Journal of Reproduction Engineering
Vol.10, Supplement, 436-448, 2007

Xenografting of porcine immature germ cells and their developmental competence

Hiroyuki Kaneko[1], Kazuhiro Kikuchi[1], Michiko Nakai[1,2], Naoki Maedomari[3] and Naomi Kashiwazaki[3]

[1] Reproductive Biology Research Unit, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan.

[2] Research Fellow of the Japan Society for the Promotion of Science, Chiyoda, Tokyo 102-8471, Japan.

[3] Laboratory of Animal Reproduction, Graduate School of Veterinary Medicine, Azabu University, Sagamihara, Kanagawa 229-8501, Japan.

ABSTRACT

Immature germ cells, for example oocytes in primordial follicles (primordial oocytes) in the female and spermatogonia in the male, are potential sources of fertile gametes for agricultural, zoological and medical purposes. Cross-species grafting (xenografting) of gonadal tissues provides an effective method for maturation of germ cells of large mammals. In this short review, we describe our trial attempts to endow immature germ cells in porcine gonads with developmental ability using a combination of xenografting and subsequent *in vitro* culture. We have successfully generated porcine blastocysts derived from oocytes or spermatozoa grown in host mice; however, further studies will be required in order to obtain progeny from these grafted germ cells.

Key words: *xenografting, immature germ cells, developmental ability*

Received for publication: Sep.10, 2007

Accepted: Oct.14, 2007

*Correspondence: Dr. Hiroyuki Kaneko

INTRODUCTION

In 1989, Gosden et al. [1] first demonstrated that sheep ovarian grafts not only survive for many months in mice with severe combined immunodeficiency (SCID) but also show development of follicles to the antral stage. This pioneering study suggested that the model might be applicable to a wide range of animals, and that follicles developing in the xenografts might contain fertile oocytes. Snow et al. [2] reported that oocytes growing within mouse ovarian tissue grafted into nude rats acquired the ability to develop into pups. Ovarian tissues have been prepared from species phylogenetically distant from the mouse, including human [3-6], dog [7], monkey [8], ox [9], pig [10, 11], tammar wallaby [12] and common wombat [13, 14], and grafted into immunodeficient mice. To our knowledge, only one study [10] of xenografting of non-rodent ovarian tissues, in which neonatal pig ovarian tissues were used, has proven that primordial oocytes can grow in host mice and acquire fertilization ability *in vitro*. Recently, our group was successful in producing blastocysts from porcine primordial oocytes grafted into nude mice [15]. On the other hand, Honaramooz et al. [16] first reported the completion of spermatogenesis in pig and goat testicular tissues that had been transplanted into host mice. Although sperm have been isolated from testicular grafts of several species grown in host mice (pig [16], goat [16], cat [17] and rhesus monkey [18]), blastocysts were obtained only from sperm isolated from grafts of monkey testis by intracytoplasmic sperm injection (ICSI) into monkey oocytes [18].

Here we describe a series of studies we have conducted to develop a method of endowing primordial oocytes or spermanogonia/gonocytes with full developmental competence as a model for gonadal xenografting of large mammals, and discuss the possibility of improving this method further.

Developmental ability of porcine primordial oocytes grafted into nude mice

1. *In vitro* maturation and fertilization

of porcine primordial oocytes after xenografting

To date, there has been little information about the developmental competence of non-rodent oocytes that have survived in host mice; one of the most probable reasons is that few viable oocytes have been recovered from xenografts. Therefore our first objective was to develop a method for obtaining a large number of oocytes with full maturation and fertilization ability from host mice [10]. In ovarian tissues obtained from 20-day-old piglets, most of the follicles were found to be primordial (Fig. 1).

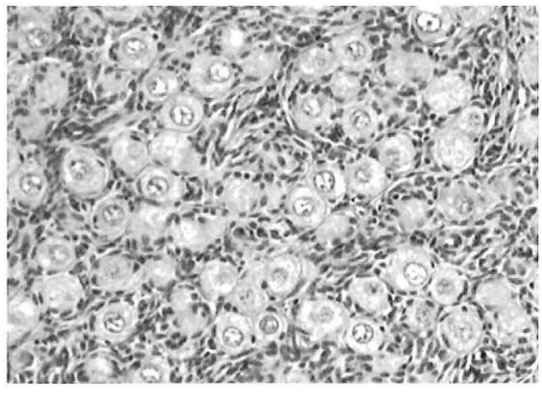

Fig. 1. Histological appearance of the cortex area of a neonatal donor porcine ovary before grafting. Bar indicates 100 μm. Modified from Kaneko et al. [10].

These tissues were transplanted under the capsules of both kidneys of ovariectomized nude mice. The host mice were treated with 5 IU of equine chorionic gonadotropin (eCG) at 10 days (eCG-10), 30 days (eCG-30), or 60 days (eCG-60) after detection of cornified epithelial cells in their vaginal smears. Oocytes were recovered from the grafts 48 h after eCG treatment and transferred to *in vitro* culture systems [19] for assessment of their maturational and fertilization abilities.

Forty-five to 70 days after grafting, the host mice in all groups showed for the first time vaginal cornification, accompanied by formation of a small number of antral follicles in the grafts. However, when the grafts were recovered after treatment of the host mice with eCG at 60 days after vaginal cornification, they contained many antral follicles (Fig. 2).

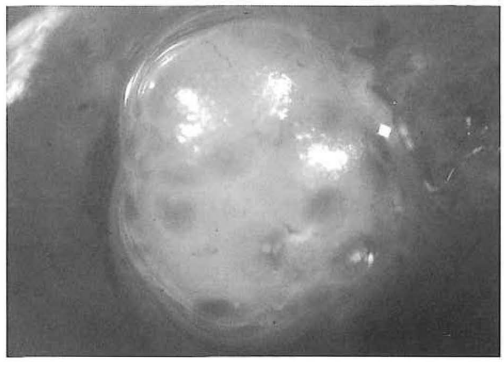

Fig. 2. Porcine ovarian tissue grafted under the renal capsule of a nude mouse in the eCG-60 group, which received 5 IU of equine chorionic gonadotropin 60 days after detection of vaginal cornification, and recovered 48 h later. Bar indicates 1 mm. Modified from Kaneko et al. [10].

We recovered large numbers of full-sized oocytes (≥115 μm in diameter) only from mice in the eCG-60 group (20 oocytes/ mouse) (Fig. 3);

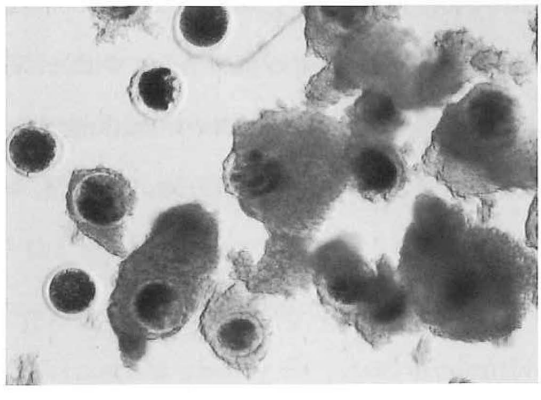

Fig. 3. Cumulus-oocyte complexes recovered from a mouse in the eCG-60 group. Bar indicates 200 μm. Modified from Kaneko et al. [10].

the numbers of full-sized oocytes in the other groups were low (2-8 oocytes/ mouse). Of 573 oocytes obtained from the eCG-60 group, 98 (17%) were at the metaphase-II stage after *in vitro* culture for maturation. Moreover, 55% (11/20) of matured oocytes showing the first polar body were fertilized *in vitro* resulting in both male and female pronucleus formation (Fig. 4).

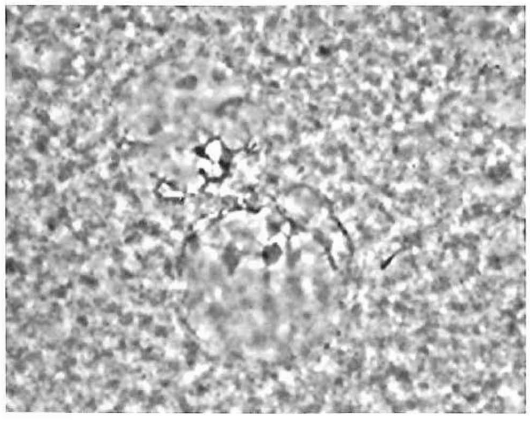

Fig. 4. Fertilization of porcine oocytes recovered from grafts in the eCG-60 mouse group. FPn, female pronucleus; MPn, male pronucleus. Arrow indicates a sperm tail associated with a male

pronucleus. Bar indicates 25 μm. Modified from Kaneko et al. [10].

We were able to obtain more oocytes from porcine ovarian grafts by optimizing the timing of eCG treatment of the host mice. These results also indicate that fertilization of porcine primordial oocytes is achievable by a combination of xenografting and *in vitro* culture.

2. Improvement of developmental competence of porcine primordial oocytes by treatment of host mice with FSH.

The above study [10] showed it was possible to recover many full-sized oocytes derived from porcine primordial follicles grafted into nude mice. However, these recovered oocytes did not reach the blastocyst stage when they were matured and fertilized *in vitro* and immediately transferred to the oviducts of estrous-synchronized recipient gilts [20]. This suggests that the oocytes grown in host mice may not acquire full cytoplasmic maturation for embryonic development, even after 2 days of eCG treatment of the host mice and *in vitro* culture of the oocytes. One strategy for improving the developmental competence of oocytes within xenografts is to facilitate oocyte development by accelerating follicular growth with a more effective hormone treatment. In the next study, therefore, we subjected host mice to several hormonal treatments to promote follicular growth in the xenografts, and evaluated the influence of these treatments on the meiotic and developmental competence of porcine primordial oocytes in *in vitro* embryo production systems [15]. Gonadotropin treatments were started around 60 days after vaginal cornification in mice that had received ovarian tissues prepared from 20-day-old piglets. Ovarian grafts were obtained 2 or 3 days after treatment with eCG (eCG-2 or eCG-3 group), or after porcine FSH infusion with osmotic pumps for 7 or 14 days, or after infusion of porcine FSH for 14 days with a single injection of estradiol antiserum (FSH-7, FSH-14, or FSH-14EA groups, respectively).

Compared with the follicle size in control mice given no gonadotropins (Fig. 5a), gonadotropin treatment, especially

treatment with FSH for 7 days or more, promoted follicular growth within the xenografts (Figs. 5b-e); the sizes of several antral follicles in the FSH groups were similar to those of antral follicles observed in the ovaries of prepubertal gilts (Fig. 5f), from which oocytes are usually used for *in vitro* production of embryos [19]. However, in the FSH-14 group, follicular antra were frequently filled with erythrocytes (Fig. 5e).

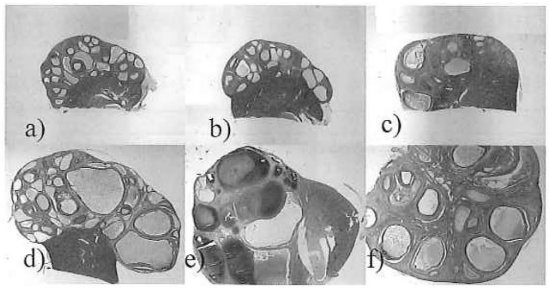

Fig. 5. Histological appearance of porcine ovarian xenografts at low magnification in host mice in the (a) control, (b) eCG-3, (c) FSH-7, (d) FSH-14 and (e) FSH-14EA groups. (f) Image of a section of the ovary obtained from a prepubertal gilt. Ovarian grafts were examined 3 days after eCG treatment (eCG-3), or after infusion of porcine FSH for 7 (FSH-7) or 14 days (FSH-14), or after infusion of porcine FSH for 14 days with a single injection of estradiol antiserum 7 days after the beginning of FSH infusion (FSH-14EA). Control mice received no gonadotropin treatment. Bars indicate 5 mm. Modified from Kaneko et al. [15].

We recovered large numbers of full-sized oocytes (≥115 μm in diameter) with meiotic competence to the mature stage from the eCG-3, FSH-7, and FSH-14EA groups, whereas small numbers of oocytes were obtained from the control group (Figs. 6 and 7).

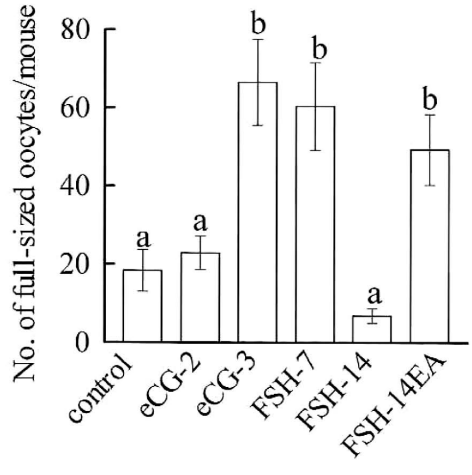

Fig. 6. Numbers of full-sized oocytes (≥115 μm in diameter) recovered from host mice that had received hormonal

treatment. The number of oocytes in each group is represented as the mean ± SEM per mouse. [a-b] Values without common superscripts are significantly ($P<0.05$) different. Modified from Kaneko et al. [15].

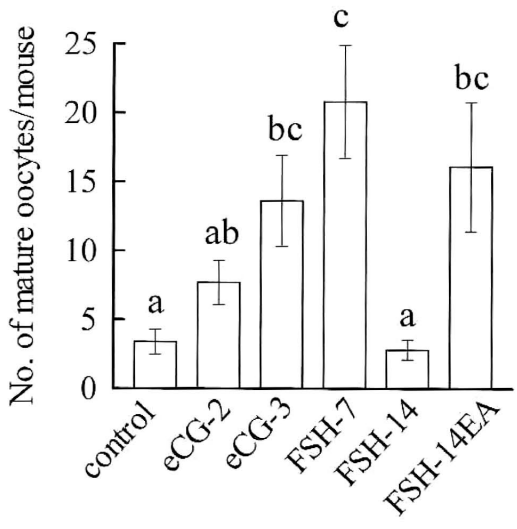

Fig. 7. Numbers of oocytes with meiotic competence recovered from host mice that had received hormonal treatment. The number of oocytes in each group is represented as the mean ± SEM per mouse. [a-c] Values without common superscripts are significantly ($P<0.05$) different. Modified from Kaneko et al. [15].

Approximately 35% of the full-size oocytes matured in the FSH-7 and FSH-14EA groups, whereas the ratio was about 20% in the control and eCG-3 groups. After *in vitro* fertilization and subsequent culture for 7 days, one blastocyst was obtained from each 100 oocytes of the eCG-3, FSH-7 and, FSH-14EA groups (Figs. 8a and b),

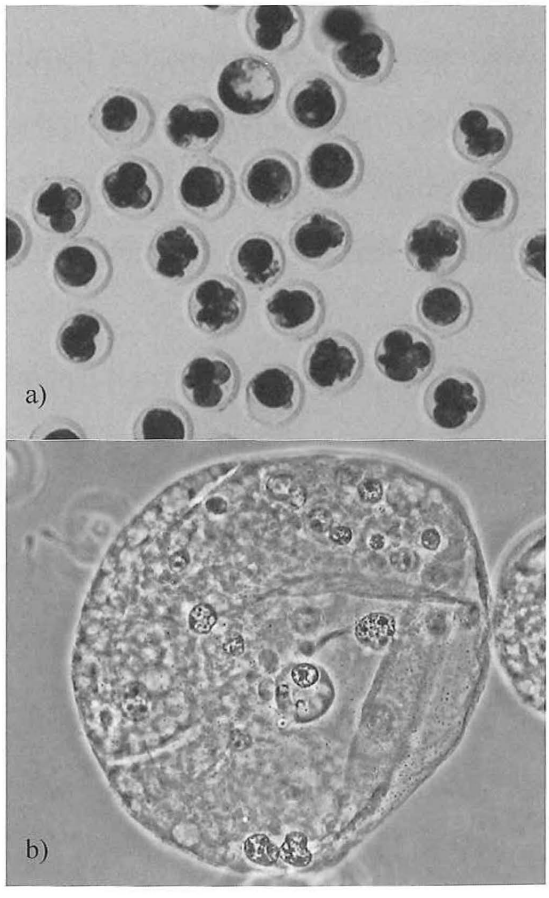

Fig. 8. Developmental competence of porcine oocytes recovered from mice

in the FSH-14EA group. (a) Results of *in vitro* fertilization of oocytes and subsequent *in vitro* culture for 7 days. (b) A blastocyst that appeared on day 7 of *in vitro* culture (day 0 = day of *in vitro* fertilization). Bars indicate 100 μm. Modified from Kaneko et al. [15].

whereas no blastocysts appeared in the other groups. These results indicate that exogenous gonadotropins stimulated the growing follicles that had developed from the primordial follicles in the xenografts: the effects were incomplete but improved to some extent the meiotic and developmental abilities of the oocytes.

Sperm production from spermatogonial cells and embryo development by ICSI

Testis obtained from 5- to 10-day-old piglets contains seminiferous cords consisting of only gonocytes/spermatogonia and Sertoli cells (Fig. 9).

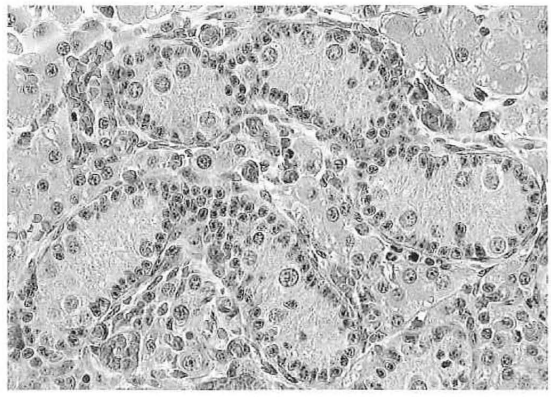

Fig. 9. Histological appearance of neonatal donor porcine testis (before grafting). Arrows indicate gonocytes in the seminiferous cord. Bar indicates 50 μm. Nakai et al. unpublished observations.

Such testicular tissues were transplanted under the back skin of castrated male nude mice, and testicular grafts were obtained between 30 and 210 days or more after xenografting. In this experiment, we assessed the development of germ cells in the grafted tissues and evaluated the ability of recovered sperm to support embryo development by injecting a single sperm into the cytoplasm of pig oocytes [21].

There was a gradual progression from the primitive seminiferous cords

into fully developed seminiferous tubules in the testicular xenografts. Spematocytes first appeared in the porcine testicular tissues 60 days after grafting, and spermatids 30 day later. Spermatozoa were first present in the seminiferous tubules 120 days after grafting (Fig. 10a), and the percentage of the tubules containing spermatozoa increased with time after xenografting. We were able to recover mature spermatozoa from the grafted tissues 120 days after grafting (Fig. 10b).

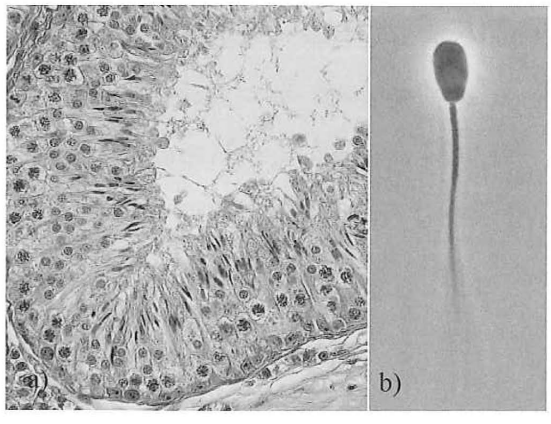

Fig. 10. Development and differentiation of grafted testicular tissues. (a) Histological appearance of seminiferous tubules recovered 120 days after grafting. Bar indicates 50 μm. (b) Sperm isolated from the grafted tissues 120 days after grafting. Bar indicates 5 μm. Nakai et al. unpublished observations.

Out of 253 oocytes in which the cytoplasm had been injected with a single sperm, 63 reached the blastocyst stage (Fig. 11).

The average total cell number of blastocysts was 41.9 ± 3.9: this number is comparable to that of *in vitro*-produced blastocysts after *in vitro* fertilization with frozen-thawed sperm obtained from boars. The system described here proved capable of producing blastocysts from spermatogonial cells of domestic animals.

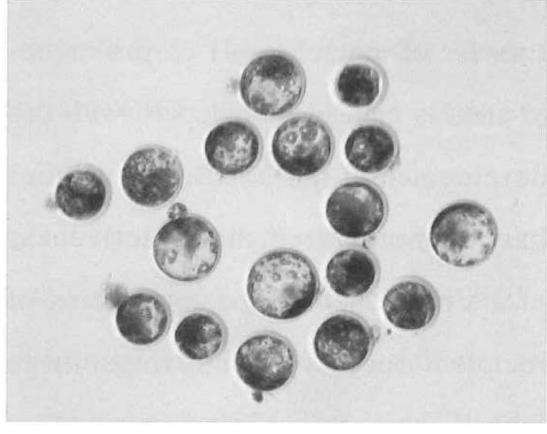

Fig. 11. Development of blastocysts from *in vitro*-matured oocytes after intracytoplasmic injection with

sperm from grafted testicular tissues. Bar indicates 100 μm. Nakai et al. unpublished observations.

Unsolved problems

It is now possible to generate blastocysts from porcine immature germ cells by combining different methods: xenografting of gonadal tissues, hormone treatment of host mice, and *in vitro* culture of recovered germ cells. However, only one per cent of mature oocytes derived from grafted primordial oocytes are able to develop to the blastocyst stage. One possible procedure for enhancing embryonic development is transfer of metaphase-II chromosomes to another enucleated cytoplast with full developmental ability. Recent studies have demonstrated the effectiveness of a simple method for enucleation of recipient oocytes by centrifugation in Percoll solution [22, 23]. At present, we have no information about the ability of blastocysts, produced by ICSI using pig sperm grown in host mice, to progress to the fetal or neonatal stage. Further studies will be necessary to evaluate the full developmental ability of these blastocysts by transfer to the oviducts of recipient gilts.

ACKNOWLEDGEMENTS

This study was supported in part by a Grant-in-Aid for Scientific Research to H.K. (17380170) from the Japanese Society for the Promotion of Science. H.K and K.K. contributed equally to the studies.

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