KIT-KIT Ligand in the Growth of Porcine Oocytes in Primordial Follicles

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Abstract. Mammalian ovaries are endowed with a huge number of small oocytes in primordial follicles (primordial oocytes). The mechanism regulating initiation of oocyte growth and follicular development is not well understood. Several growth factors and cytokines are known to be involved in oocyte growth and follicular development. Herein, the involvement of KIT, a receptor tyrosine kinase, and its ligand, KIT ligand (KL), in the initiation of porcine oocyte growth was examined. At first, KIT expression was examined immunohistochemically in primordial oocytes from neonatal (10–20 days) and prepubertal (about 6 months) pigs. Similar expression of KIT was detected in all oocytes from both the neonatal and prepubertal pigs. Next, to examine the growth of primordial oocytes, ovarian tissues containing primordial oocytes were xenotransplanted into immunodeficient SCID mice. Primordial oocytes from the neonatal pigs grew with follicular development as described previously, whereas those from the prepubertal pigs did not initiate growth in the xenografts after 2 months. To stimulate the growth of primordial oocytes from the prepubertal pigs, they were cultured in a medium supplemented with KL (50 and 100 ng/ml) for 1 or 3 days before xenografting. After 2 months, however, the oocytes did not grow, and the primordial follicles did not develop, although a higher number of primordial oocytes survived in the KL-treated tissues. These results suggest that KIT-KL might not be associated with the growth initiation of porcine primordial oocytes, although they do enhance the survival of the oocytes.

Key words: KIT, KIT ligand, Oocyte, Pig, Primordial follicle

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mordial follicles to the antral stage in nude mice 45–75 days after xenografting of ovarian tissues collected from 20-day-old pigs. We have previously demonstrated that primordial follicles from cows survive without initiating development 6 weeks after xenografting to SCID (severe combined immune deficiency) mice [5]. Similarly, our preliminary data showed that primordial oocytes from prepubertal pigs (6-month-old) did not start to grow 2 months after xenografting to SCID mice. We considered that porcine primordial oocytes from prepubertal ovaries might be deficient in certain stimulatory factors or cascades necessary for oocyte growth and follicular development.

Several growth factors and cytokines are thought to regulate the process of oocyte growth and follicular development [6, 7]. Among these, KIT (c-Kit), a tyrosine kinase receptor, and its ligand KL (KIT ligand, stem cell factor, SCF), have been suggested to play a key role in oocyte growth and follicular development in mammals [8]. There is expression of KIT protein on the surface of oocytes in mice [9] and sheep [10]. In situ hybridization has revealed the presence of mRNA for KIT in oocytes of primordial, developing and antral follicles in mice and sheep [11–13]. In sheep ovaries, KL and its encoding mRNA have been detected in the granulosa cells of follicles at all stages [10]. However, limited information is available regarding the functional significance of KIT and KL in other species.

The role of KIT in initiation of oocyte growth has been shown by inhibiting experiments using its specific antibody, ACK2. The numbers of growing oocytes decreases in ACK2-treated rodent ovaries in vitro [14, 15]. Injection of ACK2 into mice at various times during the first 2 weeks after birth causes disturbances in initial follicle recruitment [16]. Our previous results have demonstrated that KIT-deficient mice contain a number of germ cells at 13 days post-coitum, but that after birth, they have no oocytes [17]. On the other hand, KIT-deficient Fas-knockout Wv/Wv:Fas–/– mice have been found to contain a small number of oocytes after birth, with follicles developing to the antral stage at 14 days of age. These results show that oocytes grow and follicles develop without KIT signaling, although KIT might be essential for survival of germ cells/oocytes in mice.

To examine the involvement of KIT in initiation of porcine primordial oocyte growth, we conducted two experiments. In the first experiment, KIT expression was examined in primordial oocytes from prepubertal pigs by the immunohistochemical method and compared with that of primordial oocytes from neonatal pigs because our preliminary experiments showed that primordial oocytes from prepubertal pigs exhibited a different developmental pattern after xenografting from that reported in neonatal pigs [3, 4]. However, all oocytes from both groups showed a similar pattern of KIT expression. The tissues containing primordial oocytes from prepubertal pigs were then treated with KL and xenografted into SCID mice to examine possible stimulation by KL with regard to initiation of oocyte growth.

Materials and Methods

Collection of ovarian tissues containing primordial follicles

Ovaries were collected from 10- to 20-day-old piglets and 6-month-old crossbred (Landrace, Large White and Duroc) gilts. Ovaries of gilts just before puberty were collected at a local slaughterhouse. The ovaries were washed three times in Dulbecco’s phosphate-buffered saline supplemented with 0.1% (w/v) polyvinyl alcohol (PBS-PVA), and cortical tissues with a thickness of less than 0.5 mm (approximately) were dissected with surgical blades (No. 11). The tissues were then examined under dissection and inverted microscopes, and tissues containing secondary follicles were avoided. Primordial follicles were identified as having diameters within the range of 30 to 38 μm and oocytes that included a large spherical nucleus surrounded by small lipid droplets. Ovarian tissues (approximately, 2 × 1 × 0.5 mm) that contained primarily primordial follicles were selected and immersed in TCM-199 (pH 7.4, Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% (w/v) PVA, 0.85 mg/ml sodium bicarbonate, 0.08 mg/ml kanamycin sulfate (Sigma-Aldrich, St. Louis, MO, USA) and 25 mM HEPES.

Immunohistochemistry

Cryostat sections (6 μm thick) of cortical tissues from neonatal and prepubertal ovaries were prepared on silane-coated slides, air-dried and fixed in 1% (w/v) paraformaldehyde in PBS at room temperature for 15 min. To prevent nonspecific antibody binding, the sections were blocked with
3% (w/v) bovine serum albumin (BSA; Wako Pure Chemical, Osaka, Japan) for 1 h, and immunostaining was then performed with the primary antibody, goat polyclonal anti-c-kit antibody (1:250; Santa Cruz Biotechnology, Santa Cruz, CA, USA; #sc-1494) overnight at 4 C. After washing with PBS, the sections were reacted with Alexa Fluor 488-labeled donkey anti-goat immunoglobulin antibody (1:1,000; Molecular Probes, Eugene, OR, USA) for 45 min and then counterstained with propidium iodide (PI; 100 µg/ml; Sigma) for 15 min. After washing 3 times with PBS, the sections were mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA, USA) and observed under a fluorescence microscope (U-LH100HGAPO; Olympus, Tokyo, Japan). Some sections were incubated without primary antibody as negative controls.

**KL treatment**

Ovarian tissues (approximately, 2 × 1 × 0.5 mm) collected from the prepubertal pigs were cut into 2 pieces; one was fixed for histological examinations and the other was washed 3 times in 25 mM HEPES-buffered TCM-199 containing 0.1% (w/v) PVA, 0.85 mg/ml sodium bicarbonate and 0.08 mg/ml kanamycin sulfate. After washing, the tissues were incubated with gentle agitation in TCM-199 containing 10% (v/v) fetal calf serum (FCS; Dainippon Pharmaceutical, Osaka, Japan), 0.1 mg/ml sodium pyruvate and 0, 50 and 100 ng/ml (w/v) KL (recombinant mouse SCF; R&D Systems, Minneapolis, MN, USA; #455-MC) at 38.5 C for 1 or 3 days under an atmosphere of 5% CO₂ in humidified air. In the preliminary experiment, almost all primordial oocytes in the ovarian tissues looked healthy after 3 days of culture in this condition, although a duration of more than 3 days caused degeneration of some tissues and decrease in number of viable oocytes. Therefore, we set the maximum duration for KL treatment to 3 days. After incubation, some tissues were fixed for histological examination and others were xenografted into SCID mice. Some fresh tissues were also examined and xenografted in a similar manner.

**Xenografting**

Six- to 8-week-old male SCID mice were purchased from Clea Japan (Tokyo, Japan). Prior to xenografting, the mice were anaesthetized, and the left kidney was exteriorized through a dorsal-horizontal incision. A small hole was torn in the kidney capsule using fine forceps. Three pieces of porcine ovarian tissues were inserted under the kidney capsule of each SCID mouse. The surgery was performed at room temperature, and the mice were kept on a warming plate (37 C) for 24 h. The mice were housed in filter-topped cages in a positive-pressure room, with free access to clean water and balanced feed pellets. The light cycle of the room was set at 12/12 h L/D. The duration of xenografting was 2 months. Ovarian tissues from neonatal pigs were xenografted to confirm that our xenografting conditions were reliable. This study was approved by the Institutional Animal Care and Use Committee (permission number: 15-4-05) and was carried out according to the Guidelines of Animal Experimentation of Kobe University.

**Histological examination**

Ovarian tissues before and after xenografting were fixed in 3% (w/v) paraformaldehyde in PBS, dehydrated, embedded in methacrylate resin (JB-4; Polyscience, Niles, IL, USA), serially sectioned into 5 µm thick slices, and stained with hematoxylin and eosin. The number of follicles at different stages and the diameters of oocytes were recorded. The number of follicles was counted for every section in which an oocyte nucleus was observed. Double counting in adjacent sections was avoided. The follicles were classified into the following four categories according to the number and morphology of granulosa cell layers: primordial follicles with one layer of flattened granulosa cells surrounding the oocyte, primary follicles with a single layer of cuboidal granulosa cells, secondary follicles with two or more layers of granulosa cells, and antral follicles having an antral cavity with multiple layers of granulosa cells. The ovaries from the neonatal pigs contained primordial follicles based on the fact that the oocytes were surrounded by granulosa cells and the follicles contained naked oocytes with a few granulosa cells. The latter ones were also classified as primordial follicles. The diameters of the oocytes (excluding the zona pellucida) were measured to the nearest 1 µm by taking the average of two perpendicular measurements of each oocyte showing the maximum diameters in serial sections using an ocular micrometer (Nikon, Tokyo, Japan) attached to a microscope.
The average numbers of oocytes and follicles per tissue are represented as means ± SEM (standard error of the mean). Data were subjected to one-way ANOVA, and the significance of differences among means was determined by the Tukey’s multiple range test. Differences at P<0.05 were considered statistically significant.

Results

KIT expression in primordial oocytes

KIT signals were observed in all examined primordial oocytes from the neonatal (55/55 from 3 tissues) and prepubertal (20/20 from 3 tissues) pigs (Fig. 1). The signal was strong at the surface of all oocytes, while the cytoplasm was also stained in

Fig. 1. Immunolocalization of KIT in the primordial oocytes from the neonatal and prepubertal pigs. Cryosections were treated with goat anti-KIT antibody and Alexa Fluor 488-labeled anti-goat immunoglobulin antibody (B and E) and counterstained with PI (C and F). Expression was observed in all primordial oocytes from both the neonatal and prepubertal pigs. Serial sections were incubated without primary antibody as negative controls (A and D). The scale bar represents 40 µm.

Statistical analysis

The average numbers of oocytes and follicles per tissue are represented as means ± SEM (standard error of the mean). Data were subjected to one-way ANOVA, and the significance of differences among means was determined by the Tukey’s multiple range test. Differences at P<0.05 were considered statistically significant.
the cryostat sections. Oocyte nuclei did not show any KIT signals. The surface epithelium of the ovaries, ovarian stroma and granulosa cells of the primordial follicles were also negative. This expression pattern of KIT was similar for the tissues from both the neonatal and prepubertal pigs. In the negative control, none of the oocytes and granulosa cells were stained (Figs. 1A and D).

KL-treated primordial oocytes in xenografts

We examined the development of primordial follicles from the neonatal pigs under our grafting conditions. Histological examination revealed that the ovarian tissues from the neonatal pigs contained only primordial follicles before grafting (Fig. 2A, Table 1). The ovarian tissues, which were xenografted into SCID mice, grew to approximately...
3–5 fold in size after 2 months in compared with their original sizes. In the grafts, some of the primordial follicles developed to the antral stage, and the oocyte diameters increased (Fig. 2B and Table 1). The developing follicles were evenly distributed throughout the xenografts, irrespective of their locations (near or far from the mouse kidney). The xenografts were free from necrosis and were well vascularized. No corpora lutea were observed.

In the dissected ovarian tissues from the prepubertal pigs, almost all follicles were at the primordial stage, and the oocyte diameters were less than 35 μm (Fig. 3A and Table 1). The average number of primordial follicles was 55 ± 3 per tissue. After KL treatment for 1 or 3 days, the primordial follicles did not develop to the primary stage or beyond, and the diameters of oocytes did not change (Figs. 3B, 3C and Table 1). Some granulosa cells were detached from the oocytes, swollen and degenerated, although the oocytes and their nuclei were normal. The numbers of oocytes in the tissues before and after KL treatment were similar.

After xenografting for 2 months, the size of the xenografts remained similar, and there were no necrotic regions in the xenografts. All oocytes and granulosa cells appeared to be healthy and normal (Figs. 3D–F). However, all the xenografts contained only primordial follicles, indicating that the follicles remained in a similar state without initiation of development (Table 1). Higher numbers of oocytes were obtained in the KL-treated tissues compared with the non-treated controls (0 ng/ml KL) and fresh tissues. Before and after xenografting, the oocyte diameters were unchanged, which indicated that growth of the oocytes was not initiated by KL treatment.

**Discussion**

KIT and KL have been reported to be expressed in the ovaries of several species. Both the soluble- and membrane-bound forms of KL mRNA have been detected in juvenile and adult mouse ovaries and in granulosa cells isolated from mouse preantral follicles [9, 18]. The expression of KIT mRNA and KIT protein has been detected in the primordial, growing and full-grown oocytes of the mouse [8]. The mRNA encoding KL and KIT has also been detected in the porcine ovary; KL mRNA was localized in granulosa cells and KIT mRNA was detected in oocytes [19, 20]. In the present study, KIT was expressed in primordial oocytes from both neonatal and prepubertal pigs. This result agrees with the expression pattern in sheep [10] and humans [21], the KIT expressions of which were similarly found in primordial oocytes from fetal and postnatal ovaries.

The mechanism regulating the entrance of primordial oocytes into the growth phase remains unknown, although KIT-KL interaction is thought to play a pivotal role in this process in the mouse [8]. The role of KIT has been demonstrated by inhibiting experiments using its specific antibody, ACK2. Yoshida et al. [16] injected mice with ACK2 at various times during the first 2 weeks after birth and reported that neutralization of KIT caused disturbances in initial follicle recruitment. Furthermore, the results of in vitro studies suggest that KL promotes oocyte growth and follicular development in mice [14] and rats [15]. Packer et al. [14] reported that KIT and KL are required for mouse oocyte growth and that ACK2 inhibits this growth in vitro. Parrott and Skinner [15] suggested that KIT is necessary for primordial follicle development. Actually, in KL mutant mice, oocyte growth and follicular development stop at the primary stage [22]. On the other hand, no direct evidence of the involvement of KIT in oocyte growth has been obtained in KIT-mutant mice because the KIT mutation causes severe death of germ cells during the prenatal stage and no oocytes survive after birth [23, 24]. Fas, a member of the TNF (tumor necrosis factor) receptor superfamily containing a death domain, is involved in germ cell degeneration in the absence of KIT signaling [25]. In mutant mice that lack both KIT and Fas, the germ cells are rescued. Under such conditions, the surviving oocytes are able to grow with follicular development [17]. Thus, the importance of KIT in initiation of oocyte growth and follicular development remains controversial. Furthermore, the role of KIT and KL, especially in large animals, has not been studied because of the lack of appropriate methods for in vitro growth of primordial oocytes.

In the present study, primordial oocytes from neonatal pigs grew with follicular development in xenografts. The results and the time span for development of the primordial follicles from the neonatal pigs to the antral stage are consistent with the results of previous reports showing that antral follicles were obtained 45–75 days after xenograft-
Fig. 3. Histological sections of ovarian tissues from the prepubertal pigs before and after xenografting. Before xenografting, all of the ovarian tissues contained primordial follicles. The tissues were treated with KL (100 ng/ml) for 1 or 3 days prior to xenografting (B and C). Two months after xenografting, primordial follicles did not develop in either the treated (E and F) or non-treated (D) tissues. The scale bars represent 40 µm (A–C) and 100 µm (D–F). The letter ‘r’ indicates the renal tissue of SCID mice.
ing ovarian tissues from 20-day-old pigs [3]. On the other hand, the primordial oocytes from the prepubertal pigs did not grow after 2 months. We thought that the primordial oocytes from prepubertal ovaries might possess some deficiency or a higher demand for KL. Exogenous KL did not induce oocyte growth and follicular development after 3-day treatment of cultured ovarian tissues from the prepubertal pigs. We then conducted the xenografting experiment because we expected oocyte growth in the xenografts following KL treatment. However, no primordial follicles developed after grafting for 2 months, although a significantly higher number of primordial oocytes survived in the KL-treated tissues. This result suggests that KIT-KL is involved in survival of oocytes in pigs, although KL had no critical effect on initiation of growth of primordial oocytes from prepubertal pigs under our experimental conditions. It was unclear whether KL supported oocyte viability during in vitro treatment or in the subsequent xenografting in this experiment. Similar results have been reported by Carlsson et al. [26], who employed organ culture for human ovarian tissues. They showed that KL does not affect follicular development, although the number of viable follicles is increased.

In conclusion, KIT was expressed in the primordial oocytes of both the neonatal and prepubertal pigs. Primordial oocytes from the neonatal pigs grew with follicular development, whereas those from the prepubertal pigs did not initiate growth in xenografts after 2 months. Furthermore, treatment of primordial oocytes from the prepubertal pigs with exogenous KL did not induce oocyte growth and follicular development under our experimental conditions, although a higher number of primordial oocytes survived in the KL-treated tissues. These results suggest that KIT-KL might not be associated with growth initiation of porcine oocytes, although they do enhance oocyte survival.

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