Germ stem cells in the mammalian adult ovary: considerations by a fan of the primordial germ cells

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ABSTRACT: At or early after birth, mammalian ovaries are filled with primordial follicles each composed by an oocyte blocked at the end of prophase I surrounded by a single layer of granulosa cells. The doctrine that female mammals are born with a finite number of oocytes fated to be exhausted with the age has been challenged by recent results claiming that new oocytes can be continuously formed in the post-natal mouse ovary. In my view, this notion, termed neo-oogenesis, is strictly linked to the process of the germline specification which presents unique features. Therefore, in the present paper, I am going to discuss two aspects of neo-oogenesis related to this process: first, evidence showing that adult mammalian ovary contains cells able to undergo germline specification and produce new oocytes; and second, the possible origin of such cells. In conclusion, I favour the possibility that a small number of primordial germ cells (PGCs)/oogonia or of PGC-derived undifferentiated cells with stem cell characteristics could remain in the post-natal ovary and under certain conditions may resume mitosis, enter meiosis and give rise to oocytes.

Key words: oogonia / PGC / mammalian

Introduction

The extragonadal origin of mammalian primordial germ cells (PGCs), considered the gamete precursors, has been demonstrated by studies performed on a variety of mammalian species (for a review see, Baker and Eastwood, 1983). In the early 1950s, the use of the histochemical detection of the alkaline phosphatase enzyme allowed the localization of PGCs in the posterior region of the embryo of both sexes at the angle between the allantois and the wall of the yolk sac of mouse and human embryos and to follow their movement into the forming gonadal ridges (for a review, see Eddy et al., 1981). It is generally believed that PGCs will eventually differentiate into oogonia/oocytes or degenerate in extragonadal regions (Pesce et al., 1993; Stallock et al., 2003).

We are now starting to understand that the reason for this singular beginning of gametogenesis in mammals is to be traced in a complicated genetic and epigenetic program underlying the germline formation aimed to preserve the unique characteristics of the germline. During the pre-gastrulation period, somatic lineages are beginning to be determined in the epiblast cells. In a small number of epiblast cells, however, somatic lineages must be repressed so that they can enter the germline. This occurs in about six cells located in the posterior region of the epiblast which receiving higher amount of the growth factor BMP-4 and expressing the transcription factor BLIMP-1 become the PGC precursors. At the same time, pluripotency genes such as Oct4, Nanog and Sox2 are reactivated in the specified PGCs located in the extraembryonic mesoderm of the wall of the yolk sac before they start migration toward the gonadal ridges (Ohinata et al., 2009 and references herein). We know that the genome of PGCs during migration and after gonad colonization undergoes complex rounds of epigenetic changes. Together with the activity of pluripotent genes, epigenetic changes are crucial to maintain and/or regain unique features of germ cells focused to preserve totipotency within a specific differentiation program. At the same time, they involve the erasing of the sex-dependent genomic imprinting imposed in germ cells during previous gametogenesis, an exclusive PGC capability (for a review, see Hayashi et al., 2007).

In the females of most mammalian species, PGCs and oogonia coming from them apparently disappear within the fetal ovaries as meiosis begins and they differentiate into primary oocytes. These become progressively enclosed by somatic cells and form the primordial follicles. At birth, ovaries are filled with primordial follicles each composed by an oocyte blocked at the end of prophase I (the dictyate period) surrounded by a single layer of flat granulosa cells. Is this follicle pool sufficient to sustain the production of fertilizable oocytes for the entire fertility period of a female? Is this pool of oocytes renewable in physiological conditions or after ovary damage?
I will not enter directly in the debate on these subjects because after the controversial studies by the Jonathan Tilly’s group (Johnson et al., 2004, 2005), several papers have addressed these topics (see the recent reviews by Abban and Johnson, 2009; Tilly and Telfer, 2009; Tilly et al., 2009). Briefly, according to a long-held doctrine of the biology of reproduction the pool of not-renewable primordial follicles serves as a source of developing follicles and oocytes that declines with age. This view has been challenged by research results from the Tilly’s group that neo-oogenesis actually takes place during adult life in the mouse ovary from germline stem cells (GSCs) located in the surface epithelium of the ovary (OSE) and under the crucial influence of bone marrow (BM) cells (Johnson et al., 2004, 2005).

In the present short review, I am going to consider only two points of this debate: evidence showing that adult mammalian ovary contains cells able to produce new oocytes and in that case, the possible origin of such cells.

### Evidence of neo-oogenesis

Having clearly established that in mammals the germ line originates from the somatic cells of the epiblast and that mouse embryonic stem (ES) cells and different types of somatic tissues, such as BM and peripheral blood (PB), fetal pig skin cells and adult rat pancreatic cells, are all capable of forming in vivo (BM and PB) or in vitro (all the other cell types) oocytes or oocyte-like cells, I do not see in principle any difficulty to believe that the adult ovarian tissues may also contain oocyte-forming cells. But what evidence exists to support such a notion? In the adult ovaries of some species of the prosimian primates, mitotically active germ cells have been described (Duke, 1967; David et al., 1974). More recently, Johnson et al. (2004) reported the presence of presumptive mitotically active GSCs, expressing the germ cell-specific VASA (MVH) protein, in or proximal to the OSE of juvenile and adult mice. The number of these cells, however, decreases sharply as females transit through puberty (from ~63 cells/ovary at 30 days post-partum to 6 cells/ovary at 40 days post-partum) making it unlikely in the opinion of the authors themselves that these cells can represent adult GSCs (Johnson et al., 2005). Follow-up studies by the same group suggested a possible extra-ovarian origin of GSCs in adult mice, namely from the BM (Johnson et al., 2005). Other studies from the same and an other groups have shown, however, that BM cells are not involved in the formation of mature, ovulated oocytes (Egkan et al., 2006; Lee et al., 2007), but are crucial for adult neo-oogenesis at least after ovary damage (Lee et al., 2007). Bukovsky et al. (2005) scraping OSE cells from adult human ovary claimed that in culture some of these cells form follicle cells and large cells with oocyte phenotype as well. Small (diameter 2–4 μm) round putative stem cells also able to forming oocyte-like cells in vitro have recently been isolated from adult human ovarian tissues (Virant-Klun et al., 2008). Moreover, proliferative large cells expressing the VASA protein have been very recently purified from neonatal or adult mouse ovaries and maintained in vitro for months. Strikingly, these cells, after transplantation into ovaries of chemotherapy-sterilized recipients, generate oocytes that can be fertilized and produce viable offspring (Zhou et al., 2009). With this last noteworthy exception, however, the oocyte-like cells described in all other works cited above were not proven to be able to possess the unique developmental oocyte capability to form a new individual after fertilization.

It is possible that in some types of pluri- or multipotent stem cells, genes encoding proteins typical or exclusive of the germ line and even of oocytes are activated in certain in vivo or in vitro conditions. This could be sufficient for the formation of cells phenotypically resembling oocytes but insufficient for creating proper oocytes. In fact, we know that the correct female germ line development requires a sequence of complex and long-lasting events and interactions with the surrounding somatic cells starting from the formation of PGCs and crucial for acquiring not only the typical oocyte phenotype and meiotic abilities but also the proper genomic activity and epigenetic configuration necessary to sustain the development of a new individual.

Therefore, for now, the only source of cells able to form after birth new bona fide oocytes suitable to be ovulated and/or fertilized remains the ovary.

### PGCs or PGC-derived cells as possible source of GSCs

Where might such putative GSCs come from? Excluding the extra-ovarian origin demonstrated by papers reported above, including results from the Tilly’s group itself, there are of course basically two possibilities (Fig. 1). They could arise from ovarian somatic cells or from PGCs/oogonia remained in the ovary or from PGC-derived GSCs. As reported above, Bukovsky et al. (1995, 2004), using morphological and immunohistochemistry observations, described the formation of putative germ cells within the OSE of adult human ovary. They postulated that such germ cells originate from OSE stem cells which differentiate from mesenchymal progenitors in the ovarian tunica albuginea. In line with this possibility, Virant-Klun et al. (2008) isolated small round c-KIT and SSEA-4 positive cells from OSE cells of human ovaries. These cells expressed mRNA for several protein typical of ES cells and early PGCs such as OCT-4, NANOG, SOX-2 and after 20 days of culture formed growing oocyte-like cells expressing germ cell-specific Mvh (Vasa) and zona pellucida (Zp2) genes. Within the same cultures, other cells appeared to be able to differentiate into granulosa cells. Since similar cells called very small embryonic-like (VSEL) stem cells have been found in different human and animal adult tissues including blood (Ratajczakz et al., 2007), blood cell contamination in such cultures cannot be excluded. Recently, Zhang et al. (2008) reported, however, the isolation from mouse ovaries of small cell aggregates containing cells similar to VSEL and expressing besides OCT-4 and SSEA-1 also the germ cell-specific protein VASA. Additional evidence about the presence in the OSE of cells able to give rise to bona fide oocytes in the adult mouse ovary have recently been provided by the already mentioned paper by Zhou et al. (2009). In this work, proliferating (BrdU positive) VASA-positive cells were immunolocalized in the OSE of 5-day-old female mice. When isolated by immunomagnetic selection with an antibody against VASA, ~20 cells from 5-day-old mouse ovary and 8 cells from adult ovary, such cells appeared to be round and large (diameter 15–20 μm) and were able to give rise in co-culture onto an STO cell monolayers highly proliferating cell lines expressing besides Mvh (Vasa) several ES cell and early PGC genes (APase, Oct-4, Dazl, Blimp-1, Fragilis, Stella and Rex-1) but not others (c-Kit, Sox-2 and Nanog) and not meiotic (Scp1-3) or growing oocyte (Figla and Zp3) genes. Such cells were chromosomally normal and...
showed a female imprinting pattern. Most extraordinary, after transplantation into ovaries of chemotherapy-sterilized recipients cells of these cell lines generated oocytes that can be fertilized and produced viable offspring. Which is, if any, the relationship between the small VASA negative VSEL-like cells isolated from the human ovary (Virant-Klun et al., 2008), the similar but VASA-positive cells identified in the mouse ovary (Zhang et al., 2008) and the large VASA-positive cells isolated from the mouse ovary (Zhou et al., 2009)? Considering the normal development of mouse and human PGCs, c-Kit (mouse and human), SSEA-1 (mouse) and SSEA-4 (human) found in the VSEL-like cells are expressed by the majority of pregonadal PGCs and are progressively down-regulated when PGCs entering into meiosis within the embryonic ovary differentiate into primary oocytes. On the contrary, the VASA protein is detectable only when PGCs enter into the gonadal ridges and remains elevated in fetal and post-natal oocytes (Castrillon et al., 2000; Toyoooka et al., 2000). This suggests that the large VASA-positive cells might derive from the small VSEL-like cells. Moreover, the VSEL-like cells isolated by Virant-Klun et al. express genes typical of ES cells and early PGCs such as Oct-4, Nanog and Sox-2 indicating undifferentiated status. Taken together, these last observations suggest that cells with the characteristics of early PGCs are present or are generated in the adult mammalian ovary.

I favour the possibility that a small number of PGCs/oogonia or of PGC-derived undifferentiated cells with GSC characteristics remains in the post-natal and adult ovary and under certain conditions may resume mitosis, enter meiosis and give rise to oocytes. My view is based on the following considerations: (i) in the adult male, spermatogenesis starts from GSCs derived from PGCs, (ii) the occurrence of oogenesis from PGC-derived GSCs in the ovaries of adult flies (for a review, see Krilly and Xie, 2007) and non-mammalian vertebrates (Draper et al., 2007) is well established; (iii) PGCs are able to give rise to pluripotent stem cells (embryonal carcinoma, EC, and embryonal germ, EG, stem cells, Stevens, 1967; Matsui et al., 1992; Resnick et al., 1992); (iv) relatively high number of PGCs/oogonia which do not enter into meiosis are present in the prenatal mouse ovary (~10% of the total germ cells) (McClellan et al., 2003) and are mitotically activatable in the post-natal ovary (Bristol-Gould et al., 2006); (v) mitotic PGCs/oogonia have been described also in rat, prosimian and human adult ovary (Duke, 1967; David et al., 1974; Bukovsky et al., 2004; Bukovsky and Virant-Klun, 2007). A last but not least consideration is that it is difficult to realize how somatic cells in the adult ovary environment could undergo the same rounds of genetic and epigenetic changes necessary to establish the germline in the embryo and give rise to oocytes with the correct epigenetic status of chromatin, in particular those associated with totipotency and gene imprinting. In fact, we know that this is established in PGCs and growing oocytes as a consequence of complex genome-wide reprogramming throughout various developmental stages and requires at least in part interactions with somatic cells of the embryonic ovary (Seki et al., 2005, 2007; Hajkova et al., 2008). Therefore, in my view, it is more acceptable to think that neo-oogenesis begins from cells which already underwent some of such changes.

**Perspectives**

An important corollary of my favourite possibility is to verify if PGCs transplanted into an adult ovary are able to develop normally and give rise to growing oocytes. In this regard, it should be crucial to verify the presence in the transplanted cells of morphologically recognizable stages of the meiotic prophase I. Moreover, it should be worthy to investigate if the PGC population contains a subfraction of cells with the morphological characteristics of VSEL or if PGCs are able to transform in such cell type.

Having established that evidence exists for the presence of putative GSCs within the post-natal ovary and their more likely origin from PGCs, a final important aspect of neo-oogenesis, I only touch on here, is to establish if it occurs under normal physiological conditions or as a stress response of the ovary only. At least in humans, I favour this last possibility. In fact, although the kinetics of folliculogenesis in the human female ovary has not been completely elucidated, a reserve of almost 400 000 follicles per ovary at puberty should be largely sufficient to assure about 400 ovulations occurring during the fertile life. In fact, during the reproductive years in women, the estimated decline in the number of primordial follicles remains steady at
about 1000 follicles per month and accelerates after the age of 37 years (Broekmans et al., 2007). On the other hand, in my view the clearest evidence of neo-oogenesis in the mouse ovary was obtained after depletion of the follicle pool of the ovary by the chemotherapeutic agent doxorubicin or after the injection of the histone deacetylase inhibitor trichostatin (Johnson et al., 2005). Further studies are needed, however, to clarify such aspects. In this regard, an undocumented merit of the renaissance of the neo-oogenesis idea has been to have stimulated new investigations on the ovary physiology.

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