Knockdown of FOXO3 induces primordial oocyte activation in pigs

Mohammad Moniruzzaman, Jibak Lee, Mai Zengyo and Takashi Miyano

Graduate School of Agricultural Science, Kobe University, Nada-ku, Kobe 657-8501, Japan

Correspondence should be addressed to M Moniruzzaman; Email: monir@silver.kobe-u.ac.jp

Abstract

Mammalian ovaries are endowed with a large number of primordial follicles, each containing a nongrowing oocyte. Only a small population of primordial oocytes (oocytes in primordial follicles) is activated to enter the growth phase throughout a female's reproductive life. Little is known about the mechanism regulating the activation of primordial oocytes. Here, we found that the primordial oocytes from infant pigs (10- to 20-day-old) grew to full size at 2 months after xenografting to immunodeficient mice, whereas those from prepubertal pigs (6-month-old) survived without initiation of their growth even after 4 months; thereafter, they started to grow and reached full size after 6 months. These results suggest that the mechanism regulating the activation of primordial oocytes in prepubertal pigs is different from that in infant pigs. In this regard, the involvement of FOXO3, a forkhead transcription factor, was studied. In prepubertal pigs, FOXO3 was detected in almost all (94±2%) primordial oocyte nuclei, and in infant pigs, 42±7% primordial oocytes were FOXO3 positive. At 4 months after xenografting, the percentage of FOXO3-positive primordial oocytes from prepubertal pigs had decreased to the infant level. Further, siRNA was designed to knock down porcine FOXO3. FOXO3-knockdown primordial follicles from prepubertal pigs developed to the antral stage accompanied by oocyte growth at 2 months after xenografting. These results suggest that primordial oocytes are dormant in prepubertal pigs by a FOXO3-related mechanism to establish a nongrowing oocyte pool in the ovary, and that a transient knockdown of the FOXO3 activates the primordial oocytes to enter the growth phase.


Introduction

The mammalian ovary contains a large number of nongrowing small oocytes (primordial oocytes). Each oocyte is surrounded by a single layer of flattened granulosa cells. These granulosa cells, together with a small oocyte, are called the primordial follicle. Activation of primordial follicles causes the transformation of granulosa cells into a cuboidal shape (Lintern-Moore & Moore 1979). The follicle at this stage is called a primary follicle. The granulosa cells then proliferate, and the follicles develop to the secondary, antral, and finally Graafian stages. The time point at which oocyte growth and follicular development begin depends on the species. In mice, oocyte growth begins within a few days after birth (Peters 1969, Hirshfield 1991), whereas in ruminants and primates, growth begins before birth (Russe 1983, Gougeon 2004). The ovaries of infant pigs contain a large number of primordial follicles and also a small population of developing follicles that contain growing oocytes. Primordial follicles represent more than 95% of the total follicular proportion in porcine ovaries at 2 weeks after birth (Black & Erickson 1965, Oxender et al. 1979). The ovaries of prepubertal and adult animals possess numerous growing and fully grown oocytes in the developing and mature follicles respectively, as well as a number of nongrowing primordial oocytes as the future stock.

The mechanism regulating the initiation of oocyte growth is not well understood. Primordial follicles from newborn rodents are activated, and oocytes grow to full size in culture (Eppig & O’Brien 1996). For humans and large domestic animals, however, in vitro growth of primordial oocytes is hardly applicable (Miyano 2003). The xenografting of ovarian tissues to immunodeficient animals can be a substitute for an effective long-term culture system. Mice homozygous for the Scid mutation lack both humoral and cell-mediated immunity due to the absence of mature T and B lymphocytes that render them able to accept the xenografts (Bosma et al. 1983, Bosma & Carroll 1991). Gosden et al. (1994) have developed a method of xenografting mammalian follicles to severe combined immune deficiency (SCID) mice as a model for investigating the early stages of follicular development. Follicular development in xenografts in immunodeficient mice has been reported in different species, including cats (Gosden et al. 1994).

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The duration of ovarian xenografting depends on the time course necessary for the follicular development of the donors. Primordial follicles developed to the antral stage at 45–75 days after xenografting of the ovarian tissues from 20-day-old piglets (Kaneko et al. 2003). On the other hand, the development of primordial follicles from cows (Senbon et al. 2003) and prepubertal pigs (Moniruzzaman & Miyano 2007) was not initiated at 6–8 weeks after xenografting. Primordial oocytes, therefore, are thought to differ between infant and prepubertal/adult animals. Accelerated development of xenografted ovarian follicles has been reported in younger pouch wallabies in comparison to older ones (Mattiske et al. 2002). We hypothesized that adult ovaries that contain growing oocytes require a mechanism to establish a pool of nongrowing oocytes as a reserve for the future, and that the growth of primordial oocytes in adult ovaries is restrained by a certain inhibitory mechanism. The recruitment of nongrowing oocytes might result from the release of the inhibitory mechanism that maintains the resting follicles in stasis (McGee & Hsueh 2000).

It has been suggested that FOXO3, a forkhead transcription factor, regulates the activation of primordial follicles in the mouse ovary (Brenkman & Burgering 2003). Forkhead transcription factors are characterized by the presence of a highly conserved, monomeric DNA-binding domain, also known as the forkhead box or FOX (Weigel & Jackle 1990). In mammalian somatic cells, FOXO factors induce cell cycle arrest (in G1/S transition) and apoptosis (Brunet et al. 1999, Nakamura et al. 2000, Alvarez et al. 2001). FOXO3 expression has been observed in the nuclei of mouse and rat oocytes (Reddy et al. 2005), and Foxo3−/− mice show global follicular activation, which leads to the early depletion of ovarian follicles (Castillon et al. 2003). These studies suggest that FOXO3 inhibits the primordial oocyte activation in newborn rodents. However, it has not been shown whether FOXO3 regulates the dormancy and reactivation of primordial oocytes in adult ovaries, since all oocytes are activated and depleted before puberty in the Foxo3-knockout mice.

The present study aimed to elucidate the primordial oocytes growth in pigs. We found that primordial oocytes in prepubertal pigs took much longer time to initiate growth than did those in infants. To determine the molecular mechanism underlying the difference between infant and prepubertal pigs, we examined the expression level of FOXO3 in primordial oocytes from infant and prepubertal pigs. Further, FOXO3 was knocked down by small RNA interference prior to xenografting to SCID mice in order to determine its effect on the activation of porcine primordial oocytes.

Results

Growth of primordial oocytes from infant and prepubertal pigs

Pig ovarian tissues dissected for xenografting contained primordial oocytes (Fig. 1A). Histological examination confirmed that the ovarian tissues dissected from both infant and prepubertal pigs contained mainly primordial follicles before xenografting (Figs 2A and B, 3A and C). The tissues did not contain any necrotic region. Each oocyte contained a large spherical nucleus. In infant pigs, some of the oocytes were gathered together, making a cluster- or nest-like appearance (Fig. 2A). In prepubertal pigs, the oocytes were scattered uniformly throughout the tissues (Fig. 2B), with a few exceptions of 1–2 cluster formations in a few tissues. The average numbers of oocytes per tissue were higher in infant (154 ± 6; n = 65) than in prepubertal pigs (78 ± 8; n = 116). The oocyte diameters were < 35 μm in both the infant and prepubertal pigs before xenografting (Fig. 4A and C).

After 2 months, ovarian xenografts from infant pigs grew approximately three- to five-fold in comparison to their original sizes, and some large antral follicles were visible. Histological examination revealed the development of follicles at various stages, including antral follicles, in all types of recipients, i.e. male (n = 7), female (n = 7), and ovariectomized (n = 6) SCID mice (Figs 2E and 3B). Average numbers of follicles obtained in the xenografts were 116 ± 8, 108 ± 5, and 117 ± 5 in male, female, and ovariectomized SCID mice respectively. The developing follicles were evenly distributed throughout the xenografts. The xenografts were free from necrosis and were well vascularized. Some follicles were hemorrhagic. The corpus luteum was not observed.

Figure 1 Primordial oocytes are visible in a porcine ovarian tissue under the inverted microscope. Arrows indicate the cytoplasmic lipid droplets at the peri-nuclear region of the oocytes (A). Primordial oocytes were collected enzymatically from ovarian tissues for western blot analysis (B). Scale bar represents 40 μm.
In the xenografts from prepubertal pigs, 54\(G2\), 66\(G3\), and 60\(G3\) primordial follicles survived in male, female, and ovariectomized mice respectively, but none of them developed to the primary stage or beyond after 2 months (Figs 2C and 3D), irrespective of the type of recipient mice (male \(n=6\), female \(n=7\), and ovariectomized \(n=6\)). The diameters of the oocytes did not increase (Fig. 4D). The xenografts were well vascularized and free from necrosis. These results indicate that the initiation of oocyte growth and the pattern of follicular development are different between infant and prepubertal pigs. The duration of xenografting was prolonged in the case of prepubertals. At 4 months after xenografting, 52\(G3\), 47\(G2\), and 32\(G3\) oocytes survived in male (\(n=3\)), female (\(n=3\)), and ovariectomized (\(n=3\)) mice respectively. In the xenografts, oocytes did not grow and follicles did not develop with a few exceptions. Three developing follicles (two primary and one secondary) were observed in three different xenografts, and the diameters of the oocytes ranged from 40 to 50 \(\mu\)m. All the other 15 xenografts contained only primordial follicles, and the diameters of the oocytes did not increase.

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increase (Fig. 2D). When the duration was prolonged to 6 months, oocytes grew and follicles developed to various stages, including antral follicles, in all three types of recipient mice (male n = 6, female n = 5, and ovariectomized n = 5) (Figs 2F, 3E and 4E). In this case, the numbers of follicles obtained in the xenografts in male, female, and ovariectomized mice were 43, 34, and 32 respectively. There were no ovulated follicles or corpora lutea in the xenografts, although some large follicles were hemorrhagic.

The patterns of oocyte growth and follicular development in infant pigs at 2 months after xenografting were similar to those in prepubertals after 6 months, although they differed among the types of recipients (Figs 3 and 4). The percentage of primordial follicles was highest in females, and lowest in male SCID mice. In contrast, the proportions of antral follicles and oocytes with a diameter of more than 95 μm were highest in male mice. In both prepubertal and infant porcine ovarian xenografts, higher percentages of antral follicles were recorded in ovariectomized recipients than in intact females.

**FOXO3 expression in primordial oocytes from infant and prepubertal pigs**

Primordial oocytes were collected from ovarian tissues by enzyme treatment for western blot analysis (Fig. 1B). FOXO3 expression was stronger in the primordial oocytes from prepubertal pigs than in those from infants, while the expression levels of mouse vasa homolog (MVH) as well as of β-actin, as internal controls, were similar between those oocytes (Fig. 5a). MVH, a germ cell-specific marker, was expressed only in oocytes not

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**Figure 4** Distributions of porcine oocytes on the basis of their diameters before and after xenografting. Ovarian tissues before and after xenografting were embedded in methacrylate resin, serially sectioned, and stained with hematoxylin and eosin. Before xenografting, diameters of oocytes in both infant and prepubertal ovarian tissues did not exceed 35 μm (A and C). At 2 months after xenografting, the oocytes of infant pigs grew, and some of them reached full size (B), while the oocytes of prepubertals did not grow (D). After 4 months, diameters of oocytes from prepubertal pigs remained unchanged. After 6 months, oocytes grew, and some of them reached full size (E). Each bar represents the mean ± S.E.M. of oocytes recorded from at least 11 separate tissues. Proportions of oocytes of different diameters were compared among recipients; male, female, and ovariectomized SCID mice. Bars with different letters within each category differ significantly (P < 0.05).

**Figure 5** (a) Expression of FOXO3 in primordial oocytes from prepubertal and infant pigs by western blotting. Ovarian tissues were treated with actinase, and oocytes of around 30 μm were collected. A number of 500 primordial oocytes or 5200 granulosa cells were prepared for each sample to be run on SDS-polyacrylamide gel. In each sample, a single FOXO3 band of ~95 kDa was detected by rabbit anti-FOXO3 antibody (Cell Signaling Technology). FOXO3 level was higher in the primordial oocytes from prepubertal pigs than in those from infants. Mouse vasa homolog (MVH) and β-actin were used as internal control. The experiment was repeated thrice with similar results. (b) Immunolocalization of FOXO3 in primordial oocytes of infant and prepubertal pigs. Cryosections were treated with rabbit anti-FOXO3 antibody (Upstate Biotechnology) and Alexa Fluor 488-labeled anti-rabbit immunoglobulin antibody (green; D–F) and counterstained by PI (red; G–I). Serial sections were incubated without primary antibody as negative controls (A–C). The nuclei of some oocytes showed FOXO3 expression in infant porcine ovaries before xenografting (D). In contrast, all primordial oocytes were positively stained in prepubertal pigs (E). After 4 months of xenografting, some of the oocytes became FOXO3 negative (F). Arrow heads indicate the nuclei of oocytes showing FOXO3 signals. Scale bar represents 100 μm.
Table 1 Reduction of FOXO3-positive primordial oocytes in prepubertal porcine ovarian tissues after 4 months of xenografting.

<table>
<thead>
<tr>
<th>Pig ovarian tissues</th>
<th>Recipient mice</th>
<th>Number of tissues examined</th>
<th>Number of oocytes</th>
<th>Number (%) of FOXO3-positive oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infants</td>
<td>Male SCID</td>
<td>6</td>
<td>90</td>
<td>73 ± 10 (21 ± 10)</td>
</tr>
<tr>
<td>Prepubertals</td>
<td>Female SCID</td>
<td>5</td>
<td>65</td>
<td>55 ± 10 (19 ± 10)</td>
</tr>
<tr>
<td>Xenografts from prepubertals</td>
<td>Female SCID</td>
<td>3</td>
<td>45</td>
<td>35 ± 10 (12 ± 10)</td>
</tr>
</tbody>
</table>

* † Values with different superscripts are significantly different (P<0.05).

FOXO3 knockdown and growth of porcine primordial oocytes

A partial nucleic acid sequence of porcine FOXO3 was determined to design effective siRNAs. PCR amplification of porcine mRNA generated a 1128-bp cDNA, which was highly similar to the corresponding region of mouse and human FOXO3 (Fig. 6). To determine the efficiency of siRNAs to knock down FOXO3, porcine somatic cells LLC-PK1 were transfected with siRNAs. The results of western blot analysis showed that the expression of FOXO3 was decreased in the cells treated with two siRNAs (No. 1 and No. 3 in Fig. 7a).

In preliminary experiments, different concentrations of transfectant reagent Lipofectamine 2000 and siRNAs were applied to knock down FOXO3 in primordial oocytes in ovarian tissues. Lower concentrations (0.25–0.5%) of Lipofectamine 2000 did not result in significant change in FOXO3 expression in primordial oocytes in immunohistochemistry, while higher concentrations (≥1.5%) caused the degeneration of oocytes. Finally, a 1% concentration was used for the transfection of siRNAs. To determine the efficiency of siRNAs to transf ect the oocytes in ovarian tissues, Alexa Fluor 546-labeled siRNAs were used. Oocytes and other ovarian cells in the tissues exhibited a red color, which indicated the penetration of siRNA into the oocytes (Fig. 7c).

Western blot analysis of total protein extracted from ovarian tissues showed that the expression of FOXO3 decreased after treatment with 0.4 μM siRNA No. 1 or siRNA No. 3, while control siRNA-treated tissues still expressed FOXO3 (Fig. 7b). Further, immunofluorescent staining confirmed the reduction of FOXO3 expression in oocytes in the ovarian tissues by siRNAs (Fig. 8a and Table 2). The percentage of FOXO3-positive oocytes in siRNA-treated tissues depended on the concentration of siRNAs. A lower concentration (0.2 μM) of siRNAs did not interfere with FOXO3 expression in oocytes. FOXO3 expression was reduced in half of the primordial oocytes treated with 0.4 μM siRNA, while almost all primordial oocytes were FOXO3 positive in the controls. Higher concentrations (≥0.6 μM) of siRNAs caused the degeneration of oocytes (data not shown).

Following siRNA treatment, ovarian tissues containing primordial follicles were xenografted to SCID mice. In the histological examination, all tissues contained only primordial follicles before xenografting (Fig. 8G–I and Table 3). At 2 months after xenografting, oocyte diameters did not increase and follicles remained in the primordial stage in the controls (Fig. 8) and Table 3). Follicles developed to the secondary and antral stages in siRNA No. 1- and siRNA No. 3-treated tissues, accompaniment with the oocyte growth (Fig. 8K and L and Table 3).

Discussion

In mammals, a small population of primordial oocytes enters the growth phase at one time, while large numbers of them remain quiescent throughout a female’s reproductive life (Hirshfield 1991). The initiation of primordial follicle development is known to be independent of gonadotropin stimulation (Fortune et al. 2000). Factors concerning the recruitment of primordial oocytes to enter the growth phase are not understood. Recently, xenografting of ovarian tissues or follicles has begun to be used as a method to study oocyte growth and follicular development. Kaneko et al. (2003) reported the development of primordial follicles to the antral stage at 45–75 days after xenografting ovarian tissues from 20-day-old pigs. The duration of follicular development in infant pigs in
the present experiment is consistent with the result of Kaneko et al. (2003). However, in similar conditions, primordial follicles from prepubertal pigs had not started to develop even after 4 months. Similarly, our previous reports revealed that the primordial follicles from cows (Senbon et al. 2003) and prepubertal pigs (Moniruzzaman & Miyano 2007) survived without initiating development at 6–8 weeks after xenografting. In the present study, a period of 6 months was required for development to the antral stage in the xenografts from prepubertal pigs. These results suggest that primordial oocytes in prepubertal pigs remain dormant for a long period before the growth is initiated.

Dormancy of primordial follicles is perhaps required to reserve nongrowing oocytes for their long reproductive life, because depletion of this oocyte reserve causes premature ovarian failure. The development of primordial follicles is prevented by some inhibitory factors in the ovary (McGee & Hsueh 2000, Brenkman & Burgering 2003). Castrillon et al. (2003) revealed that FOXO3 is a critical regulator that suppresses the initiation of primordial follicle development in the newborn mouse. In the present study, we observed FOXO3 in the nucleus of porcine primordial oocytes in agreement with previous results observed in mouse oocytes (Reddy et al. 2005). In prepubertal pigs, FOXO3...
was expressed in almost all primordial oocytes, and in infant pigs, a number of primordial oocytes did not exhibit FOXO3. The results of western blots reflected the higher proportion of FOXO3-positive primordial oocytes from prepubertal pigs than from infants. Further, at 4 months after xenografting, some oocytes from prepubertal pigs became FOXO3 negative, and some oocytes grew in the subsequent 2 months. These results imply that FOXO3 may be involved in the dormancy of primordial oocytes in prepubertal and perhaps adult porcine ovaries, and that the oocytes start to grow when they are released from the inhibitory action of FOXO3.

To clarify the involvement of FOXO3 in the suppression of oocyte growth in prepubertal pigs, we conducted a FOXO3 knockdown experiment using siRNAs. RNA interference by siRNAs is a well-established method for gene knockdown in cultured cells (Gampel & Mellor 2002, Abdelrahim et al. 2003), and has recently been applied for oocytes (Lee et al. 2008), cultured hamster ovaries (Wang & Roy 2006), and fetal mouse ovaries (Yamaguchi et al. 2006). In the present experiment, we knocked down FOXO3 in primordial oocytes in ovarian tissues from prepubertal pigs by siRNA transfection. Since siRNA mediates sequence-specific mRNA degradation, the transfected FOXO3 siRNAs worked transiently to knock down the target FOXO3 in the present experiment. After the knockdown of FOXO3, a proportion of primordial oocytes became activated and entered the growth phase in the xenografts. This result suggests that FOXO3 induces at least in part the dormancy of primordial oocytes in the prepubertal pig ovary. The ovaries of Foxo3<sup>−/−</sup> mice exhibit several fold increases in the number of developing follicles (Castrillon et al. 2003). At 8 days post partum, an enlargement of volume occurred in the majority of the oocytes, including those in primordial follicles. The pool of nongrowing oocytes was depleted in the ovaries of the mutant mice at 14 days post partum. Unlike the phenotypes of the Foxo3<sup>−/−</sup> mice, a proportion of follicles developed, while the majority of them remained in the primordial stage with nongrowing oocytes in the xenografts of FOXO3 siRNA-treated tissues in the present experiment. Here, the efficiency of siRNA to knock down the FOXO3 expression was low. FOXO3 knockdown might be related to the efficiency of siRNA transfection into the oocytes in ovarian tissues. However, Alexa Fluor 546-labeled FOXO3 siRNA indicated the penetration of siRNA into the oocytes as well as into other cells in the ovarian tissues. siRNA treatment caused the degeneration of oocytes in the ovarian tissues, although there was no significant difference between control siRNA and FOXO3 siRNA-treated tissues in the present experiment.

Figure 7 (a) Porcine LLC-PK1 cells were treated for 6 h with three different FOXO3 siRNAs; No. 1: 5'-UCAGAUUACUGGCUCGCCGCAUGUC-3', No. 2: 5'-UUCAAGACGGAGGUCCAAAACUG-3', No. 3: 5'-UUCAUGCCUGULUGAAACUGCA-3', and control: 5'-GACCGCG AAGGAUCUAAACUGCA-3'. Cells were further cultured in TCM-199 containing 5% FCS for 36 h and were subjected to western blot analysis by using rabbit anti-FOXO3 antibody (Upstate Biotechnology). Expression of FOXO3 was decreased by two siRNAs (No. 1 and No. 3). Levels of β-actin were used as internal control. The experiment was repeated thrice with similar results. (b) Ovarian tissues containing primordial follicles from prepubertal pigs were treated with FOXO3 siRNAs (No. 1 and No. 3) for 6 h and further cultured in TCM-199 containing 5% FCS for 36 h. Same amount of proteins extracted from treated tissues were separated in SDS-polyacrylamide gel, and western blotting was performed by anti-FOXO3 antibody (Cell Signaling Technology). FOXO3 level was decreased by two siRNAs (No. 1 and No. 3). Levels of β-actin were used as internal control. The experiment was repeated thrice with similar results. (c) Ovarian tissues containing primordial follicles from prepubertal pigs were treated with Alexa Fluor 546-labeled FOXO3 siRNAs for 6 h, cryosectioned, and observed under the fluorescence microscope. Red-colored cells are considered to be transfected with siRNAs (A, C, and E). Sections were counterstained with DAPI (blue; B, D, and F). The experiment was repeated thrice with similar results. Oocytes were indicated by arrow heads. Scale bar represents 60 μm.
FOXO3 siRNAs. The degeneration was increased dose dependently by the transfection reagent even in the absence of siRNA (data not shown), indicating that the degeneration was at least partly induced by the toxicity of the transfection reagent. Alternatively, it is possible that FOXO3 is not the sole factor responsible for the dormancy of primordial oocytes in the pig, and some other treatment might be required for the full activation of those oocytes.

The mechanisms associated with the regulation of FOXO factors have been characterized mainly in somatic cells in response to growth factor signaling via the phosphatidylinositol 3-kinase–protein kinase B (PKB) signaling pathway (Tran et al. 2003). PKB-induced phosphorylation inhibits transcriptional activity of the FOXO members (Biggs et al. 1999, Brunet et al. 1999, Kops et al. 1999, Rena et al. 1999). In the absence of PKB activity, the FOXO members are predominantly localized in the nucleus and are presumed to be active, and PKB-mediated phosphorylation induces their relocalization from the nucleus to the cytoplasm (Biggs et al. 1999, Brunet et al. 1999, Brownawell et al. 2001). Functions of FOXO factors are regulated at multiple levels, which include phosphorylation, ubiquitylation, and acetylation (Huang & Tindall 2007). However, phosphorylation-dependent exclusion of FOXO3 from the nucleus has not been known to be involved in oocytes. How the FOXO3 level increases in prepubertal porcine ovaries is not well understood. Prepubertal porcine ovaries contain a large number of developing follicles (Black & Erickson 1965). Developing follicles are found in well-vascularized cortical–medullary borders, whereas primordial follicles are located in a relatively avascular region in the cortex (Gougeon 2004). During ovarian development, primordial follicles are placed at the periphery of the ovary, and they might suffer from a shortage of nutrients and growth factors due to poor blood supply. In a worse nutritional condition, the proliferation of cells is arrested in G1 or

![Figure 8](image-url)

**Figure 8** (a) Immunohistological examination of FOXO3 expression in siRNA-treated primordial oocytes in prepubertal pigs. Porcine ovarian tissues containing primordial follicles were treated with FOXO3 siRNAs (No. 1 and No. 3) for 6 h, and further cultured in TCM-199 containing 5% FCS for 36 h. Cryosections were treated with anti-FOXO3 antibody (Sigma) and Alexa Fluor 488-labeled secondary antibody (green; A–C) and counterstained with PI (red; D–F). The experiment was repeated thrice with similar results. Oocytes were indicated by arrow heads. Scale bar represents 60 μm. (b) Histological sections of FOXO3 siRNA-treated porcine ovarian tissues before and after xenografting. Ovarian tissues containing primordial follicles were treated with control (G) or FOXO3 siRNAs (H and I) and xenografted to SCID mice. The tissues were fixed in paraformaldehyde, embedded in methacrylate resin, serially sectioned by 5 μm, and stained with hematoxylin and eosin. At 2 months after xenografting, follicles developed to the secondary and antral stage in siRNA No. 1- and siRNA No. 3-treated tissues, accompaniment with the oocyte growth (K and L). In the similar conditions, primordial follicles did not develop in control siRNA-treated tissues after xenografting (J). ‘r’ indicates mouse renal tissues (J and K). Scale bars represent 40 (G–I), and 200 μm (J–L).

**Table 2** FOXO3 knockdown in porcine primordial oocytes.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of tissues examined</th>
<th>Number of oocytes</th>
<th>Number (%) of FOXO3-positive oocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control siRNA</td>
<td>4</td>
<td>10 ± 2*</td>
<td>10 ± 2 (96 ± 2*)</td>
</tr>
<tr>
<td>siRNA # 1</td>
<td>4</td>
<td>14 ± 4*</td>
<td>4 ± 1 (27 ± 3)</td>
</tr>
<tr>
<td>siRNA # 3</td>
<td>5</td>
<td>19 ± 3*</td>
<td>7 ± 1 (38 ± 4)</td>
</tr>
</tbody>
</table>

*Values with different superscripts are significantly different (P < 0.05).
*Ovarian tissues measuring $\sim 1 \times 1 \times 0.5$ mm were collected from prepubertal pigs. After FOXO3 siRNA treatment, tissues were cultured for 36 h. Serial cryosections were prepared, reacted with anti-FOXO3 antibody and Alexa Fluor 488-labeled secondary antibody. Sections were counterstained with PI, and FOXO3-positive oocytes were counted throughout the tissues. Each value represents the mean ± S.E.M.
Table 3 Oocyte growth and follicular development in FOXO3 siRNA-treated porcine ovarian tissues at 2 months after xenografting.

<table>
<thead>
<tr>
<th>Xenografting</th>
<th>Treatment</th>
<th>Number of SCID mice</th>
<th>Number of tissues examined</th>
<th>Number of primordial follicles</th>
<th>Number of primary follicles</th>
<th>Number of secondary follicles</th>
<th>Number of antral follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before</td>
<td>Control</td>
<td>9</td>
<td>2</td>
<td>15±2 (100±0)</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td></td>
<td>siRNA No. 1</td>
<td>9</td>
<td>2</td>
<td>29±3 (100±0)</td>
<td>0±0</td>
<td>0±0</td>
<td>0±0</td>
</tr>
<tr>
<td></td>
<td>siRNA No. 3</td>
<td>5</td>
<td>4</td>
<td>6±1 (48±5)</td>
<td>6±1 (12±4)</td>
<td>3±1 (12±3)</td>
<td>1±1 (4±3)</td>
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<tr>
<td>After</td>
<td>Control</td>
<td>2</td>
<td>4</td>
<td>41±1 (29±2)</td>
<td>5±1 (10±3)</td>
<td>3±1 (12±3)</td>
<td>1±1 (4±3)</td>
</tr>
<tr>
<td></td>
<td>siRNA No. 1</td>
<td>5</td>
<td>4</td>
<td>41±1 (29±2)</td>
<td>5±1 (10±3)</td>
<td>3±1 (12±3)</td>
<td>1±1 (4±3)</td>
</tr>
</tbody>
</table>

*Values with different superscripts in the same column are significantly different (P<0.05).

In conclusion, our results suggest that primordial oocytes are dormant in the prepubertal pigs by FOXO3-related mechanism that functions to establish the nongrowing oocyte pool in the ovary, and that a transient knockdown of FOXO3 activates the primordial oocytes to enter the growth phase.

Materials and Methods

Collection of ovarian tissues containing primordial follicles

Ovaries were collected from 10- to 20-day-old and 6-month-old crossbred (Landrace, Large White and Duroc) gilts. Six- to 8-week-old male and female SCID mice were purchased from Clea Japan, Inc., Tokyo, Japan. Prior to xenografting, the mice were anesthetized, and the left kidney was exteriorized under the kidney capsule where they were exposed to circulatory blood of host mice. The blood provides the factors essential for the metabolic activities of oocytes as well as of other cells in the xenografted tissues. The blood/serum contents (especially growth factors) of host mice may have downregulated FOXO3 in oocytes in xenografts. Since prepubertal pig oocytes contained a higher level of FOXO3, they are thought to require a longer period to reduce the FOXO3 in xenografts, and FOXO3 siRNA facilitates this reduction.

Xenografting

Six- to 8-week-old male and female SCID mice were purchased from Clea Japan, Inc., Tokyo, Japan. Prior to xenografting, the mice were anesthetized and the left kidney was exteriorized.
through a dorsal-horizontal incision. A small hole was torn in
the kidney capsule using fine forceps. Three to five pieces of
porcine ovarian tissues were inserted underneath the capsule.
Half of the female SCID mice were ovarioctomized during
xenografting. 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-aged 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 h
light:12 h darkness.

Ovarian tissues from both infant and prepubertal pigs were
xenografted into male, female, and ovarioctomized SCID mice
for 2 months at the first phase. However, the xenografting was
prolonged up to 6 months in the case of prepubertal pigs. This
study was approved by the Institutional Animal Care and Use
Committee (Permission number: 15-4-05, 19-5-09 and 19-5-
45) and was carried out according to the Guidelines of Animal
Experimentation of Kobe University, Japan.

**Histological examination**

Ovarian tissues before and after xenografting were fixed in 3%
(w/v) paraformaldehyde in PBS, then dehydrated, embedded in
methacrylate resin (JB-4; Polysciences, Inc., Warrington, PA,
USA), serially sectioned by 5 μm, and stained with hematoxy-
lin and eosin. The number of different stages of follicles and
diameters of the oocytes were recorded. The follicles were
counted in every section where the oocyte nucleus was seen.
Double counting in adjacent sections was avoided. The
follicles were classified into four categories according to the
number and morphology of granulosa cell layers: primordial
follicles were classified into 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 but no antrum, and antral follicles having an
antral cavity with multiple layers of granulosa cells. The ovaries
from infant pigs contained primordial follicles in that the
oviducts were surrounded by the granulosa cells, and in
addition, they 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 by taking the average of two perpendicular
measurements of each oocyte, which showed the maximum
diameter in serial sections, to the nearest 1 μm with an ocular
micrometer (Nikon, Tokyo, Japan) attached to a microscope.

**Collection of primordial oocytes**

Ovarian cortical slices of about 1 mm thickness were minced
into 1-mm square pieces. After washing thrice in 25 mM
HEPES-buffered TC199 containing 0.1% (w/v) PVA, 0.85 mg/ml sodium bicarbonate, and 0.08 mg/ml kanamycin
sulfate, the tissues were incubated for 2.5 h with gentle
agitation in TC199 containing 2.5 mg/ml pronase (actinase
E, Kaken Pharmaceutical Co., Tokyo, Japan) at 38.5 °C under
an atmosphere of 5% CO₂ in humidified air. After washing
thrice in HEPES-buffered TC199, the tissues were gently
pipetted for 15–20 min in HEPES-buffered TC199 containing
10% (v/v) FCS (Dainippon Pharmaceutical Co. Ltd,
Osaka, Japan). Oocytes with diameters ranging from 30 to
35 μm were picked up using a glass pipette (Fig. 1B). After
washing thrice in PBS–PVA, each group of 500 oocytes was
transferred into an Eppendorf tube with 2–3 μl of PBS–PVA.
Then, equal volume of two-times-concentrated SDS sample
buffer (Laemmli 1970) was added, and the samples were boiled
for 5 min and kept at −20 °C before use.

**Preparation of ovarian extracts**

Ovarian cortical tissues containing primordial follicles were
collected from prepubertal pigs, and were homogenized by
a glass homogenizer by keeping on ice in a lysis buffer containing
50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM
EDTA, 0.1% SDS, 0.5 mM p-aminophenyl methanesulfonyl
fluoride hydrochloride (p-APMSF; Wako Pure Chemical
Industries Ltd, Osaka, Japan), and 1% Triton X-100. Lysates
were transferred to an Eppendorf tube to be sonicated by
Vibra cell (Sonics & Materials, Inc., Danbury, CT, USA) with
three short bursts of 10 s at 20% amplitudes followed by
intervals of 1 min on ice. After centrifugation at 11 000 g
for 10 min at 4 °C, supernatants were collected and protein
concentrations were measured using the bicinchoninic acid
protein assay. Samples were prepared for Western blotting as
described above.

**Western blotting**

The samples were run on 10% SDS-PAGE, and the proteins
were transferred to hydrophobic PVDF membranes (Immo-
bilon; Millipore Co., Bedford, MA, USA). The membranes were
cut into two pieces: one part contained >50 kDa and the other
part contained 50–25 kDa. The membranes were blocked with
10% (v/v) FCS in PBS containing 0.1% (v/v) Tween
20 (PBS–Tween) for 1 h, and were incubated overnight at 4 °C in a
humidified chamber with rabbit polyclonal anti-human
FKHRL1/FOXO3 antibody (1:1000; # 27567, Upstate Bio-
technology, New York, NY, USA; or # 9467, Cell Signaling
Technology, Danvers, MA, USA). It is noted that the FOXO3 is
highly conserved between humans and pigs (Fig. 6). After
washing thrice in PBS–Tween, the membranes were treated
with HRP-conjugated goat anti-rabbit immunoglobulin
antibody (1:1000; Pierce Biotechnology, Inc., Rockford, IL,
USA) for 1 h at room temperature. For control, the membranes
containing 50–25 kDa were probed with mouse monoclonal
anti-β-actin antibody (1:50 000; # A2228, Sigma) and sub-
sequently with HRP-conjugated goat anti-mouse immunoglobulin
antibody (1:1000; Pierce Biotechnology, Inc.). In some
experiments, membranes containing >50 kDa were re-blotted
to examine the expression of MVH, a germ cell-specific marker,
as an internal control. Membranes were treated with stripping
buffer (Re-Blot Plus Strong Solution, Millipore, CA, USA)
according to the manufacturer’s instructions and reprobed
with rabbit polyclonal DDX4/MVH antibody (1:1000; #13840,
Abcam, Cambridge, MA, USA). The granulosa cells were
collected from antral follicles and blotted as the control.
After washing in PBS–TWEEN, the peroxidase activity was
visualized using the Super Signal detection system (Pierce
Biotechnology, Inc.).
Immunohistochemistry

For immunohistochemistry, cryostat sections of 6 μm of cortical tissues from infant and prepubertal ovaries, xenografts (after 4 months), and siRNA-treated ovarian tissues were prepared on silane-coated slides. Sections were dried in air and fixed in 3% (w/v) paraformaldehyde in PBS at room temperature for 15 min. To prevent nonspecific antibody binding, blocking was done by 3% (w/v) BSA (Wako Pure Chemical Industries Ltd) for 1 h, and immunostaining was performed with rabbit anti-human FKHR/FOXO3 antibody (1:250; #27567, Upstate Biotechnology or #2178, Sigma) overnight at 4°C. After washing with PBS, the sections were reacted with Alexa Fluor 488-labeled goat anti-rabbit immunoglobulin antibody (1:1000; Molecular Probes Inc., Eugene, OR, USA) for 45 min, and counterstained with propidium iodide (PI:100 μg/ml; Sigma) for 15 min. Again, after washing thrice with PBS, the sections were mounted with Vectashield Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA) and observed under a fluorescence microscope (U-LH100HGAP; Olympus Optical Co., Tokyo, Japan). Some sections were incubated without a primary antibody as negative controls.

Cloning of partial sequence of FOXO3 and designing FOXO3 siRNA

Total RNAs were extracted from porcine fetal testes using ISOGEN RNA extraction reagent (Nippon Gene Inc., Tokyo, Japan), and then total RNAs were reverse-transcribed to cDNA. The cDNAs were amplified by PCR to obtain the fragment of porcine FOXO3. Primers were designed on the basis of human FOXO3 sequence and the available similar sequence in pigs (BP146966 and BX673604): forward 5’-TCTGGCGGCTGGAAGAACTC-3’ and reverse 5’-CTACGGATAATGGGACCCAT-C3’. PCR amplification conditions consisted of an initial denaturation step at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 1.5 min; a final extension period at 72 °C for 6 min completed the amplification. PCR product was sequenced by Takara Bio Inc. (Shiga, Japan). Using the partial sequence as a template, three sequences of siRNA senses and a nontargeted RNA sequence as a control were designed with online RNAi Designer of Invitrogen as follows: No. 1: 5’-UGAGAUUCAUGGUUCCGCGGCAUGUC-3’; No. 2: 5’-UUCAAGAGCGAGGUGUCCAAACACUG-3’; No. 3: 5’-UUCAGCGUCCGGUGAACACUGUGCA-3’; and control: 5’-GACCGGGAAGGCUACCUAACGCUAUCA-3’.

siRNA treatment

At first, the efficiency of siRNA-induced knockdown was examined in porcine kidney epithelial cell line LLC-PK1 (Dainippon Sumitomo Pharma Co., Ltd, Osaka, Japan). Cells were cultured in TCM-199 containing 5% (v/v) FCS, 0.1 mg/ml sodium pyruvate, and 2 mM glutamine (Sigma) at 38.5 °C under an atmosphere of 5% CO₂ in humidified air. When the cells reached about 50% confluency, the medium was replaced by a serum-free medium Opti-MEM (Invitrogen) and incubated for 30 min before transfection. For transfection, 0.25% Lipofectamine 2000 (Invitrogen), and 0.1 μM of each siRNA were added, and the cells were incubated for 6 h according to the manufacturer’s instruction. After transfection, cells were cultured for 36 h in normal medium. After washing twice with PBS–PVA, cells were detached from the dish by treating with 0.25% trypsin–EDTA solution (Sigma). They were washed once in normal medium, and thrice in PBS–PVA by centrifugation at 100 g for 2 min, and were subjected to prepare the sample for western blotting as described above.

Ovarian tissues were treated with 1% Lipofectamine 2000 and 0.4 μM siRNAs following the methods used for cultured cells as described above. Ovarian tissues (~2×1×0.5 mm) containing primordial follicles were collected from prepubertal pigs. Each tissue was cut into two pieces: one was fixed for immediate histological examination, and the other was allocated for siRNAs treatment (siRNA No. 1 and No. 3). After siRNA treatment for 6 h, some tissues were xenografted to male SCID mice, and the others were further cultured in TCM-199 containing 5% (v/v) FCS for 36 h and prepared to detect the FOXO3 expression by immunohistochemistry.

To know the penetration of siRNAs into the ovarian tissues, Alexa Fluor 546-labeled FOXO3 siRNAs (Invitrogen) were transfected in a similar manner. After transfection for 6 h, cryosections were prepared, counterstained with 4,6-diamidino-2-phenylindole (Molecular Probes Inc.), and observed under a fluorescence microscope.

Statistical analysis

The average numbers of follicles per tissue and average diameters of the oocytes were represented as the mean ± S.E.M. All data were subjected to one-way ANOVA, and the significance of difference among means was determined by the Tukey’s multiple range test. All percentile data were transformed to arc sin values before analysis. Differences at P<0.05 were considered statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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