unable to proliferate and differentiate into oocytes during aging. When transplanted into a young host, these germ cells resume differentiation to form oocytes, suggesting that ovarian aging may be reversible (Niikura *et al.* 2009). Thus, from a reproductive aging perspective, the main question is what causes the debility in transition of OSCs into oocytes.

Evidence for ongoing oogenesis in adult postnatal ovaries comes from reports demonstrating the presence of the germ cell specific gene stimulated by retinoic acid gene 8 (*Stra8*), a regulator of meiotic initiation and progression in both spermatogenesis and oogenesis (Niikura *et al.* 2009). *Stra8* is required for premeiotic DNA replication and progression into meiotic prophase (Baltus *et al.* 2006; Anderson *et al.* 2008). *Stra8*^{-/-} germ cells do not undergo premeiotic DNA replication and do not enter meiotic prophase (Baltus *et al.* 2006). Induction of *Stra8*, in both male and female germ cells, occurs in response to retinoic acid (RA) signalling in the somatic cells (Anderson *et al.* 2008). An important step in the regulation of mammalian oogenesis is the sex-specific timing of RA-mediated *Stra8* transcription. Recently, it was shown that, in adult mammalian ovaries, RA-induced *Stra8* expression is epigenetically regulated at the RA response elements (RARE) region of the *Stra8* promoter (Wang and Tilly 2010). Based on the pivotal role of *Stra8* in male spermatogenesis and some initial evidence of expression in the adult ovary (Niikura *et al.* 2009), *Stra8* is a good marker for postnatal oogenesis and should be investigated in OSCs. In addition, genes for meiosis, including *Stra8*, serve as good indicators of neo-oogenesis in postnatal mammalian ovaries. It is noteworthy to mention that only a fraction of the OSCs in culture undergo meiosis to form oocytes (Woods and Tilly 2013). Why only a few cells express *Stra8* and undergo differentiation remains unknown. Thus, the identification of a molecular clock regulating differentiation in OSCs is awaited.

Delaying reproductive aging

According to the ‘disposable soma theory’ of aging, more energy is invested by animals into reproduction than for the maintenance of the soma. This results in fewer resources available for protecting the soma for indefinite survival, thereby driving aging (Kirkwood and Holliday 1979). Conversely, calorie restriction (CR), a dietary regimen that involves consumption of a diet approximately 30%–40% reduced in calories compared with *ad libitum* diet, redirects energy required for reproduction towards somatic maintenance, thus retarding aging at the cost of reduced fertility. This temporary hiatus from fertility permits

an individual to survive and preserve fertility for when resources become available (Shanley and Kirkwood 2000).

Of the several interventions studied to prevent aging, CR by far stands out to be the most promising in lifespan extension (Holliday 1989) in diverse species and protects against age-related pathologies. In lower organisms such as *Drosophila* and *Caenorhabditis elegans*, nutritional status determines the fate of germline stem cells (LaFever and Drummond-Barbosa 2005; Angelo and Van Gilst 2009; Hsu and Drummond-Barbosa 2009). Most of the germline degenerates when resources are scarce. However, a small pool of germline stem cells is maintained that retains its ability to repopulate and reproduce when normal feeding resumes (Angelo and Van Gilst 2009; Mair *et al.* 2010). Thus, the maintenance of a germline stem cell pool during CR to extend reproductive period is a potential mechanism by which CR may delay aging in lower organisms (Mair *et al.* 2010).

In mice, restricting calories has been shown to prolong lifespan by almost 50% and retard the onset of age-related diseases (Sohal and Weindruch 1996). Although female mice experience reduced fecundity while on the CR diet, age-associated loss of oestrous cyclicity and follicular reserve are delayed and, upon resumption of *ad libitum* diet, mice remain fertile for an extended period of time (Nelson *et al.* 1985). In aging rodents, CR has been shown to extend fertility (Selesniemi *et al.* 2008). An adult onset of CR for a brief period of time diminished age-related chromosomal abnormalities and mitochondrial dysfunction in oocytes and improved postnatal survival of offspring delivered by aged female mice (Selesniemi *et al.* 2008; Selesniemi *et al.* 2011). Although CR is an effective way to delay ovarian aging, little is known about the underlying molecular mechanisms by which CR delays aging and reproductive senescence.

Of the several pathways mediating CR, the nutrient sensing pathways of insulin/insulin-like growth factor-1 (IGF-1), mammalian target of rapamycin (mTOR), sirtuins and AMP-activated kinase (AMPK) are well established (Tilly and Sinclair 2013). The metabolic and energy sensors sirtuin 1 (SIRT1) and AMPK operate as a synchronised network in response to environmental signals and interact with insulin/IGF-1 and mTOR to regulate cellular metabolism, growth and energy intake. The phosphatase and tensin homologue (PTEN)/phosphatidylinositol 3-kinase (PI3K) pathway has been shown to be functional in regulating oocyte growth (Reddy *et al.* 2008; Li *et al.* 2010) and the mTOR/PI3K/PTEN pathway plays a role in meiotic progression and embryonic genome activation in embryos (Zheng *et al.* 2010; Lee *et al.* 2012). Recently, a regulatory role for hypoxia-mediated IGF-I receptor (IGF-IR)–PI3K/Akt–mTOR–hypoxia inducible factor (HIF)-2 α signalling was demonstrated to mediate proliferation and maintenance of pluripotency in mouse germline stem cells (Huang *et al.* 2014).

Some of the beneficial effects of CR are mediated in a SIRT1, an NAD⁺ dependent deacetylase and nutrient sensing protein, dependent manner (Guarente and Picard 2005; Guarente 2013). Alternatively, increasing SIRT1 activity or raising intracellular levels of NAD⁺ has also been shown to mimic CR-like physiology and protection from several age-associated diseases in mice (Howitz *et al.* 2003; Gomes *et al.* 2013). Moreover, increased SIRT1 activity, as depicted by use of small molecule activators of SIRT1, such as resveratrol,

has been shown to increase the number of eggs laid per organism in *C. elegans* and *Drosophila* (Wood *et al.* 2004) and both the number and quality of oocytes in aged mice compared with age-matched control mice (Liu *et al.* 2013). Considering OSCs accumulate in the ovaries of aged mice with an apparent block in differentiation, it is reasonable to postulate that raising NAD⁺ levels of these cells, thereby activating SIRT1, will promote differentiation. Thus, compounds that raise NAD⁺ levels may serve as molecules with the potential to delay ovarian aging and extend female reproductive lifespan. Whether this occurs through SIRT1-mediated regulation of *Stra8* is yet to be investigated. Identification of the pathways by which SIRT1 regulates the transition of OSCs into oocytes will help open new dimensions in the understanding of reproductive aging.

Mitochondria and energetics

The oocyte has the largest number of mitochondria and mitochondrial (mt) DNA per cell necessary for maintaining energy requirements during proper oocyte maturation, spindle formation, fertilisation and embryo development (Bentov *et al.* 2011; Tilly and Sinclair 2013). Mitochondrial function in oocytes declines with age and, consistent with this, oocytes from older women have decreased ATP levels, low mtDNA copy number and intracellular clusters of aggregated mitochondria that are increased in size and density (Bentov *et al.* 2011; Selesniemi *et al.* 2011; Tilly and Sinclair 2013). An energy deficit in the aging oocyte also disables the formation of the meiotic spindle, a high energy-consuming process, and results in chromosomal misalignment and aneuploidy, which are closely linked to trisomic conceptions, implantation failures and miscarriages (Hassold and Chiu 1985; Munné and Cohen 1998; Tilly and Sinclair 2013). Because mitochondria are solely inherited from the mother, the embryo also inherits a dysfunctional energy system, which then poses a threat to embryonic development (Tilly and Sinclair 2013). Conversely, increasing mitochondrial function and energy production have a positive impact on the overall fertility outcome in women of advanced maternal age. Being natural oocyte progenitors, OSCs in culture can be used to identify compounds that, by raising energy levels, facilitate the formation and maintenance of meiotic spindles, thereby reducing the occurrence of oocyte aneuploidy. One such method of increasing energy levels is by raising NAD⁺, which has emerged as a potent mitochondrial booster (Lin and Guarente 2003; Gomes *et al.* 2013). Thus, it is reasonable to believe that using mitochondrial activators as *in vivo* agents would benefit women with energetically compromised eggs or embryos, especially those undergoing IVF.

Perspective

In the past few years a lot of interest has been generated in the field of stem cell aging. Aging seems to be a consequence of diminished stem cell function or a decline in the function of other self-renewing cells, and understanding how age-dependent changes in stem cell function contribute to human aging may aid in the development of new anti-aging therapies. An outstanding example of cellular aging, separate from the aging of the rest of the organism, is the aging of germ cells within the ovaries of postmenopausal women (Niikura *et al.* 2009). Women struggling with infertility, due to either advanced age or ovarian insufficiency, have little option but to rely on egg donation to achieve a successful pregnancy. However, in such a case the child is not their biological child (Tilly and Sinclair

2013). With the recent discovery of postnatal oogenesis and mitotically active germ cells or OSCs, there may be a possibility of coaxing these cells into forming healthy oocytes in women of advanced age. However, the reason OSCs fail to produce functional oocytes in older women is not known. Alternatively, it is possible that OSC aging is a result of impaired DNA double-strand break repair, a recently identified cause of aging in mammalian oocytes (Titus *et al.* 2013; Govindaraj *et al.* 2015). Elucidating the mechanisms that cause OSCs to age could lead to new treatments that could delay ovarian aging and slow infertility.

It is now well recognised that CR results in extended lifespan and rebound fertility. However, the role of SIRT1, as a mediator of the CR response, has yet to be linked to the longevity of germ cells. The cell autonomous function of SIRT1 in germline stem cells and reproductive aging, if any, is yet to be determined. One of the theories of reproductive aging that we think can explain the role of SIRT1 in germ cells, or more so in the germline stem cells, links to its epigenetic regulation of *Stra8* expression. The OSCs are natural precursor cells for oocytes and thus any manipulation or testing done in these cells should, theoretically, have a similar effect on oocytes. Moreover, unlike oocytes, OSCs can be propagated and cultured *in vitro* for long periods of time and in seemingly infinite numbers. Thus, they can be exploited as a platform to screen for compounds that, by raising NAD⁺ levels, may potentially rescue the OSCs from their dormant state and facilitate the production of more oocytes (Tilly and Sinclair 2013).

The field of female germ cell biology is still in its infancy, and many questions remain. If human OSCs can be proven to generate fertilisable eggs and eventually offspring, they hold considerable promise in counteracting the effects of chemotherapeutic drugs and aging on fertility. The OSCs may allow for germline correction of inherited dominant human diseases and, eventually, it may be possible to differentiate OSCs *ex vivo* using tissue explants or cell coculture, allowing them to be fertilised. In this way, dozens of healthy oocytes could be produced from an infertile woman and screened before implantation, thereby revolutionising human fertility. There may be other applications as well that, at present, we can only see, such as tissue regeneration and organ replacement.

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