OVOLUTION?

An overview on the mammalian ovarian reserve and the accuracy of our current conception

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Preface

My master in Biomedical Science started with a pilot study on ovarian material of perinatally smoke exposed mice. I counted oocytes in different stages of development and found that primordial follicles, being the least developed, have the tendency to reside in the epithelium of the ovary. During background studies I stumbled upon the possible existence of ovarian stem cells, being a source for primordial follicles. As I continued reading, I quickly found out that the controversy was too big to address shortly in my report in that study. However, during this thesis, more time is given to do literature study and decided to focus on the current facts and discussion on the (in)finite follicle pool. Developmental and stem cell biology, though being subjected to more than 100 years of devoted research, is still an area that surprises researchers regardless of the ever expanding possibilities biotechnology offers.

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Introduction

It is the general view of biologists and physicians that, in contrary to men, in women the pool of germ cells is finite. The central dogma is that oogenesis in humans, the making of new oocytes, only occurs before birth[1]. Throughout the female life, a decline in oocyte number eventually leads to depletion and fertile senescence[2]. The first to make this statement was the German anatomist Waldeyer in 1870. Sixty years later, Zuckerman and colleagues were interested in the ovary and spend 20 years exploring the previously conducted experiments and reported on new work. In 1950, Zuckerman summarized his work at a presentation at the Laurentian hormone conference, all validating the previously performed work of the German anatomist Waldeyer[3], further strengthened with his own observations. The debate was settled for decades and developed fertility treatment was based on the conception on a finite oocyte pool.

In general, age is an excellent predictor of the pregnancy potential in healthy women[4]. It is known that fecundity in both natural cycles and during fertility treatment with the help of gonadotropin stimulated ovarian cycles declines with maternal age, beginning in the late 20s and becoming more abrupt in the late 30s. This is observed in natural pregnancies based on population studies, and in IVF in clinics investigating patient data[4]. As women the last decades receive their first child at increased age[5], prolonged female fertility has received renewed interest.

The research group of Tilly published work in 2004 in which the finity of the oocyte pool was challenged again, pivotally supported by the presence of oocytes in ovaries of busalphan-treated mice[6]. This study received plenty attention, even from outside the scientific community[7]. Many journals received letters from critics, others performed experiments opposing Tilly's work. Also, new evidence was generated against the dogma, as human embryonic stem cells were found to give rise to primordial germ cells *in vitro*[8].

To understand the debate on presence of female oogonial stem cells (OSC) replenishing the ovarian reserve, this overview displays the experiments performed on ovaries and the dynamics in follicle numbers. A summary on the current knowledge on ovarian development from mammalian early life to maturity is given below. Subsequently, the experiments will be elaborated upon to discuss the possible existence of OSC.

Mammalian ovarian development

Union of a haploid germ cell of a male and female leads to the formation of a diploid cell that has the ability to develop into a unique individual, in which already during early development the gonads are formed. This is a prerequisite for reproduction during later life. Differentiation and migration of primordial germ cells (PGC) is coerced by the developing gonads in the embryo. Interestingly, the PGCs are identical in males and females and will give rise to the germ cells, the most important cells in reproduction. After fertilization, they eventually give rise to the totipotent zygote and transfer the required genetic information to form a new human being.

Early development: formation and migration of primordial germ cells

After formation, 2 weeks post-ovulation, the PGCs are formed and migrate through the human embryo until they reach the presumptive gonads. Here, the PCGs are nurtured by the surrounding somatic cells and coerce the PGCs towards either oogenesis or spermatogenesis[9]. The PGC initially progress by mitotic divisions in gestational week 10 and are connected by intracellular bridges, formed by incomplete cytokinesis[10]. By doing so, an excess of interconnected oogonia is produced in

the case of women. Mitotic divisions then cease and the germ cells enter meiosis I after 11-12 weeks of gestation, progressing through the first few stages of prophase I before arrest. The cells are now clustered and undergo 'breakdown' in gestational week 16, where most of the oocytes die through apoptosis[11]. The cells now present in the ovaries are primary oocytes. The development is summarized in figure 1.

It is speculated that the large breakdown of only recent formed oocytes might be a means of selection, as correct interaction with its surrounding is crucial for propagation from PGC to primary oocyte. It is observed that during formation of oocytes, a large percentage of abnormal female gametes is produced[12]. Therefore, the large breakdown in gestational week 16 is often regarded as quality check of the produced oocytes, increasing the overall health of the ovarian reserve[13].

The remainder of the oocytes becomes surrounded by a layer of somatic pre-granulosa cells. Oocytes and the granulosa cells communicate, which is necessary for growth and development of the follicle and oocyte after birth[13]. Though the unraveling of the complex regulation at transcriptional level only just started, it can be concluded that strong regulation of meiotic competence exists in order to achieve an oocyte reserve constituted of healthy follicles.

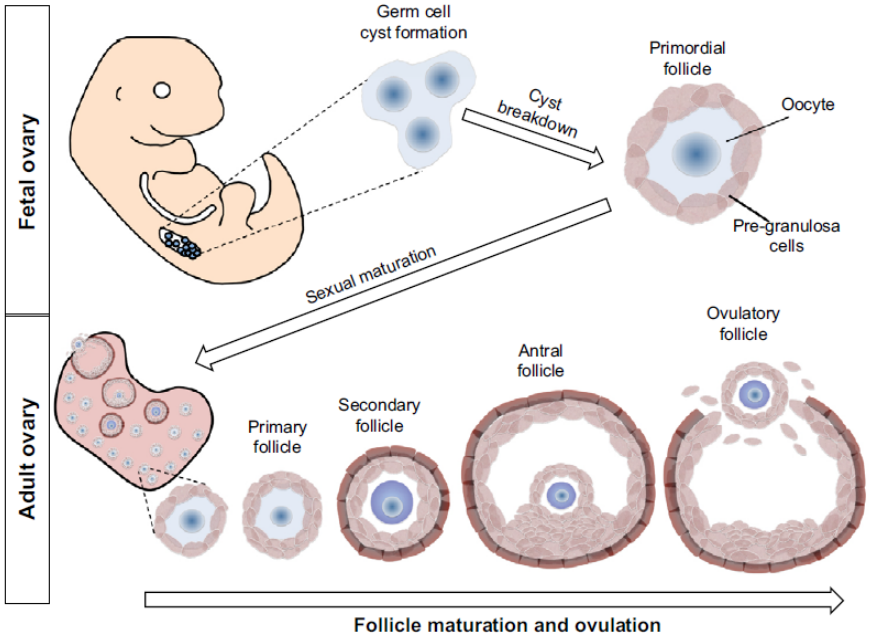

***Fig. 1**. Timeline of development of female germ cells in the fetal ovary and progression of primordial follicles to ovulation*[13].

The role of meiosis in females

The central characteristic of germ cell is haploidicity, enabling formation of a genetic recombination of two haploid parent cells to form a genetically unique individual. Haploid cells are formed when a cell divides meiotically, which is evolutionary highly conserved. All eukaryotes reproduce meiotically and correct execution of the meiotic program is crucial for reproduction. Approximately 50% of all miscarriages in humans is caused by nondisjunctions in the first phase of meiosis. Interestingly, in 90% of the cases of miscarriage, the error has arisen in the maternal germ cells[14].

Though the timing of meiosis during development is different between males and females, it occurs in both sexes in the primordial germ cells, summarized in figure 2. Meiosis consists of two rounds of cell division. In meiosis I the cell forms two haploid cells from one diploid cell, segregating homologous chromosomes. New combinations of genes are formed as recombination is possible before segregation. Meiosis II is mechanically similar to mitosis, apart from the result with respect to genetics, as it produces two haploid cells. During male differentiation the cells remain in mitotic arrest until just prior to puberty, when meiosis (and spermatogenesis) starts. This early commitment to mitosis determines the male germ cells to differentiation and thus start of spermatogenesis[15].

In females meiosis starts in early fetal development. Meiotic arrest takes place later in fetal development to allow for chromosomal recombination. The onset of puberty, indicated by a surge of luteinizing hormone, causes meiosis I to complete in oocytes of dominant antral follicles. Rearrest takes place in meiosis II when they are ovulated.

Another difference between males and females with respect to meiosis is the number of germ cells that are formed. In male germ cell formation, one germ cell can lead via meiosis to the formation of four sperm cells whereas in females only one oocyte is formed. The excess genetic information is discarded via polar bodies.

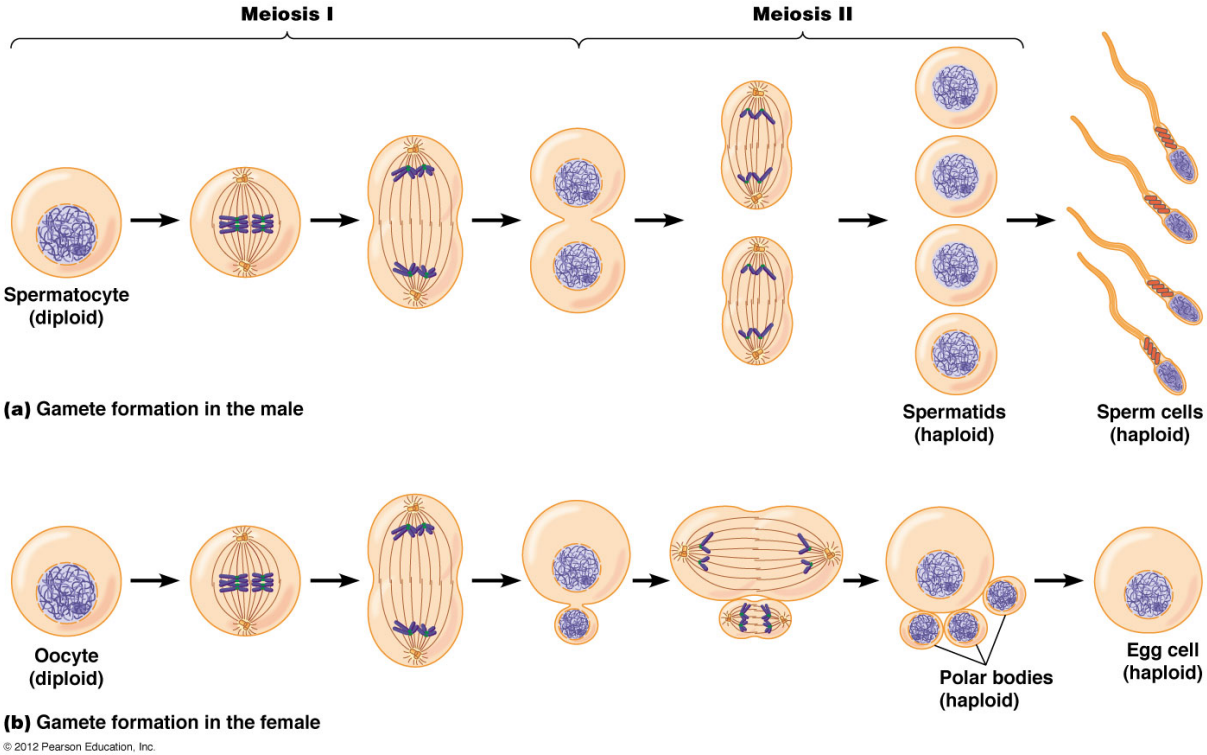

Fig. 2. *Meiosis in males and females. Note the difference in formation of 4 spermatids in males and 1 egg cell with 3 polar bodies in females (Pearson Education 2012).*

PGCs in human females enter meiosis in gestational week 11-12, and not mitosis as in males, and thereby commit to oogenesis. Often they stay connected by incomplete cytokinesis, forming clusters of immature oocytes. After DNA recombination, they arrest in the diplotene stage at around

gestational week 15. One week later, most of the oocytes are lost via caspase-2 dependent apoptosis[16].

Also many other species, such as xenopus and mice, know the interconnection of germ cells to facilitate exchange of organelles and RNA. However, these connected germ cells do not undergo massive breakdown, leaving cysts of oocytes that can develop into oocytes during later life. Therefore, also females of these species can remain fertile during their mature life, unlike human females. The mouse is often used as a model for human fertility, as they are thought to similarly have an established reserve of oocytes. In fig. 3 a comparison in development of oocytes in human and mouse is given.

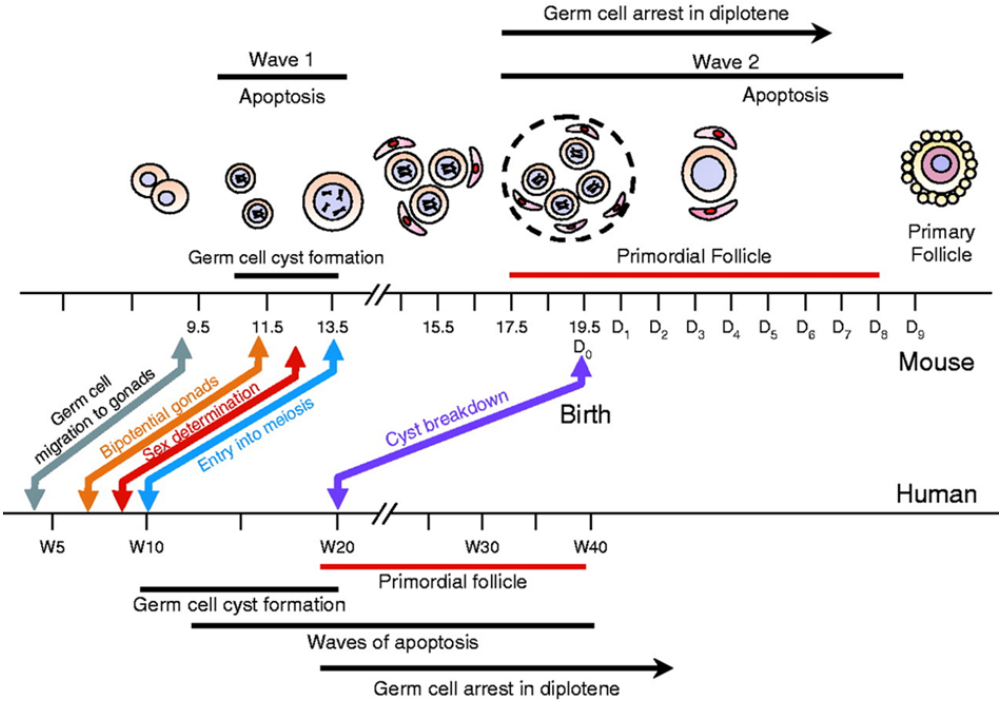

Fig. 3. *Comparison between mouse (top) and human (bottom) oocyte development.*

Ovulation and ovarian ageing

In the developing ovary, PGCs arrest at meiosis in gestational week 10, awaiting signals for further development. During sexual maturation, primordial follicles can mature into the primary and secondary follicle stages until the antral follicle during infant years (see fig. 1). This is called initial recruitment. After puberty, matured dominant follicles are activated by a peak in luteinizing hormone occurring once a month, therefore called cyclic recruitment. The LH peak results in breakdown of the germinal vesicle, nuclear maturation and completion of the first meiotic division with extrusion of the first polar body. These oocytes then re-arrest in metaphase II of meiosis II and are ovulated.

As the chronological age of the woman increases, the ovarian reserve decreases, known as ovarian ageing[17]. It is remarkable how this ageing differs from the ageing of other organs, as its function changes at the fifth decade of the female life span. The immature, fully colonized ovary is estimated to contain 6-7x10^6 oocytes at gestational week 16, of which only 2-3x10^5 primordial follicles present at the onset of puberty. In between puberty and menopause, only +- 400 oocytes will ever reach

maturity between puberty and menopause, as a result of ovulation. The oocytes are not only lost during ovulatory menstrual cycles but also pivotally via apoptosis and also during pregnancy, breastfeeding or during use of contraceptives. The rate of apoptosis increases in the last 10-15 years before menopause, which starts when there are approximately 1000 remaining oocytes.

Multiple clinical tests are developed to estimate the female ovarian reserve particularly in cases of IVF treatment. There tests are based on hormone levels such as anti-Mullerian hormone AMH, which is only high during the fertile period of the female life span. Furthermore, it can be measured in blood serum regardless of phase of menstrual cycle. Another assessment of female overian reserve is measurement of FSH serum concentration, which can be performed during day 2 or 3 of menses. It is used for the estimation of oocyte yield after ovum pick up in women undergoing IVF treatment. Although age remains the most important predictor of ovarian ageing, recent studies suggest that antral follicle count potentially reflects ovarian biological age more precise[18].

Eventually, all women will reach reproductive senescence in the fifth decade of their life. The function of this is speculated upon, as it is not a common phenomenon in the kingdom of animals, where most females remain fertile throughout their life. When looking at other female individuals from species such as flies and fish, it is observed that such a depletion does not take place. Here, individuals contain stem cells replenishing their oocyte reserve[13].

Considering that the embryonic origin of germ cells is identical in both sexes, it might be surprising that in contrary to women, men do not lose their fertility over age. The PGCs in males differentiate into spermatogonia: stem cells located in the basal lamina of the seminiferous tubules of the testes. Though their numbers are low, they continue to proliferate and differentiate into sperm throughout the mature male life. A protein that is present on germ cells in both sexes, at the connecting part between two dividing cells, is testis-expressed protein 14 (TEX14). In males, this protein is essential for fertility, whereas TEX14-null females only have reduced litter size and possess fewer oocytes. Although there may be reasons to doubt it, the current conception is that female germ cells lose their capacity to proliferate after birth.

A decade of research: a challenge for the dogma?

It is generally agreed upon that the regulation of primordial follicle assembly during the fetal and neonatal periods distinctly determines the long-term reproductive capacity of female mammals. Although the development from germ cell to depleted ovary as depicted above may seem straightforward and the restriction of fertile life span of women is well-documented, the limitation of female fertility has been questioned by multiple researchers. A research group from Boston has reopened the debate by publishing a study on presence of oocytes after gonadoxotic treatment[19]. As this contradicts the long-held believe of many developmental researchers and clinicians, this evoked multiple experiments on the dynamics of the ovary, keeping the discussion going for more than a decade at this moment. In this thesis I aim to give a comprehensive overview of the results of experiments performed on the ovarian oocyte reserve and the conclusions based on these results.

Experiments against the finite oocyte pool

In 2004, a research group form Boston Medical School published a study suggesting presence of mitotically active germ cells supporting replenishment of the ovarian follicle pool, based on several experiments[6]. As these results were perpendicular to current fundamental reproductive biology as well as to clinical practice in infertility treatment, attention was drawn to the research field and others focused their scope on the possibility of existence of the female germline stem cell.

The study from Tilly's research group entailed multiple experiments[6]. Firstly, it compared healthy (non-atretic) and degenerating (atretic) follicles of mice to assess the line of decrease of follicle numbers over time. The rate of atresia measured indicated a depletion long before the fifth decade of the human female life, indicating the need for post-natal renewal to support fertility. However, several months later, Tilly admitted that these calculations were based on a misconception. Regardless of this, further histological analysis revealed presence of ovoid-like structures close to the ovarian surface epithelium. Presence of Mouse Vasa Homologue (MVH) in these structures was assessed as it is known to be exclusively expressed in germ cells. Furthermore, injection with 5-bromodeoxyuridine (BrdU) 1h prior to ovary harvesting demonstrated incorporation of BrdU is MVH positive cells, suggesting proliferative activity.

A second line of evidence is formed by expression of three markers required for the initiation of meiosis (zygotene and pachytene stages). Furthermore, to asses fertility, female mice were injected with busalphan, a treatment known in male mice to target germ stem cells and spermatogonia, avoiding post-meiotic cells. In i*n utero* exposed female mice this treatment also selectively targets pre-meoiotic cells. However, the mice in this study all displayed presence of healthy maturing follicles with non-degenerative oocytes. This confirms, according to Tilly[6], the concept that proliferative germ cells not only exist in the postnatal ovary, but also are required for continuous follicle renewal.

To further support these findings, a grafting experiment was excluded in the same study[6]. Ubiquitously green fluorescent protein (GFP) expressing mice were used as a host after removal of 50% of the ovary. These mice received ovaries from wild-type mice, which were collected again 3-4 weeks after grafting for analysis of GFP expression. The grafted fragments contained follicle-enclosed GFP positive oocytes, similar to the oocytes found in the ovaries from transgenic mice. When these follicles developed, the surrounding granulosa cells were GFP-negative, indicating their origin from the wild-type mouse. In this study, this is explained as stem-cell migration to their natural niches after introduction to a host.

Though some suggestions were made, Tilly's study was not conclusive on the location of the contentious female germline stem cells[6]. This was addressed in a successive study, also from the group of Tilly et al., proposing the bone marrow (BM) as a source for germ cells[20]. The foundation of this line of reasoning is that BM and primordial germ cells (PGC) share their embryological origin. Furthermore, previous studies have shown that PGC can differentiate into hematopoietic stem cells[21], known for their multiple lineage potential. Again, multiple experiments were performed to test this hypothesis, discussed below.

Mice ovaries were immunohistologically examined for expression of stage-specific embryonic antigen-1 (SSEA-1), which is, amongst others, found on PGCs. Some clusters of SSEA-1 positive cells were found in the medulla, but their numbers were considered too low to represent PGCs. As their origin may be found elsewhere, bone marrow was examined and appeared to express several germ line specific markers. Of these markers, Mvh expression was assessed quantitatively using PCR to assess potential changes in expression in bone marrow, during the female reproductive cycle. This was found to be strongly correlated, indicating a connection between bone marrow and the female ovary[20].

To test this finding *in vivo,* female mice were treated with cyclophosphamide and busalphan to deplete the oocyte pool, both pre- and postmeiotic. Subsequently these mice received BM from adult wild-type mice after which their ovaries were collected. Histological analysis accompanied by germ-cell and oocyte-specific markers showed that mice receiving BM after pretreatment had more oocytes than the mice only receiving pretreatment. These oocytes were found to be in all stages of development and remained present even after 11 months post-transplantation.

Another mouse model was used to confirm this finding. A mutant mouse, known for their lack of ability of execution of meiosis received BM transplantation, inducing fertility[20]. It was thus assumed that BM must contain female GSCs. Peripheral blood cell transplantation (PBCT) therefore should be sufficient to achieve oocyte renewal. This was executed by a blood transfer to wild-type mice from GFP expressing mice driven by the Oct-4 promoter, which is known to be exclusively expressed in germ cells. GFP-positive oocyte were found already 28-30h later in the ovaries of the recipient mice. Though this study did not demonstrate fertilizability of these oocytes, Tilly argues that oocytes do have more purposes than procreation as also propagation to the next estres cycle is achieved by oocytes[20].

In a study conducted in 2009, life offspring was produced from a female germline stem cell (FGSC) line derived from neonatal ovaries in that study[22]. Mice, injected with BrdU, were immunostained for Mvh and BrdU, to identify germ cells and proliferation, respectively. Double staining indicated presence of FGSCs and these cells were isolated by the use of magnetic bead sorting. The selected cells were passaged on for 15 months, without displaying change in morphology compared to the freshly isolated cells indicating self-renewal capacity. Characterization showed expression of Oct4, Mvh and other factors associated with stem and germ cells, including high telomerase activity and normal karyotype.

This FGSC line was further studied by induction of GFP using a retrovirus and subsequent transplantation in sterilized recipient female mice. Sterilization was performed by pre-treatment with cyclophosphamide and busulphan as performed in the grafting study of Tilly et al.[22]. Two months after transplantation, ovaries were harvested and presence of GFP-positive oocytes was observed. Furthermore, fertility after transplantation was assessed by natural mating with a wild-type male. Around 25% of the F1 progeny was GFP transgene, indicating their origin of the FGSC line. These results indicate that the FGSC cell line can not only be used to produce offspring, but also to generate transgenic progeny[22].

In addition to previous studies performed in mouse, Tilly reported on a study in human ovarian cortical tissue[23]. Mitotically active germ cells, with a gene expression profile consistent with primitive germ cells were isolated using fluorescence-activated cell sorting. Spontaneously *in vitro* generated oocytes subsequently were engineered to express GFP and placed into human ovarian cortical biopsies. GFP positive oocytes were formed 1-2 weeks after xenotransplantation in immunodeficient female mice. Hereby Tilly concludes that mitotically active germ cells can be obtained from ovaries form reproductive-age women and furthermore, that they can generate oocytes both *in vitro* and *in vivo*[23]. It should be noted that a conflict of financial interest was reported.

Experiments in response

The unexpected suggestion that the mammalian ovary possesses regenerative capacity, created speculations on a broadened therapeutic horizon for women diagnosed with premature menopause and chemotherapy-induced sterility due to oocyte loss. However, as it is perpendicular to the current concept on follicle dynamics, these findings were refuted by multiple researchers, based on the experiments described below.

In 2006, a research group responded with experiments performed in parabiotic mice[24]. Pairs of wild-type and GFP-expressing under the β-actin promoter were created and remained joined for 6-8 months to ensure the establishment of a joined circulatory system. Mice were superovulated and metaphase II oocytes were collected from the oviduct of each partner and analyzed for GFP expression: no chimaerism of ooctyes in these mice was observed. Occasionally observed GFP positive cells were found in association with the ovulated oocytes, later on confirmed to be pan-haemotopoietic marker $CD45^+$, thus circulating blood cells. It was therefore concluded that, although blood cells can enter the ovary and associate with oocytes, their haematopoietic fate is remained.

It may be possible that an insult would 'activate' the BM derived cells to form oocytes. Therefore, again a non-transgenic and transgenic mouse were paired, the non-transgenic one day after pretreatment with cyclophosphamide and busalphan[24]. After superovulation, the mice were euthanized and analyzed for chimerical oocytes. Though a shared blood stream was observed by both GFP positive and negative BM cells, this was not reflected in the ovaries: ovulated oocytes from both treated and untreated mice all possessed the phenotype of the host animal. Thorough analysis revealed that the used chemoablative therapy does not eliminate all oocytes, as the numbers of oocytes ovulated remain similar between mice from each pair. Incomplete depletion was also apparent upon histological analysis.

The research group of Honda et. al. aimed, in response to the 2004 and 2005 studies[6,20]., to isolate and characterize putative oocyte stem cells as a cell line[25], as was performed by Zou et al[22]. During the first passages after isolation, it appeared that oocytes were growing on the colony surface. Examination using Mvh and BrdU double staining indicated presence of pre-existing post-meiotic oocytes only. Cells that persisted in culture appeared to be thecal cells, thus by serendipity a thecal cell line was discovered. As the interaction between germ cells, granulosa cells and thecal cells is only poorly understood, this offers a new strategy for future research.[25]. This study was not conclusive on the existence of female germ line stem cells, but the results of Zou et al[22] could not be reproduced.

In addition to the mouse studies described above, Byskov et al. performed a study on human ovaries, 59 from 5 weeks post conception (wpc) to 2 years wpc, and 23 ovaries from 2 to 32 years[26]. Measurement of expression of PGC and oogonia specific markers was performed. No cells in post-natal human ovaries could be recognized as oogonia, either by histological or immunohistological analysis. It was found that a resemblance exists between humans and other species in the formation of clusters: in the neonate's ovary, up to 2 years old, large numbers of oogonia-like cells were found to be positive for the tested markers. Fruit flies and fish, having continuous follicle renewal throughout the mature life, display comparable clusters. However, they do not match the location of the suggested *de novo* follicle renewal, as suggested previously[27]. Therefore, this study resulted in no evidence for the presence of oogonia in the human ovary after their final clearance during the first two years of life[26]. This parallel can also be drawn from a more molecular perspective. Especially mice and fruit flies have been studied extensively the past 15 years, all suggesting a conserved mechanism in genetic and hormonal regulation in oocytes. It is known that many other factors play their part but the exact functions and interactions are yet unknown[13].

A study performed on human material[23], reporting on mitotically active germ cells isolated from reproductive-age women, was reproduced by Jones et al[28]. The provided protocol lead to the isolation of presumed oogonial stem cells. After analysis of these cells, multiple questions on the characteristics were raised. The sorting method used relies on a membrane-bound marker, Ddx4+. The marker used for isolation however was previously reported to be cytoplasmic. When isolated, expression of this marker could not be confirmed. In culture, some pre-meoitic markers were observed, but never the oocyte-specific markers. Moreover, immortality was not observed, as cells died after several months in culture. Therefore, this study suggests that pre-meiotic markers may be induced by culture. Although the nature of the isolated cells was not confirmed, the possibility that these were oogonial stem cells was excluded[28].

Discussion

Although the aim of the experiments mentioned above is to elucidate the origin of ovarian reserve throughout the life span, it remains difficult to reach an unambiguous conclusion on this topic. Some persuasive arguments are found in both of the opposing groups. The initial study, starting the discussing, showed results grafting GFP-expressing ovarian material after partial removal of the host's ovary[6]. GFP-expressing oocytes were found in the host's remaining part of the ovary, indicating potential post-partum germ cell proliferation. The origin of these cells is in subsequent studies suggested to be bone marrow[20]. However, no further justification for this in subsequent studies is found, from neither Tilly nor other groups. Opposing findings are summarized in table 1.

The parabiotic study was an extraordinary one and enabled studying the isolated effect of whole blood on the ovarian reserve[24]. It should be noted that this type of experimentation, joining pairs of animals, is no longer allowed by ethical committees. Therefore, an extra careful examination is needed, as repetition for validation is not possible. With this experiment the possibility of contribution of the bone marrow to the ovarian reserve was examined. This hypothesis was supported by Mvh expression found in the bone marrow having synchronized expression levels with the oestrus cycle. However, no results indicating contribution from circulating blood cells to the formation of mature oocytes was found, also not after inducing damage using cyclophosphamide and busalphan. The bone marrow derived cells that were found in the ovary resembled blood leukocytes, which might explain the suggestion of bone marrow. After the parabiotic study, no other responses were made on the bone marrow as potential source of female germ line stem cells, also not by the research group of Tilly, initiating the hypothesis.

Chemotherapy (Cy/Bu administration) did substantially decrease the number of ovulated oocytes, but did not diminish the reserve[24],[29]. In the mouse strain used by Tilly[6] it is known that Cy/Bu treatment depletes 75% of the oocyte pool, after 3-4 days of treatment. The Cy/Bu treated mouse in the parabiotic mice did not significantly differ from the unpaired mice that were Cy/Bu exposed, indicating no healing effect of factors from the non-treated paired mouse. As radiation does induce complete sterility[30], this might offer a more convincing model for the grafting study.

Limited: no renewing after birth	Renewable, continuous throughout life
Parabiotic: no chimearism after joined blood stream	Comparison atresia and non-atretic follicles
Study of human material in different stages of life: no proliferation 2 years after birth	Stem cell protein expression in ovary
Characterization of cell sorting based on markers did not confirm existence of germ stem cells	Chimearism after GFP-expressing graft
	Blood transplantation
	Germline stem cell isolation
	GFP-positive progeny after mating
	Isolation of mitotically active germ cells from human females

Table 1. *In this table, the previously discussed studies are opposed in their opinion on the presence of post-natal female oocyte production.*

Using mouse models is a common strategy to overcome ethical objections that do apply on human fetal tissue. However, the small size of fetal ovaries of mice renders high-throughput analysis a difficult task[31]. Also *in vitro* options could be considered to avoid ethical objections. As previously described, GFP-expressing female germ line stem cells were isolated from adult mice, and after transplantation in a WT mouse and mating with a WT mouse, GFP-expressing offspring was reported[22]. Although attempted, this study was not successfully repeated, but lead to the creation of a thecal cell line and reported on the presence of premature oocytes after isolation[25]. However, these oocytes did not survive culture. Furthermore, it is important to note that identification of an either natural or artificial ovarian stem cell does not entail that follicle renewal occurs to contribute to fertility.

The contribution of these potential cells to ovulation and fertility has to be tested before any conclusions can be drawn in this direction. However, it can be concluded that many of the complex interactions between PGCs and their surrounding remain unknown and therefore, *in vitro* production of human female germ cells can be considered futuristic.

Although the complexity of differentiation of primordial germ cells is often regarded as quality control, the majority of miscarriages is founded in the maternal line, indicating that many other factors influence in the success rate of a pregnancy. These factors, for example environmental toxins, metabolic disorders and autoimmunity, presumably reducing the oocyte pool, result in premature menopause[32]. Reduced quantity is also reflected in quality, as oocytes are reported to miss check points preventing incorrect execution of the meiotic program, critically influencing chromosome distribution and affecting fertility[33].

Assuming that ovarian stem cells contribute to replenish oocytes throughout life, the reason for menopause becomes unclear. Multiple theories prevail on the mechanism of ovarian ageing, being either in the ovary itself or as a result of deregulation in the neuroendocrinology. Cause and effect is hard to distinguish as both are influencing the same hormone system[32]. It is clear that depletion of the oocyte pool occurs in the ovary, resulting in a post-fertility life span that is uncommon in most species. Therefore, it is thought to be an evolutionary advantage of humans[34]. It is suggested that this post-fertility life-span is beneficial for the lifetime reproductive success of their offspring, reflected in earlier, more frequently and more successful breeding, as found by studying demographic records of pre-modern populations[35]. These fitness benefits reach until the reproductive output of their offspring declines, which is known as the “grandmother hypothesis.” Taking this into account, it appears to be undesirable to have ongoing oocyte replenishment as seen in fish and flies. Post-fertility lifespan should not decrease in humans as a result of activation of female germ line stem cells. However, these studies are only indicating benefits of our current conception and are not conclusive on the actual existence of female germ line stem cells.

Can we settle the debate?

The implications of the suggested paradigm shift have a large magnitude, reaching from fundamental biology to clinical applications. If the existence of female germ stem cells can be proven, science and the clinical treatment based on it will change drastically.

After more than a decade of renewed interest in the topic, still no unambiguous conclusion on the existence of oogonial stem cells is made. Most studies in favour of the existence used the mouse as a model. Many dissimilarities can be found especially with respect to the reproductive cycle in human and mouse females. The critics say that oogonial stem cells isolated *in vitro* are a culturing artefact, and do not contribute to oocyte replenishment in mature life. Tilly, igniting the debate, regards this as a matter of 'ovarian optimism or pessimism[19].' It appears that he find his study supportive for starting a company in offering fertility treatment in selected IVF clinics, based on his own research. According to others, this is too early as there are many uncertainties in his experiments. Being the one who ignited the debate, the low number of new supportive studies on the topic by Tilly is remarkable. For a research group in Copenhagen, the absence of evidence in human ovaries studied by them is enough to shift their interest to other topics[26].

Formation of an ultimate experiment is complicated: in what setup can we conclude on the existence of these cells? Experimentation *in vivo* with human oocytes is ethically very complex. Lineage tracing of implanted expected oogonial stem cells would render it impossible to test the possibility of fertilization, as it would result in genetically modified offspring. Therefore we rely on the test performed *in vivo* in mouse and only *ex vivo* in humans. Having these debates in science can lead to renewed insight, especially when supported by experiments. Anyone interested in female fertility is encouraged to interpret the studies performed on the existence of oogonial stem cells.

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