

# Foxo3 negatively regulates the activation of mouse primordial oocytes

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## Abstract

**Purpose** The objective of this study is to know the role of Foxo3, a forkhead transcription factor, in the growth initiation of primordial oocytes in neonatal mice.

**Methods** We studied the expression of Foxo3 in 0-, 1-, 2-, 7- and 21-day-old mouse ovaries by immunohistochemistry. Ovaries from 1-day-old mice were treated with *Foxo3* siRNAs (small interfering RNAs) and subsequently organ-cultured for 6 days, and the oocyte growth was examined histologically.

**Results** Expression of Foxo3 was low in newborn mouse ovaries. In 1-day-old ovaries, Foxo3 was expressed in the nuclei of  $20 \pm 7$  % primordial oocytes. The percentage of Foxo3-positive primordial oocytes was increased to  $48 \pm 8$ ,  $37 \pm 2$  and  $47 \pm 4$  in 2-, 7- and 21-day-old mice, respectively. After treatment of ovaries with *Foxo3* siRNAs, higher proportion of oocytes entered the growth phase in cultured ovaries than that in control.

**Conclusions** These results suggest that Foxo3 negatively regulates the growth initiation of primordial oocytes and knockdown of Foxo3 leads primordial oocytes to the growth phase in vitro.

**Keywords** Foxo3 · Mouse · Oocyte · Organ culture · Primordial follicle

## Introduction

After differentiation of primordial germ cells to oogonia, they undergo mitotic proliferation and enter meiosis to become oocytes. In mice, these oocytes are surrounded by a single layer of flat-shaped pre-granulosa cells around the time of birth. This unit, consisting of an oocyte and granulosa cells, is called the primordial follicle and the oocytes in primordial follicles are called primordial oocytes [1]. Activation of primordial oocytes causes their growth initiation and the transformation of their surrounding granulosa cells to cuboidal shape [2]. The follicles at this stage are called primary follicles that contain growing oocytes. The granulosa cells proliferate and become multilayered to form secondary follicles. As follicles develop through the primary, secondary and antral stages, they gain successive layers of granulosa cells and theca layers, and oocytes increase the size towards 70–75  $\mu\text{m}$  in rodents and 120–125  $\mu\text{m}$  in humans, cows and pigs. Finally, a large fluid-filled antral cavity is formed, and the follicles are called antral follicles.

Primordial follicles start to develop at the time of birth in mice [3]. A small proportion of primordial oocytes enter the growth phase, while a large number are quiescent. The mechanism regulating this selection of primordial oocytes are not well understood. The forkhead transcription factors Foxo1 (fkhr), Foxo3 (fkhr11) and Foxo4 (afx) are characterized by the presence of a highly conserved, monomeric DNA-binding domain, also known as the forkhead box or Fox [4]. In mammalian somatic cells, Foxo factors induce cell cycle arrest (in G<sub>1</sub>/S transition) and apoptosis [5–7].

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Foxo3<sup>-/-</sup> mice show global follicular activation, which leads to the early depletion of ovarian follicles [8]. In prepubertal pigs, FOXO3 was detected in almost all primordial oocytes, although in infant pigs, around a half of primordial oocytes were FOXO3-negative [9]. In the xenotransplantation experiment, primordial oocytes from prepubertal pigs took a longer time to initiate the growth compared with those from neonatal pigs. However, after transient knockdown of FOXO3 by the small interfering RNA (siRNA) interference, a proportion of primordial oocytes from prepubertal pigs became activated and entered the growth phase in the xenografts [9].

To study the involvement of Foxo3 in the activation (growth initiation) of primordial oocytes, we treated newborn mouse ovaries with *Foxo3* siRNAs and oocyte growth was examined in subsequent organ-culture.

## Materials and methods

### Mice

C57BL/6 mice (SLC, Shizuoka, Japan) were housed in a positive pressure room with controlled temperature at 22–24 °C. The light cycle of the room was set at 12/12 h L/D. One male and one female mouse were kept in a single cage for mating. At the day of birth, the mouse was considered newborn or 0-day-old. Mice had free access to clean water and balanced feed pellets.

This study was approved by the Institutional Animal Care and Use Committee (permission number: 19-5-35 and 21-05-03) and carried out according to the Guidelines of Animal Experimentation of Kobe University, Japan.

### Immunohistochemistry

Ovaries were collected from 0-, 1-, 2-, 7- and 21-day-old mice for immunohistochemistry. Cryostat sections of 5 µm thickness were prepared from ovaries. From the central region of each ovary, a largest section was selected. They were dried in air and fixed in 1 % (w/v) paraformaldehyde in PBS at room temperature for 5 min. To prevent non-specific antibody binding, blocking was done by 3 % (w/v) bovine serum albumin (BSA; Wako Pure Chemical Industries Ltd., Osaka, Japan) in PBS for 1 h and immunostaining was performed with rabbit polyclonal anti-human Foxo3a antibody (1:1,000; # 9467, Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. After washing with PBS, the sections were reacted with Alexa Fluor 488-labeled goat anti-rabbit immunoglobulin antibody (1:1,000; Molecular Probes Inc., Eugene, OR, USA) for 1 h, and counterstained with propidium iodide (PI; 200 µg/ml; Sigma, St. Louis, MO, USA) for 10 min.

Again, after washing three times with PBS, the sections were mounted with Vectashield Mounting Medium (Vector Laboratories, Inc., Burlingame, CA, USA) and observed under a fluorescence microscope (U-LH100HGAPO; Olympus Optical Co., Tokyo, Japan).

The 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. Ovaries from newborn mice contained primordial follicles in that the oocytes were surrounded by granulosa cells, and in addition, they contained naked oocytes with a few granulosa cells. The latter ones were also classified as primordial follicles. All oocytes in every category of follicles showing green fluorescence, considered as Foxo3-positive, were counted in each ovary.

### siRNA treatment and cell culture

Three *Foxo3* siRNAs and a non-targeted control siRNA were purchased from Invitrogen (Tokyo, Japan). siRNAs were as follows:

- No. 1: 5'-CCGGCACCAUGAAUCUGAAUGAUGG-3'
- No. 2: 5'-CAGUACCGUGUUUGGACCUUCGUCU-3'
- No. 3: 5'-CCAGUGACUUGGACCUGGACAUGUU-3'

At first, the efficiency of siRNA-induced knockdown was examined in mouse fibroblast NIH/3T3 cells. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; GIBCO Invitrogen, CA, USA) containing 10 % (v/v) fetal calf serum (FCS, ICN Biomedicals, Inc., OH, USA) at 37.5 °C under an atmosphere of 5 % CO<sub>2</sub> in humidified air. When the cells reached about 50 % confluency, the medium was replaced by serum free Opti-MEM (Invitrogen) and incubated for 1 h before siRNA treatment. Cells were treated with 0.25 % Lipofectamine 2000 (Invitrogen) and 0.1 µM of each siRNA, and incubated for 6 h according to the manufacturer's instruction. After siRNA treatment, cells were cultured for 42 h in normal medium. After washing two times with PBS, cells were detached from the dish by treating with 0.25 % (w/v) trypsin-EDTA solution (Sigma). They were washed two times in PBS by centrifugation at 90g for 2 min. Then, equal volume of two-times-concentrated SDS sample buffer [10] was added, and the samples were boiled for 5 min and kept at -80 °C before use.

## Western blotting

The samples from siRNA-treated cells were run on 10 % SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and the proteins were transferred to hydrophobic polyvinylidene difluoride membranes (Immobilon P; Millipore Co., Bedford, MA, USA). The membranes were cut into two pieces; one part contained >65 kDa and the other <65 kDa. The membranes (>65 kDa) were blocked with 10 % (v/v) FCS in PBS containing 0.1 % (v/v) Tween 20 (PBS–Tween) for 1 h, and incubated with rabbit polyclonal anti-human Foxo3 antibody (1:1,000) for 3 h at room temperature. After washing three times in PBS–Tween, the membranes were treated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin antibody (1:1,000; Thermo Fisher Scientific, Rockford, IL, USA) for 1 h at room temperature. For control, the membranes containing <65 kDa were probed with mouse monoclonal anti- $\beta$ -actin antibody (1:10,000; # A2228, Sigma) and subsequently with HRP-conjugated goat anti-mouse immunoglobulin antibody (1:2,000; Thermo Fisher Scientific). After washing in PBS–Tween, the peroxidase activity was visualized using Western blotting Luminol Reagent (SuperSignal West Femto Maximum Sensitivity substrate, Thermo Fisher Scientific).

## siRNA treatment and organ culture of ovaries

Ovaries collected from 1-day-old mice were treated with 0.25 % Lipofectamine 2000 and 0.1  $\mu$ M siRNAs (No. 1 and 3) following the methods used for cultured cells as described above. After siRNA treatments for 6 h, ovaries were cultured in alpha minimum essential medium ( $\alpha$ -MEM; Invitrogen) supplemented with 5 % (v/v) FCS, 2.2 mg/ml sodium bicarbonate and 80  $\mu$ g/ml kanamycin sulfate (Sigma) for 6 days in center-well organ culture dishes (Falcon # 3037; Becton–Dickinson and Company, NJ, USA), and follicular development and oocyte growth were examined by histological procedures.

To know the penetration of siRNAs into the ovarian tissues, Alexa Fluor 546-labeled *Foxo3* siRNAs (No. 1 and 3; Invitrogen) were transfected in a similar manner. After transfection for 6 h, ovaries were washed and cultured in DMEM for 18 h, and cryosections were prepared. The sections were counterstained with 4,6-diamidino-2-phenylindole (Molecular Probes Inc.) and observed under a fluorescence microscope.

## Histological examination

Fresh and cultured ovaries 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  $\mu$ m, and stained with hematoxylin and eosin. From the central region of each ovary, two largest sections being 20  $\mu$ m apart from each other were selected and the diameters of the oocytes (excluding the zona pellucida) were measured, where oocyte nucleus was seen, to the nearest 1  $\mu$ m with an ocular micrometer (Nikon, Tokyo, Japan) attached to a microscope. The oocytes were classified into two categories, <20 and  $\geq$ 20  $\mu$ m (growing oocytes) in diameter.

## Statistical analysis

The average numbers of oocytes or follicles was represented as the mean  $\pm$  SEM (standard error of the mean). Data were arcsine transformed and compared by the Student's *t* test. Differences at *P* < 0.05 were considered statistically significant.

## Results

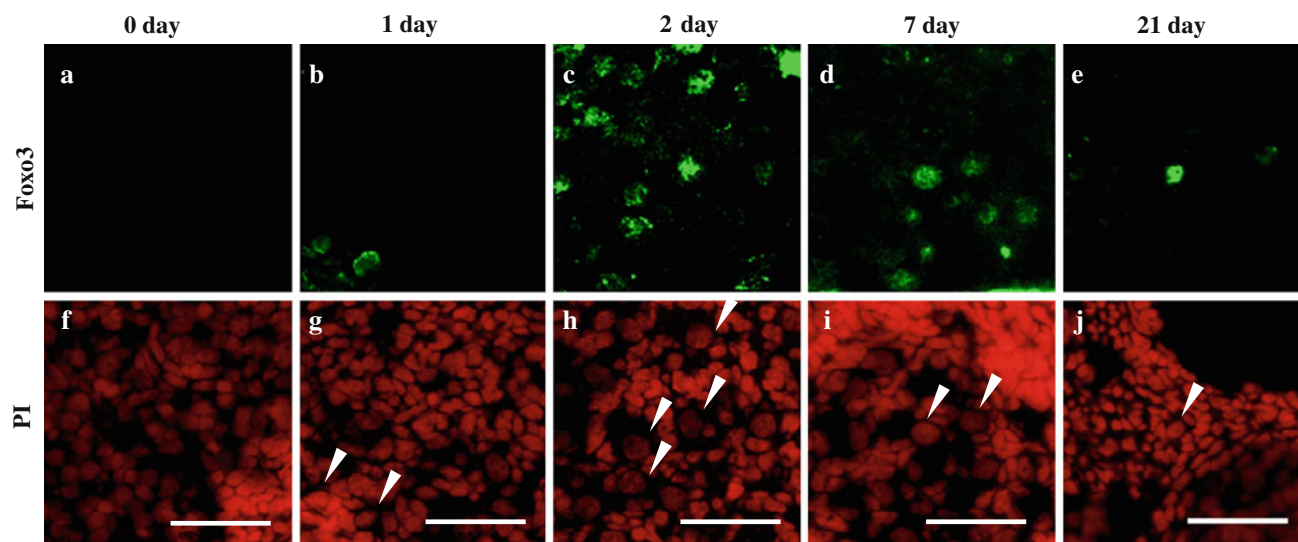
### Expression of Foxo3 in mouse oocytes

In newborn mice, the expression of Foxo3 was scarcely observed in primordial oocytes (Table 1; Fig. 1). Its

**Table 1** Expression of Foxo3 in mouse oocytes

Age (days)	No. of ovaries examined <sup>a</sup>	No. (%) of Foxo3-positive oocytes/examined oocytes in follicles at different stages		
		Primordial	Primary	Secondary
0	3	1 $\pm$ 1/56 $\pm$ 5 (1 $\pm$ 1) <sup>a</sup>	0/0	0/0
1	3	7 $\pm$ 3/40 $\pm$ 8 (20 $\pm$ 7) <sup>b</sup>	0/0	0/0
2	3	17 $\pm$ 5/33 $\pm$ 5 (48 $\pm$ 8) <sup>bc</sup>	0/2 $\pm$ 1 (0)	0/0
7	3	13 $\pm$ 3/38 $\pm$ 10 (37 $\pm$ 2) <sup>bc</sup>	2 $\pm$ 1/8 $\pm$ 2 (15 $\pm$ 6) <sup>a</sup>	0/0
21	3	8 $\pm$ 2/17 $\pm$ 4 (47 $\pm$ 4) <sup>c</sup>	1 $\pm$ 0/3 $\pm$ 0 (22 $\pm$ 9) <sup>a</sup>	0/2 $\pm$ 1 (0)

<sup>a</sup> Cryosections were fixed and reacted with rabbit anti-Foxo3 antibody and Alexa Fluor 488-labelled secondary antibody. Sections were counterstained with PI and Foxo3-positive oocytes were counted in the section prepared from each ovary. Each value represents the mean  $\pm$  SEM. Values with different superscripts in the same column differ significantly (*P* < 0.05)



**Fig. 1** Immunolocalization of Foxo3 in mouse ovaries. Cryosections were treated with rabbit anti-Foxo3 antibody and Alexa Fluor 488-labeled anti-rabbit immunoglobulin antibody (green; **a–e**) and counterstained with PI (red; **f–j**). The nuclei of some oocytes showed

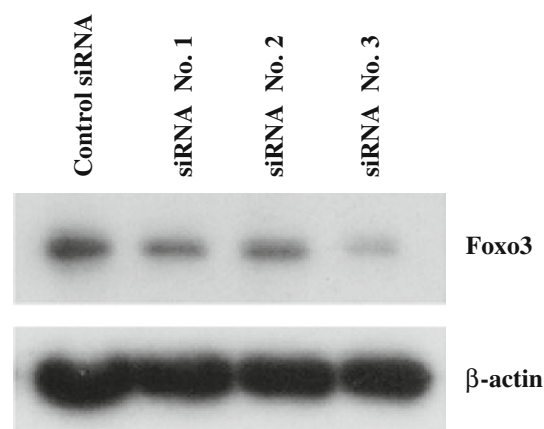
Foxo3 expression. Oocytes showing Foxo3 expression were indicated by arrowheads. Scale bars represent 60  $\mu$ m (**a–d**, **f–i**) and 100  $\mu$ m (**e**, **j**)

expression was faint in ovarian interstitial cells and flat-shaped pre-granulosa cells in newborn ovaries. The expression was found in the nucleus of 20 % of primordial oocytes at 1-day-old (Fig. 1b), and the percentage of Foxo3-positive oocytes increased at 2-day-old (Fig. 1c; Table 1). There was no significant difference among the percentages of Foxo3-positive primordial oocytes at 2-, 7- and 21-day old. Foxo3 was expressed in granulosa cells and ovarian interstitial cells faintly in ovaries of different ages, although there were no differences in Foxo3 expression in ovaries of different aged groups.

#### Knockdown of Foxo3 and growth of mouse oocytes

To determine the efficiency of siRNAs to knock down Foxo3, mouse fibroblasts were treated with siRNAs. The results of Western blot analysis showed that the expression of Foxo3 was decreased in the cells treated with three kinds of *Foxo3* siRNAs, but not with control siRNA (Fig. 2). Two *Foxo3* siRNAs with higher efficacy (No. 1 and 3) were selected to use the following experiment. To determine the transfection of siRNAs to the oocytes in ovarian tissues, Alexa Fluor 546-labeled control and *Foxo3* siRNAs were used. Oocytes and other ovarian cells in the tissues exhibited a red color, which indicated the penetration of siRNAs into the oocytes (Fig. 3).

In the histological examination, 1-day-old ovaries contained only primordial follicles and a few of oocytes were more than 20  $\mu$ m in diameter before culture (Table 2). After culture for 6 days, a number of oocytes grew to 20  $\mu$ m or more in diameters (growing oocytes), while the



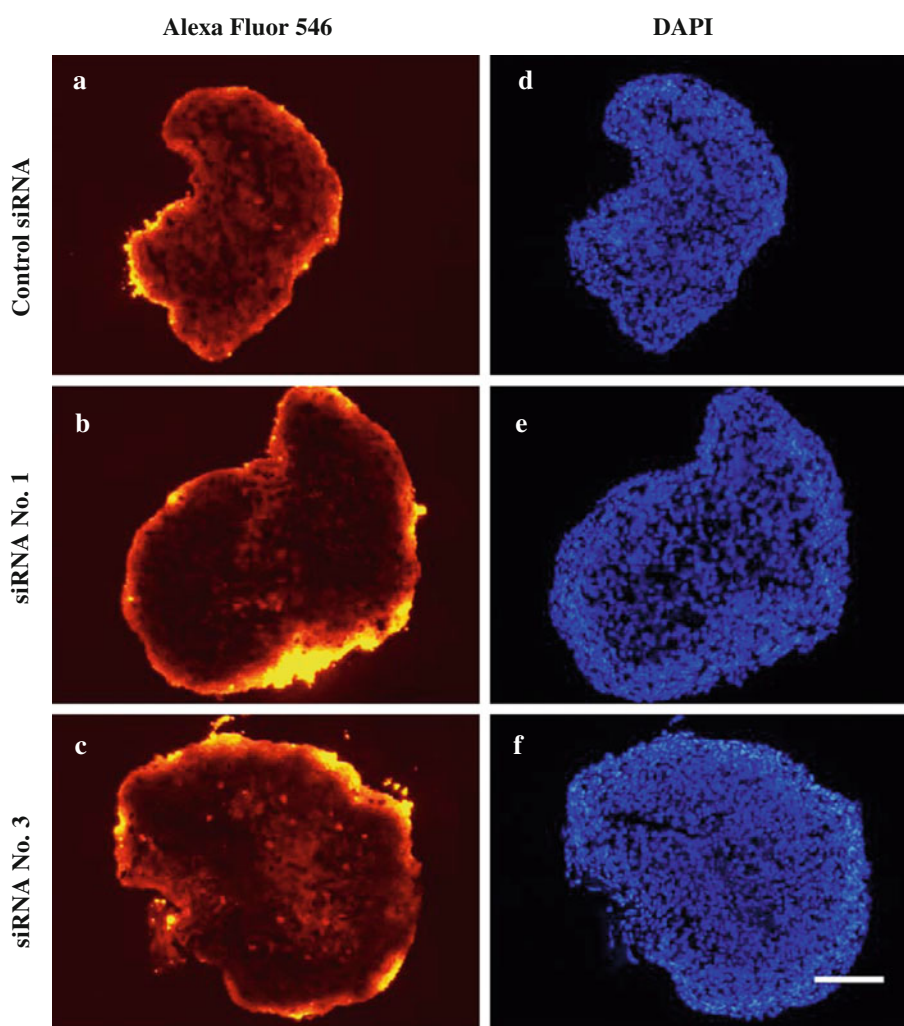
**Fig. 2** Mouse fibroblasts (3T3 cells) were treated for 6 h with three different *Foxo3* siRNAs; No. 1: 5'-CCGGCACCAUGAAUCUGAAUGAUGG-3', No. 2: 5'-CAGUA CCGUGUUUGGACCUUCGUCU-3', No. 3: 5'-CCAGUGACUUGGACCUGGACA UGUU-3' and control siRNA. Cells were subjected to Western blot analysis by using anti-Foxo3 antibody. Levels of  $\beta$ -actin were used as internal control. The experiment was repeated three times with similar results

follicles remained in the primordial or primary stage in both control and *Foxo3* siRNA-treated ovaries (Fig. 4). The percentages of growing oocytes were higher in *Foxo3* siRNA-treated ovaries than in control siRNAs-treated ovaries (Table 2).

#### Discussion

The mechanisms concerning the entrance of primordial oocytes into the growth phase remain largely unknown.

**Fig. 3** Newborn mouse ovaries were treated with Alexa Fluor 546-labeled control and *Foxo3* siRNAs (No. 1 and No. 3) for 6 h, cryosectioned and observed under the fluorescence microscope. Red colored cells are considered to be transfected with siRNAs (a–c). Sections were counterstained with DAPI (blue; d–f). The experiment was repeated three times with similar results. Scale bar represents 100  $\mu$ m



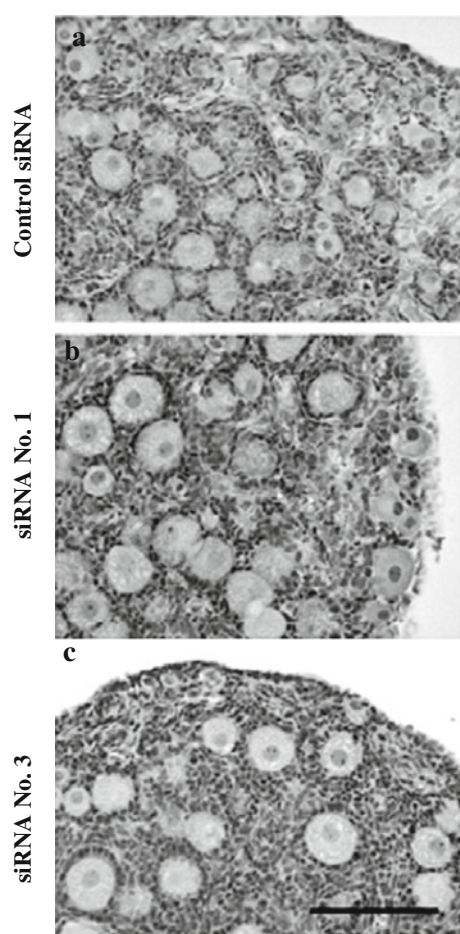
**Table 2** Oocyte growth in *Foxo3* knocked-down mouse ovaries after culture

Treatment	No. of ovaries examined	No. of oocytes observed per ovary <sup>a</sup>	No. (%) of growing oocytes ( $\geq 20$ $\mu$ m in diameter)
1-day-old fresh ovaries	3	253 $\pm$ 15	5 $\pm$ 4 (2 $\pm$ 2) <sup>a</sup>
Control siRNA	3	184 $\pm$ 10	69 $\pm$ 2 (38 $\pm$ 2) <sup>b</sup>
siRNA No. 1	3	120 $\pm$ 26	80 $\pm$ 8 (72 $\pm$ 8) <sup>c</sup>
siRNA No. 3	3	128 $\pm$ 3	88 $\pm$ 1 (69 $\pm$ 1) <sup>c</sup>

<sup>a</sup> Ovaries from 1-day-old mice were treated with *Foxo3* siRNAs (No. 1 and No. 3) and cultured for 6 days. Fresh and cultured ovaries were fixed, dehydrated and embedded, and the serial sections were stained with H&E. From the central region of each ovary, two largest sections being 20  $\mu$ m apart were selected and the diameters of the oocytes (excluding the zona pellucida) were measured. Each value represents the mean  $\pm$  SEM. Values with different superscripts in the same column differ significantly ( $P < 0.05$ )

Several factors including KIT, KL (KIT ligand), GDF9 (growth differentiation factor 9) and NGF (nerve growth factor) are known to be involved in oocyte growth and follicular development in mammals [11]. *Foxo3* has been suggested to be a suppressor of primordial oocyte activation because *Foxo3*-deficient mice exhibit excessive activation of primordial follicles [8]. In our previous study, *FOXO3* was expressed in the nucleus of a proportion of

primordial oocytes in neonatal (10- to 20-day-old) and prepubertal (6-month-old) pigs [9]. In the present study, *Foxo3* expression was low in the primordial oocytes in newborn mice. The expression was found in the nucleus of a small number of oocytes at 1-day-old. The percentage of *Foxo3*-positive oocytes increased at 2-day-old. The proportion of *Foxo3*-positive primordial oocytes was similar in the ovaries from 2- to 21-day-old mice. Since, mouse



**Fig. 4** Histological sections of 1-day-old mouse ovaries after organ culture for 6 days. Ovaries were treated with control siRNA (**a**), *Foxo3* siRNA No. 1 (**b**) and *Foxo3* siRNA No. 3 (**c**), and cultured for 6 days. Ovaries were fixed, embedded, serially sectioned by 5 μm, and stained with H&E. After culture, increased number of oocytes grew in *Foxo3* siRNA-treated ovaries (**b**, **c**) than control siRNA treated groups (**a**). Scale bar represents 100 μm

oocytes start to grow after birth, this pattern of *Foxo3* expression indicates its possible involvement in the growth of primordial oocytes in mice.

RNA interference by siRNAs is a well-established method for gene knockdown in cultured cells [12, 13], and has been applied for oocytes [14], cortical slices of porcine and bovine ovaries [9, 15] and mouse ovaries [16]. Since siRNA mediates sequence-specific mRNA degradation, the *Foxo3* siRNAs worked to knock down the target *Foxo3* protein in the present experiment. After the treatment with *Foxo3* siRNAs, the proportion of growing oocytes ( $\geq 20$  μm in diameter) increased significantly and that of non-growing oocytes ( $< 20$  μm in diameter) decreased compared with the control in cultured mouse ovaries. Thus, the present experiments suggest that primordial oocytes entered the growth phase in vitro by the knockdown of *Foxo3*. In previous experiments, we found that primordial follicles from neonatal pigs developed to the antral stage

along with oocyte growth at 2 months after xenografting, whereas those from prepubertal pigs survived without initiation of development [17]. After knockdown of *FOXO3*, a proportion of primordial oocytes from prepubertal pigs became activated and entered the growth phase in the xenografts [9]. The similar results have been obtained in bovine primordial oocytes [15]. In the present study, the role of *Foxo3* in survival of mouse primordial oocytes was not elucidated. In our preliminary experiments, different concentrations of transfection reagent Lipofectamine 2000 and siRNAs were applied to knock down *Foxo3* in primordial oocytes in ovarian tissues. Higher concentrations ( $\geq 0.5$  %) of the reagent caused the degeneration of oocytes (data not shown).

The mechanisms associated with the regulation of *FOXO* molecules have been characterized mainly in somatic cells in response to growth factor signaling via the phosphatidylinositol 3-kinase (PI3K)—protein kinase B (PKB) pathway [18]. PKB-induced phosphorylation inhibits transcriptional activity of the *FOXO* members [5]. In mice, the oocyte-specific deletion of *Pten* (phosphatase and tensin homolog deleted on chromosome 10), which negatively regulates the action of PI3K, causes excessive activation of primordial follicles [19, 20]. Li et al. [21] treated human ovarian fragments and mouse ovaries with a *PTEN* inhibitor and transplanted them to SCID mice. After transplantation, increased numbers of antral follicles were obtained from inhibitor-treated groups than the control. Reddy et al. [20] suggest that the KIT-induced PI3K pathway regulates primordial follicle activation. However, the role of PI3K in the activation of primordial follicles is still controversial, since it has been found that KIT-deficient mice possessed growing oocytes in developing follicles [22, 23].

In summary, *Foxo3* expression was low in primordial oocytes in newborn mouse ovaries. The expression was found in nuclei of a proportion of primordial oocytes in 1-day-old mice, and the percentage of *Foxo3*-positive primordial oocytes increased at 2-day-old mice. After treatment with *Foxo3* siRNAs, higher proportion of oocytes entered the growth phase in organ-cultured ovaries compared to the control. These results suggest that *Foxo3* negatively regulates the growth initiation of primordial oocytes and knockdown of *Foxo3* leads primordial oocytes to the growth phase in vitro.

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