Pluripotent Stem Cells from Maturing Oocytes

Alena Langerova,¹ Helena Fulka,² and Josef Fulka, Jr.²

Abstract

Embryonic stem cells are mostly derived from mature oocytes that were either fertilized or activated parthenogenetically and then reached the blastocyst stage. From the cell cycle perspective, fertilization or activation induces the exit from meiosis, decondensation of oocyte chromosomes, and the entry into mitosis. Decondensation of oocyte chromatin with subsequent formation of nuclei can be, however, induced at any postgerminal vesicle breakdown meiotic maturation stage. In this article, we discuss the possibility of cleavage of transformed maturing oocytes and whether they can reach the blastocyst stage, from which pluripotent stem cell lines could be derived.

Introduction

FINAL STAGES OF OOCYTE MATURATION cover the period when germinal vesicle breakdown (GVBD) occurs and chromosomes condense with subsequent formation of the first metaphase spindle. Then a short anaphase-to-telophase transition can be detected, and chromosomes are arranged in metaphase II plate with the first polar body extruded. Here the oocyte awaits the fertilizing sperm. Once the sperm penetrates the oocyte, it initiates the anaphase-to-telophase II transition with accompanying extrusion of the second polar body (Fulka Jr. et al., 1998). This is then followed by extensive oocyte and sperm head chromatin decondensation and formation of a male (paternal) and female (maternal) pronucleus.

Eventually, the metaphase II oocyte can be activated parthenogenetically, but here only a maternal pronucleus (i) is formed in the cytoplasm. DNA replication in pronuclei begins approximately 6–8 h postfertilization, and, once completed, the chromatin condenses and the first mitotic chromosome group is formed. In normally fertilized oocytes, this mitotic group is diploid (McLay and Clarke, 2003). If the extrusion of the second polar body is prevented in parthenogenetically activated oocytes by incubating them in cytochalasin B (or D), two pronuclei are usually formed in the oocyte cytoplasm and the first mitotic group is also diploid. In parthenogenetically activated oocytes with the second polar body extruded, the mitotic group is haploid.

The difference between the first and a second polar body is interesting. The first polar body disappears soon after its extrusion, but the second polar body persists for several embryonic cleavages. The second polar body contains a

pronucleus-like structure, and DNA replication also occurs in it. The length of oocyte maturation is species specific. For example, in the mouse it lasts approximately 10–12 h, bovine oocytes need approximately 20 h, pig oocytes require 40 h, and human oocytes require more than 30 h. The process of oocyte maturation proceeds in ovarian follicles after a luteinizing hormone (LH) surge. If oocytes are, however, isolated from large follicles and cultured under appropriate conditions, they begin to mature and reach metaphase II stage *in vitro* as well. In general, their quality is typically lower when compared to *in vivo*-matured oocytes.

Oocyte Maturation: The Chromosome (Nuclear) Perspective

Immature oocytes contain a prominent nucleus, the germinal vesicle (GV), in which a specific organelle, the nucleolus, is visible in some species (rodents, pig, humans). This nucleolus is morphologically different from nucleoli that can be found in differentiated cells. In somatic cells, nucleoli contain three compartments—dense fibrillar centers and fibrillar and granular components. These differentiated nucleoli are engaged in many cellular processes, *i.e.*, cell cycle control, transcription, differentiation, etc. On the other hand, nucleoli in fully grown oocytes are spherical and contain only a dense fibrillar material. For this reason, they are called as the “nucleolus precursor bodies” (NPBs). In most developmentally competent oocytes, NPBs are enclosed [surrounded nucleoli (SN) oocytes] with a ring of chromatin (heterochromatin). The developmentally incompetent, fully grown oocytes, where the nucleolus is not surrounded with

¹GENNET, Prague, Czech Republic.

²Institute of Animal Science, Prague, Czech Republic.

chromatin [nonsurrounded nucleoli (NSN)], are able to mature, but after fertilization they typically cleave only to the two-cell stage (Bonnet-Garnier et al., 2013). As mentioned above, following a gonadotropin surge or after liberation of oocytes from follicles, the process of maturation begins; the nucleus (GV) slowly disappears (in mouse at 1.5 h, in pig at 18 h) and the chromosomes condense. During the first meiotic division, the homologous chromosomes are segregated while the sister chromatids remain still attached to each other (until metaphase II). The sister chromatids are separated during the second meiotic division, which occurs after the oocyte becomes fertilized or activated parthenogenetically (Li and Albertini, 2013).

Oocyte Maturation: The Cell Cycle Control Perspective

The onset of oocyte maturation is under the control of so called maturation-promoting factor (MPF), which is a heterodimer composed of a catalytic (CDK1) and a regulatory (cyclin B) subunit. The onset of MPF activity is regulated by Wee 1/Myt 1 kinases that phosphorylate CDK1, thus keeping MPF inactive. On the other hand, CDC25 phosphatases dephosphorylate CDK1 and MPF becomes active. The activity of MPF becomes high shortly before the onset of GVBD. High activity persists until the metaphase I stage, with its drop during the anaphase to telophase I transition. Following this transition, MPF activity rises again and declines after fertilization or parthenogenetic activation (Jones, 2004).

Induced Chromosome Decondensation in Maturing Oocytes

In this article, the term “maturing oocytes” refers to those oocytes in which GVBD has already occurred but have not yet reached the metaphase II stage. As far as we are aware, the first papers dealing with induced chromosome decondensation were published by Clarke and Masui (1983, 1985). These authors treated mouse oocytes at different stages of maturation with the protein synthesis inhibitor puromycin. When puromycin was added to oocytes shortly after GVBD, it prevented the progression of oocyte maturation, and the chromosomes formed a cluster. Nuclei (decondensed chromosomes) in these oocytes are formed only exceptionally. Quite a different situation was, however, observed in oocytes that were more advanced in maturation, *i.e.*, in those oocytes that already reached the metaphase I stage with a well-formed spindle. When these oocytes were treated with puromycin, the first polar body was rapidly expelled and chromosomes that remained in the oocyte cytoplasm decondensed and formed a nucleus with a very visible nucleolus. This nucleus was typically detectable after 6 h. The minimum puromycin treatment time that is necessary for the formation of a stable nucleus was approximately 8–9 h. If shorter, the nucleus broke down and chromosomes recondensed. When metaphase I oocytes treated by puromycin for 9 h were transferred into a puromycin-free medium, their nuclei persisted, but DNA replication occurred only exceptionally in them.

The same results were published also by Hashimoto and Kishimoto (1988). These experiments were later expanded by Clarke et al. (1988). Mouse metaphase I oocytes were treated with puromycin for 9 h and then transferred into an inhibitor-free medium. Nuclei in these oocytes remained very visible

for 8–10 h, but after this interval chromosome condensation occurred. If, however, the oocytes with nuclei were transferred into a medium supplemented with dibutyryl cyclic adenosine monophosphate (dbcAMP), nuclei did not break down and began to replicate DNA. After transfer into oviducts of host mice, they even reached the blastocyst stage. No attempts to establish embryonic stem cell (ESC) lines were performed. Karyotyping demonstrated that these blastocysts were mostly diploid.

Another group of drugs that were used for induced chromosome decondensation were inhibitors of phosphorylation (kinases inhibitors). In general, 6-dimethylaminopurine (6-DMAP) inhibits the maturation progression when applied at all stages post-GVBD. If treatment is sufficiently long, chromosomes (chromatin) decondense and a nucleus is formed. These nuclei were characterized only by electron microscopy (EM), which showed well-developed membrane and the presence of NPBs that were morphologically similar to NPBs found in GVs. DNA replication was not studied (Szollosi et al., 1991).

The effect of another kinase (CDK1) inhibitor butyrolactone 1 (BL1) on metaphase I mouse oocytes has been studied by Fulka Jr. et al. (1999). Under our conditions, GVBD occurred approximately within 60 min after the beginning of oocyte culture (time T1). Well-formed metaphase I spindles were detected after 7–8 h (T7–8) and anaphase-to-telophase I transition was detectable after about 9 h (T9). The oocytes reached the MII stage at 11–12 h after the start of their culture (T11–12). For the BL1 treatment, the oocytes were first cultured with cumulus cells, which were removed by pipetting at T1.5. Those oocytes with GVs were discarded, and the remaining oocytes were cultured further. At T7.5, these oocytes were transferred into BL1-supplemented medium (75–100 μ M). The oocytes exited from MI normally and the polar body was extruded, but MII spindle was not formed in them. Instead, chromosomes decondensed and formed a very visible nucleus with a prominent nucleolus. These nuclei (lamin A/C-positive) are typically detectable in the cytoplasm approximately after 6 h of incubation in BL1-supplemented medium. We observed an unexpected behavior of oocytes when they were removed from the BL1 medium (approximately after 12 h) and transferred into a normal medium. In this medium, the BL1-treated oocytes cleaved directly into a two-cell embryo-like stage.

This unexpected behavior prompted us to study the effect of BL1 in more detail. The cleavage into a two-cell embryo-like stage suggested that DNA replication occurred in BL1-treated oocytes. This was confirmed when BL1-treated oocytes were incubated with bromodeoxyuridine (BrdU). Interestingly, polar bodies were also positively labeled, and this indicated that they rather represent second polar body-like structures. Moreover, these polar bodies did not disintegrate rapidly and persisted for several further cleavages. Also chromosomes of first mitotic cleavage plates were morphologically similar to chromosomes that can be found in normally fertilized embryos. These results clearly indicated that BL1 treatment of metaphase I oocytes directly converts a meiotic division into a mitotic one. A similar effect was observed when oocytes were incubated in roscovitine, but their response to this drug was very inconsistent.

The detailed examination of chromosomes in BL1-treated oocytes supported the view that BL1 induces a metaphase

II-like chromosome morphology. This was especially striking when we analyzed the presence of REC8, a meiosis-specific cohesin. Similar to normal metaphase II chromosomes, the BL1-treated oocytes showed the loss of REC8 along the chromosome arms and retained only the centromeric REC8. Even more interesting was the behavior of BL1 oocyte-treated chromatin with respect to replication licensing. Although the BL1 oocytes never really reach the metaphase II stage, their chromatin is replication competent. Under normal circumstances, the key licensing factor CDC6 is not present in oocytes and associated with chromosomes until the metaphase II stage. However, in the BL1-treated oocytes, CDC6 can be found on chromosomes as early as 1.5h after the beginning of treatment. This is followed by loading of different minichromosome maintenance (MCM) proteins onto the oocyte chromosomes, which leads to the replication licensing of BL1-treated maturing oocytes.

Development of BL1-treated oocytes

The first article in which we described the effect of BL1 on metaphase I mouse oocytes was not focused on the development of these cells. We began to study this much later. First, we wanted to know whether these converted oocytes could reach the blastocyst stage. When the experimental conditions were perfected, almost all metaphase I oocytes responded to BL1 treatment, *i.e.*, they formed a nucleus in the cytoplasm and gave off the polar body (80–90%). From these oocytes, approximately 80% cleaved and about 30% reached the blastocyst stage. It must be noted here that this percentage was even higher (more than 50%) in some experiments. The BL1 blastocysts were indistinguishable from blastocysts originating from normally fertilized oocytes. They exhibited the presence of inner cell masses positive for Oct 4 and Nanog, whereas their trophoctoderms were Cdx2 positive. In all cases, these blastocysts were diploid.

Next we tested if we could establish ESCs from them. Interestingly, we found that BL1 blastocysts showed a higher rate of expanded inner cell mass formations when compared with the parthenogenetic blastocysts. This phenomenon cannot be simply explained by the absence of the paternal genome in BL1 blastocysts because it is also absent in the parthenogenetic embryos. Even more interesting is the fact that when the ESC lines were analyzed by low-density arrays, BL1 ESC lines were more similar to lines obtained from fertilized oocytes when compared to parthenogenetic ESC lines. This indicates that some changes must occur during the final phases of oocyte maturation that might lead to the establishment of an oocyte-specific developmental program.

Differentiation of BL1 ESC lines

In vitro-induced BL1 ESC differentiation resulted in the formation of embryoid bodies containing a wide range of cells of ectodermal, mesodermal, and even endodermal origin. When BL1 ES cells were injected into severe combined immunodeficiency (SCID) mice, they formed teratomas. Histological evaluation showed that these teratomas contained a wide range of somatic tissues, again originating from all three embryonic layers. BL1 ESCs were also injected into ICR blastocysts that were transferred to recipient females, and we obtained several chimeric offspring. No germ-line transmission was, however, observed. We cannot

exclude the possibility that the production of more mice and their extensive breeding would result in the birth of such offspring. These findings are interesting with respect to the fact that parthenogenetic ESC lines were originally described to be excluded from tissues originating from mesoderm and endoderm and thus were considered to be inferior to ESCs obtained from fertilized oocytes (Nagy et al., 1987; Paldi et al., 1989). However, more recent reports have shown that even parthenogenetic ESCs can exhibit full developmental and differentiation competence and even contribute to the germ line in chimeric assays (Chen et al., 2009). These findings are indeed very exciting (Fulka et al., 2011).

The effect of BL1 on oocytes of other species

We have also tested the effect BL1 on oocytes of other species. We used bovine oocytes. Compared to the mouse, the main disadvantage here is the lack of transparency of these oocytes. We have transferred metaphase I oocytes into BL1-supplemented medium, and their response to this drug was similar to mouse oocytes. The polar body was extruded and the cytoplasm contained a nucleus that replicated DNA. Further culture of BL1-treated oocytes in synthetic oviductal fluid (SOF) medium resulted in a production of blastocysts with an excellent morphology. Unfortunately, no similar markers that are commonly used in the mouse can be used for labeling of bovine embryos because OCT4 and CDX2 are found in both the inner cell mass and trophectoderm. Also, there is no method for derivation of ESC lines in bovine.

Logically, the most attractive model for our approach would be human. First, in most *in vitro* fertilization (IVF) clinics, the oocytes that are not metaphase II staged when collected from stimulated ovaries are discarded. Thus, a potentially very valuable material is lost. Second, because offspring would never be born from these oocytes (if they respond to BL1), the possibility of deriving ESC lines from them would represent an ethically acceptable approach. The main disadvantage is that we do not know the exact stage of maturation at which these oocytes are collected (these can be shortly after GVBD, early metaphase I, or late metaphase I).

FIG. 1. The nucleus (N) with several nucleoli in a human oocyte that was incubated from metaphase I stage in BL1-supplemented medium. PB, Polar body. Magnification, 600 $\times$.

FIG. 2. DNA replication in nuclei (N) formed in maturing oocytes after BL1 treatment. Note that DNA replication is also detectable in a polar body (PB). Magnification, 600 $\times$.

The other disadvantage is the low number of oocytes available, and this does not allow us to test, for example, different concentrations of BL1. So, in humans we have applied the scheme that was used in the mouse. Nevertheless, even without testing, human oocytes also responded to BL1—*i.e.*, the polar body was extruded and a nucleus was formed in the cytoplasm. In the mouse, a single nucleolus is typically visible in the nucleus, whereas in humans nuclei contained several nucleoli (Fig. 1). These nuclei also replicated DNA (Fig. 2). Further development of BL1 oocytes was, however, compromised, and only few of them cleaved and exceptionally reached the eight-cell stage (Langerova, unpublished results).

The explanation for this poor development is rather difficult. First, it must be noted that we have used oocytes that were collected along with the population of already matured oocytes (metaphase II), and this may indicate that their quality was somewhat compromised. Second, the oocytes were collected from patients treated for different forms of infertility. Third, it is well known that the quality of human oocytes when compared to oocytes of other species is much lower. For example, the frequency of aneuploidies in human oocytes is very high—up to 60%—compared with 5% in mouse and 10% in cattle and pig (Nagaoka et al., 2012). Also the evaluation of epigenetic changes accompanying the process of maturation and related to the process of chromosomes segregation (acetylation of chromatin) clearly showed, that when compared to other species (mouse), the pattern of labeling is very inconsistent (Langerova, 2013; van den Berg et al., 2011) and this may indicate that the oocytes used might have been developmentally handicapped. Logically, the best solution would be to use high-quality oocytes from paid donors. We believe that further experiments will lead to the production of viable blastocysts from which ESC lines will be established sooner or later.

Conclusions

The oocyte is an exceptional cell, and the results obtained so far clearly show that even the oocyte (meiotic cell) that did

not reach yet the metaphase II stage can be converted into a mitotic cell without a typical activation stimulus. Moreover, in the mouse, where we can select the best cells, ESC lines can be obtained. Thus, even with the advent of induced pluripotent stem cell (iPSC) technologies, the oocyte remains an attractive model for different studies (Morris and Daley, 2013; Yamanaka, 2012).

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

J. Fulka, Jr.

Institute of Animal Science

Pratelstvi 815

104 00 Prague 10, Czech Republic

E-mail: fulka@vuzv.cz