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## Fertilization and development of bovine oocytes grown in female SCID mice

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### Summary

We previously reported that xenografted bovine secondary follicles developed to the antral stage in severe combined immunodeficient (SCID) mice. In the present study, bovine secondary follicles 100–240 µm in diameter were xenografted under the kidney capsules of female SCID mice for 6 and 8 weeks, and we examined the oocytes' fertilization and developmental abilities. Bovine follicles developed with prolongation of grafting and became significantly larger than those before grafting. Injection of equine chorionic gonadotropin (eCG) into host mice made some surviving follicles develop larger than the other follicles. Furthermore, bovine oocytes grew in the follicles, and the mean diameter of the oocytes was 100 µm or more at 6 and 8 weeks of transplantation. Bovine oocytes that had grown in eCG-stimulated SCID mice 8 weeks after grafting were subjected to maturation culture. Some of the oocytes that had grown to 110 µm or more matured to the second metaphase (7% of oocytes 110–119 µm and 44% of those >120 µm). When the oocytes were inseminated with bovine spermatozoa, 15% (6/39) formed a female and a male pronucleus, and 2 days after insemination 24% (18/75) of oocytes cleaved and 2% (2/75) developed to the 5- to 8-cell stage. However, no embryo reached the blastocyst stage. These results indicate that bovine oocytes grown in SCID mice could be fertilized but acquired insufficient competence for embryonic development in the present conditions.

Keywords: Cow, Fertilization, Oocyte, SCID mouse, Xenografting

### Introduction

Ovarian grafting has been used in attempts to develop small follicles. Cryopreservation methods for ovarian tissues have been improved in parallel with the development of grafting techniques. The use of these

techniques in combination is expected to become a new clinical therapy for infertility and a method for rescuing threatened and endangered animals. A monkey recently gave birth to a healthy baby that originated from an oocyte collected from autografted ovarian tissues (Lee *et al.*, 2004). Furthermore, the first human live birth after orthotopic autografting of cryopreserved ovarian tissues has been reported (Donnez *et al.*, 2004). Among the grafting techniques, xenografting into immunodeficient animals can be used as a model to verify follicular viability and to study the dynamics of follicular development (Gosden *et al.*, 1994; Candy *et al.*, 1995; Gunasena *et al.*, 1998; Oktay *et al.*, 1998; Weissman *et al.*, 1999). In 1994, it was first reported that sheep and cat follicles survived and developed to the antral stage in immunodeficient mice after several months of their grafting (Gosden *et al.*, 1994). Since that time, frozen-thawed ovarian tissues have been shown to survive and to develop into antral

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follicles in immunodeficient mice (Oktay *et al.*, 1998; Gook *et al.*, 2001; Van den Broecke *et al.*, 2001). Recently, it was reported that mouse oocytes in xenografted ovaries grew to their final size in nude rats, matured, were fertilized *in vitro*, and resulted in the successful production of live pups (Snow *et al.*, 2002). We also showed that bovine oocytes in secondary follicles grafted into female severe combined immunodeficient (SCID) mice grew to the final size, and that the grown oocytes matured to the second metaphase (Senbon *et al.*, 2003). In the present study, bovine secondary follicles were xenografted into female SCID mice for 6 and 8 weeks, and the oocytes' fertilization and developmental abilities were examined.

## Materials and methods

### Xenografting of bovine follicles into SCID mice

Approximately 0.5 mm × 0.5 mm × 0.5 mm slices of bovine ovarian cortex, each containing only one secondary follicle 100–240 µm in diameter, were dissected using fine blades while immersed in TCM199 (pH 7.4; Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% polyvinylalcohol, 0.85 mg/ml NaHCO<sub>3</sub>, 0.08 mg/ml kanamycin, and 25 mM HEPES (HEPES-buffered TCM199). Secondary follicles were randomly separated into two groups, one for measuring diameters of follicles and oocytes, and the other for xenografting. Groups of 10 to 100 secondary follicles enclosed by connective tissue were inserted under the kidney capsule of each female SCID mouse (8–13 weeks old; C. B-17/Icr-*scid* Jcl, Clea Japan, Tokyo, Japan) using a pipette.

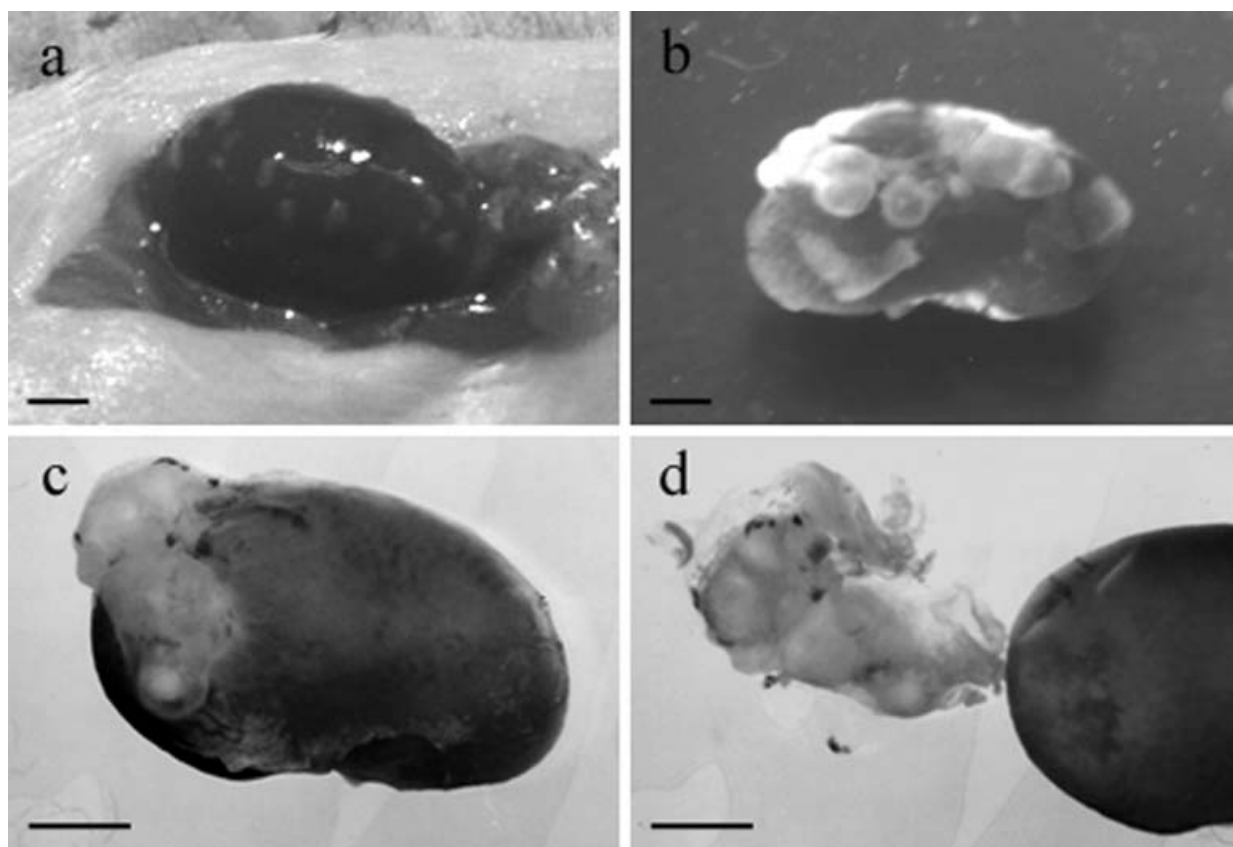
Bovine secondary follicles were grafted into 43 mice, 19 of which were killed 6 weeks after grafting and 24 of which were killed 8 weeks after grafting, by cervical dislocation, the grafts then being carefully separated from the kidneys with fine forceps. Forty-eight hours before slaughter, 4 and 7 mice were injected intraperitoneally with 10 IU of equine chorionic gonadotropin (eCG, Serotropin; Teikoku Hormone, Tokyo, Japan) after 6 and 8 weeks of grafting, respectively. The morphologically normal bovine follicles in the grafts were collected with a pair of fine blades under a dissection microscope. During recovery of these follicles, some follicles were ruptured. Morphologically normal oocytes released from the ruptured follicles were used in the experiments. The diameters of intact follicles were measured by taking two perpendicular measurements to the nearest 1 µm with an ocular micrometer (Nikon, Tokyo, Japan) attached to an inverted microscope. These follicles were then opened with fine needles, and cumulus-cell-enclosed or denuded oocytes were recovered. After denuding the oocytes by pipetting, the diameters of oocytes

(excluding the zona pellucida) together with the oocytes from ruptured follicles were measured.

### *In vitro* maturation and fertilization

Bovine secondary follicles were grafted in 14 SCID mice as described above. After 8 weeks, all recipient mice were injected with 10 IU of eCG, and their kidneys were removed 48 h after the injection. After the oocytes were recovered, they were washed in HEPES-buffered TCM199. Seven to 15 oocytes were then cultured in 500 µl of maturation medium (IVMD101; Research Institute for the Functional Peptides, Yamagata, Japan) in four-well dishes (Nunclon Multidishes; Nalge Nunc International, Denmark) for 20–22 h at 38.5 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. After culture, some oocytes enclosed by cumulus cells were completely denuded by pipetting. They were mounted onto slides, fixed in acetic ethanol (1:3), stained with 1% aceto-orcein, and examined under a differential interference microscope at ×400 magnification. The maturational stage of each oocyte was determined based on the changes in configuration of chromosomes and nuclear membranes.

After maturation culture, other oocytes were inseminated with bovine spermatozoa. Frozen bovine spermatozoa were thawed in water at 38.5 °C and suspended in fertilization medium (IVF100; Research Institute for the Functional Peptides). Spermatozoa were washed twice in this medium and then introduced into 100 µl drops of fertilization medium containing 10 mg/ml bovine serum albumin (BSA, International Regents Corporation, Kobe, Japan), 10 µg/ml heparin (Novo Heparin, Novo Nordisk Pharma, Denmark), and 5 mM theophylline to give a final concentration of 5 × 10<sup>6</sup> spermatozoa/ml. The cumulus-cell-enclosed oocytes were placed in the droplets in which the spermatozoa were suspended and co-cultured for 5 h at 38.5 °C in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air. Then the oocytes were denuded and washed several times in development medium (IVD101; Research Institute for the Functional Peptides). The denuded oocytes were then transferred into 500 µl of fresh development medium. After 10–12 h, some oocytes were mounted onto slides, fixed, stained, and examined under a differential interference microscope to check sperm penetration. Oocytes with a sperm head(s) or male pronucleus(ei) and corresponding sperm tail(s) in the ooplasm were judged to be penetrated. Penetrated oocytes were categorized into normal or polyspermic oocytes according to the number of spermatozoa and male pronuclei in the cytoplasm. As the control, fully grown oocytes collected from ovarian large antral follicles (3–5 mm) were cultured, inseminated, and examined in the same manner. The other inseminated oocytes were further cultured for up to 8 days. Cleavage of the embryos was checked after 2 days,



**Figure 1** Bovine follicles immediately after xenografting and 8 weeks after grafting. Bovine secondary follicles were xenografted under kidney capsules of SCID mice (*a*), and they developed large antral follicles after 8 weeks (*b*). Morphologically normal follicles in the grafts were recovered from mouse kidney capsules under a dissection microscope (*c*, *d*). Scale bars represent 2 mm.

and development to blastocysts was examined after 8 days.

This study was approved by the Committee on Animal Experimentation of Kobe University, Rokkodai Campus, Japan.

### Statistical analysis

The results from all replications were pooled and analysed. Statistical differences in the mean diameters of follicles and oocytes were analysed using Student's *t*-test. Other values were analysed by chi-square analysis. A probability of less than 0.05 was considered significant.

## Results and discussion

### Development of bovine follicles in SCID mice

Bovine secondary follicles were collected from ovarian tissues and 10–100 follicles were xenografted into each SCID mouse (Fig. 1a). At 6 and 8 weeks after grafting, they developed to large antral follicles (Fig. 1b, c). In general, follicles are lost due to ischemic conditions that occur immediately after transplantation, with

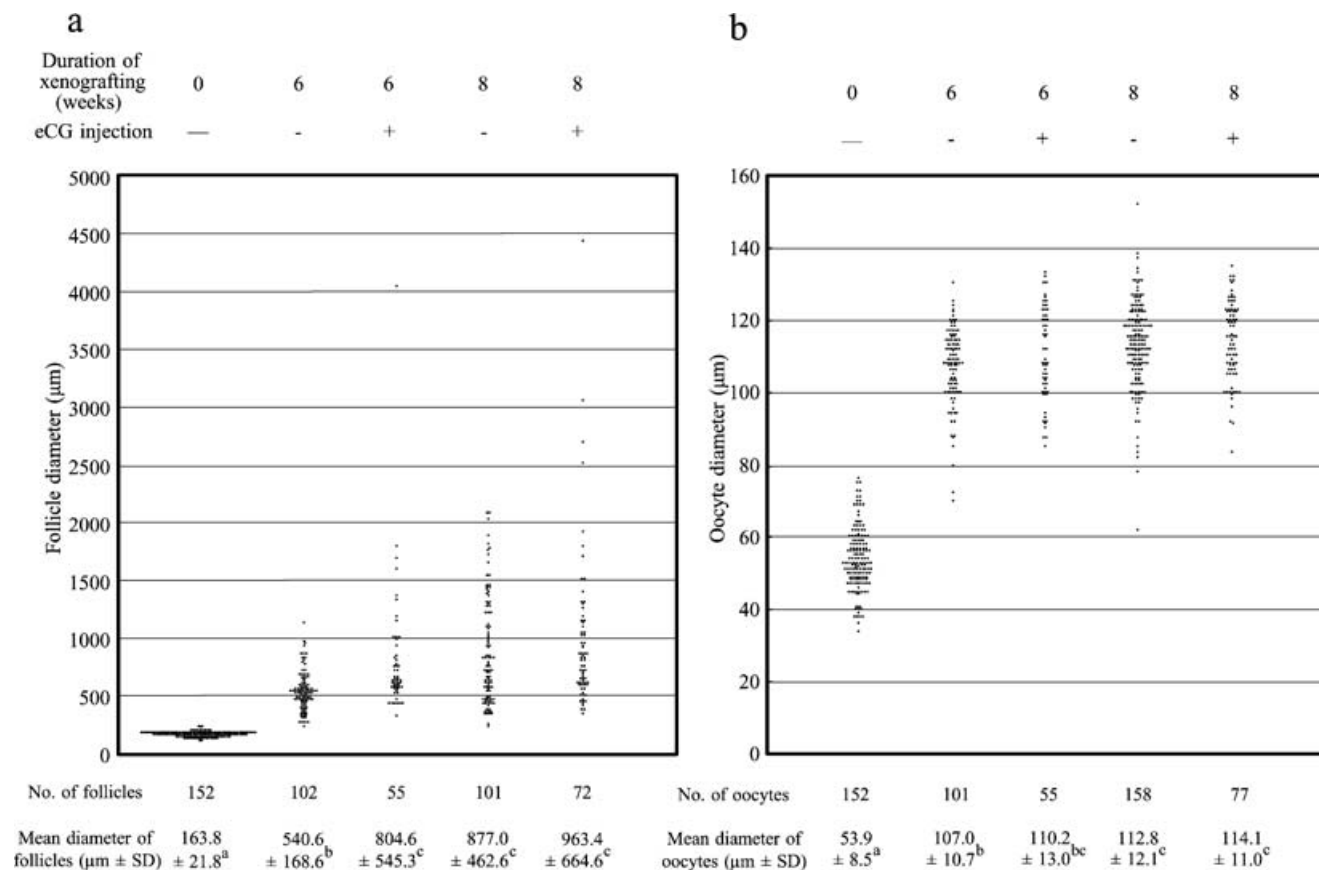
**Table 1** The number of bovine follicles and oocytes recovered from SCID mice after xenografting

Duration of xenografting* (weeks)	eCG*	No. of mice	No. of grafted follicles	No. (%) of recovered follicles**	No. (%) of recovered oocytes**
0	–	–	152	152 (100)	152 (100)
6	–	15	306	102 (33) <sup>a</sup>	101 (33) <sup>a</sup>
6	+	4	260	55 (21) <sup>bc</sup>	55 (21) <sup>b</sup>
8	–	17	570	101 (18) <sup>b</sup>	158 (28) <sup>a</sup>
8	+	7	264	72 (27) <sup>ac</sup>	77 (29) <sup>a</sup>

\*Groups of 10–100 bovine secondary follicles 140–200  $\mu\text{m}$  in diameter were xenografted under the kidney capsule of each SCID mouse. In hormonal treatment groups, mice were injected with 10 IU of equine chorionic gonadotropin (eCG) 48 h before recovering kidneys. The morphologically normal bovine follicles in the grafts were collected and diameters of the follicles were measured. These follicles were opened with fine needles, and cumulus-cell-enclosed or denuded oocytes were recovered.

\*\*The morphologically normal bovine follicles in the grafts were collected with a pair of fine blades under a dissection microscope. At the time, some follicles were ruptured and released morphologically normal oocytes. The released oocytes were included in the data "recovered oocytes".

<sup>a–c</sup>Values with different superscripts in the same column differ significantly ( $p < 0.05$ ).

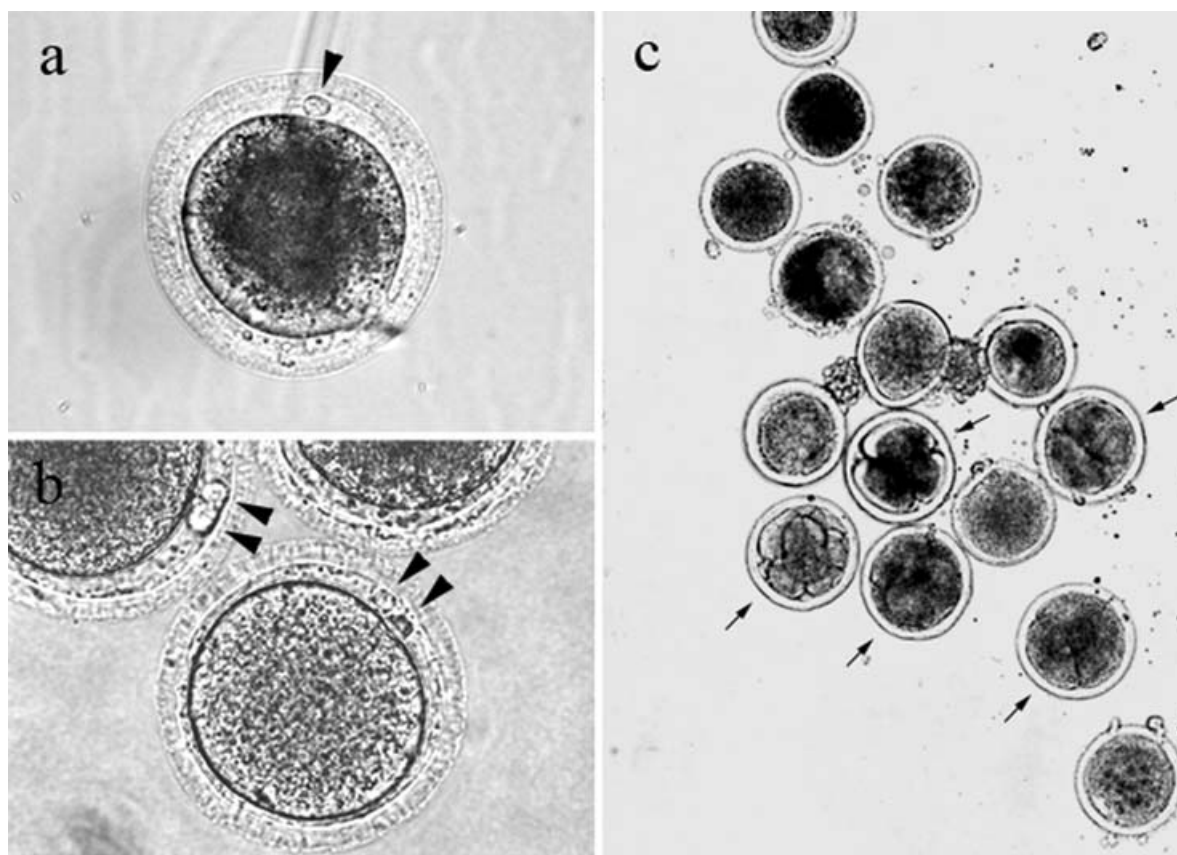


**Figure 2** Effect of eCG on the development of bovine follicles (a) and oocytes (b) xenografted in SCID mice. Forty-eight hours before recovery of the grafts, mice were injected with 10 IU eCG after 6 and 8 weeks of grafting. The morphologically normal bovine follicles in the grafts were collected and their diameters were measured. The follicles were then opened with fine needles, cumulus-cell-enclosed or denuded oocytes were recovered, and their diameters (excluding the zona pellucida) were measured. Each dot represents the diameter of a morphologically normal follicle and oocyte. Note that follicles grew larger in eCG-treated mice compared with non-treated mice.

follicles succumbing to apoptosis (Liu *et al.*, 2002). In an experiment on autografting in rats, revascularization of ovarian grafts was estimated to occur within 48 h (Dissen *et al.*, 1994). During this time, the majority of growing follicles within ovarian grafts are lost and up to 50% of the primordial follicle population do not survive (Dissen *et al.*, 1994; Liu *et al.*, 2002). In the present study, we had 3–5 h to isolate and to graft bovine follicles into SCID mice. During this time, these follicles and oocytes may be damaged. Furthermore, as individual follicles were cut from ovarian tissues using fine blades, rude handling of blades easily breaks three-dimensional structures of follicles. However, although it was unclear how many bovine secondary follicles survived immediately after grafting, some xenografted follicles survived the ischemic conditions and developed during 8 weeks of grafting.

The follicles were isolated from mouse kidney capsules under the microscope (Fig. 1d), but some follicles were ruptured and oocytes were released. After 6 and 8 weeks of grafting, 18–33% of surviving follicles

were recovered (Table 1). We first measured diameters of the follicles that maintained intact structure. Bovine follicles developed with prolongation of the grafting and became significantly larger compared with those before grafting (Fig. 2a). The follicle diameters were  $540.6 \pm 168.6 \mu\text{m}$  ( $n = 102$ ) and  $877.0 \pm 462.6 \mu\text{m}$  ( $n = 101$ ) after 6 and 8 weeks, respectively, which suggests that prolongation of the grafting term made grafted follicles larger in SCID mice. By injection of eCG into host mice, some surviving follicles had developed to the point at which they were obviously larger than the other follicles at 6 and 8 weeks. The largest follicle developed to over 3 mm in diameter in the eCG treatment group. The result was consistent with previous reports that stimulation of exogenous gonadotropins against hosts promotes the development of xenografted human (Oktay *et al.*, 1998) and pig follicles (Kaneko *et al.*, 2003). Furthermore, diameters of the oocytes were measured. More than 20% of grafted oocytes were recovered in each group (Table 1). After 6 weeks of xenografting, mean diameter of



**Figure 3** Bovine oocytes grown in SCID mice were subjected to maturation culture and then inseminated with spermatozoa. Oocytes that had grown in eCG-treated mice matured to the second metaphase after maturation culture for 22 h (a). The oocytes had extruded the first polar body (arrowhead). After insemination, some oocytes had two polar bodies (arrowheads), which suggested that they had been penetrated by sperm and resumed meiosis (b). Two days after insemination, some oocytes had cleaved and developed to 2- to 8-cell stage embryos (c; arrows).

oocytes increased by 50  $\mu\text{m}$  compared with that before transplantation. The mean size increased by 5  $\mu\text{m}$  in the following 2 weeks (Fig. 2b). After 8 weeks, the mean diameter of the oocytes recovered from eCG-injected mice was  $114.1 \pm 11.0 \mu\text{m}$  ( $n = 77$ ). Injection of eCG had no significant effect on growth of xenografted oocytes.

#### Meiotic, fertilization and developmental competence of bovine oocytes grown in SCID mice

To assess the maturational competence, we recovered 26 morphologically normal bovine oocytes from 2 eCG-injected SCID mice after 8 weeks of grafting. After maturation culture, some oocytes extruded the first polar body (Fig. 3a). Table 2 shows the nuclear morphology of the oocytes after maturation culture. Sixty-nine per cent (18/26) of the oocytes had undergone germinal vesicle breakdown (GVBD), and 20% (5/26) had reached the second metaphase. When the oocytes were classified according to diameter (<109, 110–119 or >120  $\mu\text{m}$ ), they showed a size-dependent ability to undergo GVBD and to mature

**Table 2** Maturational competence of bovine oocytes grown in SCID mice

Oocyte diameter* ( $\mu\text{m}$ )	No. of oocytes examined	Nuclear morphology of surviving oocytes** (%)			No. (%) of oocytes degenerating
		GV	MI	MII	
<109	3	1 (33)	1 (33)	0 (0)	1 (33)
110–119	14	2 (14)	8 (57)	1 (7) <sup>a</sup>	2 (14)
120 <	9	0 (0)	4 (44)	4 (44) <sup>b</sup>	1 (11)

\*After 8 weeks of xenografting, oocytes were recovered from eCG-treated SCID mice. The oocytes were classified into three groups on the basis of size (<109, 110–119 and >120  $\mu\text{m}$ ), cultured in maturation medium for 20–22 h, and their nuclear morphology examined.

\*\*GV, germinal vesicle stage; MI, first metaphase; MII, second metaphase.

<sup>a,b</sup>Values with different superscripts in the same column differ significantly ( $p < 0.05$ ).

to the second metaphase. This result indicates that the proportion of oocytes that undergo GVBD and reach the second metaphase increases with the increase

**Table 3** *In vitro* fertilization of bovine oocytes grown in SCID mice

Oocyte growth*	No. of oocytes inseminated	No. (%) of non-penetrated oocytes**				No. (%) of penetrated oocytes***			No. (%) of oocytes degenerating
		GV	MI	MII	Abnormal	FP + SH	FP + MP	Abnormal	
<i>In vivo</i>	98	0 (0)	0 (0)	6 (6)	1 (1)	7 (7)	63 (64) <sup>a</sup>	15 (15) <sup>a</sup>	6 (6) <sup>a</sup>
Xenografted	39	2 (5)	2 (5)	2 (5)	0 (0)	0 (0)	6 (15) <sup>b</sup>	20 (51) <sup>b</sup>	7 (18) <sup>b</sup>

\*Bovine oocytes collected from ovarian follicles 3–5 mm in diameter (*In vivo*), and oocytes recovered from xenografted follicles in SCID mice after 8 weeks (Xenografted) were cultured in maturation medium for 20–22 h, and then inseminated with frozen-thawed bovine spermatozoa. Oocytes were fixed, stained and examined 10–12 h after insemination.

\*\*GV, germinal vesicle stage; MI, first metaphase; MII, second metaphase; Abnormal, chromosomes were scattered in cytoplasm.

\*\*\*FP + SH, oocytes had a female pronucleus and an enlarged sperm head; FP + MP, oocytes had a female and a male pronucleus; Abnormal, MII oocytes penetrated by multiple spermatozoa, or MI oocytes penetrated by spermatozoa.

<sup>a,b</sup>Values with different superscripts in the same column differ significantly ( $p < 0.05$ ).

**Table 4** Embryonic development of bovine oocytes grown in SCID mice

Oocyte growth*	No. of oocytes inseminated	No. (%) of cleaved embryos**					No. (%) of blastocysts**
		Total	2 cell	3–4 cell	5–8 cell	9–16 cell	
<i>In vivo</i>	120	86 (72) <sup>a</sup>	9 (8)	20 (17)	51 (43) <sup>a</sup>	6 (5)	40 (33)
Xenografted	75	18 (24) <sup>b</sup>	5 (7)	11 (15)	2 (2) <sup>b</sup>	0 (0)	0 (0)

\*Bovine oocytes collected from ovarian follicles 3–5 mm in diameter (*In vivo*) and oocytes recovered from xenografted follicles in SCID mice after 8 weeks (Xenografted) were cultured in maturation medium for 20–22 h, and then inseminated with frozen-thawed bovine spermatozoa. Oocytes were fixed, stained and examined 10–12 h after insemination.

\*\*Cleavage of embryos was checked 2 days after insemination, and development to the blastocyst stage was examined after 8 days.

<sup>a,b</sup>Values with different superscripts in the same column differ significantly ( $p < 0.05$ ).

in the diameter of oocytes grown in SCID mice, which is consistent with the relationship between oocyte diameter and maturational competence noted in studies of bovine oocytes grown *in vivo* (Fair *et al.*, 1995) and *in vitro* (Otoi *et al.*, 1997).

After insemination, some oocytes extruded the second polar body (Fig. 3b). Of 39 oocytes, 6 (15%) formed a female and a male pronucleus (Table 3) and half the oocytes had been penetrated by multiple spermatozoa. In contrast, the rate of polyspermic oocytes grown *in vivo* was low. Two days after insemination, 24% (18/75) of oocytes cleaved and 2% (2/75) developed to the 5- to 8-cell stage (Fig. 3c, Table 4). However, no embryo reached the blastocyst stage. On the other hand, 72% (86/120) of *in vivo* grown oocytes cleaved, and 33% developed to the blastocyst stage.

There are several potential causes of maturational and developmental failure of bovine oocytes grown in SCID mice. Some oocytes competent to reach metaphase II are not able to develop to the blastocyst stage, which is indicative of deficient or defective cytoplasmic maturation (Eppig *et al.*, 1994). In the present study, xenografted oocytes were exposed to inappropriate conditions in SCID mice for 8 weeks. Considering the difficulties of maintaining oocyte

viability *in vitro*, it was remarkable that bovine oocytes survived and grew to nearly full size during 8 weeks of grafting. However, placing the oocytes in non-optimal circumstances in SCID mice might have resulted in the acquisition of incomplete developmental competence. In addition, it is unclear how far the ageing of these oocytes proceeded. To achieve a high efficiency of oocyte fertilization and embryonic development it may be necessary to control and to synchronize the ageing steps of recovered oocytes.

This study demonstrates that bovine oocytes grown in SCID mice can mature and be fertilized, although they do not acquire full developmental competence. We need to improve the technology for producing the oocytes to allow them to acquire full developmental competence, and verify the mechanisms underlying folliculogenesis and oogenesis in domestic animals.

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