

IN VITRO GROWING IMMATURE PORCINE OOCYTES

Yuji Hirao
Animal Breeding and Reproduction Research Division,
NARO Institute of Livestock and Grassland Science, Tsukuba, Ibaraki, Japan
e-mail: yujih@affrc.go.jp

ABSTRACT

Porcine ovaries contain a large number of oocytes at different stages of growth, most of which degenerate either before or at various stages of growth. To utilize those potential female gametes stored in the ovary, it is important to develop culture system that can provide a suitable alternative environment for oocytes to achieve full growth and competences to undergo meiotic maturation, fertilization, and embryonic development. Several culture systems have been developed for the porcine oocytes, and studies have demonstrated that a proportion of the oocytes grown in vitro can acquire the competence to undergo meiotic maturation.

Keywords: Porcine Oocyte, Oocyte–granulosa Cell Complexes, Oocyte Growth, *In vitro*

INTRODUCTION

In the mammalian ovary, a small proportion of primordial oocytes periodically enter the growth phase, of which even fewer grow and mature. Because of this reduction in the number of oocytes before acquiring the competences to undergo meiotic maturation, fertilization, and embryogenesis, it is impossible to utilize oocytes in the same manner in which we use sperms. To utilize the potential female gametes stored in ovaries, it is important to develop culture system that can provide a suitable alternative environment for oocytes to survive by escaping degeneration in the ovary.

In the pig, several culture systems have been developed for oocyte growth in vitro. The starting materials used for those cultures were usually secondary follicles or early antral follicles containing oocytes that were still necessary to grow for the latter half of the growth period. Although normal offspring have been produced from such oocytes in mice (Eppig and Schroeder 1989) and cattle (Hirao *et al.* 2004), no piglets have been produced to date from oocyte grown in vitro.

Three Types of Culture Systems for Growing Porcine Oocytes

The follicles used for culture are isolated from the ovary either mechanically or enzymatically. When collagenase is used on the secondary follicles, theca cell layers become generally removed, leaving behind “oocyte–granulosa cell complexes.” Early antral follicles are dissected from the surfaces of the ovaries, and complexes consisting of oocytes and cumulus/granulosa cells (oocyte–granulosa cell complexes) are mechanically extracted from the follicles.

The culture systems developed for the growing oocytes in pigs can be divided into two types depending on whether follicles/oocyte–granulosa cell complexes spread on a substratum or maintain a spherical shape. The typical

morphologies of follicles and oocyte–granulosa cell complexes that developed after culture are shown in Fig. 1.

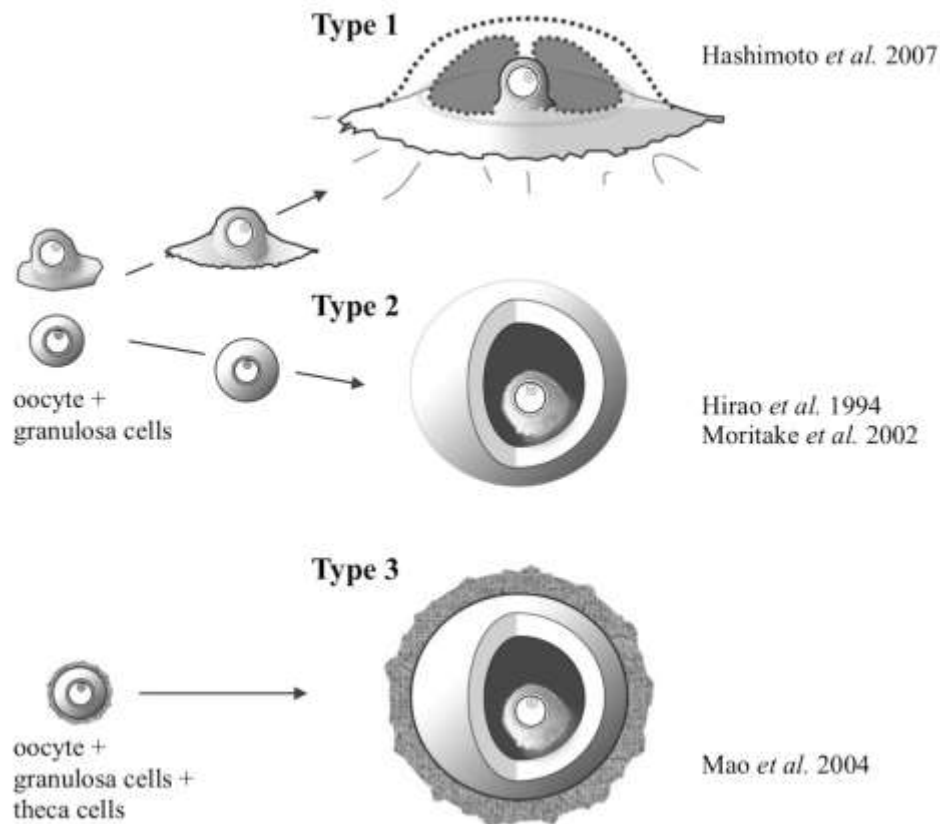


Fig. 1. Three typical morphologies of the follicle or oocyte–granulosa cell complex after long-term culture.

When a flat substratum is used, oocyte–granulosa cell complexes adhere to the substratum and growth continues until oocyte–granulosa cell complexes form a dome-like structure (Hashimoto *et al.* 2007). On a substratum to which cells hardly attach, or when matrices such as collagen are used to embed the follicle/oocyte–granulosa cell complexes, follicle/oocyte–granulosa cell complexes grow in size (usually turn into a sphere) and develop an antral cavity when subjected to appropriate stimulation (Hirao *et al.* 1994; Mao *et al.* 2004). This sphere type is further divided into two types depending on whether the follicle is intact, i.e., it has the basement membrane and theca cells, or the complex consists of only granulosa cells and an oocyte (Fig. 1).

Derivations and specific characteristics of each type are as follows:

Type 1: Oocyte–granulosa cell complexes grow on the substratum and develop a dome-like structure.

Type 2: Oocyte–granulosa cell complexes or preantral follicles without theca cells grow into a sphere and form the antral cavity. Usually the follicles/complexes are entirely embedded in (and adherent to) a substrate, such as collagen or alginate-based matrices, in order to achieve a 3-D culture.

Type 3: Intact follicles sustained on a dish surface or in a 3-D matrix and form the antral cavity.

Most importantly, regardless the types listed above, the systems for oocyte growth should meet the following three

conditions (Hirao 2011): 1) oocytes remain viable and functional to pursue the intrinsic oocyte program that directs growth and development; 2) granulosa cells proliferate to avoid oocyte denudation; and 3) granulosa cells should maintain and develop functions necessary for supporting oocyte growth, with particular emphasis on establishment of appropriate oocyte–granulosa cell interactions. We consider these conditions represent the essence of the culture system established by Eppig and Schroeder (1989) to grow mouse oocytes, and if any of these conditions are negated, oocytes will not be able to achieve full growth.

Acquisition of Meiotic Competence *In Vitro*

Like most studies on the mammalian oocytes, the methods used for oocyte growth *in vitro* have progressed from laboratory mice to larger animals. However, a porcine oocyte *in vivo* eventually becomes much larger than that of mouse (about 125 μm vs. 75 μm in diameter), which continues to make it difficult to prepare fully grown oocytes *in vitro* in this species by simply applying the culture system used for the mouse oocytes.

In addition, a large amount of research indicates that oocytes acquire various essential competences during the final stage of growth. Thus, not only the mass (oocyte size) but also the quality of oocytes is crucial to obtain functional ova. The germinal vesicle of the porcine oocytes of 80 μm and smaller in diameter contained diffuse filamentous chromatin, and these oocytes are incompetent to resume meiosis (Hirao *et al.* 1995). Those of about 105 μm , containing thickened but still stringy chromatin, have only a partial competence, because those oocytes can resume meiosis (germinal vesicle breakdown), but the progression of meiosis ceases at or before metaphase I (Hirao *et al.* 1995). The competencies to reach metaphase II develop in oocytes of about 115 μm in diameter (Hirao *et al.* 1995). After reaching the final size, formation of chromatin perinucleolar rim appears to be correlated with the competence of oocytes to progress to metaphase II.

The first study of *in vitro* growth of porcine oocytes were conducted using oocytes of 70.0-89.5 μm in diameter, and a proportion of the oocytes grew up to their final size and acquired meiotic competence *in vitro* (Hirao *et al.* 1994). Preantral follicles were isolated from ovaries and cultured in collagen gel (Type 2 system) for up to 16 days, in the presence of serum, follicle-stimulating hormone (FSH) and estradiol. Thirty to forty percent of the oocytes were viable after the 16 day-culture period, and 40% of the surviving oocytes that reached 110 μm or larger were able to mature to the metaphase II stage. Some of the matured oocytes underwent fertilization, but failed to develop a male pronucleus (Hirao *et al.* 1994).

Type 1 system has also been developed for the porcine oocytes with the help of a high concentration of polyvinylpyrrolidone (PVP). PVP exerts a profound influence on the morphology of bovine oocyte–granulosa cell complexes (Hirao *et al.* 2004), which was realized using a substrate-adherent model similar to that of the rodent models (Type 1). In the pig, a dome-like structure was developed by oocyte–granulosa cell complexes isolated from early antral follicles (Hashimoto *et al.* 2007). Furthermore, 25% of the oocytes that had survived the 14-day culture period were competent to progress to the metaphase II stage (Hashimoto *et al.* 2007).

Effects of FSH and Growth Factors

It is established that FSH has essential roles in the female reproduction system. The effect of FSH in porcine oocyte–granulosa cell complexes has been suggested in a study in which the complexes cultured in Type 2 system survived better for 7 days in medium containing FSH than in medium without FSH (Moritake *et al.* 2002). In the same study, porcine oocytes grown in the presence of 2 mM hypoxanthine showed a higher competence of progression to the metaphase II stage than in medium without hypoxanthine (Moritake *et al.* 2002). However, there was no profound effect of hypoxanthine on viability during oocyte growth.

Insulin-like growth factor-I (IGF-I) is also a major growth factor known to be involved in regulation of the follicular development. It has been suggested that IGF-I modulates the responsiveness of granulosa cells to FSH (Monget *et al.* 2002). Using Type 3 culture system, Mao *et al.* (2004) found that IGF-I stimulated follicular growth. In particular,

IGF-I promoted granulosa cell proliferation, maintenance of follicular integrity, and even recovery rate of oocytes. On the other hand, IGF-I reduced apoptosis of granulosa cells. In the presence of FSH and serum, IGF-I and epidermal growth factor (EGF) exhibited a promoting effect of granulosa cell proliferation; however, this effect was negated in serum-free medium, where exposure to EGF and IGF-I resulted in increased apoptosis (Mao *et al.* 2004).

CONCLUSION

A body of research has indicated that porcine oocytes grown *in vitro* for the latter half of their growth period can acquire the competence to undergo meiotic maturation. However, the oocytes grown in any systems described above continue to be on average smaller than those fully grown in the ovary. Thus, it is clear that further improvements to existing culture systems are necessary to produce offspring derived from porcine oocytes grown *in vitro*.

REFERENCES

- Eppig, J.J. and A.C. Schroeder. 1989. Capacity of mouse oocytes from preantral follicles to undergo embryogenesis and development to live young after growth, maturation, and fertilization *in vitro*. *Biology of Reproduction* 41: 268-276.
- Hashimoto, S., K. Ohsumi, Y. Tsuji, N. Harauma, Y. Miyata, A. Fukuda, Y. Hosoi, A. Iritani, Y. Morimoto. 2007. Growing porcine oocyte-granulosa cell complexes acquired meiotic competence during *in vitro* culture. *Journal of Reproduction and Development* 53: 379-384.
- Hirao, Y. 2011. Conditions affecting growth and developmental competence of mammalian oocytes *in vitro*. *Animal Science Journal* 82: 187-197.
- Hirao, Y., T. Nagai, M. Kubo, T. Miyano, M. Miyake, S. Kato. 1994. *In vitro* growth and maturation of pig oocytes. *Journal of Reproduction and Fertility* 100: 333-339.
- Hirao, Y., Y. Tsuji, T. Miyano, A. Okano, M. Miyake, S. Kato, R.M. Moor. 1995. Association between p34^{cdc2} levels and meiotic arrest in pig oocytes during early growth. *Zygote* 3: 325-332.
- Hirao, Y., T. Itoh, M. Shimizu, K. Iga, K. Aoyagi, M. Kobayashi, M. Kacchi, H. Hoshi, N. Takenouchi. 2004. *In vitro* growth and development of bovine oocyte-granulosa cell complexes on the flat substratum: effects of high polyvinylpyrrolidone concentration in culture medium. *Biology of Reproduction* 70: 83-91.
- Mao, J., M.F. Smith, E.B. Rucker, G.M. Wu, T.C. McCauley, T.C. Cantley, R.S. Prather, B.A. Didion, B.N. Day. 2004. Effect of epidermal growth factor and insulin-like growth factor I on porcine preantral follicular growth, antrum formation, and stimulation of granulosa cell proliferation and suppression of apoptosis *in vitro*. *Journal of Animal Science* 82: 1967-1975.
- Moritake, S., Y. Hirao, T. Miyano. 2002. Hypoxanthine promotes the acquisition of meiotic competence in pig oocytes from early antral follicles during growth culture. *Journal of Mammalian Ova Research* 19: 39-45.
- Monget, P., S. Fabre, P. Mulsant, F. Lecerf, J-M. Elsen, S. Mazerbourg, C. Pisselet, D. Monniaux. 2002. Regulation of ovarian folliculogenesis by IGF and BMP system in domestic animals. *Domestic Animal Endocrinology* 23: 139-154.