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Post-natal oogenesis: a concept for controversy that intensified during the last decade

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Summary

For decades, scientists have considered that female mammals are born with a lifetime reserve of oocytes in the ovary, irrevocably fated to decline after birth. However, controversy in the matter of the possible presence of oocytes and granulosa cells that originate from stem cells in the adult mammalian ovaries has been expanded. The restricted supply of oocytes in adult female mammals has been disputed in recent years by supporters of neo-oogenesis, who claim that germline stem cells (GSCs) exist in the ovarian surface epithelium (OSE) or the bone marrow (BM). Differentiation of ovarian stem cells (OSCs) into oocytes, fibroblast-like cells, granulosa phenotype, neural and mesenchymal type cells and generation of germ cells from OSCs under the contribution of an OSC niche that consists of immune system-related cells and hormonal signalling has been claimed. Although these arguments have met with intense suspicion, their confirmation would necessitate the revision of the current classic knowledge of female reproductive biology.

Keywords: Germline stem cell, Oocyte, Oogenesis, Ovarian stem cell, Ovary

Background

At present, most scientists accept the dogma that at the beginning of life all oogonia [the female germline stem cells (GSCs)] reserves are depleted as a result of their differentiation into oocytes. However, throughout the last 150 years, there has been controversial discussion among scientists about neo-oogenesis in adult mammals. In 1870, Waldeyer-Hartz claimed that in adult mammalian species and birds, there is no possibility of new oocyte production, but that the oocytes arise from the ovarian surface epithelium (OSE; germinal epithelium) during a limited period of early life (Waldeyer-Hartz, 1870). However, in 1917, Kingery claimed that all oocytes that degenerated during fetal life were restored by oocytes that developed in the adult ovarian germinal epithelium (Kingery, 1917). This hypothesis was supported by an argument that new oocytes arise from the germinal epithelium as a result of mitotic division (Allen, 1923; Allen & Creadick, 1937). However, in 1921, Pearl & Schoppe asserted that there was no increase in the supply of primary oocytes during the life of the individual (Pearl & Schoppe, 1921). The discussion of neo-oogenesis in the adult mammalian ovary was almost ended by the studies of Zuckerman; this work resulted in the basic doctrine of reproductive biology, arguing that most mammalian females have the potential of generating a limited reserve of oocytes during fetal development only, oocytes enclosed by somatic cells (granulosa cells) that are described as ovarian follicles (Zuckerman, 1951; Zuckerman & Baker, 1977). In spite of some criticism (Pansky & Mossman, 1953; Vermandevaneck, 1956; Artem'eva, 1961), in 1951 Zuckerman put an end to the argument in favour of a definite quantity of oocytes at birth, concluding that during the lifetime there is no increase in the number of primary oocyte reserve beyond those cells initially present when the ovary developed (Zuckerman, 1951). In parallel with this theory, Peters' group demonstrated that in mouse ovaries oocyte generation took place only during a limited prenatal period, the pre-meiotic S phase, and

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that these oocytes remained throughout adulthood (Peters *et al.*, 1962). Accordingly, a central dogma of mammalian reproductive biology was introduced based on this principle: female mammals are born with a limited, non-renewing supply of germ cells, all of which are arrested in meiosis I and are described as follicles (Zuckerman, 1951; Borum, 1961; Franchi *et al.*, 1962; Peters, 1970; McLaren, 1984; Anderson & Hirshfield, 1992). Oocyte numbers constantly decrease during post-natal life (Faddy *et al.*, 1976, 1987; Faddy, 2000) through mechanisms involving apoptosis (Perez *et al.*, 1999; Tilly, 2001). In humans, exhaustion of the oocyte pool takes place until 50 years of age, resulting in menopause (Richardson *et al.*, 1987). Although this dogma is established (Gougeon & Notarianni, 2011), recent studies (Virant-Klun *et al.*, 2011c; Woods & Tilly, 2012; Woods *et al.*, 2013) have provided some evidence that may challenge the validity of this widespread doctrine, which represents the foundation of reproductive biology.

Controversy over limited and non-renewable ovarian follicles after birth

In the last 10 years, in contrast with the classic idea that oogenesis does not occur in post-natal mammalian ovaries, the concepts of ovarian stem cells (OSC) and neo-oogenesis, which can restore the ovarian reserve in post-natal mammals, have led to this controversial debate in reproductive biology being reopened. Primary studies have reported *ex vivo* oogenesis in the culture of mouse embryonic stem cells (ESCs; Hubner *et al.*, 2003), and mitotically active germ cells have been observed in the ovaries of adult prosimian primates (Ioannou, 1967) and mice (Johnson *et al.*, 2004). In the ovarian tunica albuginea, mesenchymal cells have been found to develop into surface epithelium and contribute to follicular renewal in adult human females (Bukovsky *et al.*, 1995, 2004). These reports have resulted in controversy about the defined dogma on the fetal origin of mammalian oocytes (Bazer, 2004; Gosden, 2004; Oktem & Oktay, 2009). In 2004, Johnson and colleagues' demonstration about the existence and proliferation of GSCs in post-natal mouse ovaries has led to an argument on the possibility of post-natal oogenesis (Johnson *et al.*, 2004). Another study based on the mouse model revealed that there is no decline in the number of primordial follicles per ovary between day 7 to day 100 after birth, and that a significant reduction only takes place 200 days after birth (Kerr *et al.*, 2006). Hence, it was suggested that immature putative germ cells in the adult may lead to the *de novo* generation of oocytes in appropriate circumstances. In contrast with these studies, Liu and collaborators reported the absence of early meiotic-specific or oogenesis-associated mRNA (SPO11, PRDM9, SCP1, TERT and NOBOX) and a lack of early meiocytes and proliferating germ cell markers (SCP3, Oct4/3 and c-KIT) in healthy adult human ovaries (28–53 years), and concluded that neo-oogenesis does not occur in the adult human ovary (Liu *et al.*, 2007). Furthermore, using statistical analyses and mathematical modelling, other groups have shown that mammalian females produce a limited number of oocytes in the prenatal phase of life, and that this production ceases after birth (Bristol-Gould *et al.*, 2006; Faddy & Gosden, 2009; Wallace & Kelsey, 2010). In parallel with these studies, Zhang *et al.* (2010, 2012) failed to demonstrate the presence of large ovoid germ cells that expressed the *VASA/DDX4* gene, the transcription of the meiotic-specific genes *SCP1*, *SCP3*, *SPO11* and the translation of DMC1, STRA8, SCP3 in the surface epithelium of adult rat ovaries (Zhang *et al.*, 2010, 2012). Byskov and collaborators were also unable to confirm the existence of GSCs by staining with pluripotent pre-meiotic germ cell markers (SSEA-4, Oct4, Nanog or MAGE-A4) and oogonia (by morphology) in the post-natal human ovary after final clearing of these cells during the first 1 or 2 years of early life (Byskov *et al.*, 2011). In other reports, neo-folliculogenesis failed in mice after sterilization by chemicals [doxorubicin (DXR)] or γ-irradiation (Kerr *et al.*, 2012), and the existence of both GSCs and *in vivo* neo-oogenesis were not confirmed in adult mouse ovaries (Lei & Spradling, 2013). Recently, Yuan *et al.* (2013) were also unable to confirm the presence of proliferative GSCs and germ cell renewal (lack of *Sox2*, *LIN28*, *VASA* and *DAZL* genes) in adult monkey and mouse ovaries (Yuan *et al.*, 2013). Unexpectedly, they found cells with characteristics of non-germline somatic stem cells in adult ovaries. As mentioned above, during the last decade the concept of neo-oogenesis has resulted in intense controversy. Some studies that either support or refute the possibility of post-natal oogenesis in mammals are summarized in Table 1.

Renewal of follicles and neo-oogenesis in the post-natal ovary

Approximately 10 years ago the hypothesis that oocyte and follicle renewal may occur in the post-natal mouse ovary was addressed by several experimental approaches (Johnson *et al.*, 2004). Mitotically active germ cells were supposed to exist in the ovaries of both young and adult mice; depending on the amount of oocyte atresia and depletion, these cells are required to continuously replenish the follicle pool. Prepubertal female mice were treated with the mitotic germ cell toxicant (busulphan), which is known to

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Table 1 Some experiments that support and refuse the putative post-natal oogenesis in female mammals

(a) Studies that support the post-natal oogenesis

References	Study highlights
Johnson *et al.* (2004)	Presence of mitotically active GSCs in young and adult mice ovary. Development of chimeric follicles following transplantation of wild-type ovarian tissue onto ovary of GFP expressing transgenic mice
Bukovsky *et al.* (2005)	Differentiation of oocyte, granulosa, neural, epithelial, and mesenchymal type cells from surface epithelium of adult human ovaries
Kerr *et al.* (2006)	Immature putative germ cells in the adult ovary may lead to *de novo* generation of oocytes in appropriate circumstances
Lee *et al.* (2007a)	CABLES1 is a crucial gene associated with constraining the rate of oocyte renewing in adult mouse ovaries
Zhang *et al.* (2008)	Germ cell and meiotic markers in specific cells aggregate in the periphery of adult mouse ovaries
Szotek *et al.* (2008)	The label-retaining cells (LRCs) population in coelomic OSE of adult H2B-GFP transgenic mouse ovary exhibited stem/progenitor cell characteristics
Virant-Klun *et al.* (2008, 2009; 2011a,b; 2013a,b,c)	Putative stem cells with germline characteristics, which spontaneously generate OLCs with capacity of undergoing parthenogenetic development to create preimplantation blastocyst-like structures *in vitro.* Naturally present stem cells from the OSE of patients with POF expressed some markers of pluripotency Using MACS and FACS a population of SSEA-4-positive cells was isolated from adult human OSE
Zou *et al.* (2009)	Proliferative MVH-positive female GSCs purified from neonatal and adult mice ovaries and maintained *in vitro* for 15 or 6 months, respectively. GSCs following transplantation into atretic ovaries of chemotherapy-sterilized recipient mice generate chimeric follicles that fertilized and produced viable offspring
Niikura *et al.* (2009)	In the OSE of aged mouse ovaries, there is a rare population of pre-meiotic germ cell with high expression of the *STRA8* and *DAZL* genes. These cells retained the capacity to develop into GFP-positive oocytes following transplantation into a young host mouse environment
Pacchiarotti *et al.* (2010)	Two different populations of GFP-Oct4 positive cells were found in mouse ovaries, dependent on their distribution and size. A small group of cells (10–15 μm) were located at the OSE and oocyte-like larger cells (50–60 μm) were enclosed by follicular structures. These ovarian GSCs produced embryoid body-like structures with differentiation into all three germ cell layers
Gong *et al.* (2010)	Two pluripotent colony-forming cell lines were isolated form adult ovarian stromal cells which expressed pluripotent markers and formed embryoid bodies and teratomas
Parte *et al.* (2011, 2013)	In adult rabbit, sheep, monkey and menopausal human OSE, there are VSELs that express pluripotent gene transcripts with the ability of differentiation into oocyte-like structures *in vitro* Both FSH- and basic fibroblast growth factor (bFGF)-stimulated stem cells exist in OSE and can initiate primordial follicles growth. Cryopreserved ovarian cortical tissue can be a supply of stem cells with the capacity for spontaneous differentiation into oocyte-like structures
White *et al.* (2012)	Isolated rare mitotically active oogonial stem cells in both mouse and human ovaries generated oocytes spontaneously and underwent meiotic division. Injection of GFP-transfected human oogonial stem cells into human ovarian cortical biopsies leads to formation of follicles that contain oocytes 1–2 weeks after xenotransplantation into immunodeficient female mice
Esmaeilian *et al.* (2012)	Presence of three well known pluripotent stem cell markers, Oct4, Nanog and Sox2, in mRNA and protein levels in prepubertal and adult mouse ovaries
Bhartiya *et al.* (2012)	Gonadotropin-induced pluripotent VSELs exist in the OSE, leading to proliferation and differentiation of GSCs to oocyte and primordial follicle assembly in adult mammalian ovaries
Patel *et al.* (2013)	FSH stimulates OSCs via the FSH receptor 3 (FSH-R3) to undergo self-renewal and differentiation into oocytes
Stimpfel *et al.* (2013)	Presence of the cells with expression of some pluripotency, germinal lineage and multipotency markers in the cortex of adult human ovaries. Isolation of small round SSEA-4-positive cells with a high degree of plasticity and differentiation into various types of somatic cells of three germ layers *in vitro*

(b) Studies that refuse the post-natal oogenesis

References	Study highlights
Bristol-Gould *et al.* (2006)	Statistical analyses and mathematical modelling showed that mammalian females produce a limited number of oocytes in the prenatal phase of life, and this production ceases after birth

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Table 1 (b) Continued

Liu *et al.* (2007)	Absence of early meiotic-specific or oogenesis-associated mRNA and lack of early meiocytes and proliferating germ cells markers in the healthy adult human ovaries resulted in the conclusion that neo-oogenesis does not occur in the adult human ovary
Zhang *et al.* (2010, 2012)	Failed to demonstrate the large ovoid germ cells with expression of *VASA* gene and the transcription of the meiotic-specific genes in the surface epithelium of adult rat ovary. Post-natal follicular renewal does not occur in mammals, and mitotically active DDX4-expressing female germline progenitors do not reside in post-natal mouse ovaries
Byskov *et al.* (2011)	There is no evidence for the presence of GSCs by staining pluripotent pre-meiotic germ cells markers and oogonia in the post-natal human ovary after final clearing of these cells during the first 1 or 2 years of early life
Kerr *et al.* (2012)	Neo-folliculogenesis does not occur in mice after eradication of the primary follicle resource by chemicals or γ-irradiation
Yuan *et al.* (2013)	There is no evidence for proliferative GSCs and germ cell renewal in adult monkey and mouse ovaries
Lie & Spradling (2013)	In adult female mice ovary there is no active GSC and production of new oocytes *in vivo*

eradicate the primordial follicle source (Hemsworth & Jackson, 1963; Burkl & Schiechl, 1978; Pelloux *et al.*, 1988; Meirow *et al.*, 1999; Shirota *et al.*, 2003; Jiang *et al.*, 2013) without stimulating atresia. After treatment with busulphan, cells expressed the meiotic entry marker (SCP3) in juvenile and adult mouse ovaries. However, the ovaries of females that were treated with busulphan included <5% of the normal primordial follicle pool. These data supported the theory that proliferative germ cells do not only reside in the post-natal ovary but are also needed to restore the follicle source. Subsequently, wild-type ovaries have been grafted into transgenic female mice that expressed green fluorescent protein (GFP). Grafted wild-type ovarian fragments penetrated into the GFP-positive ovarian tissue and follicle-enclosed wild-type germ cells and eventually became indistinguishable from the GFP-positive germ cells that formed follicles. Previous studies have suggested that mammalian stem cells migrate to their natural niche after introduction into a host (Nagano, 2003; Oh *et al.*, 2003; Szilvassy *et al.*, 2003; Torrente *et al.*, 2003). These data provided remarkable evidence for the presence of proliferative germ cells that maintain oocyte and follicle production in the post-natal mammalian ovary. Accordingly, the hypothesis that GSCs exist in adult mammals has been strengthened by detection of germ cell markers [OCT-3/4, mouse VASA homologue (MVH), SCF-R and SSEA-1] and meiotic markers (DMC1 and SCP3) in specific cells that aggregate in the periphery of adult mouse ovaries (Zhang *et al.*, 2008). Pacchiarotti *et al.* (2010) reported the isolation and characterization of GSCs in post-natal mouse ovaries using transgenic mice that expressed GFP under the control of the *Oct4* promoter (Pacchiarotti *et al.*, 2010). Two different populations of GFP–Oct4-positive cells were found in mouse ovaries, dependent upon their distribution and size; a small group of cells (10–15 μm) was located at the OSE and larger oocyte-like cells (50–60 μm) were enclosed by follicular structures. These ovarian GSCs sustained their stem cell characteristics, high telomerase activity, and normal karyotype after many passages for more than 1 year. They produced embryoid body-like structures with differentiation into all three germ cell layers. Meanwhile, Gong *et al*. (2010) isolated two pluripotent colony-forming cell lines from adult ovarian stromal cells, which also formed embryoid bodies and teratomas after injection into non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice. Gong *et al*. (2010) also reported that a small subgroup of the isolated cells from adult ovaries is immunoreactive for Oct4 and Nanog. Reverse transcription polymerase chain reaction (RT-PCR) results also revealed the presence of transcripts for both Oct4 and Nanog in adult ovaries (Gong *et al.*, 2010). In another study, scientists isolated OSCs from a newly born piglet with expression of transcription factors such as Oct-3/4, Nanog, and Sox2 (Song *et al.*, 2011). These cells displayed a remarkable ability to differentiate into putative oocyte-like cells (OLCs) *in vitro* and expressed VASA, DAZL and ZPC. Furthermore, putative GSCs from neonatal and adult mouse ovaries formed compact round colonies with unclear borders, ESC characteristics, alkaline phosphatase (AP) activity, and expression of a germ cell marker (VASA) and stem cell markers (Oct4, Klf4, c-Myc, Nanog, CD49f, Sox2, CD133, SSEA-1 and SSEA-4). These cells also had the ability to differentiate into OLCs *in vitro* following porcine follicular fluid treatment and form embryoid bodies, which expressed specific markers for all three germ layers (Hu *et al.*, 2012). In an outstanding study, White *et al.* (2012) reported that a rare population of mitotically active oogonial stem cells (OSCs) can be isolated by fluorescence-activated cell sorting (FACS) with DDX4 expression on the cell surface of adult mouse ovaries and human ovarian cortical tissues. The OSCs were expanded for months on mouse embryonic

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fibroblast (MEF) feeders and formed spontaneously oocyte-like structures, based upon morphology, gene expression (expression of DDX4, KIT, NOBOX, LHX8, GDF9, ZP1, ZP2, and ZP3) and haploid status. Injection of the OSCs into human ovarian cortical biopsies led to formation of follicles that containing oocytes 1–2 weeks after xenotransplantation into immunodeficient female mice (White *et al.*, 2012). Additionally, cultured OSCs besides sustaining their germline gene expression profile (Blimp1, Stella, Fragilis, DDX4, and DAZL) obtained expression of pluripotency-associated transcripts (Oct4, Nanog, and Sox2) and the meiotic marker STRA8. Expression of the meiotic commitment gene *STRA8* in cultured OSCs and the absence of *STRA8* in freshly isolated OSCs revealed the undifferentiated (pre-meiotic) situation of these germ cells before *in vitro* development (Imudia *et al.*, 2013).

Esmaeilian *et al*. (2012) reported the presence of three well known pluripotent stem cell markers, Oct4, Nanog and Sox2, in the mRNA and protein levels in 2-week-old (pre-puberty) and 8-week-old (adult) mouse ovaries (Esmaeilian *et al.*, 2012). Expression of these transcripts was observed in the ovaries of two different age groups by real-time quantitative RT-PCR (qRT-PCR), and immunohistochemistry results showed the presence of both Sox2 and Oct4 proteins in the cytoplasm of ovarian epithelial cells, granulosa cells, oocytes and theca cells. Nanog protein was observed only in the nucleus of the oocytes, and the expression of this protein was higher in 8-week-old samples compared with 2-week-old ones according to qRT-PCR results. As Oct4, Nanog and Sox2 have transcriptional regulator functions, they are expected to be found in the nucleus of the cells, however in several studies (Avilion *et al.*, 2003; Cauffman *et al.*, 2005; Parte *et al.*, 2011; Esmaeilian *et al*. 2012; Hu *et al.*, 2012) Oct4 and Sox2 proteins have been observed in the cytoplasm of adult ovary cells. During localization of these proteins in the cytoplasm of cells, pluripotential capacity of these cells can be questionable. Nevertheless, dependent on their cellular regulation, transcription factors can pass from the cytoplasm to the nucleus using a nuclear localization sequence (NLS) and pass in the opposite direction through the nuclear export sequence (NES; Whiteside & Goodbourn, 1993).

Besides GSCs, somatic stem cells also have been observed in post-natal ovaries; putative thecal stem cells have been isolated with the potential of self-renewal and differentiation *in vivo* and *in vitro* (Honda *et al.*, 2007). These cells, with fibroblast morphology and the potential to differentiate into steroidogenic cells in response to suitable hormone and growth factor stimulation, expressed the anticipated genes and morphological markers, and later also secreted androstenedione. Furthermore, theca-derived multipotent stem cells (TSCs) were isolated successfully and characterized from the thecal layer of porcine ovarian follicles and differentiated into osteocytes, adipocytes and OLCs *in vitro*. The TSCs expressed AP and mesenchymal stromal/stem cell markers CD29, CD44 and CD90 and were positive only for Sox2, whereas induced OLCs expressed Oct4, Nanog and Sox2, both at the mRNA and protein levels. The OLCs also expressed the oocyte-specific marker genes (ZPC, SCP3, Stella, DAZL, VASA, GDF9B and C-MOS) and the folliculogenesis marker follicle stimulating hormone (FSH) receptor (Lee *et al.*, 2013). Otherwise, a multipotent subpopulation of luteinizing granulosa cells was isolated from the ovarian follicles of infertile patients (Kossowska-Tomaszczuk *et al.*, 2009). These cells, cultured for a long period of time, expressed mesenchymal lineage markers (CD29, CD44, CD90, CD105, CD117 and CD166), POU5F1 (POU domain, class 5, homeobox 1) and differentiated into other cell types such as neurons, chondrocytes, and osteoblasts. After their transplantation into SCID mice, follicle-derived stem cells survived and produced tissues of mesenchymal origin. Recently, Stimpfel *et al.* (2013) observed cells with expression of some pluripotency-associated transcripts (Oct4, Sox2, Nanog, Stella, SSEA-4, AP and LIN28), germinal lineage (DDX4) and multipotency markers (MCAM/CD146, Thy-1/CD90 and STRO-1) in the cortex of adult human ovaries (Stimpfel *et al.*, 2013). They isolated small round SSEA-4-positive cells (4 μm) with a high degree of plasticity and differentiation into various types of somatic cells of three germ layers *in vitro*: neuronal-like cells (ectoderm), adipogenic and osteogenic cells (mesoderm), and pancreatic-like cells (endoderm).

The ovarian surface epithelium as a source of germline stem cells

Histological analyses of young and adult mouse ovaries have identified large oval cells in the OSE, similar to germ cells of fetal mouse ovaries (Crone *et al.*, 1965; Morita *et al.*, 1999). The OSE was supposed to be a source of germ cells, and new primordial follicles were developed by the accumulation of oocytes with nests of primitive granulosa cells in the ovarian cortex (Bukovsky *et al.*, 1995). In 2005, a study of the possibility of neo-oogenesis and granulosa cell generation was done on cultures derived from the surface epithelium of adult human ovaries (Bukovsky *et al.*, 2005). Ovarian surface epithelium cells that were cultured in medium without oestrogenic stimulus differentiated into small cells of granulosa phenotype, neural, epithelial and mesenchymal type cells. In contrast, OSE cells cultured

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in the presence of an oestrogenic stimulus led to the generation of OLCs. These cells displayed germinal vesicle breakdown (GVBD), release of the polar body, and protein characteristics of secondary oocytes. The label-retaining cell (LRC) population in coelomic OSE of adult H2B–GFP transgenic mouse ovaries exhibited stem/progenitor cell characteristics, including dye retention that provided evidence for putative somatic stem/progenitor cells (Szotek *et al.*, 2008). These cells showed quiescence, slow cycling, asymmetric cell division and an increased growth potential *in vitro*. The existence of MVH-positive cells in the OSE of neonatal mouse ovaries has been reported by Zou *et al.* (2009). After immunomagnetic isolation, they cultured neonatal mouse GSCs for more than 15 months and adult mouse GSCs for more than 6 months. These GSCs maintained high telomerase activity and a normal karyotype during long-term culture. After transfection of such cells with a GFP virus and their transplantation into the busulphan-treated mouse ovaries, transplanted GSCs underwent oogenesis and the host mice generated offspring that expressed the GFP transgene. At the same time, Niikura *et al.* (2009) demonstrated that there is a rare population of pre-meiotic germ cells in the OSE of aged mouse ovaries with high expression of the *STRA8* and *DAZL* genes. These cells retained the capacity to develop into GFP-positive oocytes (increased expression of Oct4–GFP, c-KIT, MVH and SSEA-1) following transplantation into a young host mouse environment (Niikura *et al.*, 2009). Indeed, subsequent studies have provided surprising results about the presence of rare stem-like cells with germline features in the OSE of women with no natural oocytes and follicles. Virant-Klun and collaborators scraped the OSE in post-menopausal women and young women with premature ovarian failure (POF). They have reported the isolation of putative stem cells with germline characteristics that spontaneously generated OLCs with the capacity of undergoing parthenogenetic development to create preimplantation blastocyst-like structures *in vitro* (Virant-Klun *et al.*, 2008, 2009). The same group concluded that stem cells naturally present in the OSE of patients with POF expressed some markers of pluripotency, such as Oct4, Sox2, Nanog, SSEA-4, KLF4, and MYC, just after scraping and during culture (Virant-Klun *et al.*, 2011b). They also revealed the presence of round (10–15μm) SSEA-4-, Sox2-, VASA- and ZP2-positive primitive OLCs in the adult OSE of a patient with serous papillary adenocarcinoma (Virant-Klun *et al.*, 2011a). Additionally, using magnetic-activated cell sorting (MACS) and FACS, a population of SSEA-4-positive cells was isolated from adult human OSE (Virant-Klun *et al.*, 2013a,c). The immunocytochemistry and genetic approaches showed that these small putative stem cells expressed the markers of primordial germ cells (PGCs) (PRDM1, PRDM14, and DPPA3) – the *PRDM1* gene is the key determinant of PGCs and plays a important role along with PRDM14 during PGC specification from post-implantation epiblast cells and is critical for the maintenance of unipotent germ cells (Bao *et al.*, 2012) – and pluripotency (Oct4A, Sox2, SSEA-4, SALL4, CDH1, and LEFTY1). In relation to the *in vitro* development of OLCs, some oocyte-specific transcription factors (ZP3, SCP3, and c-KIT) were also expressed in the presence of donated follicular fluid, including several crucial factors for oocyte growth and maturation (Virant-Klun *et al.*, 2013a,b). In another study, researchers demonstrated that in adult rabbit, sheep, monkey and menopausal human OSE, there are very small embryonic-like stem cells (VSELs) that express pluripotent gene transcripts of Oct4, Oct4A, Nanog, Sox2, TERT and Stat-3 with the ability to differentiate into oocyte-like structures that express c-KIT, DAZL, VASA and ZP4 in 3-week cultures (Parte *et al.*, 2011). In regards to analyses of FSH effects on GSCs, gonadotropin treatment through induction of FSH and FSH receptor action stimulated the pluripotent VSELs (upregulation of Oct4A and Nanog) that exist in the OSE, leading to proliferation (increased PCNA staining and Oct4A expression) and differentiation (upregulation of Oct4, MVH, Stella and Fragilis) of GSCs into oocytes and primordial follicle assembly in adult mammals (Bhartiya *et al.*, 2012; Parte *et al.*, 2013; Patel *et al.*, 2013). These findings led to the hypothesis that epithelial mesenchymal transition develops the granulosa-like cells, whereas VSELs undergo neo-oogenesis.

There may be a reasonable explanation for the possible existence of GSCs in the OSE. Hummitzsch *et al.* (2013) demonstrated that during development of bovine fetal ovaries, proliferation of a novel cell type called gonadal ridge epithelial-like (GREL) cells at the surface epithelium of the mesonephros led to formation of the gonadal ridge/ovarian primordium (Hummitzsch *et al.*, 2013). Migration of PGCs into the ovarian primordium has been observed before 70 days of gestation. Thus, in contrast with the widespread theory, the OSE cells do not penetrate into the ovary to form the granulosa cells of follicles, and OSE cells and granulosa cells have a common precursor, the GREL cell.

The extra-gonadal source of female germline stem cells after birth

Johnson and his collaborators hypothesized that bone marrow transplantation (BMT) may restore the generation of oocytes in wild-type mice sterilized by chemotherapy and mice with a genetic disorder

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that rendered them incapable of oocyte production (Johnson *et al.*, 2005a). Based on gene expression analyses and BMT studies using chemotherapy-sterilized mice, these authors suggested that a putative GSC supply in BM supported oogenesis in adult female mice. They also claimed that peripheral blood included an additional source of GSCs in female mice. After transfusion of peripheral blood collected from transgenic females with germline-restricted GFP expression, GFP-positive oocytes were observed in the ovaries of chemotherapy-sterilized recipient females. These results led to the hypothesis that putative germ cells in BM release progenitor cells into the peripheral circulation that then may migrate to the ovaries (Johnson *et al.*, 2005b). However, to investigate the potential of naturally circulating peripheral blood cells to engraft in the ovary and contribute to oogenesis, Eggan and colleagues analysed ovulated oocytes from adult female mice that were surgically joined by parabiosis (Eggan *et al.*, 2006). Parabiotic mice develop a common circulatory system and reveal a continuous, rapid exchange of cells and other circulating factors through the bloodstream (Wright *et al.*, 2001a,b; Bunster & Mayer, 2005). Although circulating cells had the capacity to enter the ovary and associate with ovulating oocytes, they sustained all haematopoietic characteristics in this environment and did not contribute to the production of ovulated oocytes (Eggan *et al.*, 2006). Moreover, in another study, 819 oocytes were examined in 30 ovarian grafts: GFP-negative ovaries were transplanted into GFP-positive transgenic hosts to test whether circulating germ cell progenitors could colonize the ovaries and organize new follicles. There was no evidence to support the hypothesis that progenitor cells from extra-ovarian sources could replenish the oocytes in adult ovaries (Begum *et al.*, 2008). However, in contrast with this result, Lee and collaborators observed that BMT into adult female mice treated with cytotoxic chemotherapy resulted in a restoration of follicle production, compared with continued sterility in chemotherapy-treated mice without receiving BMT. They suggested that a supply of GSCs resides in the BM, and that BMT triggers host neo-oogenesis by introducing oocyte precursors (Lee *et al.*, 2007b). By using double-colour immunohistochemistry, Bukovsky and his group have claimed that BM-derived cells (monocyte-derived cells and T cells) contribute to the origin of putative germ cells from the OSE stem cells in normal adult rat females and from the medullary somatic stem cells in neonatally estrogenized mature female rats without OSE. They have argued that an alternative origin of putative germ cells from the medullary region may describe why ovaries with destructed OSE are still capable of forming new primordial follicles (Bukovsky *et al.*, 2007, 2009; Bukovsky, 2011a; Bukovsky & Caudle, 2012). Furthermore, vascular pericytes and BM-derived monocytes have been observed in association with the initiation of follicular development, selection and pre-ovulatory maturation of autologous oocytes (Bukovsky *et al.*, 1995; Bukovsky, 2006, 2011b). It has also been demonstrated that a once-monthly infusion of BM-derived cells into young adult female mouse ovaries maintained the fertility of ageing females long past the time of normal reproductive failure (Selesniemi *et al.*, 2009). This effect was attributed to the development of mature oocytes from host germline cells, sustained by a beneficial effect of BM-derived cell infusions on the ovarian environment (Niikura *et al.*, 2009; Selesniemi *et al.*, 2009). Hence, age-related reproductive failure may be related to deterioration of somatic microenvironments (niche) that support stem cell capacity. Interestingly, some regenerative signals in young and aged male blood can rejuvenate follicular dynamics in an aged ovary. The blood of male mice contains STRA8, which can induce ovarian expression of the germ cell-specific meiosis genes (Anderson *et al.*, 2008) and leads to a significant increase in the ovarian follicle reserve through enhanced oogenesis (Niikura *et al.*, 2010). In continuation of a study on putative BM-derived GSCs after birth, BM stem cells were injected intravenously into follitropin receptor knockout (FORKO) mice and penetrated into ovaries. These cells triggered the expression of the FSH receptor gene, synthesis of FSH receptors, oestrogen hormone production, and folliculogenesis in ovaries of FORKO mice (Ghadami *et al.*, 2012). However, Santiquet *et al.* (2012) reported that there was no evidence that BMT or bovine embryonic ovarian tissue grafts led to the production of new oocytes in PU.1 and SCDI mice following treatment by chemotherapeutic agents. Nevertheless, the influence of BM cells improved the fertility of SCID mice that had been previously exposed to chemotherapeutic agents (Santiquet *et al.*, 2012). Thus, Santiquet *et al.* (2012) have suggested that the positive effects of transplanted bone marrow (BM) cells on the fertility of female mice could be due to renewal of self-tolerance of ovarian antigens; thereby, follicles are not demolished by chemotherapeutic agent treatment as a result of autoimmune damage. Additionally, Notarianni (2011) hypothesized that chemotherapeutic treatments led to ovarian failure, cellular apoptosis and reduction of ovarian antigen-specific regulatory T cells, and eventually to an autoimmune response in the ovary (Notarianni, 2011). In conclusion, it is proposed that BMT decreases autoimmune responses induced by chemotherapy, and this change may occur by production of regulatory T cells in the ovary to improve restoration of self-tolerance. Some studies that report the role of an estimated extra-gonadal source of female GSCs in post-natal oogenesis are summarized in Table 2.

Table 2 Some experiments that observe the role of an estimated extra-gonadal source of female GSCs in post-natal oogenesis

(a) Studies that support the role of an estimated extra-gonadal source of female GSCs in post-natal oogenesis

References	Study highlights
Johnson *et al.* (2005a)	Putative GSCs in BM and peripheral blood may support oogenesis in adult female mice
Lee *et al.* (2007b)	Putative GSCs reside in the BM, and BMT can stimulate host neo-oogenesis by introducing oocyte precursors in adult female ovary
Bukovsky (2011b); Bukovsky & Caudle (2012); Bukovsky *et al.* (2007, 2009)	BM-derived cells contribute to the origination of putative germ cells from the OSE stem cells in normal adult rat females and from the medullary somatic stem cells in the neonatally estrogenized mature female rats without OSE
Selesniemi *et al.* (2009)	Once-monthly infusion of BM-derived cells into young adult female mice ovary maintained the fertility of ageing females long past the time of normal reproductive failure
Ghadami *et al.* (2012)	Intravenously injection of BM-derived GSCs into the FORKO mice led to expression of the FSH receptor gene, synthesis of FSH receptors, oestrogen hormone production, and folliculogenesis in the ovaries

(b) Studies that refuse the role of an estimated extra-gonadal source of female GSCs in post-natal oogenesis

References	Study highlights
Eggan *et al.* (2006)	There is no evidence that circulating or BM cells contribute to the generation of oocytes or enhance ovulation of endogenous oocytes in transplanted mice
Begum *et al.* (2008)	There is no evidence to support the hypothesis that progenitor cells from extra-ovarian sources can replenish the oocytes in adult ovary
Santiquet *et al.* (2012)	Chemotherapy-sterilized SCID mice cannot produce new oocytes after BMT but fertility of mice improved

Conclusions

Remarkable studies on stem cell biology have revealed that most stem cells from different resources possess the same features such as multipotency (or sometimes pluripotency), self-renewal and regenerative potential in adulthood. The field of mammalian OSC biology has arguably been continuing, as the controversy of putative neo-oogenesis and follicular renewing in post-natal ovaries has intensified in the last 10 years. The basic doctrine that post-natal oogenesis occurs in lower vertebrates but not in mammals still prevails. However, the results of several studies have indicated that post-natal mammalian ovaries contain GSC precursors that are capable of renewing the oocyte pool and follicles.

The current authors believe that epigenetic mechanisms, such as histone modifications, DNA methylation, chromatin remodelling, and non-coding transcripts, play a determinative role in oogenesis and putative neo-oogenesis in post-natal ovaries. In studies that consider the epigenetic mechanisms in oogenesis, histone acetylation has been demonstrated to be involved in transcriptional regulation of germ cell development, including meiotic entry (Wang & Tilly, 2010) and meiotic continuation (Kim *et al.*, 2003). The activation of the *STRA8* promoter in pre-meiotic germ cells was repressed by epigenetic factors that involved histone deacetylation (Wang & Tilly, 2010), and deacetylating histones of oocyte cytoplasm induced reprogramming of gene expression and resulted in the resumption of meiosis (Kim *et al.*, 2003). Wang & Tilly (2010) also revealed that the class I/II histone deacetylase (HDAC) inhibitor, trichostatin-A (TSA), is associated with STRA8 activation, and that STRA8 expression is detectable and regulated physiologically in adult mouse ovaries. Cdk5 and the Abl enzyme substrate 1 (*CABLES1*) gene, which encodes a protein involved in the regulation of the cell cycle through interactions with several cyclin-dependent kinases, is a gene crucial for constraining the rate of oocyte renewal in adult mouse ovaries (Lee *et al.*, 2007a). *LIN28A* also is a critical gene that affects PGC proliferation during embryogenesis and the size of the germ cell pool (Shinoda *et al.*, 2013); FOXO3 (Forkhead box O3) has been described as a determinative gene in the development of the follicle pool size (Pelosi *et al.*, 2013). Over-expression of FOXO3 can lead to augmentation of ovarian reproductive capacity in adulthood. In addition to these arguments, further studies are needed to clarify the effects of epigenetic mechanisms on putative neo-oogenesis in adulthood.

The controversy over the existence of OSCs and neo-oogenesis in adult mammals continues, and much more work is required to fully approve the concept that mammalian ovaries include cells with OSC-like characteristics that can be stimulated to enter

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a differentiation process and generate new oocytes. Nevertheless, there are some epigenetic and genetic concerns related to the *in vitro* differentiation of GSCs into oocytes, culture of OSE to produce autologous oocytes and growth of primordial follicles *in vitro*. In particular, stem cell dysfunction may result in some disorders such as ovarian cancer, polycystic ovary syndrome and fetal chromosomal abnormalities. In spite of these concerns, new outcomes in putative post-natal neo-oogenesis studies may lead to efficient therapy in female infertility and autologous regenerative medicine. Genetic disorders (such as POF), cancer treatment by chemotherapeutic agents and exposure to radiation can lead to infertility and inability to conceive. The differentiation of autologous GSCs into mature oocytes would enable *in vitro* fertilization and provide a new and novel expectancy for these sterile patients to produce offspring and delay menopause. It has also been postulated that putative stem cells in OSE can be involved in tumorigenesis and may be a therapeutic target in patients to offer more efficient therapy in the future.

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