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Opinions and Hypotheses

Developmental competence of oocytes grown *in vitro*: Has it peaked already?

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Abstract. *In vitro* growth of immature oocytes provides opportunities to increase gametic resources and to understand the mechanisms underlying oocyte development. Many studies on the *in vitro* growth of oocytes have been reported thus far; however, only a few cases have been reported, which demonstrated that oocytes can support full-term development after *in vitro* fertilization. Our research group recently found that culture of mouse neonatal primordial follicles increased the birthrate; however, the establishment of an *in vitro* system that can completely mimic follicle or oocyte growth *in vivo* and control oogenesis remains an ongoing challenge.
Key words: Follicle culture, *In vitro* growth, Oocyte competence, Organ culture

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Female germ cells in mammals mitotically divide and increase in number in the order of 1×10^4–10^6, and then almost all cells enter meiosis in the fetal stage. Oocytes assemble within the follicles around the time that they undergo meiotic arrest at the diplotene stage of prophase during the first meiotic stage. Meiosis does not resume until just prior to ovulation. Most ovarian follicles are dormant at the primordial stage, and a portion of the follicle pool is introduced into the growth phase. Once follicular growth is activated, only a few follicles are selected to achieve full growth. Consequently, only a small proportion of competent oocytes from the abundant primordial follicles ovulate during a reproductive period. The resulting scarcity of functional oocytes is a major obstacle for the use of oocytes in research and other applications: large-scale omics studies and the preservation of oocytes as gametic resources for livestock and endangered animals are limited. For this reason, the development of *in vitro* systems for the production of competent oocytes from latent ovarian follicles from livestock and rodents has been underway [1–4].

A chronicle of successful production of mammalian functional oocytes *in vitro*

Oocyte growth is accompanied by follicular growth. Once the primordial follicle is activated, a few flattened granulosa cells become cuboidal and increase in number, while the oocytes increase in size. Follicles are classified based on morphology and size: primary follicles are surrounded by a single layer of columnar granulosa cells, while secondary follicles are surrounded by two-layered, preantral follicles are surrounded by multiple layers, and antral follicles are characterized by the formation of an antrum within multiple layers of granulosa cells. *In vitro* growth of immature follicles has been studied mainly in juvenile rodents whose ovaries are large sources of immature follicles that grow synchronously at the same stage until puberty. Based on the follicle stage, *in vitro* systems for oocyte culture require optimal culture conditions and growth factors (Tables 1 and 2).

Secondary or later follicles isolated from ovaries are cultured for 6–17 days in droplets of medium, on a membrane insert set on a well or in gels such as alginate and collagen [5–10]. O'Brien *et al*. demonstrated culture of secondary or preantral follicles of 13 days postnatal (dpn) mice for 10 days on Transwell membrane inserts coated with collagen [7]. Mochida *et al*. also demonstrated embedding and culture of primary or secondary follicles of 6 dpn mice in collagen gels for 9 days to prevent denudation of granulosa cells followed by 8 days of culture on a collagen-coated membrane [5]. In both studies, live mice were successfully obtained from *in vitro*-grown oocytes after *in vitro* fertilization and embryo transfer. The keys to success in these cases were maintenance of the 3-dimensional structure of oocytes and adhesion between the oocyte and granulosa cells. Transwell membrane inserts have frequently been used for follicle culture, and a recent report has also shown that culture of preantral follicles on plastic dishes with a low-attachment surface is also effective for antrum formation of follicles and for eliciting developmental competence of oocytes [11]. In bovines, early antral follicles cultured in a medium supplemented with 4% polyvinylpyrrolidone (PVP) exhibited increased cumulus cell-oocyte complex recovery rates after 14 days of culture, and eventually the authors obtained a live male calf [12]. The developmental abilities of these bovine oocytes were sustained even after somatic cell nuclear transfer [13]. The addition

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Table 1. Culture systems for the production of fertile oocytes in mice

Follicle stages	References	Culture methods	Culture periods (days)	Culture media	
				Base	Supplements
Primordial follicle	[7]	OC on Transwell-COL membrane	8	Waymouth	FBS
		FC on Transwell-COL membrane	8	Waymouth	BSA, fetuin, ITS, FSH, EGF
			6	α-MEM	BSA, fetuin, ITS
	Morohaku *et al*., 2016 (present study)	OC on Transwell-COL membrane	10	α-MEM	FBS, Ascorbic acid
		FC on Millicell membrane	14	α-MEM	FBS, PVP, FSH Ascorbic acid
Primary to secondary follicle	[5]	FC in collagen gel	9	α-MEM	FBS, ITS, FSH, EGF
		FC on Transwell-COL membrane	8	α-MEM	FBS, ITS, FSH
Secondary to preantral follicle	[28]	FC on Transwell-COL membrane	10	α-MEM	FBS, Pyruvic acid
	[6]	FC on Transwell-COL membrane	6	α-MEM	BSA, ITS, fetuin Sodium pyruvate

OC, organ culture; FC, follicle culture; COL, collagen; FBS, fetal bovine serum; BSA, bovine serum albumin; ITS, Insulin-Transferrin-Selenium; PVP, Polyvinylpyrrolidone.

Table 2. Key factors associated with folliculogenesis in mice

Follicle stage	Key factors	References
Primordial follicle	KIT/KL, AMH, PTEN, GREM2	[2, 29, 30]
Primary follicle	BMP-4, BMP-7, GDF-9, GREM2 activin, AMH, NGF	[29–32]
Preantral follicle	BMP-4, BMP-7, GDF-9, NGF activin, EGF, FSH, FGF, TGF-beta	[30–32]
Antral follicle	BMP-4, BMP-7, GDF-9, activin, BMP-6, inhibin, FSH, TGF-beta	[31]

of PVP to the medium used for follicle culture was shown to affect the later stage of follicular development and increased embryo cleavage rates [12]. The PVP, a viscous reagent, appears to prevent diffusion of cytokine and growth factors from around the follicles into the culture medium, thereby facilitating oocyte competency.

For culture of primordial follicles, a combination of organ culture and follicle culture techniques was developed, which was referred to as 2-step culture [14]. Ovarian culture aimed at growing immature follicles has been reported since the late 1960s, which is when Baker and Neal demonstrated that human ovarian pieces and follicles could be cultured for 12 days on lens tissue supported on stainless steel grids in plastic dishes using Eagle's minimal essential medium supplemented with calf serum, gonadotrophins and hormones. The resultant oocytes, however, did not grow *in vitro* [15]. These results indicated that secondary or later follicles cannot be cultured beyond several days due to degeneration of large parts of follicles. Ovarian culture is more effective for primordial follicle culture: Eppig's group cultured newborn mouse ovaries abundant in primordial follicles for 8 days in a thin layer of medium on a Transwell membrane, i.e., using the gas-liquid interphase method (Table 1). Follicles were then isolated from *in vitro*-derived ovaries by collagenase treatment and cultured for 14 days. Using the 2-step culture method, pups were successfully obtained; however, the rate of development of the pups in the first report was low (2/190, pups/2-cell embryos). One of the pups died immediately after birth, while the other, named Eggbert, suffered from obesity in later life. In 2003, Eppig's group revised their culture protocol by reducing the FSH and glucose concentrations in the last step of follicle culture [7]. High glucose concentrations and the combination of FSH and insulin are known to affect preimplantation development *in vitro*. The basal medium used (Waymouth's medium, 27.8 mM glucose) was therefore substituted with alpha-MEM medium (5.5 mM glucose), and the combination of FSH and insulin was eliminated [16]. These changes resulted in the developmental ability of oocytes grown from primordial follicles *in vitro* increasing to 5.7% (66/1160, pups/2-cell embryos), which is comparable to that of oocytes grown *in vitro* from secondary follicles (5.1%, 7/137, pups/2-cell embryos).

On the other hand, a recent study in mice deficient in phosphatase and tensin homolog (PTEN), a major negative regulator of PI3K, reported that the ovaries of the mice showed accelerated follicle activation of all primordial follicles [17]. Consistent with this finding, addition of a PTEN inhibitor in the organ culture media was shown to result in transient oocyte growth in normal neonatal mouse ovaries [18, 19]. These findings have already been applied in reproductive medicine and have helped to generate fertile human oocytes after grafting of PTEN inhibitor-treated ovarian tissues in patients with primary ovarian insufficiency [20].

Recent advance in *in vitro* production of functional oocytes

Based on previous findings [7, 12], we examined a newly assembled system for primordial follicle culture. Ovaries were collected from newborn BDF1 mice and subjected to organ culture for 10 days on Transwell-COL membranes with alpha-MEM containing 10% fetal bovine serum (FBS)

in 5% CO_2 at 37 C. After culture, a total of 635 isolated secondary follicles were subsequently cultured on Millicell membranes with alpha-MEM containing 5% FBS, 0.1 IU/ml FSH and 2% PVP. After 14 days of follicle culture and *in vitro* maturation for 17 h, 526 oocytes underwent germinal vesicle breakdown (GVBD). After *in vitro* fertilization, 162 oocytes that underwent GVBD showed cleavage to the 2-cell stage (Fig. 1). A total of 134 2-cell embryos were transferred to pseudopregnant mice, and 37 live pups were delivered (Figs. 1 and 2). Three pups died after birth, but the others survived to adulthood and exhibited a normal phenotype. Compared with the data from Eppig's group [7], our *in vitro* system for growth of primordial follicles was more successful in the production of offspring. Thus, day by day, a system for *in vitro* growth of oocytes is progressing.

Problems of *in vitro* production of functional oocytes

The oocyte culture system still has some limitations. In the experiments described above, embryo transfer at the blastocyst stage was significantly less likely to result in birth than transfer at the 2-cell stage (14% *vs*. 28%, pups/transferred embryos). Although oocytes were cultured for 26 days from primordial follicles up to the 2-cell stage, another 3 days of culture to the blastocyst stage proved to be more detrimental for the *in vitro*-produced oocytes: approximately half of the 2-cell embryos were found to exhibit arrest of development *in vitro* before embryo transfer at the blastocyst stage, and hence the birth rate calculated from the 2-cell stage was less than 10%. *In vitro*-grown oocytes are therefore highly susceptible to stress during a specific developmental window.

Prior to the revision of our culture method [21], on the other hand, primordial follicles from newborn mice were cultured for 21 days, after which *in vitro*-grown oocytes were subjected to *in vitro* maturation. Most oocytes were, however, unable to resume meiosis unless the nucleus was transferred into the cytoplasm of an enucleated *in vivo*-grown oocyte. A proportion of these reconstituted oocytes did complete meiosis and develop to term after *in vitro* fertilization. This result indicated that nuclei of *in vitro*-grown oocytes can establish genomic imprinting and acquire meiotic and developmental competence, whereas

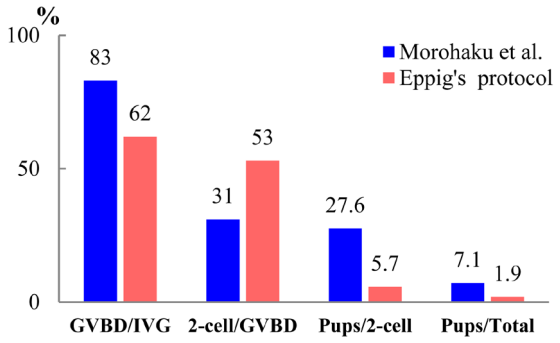

Fig. 1. Developmental competence of mouse oocytes grown *in vitro* from primordial follicles.

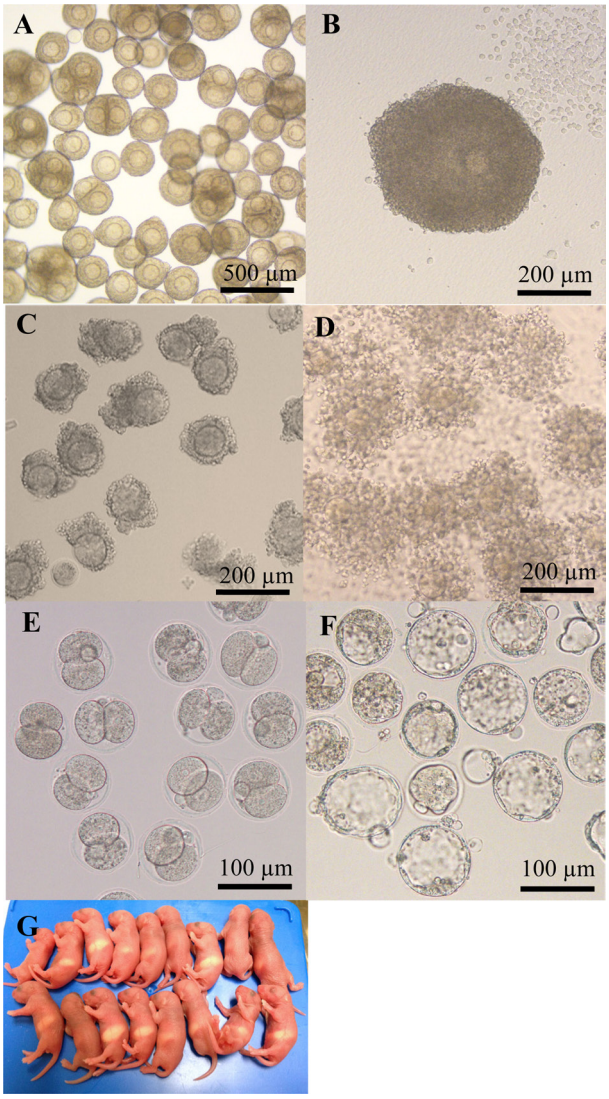

Fig. 2. Development of embryos and offspring derived from a new 2-step culture system. A: Follicles cultured for 3 days on a Millicell membrane following isolation (i.e., day 13 of the total culture period). B: A follicle cultured for 14 days following isolation (at day 24 of the total culture period). C: Isolated cumulus cell-oocyte complexes (COCs) from cultured follicles at day 24. D: Expanded COCs after 17 h of *in vitro* maturation. E, F: Embryonic development after *in vitro* fertilization with BDF1 sperms: 2-cell embryos (E) and blastocysts (F). G: Offspring delivered by caesarean section at 19.5 days post coitum.

the cytoplasm of *in vitro*-grown oocytes lack some materials needed for resumption of meiosis and initiation and maintenance of embryonic development. Although the precise mechanisms underlying the acquisition of developmental competence remain unclear, our current culture method would improve the quality of oocyte cytoplasm to some extent. Other than cytoplasmic maturation, it is possible that epigenetic mutation may occur during the culture period, as suggested by Eggbert's phenotype and the loss of embryos during implantation.

Future prospects

To understand the characteristics of oocytes grown or matured *in vitro*, transcriptome analyses have been performed using microarrays. There has, however, been little evidence to explain low competence of *in vitro*-produced oocytes. This is presumably due to the characters of individual oocyte being masked in analyses of pools of competent and incompetent oocytes [22, 23] and/or to mRNA levels being less faithful to protein levels, since the length of the poly(A) tail modulates maternal mRNA translation [24, 25]. Refinements of the methods used for transcriptome analyses may reveal the molecular basis for acquisition of cytoplasmic competence.

Most recently, Pfender *et al*. reported the application of a live-cell imaging system using *in vitro* growth of oocytes and RNAi screening to recognize genes essential for meiosis [26]. In order to understand the entire developmental processes of oocytes, complete recapitulation of oogenesis is required, and although several attempts to culture primordial germ cells (PGCs) have been reported, there is no paper demonstrating resultant oocytes that were competent to develop to term [27]. The production of the functional oocytes *in vitro* from PGCs in fetal mouse ovaries is therefore challenging.

Another new challenge is to grow primordial follicles isolated from adult rodents and other animals and not from neonatal ones. This would be highly applicable in human reproductive medicine, as it would enable the recruitment of non-growing oocytes from ovaries with premature ovarian failure and subsequent *in vitro* growth without graft surgery. *In vitro* oogenesis is therefore of great interest for future investigations.

The culture methods for the production of functional oocytes *in vitro* have not changed much since they were first reported in the late 1980s. The culture medium used is one of several important conditions that must be optimized for *in vitro* growth of oocytes, and there is certainly still room for improvement. A chemically defined medium, in particular, may be needed to determine factors essential for functional oocytes. Further studies are required to establish an *in vitro* system that physiologically mimics follicle/oocyte growth *in vivo* and can control oogenesis completely.

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