—Mini Review—

Meiotic Resumption and Spindle Formation of Pig Oocytes

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Abstract: *Fully grown mammalian oocytes arrested at prophase I resume meiosis after gonadotropic stimulation. Oocytes undergo a series of changes including chromosome condensation, nucleolus disassembly, germinal vesicle breakdown (GVBD), and spindle formation. The mouse is the best model for studying the molecular mechanisms underlying the maturation of mammalian oocytes. However, some of the maturational events are different from those in other mammalian species. To study these events, we use pig ovaries, which are available as a byproduct from local slaughterhouses. It has long been known that pig oocytes have a dependence on de novo protein synthesis for GVBD, whereas GVBD in mouse oocytes occurs independently of protein synthesis. The reason seems to be the lack of Cyclin B1 molecules in pig GV-oocytes, although the synthesis of other protein(s) may be required for the GVBD. In mouse oocytes, the spindle is formed through the action of cytoplasmic microtubule organizing centers (MTOCs), and the oocytes are able to form the spindle without chromosomes. However, pig oocytes don't have such distinguished cytoplasmic MTOCs and never form the spindle without chromosomes. In this species, the condensing chromosome plays the role of the organizer nucleating spindle microtubules. We should develop some other mammalian models that will help us understand the mechanisms underlying oocyte maturation.*

Key words: *Oocyte maturation, Germinal vesicle breakdown, Spindle, Mouse, Pig*

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Introduction

Fully grown oocytes in mammalian ovaries undergo a process called "maturation" before successful fertilization. The oocytes are arrested at the diprotene stage of prophase I (this oocyte stage is called the germinal vesicle stage or GV-stage), and some of them resume meiosis and mature to metaphase II in response to the periodic surge of gonadotropins during estrus cycles. After stimulation, oocytes undergo a series of changes: condensation of chromosomes, disassembly of nucleoli, breakdown of the nuclear envelope (germinal vesicle breakdown: GVBD), and assembly of a spindle of metaphase I (MI). The first meiotic division is completed when the first polar body is extruded after anaphase I (AI) and telophase I (TI). The oocytes then enter metaphase II (MII) without an intervening interphase and arrest at MII, when they are ovulated into oviducts for the fertilization.

The study of the maturation of mammalian oocytes has a relatively long history. Since Pincus and Enzmann [1], and Edwards [2] showed that mammalian GV-oocytes from ovaries were able to mature under the appropriate culture conditions, a huge number of studies have been conducted using various *in vitro* maturation systems. The studies have been aimed not only at the production of high-quality mature oocytes for fertilization as well as somatic cell nuclear transfer, but also at improving understanding of the mechanisms of oocyte maturation in mammals. Oocyte maturation is a series of dramatic events ranging from meiotic prophase I (equivalent to G2-phase) to MII-arrest, and it is a good model for studying the cell cycle, because oocytes are naturally arrested at the G2-phase and take a relatively long time to progress to the M-phase.

The mouse, by the accomplishment of its genome

project, has become the best model for studying the molecular mechanisms underlying the maturation of oocytes. However, we should develop other mammalian models for understanding these mechanisms, because some of the maturational events in the mouse oocytes are different from those in other mammalian species. We have been using pig oocytes as another model of studying oocyte maturation. In this short review, we focus on the early events of maturation in pig oocytes. They show protein synthesis-dependent GVBD and chromosome-dependent spindle formation that are different from those in mouse oocytes.

Maturation of Pig Oocytes *In Vitro*

Pig ovaries are available as a byproduct from local slaughterhouses. Most of the pigs are slaughtered at about 100 kg body weight at 6 months of age in Japan. Female pigs reach puberty around 200 days of age. Therefore, most of the female pigs are nearing or have just reached puberty. The pig is a multiparous animal, and a number of large antral follicles are visible on the ovary. Oocytes grow as the follicles develop. In the mouse, oocytes reach nearly full size around antrum formation. On the other hand, oocytes in the pig, like other large animals, keep on growing after antrum formation. In the pig, large antral follicles at 4–6 mm in diameter contain fully grown oocytes at 120–125 μm in diameter (without zona pellucida).

We dissected the large antral follicles from the ovaries, and collected the oocyte-cumulus-granulosa cell complexes (or oocyte-cumulus complexes depending on the experimental design). They were cultured in TCM199 containing 10% fetal calf serum and gonadotropin at 38.5–39.0°C, as described elsewhere [3–5]. Pig oocytes enclosed with cumulus cells from healthy antral follicles 4–6 mm in diameter were at the GV stage, and over 90% of them progressed to MII synchronously under our culture conditions. Soon after the beginning of culturing, oocyte chromatin started to condense to form chromosomes. After the oocyte nucleolus became invisible, the oocytes underwent GVBD and reached MI by 27 hr. Thereafter, they passed through AI and TI at 30–33 hr, and maturation was completed at MII after 36 hr of culture. Pig oocytes take longer to mature than do mouse oocytes (MI: 4–6 hr, and MII: 12 hr) or bovine oocytes (MI: 12 hr, and MII: 21–22 hr).

Initiation of oocyte maturation corresponds to the G2/M transition in somatic cells. In both somatic cells and oocytes, similar sequential changes occur at the transition, which includes the chromosome condensation, nucleolus disassembly, nuclear envelope breakdown, and spindle formation. In somatic cells, these changes occur in a short time within an hour, while oocytes take much longer to undergo these changes, as described above. These slower changes make the analysis of sequential events and examination of the relationships among events easier in oocytes, especially pig oocytes, than in somatic cells. All of these events occur in both mouse and pig oocytes, although there are some intrinsic differences between these two animals.

Protein Synthesis-Dependent GVBD

It has long been known that oocytes from domestic species including pigs [6], sheep [7], and cows [8] depend on *de novo* protein synthesis for normal progression of oocyte maturation. On the other hand, GVBD in mouse [9] and rat [10] oocytes occurs independent of protein synthesis. Pig oocytes cultured in the medium containing a protein synthesis inhibitor, cycloheximide, remained in the GV stage, regardless of the presence of cumulus cells and gonadotropin in the culture medium (Fig. 1). However, the treated oocytes had highly condensed chromosomes in the GVs. When these treated oocytes were washed and cultured in control medium, they resumed meiosis and matured to MII [11]. The results indicate that inhibition of new protein synthesis prevents GVBD completely but does not prevent chromosome condensation.

The G2/M transitions of both oocytes and somatic cells are controlled by a protein kinase known as Cdc2 kinase (Cdk1). Cdc2 kinase was first described in frog oocytes as MPF (maturation-promoting factor) [12], and it is now generally accepted as a key molecule controlling the cell cycle. Cdc2 kinase is a serine/threonine protein kinase consisting of a 34-kDa catalytic subunit Cdc2 ($p34^{cdc2}$) and a Cyclin B regulatory subunit [13–16].

Cdc2 kinase is activated around GVBD in all mammalian species examined so far [17, 18]. Increased activity of Cdc2 kinase around GVBD is maintained during MI and MII with a temporal decrease at AI/TI. Mouse GV-oocytes have been suggested to contain Cdc2 and a small amount of Cyclin B that is just enough to induce GVBD [19, 20]. After GVBD, the synthetic activity of cyclin B increases progressively, reaching the maximum at MI. In mouse GV-oocytes, the Cdc2-Cyclin B complex accumulates as an inactive form, which becomes activated through

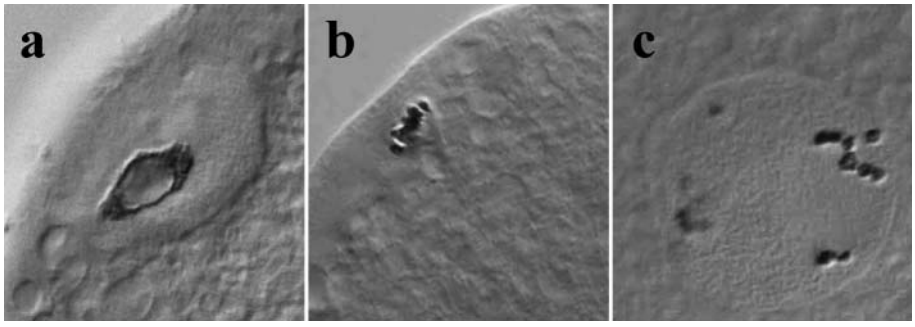

Fig. 1. Pig oocytes cultured in the medium containing cycloheximide for 27 hr. Ring-like heterochromatin was stained with aceto-orcein around the nucleolus in the pig GV-oocyte before culture (a). Oocytes cultured in cycloheximide-free medium reached MI (b), while oocytes cultured in cycloheximide-supplemented medium had condensed chromosomes in the intact GV (c).

dephosphorylation of Cdc2, perhaps by Cdc25b [21].

In pig oocytes, synthesis and degradation of Cyclin B1 molecules corresponded well to the fluctuation of Cdc2 kinase activity [22, 23]. Pig GV-oocytes just after collection from the ovary had no, or very little, Cyclin B1 and expressed a low activity of Cdc2 kinase. Following stimulation with gonadotropins, oocytes started to accumulate Cyclin B1, and Cdc2 kinase activity gradually increased around GVBD and reached a maximum level at MI. When pig oocytes were treated with protein synthesis inhibitors, Cyclin B1 was not synthesized, and the oocytes never underwent GVBD. Oocyte GVBD is also inhibited by the Cdc2 kinase inhibitors, such as olomoucine and butyrolactone I [24]. These results seem to support the conclusion that Cyclin B1 is the protein whose synthesis is required for the GVBD in pig oocytes. However, recently Kuroda *et al*. [25] conducted an experiment about pig oocyte maturation using anti-sense RNAs against Cyclin B1 and B2. Both anti-sense RNAs inhibited Cyclin B1 and Cyclin B2 syntheses, respectively, although injected oocytes underwent first meiosis normally. Kuroda *et al.* concluded that in pig oocytes, Cyclin B synthesis is not necessary for GVBD induction itself. It may be possible that the synthesis of some other protein(s) is required for GVBD in pig oocytes.

In pig oocytes, chromosome condensation progresses in the GV before Cdc2 kinase activation, and it is not prevented by protein synthesis inhibition. This implies that neither newly synthesized proteins nor Cdc2 kinase activation is required for chromosome condensation. During pig oocyte maturation, histone H3, which is one of the core histones in the nucleosome, became phosphorylated at serine 28 (Ser 28) and then at Ser 10 [26, 27]. Histone H3 kinase activity increased over a similar time course to that of the phosphorylation of histone H3 during oocyte maturation. This phosphorylation is thought to be essential for proper chromosome condensation and segregation in somatic cells [28, 29]. Actually, phosphorylation of histone H3 occurred before Cdc2 activation and GVBD in normal maturation of pig oocytes [27]. However, in cycloheximide-treated pig oocytes, neither increase in histone H3 kinase activity nor phosphorylation of histone H3 at Ser 10 and Ser 28 occurred in the condensed chromosomes [30 and Bui *et al.*, unpublished data]. It remains to be determined whether other histone molecules are phosphorylated, and other potential kinase is activated prior to Cdc2 kinase activation or activated even under the inhibition of new protein synthesis.

Chromosome-Dependent Spindle Formation

Establishing a bipolar spindle is an essential event of both mitosis in somatic cells and meiosis in oocytes. However, the oocyte spindle differs in shape from that in somatic cells. Moreover, oocyte spindle shapes differ slightly among mammalian species. Somatic cells form rugby ball-shaped spindles (Fig. 2a), while mouse oocyte spindles are barrel shaped (Fig. 2c), as first schematically represented by Szöllösi *et al.* [31]. The spindles in pig oocytes are more spherical (Fig. 2b). These differences seem to come from how these cells make their own spindles at the G2/M transition.

In somatic cells, bipolarity of the spindle is determined by two centrosomes, each of which is comprised of a pair of centrioles surrounded by electron-dense

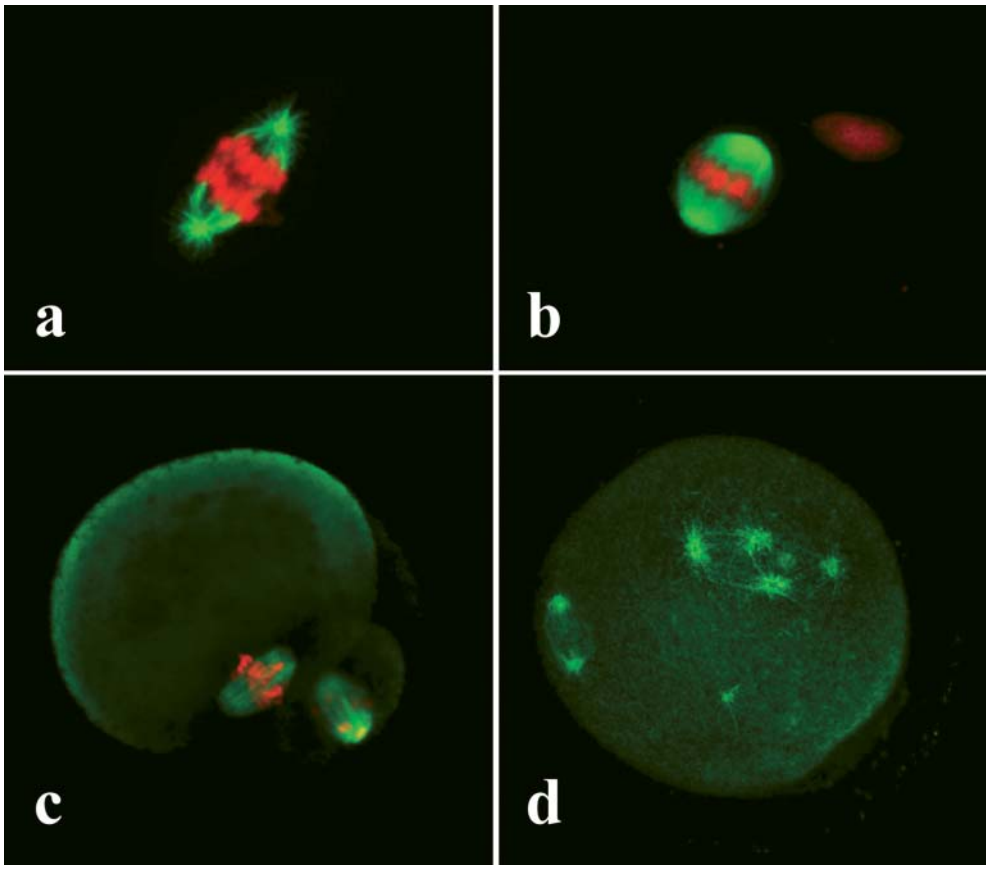

Fig. 2. Spindles of the pig cumulus cell (a) and MII-oocyte (b). Microtubule organization in mouse control (c) and enucleated oocytes (d) after maturation culture. Microtubules appear in green by anti-α tubulin antibody and Alexa fluor 488, and DNA appears in red by propidium iodide. Photos a) and b) were taken by N. Ueto, and c) and d) by S. Ogushi.

pericentriolar materials. A centrosome is replicated during the interphase. As mitosis proceeds, the replicated centrosomes separate and move away from each other to serve as the two spindle poles. A large number of microtubules radiating from the opposite spindle poles search and capture the two kinetochores of sister chromatids, which are located on opposite sides of each condensed chromosome [32]. Consequently, the polar and kinetochore microtubules form a rugby ball-shaped spindle. A combination of microtubule motor proteins at the kinetochore and microtubule dynamics is thought to align the chromosomes at the spindle equator. Thus, the interactions between the microtubules nucleated by centrosomes and the chromosomal kinetochores enable the formation of the bipolar spindle.

The formation of the MI spindle in mammalian oocytes is different from that in somatic cells. The difference is caused by the lack of centrosomes in oocytes. The primary oocyte initially has a typical centrosome with a pair of centrioles until the pachytene stage in prophase I but lacks it in the subsequent stages [31]. Since a centriole is brought to the oocyte by a spermatozoon at fertilization, it is thought that this oocyte centrosome reduction plays a role in preventing parthenogenetic embryogenesis and balancing the centrosome number in the embryonic cells [33]. In mouse oocytes, the MI spindle is formed through the action of multiple cytoplasmic microtubule organizing centers (MTOCs). During oocyte maturation, the number of MTOCs increases from diakinesis to MI [34]. During later diakinesis, numerous MTOCs are associated with the chromatin strands from which microtubules radiate, and several distinct MTOCs are located in the cytoplasm away from the chromatin region. After that, there is a dramatic increase in the

microtubule nucleating capacity of the MTOCs associated with the developing spindle and in the cytoplasm, and the number of cytoplasmic MTOCs further increases.

Pig oocytes don't have distinguished MTOCs in the cytoplasm as those in other domestic species, such as sheep [35] and cows [36]. Just after GVBD, bivalent chromosomes make a single clump at the late diakinesis stage [37]. Microtubules are nucleated toward random directions around the clump of chromosomes [5]. Although it is unknown how microtubules are nucleated in the absence of centrosomes and distinct MTOCs, the randomly arrayed microtubules may initially be nucleated from the microtubule-nucleating factor containing γ-tubulin [5], which is found in the centrosomes in somatic cells [38, 39]. At this stage, the nuclear mitotic apparatus protein (NuMA), which is associated with the dynein/dynactin motor complex [40], is found in association with the randomly arrayed microtubules. As meiosis proceeds, NuMA moves away from the condensed chromosomes and bundles the microtubules at the minus ends to integrate both spindle poles and form a round-shaped spindle. Thus, in pig oocytes it seems likely that the condensing chromosome plays the organizer nucleating spindle microtubules without MTOCs in the cytoplasm.

The chromosome-dependent spindle formation has been suggested even in the mouse oocyte. It was observed in nocodazole-treated mouse oocytes that dispersed groups of chromosomes resulted in the formation of multiple independent spindles after removal from nocodazole, which had induced chromosome dispersal by dissolution of the metaphase spindle [41]. This suggests that the chromosome promotes microtubule organization. However, mouse oocytes are able to form spindles without chromosomes. Maro and his coworkers examined spindle formation in chromosome-free mouse oocytes made by bisection of the oocytes with a glass needle [42]. In these oocytes, cytoplasmic MTOCs nucleated the microtubules, and the formation of bipolar and multipolar spindles was observed in the absence of chromosomes. When the germinal vesicles were sucked out from the mouse GV-oocytes followed by maturation culture, the enucleated oocytes made spindles (Fig. 2d). These results show that the formation of a spindle in mouse oocytes is not impaired by the absence of chromosomes. When enucleated pig GV-oocytes were cultured for maturation, they activated cell cycle molecules, such as CDK1 and MAP kinase [43]. However, enucleated pig oocytes never formed the spindles without chromosomes. The chromosomes seem to play a major role in spindle formation by promoting microtubule nucleation and directing the appropriate spindle formation in this species.

Conclusion

The mouse is the best model for studying the molecular mechanisms underlying the maturation of oocytes. However, some of the early events of oocyte maturation are different from those in other mammalian species. Here, we have briefly described two intrinsic differences in pig oocytes, which are protein synthesis dependency for GVBD and chromosome dependency for spindle formation. Pig oocytes have a dependence on *de novo* protein synthesis for GVBD, whereas GVBD in mouse oocytes occurs independent of protein synthesis. The reason seemed to be the lack of Cyclin B1 molecules in pig GV-oocytes, although the synthesis of other protein(s) may be required for the GVBD. In mouse oocytes, the spindle is formed through the action of cytoplasmic MTOCs, and the oocytes are able to form the spindles without chromosomes. However, pig oocytes don't have such distinguished cytoplasmic MTOCs and never form the spindles without chromosomes. In this species, the condensing chromosome plays the organizer nucleating spindle microtubules. Protein synthesis dependency for GVBD is common in domestic species. Furthermore, no cytoplasmic MTOC is common in these species. The latter may explain the difference in centrosome inheritance during fertilization between mouse oocytes and other mammalian oocytes. In most mammalian species, the spermatozoon introduces the centriole into the zygotes, although mouse spermatozoa lack the centriole [33]. We should have mammalian models in addition to the mouse for understanding the mechanisms underlying oocyte maturation. The accumulation of findings in different species is important for improving the reproductive technologies in domestic animals as well as in humans.

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