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—Mini Review—

Production of Functional Oocytes In Vitro

Yayoi Obata[1]* and Tomohiro Kono[1]

[1]Department of BioScience, Tokyo University of Agriculture 1-1-1 Sakuragaoka, Setagaya-ku, Tokyo 156-8502, Japan

Introduction

Although the ovaries of mammals contain thousands or millions of immature oocytes, few of these ever mature to the point at which reproduction *in vivo* is possible. Ovarian oocytes therefore constitute a large and potentially valuable resource for clinical and zoological application. However, the developmental program of oocytes is not fully understood. If oogenesis is completed *in vitro*, such *in vitro* systems are available for applicative and fundamental studies. In this review, focused on mouse oogenesis, we describe currently optimal *in vitro* systems for the production of functional oocytes. Finally, some potential future applications of these *in vitro* systems are discussed.

Oogenesis

Germ cells are uniquely specialized to transmit the genome to succeeding generations. In mice, primordial germ cells (PGCs) arise at 7.0 days post coitum (dpc), and are presumably specified by the expression of *fragilis* and *stella* genes [1]. PGCs migrate, increase in number and reach the genital ridges, after which the transformation of meiotic oogonia into oocytes by entrance into the meiotic prophase is initiated from 14.5 dpc. By the time of birth, oocytes have reached the diplotene stage of the first meiotic prophase, which lasts until the time of ovulation. Non-growing oocytes become enclosed in a follicle together with single layers of flattened granulosa cells to form primordial follicles. It is known that the factor in germline α (FIGα), a germ cell-specific transcription factor, is required in this process [2]. Primary follicles are recruited from the resting primordial pool as cohorts, enter the growth phase and the surrounding granulosa cells become cuboidal and proliferative. Oocytes promote the proliferation of granulosa cells by the expression of growth differentiation factor 9 (GDF-9) and bone morphogenetic protein 15 (BMP-15), resulting in follicular growth [3, 4]. In mice, the first synchronous follicular growth wave is observed around 5 days post-partum (dpp). A cohort of primary follicles develop into secondary follicles (preantral follicles), which are surrounded by two or more layers of granulosa cells until 8–10 dpp, and KIT ligand is secreted from the granulosa cells [5]. During the early growth stage, stroma cells near the basal lamina of the follicle are arranged parallel to each other to form a theca layer. The cells of the theca layer play a critical role in follicular development and steroidgenesis by secreting the androgens necessary as substrates for granulosa cell estradiol production. Once a follicle reaches a certain size it forms a fluid filled space (antrum) in the transition from preantral to antral follicle. After antrum formation, follicles are acutely dependent upon gonadotrophins for further growth and development. At the time of puberty, follicles developing into Graafian follicles contain fully grown oocytes. Ovulation is induced by the LH surge, and consequently fully grown oocytes resume meiosis and reach the metaphase in the second meiosis. After fertilization, the second meiosis is completed and embryonic development is initiated.

Functional Oocytes

To produce the next generation, the requirement for functional oocytes is critical because oocytes must support many events during preimplantation development (e.g. male pronuclear formation, maintenance of gametic DNA methylation pattern, a burst of gene expression). The competence of the oocytes is mainly classified into three categories: nuclear maturation, genomic or gametic imprinting, and cytoplasmic maturation. Nuclear maturation signifies

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*To whom correspondence should be addressed.

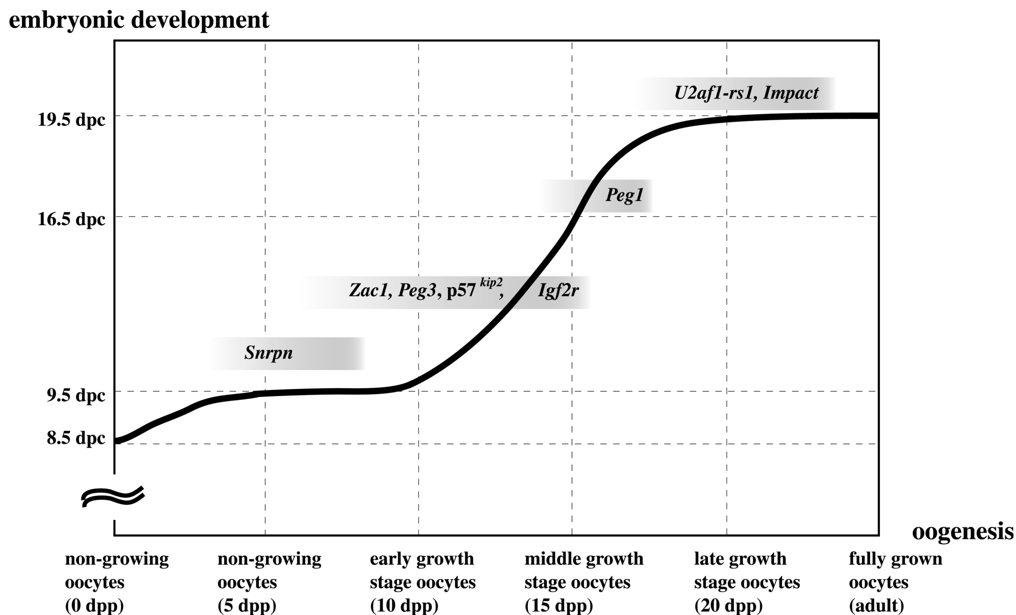

Fig. 1. Developmental competence of the embryos reconstituted with nuclei from growth stage oocytes and progress in oocyte-specific imprinting. The development of embryos reconstituted with nuclei from growth stage oocytes was extended with the oocyte growth. Paternally expressed *Snrpn*, *Zac1*, *Peg3*, *Peg1*, *U2af1-rs1* and *Impact* genes and maternally expressed p57^{kip2} and *Igf2r* genes were imprinted during oocyte growth. Imprinting for each gene was not imposed together at a specific time during oocyte growth but rather occurred throughout the period from primary to antral follicle stage oocytes.

the production of haploid gametes by meiotic division and the accumulation of the components of pronuclei, which upon fertilization give rise to the totipotent embryo [6]. Genomic imprinting leads to parental allele-specific gene expression during ontogeny due to oocyte- and sperm-specific epigenetic modification (e.g. DNA methylation). Therefore, in mammals, sexual reproduction is required, and a lack of genomic imprinting during oogenesis induces embryonic lethality (Fig. 1) [7, 8]. Cytoplasmic maturation is the most critical point affecting developmental competence. During oocyte growth, various mRNAs are transcribed and proteins are produced and accumulated in the cytoplasm. For example, DNA methyltransferase (*Dnmt*) 1 is produced and localized in the oocyte nucleus and/or cytoplasm during oocyte growth. *Dnmt1* is required to maintain embryonic DNA methylation patterns until 7.5 dpc [9]. Furthermore, maturation promoting factor (MPF) and cytostatic factor/*Mos* (CSF; c-mos proto-oncogene) are required for the resumption of the first meiosis and for the arrest at the metaphase in the second meiosis [10]. Also, nucleoplasmin 2 that is produced in the oocyte growth phase is crucial for the chromatin and nucleora organization in the oocyte and the embryo [11]. Previously, we reported that when the nucleus from the late-growth-stage oocyte was introduced into an enucleated fully grown oocyte, these reconstituted oocytes were able to develop into living pups after meiotic division, fertilization and embryo transfer, although the growth-stage oocyte itself was incompetent [8]. This finding indicates that cytoplasmic maturation is the last to be accomplished of the three criteria for functional oocytes.

In Vitro Development of Oocytes

Recently, several studies of the *in vitro* development of mouse oocytes have been reported [13–15]. Eppig *et al.* have established a fundamental technique for the *in vitro* culture of immature mice oocytes. They achieved complete oocyte development *in vitro* beginning with the oocytes in the primordial follicles of newborn mouse ovaries. Their *in vitro* culture system is a two-step strategy. The ovaries of newborn mice, which contain only primordial follicles, are cultured intact for 8 days, and then the differentiated secondary follicles are isolated and cultured for a further 14 days. However, in spite of revisions to their protocol, the developmental

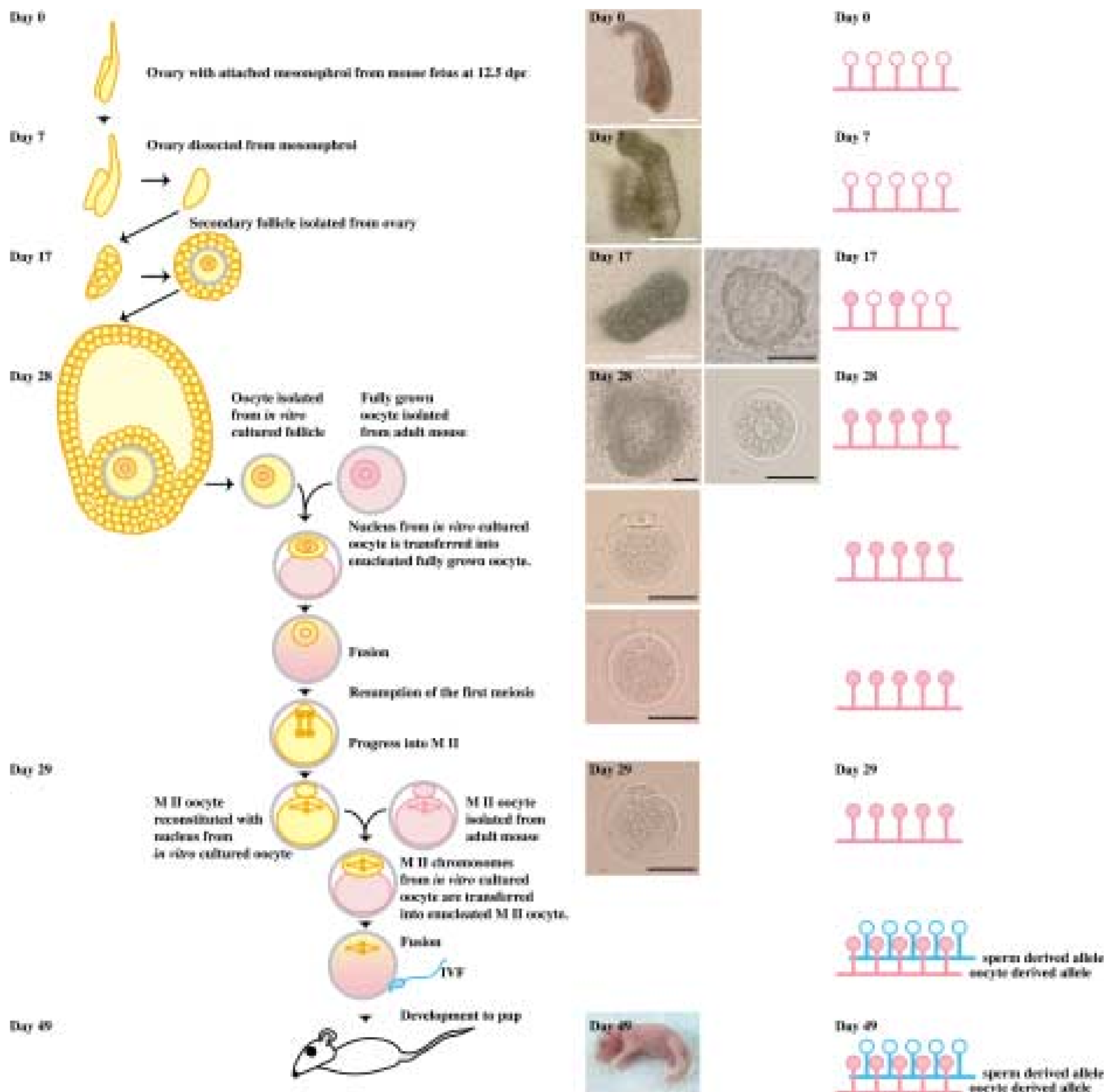

Fig. 2. Schematic diagram showing the production of functional oocytes from premeiotic female germ cells. Yellow oocytes and orange nuclei and chromosomes were derived from *in vitro* cultured oocytes. Pink oocytes, nuclei and chromosomes were derived from adult mice. MII, metaphase in the second meiosis; IVF, *in vitro* fertilization; white bar, 500 μm; black bar, 50 μm; ○ and ○, genes that have not established oocyte-specific imprinting; ●, a gene that has established oocyte-specific imprinting.

competence of these oocytes is quite limited (1.9% of *in vitro* grown oocytes developed to term). On the other hand, we reported that premeiotic female germ cells derived from mouse fetuses as early as 12.5 dpc are able to complete meiosis and genomic imprinting *in vitro*, and that these oocytes are highly competent in supporting the development to full term after nuclear transfer and *in vitro* fertilization (14.8% of reconstituted oocytes developed to term). In our strategy (Fig. 2), the key to success is nuclear transfer, but the culture system is basically the same as Eppig's. Actually, the oocytes isolated from *in vitro* cultured follicles were

small (their mean diameter was 63.9 μm), and these oocytes were not able to resume the first meiotic division. However, we transferred the nuclei into enucleated fully grown oocytes from adult mice, and these reconstituted oocytes were able to mature to the metaphase in the second meiosis. Further, *Igf2r* gene, which is imprinted in the oocyte growth phase, acquired oocyte-specific methylation patterns during the culture period. Consequently, we demonstrated that the nuclei of *in vitro* cultured oocytes are highly competent at supporting full-term development. Very surprisingly, the most recent report showed the derivation of oocytes from embryonic stem (ES) cells *in vitro*. ES cells transfected with *Oct4*, from which the proximal enhancer was deleted to restrict expression in germ cells, were cultured and maintained in ES cell medium without any feeder cells or growth factor. Twelve days later, colonies of variable size had formed, and some of them had formed small aggregates in the supernatant. These aggregates were collected by centrifugation and cultured on a new plate. During the next two weeks, follicle-like structures were formed, produced estradiol, and about 20% of these follicles sustained oocytes larger than 40 μm. Upon further culture, these oocytes formed abnormal zona pellucida and underwent oocyte growth. Finally these oocytes developed to blastocysts parthenogenetically until about the 46th day of the culture period. The authors showed that *Vasa* and *Oct4* genes and proteins were expressed during the early culture period in differentiated ES cells, which correspond to postmigratory germ cells. Furthermore, the transit to meiosis was analyzed by two meiosis-specific markers, the synaptonemal complex protein 3 (SCP3) and the mouse homologue of the yeast meiosis-specific homologous recombination gene (DMC1). However, this report did not demonstrate the factors that directly trigger the differentiation of ES cells into oocytes, and besides, the genotype of the ES cells was XY.

Outlook

We have shown that the most primitive mouse fetal oocytes can differentiate into competent oocytes *in vitro* with high efficiency. Our system and others might eventually contribute to reproductive biology as well as offer an opportunity to analyze the mechanisms behind oogenesis combined with transfection and/or RNAi.

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