In vitro growth of bovine oocytes

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Abstract

A large number of small oocytes are contained in the mammalian ovary. However, only a small number of oocytes grow to their final size, mature, and are ovulated. In vitro growth (IVG) of small oocytes would provide a potential source of mature eggs for livestock production and contribute to assisted reproduction in humans. In 2016, baby mice were successfully produced from oogonia in fetal gonads, and even from ES cells and iPS cells. On the other hand, there are significant hurdles to overcome before full establishment of IVG systems for domestic species. IVG culture systems have been improved, and now bovine growing oocytes collected from early antral follicles are able to grow to their final size and acquire full developmental capacity. IVG of small oocytes will provide a new understanding of the mechanisms regulating oogenesis and folliculogenesis in the complex mammalian ovary.

Introduction

Female amphibians and fish spawn thousands of eggs which have grown in their ovaries as oocytes. Female mammals also contain a huge number of oocytes in their ovaries. However, as the mammals are viviparous and have a long reproductive life, only a selected number of oocytes grow up to the final size, mature and are ovulated.

Oocytes grow in the ovarian follicles. In the ovary, follicles vary in size and morphology, and the majority of the follicles have a small uniform size. These are primordial follicles composed of an oocyte and a layer of flat-shaped granulosa cells (pre-granulosa cells). Larger-sized follicles are developing follicles which contain a growing oocyte and multi-layered cuboidal granulosa cells. In cattle, approximately a hundred thousand primordial follicles are contained in a female animal (Erickson, 1966; Gosden and Telfer, 1987). A small population of oocytes in the primordial follicles grow and reach the final size, in turn, a species-specific number of fully grown oocytes resume meiosis and mature to the second metaphase (MII) after gonadotropic stimulation. Thus, only a limited number of oocytes are ovulated from the ovaries throughout the female reproductive life.

We don't know whether all of the mammalian oocytes in the ovaries have the ability to grow up and develop to babies as in amphibians and fish. *In vitro* growth (IVG) culture systems of mammalian small oocytes are thought to provide new fundamental knowledge that may help to answer the question. Furthermore, IVG of small oocytes would provide a new source of mature eggs for livestock production by using existing assisted reproductive technologies, such as *in vitro* maturation (IVM) and *in vitro* fertilization (IVF) of oocytes (Fig. 1). In this review, I introduce the recent progress in IVG systems, and discuss about the hurdles have yet to overcome before establishment of IVG systems for domestic species.

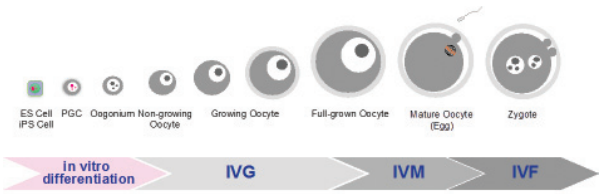

Figure 1. Schematic flow of *in vitro* growth (IVG), *in vitro* maturation (IVM) and in *vitro* fertilization (IVF) of mammalian oocytes.

Baby mice production by IVG

In 1996, Eppig and O'Brien demonstrated that mouse oocytes from primordial follicles grew to the final size by IVG, undergo fertilization *in vitro*, and develop to a live born pup (Eppig and O'Brien, 1996). They used a combination of two culture systems for the oocyte growth. First, they cultured newborn mouse ovaries containing only primordial follicles in organ culture for 8 days and recovered growing oocytes surrounded by granulosa cells (OGCs). The OGCs were further cultured for 14 days, and recovered fully-grown oocytes were matured, and fertilized *in vitro*. The resulting 2-cell embryos derived from non-growing oocytes in primordial follicles were then transferred into pseudo pregnant females, and one baby mouse was obtained. The efficiency of the procedure was low, but the birth suggested the possibility that small oocytes in primordial follicles provide a potential source of oocytes for IVF. The culture methods have been improved. In 2016, baby mice were produced from oogonia in fetal gonads (Morohaku et al., 2016), and from ES cells and iPS cells (Hikabe et al., 2016) ("*in vitro* differentiation" in Fig. 1).

Calf production by IVG

In contrast to the mouse results, there are significant hurdles to overcome before full establishment of IVG systems for domestic species. Mouse oocytes grow from 15-20 μm in diameter (excluding the zona pellucida) to 75 μm, while bovine oocytes grow from 30 μm to 120-125 μm (Hyttel et al., 1997). We developed an IVG system that supported growth of bovine growing oocytes (90-99 μm) for 2 weeks and produced a baby calf in 1999 (Yamamoto et al., 1999). Briefly, early antral follicles (0.5-0.7 mm in diameter) were dissected from bovine ovaries, and oocyte-cumulus complexes which contained pieces of parietal granulosa cells (OCGs) were collected from the follicles. OCGs were embedded in collagen gels and cultured for 2 weeks in TCM199 containing fetal calf serum and hypoxanthine. After IVG culture, surviving oocytes were further cultured for maturation and subsequently inseminated with spermatozoa. Resulting blastocysts were transferred to recipient cows and one cow delivered a live calf. The results first demonstrated that *in vitro*-grown bovine oocytes acquired full developmental ability to produce live young.

Problems in the IVG system

For the large species, no IVG systems supporting the entire developmental course from the primordial to the fully-grown stage have yet been established. Although production of calves by IVG using growing oocytes from early antral follicles has been reported, the yield was quite low. To establish the IVG systems which enable growing oocytes even at the mid-growth stage to grow to their final size and acquire full developmental capacity, we have to overcome some problems as follows.

1) Detachment of granulosa/cumulus cells from oocytes

In the ovary, oocytes grow accompanied with follicular development. In the follicle, there is bidirectional communication between oocytes and surrounding granulosa cells (cumulus cells after antral stage) (Matzuk et al., 2002). These somatic cells contact with oocytes through cytoplasmic processes penetrating the zona pellucida and establish gap junctions with the oolemma. Gap junctions efficiently transfer small molecules such as energy substrates, nucleotides, and amino acids into the oocyte (Brower and Schultz, 1982). On the other hand, oocytes secrete oocyte specific growth factors, growth differentiation factor 9 (GDF9) and bone morphogenetic protein 15 (BMP15) to regulate granulosa cells.

Therefore, it is essential for IVG of oocytes to maintain the communication between the oocyte and its companion granulosa/cumulus cells during the culture period.

Cultured granulosa cells prefer the plastic dish rather than the oocyte. When bovine OCGs are cultured in a dish, granulosa cells migrate away from the oocytes and spread onto the bottom of the dish, with the oocytes subsequently becoming denuded and degenerated. When OCGs are embedded in collagen gels and cultured, granulosa cells surround cumulus cells to continue enclosing the oocytes, and the OCGs form antrum-like structures in the gels. Hirao et al. (2004) improved IVG culture medium which contained a high concentration of polyvinylpyrrolidone (PVP). In the medium, granulosa cells proliferated and the complexes made dome-like structures, and a high percentage of oocytes grew to the full size in the structures. Although both collagen gels and PVP prevent the detachment of granulosa/cumulus cells from the oocytes, PVP medium is superior to the collagen-gel system. Collagen gels lose transparency and shrink after a long-term culture. His group reported the second successful production of baby calf by this new culture system.

2) Oocytes resume meiosis before growing to the full size

In the IVG culture of bovine oocytes, supplementation with hypoxanthine is essential (Harada et al., 1997). Hypoxanthine is a component of follicular fluids and is identified as a meiotic-arresting substance by means of an inhibitory action on cAMP-phosphodiesterase (Downs et al., 1985; Eppig and Downs, 1987). Hypoxanthine promotes association between oocytes and granulosa cells, and increases the percentages of surviving oocytes. Furthermore, it maintains oocytes are at the germinal vesicle stage. If growing oocytes resume meiosis before growing to the full size during IVG, they degenerate. Therefore, oocyte meiotic arrest must be maintained in IVG. For bovine growing oocytes having a larger size (105-115 μm), hypoxanthine is not enough to maintain the meiotic arrest. They require additional oocyte-specific phosphodiesterase 3A inhibitor, such as cilostamide and milrinone (Alam et al., 2018).

3) Low survival rate and maturation rate of oocytes

PVP medium maintains viability of bovine growing oocytes to some extent. Steroid hormones, such as 17β-estradiol and androstenedione further increase oocyte viability (Taketsuru et al., 2012). Androstenedione also promotes the acquisition of oocyte meiotic competence efficiently (Makita and Miyano, 2015). Gaseous phase in the IVG culture (20% O_2 or 5% O_2) also affects oocyte viability (Hirao et al., 2012). Now, we culture bovine growing oocytes in the medium containing a mixture of estradiol and androstenedione (Makita and Miyano, 2014), under the 5% O_2 for the first-half period of IVG then in 20% O_2. In the system, 70-80% of oocytes grow to the full size after 2 weeks; about 80% of the *in vitro* grown oocytes mature to MII, fertilize normally, and develop to blastocysts at the similar percentage to *in vivo* grown oocytes (Makita et al., 2016).

Conclusions

As described here, *in vitro*-grown bovine oocytes from early antral follicles acquire full developmental ability to produce live young. Although IVG technologies are still at the experimental stage, the efficiency is increasing. As IVM and IVF systems have provided new understandings of the mechanisms regulating maturation and fertilization of mammalian oocytes, IVG systems will provide the answer to the basic questions in oocyte/ follicle physiology including a classical question of "oocyte/ follicle selection" in the ovary.

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