Anim. Reprod., v.12, n.1, p.46-51, Jan./Mar. 2015

Current status of *in vitro* differentiation of stem cells into gametes

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Abstract

From an evolutionary point of view, the gametes are the cells in the body that matter the most as they are the ones who transmit their genes to the next generation ensuring continuation of the species. Being able to generate mature oocytes *in vitro* is of great interest. Oocytes are the key to totipotency and are able to reprogram somatic cells in approximately one day. In addition, in contrast to a clump of pluripotent stem cells, once the developmental program has started, fertilized oocytes develop into a clump of cells with positional information and the possibility to differentiate into both the embryonic and the extraembryonic lineages that form a complete developing and viable organism. How to instruct pluripotent stem cells to become oocytes *in vitro* is still unclear and even though the first steps to obtain mouse oocytes have recently been successfully demonstrated, inducing meiosis progression and folliculogenesis *in vitro* are still far from being understood and have not yet been accomplished. In humans, the specific molecular niche that leads to correct oogenesis is less understood. Here, we discuss the current status of *in vitro* differentiation of human pluripotent stem cells into gametes, in particular to oocytes.

Keywords: differentiation, human, oocytes, pluripotency, stem cells.

The biology of the gametes

It is known that the human embryo implants around 10 days post-conception, develops an amniotic cavity and a yolk sac cavity and produces considerable amounts of extraembryonic mesoderm without having specified primordial germ cells (PGCs). In fact, in humans it is still unclear when and where the PGCs are specified, but PGCs have been observed by several scientists in the 50's-60's in human embryos around 24 days post conception in the posterior wall of the yolk sac, close to the allantois, using alkaline phosphatase activity as marker (Chuva de Sousa Lopes and Roelen, 2010). It is extremely difficult to investigate this issue in humans as during this period of pregnancy women themselves have not realized they are pregnant. Therefore, also the extrinsic inductive signals needed for PGC specification during human embryogenesis are unknown. By contrast, in the mouse, at least one extrinsic signaling pathway has been identified and extensively studied, the bone morphogenetic protein (BMP) signaling pathway. Without BMPs being produced by the extraembryonic tissues, both the extraembryonic ectoderm and the visceral endoderm, and the whole signaling cascade being in place in the mouse posterior epiblast, PGCs will not be specified (Lawson *et al*., 1999; Chuva de Sousa Lopes and Roelen, 2010).

Once specified, the PGCs enter a dynamic and complex developmental program that involves a strong component of epigenetic reprogramming while the cells proliferate and migrate towards the future gonadal ridges (Roelen and Lopes, 2008; Fig. 1). We can only assume that the developmental program during this migratory period is similar between mice and humans. However, major differences regarding such fundamental issues as PGC markers between mice and humans exist, reminding us that an extrapolation between mice and humans should be considered with great caution. For example, human PGCs do not express two of the most characteristic mouse PGC markers, SOX2 (Perrett *et al*., 2008) and SSEA1 (or FUT4; Chuva de Sousa Lopes, 2014, Leiden University Medical Center, The Netherlands, unpublished results). Moreover, from week 4 until week 10 post-conception (or week 6 - week 12 of gestation) both male and female post-migratory human PGCs do not express VASA (or DDX4), and interestingly, once the human PGCs start expressing VASA they completely downregulate OCT4 (or POU5F1) and NANOG expression (Anderson *et al*., 2007); whereas in mice, post-migratory PGCs co-express VASA, OCT4 and NANOG for some time.

In addition, the population of murine PGCs is rather homogeneous regarding their developmental behavior that is strongly determined by time. As an exception, in female mice meiosis entry has a clear spatial component occurring in a rostro-caudal or anterior-posterior wave (Menke *et al*., 2003; Bullejos and Koopman, 2004). In humans, the spatial component of germ cell development in females is dominant and during the second and third trimester of human development there is a pronounced developmental wave from the inner part of the ovarian cortex (containing more mature PGCs) to the outer part of the ovarian cortex (which will contain the least mature PGCs and

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Received: June 2, 2014
Accepted: August 25, 2014

the last ones to mature). Between week 17-35 of gestation, the heterogeneous population of germ cells in human female ovaries consists of at least three distinct populations: oocytes encapsulated in primordial follicles localized in the inner part of the cortex, VASA-positive/OCT4-negative PGCs and OCT4-positive/VASA-negative PGCs, all observed together in the developing ovary in relatively concentric circles (Kurilo, 1981; Konishi *et al*., 1986; Anderson *et al*., 2007).

In women and female mice, at birth the oocytes are arrested at the prophase of the first meiotic division and it is only during puberty that folliculogenesis is resumed and that follicle growth and maturation occurs resulting in cyclic ovulations during the female's fertile period. In males, after birth, spermatogenesis slowly resumes and during puberty the spermatogonia start proliferating and undergo continuous cycles of differentiation to sperm, in both mice and humans.

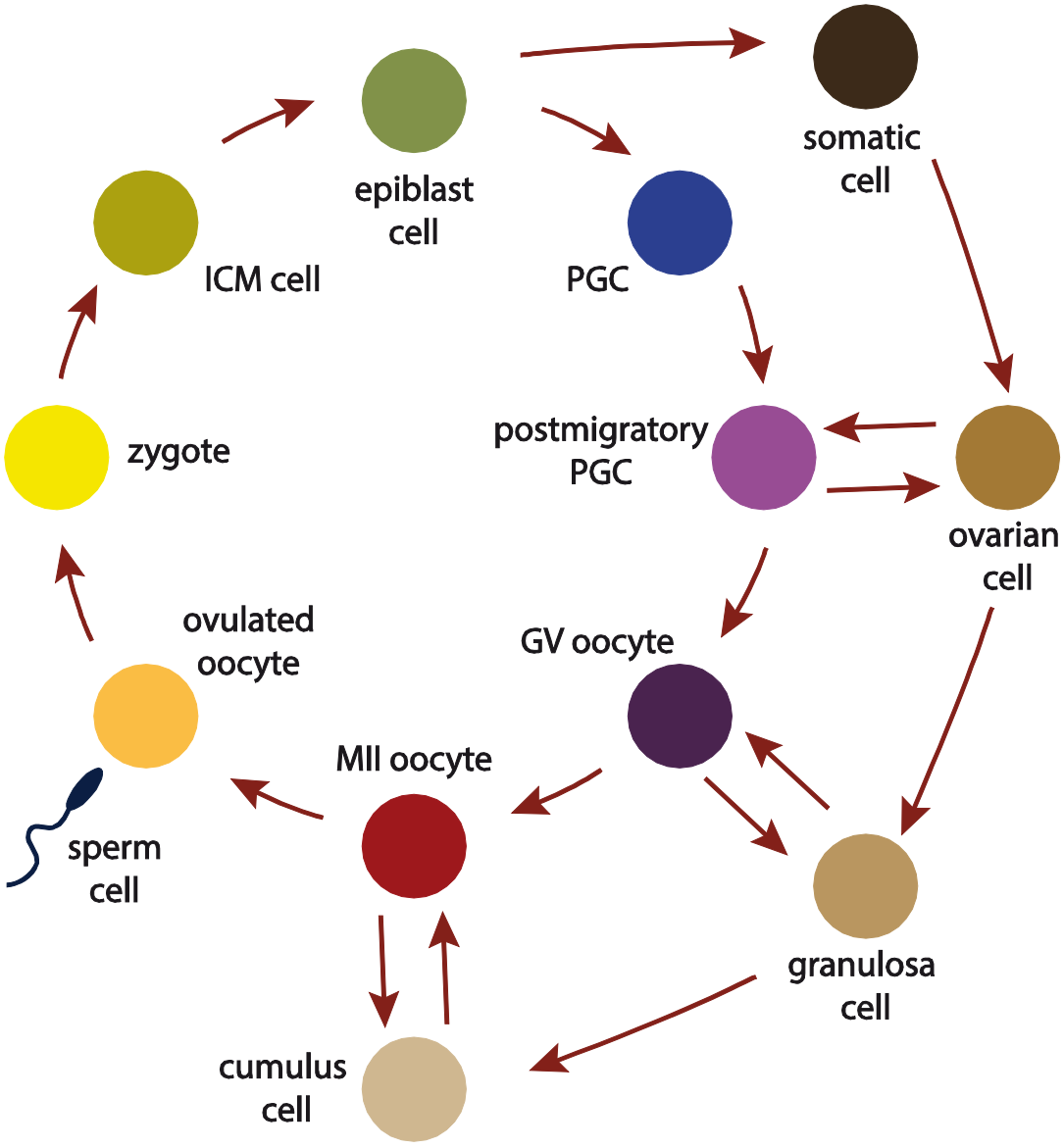

Figure 1. Schematic representation of oocyte development. After fertilization, the totipotent zygote gives rise to a pluripotent inner cell mass. Shortly after formation of the epiblast, segregation of primordial germ cells and somatic cells takes place. The primordial germ cells migrate to the developing gonads and signals from the somatic ovarian cells will stimulate meiosis entry and further specification of the oocyte. The oocyte enters meiotic arrest, remains at the germinal vesicle stage and together with the ovarian granulosa cells forms a follicle. Shortly before ovulation, as a result of hormones and cumulus-oocyte interactions the oocyte resumes meiosis and is released from the ovary at the metaphase II stage. After fertilization by a sperm cell a new zygote is formed. ICM = inner cell mass; PGC = primordial germ cell; GV = germinal vesicle; MII = metaphase II.

Why is the oocyte so special?

The mature oocyte is an extremely potent cell. Why? For one thing, the oocyte is the only cell of which the cytoplasm is capable of accepting a male haploid (n) nucleus and to orchestrate the formation of a new diploid nucleus from the haploid male and female pronuclei. After a diploid (2n) nucleus is formed this single cell, now called a zygote, can develop into a complete fetus plus all extraembryonic structures that include the fetal part of the placenta, the supporting membranes (amnion, chorion, yolk sac) and the umbilical cord/allantois. Even more remarkably, the cytoplasm of a mature oocyte is capable of reprogramming any diploid nucleus, including that of a fully differentiated somatic cell, within 24 h towards an epigenetic state from which a complete organism can be formed. This has been first demonstrated by the birth of the sheep Dolly (Wilmut *et al*., 1997), but after the birth of Dolly various animals have been successfully cloned by somatic cell nuclear transfer using many different somatic cells types. Indeed nuclei have even been successfully reprogrammed using mature enucleated oocytes of species different from that of the nucleus-donor cell (Narbonne *et al*., 2012). Exactly which components of the mature oocyte cytoplasm are responsible for its nuclear reprogramming capacity is not known.

Secondly, the (totipotent) fertilized/diploid oocyte can not only give rise to all the different cell types that are present in the fetus and the adult, but it does so orchestrating the positional information needed in a way that all the tissues are instructed to be formed in their correct places. And this is extraordinary. Although pluripotent embryonic stem cells (ESCs) also have the capacity to form all the different cell types present in the fetus and adult they lack the ability to organize axial polarity. When pluripotent stem cells are aggregated and triggered to differentiate into so-called embryoid bodies, different tissues can be formed, but these will be randomly distributed and the result is a disorganized mass of differentiated cells.

But behind every great oocyte are great granulosa cells

During maturation, the oocyte itself acquires this tremendous developmental potential via interactions with the somatic cells in the ovary. After PGCs have colonized the developing ovaries, they will become surrounded by somatic cells, the granulosa cells, to form primordial follicles. Those can stay dormant as primordial follicles for decades, but as the oocyte starts to grow in size the interactions with the surrounding somatic cells become more complex and the follicles change from primordial to primary, secondary and antral follicles (Li and Albertini, 2013). Although during follicular development the oocyte secretes proteins to form an acellular protective shell, called the zona pellucida, that surrounds the oocyte, this does not prevent interactions between granulosa cells and the oocytes. Rather, cytoplasmic transzonal projections from the granulosa cells contact the oocyte membrane via gap junctions and through these connections communication between the oocyte and surrounding cells is ensured. Indeed the number of these projections increases as the ovarian follicle develops and without a complex dialogue between the oocyte and surrounding somatic cells the oocyte cannot acquire developmental competence. During follicular growth, the oocyte is arrested at the prophase of the first meiotic division and it is only after a sudden rise in the concentration of luteinizing hormone (LH) that meiosis is resumed. The oocyte then progresses to the metaphase of the second meiotic division, when a second meiotic arrest occurs. The oocyte ovulates in meiotic arrest and it is only after fertilization by the sperm cell that meiosis in the oocyte is completed and male and female pronuclei are formed.

Apart from this nuclear maturation, the oocyte cytoplasm also needs to mature. This cytoplasmic maturation is much more difficult to monitor and visualize than the changes that occur at the nuclear level and it is poorly understood at the molecular level. Clearly, the oocyte needs to build up a reservoir of mRNA and proteins important for events related to fertilization and early development before the embryonic genome is activated.

When more knowledge is available about the molecular mechanisms important for cytoplasmic and nuclear maturation, we may better understand why some oocytes can be successfully fertilized *in vitro* while others that appear morphologically normal do not form viable embryos after fertilization. Also investigating the characteristics of mature oocytes would teach us about how to correctly reprogram a (somatic) nucleus in 24 h, knowledge that could be used to generate patient-specific stem cells lines needed for future applications in regenerative medicine. However, mature oocytes are very difficult to obtain in large number to study and consequently it is quite difficult to study some of their extraordinary properties. Therefore, there is a huge interest in learning how to generate large amounts of mature oocytes.

Differentiation of stem cells into oocytes: the germ cells

We know that mouse ESCs have the potency to form mature oocytes, because when mouse ESCs are introduced in mouse blastocysts to generate adult animals, a technique used world-wide that resulted in the Nobel prize in 2007, those ESCs are able to differentiate into the germline and to form functional mature oocytes (or sperm). So if mouse ESCs have this capacity *in vivo*, we should be able differentiate mature oocytes at least from mouse ESCs *in vitro*. But this has

proven to be very challenging indeed. There have been several studies reporting the generation of "mature" oocytes from mouse ESCs *in vitro*, based on morphology and gene expression, but all without showing functionality (Duggal *et al*., 2014; Sun *et al*., 2014). Moreover, these research papers have basically failed to show normal meiosis progression in those *in vitro*-derived germ cells (Novak *et al*., 2006; Nicholas *et al*., 2009; Sun *et al*., 2014). This block in nuclear differentiation with arrest at meiotic entry or progression, could perhaps be a consequence of the fact that the germline undergoes a complex cascade of epigenetic programming events, that includes DNA demethylation, rearrangement of histone marks, imprint erasure and X chromosome reactivation, that has proven difficult to mimic *in vitro* (Roelen and Lopes, 2008; Chuva de Sousa Lopes and Roelen, 2010). Moreover, it is becoming obvious that the germ cells need a correct molecular niche provided by the somatic compartment of the ovary to undergo their developing program correctly. Hence, specifying primordial germ cells *in vitro* may not be sufficient to ensure progression to the oocyte. In agreement, several reports have shown that female germ cells cultured in a male somatic environment develop as male germline and vice versa (McLaren and Southee, 1997; Adams and McLaren, 2002), suggesting that the germline is particularly sensitive to the molecular niche and the somatic environment that leads to sex determination.

For the development of *in vitro* differentiation protocols to differentiate stem cells into gametes, the focus is currently changing from the induction of germ cells to recreating the correct cellular niche necessary for each step of development. The importance of somatic cells in the differentiation of functional germ cells has been further illustrated by germ-soma co-culture experiments. The formation of functional gametes from *in vitro* cultured PGCs has not been successful. However, when germ cells from embryonic day (E)12.5 mouse gonads were associated together with gonadal somatic cells and transplanted under the kidney capsule of adult mice, testis- and ovary-like tissues formed. Most importantly, these structures were demonstrated to contain functional sperm cells and oocytes respectively that could give rise to healthy offspring (Matoba and Ogura, 2011).

Differentiation of stem cells into oocytes: recreating the niche

Based on the work of Nicholas and colleagues (2009) and Matoba and Ogura (2011) that underscored the importance of the ovary somatic niche to differentiation towards oocytes, Hayashi and colleagues (2012) have combined a first period of differentiation of mouse ESCs *in vitro*, followed by the ex-vivo co-culture with fetal female gonadal tissue and finally the transfer to the ovary of female mice *in vivo* (Nicholas *et al*., 2009; Matoba and Ogura, 2011; Hayashi *et al*., 2012). In this mixed "*in vitro-in vivo*" protocol, mouse ESCs were differentiated first to epiblast-like cells, followed by exposure to BMP4 and BMP8b to induce the formation of primordial germ cell-like cells *in vitro*. To overcome the meiotic arrest, these primordial germ cell-like cells were then aggregated with female fetal gonadal cells, in their natural cellular and molecular niche and that resulted in meiotic entry. Moreover, the procedure introduced the natural cellular somatic environment that contributed with the developing granulosa and theca cells important to the assembly of follicles. Thereafter, to promote folliculogenesis, this "gonadal aggregate" was transplanted to the ovary of immunocompromised adult females to be subjected to the natural hormonal cycle that would lead to follicle maturation.

Interestingly, using this protocol, all the germ-like cells introduced matured after several weeks to the germinal vesicle stage in one single cycle and were retrieved from the ovary before natural ovulation. Maturation was then induced *in vitro*, followed by *in vitro* fertilization of the generated oocytes and transfer of the resulting embryos to a foster mother. The resulting progeny developed to adulthood and was fertile, demonstrating that this mixed "*in vitro-in vivo*" protocol, including this first part of differentiation *in vitro*, was successful and opened a new door to explore the molecular niche necessary to mimic the whole process of gametogenesis. The same research group reported a similar approach using a mixed "*in vitro-in vivo*" protocol to generate mature functional sperm from mouse ESCs (Hayashi *et al*., 2011).

The future of artificial gametes

It remains to be demonstrated that ESCs (or other source of pluripotent stem cells including induced pluripotent stem cells) can be used to derive mature functional gametes *in vitro*, even though several attempts have been reported (Duggal *et al*., 2014; Sun *et al*., 2014). The generation of functional human germ cells *in vitro* has proven to be even more difficult, because we know much less about human gametogenesis and the genetic tools available to study humans are simply fewer than in mice. The use of human stem cells to produce chimera is subjected for obvious reasons to strong regulations world-wide and has been reported by few groups that have inserted human ESCs in mice blastocysts and transferred those blastocysts into foster mice for up to a week (James *et al*., 2006; Gafni *et al*., 2013). Moreover, human ESCs are more closely related to mouse epiblast stem cells (EpiSCs) than to mouse ESCs (Brons *et al*., 2007; Tesar *et al*., 2007), and EpiSCs are more reluctant to differentiate to PGCs *in vitro*. In this respect, using the recently published protocols to bring human ESCs from their primed pluripotency state to a more naïve

pluripotency state (Chan *et al*., 2013; Gafni *et al*., 2013) offers new exciting possibilities to generate human primordial germ cell-like cells.

Although patient-specific pluripotent stem cells are far from being considered as an alternative to treat infertility, there is a great societal interest in achieving artificial gametes in the dish, as infertility associated with the aging of the population is a growing concern. The societal or family pressure for couples to conceive children can be perceived as a huge stress factor. In addition, the use of gamete donation, in the case of aging women, gender issues or infertility is not always a legal option. Moreover, even in countries where gamete donation is possible, there is a strong preference for parents to have a genetic link with their children and to be able to recognize own traits and transmit their own genetic material to future generations. Families that have an IVF-conceived child prefer the following IVF-conceived children to have a genetic link with the previous (genetic brothers or sisters).

In conclusion, being able to generate patient-specific artificial gametes in a dish and providing this would occur in an efficient, safe and ethically acceptable way, would accelerate the generation of patient-specific stem cell lines needed for regenerative medicine, allow us to understand totipotency and would revolutionize the field of human reproduction, presenting a solution for psychological and ethical problems associated with gamete donation and infertility.

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