Effects of gonadotrophin treatments on meiotic and developmental competence of oocytes in porcine primordial follicles following xenografting to nude mice

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Abstract

Our objective was to improve the developmental ability of oocytes in porcine primordial follicles xenografted to nude mice, by treating the host mice with gonadotrophins to accelerate follicular growth. Ovarian tissues from 20-day-old piglets, in which most of the follicles were primordial, were transplanted under the kidney capsules of ovariectomized nude mice. Gonadotrophin treatments were commenced around 60 days after vaginal cornification in the mice. Ovarian grafts were obtained 2 or 3 days after treatment with equine chorionic gonadotrophin (eCG-2 and eCG-3 groups), after porcine FSH infusion 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 and FSH-14EA groups, respectively). Gonadotrophin treatments accelerated follicular growth within the xenografts compared with that in control mice given no gonadotrophins, consistent with higher ($P < 0.05$) circulating inhibin levels in the gonadotrophin-treated mice. In contrast, circulating mouse FSH levels were significantly ($P < 0.05$) depressed. We recovered large numbers of full-sized oocytes with meiotic competence to the mature stage from the eCG-3, FSH-7, and FSH-14EA, unlike in the control group. Moreover, 56% of matured oocytes with the first polar body ($n = 39$) were fertilized *in vitro* in the FSH-14EA group. After *in vitro* fertilization and subsequent culture for 7 days, one blastocyst was obtained from each of the eCG-3, FSH-7 and, FSH-14EA groups, whereas no blastocysts appeared in the other groups. Exogenous gonadotrophins – not mouse FSH – 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.

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Introduction

Primordial follicles act as stores for ovarian follicles and are a potential resource of oocytes for medical, agricultural, and zoological purposes. Success in the culture of primordial follicles as a method of oocyte maturation has been limited to mice (Eppig & O'Brien 1996, O'Brien *et al.* 2003), since it is difficult to establish an effective culture system for large mammals, whose primordial follicles need an extremely long time to mature (Miyano 2005). Ovarian grafting provides an alternative method for the maturation of oocytes in primordial follicles (primordial oocytes) of large mammals. Recently, grafting of ovarian tissues to another site in the body (autografting) was successful in endowing the growing follicles with full developmental competence to birth in

humans (Donnez *et al.* 2004) and primates (Lee *et al.* 2004). Cross-species ovarian grafting (xenografting) is advantageous for multiplication and conservation of domestic or endangered animals, because the ovaries can be collected from deceased animals and the oocytes can potentially be grown in a different species. Snow *et al.* (2002) demonstrated that oocytes that grow within mouse ovarian tissue xenografted to nude rats acquire the ability to generate pups. Ovarian tissues have been prepared from species phylogenetically distant from mice, including humans (Oktay *et al.* 1998, Weissman *et al.* 1999, Kim *et al.* 2002, Gook *et al.* 2003), dogs (Metcalf *et al.* 2001), monkeys (Candy *et al.* 1995), sheep (Gosden *et al.* 1994), cows (Senbon *et al.* 2003), pigs (Kaneko *et al.* 2003, Kagawa *et al.* 2005), tammar wallaby (Mattiske *et al.* 2002) and common wombats

(Cleary *et al.* 2003, 2004), and xenografted to immunodeficient mice. To our knowledge, only one study (Kaneko *et al.* 2003), in which neonatal pig ovarian tissues were xenografted, has proven that primordial oocytes can develop in the host mice and acquire fertilizing ability *in vitro*.

At present, no xenografting study has been successful in producing blastocysts from primordial or growing follicles of large mammals, indicating that optimal xenografting methods need to be established. One strategy for improving the developmental competence of oocytes within xenografts is to facilitate oocyte development by accelerating follicular growth with exogenous hormones. Cleary *et al.* (2003) indicated that greater numbers of morphologically normal oocytes were recovered from ovarian grafts of common wombats after the host mice were given follicle-stimulating hormone (FSH) for 4 or 7 days. Treatment of host mice with FSH for over 20 weeks (Gook *et al.* 2003), equine chorionic gonadotrophin (eCG) for 4 weeks (Kim *et al.* 2002) or human menopausal gonadotrophin (hMG) for 14 days (Weissman *et al.* 1999) increased the number of antral follicles within human ovarian xenografts, and the former two studies showed that some antral follicles formed early corpora lutea in response to human chorionic gonadotrophin (hCG; Kim *et al.* 2002, Gook *et al.* 2003) with resumption of meiosis of the oocytes (Gook *et al.* 2003). We previously optimized the timing of eCG treatment of host mice in terms of follicular growth and oocyte recovery, and found that more oocytes with fertilizing ability were collected from antral follicles when eCG was given 60 days after estrus was first detected in the host mice (Kaneko *et al.* 2003). 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 recipients gilts (Kikuchi *et al.* 2006). According to findings obtained in prepubertal gilts, oocytes isolated from antral follicles at least 2 mm in diameter resume meiosis at a higher rate than those from smaller follicles (Motlik *et al.* 1984), and oocytes from follicles between 3 and 5 mm in diameter have been proven to acquire the ability to develop to the blastocyst stage *in vitro* (Kikuchi *et al.* 2002, Marchal *et al.* 2002). On the other hand, the antral follicles in xenografts examined 48 h after eCG injection did not exceed 2 mm in our previous study (Kaneko *et al.* 2003). The above findings strongly suggest that a more effective hormone treatment to promote follicular development should be established. We therefore gave host mice several hormonal treatments, taking into account the size of the antral follicles in the xenografts at oocyte recovery, and evaluated the influence of the hormone treatments on the meiotic and developmental competence of porcine primordial oocytes under *in vitro* embryo-production systems.

Materials and Methods

Ovarian xenografting

Protocols for the use of animals were approved by the Animal Care Committee of the National Institute of Agrobiological Sciences, Japan. Donor ovaries were dissected from 20-day-old piglets of crossbreeds (Landrace × Large White × Duroc, born at the National Institute of Livestock and Grassland Science, Tsukuba, Japan) as previously reported (Kaneko *et al.* 2003). At this age, primordial follicles accounted for 96% of the total number of follicles in the ovary and the rest were almost all primary follicles based on histological examinations (Kaneko *et al.* 2003). Immediately after removal of the ovary the cortex was cut into small pieces; these pieces were further minced into pieces of approximately 1.5 × 1.5 × 1.5 mm in saline supplemented with 668 units/ml penicillin (Sigma Chemical Co., St Louis, MO, USA) and 0.2 mg/ml streptomycin sulfate (Sigma). As recipients, 5-6-week-old female immunodeficient mice (Crlj:CD1-Foxn1^{nu}; Charles River Japan, Yokohama, Japan) were anesthetized and ovariectomized. Small holes were made in the mouse kidney capsule with a pair of fine forceps, and approximately 10 fragments of ovarian tissue were inserted under the capsule of each kidney. To detect the onset of vaginal cornification in the mice, vaginal smears were taken every day from 40 days after grafting.

Experimental design

Kinetic analyses of follicular growth in the prepubertal gilts estimate that growth of follicles from antrum formation to a diameter of 3 mm or more requires approximately 14 days (Morbeck *et al.* 1992). In this study, therefore, we stimulated follicular growth in the xenografts with porcine FSH for 1 or 2 weeks and the resultant follicular growth and oocytes viability were compared with those after eCG treatment described in our previous studies (Kaneko *et al.* 2003, Kikuchi *et al.* 2006). To estimate an appropriate dose of porcine FSH, we carried out a pilot study using four host mice that showed vaginal cornification 60 days before. Two host mice, implanted with one Alzet osmotic pump (model 2004, DURECT Corp, Cupertino, CA, USA) containing 31.25 U/ml porcine FSH (Sigma) for 1 week, had no clear enhancement of follicular growth in the xenografts. However the other two mice, implanted with a osmotic pump containing 62.5 U/ml porcine FSH, showed the accelerated follicular growth. Therefore, a dose of 62.5 U/ml porcine FSH was chosen in this study. We also chose a dose of 4 IU eCG (PMS 1000; Nihon Zenyaku Kogyo, Koriyama, Japan), since 8–10 IU eCG injection caused an overdose and resulted in formation of many hemorrhagic follicles in the xenografts.

In the main experiment, around 60 days after the first detection of vaginal cornification in the host mice, we started giving the mice hormone treatments on the basis of our previous findings that good performance in terms of

follicular growth and oocyte recovery was achieved when eCG was given to the host mice 60 days after first detection of vaginal cornification (Kaneko *et al.* 2003). Mice were randomly assigned to the following experimental groups: control ($n = 11$), eCG-2 ($n = 15$), eCG-3 ($n = 16$), FSH-7 ($n = 14$), FSH-14 ($n = 11$) and FSH-14EA ($n = 10$). Control mice received no hormone treatment. From the eCG-treated groups, grafts were recovered 2 days (eCG-2 group) or 3 days (eCG-3 group) after a single intraperitoneal injection of 4 IU eCG. Each mouse in the FSH-treated groups was implanted with one Alzet osmotic pump (model 2004) under the skin of the back. The pump was filled with saline containing 62.5 U/ml porcine FSH (Sigma). Grafts were recovered from the mice after infusion of FSH for 7 days (FSH-7 group) or 14 days (FSH-14 group). To inhibit a surge-like release of luteinizing hormone, 7 days after the beginning of FSH infusion the mice in the FSH-14EA group received an intraperitoneal injection of 100 μ l estradiol antiserum (EA) raised in a goat (Kaneko *et al.* 1995, 2002a), and their grafts were recovered 14 days after the beginning of FSH infusion.

Blood collection and graft recovery

Before graft recovery, mice in each group were anesthetized and bled by cardiac puncture. We also collected blood samples from five ovariectomized mice that received no ovarian grafts (OVX group). Serum was stored at -30°C until it was assayed for total inhibin and FSH. After blood sampling, cumulus-oocyte complexes (COCs) were isolated mechanically with a surgical blade in Medium 199 (with Hank's salts; Sigma; Kikuchi *et al.* 2002) from antral follicles in the tissue grafted under the kidney capsules. Oocytes larger than 115 μm in diameter (full-sized oocytes) were selected for *in vitro* maturation (IVM), since oocytes larger than 115 μm obtained from prepubertal gilts acquire meiotic competence (Motlik *et al.* 1984, Hirao *et al.* 1995). Ovarian grafts in the left kidney were obtained from the two mice in each group and fixed in Bouin's solution and embedded in paraffin for histological examination.

In vitro maturation, in vitro fertilization (IVF) and in vitro culture (IVC) of oocytes

Recovered COCs were matured *in vitro* as described previously (Kikuchi *et al.* 2002). Briefly, COCs were cultured for 20–22 h in modified North Carolina State University-37 (NCSU-37) solution (Petters & Wells 1993) supplemented with 10% porcine follicular fluid, 0.6 mM cysteine, 50 μM β -mercaptoethanol, 1 mM dibutyl cAMP (Sigma), 10 IU/ml eCG (PMS 1000) and 10 IU/ml hCG (Puberogen 500 U; Sankyo, Tokyo, Japan). Subsequently, they were cultured for 24 h in IVM medium without the dibutyl cAMP and hormones. Maturation culture was carried out at 39°C under conditions of $\text{O}_2/\text{CO}_2/\text{N}_2$ adjusted to 5, 5 and 90% respectively. After IVM, cumulus cells were removed by hyaluronidase treatment (150 IU/ml;

Sigma) and gentle pipetting. Oocytes with the first polar body were harvested as matured oocytes and placed in modified Pig-FM (Suzuki *et al.* 2002) supplemented with 2 mM caffeine and 5 mg/ml BSA (fraction V; Sigma). Frozen epididymal spermatozoa (Kikuchi *et al.* 1998) were thawed and then preincubated for 15 min at 37°C in Medium 199 (with Earle's salts; Gibco, Life Technologies, Grand Island, NY, USA) adjusted to pH 7.8 (Nagai *et al.* 1988). A portion (10 μl) of the preincubated spermatozoa was introduced into 90 μl fertilization medium containing about 10 matured oocytes. The final sperm concentration was adjusted to $5 \times 10^5/\text{ml}$. The day of IVF was defined as day 0. After co-incubation at 39°C under 5% O_2 for 3 h, the oocytes were freed from the attached spermatozoa by gentle pipetting and transferred to IVC.

IVC was performed in IVC-PyrLac for days 0–2 and in IVC-Glu for days 2–7 (Kikuchi *et al.* 2002). The IVC-PyrLac consisted of NCSU-37 solution (Petters & Wells 1993) without glucose but supplemented with 50 μM β -mercaptoethanol, 0.17 mM sodium pyruvate and 2.73 mM sodium lactose. The IVC-Glu was NCSU-37 solution supplemented with 4 mg/ml BSA and 50 μM β -mercaptoethanol.

Assessment of fertilization

To confirm the fertilization status of the recovered oocytes, 39 mature oocytes obtained from the FSH-14EA group were co-incubated with the frozen-thawed boar spermatozoa for 3 h and were cultured for a further 5 h in IVC-PyrLac. They were whole-mounted on glass slides and fixed in acetic alcohol (acetic acid/methanol, 1:3). After the specimens had been stained with 1% aceto-orcin (Sigma), the nuclear status and extrusion of polar bodies were examined by phase-contrast microscopy. Fertilization of oocytes obtained from eCG-treated mice was assessed elsewhere (Kaneko *et al.* 2003).

Embryo evaluation

Development of IVF oocytes to the blastocyst stage was evaluated on day 7. An embryo with a clear blastocoele was defined as a blastocyst, and IVF oocytes that remained at the mono-cell stage or fragmented were defined as degenerated oocytes or embryos. Embryos were fixed with acetic alcohol and stained with 1% aceto-orcin (Sigma), and the total number of cells in each blastocyst was counted.

Histological analysis

Ovarian grafts from each group were sectioned at 7 μm and stained with hematoxylin and eosin, to examine the morphological changes. Ovaries from the prepubertal gilts were examined histologically, to compare their follicular growth with that in the xenografts.

Fluoroimmunoassay for total inhibin

Concentrations of total inhibin in the plasma of the host mice, as a marker of follicular growth, were determined by competitive fluoroimmunoassay (FIA) using europium (Eu)-labeled inhibin A as a probe (Kaneko *et al.* 2002b). In the FIA of total inhibin, anti-bovine inhibin serum (TNDH-1; Hamada *et al.* 1989) was used as a primary antibody. Bovine 32 kDa inhibin A was used for Eu-labeling and as a reference standard. Anti-inhibin serum was provided by Dr K Taya, Tokyo University of Agriculture and Technology, Fuchu, Tokyo, Japan; bovine 32 kDa inhibin was provided by Dr Y Hasegawa, Kitasato University, Towada, Aomori, Japan. The detection limit of the FIA was 0.078 ng/ml. The intra- and interassay coefficients of variation (CVs) were 10.5 and 15.0%, respectively.

FIA for mouse FSH

Concentrations of FSH in the plasma of the host mice were determined by competitive FIA using Eu-labeled rat FSH as

a probe (Kaneko *et al.* 2002b), to assess the changes in endogenous FSH secretion after the gonadotrophin treatments. In the FIA for mouse FSH, anti-rFSH-S-11 was used as a primary antibody, rFSH-I-9 for Eu labeling and rFSH-RP-2 as a reference standard (as an assay material, a rat FSH RIA kit was provided by Dr A F Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA, USA). Anti-rabbit immunoglobulin G (Chemicon International, Temecula, CA, USA) was used as the secondary antibody. The detection limit of the FIA was 0.39 ng/ml. The intra- and interassay CVs were 10 and 12.5%, respectively.

Data analyses

All data were subjected to analysis of variance (ANOVA), and the significance of the difference among means was determined by Duncan's multiple range test. The general linear models procedure of SAS (SAS/STAT 1988) was

Figure 1 Porcine ovarian tissue under the renal capsule of host mice in the (A) control, (B) eCG-2, (C) eCG-3, (D) FSH-7, (E) FSH-14 and (F) FSH-14EA groups. Ovarian grafts were examined 2 (eCG-2) or 3 days (eCG-3) after eCG treatment, 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 gonadotrophin treatment. Scale bars, 5 mm.

Figure 2 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 an ovary section obtained from a prepubertal gilt. Examination of ovarian grafts was following treatments as described for Fig. 1. Scale bars, 5 mm.

used for the analyses. Differences with P values of < 0.05 were considered to be significant.

Results

Morphology of ovarian grafts

Low-magnification representative features of the gross morphology and histology of the ovarian xenografts obtained from each group are shown in Figs 1 and 2. Ovarian grafts grew and fused together under the kidney capsules of the host mice with or without gonadotrophin treatments (Fig. 1). Compared with the grafts in the control mice (Fig. 1A), ovarian grafts in mice given gonadotrophin treatments (especially FSH treatment for 7 days or more) were larger in size (Figs 1B–F). The diameters of the antral follicles in the xenografts of control and eCG-treated mice were similar and did not exceed 2 mm (Figs 2A and B). In contrast, treatment of host mice with FSH enhanced the growth of antral follicles (Figs 2C–E); the sizes of several antral follicles were similar to those of antral follicles observed in the ovaries of prepubertal gilts (Fig. 2F).

However, in the FSH-14 group, follicular antra were frequently filled with erythrocytes (Fig. 2D). In the xenografts of the control and eCG-treated mice, the antral follicles had numerous concentric layers of mural granulosa cells and oocytes at the germinal vesicle stage surrounded by a few layers of cumulus cells (Figs 3A and B). In the antral follicles of the FSH-7 and FSH-14EA groups, germinal vesicles were also observed in the oocytes, which were surrounded by a few layers of cumulus cells (Figs 3C and D). However, in the FSH-14 group, disorganization of cumulus cells in the larger antral follicles was evident, and the oocytes in some follicles showed germinal vesicle breakdown (Fig. 3E). The theca cells of hemorrhagic follicles in the FSH-14 group were partly luteinized (Fig. 3F).

Changes in concentrations of total inhibin and FSH in the circulation of host mice

Circulating levels of total inhibin in ovariectomized mice that received no ovarian xenografts were close to the detection limit of the FIA (Fig. 4A). However, inhibin levels increased significantly ($P < 0.05$) in the control mice that had received xenografts. Inhibin levels in the

Figure 3 Histological appearance of COC and granulosa layers in xenografts of the (A) control, (B) eCG-3, (C) FSH-7, (D) FSH-14EA and (E) FSH-14 groups. (F) Image of a follicular wall obtained from the FSH-14 group. Examination of ovarian grafts was following treatments as described for Fig. 1. The arrowhead indicates resumption of meiosis. The asterisk indicates luteinization of theca layers. Scale bars, 100 μ m.

eCG-2 and eCG-3 groups did not show a significant increase compared with those in the control mice. FSH treatment for 7 days raised inhibin levels dramatically ($P < 0.01$), and a further increase ($P < 0.05$) was noted in the FSH-14EA group. Mouse FSH levels in the circulation of ovariectomized mice were high (Fig. 4B), but significantly decreased ($P < 0.01$) in the control mice. A further decline ($P < 0.05$) in FSH concentrations was observed in the eCG-3 and FSH-treated groups, with the lowest levels in the FSH-7 and FSH-14EA groups.

Growth and maturation of oocytes

The number of oocytes recovered or the number of full-sized oocytes larger than 115 μ m per mouse was low in the control group but increased dramatically ($P < 0.01$) in the eCG-3, FSH-7, and FSH-14EA groups (Table 1).

The ratio of full-sized oocytes to the total number of oocytes ranged from 33 to 43% in these three groups but was 26% in the control group. In the control mice after IVM, a small number of oocytes had the ability to resume meiosis to the mature stage, as determined by extrusion of the first polar body. However, much greater numbers of matured oocytes were obtained from the mice in the eCG-3, FSH-7 and FSH-14EA groups ($P < 0.05$). 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. On the other hand, the number of oocytes was very low in the FSH-14 group, mainly because of the difficulty in collecting oocytes from hemorrhagic follicles. When 39 mature oocytes obtained from the FSH-14EA group were coincubated with frozen/thawed boar spermatozoa, 22 oocytes (56%)

Figure 4 Circulating levels of (A) total inhibin and (B) mouse FSH in mice of the OVX, control, eCG-2, eCG-3, FSH-7, FSH-14 and FSH-14EA groups. Blood samples were taken following treatments as described for Fig. 1. Samples were also collected from a control group that received no gonadotrophin treatment and from ovariectomized mice that received no ovarian grafts (OVX). Dotted lines represent the detection limits of the assays.

were penetrated by a spermatozoon (or -zoa), formed a female pronucleus and extruded the second polar body. In 19 out of 22 fertilized oocytes (86.4%), the a sperm nucleus (or nuclei) were transformed into a male

pronucleus (or pronuclei). Monospermy was noted in 12 of 22 fertilized oocytes (55%).

Development of oocytes after IVF

Each mature oocyte obtained from the eCG-3, FSH-7 or FSH-14EA groups reached the blastocyst stage showing a clear blastocoele on day 7 of IVC, but the other oocytes from the same groups remained at the mono-cell stage or showed fragmentation (Table 2 and Fig. 5A). No blastocysts appeared in the control and eCG-2 groups. The total numbers of cells in these blastocysts ranged from 16 to 30 (Table 2 and Fig. 5B).

Discussion

A major obstacle to the xenografting of primordial oocytes in large mammals is the difficulty in collecting viable oocytes from the host mice for subsequent *in vitro* maturation and fertilization. Therefore, it has been unclear whether primordial oocytes grown in xenografts can develop to the blastocyst stage after IVC. We previously reported that xenografting of neonatal pig ovarian tissue with subsequent eCG treatment of the host mice enabled us to obtain *in vitro*-fertilizable oocytes from xenografted primordial follicles. 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 recipients gilts (Kikuchi *et al.* 2006). To our knowledge, this study has shown for the first time that primordial oocytes of large mammals can be developed to the blastocyst stage by a combination of xenografting and culture by adding hormone treatment of the host mice. As a first step, we tried several hormonal treatments of the host mice to promote oocyte growth by acceleration of follicular growth in the grafted porcine neonatal ovarian tissue. Then we assessed the developmental competence of the recovered oocytes with culture.

Many more full-sized oocytes ($\geq 115 \mu\text{m}$) with meiotic competence were recovered from the mice in the eCG-3, FSH-7 and FSH-14EA groups than from those in the control group. These results clearly indicate that the three gonadotrophin treatments of the host mice improved the

Table 1 Numbers and meiotic competence of porcine oocytes recovered from host mice that had received hormonal treatment.

Group ^a	No. of mice	No. of oocytes recovered ^b	No. of oocytes larger than $115 \mu\text{m}$ ^c	No. of oocytes in metaphase II stage ^d
Control	9	70.7 $\pm$ 16.8 ^e (636)	18.4 $\pm$ 5.3 ^e (166)	3.4 $\pm$ 0.9 ^e (31)
eCG-2	13	76.5 $\pm$ 14.8 ^e (995)	22.9 $\pm$ 4.3 ^e (297)	7.7 $\pm$ 1.6 ^{ef} (100)
eCG-3	14	176.3 $\pm$ 31.7 ^{fg} (2468)	66.5 $\pm$ 11.0 ^f (931)	13.6 $\pm$ 3.3 ^{fg} (191)
FSH-7	12	180.3 $\pm$ 23.8 ^g (2164)	60.3 $\pm$ 11.2 ^f (723)	20.8 $\pm$ 4.1 ^g (250)
FSH-14	9	37.3 $\pm$ 9.4 ^e (336)	6.9 $\pm$ 1.9 ^e (62)	2.8 $\pm$ 0.7 ^e (25)
FSH-14EA	8	114.0 $\pm$ 21.2 ^f (917)	49.3 $\pm$ 9.1 ^f (394)	16.1 $\pm$ 4.7 ^{fg} (129)

^a The eCG-2 or -3 group received 4 IU eCG and the grafts were examined 2 (eCG-2) or 3 days (eCG-3) later; the FSH-7 or -14 group received porcine FSH for 7 (FSH-7) or 14 days (FSH-14); the FSH-14EA group received porcine FSH for 14 days with a single injection of estradiol antiserum 7 days after the beginning of FSH infusion; control mice received no hormone treatment.

^{b-d} The number of oocytes in each category is represented by mean $\pm$ S.E.M. per mouse followed by the total number (in parentheses).

^{e-g} Values in the same column without common superscripts are significantly different.

Table 2 *In vitro* development of porcine oocytes recovered from host mice that had received hormonal treatment.

Group ^a	No. of mature oocytes for IVF ^b	No. of oocytes developed to blastocyst ^c
Control	31	0
eCG-2	100	0
eCG-3	115	1 (23)
FSH-7	163	1 (16)
FSH-14EA	113	1 (30)

^a The eCG-2 or -3 group received 4 IU eCG and the grafts were examined 2 (eCG-2) or 3 days (eCG-3) later; the FSH-7 group received porcine FSH for 7 days; the FSH-14EA group received porcine FSH for 14 days with a single injection of estradiol antiserum 7 days after the beginning of FSH infusion; control mice received no hormone treatment.

^b Oocytes with the first polar body were subjected IVF.

^c The no. of oocytes is followed by total number of cells in the blastocysts (in parentheses).

meiotic competence of the oocytes by promoting oocyte growth within the xenografts. These treatments probably induced a similar increase in the total number of antral follicles, judging from the total numbers of oocytes recovered; however, about 35% of the full-size oocytes matured

in the FSH-7 and FSH-14EA groups, whereas this ratio was 20% in the eCG-3 group. The difference in maturation rates between FSH and eCG treatments may be attributed to the difference in the follicular response after treatment. It has been demonstrated that larger-diameter follicles contain oocytes with higher meiotic or developmental potential in pigs (Motlik *et al.* 1984, Luca *et al.* 2002, Marchal *et al.* 2002), cattle (Lonergan *et al.* 1994, Blondin & Sirard 1995, Lequarre *et al.* 2005) and monkeys (Gilchrist *et al.* 1997). The ability of the oocyte to develop to the blastocyst stage is thought to be a suitable indication of competence. The appearance of blastocysts in the eCG-3, FSH-7 and FSH-14EA groups suggests that gonadotrophin treatment of the host mice can endow oocytes grown in the xenografts with developmental ability. The oocyte accumulates RNA molecules and proteins in its cytoplasm during the growth phase, and timely use of such stored molecules is essential for oocyte maturation, fertilization and subsequent embryonic transcription before genomic activation (Gandolfi & Gandolfi 2001). Gonadotrophin treatments may improve – albeit inadequately – this cytoplasmic function of oocytes within the xenografts by promoting follicular growth.

Our results clearly indicate that gonadotrophin treatments alter the hormonal environment in the host mice. It is apparent from our results that endogenous FSH secretion in control mice was regulated by inhibin produced from the surviving ovarian grafts. FSH levels in the control mice were likely to be within a range that maintained early growth of the antral follicles in the xenografts, since several antral follicles were morphologically normal and some oocytes had meiotic ability *in vitro*. After treatment with gonadotrophins, especially with FSH, the ovarian grafts showed increased inhibin production, probably in association with increased estradiol production, and this had further negative-feedback effects on mouse pituitary FSH secretion. A similar reciprocal relationship between inhibin and FSH has been observed in FSH- or eCG-treated domestic animals (McNeilly *et al.* 1989, Kaneko *et al.* 1992). The fact that FSH secretion was severely suppressed in mice of the eCG-3- and porcine FSH-treated groups strongly suggests that, in these groups, follicular growth is stimulated or maintained largely by exogenous gonadotrophins, not by endogenous mouse FSH.

It is widely accepted that the occurrence of a surge of luteinizing hormone, triggered by increased circulating estradiol levels, induces preovulatory changes in large antral follicles, including invasion of blood into the antrum, luteinization of the follicular wall and resumption of meiosis of oocytes. In the present study, luteinization of granulosa and theca cells, invasion of blood into the follicular cavity and germinal vesicle breakdown of oocyte nucleus were frequently observed within the xenografts of the FSH-14 group. These histological changes are similar to those that occur in the large antral follicles of human xenografts after treatment with hCG (Kim *et al.* 2002,

Figure 5 Developmental competence of porcine oocytes recovered from mice in the FSH-14EA group. (A) Results of IVF/IVC on day 7 of IVC (day 0 = day of IVF). (B) A blastocyst appeared on day 7 of IVC. Scale bars, 100 µm.

Gook *et al.* 2003). The observations made in the FSH-14 group suggest that a surge-like release of luteinizing hormone might occur during the 14 days of FSH treatment, probably in response to increased estradiol levels in the circulation. This hypothesis is supported by the finding that injection of estradiol antiserum (in the FSH-14EA group) reduced the rate of occurrence of hemorrhagic follicles.

Although our results show that exogenous hormone treatments have positive effects on the developmental ability of porcine primordial oocytes xenografted to host mice, there remain many problems to be resolved. The ratio of mature oocytes to full-size oocytes was about 35% and the percentage of mature oocytes that could develop to the blastocyst stage was about 1%. These ratios were lower than we found previously after IVM (70%; Kikuchi *et al.* 1999a, 2002) or IVC (20% in Kikuchi *et al.* 2002; 17% in Suzuki *et al.* 2006) of oocytes collected from the prepubertal gilts using the same culture systems. The present and previous (Kaneko *et al.* 2003) studies confirm that more than 50% of mature oocytes, obtained from host mice that received eCG or porcine FSH treatment, had the abilities of fertilization and of transformation of a sperm nucleus to a male pronucleus. Furthermore, we observed a higher incidence of monospermic fertilization. The above results suggest that the nuclear maturation of oocytes grown in the xenografts can be attained but cytoplasmic maturation of the oocytes to the blastocyst stage is difficult to be accomplished by the present hormone treatments. The morphology of COCs in the xenografts may partly account for the low competence of the oocytes grown in host mice. In our xenografts, the oocytes were surrounded by one to three layers of cumulus cells, whereas the COCs obtained from prepubertal gilts and used for *in vitro* production of blastocysts have more than several layers of cumulus cells (Kikuchi *et al.* 1999b, Kikuchi *et al.* 2002). A clear relationship between the quality of the COCs (number of layers or number of follicle cells around the oocytes) and fertilization or embryo development following culture has been observed in cows (Lonergan *et al.* 1994) and pigs (Nagai *et al.* 1993).

In conclusion, we demonstrated that gonadotrophin treatment of host mice increased the meiotic ability of the oocytes grown from the porcine primordial oocytes within the xenografts. Oocytes that matured after IVM could reach the blastocyst stage after IVC, although their rate of occurrence was very low. The above results suggest that ovarian xenografting, in combination with hormone treatment of the host mice and subsequent culture of the oocytes, may be able to endow primordial oocytes with developmental competence.

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