Neo-oogenesis in mammals

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Summary

Recently, the existence of a mechanism for neo-oogenesis in the ovaries of adult mammals has generated much controversy within reproductive biology. This mechanism, which proposes that the ovary has cells capable of renewing the follicular reserve, has been described for various species of mammals. The first evidence was found in prosimians and humans. However, these findings were not considered relevant because the predominant dogma for reproductive biology at the time was that of Zuckerman. This dogma states that female mammals are born with finite numbers of oocytes that decline throughout postnatal life. Currently, the concept of neo-oogenesis has gained momentum due to the discovery of cells with mitotic activity in adult ovaries of various mammalian species (mice, humans, rhesus monkeys, domestic animals such as pigs, and wild animals such as bats). Despite these reports, the concept of neo-oogenesis has not been widely accepted by the scientific community, generating much criticism and speculation about its accuracy because it has been impossible to reproduce some evidence. This controversy has led to the creation of two positions: one in favour of neo-oogenesis and the other against it. Various animal models have been used in support of both camps, including both classic laboratory animals and domestic and wild animals. The aim of this review is to critically present the current literature on the subject and to evaluate the arguments pro and contra neo-oogenesis in mammals.

Keywords: Germ line stem cells, Mammalian ovary, Neo-oogenesis, Ovarian stem cells, Primordial germ cells

Introduction

The ovaries are the female sex glands that produce hormones responsible for ensuring the proper functioning of all sexual organs in females. During their development, the ovaries fulfill two fundamental processes: (1) oogenesis, in which female germ cells (oocytes) are formed and mature; and (2) folliculogenesis, in which somatic cells (granulosa and theca) surround the oocyte and form ovarian follicles. Because of cellular interactions that take place between the oocyte and somatic cells and hormonal influences, the ovary becomes endowed with a finite number of germ cells at birth that diminishes with age, once the reproductive stage begins, and eventually disappears (Zuckerman, 1951). In contrast, several studies have suggested that the adult mammalian ovary is not provided with a finite number of oocytes, as has been asserted for so long. Classic and current evidence indicates that the adult mammalian ovary possesses self-renewing germline stem cells (GSCs) that form a reserve of oocytes that maintains a stable number of germ cells. This idea, in opposition to the established dogma in reproductive biology, has generated much controversy, which has intensified over the past 2 decades, creating opinions for and against the existence of a mechanism of neo-oogenesis in the adult mammalian ovary (Gosden, 2013).

In mammals, the existence of GSCs has caused great controversy. Several research groups have argued that

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GSCs do not exist in adult mammalian ovaries and are present only in the testes, in which sperm production is derived from a population of self-renewing cells known as spermatogonial stem cells (SSCs) that are present in males of all species studied (Regaud, 1901; Brinster, 2007). Considering that females ovulate only a few hundred oocytes during a portion of their life, evolutionarily, they do not require these cells (Monget *et al.*, 2012), contrary to invertebrates and fish, which require a pool of renewable GSCs due to their high reproduction rates throughout the year.

Primordial germ cells

The primordial germ cells (PGCs) are the first population of germ cells established during development and are the precursor cells of oocytes and sperm in the ovaries and testes, respectively. In mice, PGCs are first identified by their affinity for alkaline phosphatase at 7.25 embryonic day (E7.25) emerging from the posterior primitive streak in extraembryonic mesoderm (Chiquoine, 1954; Ginsburg *et al.*, 1990; Leitch *et al.*, 2013). Subsequently, PGCs migrate through the region in which the gonads are forming passing through the hindgut endoderm around E7.75 to the dorsal mesentery around E9.5 and colonizing the genital ridges at E10.5 (Saitou & Yamaji, 2012). Molyneaux and colleagues (2001) described distinct phases of PGCs migration, the first, until E9.0–E9.5, PGCs are already highly mobile, but do not leave the gut. Second, in the E9.0–E9.5 period, before the mesentery forms, PGCs very rapidly exit the gut, but do not migrate towards the genital ridges. Third, during the E10.0–E10.5 period, PGCs migrate directionally from the dorsal body wall into the genital ridges. Finally, at E11.5, PGCs are slowing and the direction of movement is dependent on the sex of the embryo. PGCs proliferate as they migrate, rapidly increasing in number to approximately 100 cells at E8.5 and to 25,000 at E13.5 (Ewen & Koopman, 2010).

Sex-specific differences in the gonads are evident by E12.5 and this represents a convenient endpoint of PGCs development (Sasaki & Matsui, 2008). In female embryos, PGCs begin the process of meiosis to reduce their genetic material. Notably, PGCs are unable to divide mitotically once they enter the first phase of meiotic division (Hilscher *et al.*, 1974; Speed, 1982). These cells undergo considerable epigenetic reprogramming, including chromatin modification, allowing the downregulation of genes from somatic cells such as those from the HOX family (*Hoxa1*, *Hoxb1*, *Lim1* and *Evx1*), which are repressed in cells destined to become germ cells (Saitou *et al.*, 2002). This downregulation permits both pluripotency in genes and activation of genes characteristic of the germ cell lineage, in particular *Prdm1* (also known as *Blimp1*) and *Prdm14*. During their specification and migration, PGCs are highly pluripotent and are characterized by the expression of genes such as *Oct-4*, *Sox2* and *Nanog*, but during ovarian development, these genes are substantially downregulated around E13.5 (Western *et al.*, 2010), just before entry into meiosis.

Oogenesis

The onset of meiosis is crucial for ovarian morphogenesis, which involves folliculogenesis and oogenesis. The onset of meiosis is executed by the action of retinoic acid (RA), which is present in the gonadal environment and activates pre-meiotic gene expression of *Stra8* (Stimulated by Retinoic Acid 8) in ovarian germ cells (Koubova *et al.*, 2006; Bowles & Koopman, 2007; Griswold *et al.*, 2012; Mu *et al.*, 2013). Recently, it has become apparent that a second gene called *Rec8* (meiotic recombination protein), which encodes a component of the cohesin complex, is essential for meiosis; it has been thought that Rec8 is another target of RA and is activated independently of Stra8 (Koubova *et al.*, 2014).

Once meiosis begins, a layer of granulosa cells begins to enclose the oocytes, forming primordial follicles. Many oocytes that are not surrounded by somatic cells undergo apoptosis, determining the pool of primordial follicles (Sanchez & Smitz, 2012). These follicles become primary follicles when granulosa cells become columnar and undergo mitotic division to form a multi-layered stratum granulosum and oocytes grow to more than 20 microns in size. At this stage of folliculogenesis, numerous genes are activated, such as the transcription factor *Figla*, which is expressed exclusively in germ cells and regulates the transcription of zona pellucida genes (*ZP1*, *ZP2* and *ZP3*) (Liang *et al.*, 1997), and *Nobox*, which restricts oocyte growth and limits the development of granulosa cells to seven layers, although primordial follicles appear histologically normal, as evidenced from mouse knockouts (Rajkovic *et al.*, 2004). During folliculogenesis, the oocyte responds to luteinizing hormone, resuming meiosis and completing maturation. Reactivation of meiosis and promotion through metaphase involves a complex signaling cascade based on epidermal growth factor (Park *et al.*, 2004). The mitogen-activated protein kinase pathway is activated in the granulosa cells of the pre-ovulatory follicle to maintain the oocyte in a state of arrest (Downs, 2010) while regulating the permeability of cyclic guanosine monophosphate through junctions between granulosa cells and the oocyte (Norris *et al.*, 2008). Oocyte maturation is completed after arrest in metaphase II, and completion of meiosis occurs exclusively with fertilization and

extrusion of the second polar body, leaving a haploid female pronucleus (Li & Albertini, 2013).

Dogma of reproductive biology

During the 1920s, Pearl and Schoppe asserted that the number of oocytes does not increase during the life of an individual (Pearl & Schoppe, 1921), an idea that was later corroborated by the British zoologist Sir Solomon Zuckerman. In 1951, Zuckerman published a report that provided a careful review of the oocyte numbers of various animal species (rat, mouse, rhesus monkey, rabbit, dog, guinea pig and human) at various ages and noted that the number of oocytes of each species decreased with increasing age. This report led to the basic doctrine of reproductive biology, which argues that during fetal development, female mammals have the potential to generate a limited stock of oocytes, which are surrounded by somatic cells (granulosa cells) and form follicles that are unable to divide (Zuckerman, 1951; Zuckerman & Baker, 1977). Likewise, another group reached the same conclusion, showing that oocyte formation in mouse ovaries takes place only during the fetal period of life, decreases with female age and is depleted in menopause (Peters *et al.*, 1962).

Currently, the central dogma of reproductive biology has been questioned because experimental evidence suggests the existence of stem cells with germ cell characteristics and mitotic activity in the ovarian surface epithelium (OSE) of the adult ovary (Johnson *et al.*, 2004). The function of these cells is to form a small reserve of undifferentiated cells that are capable of self-renewal to maintain a constant number of oocytes, a mechanism that has been called neo-oogenesis. Accordingly, there are two main stances: one that is sceptical of neo-oogenesis and supports the idea of the existence of a fixed number of oocytes established before birth (the classic idea in reproductive biology) and a second that strongly supports the existence of germ stem cells (GSCs) with mitotic activity in the germinal epithelium of adult ovaries that maintain a stable number of oocytes (neo-oogenesis). It has also been indicated in various sources that a reservoir of GSCs that migrate to the ovary exists, an assertion that has added controversy by creating greater discord concerning this hypothesis.

Evidence of neo-oogenesis

The first report to discuss the existence of a renewable source of adult stem cells in the ovaries was presented by Heinrich Waldeyer-Hartz a German anatomist and physiologist (1870). While not firmly accepting the existence of GSCs, the report indicated that the OSE is active for a limited period. Subsequently, Kingery (1917) reaffirmed this idea, stating that the oocytes degenerated during fetal life are restored by oocytes that develop in the germinal epithelium of the adult ovary. These findings were used to argue that new oocytes emerge from the germinal epithelium as a result of the mitotic division of undifferentiated cells (Allen, 1923; Allen & Creadick, 1937; Esmaeilian *et al.*, 2015). Moreover, classic histological works have showed oogonia with mitotic activity and oocytes in the early stages of meiotic prophase in adult ovaries of lower primates *Galago senegalensis* (Gerard, 1920, 1932; Gerard & Herlant, 1953; Herlant, 1961; Petter-Rousseaux, 1962; Butler, 1964), *G. crassicaudatus* (Gerard & Herlant, 1953), *G. demidoffi* (Gerard, 1932; Gerard & Herlant, 1953; Petter-Rousseaux, 1962), and *Perodicticus potto* (Gerard & Herlant, 1953), a prosimian lemuroide *Loris tardigradus lydekkerianus* (Rao, 1927; Brambell, 1930) and in *Daubentonia madagascariensis* (Petter-Rousseaux & Bourliere, 1965). It has also been argued that new germ cells are formed by direct transformation of somatic cells of the germinal epithelium of the ovary (Gerard, 1920, 1932; Gerard & Herlant, 1953; Rao, 1927).

Later, in 1967, Ioannou examined ovaries from adult prosimians and noted that oogonia at interphase or in various stages of mitosis undoubtedly occurred in adult female *Loris* and *Perodicticus* species of Galago (Ioannou, 1967). These findings and studies by Anand Kumar (1966) suggest that although few signs of mitotic activity were observed in ovaries from adult *Loris*, an examination of numerous ovaries from mature specimens of this species have shown that germ cells occur at all stages of mitosis. Oocytes at the successive stages of meiosis up to diplotene are also present, often in great numbers. While oogonia may occasionally be observed in the germinal epithelium, there is no reason to believe that such cells are derived from transformed epithelial cells. It is more likely that all germ cells in the adult prosimian ovary are derived from daughter cells of pre-existing oogonia, as is known to be the case for all other mammals studied (Franchi *et al.*, 1962). However, these findings are not currently relevant and are regarded as an exception to the rule.

The concept of neo-oogenesis resurfaced when the Johnson group proposed that mitotically active germ cells, which are capable of replacing oocytes lost through atresia and ovulation, are found in the ovaries of young and adult mice, thereby maintaining a stable pool of follicles (Johnson *et al.*, 2004). By counting follicles using mathematical models, it was estimated that up to 33% of immature follicles in adult mice are atretic at any given moment, which

depletes the ovarian reserve in a shorter time than usually occurs. This discrepancy between the rate of follicle loss and the mouse reproductive life suggest the existence of an alternative source of oocytes. Using the Vasa markers MVH or Ddx4 (DEAD-box helicase 4), which are characteristic of the germline in all vertebrates, and SCP3 (synaptonemal complex protein 3), a marker of meiotic cells in the ovaries of young and adult mice, cells were identified in the OSE. Some MVH-positive cells were also positive for bromodeoxyuridine (BrdU), a synthetic nucleoside that is incorporated into the newly synthesized DNA of replicating cells and indicates cell division rather than DNA repair, suggesting that these cells may be able to support follicular renewal. Treatment with busulphan, a chemical widely used to specifically deplete germinal cells in their migration stage, revealed ovaries possess fewer than 5% of the primordial follicle pool present in non-treated ovaries. In addition, busulphan-exposed ovaries showed healthy maturing follicles with non-degenerative oocytes and corpus luteum indicative of ovulation. Finally, ovarian fragments of wild-type mice grafted into the ovarian cavity of transgenic mice expressing the reporter gene GFP (green fluorescent protein) resulted in oocytes that express GFP surrounded by wild-type granulosa cells not positive for GFP. Taken together, these results suggest the existence of GSCs in the adult mammalian ovary that maintain folliculogenesis during postnatal life (Johnson *et al.*, 2004; Skaznik-Wikiel *et al.*, 2007; Hanna & Hennebold, 2014). Similarly, it has been demonstrated that an injection of the histone deacetylase trichostatin A rapidly and significantly increases the number of primordial follicles in young mice, young adults and adult females. These data identify epigenetic modification of chromatin structure as a key regulator for postnatal mammalian oogenesis. Furthermore, receptor signaling of RA and histone acetylation cooperatively interact to influence Stra8, which induces the expression of genes in meiosis that promote the formation of oocytes in adult mice (Wang & Tilly, 2010).

Using morphological characteristics, gene expression profiles and oestradiol synthesis, an *in vitro* study of embryonic stem cells (ESCs) from transgenic mice that express GFP indicated spontaneous generation of oocytes that remain enclosed within structures similar to developing ovarian follicles (Hübner *et al.*, 2003). Subsequent work confirmed these findings, revealing that oocytes fail to progress through meiosis and are therefore incompetent for fertilization (Novak *et al.*, 2006). Despite these results, the failure to overcome the defect in meiosis may be resolved in a matter of time, and these experiments establish a starting point, as the possibility of deriving viable sperm from ESCs has been reported (Nayernia *et al.*, 2006).

Various research groups have corroborated the hypothesis of neo-oogenesis through the detection of key markers associated with pluripotency (Oct-3/4, Sox2 and Nanos), proliferation (BrdU), germline cells (Vasa, Fragillis, Stella, and Blimp1), and meiosis (DMCI: disrupted meiotic cDNA and SCP3) in cell groups in the peripheral region of the ovary. MVH protein localization identified the presence of GSCs in the OSE of mouse ovaries. In evaluating the potential proliferation of MVH-positive cells in 5-day-old neonatal and adult mice using BrdU markers, double-positive BrdU-MVH-stained cells were observed in the surface epithelium of the ovary. Based on this result, the double-positive cells were immuno-magnetically isolated and cultured for more than 15 months and cells from adult ovaries for more than 6 months. These cells were observed to retain high telomerase activity and normal karyotype activity during long-term culture; to express germline markers such as Oct-4, MVH, Dazl, Blimp-1, Fragillis, Stella and Rex-1; and not to express markers specific to oocytes such as c-Kit, Figla, Sox-1, Nanog, SCP1–3 and ZP3. The GSCs were labeled with a reporter gene (*GFP*) and were transplanted into mouse ovaries depleted of germ cells; GSCs experiencing oogenesis were detected and produced offspring expressing the *GFP* transgene (Zou *et al.*, 2009). Moreover, another report showed a population of pre-meiotic germ cells with elevated expression of the *Stra8* and *Dazl* genes in the ovaries of OSE mice. Isolated cells marked with the *GFP* reporter gene retain the capacity to transform into oocytes after being transplanted into the ovaries of juvenile mice, thus increasing the expression of genes such as *Oct-4*, *c-Kit*, *MVH* and *SSEA-1* (Niikura *et al.*, 2009).

The purification and characterization of GSCs has been reported using transgenic mice that express *GFP* under the control of the *Oct-4* promoter. Two cell populations were identified based on their distribution and size. The first population was composed of small cells (10–15 µm) in the OSE, and the second was observed to have larger cells (50–60 µm) morphologically similar to oocytes (oocyte-like cells, OLCs) enclosed by follicular structures. Cells from the first group, or ovarian GSCs, maintained stem cell character and formed embryoid bodies presenting high telomerase activity and normal karyotypes after several stages (Pacchiarotti *et al.*, 2010). In addition to this, small round cells of unknown origin were observed among epithelial cells of OSE in adult human ovaries (Bukovsky *et al.*, 2005; Virant-Klun *et al.*, 2008). Later, these findings were confirmed in rabbit, sheep, monkey and menopausal women, in which were identified two populations of putative stem cells (PSCs), based on their distribution and size. The smaller cells (1–3 µm) expressed factors for

pluripotency as Oct-4 and SSEA-4; whereas bigger cells (4–7 μm) showed cytoplasmic localization of Oct-4 and minimal expression of SSEA-4. The small cells were called very small embryonic-like stem cells (VSELs) because these cells express gene pluripotency (*Oct-4A*, *Nanog*, *Sox2*, *TERT* and *Stat-3*). After culture, these cells were able to differentiate into structures similar to oocytes (OLCs) and expressed *c-Kit*, *Dazl*, *GDF-9*, *VASA* and *ZP4* (Ratajczak *et al.*, 2007; Parte *et al.*, 2011). VSELs are considered to be the descendants of embryonic epiblast derived pluripotency PGCs that while migrating along the dorsal mesentery to the genital ridge, also gets deposited in various somatic tissues (Ratajczak *et al.*, 2014; Bhartiya *et al.*, 2016), as in adult ovaries, bone marrow, peripheral blood and umbilical cord blood (Virant-Klun, 2015). Follicle-stimulating hormone (FSH) stimulates the VSELs, leading to upregulation in *Oct-4A* and *Nanog* expression and promoting the proliferation of these cells in the OSE (Parte *et al.*, 2013).

The search for a mechanism of neo-oogenesis in mammals has extended to domestic animals. In the pig model, experiments have suggested that skin cells from fetal pigs maintained *in vitro* can generate oocytes contained in small follicle-like structures (Dyce & Li, 2006). These presumed germ cells, formed from stem cells in the skin, manifest a meiotic blockage but coordinate the formation of follicular structures capable of secreting significant levels of oestradiol in basal conditions and in response to FSH treatment. Previous studies have also reported that potential oocytes expelled from the follicle-like structures have very similar characteristics to those of ovulated oocytes, with a structure much like the zona pellucida and the interaction and penetration of sperm (Dyce *et al.*, 2006). Another report mentioned that it is possible to isolate oogonial stem cells (OSCs), named for their expression of transcription factors such as *Oct-3/4*, *Nanog* and *Sox2*, among newborn piglets. *In vitro*, these cells showed a high capacity to differentiate into oocytes and OLCs, which express *Vasa*, *Dazl* and *ZPC* (Song *et al.*, 2011). In female adult pigs, putative stem cells (PSCs) in the ovary were isolated and characterized based on their capacity for *in vitro* proliferation and differentiation. PSCs are found in a heterogeneous population in terms of size and have affinity for pluripotent stem cell markers, such as Oct-4 and SSEA4, and germ cell markers, such as Fragillis and c-Kit. Molecular analysis of PSCs indicated that these cells undergo a cytoplasmic–nuclear translocation of Oct-4 similarly to gonadal PGCs. Thus, the cells with PGCs characteristics are present or generated in the ovaries of adult pigs, maintain their identity as germ cells under in vitro conditions, and reach a difference in OLCs in appropriate culture conditions (Bui *et al.*, 2014). The authors also suggest that PSCs can be generated from VSELs stem cells as proposed by others.

Isolated ovarian GSCs from neonatal and adult mouse ovaries and expanded them in the same culture conditions as embryonic stem cells (ESCs), were called female germline stem cells (FGSC). The FGSCs formed compact round colonies with unclear borders, maintained ESC characteristics and alkaline phosphatase (AP) activity, expressing germ cell marker Vasa, and stem cell markers: Oct4, Klf4, C-myc, Nanog, CD49f, Sox2, CD133, SSEA1 and SSEA4. These cells had the ability to form embryoid bodies (EBs), which expressed specific markers for all three germ layers. Then it was induced EBs to differentiate into neurons, cardiomyocytes, pancreatic cells and germ cells, which showed the expression of specific markers, β-III-tubulin, cardiac a-actin, Pdx1 and Zps respectively (Hu *et al.*, 2012).

The presence of GSCs with mitotically activity has found in adult mouse ovaries and human ovarian cortical tissue in which a gene expression profile is consistent with pluripotency primitive germ cells (Blimp1, Stella, Fragillis, Ddx4 and Dazl, Oct-4, Nanog, and Sox2). *In vitro*, these cells can be expanded for months and spontaneously generate oocytes. Injection of the human germline cells, engineered to stably express GFP, into human ovarian cortical biopsies leads to formation of follicles containing GFP-positive oocytes after xenotransplantation into immunodeficient female mice. Thus, ovaries of reproductive-age women, similar to adult mice, possess rare mitotically active germ cells that can be propagated *in vitro* as well as generate oocytes *in vitro* and *in vivo* (White *et al.*, 2012).

Esmaeilian and colleagues reported in ovaries from pre-puberty and adult old mice, the expression of *Sox2*, *Nanog* and *Oct-4* genes. Oct-4 and Nanog were found to be significantly differentiated between 2-week-old and 8-week-old old mice, whereas no significant difference was observed in the expression level of Sox2. However, the positive expression of Sox2 and Oct-4 protein was detected in the cytoplasm of ovarian epithelial cells, granulosa cells, oocytes and theca cells. Meanwhile, Nanog protein was observed only in the nucleus of the oocytes and it expression was higher in old ovaries. Thus, the authors suggest that pre-puberty and adult mice ovaries harboring cells with stem cells features arguing that the cytoplasmic expression of Oct-4 and Sox2 are translocated into the cytoplasm to the nucleus using a nuclear localization sequence and proceed in the opposite direction through the nuclear export sequence (Whiteside & Goodbourn, 1993; Esmaeilian *et al.*, 2012; Esmaeilian *et al.*, 2015).

Wild animals have also been used as study models for defining the existence of the neo-oogenesis mechanism. A report on three species of phyllostomid

bats (*Artibeus jamaicensis*, *Glossophaga soricina* and *Sturnira lilium*) revealed that the ovarian morphology in the three species is similar, clearly distinguishing a cortical region towards the exterior formed by a dense stroma of connective tissue that contains the ovarian follicles in various stages of development, each containing an oocyte and an internal medullar region formed of blood vessels supplying the ovary and mesenchymal cells. A small group of cells identified in the cortical region showed very similar characteristics to PGCs, including expression of specific cell markers of the germline (Fragillis, Stella, Vasa and c-Kit), stem cells (Oct-4) and cell proliferation (pH3). Thus, these cells have been called adult cortical germ cells (ACGCs). These results suggest that progenitor cells from the germline form the adult ovary in the phyllostomid bats and that the self-renewal of the germline is a possible function of progenitor cells (Antonio-Rubio *et al.*, 2013).

Human OSE, previously known as germinal epithelium, as this site was thought to serve as the origin of germ cells during embryonic development, has become very important for identifying GSCs. It was thought that the OSE might be the reservoir of undifferentiated cells capable of developing new cells. In this respect, the formation of new primordial follicles has been reported to develop with the accumulation of oocytes with nests of primitive granulosa cells in the ovarian cortex.

Research by Virant-Klun has provided surprising results and much encouragement for women who have fertility problems. This research described the presence of cells with characteristics similar to germline cells in the OSE of infertile women without oocytes and therefore without follicles. In these experiments, OSE of postmenopausal women and young women with premature ovarian failure were scraped, and PSCs with characteristics of germline OLCs that spontaneously generated the capacity to form blastocyst-like structures *in vitro* were isolated (Virant-Klun *et al.*, 2008, 2009). The authors also mentioned that these cells express some pluripotency markers, such as Oct-4, Sox2, Nanog, SSEA-4, Klf4 and c-Myc, just after scraping and during cultivation (Virant-Klun *et al.*, 2011a). In the OSE of adult women diagnosed with serous papillary adenocarcinoma, the expression of pluripotency (SSEA-4 and Sox2) and germline (Vasa and ZP2) markers was detected (Virant-Klun *et al.*, 2011b). Later, this same group published a report on the isolation of SSEA-4-positive cells from the OSE of adult females using two strategies: magnetic activated cell sorting and FACS (Virant-Klun *et al.*, 2013a; Virant-Klun *et al.*, 2013b). Immunohistochemistry showed that these small PSCs express the major markers of pluripotency (Oct-4A, Sox2, SSEA-4, Sall4, Cdh1 and Lefty1) and PGC markers (Prdm1, Prdm14 and

Dppa3). The finding of *Prdm1* is critical because is the determining gene for PGCs, plays an important role in tandem with Prdm14 for specifying the PGCs in the epiblast and is critical to the maintenance of unipotent germ cells (Bao *et al.*, 2012; Esmaeilian *et al.*, 2015). *In vitro*, these cells develop as OLCs in the presence of follicular fluid and express marker characteristics for oocytes (ZP3, SCP3 and c-Kit) (Virant-Klun *et al.*, 2013a, 2013c).

Despite evidence indicating the existence of GSCs and thus the capacity of the ovaries to produce and maintain a stable number of oocytes throughout adulthood, a question remains: why do women go through menopause? It is thought that women enter menopause when the finite supply of oocytes is exhausted. However, the existence of GSCs and the concept of neo-oogenesis do not match what happens physiologically in adult women. The justification for this discrepancy is that GSCs, like many other cells, undergo an aging process and thus lose their capacity to regenerate and differentiate. Other alternatives to this idea have been proposed. One of these arguments is that there must be some fault in the cascade of genetic and/or hormonal signaling that does not permit GSCs to differentiate into oocytes. Other alternatives are that the differentiated somatic environment does not provide the right atmosphere to trigger the development of oocytes, the GSCs do not work properly, or that no environment allows their proper differentiation (Monget *et al.*, 2012). Another is that GSCs do not exist in the adult ovary. This last explanation has had more recognition, and greater numbers of arguments demonstrate this point, as we shall see in the next section.

In summary, this evidence suggests the existence of stem cells of the germline in the postnatal mammalian ovary. However physiological relevance, ovarian function and its possible role in maintaining fertility are still unclear, and it leaves major questions to be determined. Although controversy still exists today on the biological significance of these cells, identification and isolation clearly represents a significant advance for reproductive biology, running as an attractive method for infertility (Hummitzsch *et al.*, 2015).

Arguments against neo-oogenesis

Despite the reports that corroborate the mechanism of neo-oogenesis, there are those that deny its existence because of an inability to identify and/or corroborate the published data supporting neo-oogenesis. In this regard, recent research has opened perspectives to discuss the importance of the initial follicle pool in

fertility in female adult mammals. Utilizing a mathematical model of the dynamics of follicle progression, the Bristol-Gould group examined whether the initial follicle pool is sufficient for adult fertility through reproductive senescence in mice. Establishing two mechanisms: an initial pool of primordial follicles as the only follicle source (fixed pool model) and an initial primordial follicle pool supplemented by GSCs (stem cell model), they found that the stem cell model failed to describe the observed decreases in follicles over time and did not parallel the accumulation and subsequent reduction in primary follicles during the early fertile lifespan of the mouse. Thus, they concluded that the initial endowment of ovarian follicles is not supplemented by an appreciable number of stem cells; rather, the initial pool of oocytes is sufficient to ensure the fertility of the adult female mouse, and that the loss of follicle in mouse ovary is not a stochastic process regulated both prior to puberty and again in the adult life (Bristol-Gould *et al.*, 2006). Faddy & Gosden showed that female mammals produce a limited number of oocytes, however the authors mention that the absence of evidence is not evidence of absence, the inference that total and primordial follicle numbers behave postnatally as if there is no renewal and only depletion would seem entirely reasonable, is what the statistics say (Faddy & Gosden, 2009).

Likewise, the absence of expression of early genes of meiosis, such as *Spo11*, *Prdm9*, *SCP1–3*, and *DMC1*, for germ cell development *Oct-3/4*, *c-Kit*, *Vasa* and *Nobox* and for proliferation *TERT*, *PCNA* and *Ki-67*, in healthy human ovaries between 28 and 53 years has been reported, concluding that neo-oogenesis does not take place in the adult human ovary (Liu *et al.*, 2007). Other studies that were designed to confirm the circulation of oocyte stem cells in the blood and their capacity to migrate to the ovary and form new follicles have not produced evidence to support this hypothesis (Begum *et al.*, 2008). Similarly, the Zhang group reported that it was not possible to identify the presence of Ddx4-positive germ cells or gene transcription related to SCP1, SCP3 and Spo11 meiotic activity in the OSE of adult rats (Zhang *et al.*, 2010). Later, this same group showed Ddx4-expressing cells in postnatal mouse ovaries; however, these cells were not able to divide mitotically or contribute to oocyte formation, arguing against previous results (Zhang *et al.*, 2012). In this respect and in support of the arguments against neo-oogenesis, it has also been reported that it is not possible to confirm the existence of GSCs through histological studies or the expression of markers of pluripotent cells and germ cells (SSEA-4, Oct-4 and Nanog) in the postnatal human ovary during the first 2 years of life (Byskov *et al.*, 2011). Similarly, reports by Kerr and colleagues investigated the hypothesis that adult mice have the capacity to generate new

oocytes by monitoring primordial follicle numbers throughout postnatal life and following depletion of the primordial follicle reserve by exposure to doxorubicin (DXR), trichostatin A (TSA), or whole-body γ-irradiation, in adult C57BL/6 mice between the ages of 25 and 100 days. After 2 days of treatment, primordial follicle numbers had declined to 65 with DXR and 51% with TSA, with no restoration of follicle numbers evident after 7 days for either treatment. The ovaries from mice sterilizing with γ-irradiation (0.45 or 4.5 Gy) revealed complete ablation of all primordial follicles 5 days after treatment, no indication of follicular renewal. Finally, we conclude that neo-folliculogenesis does not occur following chemical or γ-irradiation mediated depletion of the primordial follicle reserve (Kerr *et al.*, 2012).

Other studies denying the existence of GSCs and neo-oogenesis *in vivo*, except in adult mouse ovaries, were made by Lei & Spradling (2013). They measured the follicular stability in mice at 4 weeks of age by administering low doses of tamoxifen (Tmx) and analysing the ovaries at various periods by cutting them into series at each time point and estimating the total number of follicles. All germ cells appeared to be oocytes within follicles based on morphological criteria, clarifying that morphology was insufficient to rule out primordial follicles and germ cells in a state of pre-follicular development. They also calculated the volume of follicles by plotting Log_{10} [primordial follicles yellow fluorescent protein (YFP) +/ovary] over time. The slope indicated that individual follicles are slowly flushed out within a half-life (t½) of 11 months, confirming that the pool of primordial follicles together manifests stably with a half-life of 10 months. This finding suggested that there is a marked follicular stability between primordial follicles and the total number of primordial follicles and no significant production of new primordial follicles. Subsequently, the ovaries of females treated with Tmx were analysed. The number of YFP-positive cells was assessed in comparison with E10.5 embryos, in which 100% of PGCs were proliferating, and with adult male mice 4 weeks of age, in which 100% of testes showed clusters of cells marked with YFP (CGSs, spermatogonial germ cells). However, after evaluating 1000 germ cells in adult females treated with Tmx, germ cells marked on or adjacent to the surface of the ovary were never observed, indicating that the marking system functioned properly, as somatic (granulosa) cells were observed to be YFP-positive. They concluded that, together, these experimental results rule out the existence of GSCs in adult ovaries because direct measurements showed that the primordial follicles are highly stable and invalidate the inference that thousands of adult primordial follicles flow out each month. They also claimed that marked follicles are

lost at the same rate as total follicles, negating the existence of a source of new follicles in adulthood. Finally, as additional evidence, they referred to the follicles produced during fetal development, which are sufficiently stable to meet all requirements in adult life (Lei & Spradling, 2013). Parallel to these investigations, Yuan and colleagues did not identify proliferating cells or gene expression of pluripotency markers, such as Sox2 and Lin28, or the germline markers Vasa and Dazl in adult ovaries of rhesus monkeys and mice. Unexpectedly, cells with characteristics of somatic stem cells but not germline cells were found in adult ovaries (Yuan *et al.*, 2013).

With the continued publication of reports for and against possible neo-oogenesis, a final consensus on whether the mechanism of neo-oogenesis or, likewise, GSCs exist in the ovaries of adult mammals has not yet been reached. The existence of this mechanism remains unknown and still requires much more work to fully prove or disprove the notion that mammalian ovaries have cells with characteristics similar to the PGCs that can be stimulated to enter a differentiation process for the generation of new oocytes.

Studies that support and reject the role of an extra-gonadal source of germline stem cells in neo-oogenesis during adulthood

It has been suggested that GSCs that cause oocytes to emerge in postnatal stages reside in extra-gonadal reservoirs, meaning that these cells originate outside the ovary, then migrate in an undifferentiated state and when they reach the adult ovary they begin their process of differentiation and commitment towards oocytes, thus compensating for any follicles lost during each ovulatory cycle.

Reports published so far have suggested that the bone marrow acts as the GSC reservoir. This idea was derived from the fact that during early embryogenesis the hematopoietic cells and germ cells originate in the same region, so in adult life they can be found in the same niche (Johnson *et al.*, 2005b; Hanna & Hennebold, 2014). The expression of germline markers has been localized, such as Ddx4, Dazl, Stella and Fragillis in bone marrow cells and interestingly Ddx4 expression appears to fluctuate in coordination with the estral cycle of the individual and is absent in ovariectomized females. Experiments on the ovarian follicles of chemically depleted mice, subject to bone marrow transplant-ation, revealed follicle formation after treatment. These results were confirmed employing Atm mutant mice (mutated ataxia telangiectasia), which are infer-tile as they do not have the ability to produce mature germ cells, and thus do not develop oocytes. Once bone marrow transplantation had been performed, the formation of follicles was identified. These findings led to the hypothesis that GSCs reside in the bone marrow and travel through the peripheral blood to colonize the ovary (Johnson *et al.*, 2005). To test this theory, this group used Atm mice and mice that were germ cell deficient, due to chemotherapy. Both models were given a peripheral blood transfusion from a transgenic mouse (GFP), resulting in the detection of MVH-expressing GFP oocytes (*Vasa* homolog gene), *HDAC6* (histone deacetylase 6) and *NOBOX* (homeobox protein NOBOX, also known as newborn ovary homeobox protein), concluding that the GSCs are derived from the bone marrow and circulate via blood.

An analogous work derived from the same Johnson group showed that germ cell conditioned mice and monthly infusions of bone marrow-derived cells from young adult females bearing the *GFP* reporter gene were able to sustain the fertility potential of the recipient individual, extending its timespan until reproductive senescence (Selesniemi *et al.*, 2009). Contrastingly, when evaluating the reproductive capacity of females treated with cytotoxic chemicals to eliminate germ cells, they observed that mice that were undergoing chemotherapy were infertile. In contrast, mice that received a bone marrow transplant achieved term pregnancies. In comparison, there was a slight reduction in the number of offspring per litter among mice undergoing non-lethal treatment (busulfan 12 mg/kg, cyclophosphamide 120 mg/kg) plus transplantation compared with untreated controls. Although bone marrow transplantation reactivated long-term fertility in treated organisms, all offspring produced by these animals were derived from the recipient's own germ cells as all offspring were GFP negative. Therefore, the effects of bone marrow transplantation do not appear to have any positive effects on the re-establishment of germ cells, the authors proposed that the transplant acted as a chemotherapy protector and not as an activator of neo-oogenesis (Lee *et al.*, 2007). These results further increased mistrust in the theory of neo-ovogenesis, and criticisms soon emerged. One of the first works that questioned the results obtained by Johnson and colleagues (2005), which related bone marrow transplantation to restoration of fertility, was that of Eggan *et al.* (2006). This study involved parabiosis experiments with wild-type mice and *GFP* transgenic mice to determine whether circulating GSCs derived from bone marrow could cause oocytes to originate in the ovary. By surgically joining blood vessels at around 4–8 weeks of age, they observed that the highest level of peripheral blood chimerism is 65% for GFP-positive leukocytes on both sides, 6–8 months

post-surgery. Although ovulation occurred in both groups, no chimeric oocytes were detected, although GFP-positive cells were occasionally observed in the wild-type mouse group. Following treatment with cyclophosphamide and busulfan, administered 1 day prior to surgical union of blood vessels in wild-type mice, they observed a high leukocyte chimerism in blood and bone marrow but not in oocytes; however these mice manifested ovulation at around 2 weeks or 2 months after surgery. They therefore concluded that any fertility post- treatment could be attributed to follicles not affected by chemotherapy that made it possible to re-establish the number of follicles. This was corroborated by mice that were treated but surgically unattached, which showed the presence of follicles in reduced numbers, indicating incomplete oocyte ablation (Eggan *et al.*, 2006).

In order to test if oocyte progenitor cells circulate in the blood and are able to migrate to the ovary and form new follicles, they performed a positive GFP blood transfusion in wild-type ovaries. An examination of oocytes present in ovarian grafts found no evidence to support this hypothesis (Begum *et al.*, 2008). Finally, a further report (Santiquet *et al.*, 2012) that refuted the presence of GSCs in bone marrow and peripheral blood was obtained for SCDI mice, treated with chemical agents. When analysing embryonic ovarian cortex grafts there was no evidence that transplanted bone marrow cells resulted in new oocytes. However, they suggested that transplanted bone marrow cells improve fertility in SCID mice, positively influencing ovarian physiology.

The role of epigenetic mechanisms during oogenesis and neo-oogenesis

With the advent of epigenetics, some of the biochemical and cellular pathways that led to some developmental abnormalities caused by changes in chromatin have been determined that in turn control differential gene expression by DNA methylations and histone alterations. Currently, the epigenetic pathways that regulate the development of ovogenesis have been described, and disturbances associated with this process have been shown to damage postnatal health causing infertility (Bromfield *et al.*, 2008).

Epigenetics has reinforced the idea of a Lamarckian evolutionary principle because environmental conditions have led to marked changes in gene expression during mammalian development (Bromfield *et al.*, 2008). Any stress factor that affects the embryo during development may influence gene expression and thus disrupt competency for embryo development. Changes in the state of intracellular redox will alter

the expression of oxygen-sensitive genes (Harvey *et al.*, 2007), while exposure to environmental toxins will alter gene expression and capacity for embryonic developmental (Susiarjo *et al.*, 2007).

The production of fertility-competent oocytes results from good coordination between folliculogenesis and ovogenesis, a good balance of cellular interactions between the somatic and germinal components, as well as hormonal interactions and growth factors involved in the pituitary–hypothalamic–gonad axis (Combelles *et al.*, 2004). However, alterations in somatic physiology can affect the quality of the oocytes depending on the stage of development affected; fetal, prepubertal or adult (Bromfield *et al.*, 2008). Considering epigenetic regulation at a more subtle but equally important level, molecular mediators have been identified to fulfil a crucial catabolic function, in many systems. Relevant epigenetic factors in oocytes include: cMOS (r), which participates in the arrest of meiosis II and whose absence deregulates the cell cycle; E-cadherin (p), which is involved in the compaction of chromatin, whose absence causes damage to lineage assignment; NMP2 (p), which influences pronuclear maturation, whose absence causes delay in the cell cycle; and Dmnt1o (p), which is important for the methylation of chromatin and whose deficiency causes modifications in methylation; and γ-tubulin (P), also important for mitosis, as its absence results in arrest of the cell cycle. Key factors in the cell cycle assume a non-random location in order to generate fast and complete effects, ensuring the synchronized activation of the kinase and ubiquitination, for an opportune entry into the M cell phase and to ensure its exit. In contrast, the centrosomes work by limiting the diffusion capacity of components involved in the progression of the cell cycle, as the complexity of these factors or molecules are directed and maintained in the centers of microtubule organization (COMT). Finally, the spindle serves to harbor and stabilize many factors that are involved in timely cyclin degradation that provokes the metaphase–anaphase transition, during the M phase (Carmo-Fonseca *et al.*, 2000). Specific interactions between the cytoskeleton and other organelles, mRNAs and proteins can be localized and stabilized together for post-translational modification (revised by Bromfield *et al.*, 2008). Advancement in this developing area has led to the proposal of epigenetic mechanisms that may possibly be associated with neo-ovogenesis process.

It has been suggested that ovogenesis can be induced in adult females with the inhibitors histone deacetylases (HDAC) and trichostatin A (tSA), indicating that the acetylation status of histones can determine whether germ cells enter meiosis (Johnson *et al.*, 2005b). This work provided a precept for further investigation into epigenetic regulation and

Table 1 Experimental works that uphold the existence of a mechanism of neo-ovogenesis in adult mammalian females

Year	Author	Model	Relevance
1870	Waldeyer-Hartz	Theoretical	He indicates that the ovarian superficial epithelium (OSE) is active during a limited period in the early life of organisms
1917	Kingery	Mouse	He reports that oocytes that degenerated during fetal life were replaced by oocytes that evolved in the germinal epithelium of the adult ovary.
1923	Allen	Mouse	They argue that new oocytes arise from the germinal epithelium, as a result of the mitotic division of undifferentiated cells.
1965	Crone *et al.*	Mouse	Identification of oval cells in the OSE of mice, similar to germ cells of fetal ovaries.
1966	Anand Kumar	Prosimians	Observation of germ cells at different stages of mitosis (oogonias), or during the meiotic prophase (oocytes) indicating that the process of ovogenesis in prosimians continues after birth.
1967	Ioannou	Prosimians	Description of the presence of germ cells in prosimian ovaries, identifying oogonias during interphase and at different stages of mitosis, by autoradiography. However, the possibility that these germ cells are formed by the pre-existing division of oogonias is not ruled out.
1967	Duke	Prosimians	Localization of mitotically active germ cells in adult ovaries. He states that germ cells are grouped into nests within the ovarian cortex and that these are capable of incorporating tritiated thymidine.
1974	David *et al.*	Prosimians	They confirm previous observations about the active incorporation of tritiated thymidine, analysing the behavior of the germinal cells of the cortex, during a period of sexual inactivity (anestrus) in *Loris tardigradus lydekkerianus*. The appearance of marked germ cells in ovaries takes place, indicating mitosis and DNA synthesis during estrus and anestrus.
2004	Johnson *et al.*	Mouse	They suggested that there are germ stem cells GSCs in the superficial epithelium of the ovary in adult mice that express the germ cell marker Mvh and exhibit mitotic activity, evidenced by the expression of BrdU at levels that consistently indicate cell division.
2004	Bukovsky *et al.*	Human	They propose that the mesenchymal cells of the tunica albuginea are bipotential progenitors that give rise to germ cells and granulosa. The latter originate in the OSE by means of epithelial–mesenchymal transition.
2005(a)	Johnson *et al.*	Mouse	They postulate that a bone marrow transplant (BMT) can restore oocyte generation in wild-type mice sterilized by chemotherapy and in mice with a genetic disorder that made them unable to produce oocytes.
2005(b)	Johnson *et al.*	Mouse	They suggest that peripheral blood is an additional source of GSCs in mice, suggesting that putative germ cells are located in the bone marrow (BM) and released into peripheral circulation.
2005	Bukovsky *et al.*	Human	They show the potential for differentiation of superficial ovarian epithelial cells into oocytes, granulosa cells, nerve cells, epithelial cells and mesenchymal cells.
2006	Kehler *et al.*	Mouse	They demonstrate that oocytes were spontaneously generated from embryonic stem cells ESCs and then kept enclosed within structures resembling the developing ovarian follicles, determined by morphological characteristics, gene expression profiles and estradiol synthesis.
2006	Kerr *et al.*	Mouse	They mention that immature germ cells in the adult ovary can lead to the generation of new oocytes.
2006	Dyce *et al.*	Pig	They show that skin cells from cultured pig fetuses are able to generate oocytes contained in small follicle-like structures.
2007	Lee *et al.*	Mouse	They argue that bone marrow transplantation is not effective as protective chemotherapy, unless it functions as an activator of ovogenesis, caused by the action of GSCs that replenish oocytes lost from the effects of cytotoxic chemicals, such as cyclophosphamine and busulfan. *CABLES1* is an essential gene, associated with limiting the rate of oocyte turnover in adult mouse ovaries.
2008	Zhang *et al.*	Mouse	They identified the expression of meiotic and germ cell markers in specific cells grouped together at the periphery of the ovaries of adult mice.
2008	Szotek *et al.*	Mouse	They mention that a population of label-retaining cells (LRCs), located in the OSE of transgenic adult mice (H2B–GFP) exhibit stem/progenitor cell characteristics.

Table 1 Continued

Year	Author	Model	Relevance
2008	Virant-Klun *et al.*	Human	They isolated putative stem cells with germline characteristics expressing pluripotency markers (SSEA4, Oct-4, Nanog, Sox-2 and c-Kit) from postmenopausal women and those with premature ovarian failure.
2009	Zou *et al.*	Mouse	They identified GSCs in the OSE by colocalizing MVH and BrdU proteins. These cells were isolated immunomagnetically and then cultivated, revealing a normal karyotype, high telomerase activity and the expression of pluripotency and germline genes (*Oct4*, *Mvh*, *Dazl*, *Blimp1*, *Fragilis*, *Stella* and *Rex-1*). Expression of c-Kit, Figla, Sox-1, Scp1–3 and ZP3 that would indicate differentiation to oocytes was not detected.
2009	Niikura *et al.*	Mouse	They located a pre-meiotic germ cell population in the OSE of mouse ovaries with high expression of the Stra8 and Dazl gene, capable of differentiating into oocytes, once transplanted into ovaries of juvenile mice.
2009	Virant-Klun *et al.*	Human	They obtained putative stem cells from postmenopausal women that were maintained *in vitro* and that proliferated and formed structures similar to embryoid bodies, expressing Oct-4, c-Kit, Mvh and Zp2. They identified structures similar to the zona pellucida, germinal vesicle and polar bodies, concluding that differentiation to oocytes had occurred.
2010	Pacchiarotti *et al.*	Mouse	They identified two populations of GFP/Oct4 positive cells based on their distribution and size. The first group formed from small cells located in the OSE they denominated GSCs and these maintained characteristics of stem cells forming embryoid bodies, presenting high telomerase activity and a normal karyotype after several stages. The second group consisted of large oocyte-like cells (OLCs) and were encased in follicular structures.
2010	Gong *et al.*	Mouse	Adult ovarian cells cultured with fibroblasts can produce colonies that manifest similar activity to that of ESCs.
2011	Parte *et al.*	Rabbit; Sheep; Mono Rhesus; Human	They identified small cells called VSELs expressing pluripotency genes: Oct4, Nanog, Sox2, TERT and Stat-3, which having been cultured manifested an ability to differentiate into oocyte-like structures (OLCs) and which expressed c-Kit, Dazl, Mvh and ZP3.
2011a or 2011b	Virant-Klun *et al.*	Human	They identified a cell population with Sox2-positive germ cell characteristics in the OSE of women with premature ovarian failure, who had high levels of FSH and LH. They report that in culture with follicular fluid, these express alkaline phosphatase, Oct4, Sox2, SSEA-4 Nanog, Nanos, Stella, CD9, Lin28, Klf4, GDF3, and Myc.
2011	Song *et al.*	Pig	They isolated oogonial stem cells (OSCs) expressing Oct4, Nanog and Sox2. In culture these cells showed high capacity to differentiate into OLCs, evidenced by the expression of Mvh, Dazl and ZP3.
2012	White *et al.*	Mouse; Human	They report that isolated mouse and human cells in culture generate spontaneous differentiation to oocytes. Xenotransplantation experiments of human ovary cortical cells to immune-deficient mice manifest formation of chimeric follicles.
2012	Esmaeilian *et al.*	Mouse	They demonstrate the expression of pluripotency genes; Oct4, Sox2 and Nanog, in ovaries of prepubertal and adult mice.
2012	Bhartiya *et al.*	Human	By applying treatment with FSH, they report that it is possible to induce stimulus in the pluripotent VSELs, leading to the overexpression of Oct4A and Nanog and promoting the proliferation of the cells present in the OSE, evidenced by the expression of PCNA.
2012	Hu *et al.*	Mouse	They identify round and compact cells that form colonies with alkaline phosphatase (AP) activity in adult ovaries. They observe expression of germline markers (Mvh) and stem cells (Oct4, Klf4, c-Myc, Nanog, CD49f, Sox2, CD133 and SSEA1–4). *In vitro*, porcine follicular fluid induces their differentiation into oocyte-like structures.
2012	Bukovsky & Caudle	Rat	They report follicular renovation in rodents by bone marrow-derived cells, which interact with ovarian epithelial cells of adult rats, lacking OSE.
2013	Antonio-Rubio *et al.*	Bats	In the cortical region of adult ovaries, they identified a population of cells morphologically very similar to PGCs, called adult cortical germinal cells, which express proliferation markers (pH3), pluripotency (Oct4) and germ line (Dppa3, IFITM3, c-Kit and Mvh)

Table 1 Continued

Year	Author	Model	Relevance
2013	Parte *et al.*	Human	They observed proliferation of stem cells in the OSE and a transition from primordial follicles to primary follicles, following treatment with FSH and bFGF, suggesting that FSH and bFGF stimulate the stem cells present in the OSE to form follicles.
2013	Patel *et al.*	Mouse	They show that the follicle-stimulating hormone (FSH) modulates OSCs through FSH-R3 for their self-renewal, clonal expansion as 'cysts' and their differentiation into oocytes.
2013	Bhartiya *et al.*	Human	They show the presence of ovarian cysts that present self-renewal and clonal expansion, as well as incomplete cytokinesis, characteristics that are distinctive to stem cells. They indicate the expression of Oct4 and Stella, suggesting the presence of stem cells.
2013	Stimpfel *et al.*	Human	They identify the presence of cells that express pluripotency and germline markers in the ovarian cortex of adult women.
2013a	Virant-Klun *et al.*	Human	They report the isolation of SSEA-4 positive cells present in the OSE of adult women, and demonstrate that these putative small stem cells express principal pluripotency markers (Oct4A, Sox2, SSEA-4, SalI4, CDH1 and Lefty1) and markers of primordial germ cells (PRDM1, PRDM14, and Dppa3).
2013	Park *et al.*	Mouse	They suggest that the signaling of bone morphogenetic proteins (BMP4) can directly affect OSC function, producing rapid phosphorylation regulated by Smad proteins. This activates key meiosis genes (*Stra8*, *MSX1* and *MSX2*)
2014	Bui *et al.*	Pig	They identified, isolated and characterized putative ovarian stem cells (PSCs), based on capacity for in vitro proliferation and differentiation. They suggest that PSCs are generated or are present in the ovary of adult pigs, maintaining their germ cell identity.
2011	Parte *et al.*	Human; Sheep	They demonstrate a recapitulation of in vitro ovogenesis in OSE cells and show differential expression of specific markers for pluripotent VSELs (nuclear Oct4A, SSEA-4, and CD133), OSCs (cytoplasmic OCT4), primordial germ cells (Fragilis, Stella, and Mvh) and oocytes (DAZL, GDF-9 and Scp3).
2015	Sriraman *et al.*	Mouse	The culture of OSEs exposed to chemotherapy showed proliferation and formation of germ cell groups. These manifested spontaneous differentiation to structures similar to oocytes, and on day 6 were observed to be positive to Mvh, and GDF9.
2016	Ding *et al.*	Human	They demonstrate that OSCs can be recovered from follicular fluid due to the presence of traces of ovarian cortex tissue in follicular aspirates. These cells also differentiated into oocytes during the germinal vesicle stage in vitro.
2016	Guo *et al.*	Mouse	They argue that active GSCs, which are functional under physiological conditions, are present in the ovary of adult mice.

Table 2 Experimental works that refute the existence of a mechanism of neo-ovogenesis in adult mammalian females

Year	Author	Model	Relevance
2006	Bristol-Gould et al.	Mouse	They argue, through statistical analysis and mathematical models, that female mammals produce a limited number of oocytes in fetal life, ceasing production after birth.
2007	Liu et al.	Human	They do not identify the expression of pluripotency genes (Oct4 and c-Kit) and meiosis (*Spo11*, *PRDM9*, *Scp1*, *TERT* and *Nobox*) in adult ovaries of healthy women, thus refuting the existence of neo-ovogenesis.
2010	Zhang et al.	Rat	They do not identify GSCs by morphology or by the expression of characteristic germline markers; Mvh/Vasa and meiosis (*Scp1*, *Scp3* and *Spo11*).
2011	Byskov et al.	Human	They found no expression of typical pluripotency markers such as *SSEA-4*, *Oct4* and *Nanog* in 2-year-old human ovaries, so they state that there are no GSCs in postnatal ovaries.
2012	Zhang et al.	Mouse	They present evidence that *Mvh/Ddx4* positive cells are not mitotic, so they do not contribute to the formation of oocytes in the adult ovary.
2012	Kerr et al.	Mouse	They indicate that no neo-folliculogenesis occurred in mice after sterilization treatment with doxorubicin (DXR) or γ-rays, negating the existence of GSCs.
2012	Oatley & Hunt	Theorical	They mention that doubts about the existence of OSCs will persist until evidence is provided to indicate that these cells give rise to viable eggs in the adult.
2013	Yuan et al.	Mono Rhesus; Mouse	They do not identify presence of proliferative cells in the adult ovaries of Rhesus monkey and mouse. Neither do they detect the expression of pluripotency genes (*Sox2* and *Lin28*) and germ line (*Mvh/Vasa* and *Dazl*)
2013	Lei & Spradling	Mouse	Using a labeling system, they determined that the ovaries of adult mice lack stem cells, as primordial follicles generated during fetal development are stable enough to sustain folliculogenesis without requiring an alternative source of renewal.
2014	Zhang et al.	Mouse	They show that there is no mitotic activity in OSCs or folliculogenesis in the postnatal mouse ovary. They conclude that the pool of oocytes formed during the fetal development of organisms is the only source of germ cells.
2015	Zhang et al.	Mouse; Human	Human and mouse purified OSCs that were positive to DDX4 are neither cells nor do they specifically express DDX4 and nor are they functional germline stem cells.
2017	Vanii et al.	Human	They mention that the existence, origin and functionality of OSCs remain controversial and that this is not yet widely accepted in the scientific community.

how this may influence the production of oocytes during adult life. It is generally known that the meiotic cell cycle is activated by the expression of Stra8, which is induced by retinoic acid (RA) in fetal stages of development. The suppression of meiosis in male germ cells is caused by the expression of CYP26B1. When male embryonic germ cells were exposed to the class I/II inhibitors histone deacetylases (HDAC) and trichostatin A (tSA), premature activation of Stra8 was induced and therefore entry into meiosis without altering expression of CYP26B1 took place. However, the most important finding in terms of neo-ovogenesis was the physiological detection of Stra8 in ovaries of adult mice.

The induction of ovogenesis in adult females using TSA is associated with the activation of Stra8, enabling reproduction of the results with the use of HDAC inhibitor, suberoilanilida of hydroxamic acid (SAHA). This finding indicates that retinoic acid receptor and histone acetylation signaling interact cooperatively to influence Stra8 expression, which promotes the formation of oocytes in adult mice. The ability of RA to induce Stra8 expression is epigenetically controlled by co-activators upstream of RARE. Finally, the authors conclude that these events not only coordinate entry of meiosis during embryogenesis, but also contribute significantly to the regulation of ovogenesis in adult mammalian females (Wang & Tilly, 2010). Another explanation of how epigenetics can influence the production of oocytes, maintaining a stable number in adult life, indicates that this is a function of the ovarian reserve. Primordial or non-growth follicles (NGFs) are the functional unit for reproduction, constituting the ovarian reserve (OR). The dynamics of the reserve are determined by the number of NGFs formed and their subsequent destinations. During reproductive life, OR progressively decreases due to follicular atresia, as well as recruitment, maturation and ovulation. OR depletion is controlled by the menopause, when the number of primordial follicles falls below a threshold of ~1000. It is thus important to know the genes and processes involved in the development of OR (Pelosi *et al.*, 2015).

Studies have shown that OR increases dramatically from 15 weeks post-conception to 34 weeks, remaining constant, with an average of 680,000 NGF, up to the first 2 years after birth (Block, 1953; Forabosco & Sforza, 2007; Hansen *et al.*, 2008). In postnatal life, data show considerable variability between 7 and 12 years, thus no figure has been established, although limited reduction has been observed. An average of ~460,000 follicles are still present at puberty; between 12 and 14 years of age (Block, 1952; Hansen *et al.*, 2008). From this moment, OR reduce continually until menopause down to <1000 NGF (Block, 1952; Richardson *et al.*, 1987; Gougeon *et al*, 1994; Hansen *et al.*, 2008) revised

by Pelosi *et al.* (2015). Changes in follicular dynamics may result from the influence of genetic and/or environmental factors that modify the formation of new NGF or the recruitment of NGF for maturation or atresia (Kerr *et al*, 2013).

Conclusions

The controversy surrounding the existence of GSCs in adult mammals continues, thus accurate and reproducible results are necessary to demonstrate the existence of the mechanism of neo-oogenesis. If results are attained, GSCs could be used in a clinical context, promoting their isolation, growth and differentiation to provide a novel method for treating female infertility. Finally, after the review of positions both for and against the existence of a mechanism of neo-oogenesis (Table 1 and 2), whether there are cells capable of producing new oocytes in adult mammalian ovaries remains unknown. Therefore, the debate concerning the existence of GSCs in the ovary is not resolved. Before accepting or denying the existence of a mechanism of neo-oogenesis, several pieces of evidence must be provided: irrefutable results showing the existence of GSCs in the adult mammalian ovary, an indication of whether this phenomenon is a generality or represents only certain exceptions in nature; determination of whether this condition is beneficial or detrimental in evolutionary terms; and scientific characterization of GSC existence by their morphology and genetics to determine whether these GSCs play an important physiological role.

Abbreviations

ACGCs, adult cortical germ cells; AR, retinoic acid; BrdU, 5-bromo-2-bromodesoxiuridina; ESCs, embryonic stem cells; FACS, fluorescence activated cell sorting; FSH, follicle-stimulating hormone; GFP, green fluorescent protein; GREL, gonadal ridge epithelial-like; GSCs, germ stem cells; OLCs, oocyte-like cells; OSCs, oogonial stem cells; OSE, ovarian surface epithelium; PCNA, proliferating cell nuclear antigen; PGCs, primordial germ cells; PSCs, putative stem cells; SSCs, spermatogonial stem cells; Tmx, tamoxifen; VSELs, very small embryonic-like stem cells; YFP, yellow fluorescent protein.

Acknowledgements

Competing interests

The authors declare that they have no competing interests.

Author contributions

Both authors contributed to design of the study, manuscript writing and conception and final design.

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