Chapter 5

Challenging the Paradigms on the Origin, Specification and Development of the Female Germ Line in Placental Mammals

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Abstract

Most of our understanding on the origin, specification and development of the female germ line in placental mammals comes from studies in the laboratory mouse. The molecular pathway leading to the development and establishment of the female germ line in mouse has erected as the paradigm for placental mammals. It remains, however, largely unexplored whether the well-established mouse regulatory pathway is a common mechanism to other or all placental mammals. Discrete differences in mammals other than mouse reveal the existence of alternative mechanisms that challenge the currently accepted tenets on the origin and establishment of the mammalian female germinal reserve. Here, we will discuss the mouse framework in the light of emerging discrepancies seen in other placental mammals.

Keywords: placental mammals, primordial germ cells, female germ line development, germinal reserve, ovarian development

1. Introduction

Germ cells are the only cell types capable of transmitting the genetic traits of an individual. They differentiate into spermatozoa and oocytes in adult testis and ovary, respectively, and give rise to a totipotent zygote after fertilization. Germ cells guarantee the perpetuation and diversification of the genetic information across the generations in most multicellular organisms. The developmental pathways that lead to the formation of a highly specialized germ cell are long and complicated, and the molecules that are involved in this process are still a matter

of discussion. One extraordinary feature in the germ cell lineage in mammals is the fact that specification occurs far from the gonads, implying a necessary migratory phase after specification. A second feature is their unique capacity to undergo meiosis, in which chromosome recombination generates genetic variation in the haploid gametes [1–4].

Most of our understanding regarding germ cell specification and differentiation in mammals comes from studies in the laboratory mouse. It is widely accepted that specification of primordial germ cells (PGCs) in mouse takes place at a very early stage in development; more precisely, they are thought to be set apart following blastocyst implantation in the proximal epiblast of the gastrulating embryo [2]. So far, however, no lineage tracing study has shown that those early segregated PGCs finally end up in the gonads [5]. Alternatively, it has been proposed that presumptive early specified PGCs in the proximal epiblast are rather a primordial pool of stem cells from which PGCs can be specified later on in development, probably during migration toward the emerging gonad [5]. Both explanations have been raised from mouse embryo studies. Nevertheless, there are some key embryological differences between the mouse and other mammals, especially at the epiblast stage when PGCs are specified. The epiblast of the murine rodent forms a cup-shaped egg cylinder, but most other mammals have a flat disk-like epiblast. Signals from extraembryonic tissues induce germ cell fate in a subset of epiblast cell at a specific position with optimal concentration and timing of signals. As PGC specification largely depends on signals from surrounding tissues, the morphology of the embryo is crucial for dissecting out the mechanisms of germ line establishment in different mammals since tissues surrounding the epiblast in the egg cylinder are not the same in flat-disk embryos [6].

2. The mouse model for primordial germ cell specification

2.1. The egg cylinder

In mouse, the blastocyst implants in the uterus by E4.5. The inner cell mass (ICM) of blastocyst is the source of epiblast cells. The ICM is segregated into epiblast and hypoblast or the primitive endoderm. Epiblast cells are equipotent and give rise to all the somatic and germ cells. During implantation, when the syncytiotrophoblast starts to penetrate the wall of the uterus, the epiblast and hypoblast are physically constrained and form a bilaminar embryo. The internal epiblast cells reorganize from a ball of cells into a cup-shaped epithelium surrounded by hypoblast. Immediately before gastrulation (E6.0 and E6.5), the mouse embryo can be visualized as a thick-walled cup of tissue (the epiblast or embryonic ectoderm), which gives rise to the entire fetus and some of the placental membranes. A second thick-walled cup of tissue (the extraembryonic ectoderm, ExE) placed overturned on the epiblast will give rise to the main part of the placenta. Both cups are enclosed in a thin bag of primitive endoderm-derived visceral endoderm (VE) [2, 7, 8].

The embryonic disk is forced into a complex shape called the ‘egg cylinder’ in which the anterior and posterior poles of the embryo come in close proximity to each other. Around

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E4.5 and E5.5, the ExE arises from the polar trophoectoderm (TE) and makes contact with the underlying epiblast. At E6.5, gastrulation starts with the formation of the primitive streak at the posterior region of the embryo. At E7.5, epiblast cells migrating first through this structure include the PGC precursors, which form the extraembryonic mesoderm [9, 10].

2.2. Mechanism for PGC specification

In mouse, PGCs originate from the most proximal epiblast cells by induction of the ExE and VE. Both extraembryonic tissues surround the epiblast cell of the postimplantation egg cylinder at around E5.0–E6.0. The ExE and VE release the bone morphogenetic protein (BMP) 4, 8b and 2 to instruct a small number of pluripotent proximal epiblast cells to become competent to be PGCs, suppressing a somatic program that is adopted by neighboring cells [11] (**Figure 1)**.

Accordingly, BMP4 released from the ExE activates the expression of B-lymphocyte–induced maturation protein 1 (*Blimp1*), also known as PR domain-containing protein 1 (*Prdm1*), at ~E6.25 and PR domain-containing protein 14 (*Prdm14)* at ~E6.5 in a dose-dependent manner [12–16]. *Bmp2* expressed in the proximal VE enhances the same signaling pathway, ensuring that the highest levels of Bmp signaling occur in the most proximal epiblast [11, 13, 16]. Both *Blimp1* and *Prdm14* together with *Tcfap2c* (also known as *Ap2γ*) [17, 18] are required for PGC specification.

Blimp1 protein signal first appears in about 6 cells in the most proximal epiblast at the posterior side of the embryo. Blimp1$^+$ cells initially express the *Hox* genes as well as many other genes known to be involved in embryonic development and suppress the expression of genes associated with pluripotency, such as *Sox2*, *Nanog*, and *Zic3*. At around E6.75–E7.0, *Hox* genes are downregulated and Blimp1$^+$ cells regain the expression of pluripotency genes [13–15].

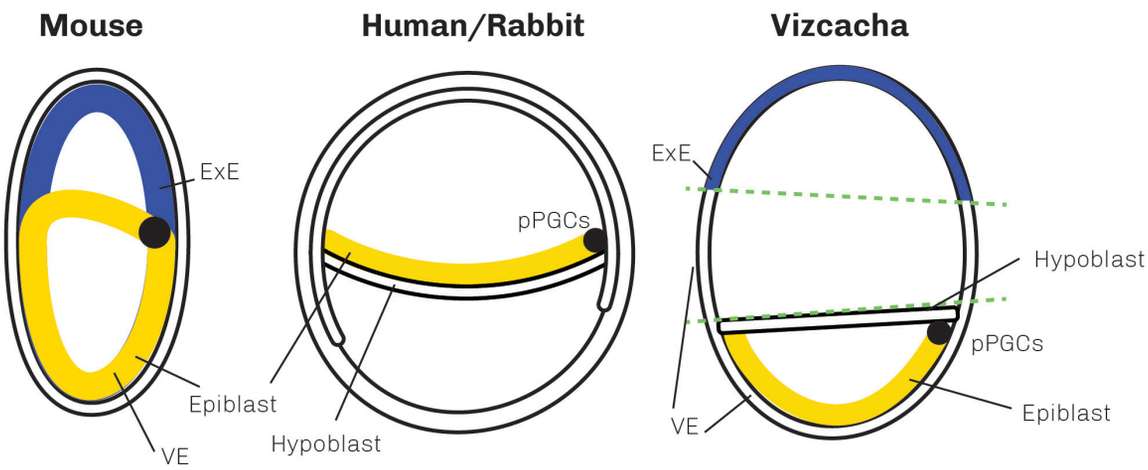

Figure 1. Schematic comparison of the early-implanted embryo in the egg cylinder of mouse and the flat disk embryo of human, rabbit and vizcacha. No extraembryonic ectoderm (ExE) is found in human/rabbit embryo; in the vizcacha, it locates far apart from the epiblast to be responsible for inducing germ cell specification as in mouse. The black circle indicates the presumptive location from which PGCs originate. PGCs: primordial germ cells, pPGC: presumptive PGC, VE: visceral endoderm.

Therefore, the PGC precursors appear to be initially induced toward a somatic mesodermal fate, but then regain their potentially pluripotent nature.

Following lineage restriction, PGC precursors initiate germ cell specification by activating *Prdm14* and *Tcfap2c* [15]. *Prdm14* plays crucial roles in two successive events characterizing the germ cell program: reacquisition of pluripotent potential and epigenetic reprogramming [12, 18]. *Prdm14* is first expressed in Blimp1$^+$ cells and later on in PGCs. Although the activation of *Prdm14* is independent of *Blimp1*, the expression of *Tcfap2c* at ~E6.75 appears to be dependent on *Blimp1* [12]. Indeed, when these factors are coexpressed they can induce PGC-like cells in the absence of cytokines, suggesting that the tripartite gene network *Blimp1*/*Prdm14* /*Tcfap2c* is sufficient for mouse PGC specification [18]. The first two genes acting in the founder PGC population are *Fragilis* and *Stella. Fragilis* (also known as mouse interferon-induced protein like gene-1 [mil-1]/interferon-induced transmembrane protein 3 [*Ifitm3]*) [19] expression marks the beginning of germ cell competence and it starts expressing at ~E6.25–E6.5 before *Blimp1* expression. *Fragilis* expression intensifies in the posterior extraembryonic mesoderm at ~E7.0–E7.25. In fact, the high levels of BMPs activate the expression of *Fragilis* and competent cells acquire the ability to form PGCs when they begin to express *Blimp1*.

Stella (also known as primordial germ cell 7 [Pgc7]/developmental pluripotency-associated 3 [*Dppa3]*) [20] begins to express specifically in *Fragilis-Blimp1* expressing cells in the extraembryonic mesoderm at ~E7.0–E7.25 and continues to be expressed in migrating PGCs. The function of *Stella* gene product is uncertain, but it has domains characteristic of proteins involved in RNA splicing. Actually, *Stella* represses homeobox genes in the nascent germ cell and as such maintains the pluripotency of PGCs during their migration toward the genital ridge. However, gene-knockout studies revealed that neither *Fragilis* nor *Stella* is essential for PGC specification [21, 22].

Blimp$^+$ PGC precursors proliferate and move into the extraembryonic mesoderm (ExM); they reexpress pluripotency-associated genes (*Oct4*, *Nanog*, *Sox2* and *Klf2*) and *Stella* at around E7–E7.75. While Klf2 and Stella are apparently dispensable proteins for PGC development [2, 23], the three core pluripotency factors Oct4, Nanog and Sox2 are essential for PGC development [24–26]. Although the specific roles of these factors during germ cell development are unclear, it is thought that their expression confers latent pluripotency to the germ line. In the case of PGCs, this regulatory network is thought to protect them from somatic-inducing signals during the extensive epigenetic reprogramming they undergo [27]. *Oct4* is uniformly expressed in postimplantation epiblast and also in nascent PGCs during specification. *Oct4* expression remains high until germ cells undergo sexual differentiation in the gonad [28, 29]. It is apparently essential for both germ cell specification [30] and maintenance [31]. *Nanog* is enriched at the proximal posterior epiblast, the position where PGCs are specified from, in E6.5 and E7.5 embryos [32]. *Nanog* appears to be dispensable for mouse PGC specification but is essential for germ cell maintenance. *Sox2* is active in mouse PGC from E7.5 forward. Conditional knockout of *Sox2* shortly after specification caused a dramatic decrease of germ cell numbers by E7.5, being undetectable by E13.5 [32].

With the establishment of germ cell fate, germ cells express factors like alkaline phosphatase (AP), Nanos3, Dazl, mouse vasa homologue (Mvh) and Dnd1 [2]. They increase in number and move out of the embryo by the primitive streak in formation toward the extraembryonic

mesoderm at the base of the allantois at E7.25. As mentioned above, PGCs form a cluster of cells 6 to 16 cells at around E6.5; then, they increase to approximately 20–28 cells, move posteriorly and develop into PGCs at E6.75-E7. During early gastrulation, the PGCs form a cluster of 40–50 cells at the base of the incipient allantois in the ExM at around E7.25 [33, 34]. Subsequently, and concomitant with an increase in their number, at around E8, they start to translocate one by one toward the developing hindgut endoderm and move through it. They then leave the endoderm to emerge in the mesentery and at around E10.5 colonize the embryonic gonads, where they proliferate and initiate a differentiation into either oocytes or spermatozoa depending on the embryo sex.

2.3. Migration of PGCs

PGCs in the mouse may be motile from their onset (E7.25) until they colonize the genital ridge (E11.5). After formation, PGCs move through the posterior primitive streak and invade the definitive endoderm and posterior extraembryonic structures. Following subsequent migration within the hindgut during its anterior extension (E8-E9.5), mouse PGCs migrate through the hindgut tissue to the mesoderm, followed by bilateral migration toward the gonadal ridges (E10.5–11.5). During this pregonadic phase, PGCs can be identified by morphological criteria and surface markers, such TNAP and SSEA-1, and the expression of pluripotent markers like Oct4, Sox2 or Nanog [35].

Six distinct stages of PGC behavior in the migratory process were identified, including: (i) invasion of the endoderm, (ii) passive or active migration into the hindgut, (iii) random migration within the hindgut, (iv) migration from the gut to the genital ridges, (v) clustering at the ridges and (vi) cell death within midline structures [36].

At E7.5, PGCs move through the primitive streak and into the definitive endoderm. Some PGCs also end up in the allantois and/or parietal endoderm. The fate of PGCs in extraembryonic structures remains uncertain, but PGCs in the definitive endoderm become incorporated into the hindgut, and at E9.0, they can be found moving within and around the cells of the hindgut epithelium [36]. At E8.5, PGCs on the lip of the hindgut pocket have a rounded non-motile morphology suggesting that PGCs are passively incorporated into the gut and then, at stage (iii), they reinitiate active motility around the epithelial cells.

Interactions between PGCs may also be important for their homing behavior. PGCs emerge from the gut individually, but during migration, they interact with each other forming a migrating network of cells [37]. This network becomes progressively aggregated into clusters of cells toward the end of migration. Antibodies against E-cadherin blocked the process of PGC aggregation in cultured embryo slices and prevented PGCs from forming tight clusters at the genital ridges [38].

At the end of their migration, PGCs presumably lose their motile properties as they associate with somatic cells in the gonad and acquire sex-specific morphologies. There does not seem to be any evidence for sex-specific differences during germ cell migration.

2.4. An alternative hypothesis for PGC specification in mouse

The mouse pathway described above is the classical currently accepted model of PGC formation. This path establishes that PGCs originate and specify as an early lineage-restricted

cluster of cells in the base of the allantois soon after implantation. Nevertheless, no definitive proof demonstrating the continuity of those presumptive early-specified PGC and the germ cells, which colonize the genital ridge, has so far been provided. In view of this, and critically reviewing the literature on PGC origin and specification in mouse, Mikedis and Downs [5] advocate in favor of an alternative hypothesis. These authors propose an alternative model in which the presumptive PGCs in the base of the allantois are instead a pool of pluripotent progenitor cells in the posterior end of the primitive streak that builds up the fetal-placental interface. The pluripotent cell pool condenses into a specific area of the proximal epiblast, namely the allantoic core domain (ACD), which extends the body axis posteriorly through the allantoic midline. The pluripotent cells in the ACD express all PGC markers and contribute to both embryonic and extraembryonic tissues. From this pluripotent population, it is suggested that PGC could be segregated later. PGC specification could take place for example during migration toward the genital ridge once evolutionarily conserved genes of germ line development, such as *VASA, Dazl* and *Nanos*, begin to be expressed. Although this alternative explanation is proposed for the mouse egg cylinder, it may well apply in flat embryos where the ExE is absent or far apart from the epiblast.

3. PGC specification and migration in mammals other than the mouse

The embryo proper of most gastrulation-stage mammals, including humans, rabbits and pigs among others, has the shape of a flat disk with two cell layers: epiblast and hypoblast (equivalent to VE in mice) [39–41]. In the flat disk of non-rodent embryos, the epiblast contacts with the VE (hypoblast), and the ExE is absent. In basal rodents of the suborder Hystricognathi such as the guinea pig (*Cavia porcellus*) and the vizcacha (*Lagostomus maximus*), the ExE persists in the flat-disk embryo, but it remains far apart from the epiblast at the time of PGC induction [42, 43]. Moreover, murine PGC nest in the growing mesodermal allantois in the proximal/posterior region of the embryo is a precocious structure found in the mouse, but not seen in nonrodent mammals. These differences have a critical effect on PGC specification factors [4].

3.1. Human

Due to ethical and technical reasons, there is limited information on the origin of human PGCs in postimplantation embryos. PGCs have been described in human embryos at early somite stage in the dorsal wall of the yolk sac near the developing allantois [44–46]. Decades later, AP activity in presumably PGCs was observed by several groups in human embryos with 5–8 somites at a similar location. Using single cell analysis, human PGCs isolated at 4 weeks of development seem to express *PRDM14* and *TFAP2C*, whereas *BLIMP1* and *FRAGILIS* are not expressed [48], suggesting that the critical molecular network in mouse and human early PGCs is divergent. A recent report showed that *BLIMP1* is activated in human PGC-like (hPGCL) cells after specification by *SOX17*, and it is suggested that its role is to inhibit the potential for somatic differentiation [47, 48]. SOX17 is the earliest marker

of hPGCL cells and it is in fact the key regulator of their fate, which is not the case in mice. *BLIMP1* is downstream of *SOX17*, and it represses endodermal and other somatic genes. Furthermore, hPGCL cells arise from precursors expressing high levels of *T* and low levels of *SOX2*, resembling posterior primitive streak-derived progenitors [32]. This suggests that human germ cell precursors may arise from a population of posterior primitive streak-derived cells that activate *BLIMP1* in response to paracrine signals, a process that occurs during postgastrulation (later than mice) [34]. The precise combination of signals that promote germ line segregation in humans is currently unknown; however, recent studies in emerging models as cynomolgus macaque, together with *in vitro* studies in human PGC-like cells derived from induced pluripotent stem cells, reveal a different molecular pathway from that evolved in mouse [46].

3.2. Rabbit

In pregastrulation rabbit embryos, *BMP2* is first expressed from the hypoblast and yolk sac epithelium at the boundary of the embryonic disk, which is equivalent to the proximal VE and extraembryonic VE in mice, respectively. In turn, rabbit *BMP4* expression is significantly delayed compared to the mouse. BMP4 marker is first detected during primitive streak formation and it is expressed peripherally in intraembryonic hypoblast and epiblast and in the mesoderm at the posterior pole of the embryonic disk. Interestingly, BLIMP1+ single PGC precursors are detected before primitive streak formation and *BLIMP1* mRNA distribution closely follows the expression pattern of BMP2. Thus, it is proposed that BMP2 may play a more essential role in rabbit PGC specification than BMP4 [49].

On the other hand, PG-2 (a germ cell epitope) and *BLIMP1*-expressing cells have been localized at early gastrulation stage in a region identified in the posterior upper layer (epiblast) and mesoderm [50]. However, *BLIMP1* shows a wider expression pattern during these developmental stages, with positive cells in the hypoblast all around the circumference of the embryo, adjacent to the site of *BMP4* expression in the extraembryonic cells surrounding the embryo. Nevertheless, from these 'blimped' pPGCs, only the posterior ones seem to become PG-2-positive [50].

3.3. Plains vizcacha

A recent study in the basal Hystricognathi rodent *Lagostomus maximus*, which develops through a flat-disk epiblast far apart from the ExE (**Figure 1**), showed that OCT4 protein seems to play an essential role in the establishment and maintenance of the germ line [43]. *OCT4* expression in the pregastrulating embryo was observed across all the epiblast cells, but after the primitive streak stage, *OCT4* was mostly downregulated, and its expression only persisted in a group of cells that was later restricted to the mesoderm of the posterior end of the embryo. It seems likely that *OCT4* expression is required for maintaining pluripotency, helping to epigenetically reprogram cells for PGC development that will be specified at a later stage, probably suppressing expression of genes involved in mesodermal specification [43]. In this model, *BLIMP1* expression has not been detected during early gastrulation

or later stages of development (migration and colonization of the genital ridges). It seems likely that *BLIMP1* would not be necessary for the specification of the germ line in the basal rodent *L. maximus*.

In an advanced stage of development, at neural plate stage, in the base of the allantois in the ectoderm and mesoderm after gastrulation, $OCT4^+$ cells become restricted in number to a group of 6–8 cells, and they begin to express *SOX17, STELLA* and *FRAGILIS*. The temporal colocalization of SOX17 and OCT4 proteins in *L. maximus* seems to play a major role in inhibiting somatic genes and maintaining pluripotency instead of the mouse alternative SOX2/OCT4 [51]. During migration through the gut, *SOX17* is downregulated, and its expression is restored in the oogonia after the colonization of the genital ridges.

Then, in the early- and late-head fold stages in mesoderm and endoderm tissues, the expression of *OCT4* and *SOX17* continues but *FRAGILIS* and *STELLA* are downregulated and turned on again during migration. Another notable protein, the germ line marker VASA, was observed early during the translocation of $OCT4^+$ cells to the hindgut. Thereafter, VASA-expressing cells were detected throughout the migration toward the genital ridges. $OCT4^+$/$VASA^+$ cells sequentially turned on *STELLA* and *FRAGILIS* during migration. Leopardo and Vitullo [43] suggested that *OCT4*/*STELLA*/*FRAGILIS*-expressing cells are finally restricted and specified to form PGCs during migration when the evolutionarily conserved germ line marker VASA is expressed.

The spatiotemporal pattern of expression of germ line markers found in *L. maximus* diverges from the currently accepted model on the origin of PGCs as a lineage-restricted cluster of cells in the base of the allantois, specified early just before, or during, gastrulation. In contrast, in this rodent, specification of germ cells seems to occur during migration of a stem cell pool derived from a pluripotent progenitor population within the embryonic axis as proposed by Mikedis and Downs as an alternative pattern of the classical mouse model [5].

4. The assembly of the mammalian ovary after PGC colonization

4.1. Germ cell proliferation in the fetal and postnatal ovary

The number of PGCs that colonize the genital ridges depends on the species. In mice, beginning with 100–145 PGCs at 8 days postconception (dpc), the number increases exponentially up to 15,000–20,000 oogonia per ovary at 15.5 dpc, the time of entry into meiosis and cessation of mitosis [33, 52–56]. A similar pattern of germ cell proliferation was described in rats [57]. In the basal rodent *L. maximus*, approximately 1000 PGCs are detected by the end of migration, rapidly increasing to 55,000 oogonia once fetal ovary colonization is finished; the number continues to increase to reach more than 3×10^6 germ cells by the end of gestation [43, 58]. The limited human data suggest that 1000–2000 colonizing PGCs reach a maximum of approximately $5–6\times10^6$ germ cells per ovary at 20 weeks of gestation [59–62].

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After a few rounds of mitosis, colonizing PGCs, now referred to as oogonia, cease proliferation and enter a premeiotic phase, with downregulation of pluripotency-associated genes such as *Oct4* and *Lin28* and upregulation of meiotic genes such as *Scp3* [63, 64]. Oogonia entering meiosis, now called oocytes, undergo prophase of the first meiotic division. Just before or early after birth, depending on the species, oocytes in diplotene stage of meiotic prophase I enter a quiescent state known as dictyate, in which they remain arrested, sometimes for years or decades, until just before ovulation [65, 66].

In mice, entry into meiosis seems to be a synchronized event, with no overlapping between mitosis and meiosis. By 17 dpc, mitotic proliferation is finished and all germ cells initiate meiosis [65] entering meiotic prophase in a wave from the anterior to the posterior end of the ovary [64]. However, there is a marked asynchrony of germ cell development in the human ovary. The onset of meiosis occurs by week 11 of gestation [55], but mitosis continues in more peripherally located germ cells for many weeks thereafter, even when primordial follicles begin to form [64, 67]. In the rat, non overlapping mitosis and meiosis of germ cells occurs as in the mouse [68]. However, the basal rodent *L. maximus* shows asynchrony and overlapping of mitotic and meiotic phases of germ cells in a comparable way as humans [58].

The persistence of PGCs or oogonia in the postnatal ovary has been a matter of discussion throughout the twentieth century since Pearl and Schoppe [69] proposed, in 1921, that postnatal oogenesis might occur in the mammalian adult ovary. Three decades later, in an extensive review of the literature of the time, Zuckerman [70] advocated for the absence of oocyte renewal in the mature mammalian ovary, proposing that mammals are born with a finite nonrenewable oocyte pool, a perspective that was widely accepted for more than 50 years generating a useful framework in advancing our knowledge of ovarian dynamics in placental mammals. Nevertheless, this long-held dogma was challenged in 2004 by Tilly's team [71] with the description of a small population of germ line stem cells in the adult ovary of the laboratory mouse. This observation refueled the possibility that neo-oogenesis could take place in the adult ovary of mammals and evidence for and against this possibility has accumulated over the recent years [72]. Although it has not been proved yet that ovarian stem cells may contribute to replenishment of the adult ovary if needed, the persistence of germ line stem cells has been independently proven in the human, mouse and rat models, as well as their ability to be manipulated *in vitro*, and to give rise to offspring following transplantation [73–75].

4.2. Germ cell death in the fetal and postnatal ovary

Death is a prominent feature of mammalian germ line development, with a predictable temporal and spatial pattern. In fetal life, direct germ cell depletion occurs by means of a constitutive massive germ cell death program, referred to as attrition [59, 60, 76–79]. In adult life, germ cell demise is mainly the result of death of the supporting follicular cells, a process known as follicular atresia [64, 76–78].The main mechanism underlying germ cell attrition and follicular atresia requires the activation of a conserved intracellular program of cell death called apoptosis. The execution of the apoptotic program depends on the coordinated

action of a group of genes that will activate as a signaling cascade in response to different stimuli. Depending on the source and type of the stimuli, apoptosis can be initiated through an extrinsic pathway, also referred to as the death receptor pathway, which includes the recognition of death ligands to their cell surface receptors [80] or the intrinsic or mitochondrial pathway, which is mainly regulated through the BCL2 protein family whose members are divided into three groups: proapoptotic proteins, antiapoptotic (or prosurvival) proteins and pore-forming proteins [81]. Extrinsic apoptosis molecules are mainly involved in final follicular regression and atresia and corpus luteum regression [82, 83]. *BCL2* gene family executing the intrinsic apoptosis path plays an essential role in the death of the germ cell proper in the antenatal ovary and of granulosa cells during follicular atresia in the adult ovary [84].

The analysis of the spatial and temporal expression of members belonging to the *BCL2* gene family in the mammalian ovary showed that, in general, the expression of proapoptotic genes is continuous throughout prenatal oogenesis, whereas antiapoptotic members are expressed in a time-restricted pattern associated mainly to differentiation and proliferation of the germ cell [85–90]. The enhanced expression of proapoptotic genes such as *BAX* in the face of antiapoptotic members like *BCL2* gives support to the high rate of apoptosis characterizing the mammalian ovary. The involvement of this biased gene balance in determining death or survival of the germ cell has been experimentally supported by showing that *Bcl2*- and *Bax*-knockout mice have decreased or increased primordial follicle reserve, respectively [91, 92].

The causes that determine massive constitutive death of mammalian female germ cells are poorly understood. This massive elimination may avoid the persistence in the ovary of germ cells exhibiting nuclear or mitochondrial chromosomal/genetic defects [93]. Alternatively, death may relate to the exhaustion of germ cells acting as nurse cells to the surviving oocyte pool [94]. Finally, it has been suggested that massive death may enable the appropriate association between germ cells and pregranulosa cells during ovigerous cords or ovarian cyst breakdown, just before primordial follicles begin to form [95]. In any case, the balance between germ cell death and survival seems to be critical to preclude ovarian dysgenesis or premature ovarian failure and to ensure reproductive success.

Germ cell elimination occurs at different points of fetal development. There are three main waves of germ cell death: (i) at prophase and metaphase of proliferating oogonia, (ii) at pachytene of meiotic prophase I oocytes and (iii) at diplotene of meiotic prophase I oocytes [57, 59, 96]. The vast majority of germ cell death occurs during the second and the third waves. Thus, germ cells entering meiosis are particularly susceptible to cell death [55, 60].

In mice, the maximum number of germ cells is registered at the time of entry of primary oocytes into meiotic prophase. However, up to two-thirds of the germ cells are lost before the ovarian reserve is established just after birth [64, 97, 98]. In rats, germ cells proliferate to reach a peak of 64,000 oogonia at 17.5 dpc, but the number of oocytes falls down to about 39,000 at birth and 19,000 at 2 dpc [57, 96]. Humans display a similar dynamics of germ-cell

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elimination. After the germ cell peak number of 5–6×10^6 oocytes that occurs at 5 months postconception, there is a dramatic decline in germ cell numbers similar to that seen in mice and rats. By the time of birth, the number of germ cells drops dramatically to 1–2×10^6 [59, 61, 64, 96] (**Figure 2**).

Moreover, the process of germ-cell apoptosis continues during postnatal life through follicular atresia. In humans, only 300,000 oocytes survive at 7 years postpartum and fewer than 1000 are present in the years just prior to menopause [59, 61, 96].

4.3. Is massive female germ cell demise a constitutive trait for all mammalian species?

Once PGCs have colonized the fetal gonad, the final endowment that will constitute the oocyte reserve seems to depend largely on the balance between cell proliferation and death. Based on the results of germ cell death displayed by mouse, rat and human, it has been widely accepted that massive intraovarian elimination of germ cells is a constitutive attribute of mammalian ovary for the final establishment of the germinal reserve. After a period of high proliferation of colonizing PGCs to reach the maximal oocyte endowment of the species, the activation of the apoptotic pathway generates a point of inflection in the growth curve of the oocyte population that eliminates from 60 to 85% of newly formed oocytes depending on the species [54, 59, 97] (**Figure 2**). The comparable pattern following the elimination of germ cells quantified in mouse, rat and human, together with the recognition that

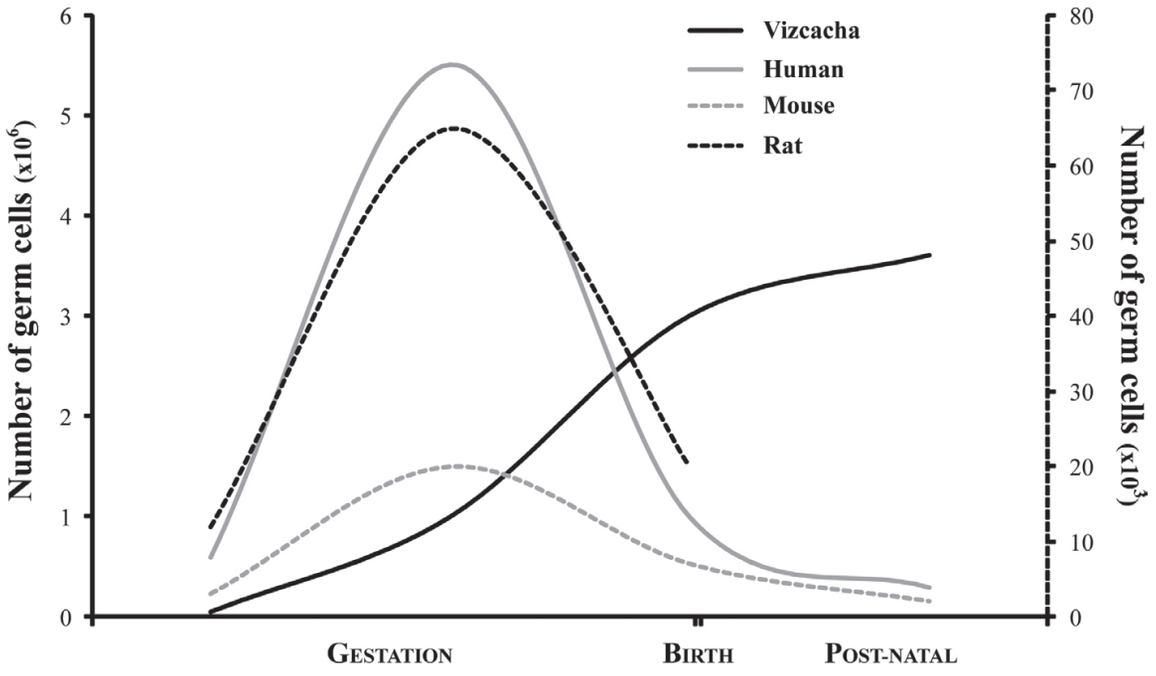

Figure 2. Germ cell growth curves in mammalian species. Human, mouse and rat share the same growth pattern with maximum germ cell endowment at approximately mid-gestation followed by a massive decline through intraovarian cell death. On the contrary, the vizcacha shows a continuous increase of germ cell population, unaffected by cell death.

apoptosis in fetal ovary is active in a few other mammals, proved sufficient to establish massive elimination as a general rule controlling the final oocyte endowment of the ovary in placental mammals.

Challenging this established rule, a quantitative estimate based on unbiased stereological methods showed that the mean germ cell number per ovary increases continuously from the early-developing fetal ovary up to 45–60 days after birth in the South American plains vizcacha, *L. maximus* [58] (**Figure 2**). Female vizcacha displays a constitutive ovary-specific overexpression of the antiapoptotic *BCL2* gene and low to absent expression of proapoptotic *BAX* gene that leads to a strong suppression of apoptosis-dependent germ cell attrition throughout fetal development [99] and apoptosis-dependent follicular atresia throughout adult life [100]. The detection of germ cells undergoing last steps of apoptosis revealed by TUNEL assay never surpasses 4% of the entire germinal population. Hence, the healthy germ cell population increases continuously from early-developing ovary reaching a 50 times higher population number by the end of gestation. Beginning with an endowment of around 56,000 oogonia at 50 dpc, total germ cell number grows up approximately to 3×10^6 by the end of gestation [58].

Whether the vizcacha is just the exception that confirms the rule or it represents another strategy for establishing the germ cell endowment in mammals, we will have to wait for quantitative studies in a more representative number of placental mammals. Until then, the vizcacha is the first mammal so far described in which female germ line develops in the absence of constitutive massive germ cell elimination since the balance between pro- and anti-apoptotic *BCL2* genes is biased in favor of suppressing apoptosis.

5. Concluding remarks

Our current knowledge regarding the origin and specification of PGCs and the establishment of the ovarian reserve in placental mammals comes by and large from model organisms, notably the mouse. The mouse model has erected as the paradigm for germ line development; however, studies in a few other species unveil differences that challenge the mouse gene network as an established path that may apply to all mammals.

The molecular pathway disclosed for the mouse embryo in the last fifteen years still lacks a final proof showing that the presumptive PGCs, originating early in the proximal epiblast of the egg cylinder, are the same cells that finally colonize the genital ridge later on development. Until this could be traced, alternative hypothesis proposing that PGCs may specify just before colonization from a migrating pluripotent cell population when evolutionarily conserved genes begin to express cannot be ruled out.

The peculiar morphology of the early-implanted mouse embryo, the egg cylinder, sets aside from most mammals that develop through a flat disk embryo. Hence, it is reasonable to suppose that the topographical difference of the gastrulating flat embryo may create a different morphological

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scenario for signaling and specification of PGCs. The current knowledge in flat embryos, such as those of human and vizcacha, supports a divergent molecular path from that of mouse.

Once the fetal gonad has been colonized by PGCs, it is widely accepted that a balance between proliferation and cell death determines the final oocyte reserve. Massive germ cell death is regarded as an intrinsic shared mechanism in the mammalian ovary regulating the establishment of the final oocyte pool. Nevertheless, only four species have been quantified at the moment and one of these four shows a continuous growth of the germinal population with a minimum cell death. If this is an exception to a general rule or an alternative strategy for establishing the oocyte pool remains unanswered for now.

At this time, we are still far from having a comprehensive knowledge on the possible variety of mechanisms regulating the origin and specification of PGCs and the establishment of the final oocyte reserve in placental mammals. The few species investigated so far seem to indicate that strategies that remain hidden in the great diversity of mammals have not yet been revealed. Comparative studies from different mammalian orders are still lacking and needed.

Abbreviations

PGCs	primordial germ cells
ICM	inner cell mass
ExE	Extraembryonic ectoderm
VE	visceral endoderm
TE	trophoectoderm
ExM	extraembryonic mesoderm
ACD	allantoic core domain
hPGCL	human primordial germ cell-like
pPGC	preprimordial germ cell

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