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Summary

Cortical tissues containing only primordial and primary follicles, or secondary follicles 140–190 µm in diameter, were collected from bovine ovaries and xenografted under the kidney capsules of female severe combined immunodeficient (SCID) mice. Histological examination revealed that all grafts were well vascularised and contained surviving follicles at 4 or 6 weeks after grafting. Primordial and primary follicles survived but did not develop beyond the one-layer stage. Secondary follicles, on the other hand, had formed antra at 4 weeks after grafting. The mean diameter of secondary follicles, which was 165.2 ± 17.0 µm ($n = 42$) before grafting, had developed to 442.9 ± 77.9 µm ($n = 37$) and 592.9 ± 116.0 µm ($n = 45$) in diameter at 4 and 6 weeks after grafting, respectively. The mean diameter of oocytes, which was 55.1 ± 4.9 µm ($n = 42$) before grafting, also increased significantly (4 weeks: 105.6 ± 6.3 µm; 6 weeks: 122.2 ± 2.6 µm; $p < 0.05$). Oocytes were recovered from follicles that had developed to more than 400 µm in diameter after 6 weeks, and were subjected to subsequent mature culture. Of these oocytes, 34% (11/32) resumed meiosis and 6% (2/32) matured to the second metaphase. Follicular fluid in bovine antral follicles developed in SCID mice had the 69 kDa protein, which was detected by anti-mouse albumin antibody but not by anti-bovine albumin antibody in immunoblotting analysis. These results demonstrated that bovine secondary follicles develop to the antral stage in SCID mice, and that the oocytes in the follicles acquire the meiotic competence.

Key words: Cow, Follicular development, Growing oocyte, Ovary, Xenografting

Introduction

Mammalian ovaries contain large numbers of follicles that are in various developmental stages (Erickson, 1966; Gosden & Telfer, 1987). Primordial follicles are formed when primary oocytes become invested with a layer of flattened pre-granulosa cells. These follicles constitute a pool from which their further development can begin. However, throughout the reproductive life span of female mammals, only a limited number of oocytes grow to final size and are ovulated. The rest of the oocytes degenerate during the growth phase or remain in the primordial follicles. Artificial growth of these oocytes will provide a new source of mature oocytes for livestock production and reproduction in humans and endangered species.

Eppig & O'Brien (1996) successfully produced live young from mouse oocytes that were grown, matured and fertilised *in vitro*. Several culture systems have been developed to support the growth of oocytes in rodent follicles, and gradual progress has been made by modifying these techniques to support the growth of oocytes from domestic animals *in vitro*. In domestic animals, growing oocytes from developing follicles have survived and been grown to their final size *in vitro* (Hirao *et al.*, 1994; Harada *et al.*, 1997; Osaki *et al.*, 1997; Yamamoto *et al.*, 1999; Gutierrez *et al.*, 2000). Yamamoto *et al.* (1999) have successfully produced live young from bovine growing oocytes collected from early antral follicles 0.5–0.7 mm in diameter, but the efficiency of the procedure was low.

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Xenografting of ovarian tissue into immunodeficient mice has been used as a model to verify follicular viability and also 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). The follicular development of sheep (Gosden *et al.*, 1994), cats (Gosden *et al.*, 1994), marmosets (Candy *et al.*, 1995) and humans (Weissman *et al.*, 1999) to the antral stage has been reported in xenografts into immunodeficient mice. Recently, frozen-thawed ovarian tissues have been shown to survive and to develop into antral follicles in immunodeficient mice (Oktay *et al.*, 1998; Gook *et al.*, 2001; van den Broecke *et al.*, 2001). These studies demonstrated that ovaries or ovarian tissue xenografted into immunodeficient mice could lead to the development of apparently normal antral follicles. However, because mammalian ovaries contain follicles that are at different developmental stages, it is unclear which follicles at which stages develop to the antral stage. Furthermore, it has been demonstrated that mammalian follicles survive and develop in immunodeficient mice, but whether or not oocytes in follicles developing in the mice acquire meiotic competence has not been determined. In the present study, we collected bovine cortical slices containing primordial and primary follicles or secondary follicles 140–190 μm in diameter, and xenografted the follicles into severe combined immunodeficient (SCID) mice. Then, oocytes in the follicles developing in the SCID mice were subjected to maturation culture, and the meiotic competence of the oocytes was examined.

Materials and methods

Xenografting of bovine follicles into SCID mice

Ovaries were obtained from pure-bred Japanese Black cows slaughtered at a local abattoir, and were washed in Dulbecco's phosphate-buffered saline three times. In the first experiment, slices of ovarian cortex measuring approximately 2.0 mm × 2.0 mm × 0.5 mm were cut off using fine blades while immersed in TCM199 (pH 7.4; Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% polyvinylalcohol, 0.85 mg/ml NaHCO$_3$, 0.08 mg/ml kanamycin and 25 mM HEPES. Cortical slices that had only primordial and primary follicles were selected under a dissecting microscope (×40). Each of these cortical slices was bisected. A bisected section from each slice was then used for xenografting into 4 female SCID mice (CLEA Japan, Tokyo, Japan), while the other section was fixed in 3% formaldehyde in phosphate-buffered saline immediately for histological examination. In the second experiment, approximately 0.5 mm × 0.5 mm × 0.5 mm slices of ovarian cortex containing only one secondary follicle 140–190 μm in diameter

were dissected. Secondary follicles were randomly separated into two groups: one for measuring the diameters of the follicles and oocytes, and the other for xenografting. Groups of 10–16 secondary follicles enclosed by connective tissue were xenografted into 8 female SCID mice.

Female SCID mice 8–15 weeks old were anaesthetised by an intraperitoneal injection of sodium pentobarbital, and the left kidney was exteriorised. Bovine ovarian slices containing primordial/primary or secondary follicles were inserted under the kidney capsule using a pipette. This study was approved by the Committee on Animal Experimentation of Kobe University, Rokkodai Campus, Japan.

Histological examination

Four or six weeks after xenografting, the mice were killed by cervical dislocation, and the kidneys containing cortical slices were carefully dissected free with fine forceps. After washing in Dulbecco's phosphate-buffered saline, the kidneys were fixed in 3% formaldehyde in phosphate-buffered saline. They were dehydrated, embedded in JB-4 (Polyscience, Niles, IL), serially sectioned at 5 μm, stained with haematoxylin and eosin, and examined. Follicles were counted and classified, according to the number and morphology of granulosa cell layers, into four categories: primordial follicles with one layer of squamous granulosa cells surrounding the oocyte, primary follicles containing a single layer of cuboidal granulosa cells, secondary follicles containing an oocyte encapsulated by two or more layers of granulosa cells but no antrum, and antral follicles having an antral cavity with multiple layers of granulosa cells. The serial sections were examined to find the largest cross-section of follicles and oocytes. The diameters of the follicles and oocytes (excluding the zona pellucida) were measured by taking two perpendicular measurements to the nearest 1 μm with an ocular micrometer (Nikon, Tokyo, Japan) attached to an inverted microscope and recording the average. For the controls, ovarian slices containing primordial/primary follicles before xenografting were examined in the same manner.

Maturation culture of oocytes recovered from SCID mice

To examine the meiotic competence of the oocytes that grew in the SCID mice, secondary follicles enclosed by connective tissue were collected and xenografted into 6 SCID mice in the same manner as described above. After 6 weeks, the kidneys containing bovine follicles were recovered. The antral follicles that had developed to more than 400 μm in diameter in the grafts were collected with a pair of fine blades under a dissecting

microscope. These follicles were then opened with fine needles, and cumulus-cell-enclosed or denuded oocytes were recovered. They were washed in HEPES-buffered TCM199, and were then cultured individually in a 10 μl drop of maturation medium, which was TCM199 containing 2.2 mg/ml $NaHCO_3$, 0.08 mg/ml kanamycin, 0.1 mg/ml sodium pyruvate and 10% fetal calf serum (FCS; Dainippon Pharmaceutical, Osaka, Japan) for 24 h at 38.5 °C in a humidified atmosphere of 5% CO_2 and 95% air. After the maturation culture, the oocytes enclosed by cumulus cells were completely denuded by pipetting. Oocytes that showed evidence of cytoplasmic degeneration were excluded from further analysis. Oocytes showing normal morphology were considered surviving oocytes. After their diameters had been measured 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 precise maturational stage of each oocyte was determined on the basis of the changes in the configuration of chromosomes and nuclear membranes. Oocytes with abnormal configurations of chromosomes were classified as degenerated oocytes. For controls, oocyte–cumulus cell complexes collected from secondary follicles 0.1–0.2 mm in diameter and late antral follicles 3–5 mm in diameter were cultured in maturation medium for 24 h, and then their nuclear morphology was examined in the same manner.

Immunochemistry of serum albumin in follicular fluid

To examine the origin of follicular fluid in the bovine follicles that developed in SCID mice, all proteins in follicular fluid were analysed by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and silver staining. Next, serum albumins in follicular fluid were detected using anti-bovine albumin and anti-mouse albumin antibodies.

Bovine secondary follicles were xenografted in SCID mice in the same manner as described above. After 4 weeks, the follicles that had grown to the antral stage were recovered. After the diameters of the follicles had been measured under an inverted microscope, morphologically normal follicles 400–500 μm in diameter were selected, and the volumes of the follicular fluid were calculated from the diameter of the antra. The follicles were then transferred onto a sheet of plastic film, and a volume of ice-cold phosphate-buffered saline was added to make a 0.1% follicular fluid. The follicles were opened, and the antral fluids with phosphate-buffered saline were collected by a micropipette and pooled in Eppendorf tubes. They were centrifuged at 400 *g* for 5 min and the supernatant was recovered and added to an equal volume of a 2× SDS-PAGE

sample buffer (Laemmli, 1970). The samples were boiled for 5 min and then stored at –20 °C before use. For controls, samples of sera and follicular fluid from cows and female SCID mice were prepared in the same manner. After the samples had been run in 13% SDS-polyacrylamide gels, the gels were fixed, silver-stained and photographed.

After electrophoresis in the same manner, proteins were transferred to hydrophobic polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA) in a Trans-Blot SD semi-dry transfer cell (Bio-Rad Laboratories, Hercules, CA) for 1 h at 2 mA/cm² in transfer buffer. The membranes were blocked with 10% skimmed milk in phosphate-buffered saline containing 0.1% Tween 20 (PBS-Tween) for 3 h, and then incubated with rabbit anti-bovine albumin antibody (batch no. 21111903, Biogenesis, Poole, UK) or rabbit anti-mouse albumin antibody (lot 062, DAKO, Glostrup, Denmark) at a dilution of 1:200 for 4 h at room temperature. After three washes in PBS-Tween, the membranes were incubated for 1 h at room temperature in blocking buffer containing horseradish-peroxidase-labelled donkey anti-rabbit immunoglobulin antibody (1:1000, Amersham Life Science, Buckinghamshire, UK). After three washes of 10 min each with PBS-Tween, peroxidase activity was visualised using the ECL Western blotting detection system (Amersham Life Science).

Statistical analysis

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

Results

Bovine primordial and primary follicles in SCID mice

In the cortical slices of bovine ovaries, some regions were rich in primordial and primary follicles but not follicles that had developed to the secondary stage or beyond (Fig. 1*a*). We confirmed that the histological sections of the slices contained only primordial and primary follicles (Fig. 1*b*). There were about 20 primordial follicles and 2–7 primary follicles per 2 mm × 1 mm × 0.5 mm of the cortical slices (Table 1). There were no degenerating follicles in these slices.

Four or 6 weeks after xenografting, histological examination revealed that all grafts were well vascularised and contained surviving follicles. However,

Table 1 Number of bovine primordial and primary follicles in xenografts

Xenografting*	No. of grafts examined	Mean no. of primordial follicles	Mean no. of primary follicles
Before	4	18.8 ± 2.1[a]	5.5 ± 1.3[a]
After	4	20.8 ± 2.6[a]	3.3 ± 1.3[a]

*Each bovine ovarian slice measuring approximately 2.0 mm × 1.0 mm × 0.5 mm was cut off and xenografted under the kidney capsule of SCID mouse for 4–6 weeks.
[a]Values with different superscripts in the same column differ significantly ($p < 0.05$).

there were only primordial and primary follicles in the grafts, and no secondary follicles (Fig. 1*c*). In the primordial and primary follicles, oocytes were surrounded closely by a layer of squamous and cuboidal granulosa cells, and were morphologically normal. Because the numbers and diameters of follicles showed no significant differences between 4 and 6 weeks after xenografting, we pooled the data in Table 1. When the number of follicles after xenografting was compared with that before grafting, no significant shift from primordial follicles to primary follicles was observed. The mean diameter of primordial follicles after xenografting was 37.9 ± 4.6 µm, which was similar to that of the follicles in the tissue before xenografting (38.5 ± 3.8
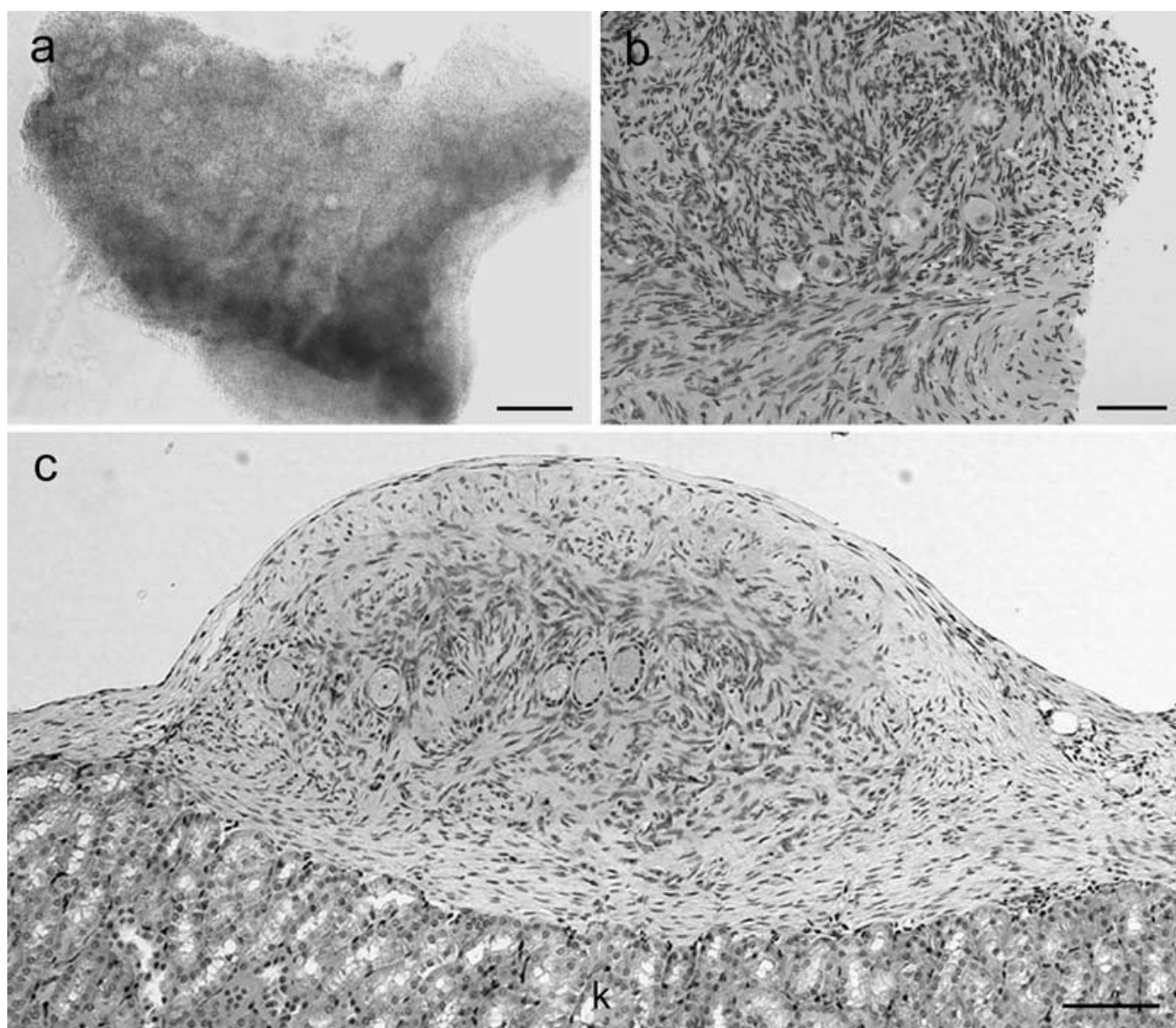

Figure 1 Cortical slices were dissected from bovine ovarian cortex, and slices containing only primordial and primary follicles were selected under a dissection microscope (*a*). In the histological sections, the slices contained only follicles at primordial and primary stages (*b*). The slices were xenografted under the kidney capsules of SCID mice. Only primordial and primary follicles were observed in the grafts 6 weeks after xenografting (*c*). Scale bars represent 200 µm in (*a*), and 100 µm in (*b*) and (*c*). k, kidney tissue.

μm) (Table 2). Likewise, no difference was observed between diameters of primary follicles before and after grafting. The oocyte diameters in the primordial and primary follicles did not change after grafting.

Development of bovine secondary follicles in SCID mice

Four weeks after grafting, antral follicles were observed on the kidney surface of the SCID mice (Fig. 2*b*). Vascularisation was seen on the surface of the antral follicles under a dissecting microscope (Fig. 2*c*). In the recovered grafts, some follicles had developed a clear antrum (Fig. 2*d*). In the histological sections, well-developed antral follicles contained oocytes that had remained at the germinal vesicle stage (Fig. 3). However, a few follicles had been invaded by many erythrocytes, and the oocytes in those follicles had degenerated.

Table 3 shows the development of follicles and oocytes after grafting. The mean diameter of secondary follicles collected from bovine ovaries before grafting

Table 2 Change in follicle and oocyte diameters of bovine primordial and primary follicles xenografted in SCID mice

Xenografting*	No. of grafts examined	Primordial follicles			Primary follicles		
		No. of follicles	Mean diameter of follicles (μm ± SD)	Mean diameter of oocytes (μm ± SD)	No. of follicles	Mean diameter of follicles (μm ± SD)	Mean diameter of oocytes (μm ± SD)
Before	4	75	38.5 ± 3.8^a	30.5 ± 3.1^a	22	56.1 ± 6.4^a	44.1 ± 4.2^a
After	4	83	37.9 ± 4.6^a	30.0 ± 3.0^a	13	56.5 ± 6.7^a	44.9 ± 3.6^a

*Each bovine ovarian slice measuring approximately 2.0 mm × 1.0 mm × 0.5 mm was cut off and xenografted under the kidney capsule of SCID mouse for 4–6 weeks.
[a]Values with different superscripts in the same column differ significantly ($p < 0.05$).
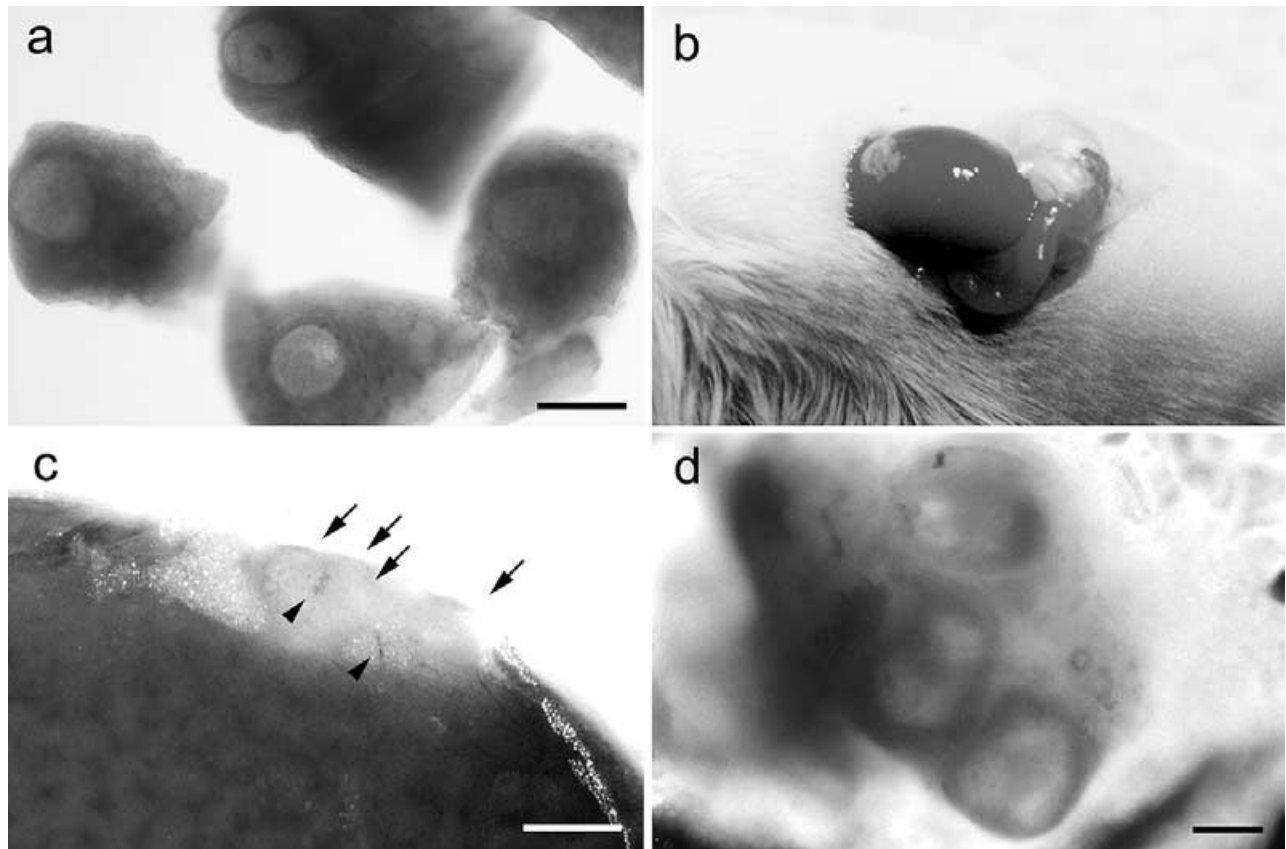

Figure 2 Bovine secondary follicles (*a*) were xenografted under the kidney capsules of SCID mice (*b*). Bovine secondary follicles developed to antral follicles (*c*, arrows) under the kidney capsule after 4 weeks and vascularisation (arrow heads) to the follicular surface was observed. Antral follicles were recovered from a kidney capsules 6 weeks after xenografting (*d*). Scale bars represent 200 μm in (*a*) and (*d*), and 500 μm in (*c*).

was 165.2 ± 17.0 µm (n = 42). The diameter increased gradually as the duration of grafting was prolonged. After 4 weeks, all surviving follicles had formed antra and developed to 442.9 ± 77.9 µm (n = 37). Thereafter, the follicles grew larger and the final diameter of morphologically normal follicles after 6 weeks was 3–4 times greater than the diameters of follicles before xenografting. After 4 and 6 weeks of grafting, 63% (34/54) and 59% (36/61) of oocytes respectively were morphologically normal, and all the oocytes were at the germinal vesicle stage. The diameters of the oocytes increased significantly, and they reached the fully grown size after 6 weeks.

Meiotic competence of bovine oocytes grown in SCID mice

After 6 weeks of grafting, 32 morphologically normal oocytes were recovered from the 6 SCID mice. All the oocytes grew to be 105 µm or more, and their mean diameter was 121.7 ± 4.0 µm, which was significantly larger than that before grafting (Table 4). After 24 h of maturation culture, 34% (11/32) of the oocytes had undergone germinal vesicle breakdown, and 6% (2/32) had reached the second metaphase. During maturation culture, no cumulus expansion was observed. When the oocytes collected from secondary follicles (140–190 µm in diameter) and late antral follicles (3–5 mm in diameter) were subjected to maturation culture in the same manner, 0 (0/41) and 80% (35/44) of the oocytes had matured to the second metaphase, respectively.

Serum albumin in follicles developed in SCID mice

Proteins in follicular fluid in the bovine antral follicles developed in SCID mice were silver-stained after electrophoresis (Fig. 4*a*). A protein of 69 kDa was detected, and proteins with similar molecular weight were clearly detected in all samples including mouse and bovine sera. Two other proteins, of 45 and 50 kDa, were detected in three kinds of follicular fluids, but not in serum samples. In immunoblotting analysis, anti-mouse albumin antibody reacted with the 69 kDa protein in follicular fluid (Fig. 4*b*, lane 2) and with mouse serum (Fig. 4*b*, lane 4). Furthermore, the 69 kDa protein in the follicular fluid in bovine antral follicles grown in the SCID mice reacted with the anti-mouse albumin antibody (Fig. 4*b*, lane 1), but bovine follicular fluid and serum did not. The 69 kDa protein in follicular

Table 3 Follicular development and oocyte growth in bovine secondary follicles xenografted in SCID mice

Duration of xenografting* (weeks)	No. of mice	No. of grafted follicles	No. (%) of surviving follicles	Mean diameter of follicles (µm ± SD)	No. (%) of surviving oocytes	Mean diameter of oocytes (µm ± SD)
Before	–	42	42 (100)	165.2 ± 17.0[a]	42 (100)	55.1 ± 4.9[a]
4	4	54	37 (68.5)	442.9 ± 77.9[b]	34 (63.0)	105.6 ± 6.3[b]
6	4	61	45 (73.8)	592.9 ± 116.0[c]	36 (59.0)	122.2 ± 2.6[c]

*Groups of 10–16 bovine secondary follicles 140–190 µm in diameter were xenografted under the kidney capsule of each SCID mouse.
[a–c]Values with different superscripts in the same column differ significantly ($p < 0.05$).

Table 4 Maturational competence of bovine oocytes grown in SCID mice

Follicles*	No. of oocytes examined	Mean diameter of oocytes (µm ± SD)	Nuclear morphology of oocytes (%)			No. (%) of oocytes degenerating
			GV	MI	MII	
In vivo (secondary)	41	55.1 ± 4.5[a]	32 (78)[a]	0 (0)	0 (0)	9 (22)
In vivo (late antral)	44	120.0 ± 4.1[b]	1 (2)[b]	4 (9)[a]	35 (80)[a]	4 (9)
Xenografted	32	121.7 ± 4.0[b]	12 (41)[c]	9 (28)[b]	2 (6)[b]	8 (25)

GV, germinal vesicle stage; MI, the first metaphase; MII, the second metaphase.
*After 6 weeks of transplantation, surviving oocytes in the xenografted follicles in SCID mice were recovered, transferred to maturation medium for 24 h, and their nuclear morphology was examined. As the controls, oocyte–cumulus complexes collected from secondary (140–190 µm) or late antral (3–5 mm) follicles were cultured in maturation medium for 24 h, and then their nuclear morphology was examined.
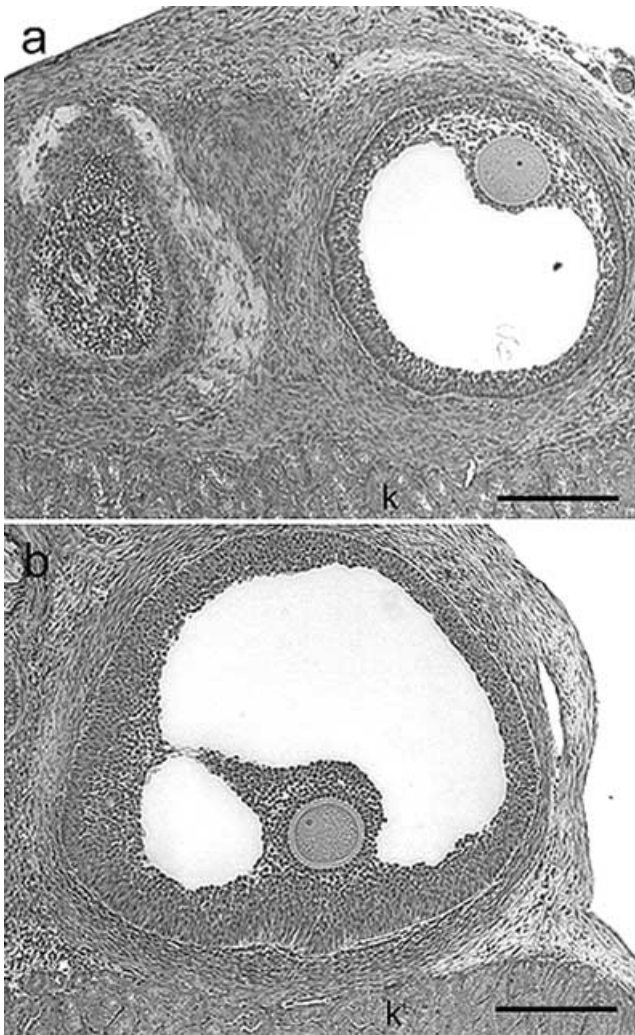[a–c]Values with different superscripts in the same column differ significantly ($p < 0.05$).

Figure 3 In the histological sections, bovine secondary follicles developed to the antral stage under kidney capsules of SCID mice at 4 weeks (*a*) and 6 weeks (*b*) after xenografting. Scale bars represent 200 μm in (*a*) and (*b*). k, kidney tissue.

fluid in bovine antral follicles grown in SCID mice was scarcely detected by anti-bovine albumin antibody, similar to the case of mouse with follicular fluid and serum (Fig. 4*c*).

Discussion

By culturing mouse newborn ovaries containing primordial follicles, Eppig & O'Brien (1996) grew oocytes in the follicles through maturity and fertilisation, obtaining a live young. Because mouse oocytes start to grow after birth, only primordial follicles are present in the ovary of the newborn infant at birth. In contrast, bovine primordial follicles start to develop in the ovaries of the fetus, and neonatal ovaries already have

follicles of various developmental stages. Although there have been several reports of the development of small follicles from various animals to the antral stages in immunodeficient mice (Gosden *et al.*, 1994; Oktay *et al.*, 1998; Liu *et al.*, 2000; Hosoe *et al.*, 2001; Metcalfe *et al.*, 2001), there have been few reports in which follicles were sorted by their developmental stage for xenografting. In the present study, pieces of ovarian cortex containing only primordial and primary follicles, as well as pieces containing only secondary follicles, were collected and grafted separately into SCID mice. The xenografted follicles survived in the SCID mice, although the rate of degeneration of the secondary follicles was a little higher than that of the primordial/primary follicles. Primordial follicles evidently survived in the grafts better than follicles at later stages, perhaps as a result of their lower metabolic activity (Krohn, 1977). Follicles that have entered their developing stage are metabolically more active and need more substances for their survival. Follicles that do not receive sufficient metabolites will degenerate by ischaemia (Bland & Donovan, 1968; Felicio *et al.*, 1984). It is possible that some bovine secondary follicles had received insufficient amounts of substances and degenerated before they became vascularised.

Bovine primordial and primary follicles did not develop to the secondary stage, although they survived for 6 weeks in SCID mice. It is unclear what triggered the oocytes to enter the growth phase, although some factors derived from follicular cells are thought to be involved in the development of primordial and primary follicles (Huang *et al.*, 1993; Dong *et al.*, 1996; Yoshida *et al.*, 1997). The KIT ligand (KL or stem cell factor), which functions in a paracrine manner, is produced by granulosa cells in primordial follicles in mouse and sheep (Clark *et al.*, 1996; Tisdall *et al.*, 1997). The KIT receptor has been expressed in mouse oocytes at the diplotene stage (Manova *et al.*, 1993) and in fetal sheep oocytes from the primordial follicle stage (Clark *et al.*, 1996). Yoshida *et al.* (1997) demonstrated the importance of KL–KIT interactions for growth initiation of mouse oocytes, based on administration of the antibody (ACK2) that blocks KL–KIT binding. By injection of ACK2 into newborn mice, the initiation of follicular development was completely inhibited. In addition, Parrott & Skinner (1999) used an *in vitro* culture model of postnatal rat ovaries in the presence of KL to demonstrate that KL supplementation markedly induced development of primordial follicles. Furthermore, the role of oocytes themselves in initiating their growth phase has been demonstrated (Eppig, 2001). Some factors secreted by oocytes have been identified as regulating follicular development and oocyte growth. Growth differentiation factor 9 (GDF-9) is an oocyte-specific member of the TGFβ family produced by ovine and bovine oocytes (Bodensteiner *et al.*,
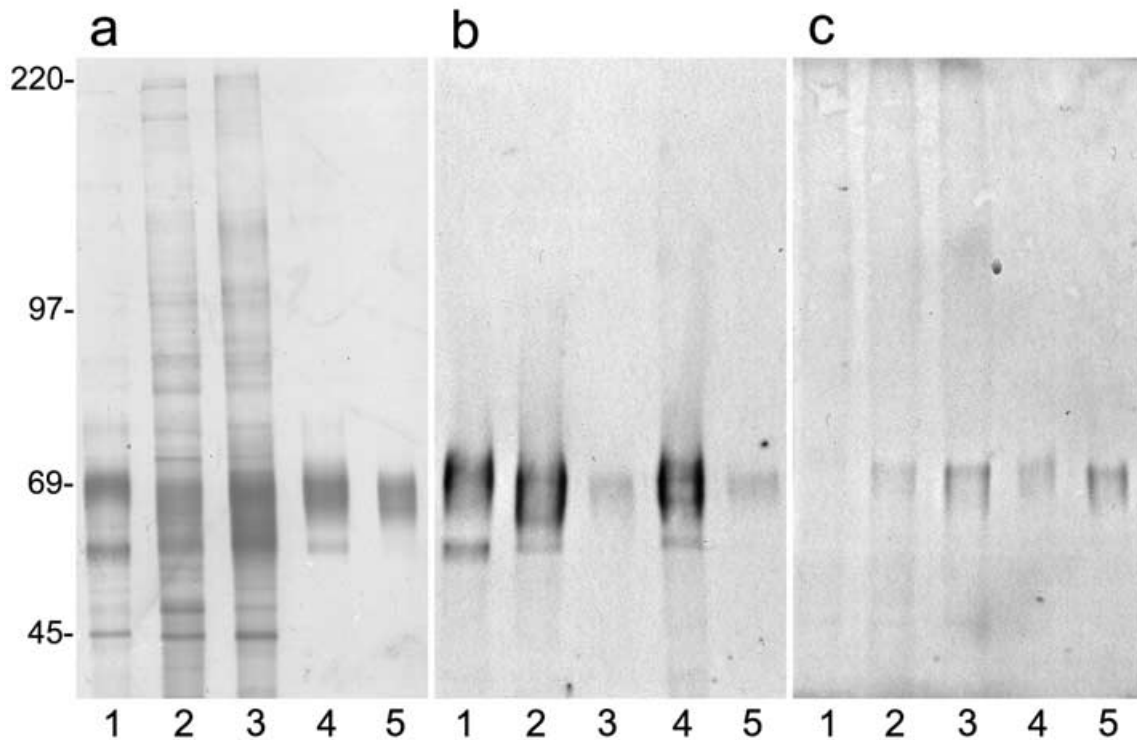
Figure 4 Immunochemical analysis of the follicular fluids accumulated in the bovine antral follicles that developed in SCID mice. Follicular fluid was collected 4 weeks after xenografting, run in 13% SDS-polyacrylamide gels and silver-stained (*a*). Albumin from the follicular fluid was Western blotted with anti-mouse albumin antibody (*b*) and anti-bovine albumin antibody (*c*). Lane 1, bovine follicular fluid in the follicles developed in the SCID mice; lane 2, mouse follicular fluid in follicles 0.4–0.5 mm in diameter; lane 3, bovine follicular fluid in follicles 0.5–0.7 mm in diameter; lane 4, mouse serum; lane 5, bovine serum. Molecular mass standards (kDa) are shown on the left.

1999). In GDF-9 knockout mice, folliculogenesis is blocked at the primary stage, as is oocyte growth (Dong *et al.*, 1996). Furthermore, granulosa cell expression of the KL gene appeared to be regulated by GDF-9 secreted by oocytes (Elvin *et al.*, 1999). Another member of the TGFβ family, BMP-15, exhibits an expression pattern similar to that of GDF-9 (Dube *et al.*, 1998; Jaatinen *et al.*, 1999; Elvin *et al.*, 2000; Otsuka *et al.*, 2000). Homozygosity of a natural mutation in sheep results in sterility due to the failure of the follicles to develop beyond the primary stage (Davis *et al.*, 1992; Galloway *et al.*, 2000), similar to that observed in GDF-9 null mice. Although we have not examined the expression of these factors by bovine primordial and primary follicles and their oocytes, it is thought that some factor or factors must exist for the initiation of the bovine oocytes in primordial and primary follicles.

In contrast, bovine secondary follicles not only survived but developed to the antral stage after xenografting. Each stage of mammalian follicular development requires various factors. Follicular development has been classified into gonadotrophin-independent and gonadotrophin-dependent phases. It is considered that secondary and antral follicles continue to develop under the control of gonadotrophins (Oktay *et al.*,

1998). In the present study, bovine secondary follicles may have developed under gonadotrophins provided by the SCID mice.

As bovine follicle size increases to 3 mm, there is a corresponding increase in oocyte diameter. Thereafter, the mean oocyte diameter reaches a plateau (120–130 µm) independent of the follicle diameter (Fair *et al.*, 1995). It takes several months for bovine secondary follicles to develop to the Graafian stages (Lussier *et al.*, 1987). Although surviving secondary follicles developed to approximately 0.6 mm in diameter 6 weeks after grafting in the present experiment, the mean diameter of oocytes in the follicles reached 122.2 ± 2.6 µm, which is comparable with that of oocytes in follicles 3–5 mm in diameter. This result demonstrates that bovine oocytes in the follicles grew to their final size, although the follicular development in the xenografts differed from that in the bovine ovaries.

Bovine follicles that developed in the SCID mice were recovered after 6 weeks of grafting, and the oocytes in those follicles were subjected to maturation culture. Thirty-four per cent (11/32) of the oocytes resumed meiosis and 6% (2/32) matured to the second metaphase. This result is the first report, using large animals, to show that oocytes grown in SCID mice

resume meiosis and mature to the second metaphase. Bovine oocytes >110 μm in the ovary acquire the ability to mature to the second metaphase (Fair *et al.*, 1995; Otoi *et al.*, 1997). In the maturation culture in the present study, 80% of oocytes that grew to their final size *in vivo* matured to the second metaphase. However, the oocytes that grew in SCID mice matured to the second metaphase at the low rate of 6%. Bovine oocytes that grew in a long-term culture acquired meiotic competence but the maturation rate was low (Harada *et al.*, 1997; Osaki *et al.*, 1997; Yamamoto *et al.*, 1999). It is considered that additional factors, which are deficient in SCID mice, are required for bovine oocytes to acquire full meiotic competence.

Oocyte growth and differentiation in follicles depend on communication between the oocytes and the granulosa cells with which they are intimately associated. Throughout oocyte growth in the ovary, granulosa cells are coupled with the oocytes via heterologous gap junctions, through which small molecules such as energy substrates, nucleotides and amino acids are transferred into the oocytes (Heller *et al.*, 1981; Brower & Shultz, 1982). Besides having a nutritional role, the meiosis-arresting effect of the follicular environment is probably mediated by the passage of substances from the granulosa cells to the oocyte through gap junctions (Tsafriri & Channing, 1975; Leibfried & First, 1980; Dekel, 1988; Racowsky & Baldwin, 1989). In the present study, all the grown oocytes were at the germinal vesicle stage in the SCID mice. This suggests that granulosa cells maintained the oocytes at the prophase of the first meiosis. However, in the maturation culture no expansion of cumulus granulosa cells was observed in the oocyte–cumulus complexes from the SCID mice. The cumulus cells in bovine Graafian follicles undergo expansion or mucification, consisting of the deposition of a hyaluronic-acid-rich extracellular matrix in response to hormonal stimulation. Although bovine oocytes in SCID mice had grown to full size 6 weeks after grafting, the follicles were smaller than those developed *in vivo*. This suggests that cumulus granulosa cells in the bovine follicles recovered from the SCID mice did not differentiate and mature sufficiently, and it is possible that the ability of the cumulus granulosa cells was not sufficient for the oocytes to acquire full meiotic competence.

Western blotting showed that the 69 kDa protein in the follicular fluid was serum albumin. The follicular fluid in the bovine antral follicles that developed in the SCID mice reacted strongly with anti-mouse albumin antibody, but not with anti-bovine albumin antibody. This result indicates that mouse serum albumin permeated into grafted bovine follicles through the vascularised capillaries surrounding the follicles. The follicular fluid that accumulated in antral follicles consisted of components that theca cells and granulosa cells secrete,

in addition to components of plasma origin (McNatty, 1978). The follicular fluid has effects that suppress oocyte meiotic resumption (Tsafriri & Channing, 1975; Nandedkar *et al.*, 1996) and that maintain oocytes for further development. In the present study, it is speculated that the components in mouse blood other than serum albumin also invaded into the bovine follicles. Although the components in mouse blood may participate in bovine follicular development and oocyte growth, it must be examined further whether bovine oocytes develop the competence for fertilisation and develop fully under such conditions. Quite recently Snow *et al.* have demonstrated that mouse ovarian tissue, when xenografted into nude rats, can produce mature oocytes that can be fertilised and develop into fertile adult mice (Snow *et al.*, 2002). It may be possible in the near future to produce live young from xenografted ovarian tissue from domestic animals.

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