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Infertility caused by retardation of follicular development in mice with oocyte-specific expression of Foxo3a

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In recent years, mammalian oocytes have been proposed to have important roles in the orchestration of ovarian follicular development and fertility. To determine whether intra-oocyte Foxo3a, a component of the phosphatidylinositol 3-kinase (PI3K) signaling pathway, influences follicular development and female fertility, a transgenic mouse model was generated with constitutively active Foxo3a expressed in oocytes. We found that the female transgenic mice were infertile, which was caused by retarded oocyte growth and follicular development, and anovulation. Further mechanistic studies revealed that the constitutively active Foxo3a in oocytes caused a dramatic reduction in the expression of bone morphogenic protein 15 (Bmp15), connexin 37 and connexin 43, which are important molecules for the establishment of paracrine and gap junction communications in follicles. Foxo3a was also found to facilitate the nuclear localization of p27^{kip1} in oocytes, a cyclin-dependent kinase (Cdk) inhibitor that may serve to inhibit oocyte growth. The results from the current study indicate that Foxo3a is an important intra-oocyte signaling molecule that negatively regulates oocyte growth and follicular development. Our study may therefore give some insight into oocyte-borne genetic aberrations that cause defects in follicular development and anovulation in human diseases, such as premature ovarian failure.

KEY WORDS: Oocyte, Foxo3a, PI3K pathway, Follicular development, Transgenic, Mouse

INTRODUCTION

Ovarian follicle activation in mammals takes place when primary follicles are recruited from the primordial pool, when oocytes grow rapidly and the surrounding granulosa cells become cuboidal and proliferative. This process is followed by further follicular development, ovulation and luteinization during each repetitive menstrual/estrous cycle (for reviews, see Matzuk et al., 2002; McGee and Hsueh, 2000; Wassarman and Albertini, 1994). In recent years, it has been suggested that oocytes may orchestrate the development of mammalian ovarian follicles, and that the rate of follicular development is based on a developmental program intrinsic to the oocyte (Eppig et al., 2002; Matzuk et al., 2002). Although gonadotropins are essential for driving the differentiation of granulosa cell phenotypes, the oocyte is probably the dominant factor within its range of influence and determines the direction of differentiation and the function of the granulosa cells associated with it (Eppig, 2001). It is generally believed that some intra-ovarian factors have important roles in regulating oocyte growth and early follicular development before the action of follicle-stimulating hormone (FSH) becomes important (Matzuk et al., 2002; Peters et al., 1975).

The bi-directional communication between oocytes and granulosa cells has been shown to be essential for follicular development. The growth and meiotic regulation of oocytes are dependent on granulosa cells; at the same time, oocytes secrete various factors that have key roles in folliculogenesis. For example, oocyte and follicular growth are dependent on kit ligand (Kitl – Mouse Genome Informatics; stem cell factor, SCF) which is produced by granulosa

cells; oocyte-derived factors such as bone morphogenic protein 15 (Bmp15) and growth differentiation factor 9 (Gdf9) also have fundamental roles in the control of granulosa cell proliferation and differentiation during follicular development (for reviews, see Albertini and Barrett, 2003; Eppig, 2001; Matzuk et al., 2002; McGee and Hsueh, 2000; Shimasaki et al., 2004).

Our previous report has shown that in cultured mouse and rat oocytes, Kitl can activate the oocyte phosphatidylinositol 3-kinase (PI3K) pathway via Kit receptor on the oocyte surface, a process that involves activation of the growth-enhancing molecule Akt and also the suppression of the Akt substrate Foxo3a (also known as FKHL1), which is a transcription factor that regulates the cell cycle and apoptosis (Reddy et al., 2005). Our next question is what functional roles the PI3K pathway plays in mammalian oocytes. Although the conventional Foxo3a-knockout mice have been shown to exhibit excessive activation of primordial follicles in a pioneer study by Castrillon et al. (Castrillon et al., 2003), the question of how Foxo3a influences follicular growth remains unanswered. This involves several issues, such as whether Foxo3a functions within the ovary itself (Brenkman and Burgering, 2003), the nature of the cells and the developmental stages in which Foxo3a plays its role, and the identity of the pathways through which Foxo3a regulates follicular growth. Based on previous reports and our own data, we have proposed that intra-oocyte Foxo3a may play an important role in the regulation of follicular activation and development (Liu et al., 2006; Reddy et al., 2005). To test this hypothesis and to learn more about the functions of intra-oocyte Foxo3a in follicular development, we generated a transgenic (Tg) mouse model where constitutively active Foxo3a is maintained in the oocytes, mediated by the oocyte-specific zona pellucida glycoprotein 3 (*Zp3*) promoter (Epifano et al., 1995). We found that the Tg mice showed infertility caused by retardation of oocyte growth and follicular development, and anovulation. The expression of several important ovarian genes, which we supposed to be responsible for the above-mentioned defects, was also studied in the Zp3-Foxo3a Tg mice.

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MATERIALS AND METHODS

Generation of Tg mice expressing constitutively active Foxo3a in the oocytes

The full coding region of mouse Foxo3a cDNA where the Akt phosphorylation sites Thr32, Ser252 and Ser314 were mutated to alanines (Foxo3a-TM, kindly provided by Drs Noboru Motoyama and Yoko Furukawa-Hibi, National Institute for Longevity Sciences, Japan) was subcloned into the *Sma*I site of a murine Zp3 promoter-BGH poly-A expression vector (kindly provided by Dr Jurrien Dean, NIH, MD). The 8.5 kb Zp3-Foxo3a-TM fragment (Fig. 2A) was excised by *I-Sce*I and *Nor*I digestion. After injection into the male pronucleus of one-cell zygotes from F2 of B6CBA×B6CBA, the embryos were transferred to oviducts of foster mothers (Umeå Transgene Core Facility, Sweden). Three founders (two male mice and one female mouse) were identified by PCR screening. The female founder mouse was apparently normal and infertile, and was sacrificed at the age of 6 months. The male founders S23 and S26 were apparently normal and fertile, and were backcrossed three times to C57BL/6J wild-type (WT) mice (Charles River Laboratory, Sulzfeld, Germany) before experiments were performed. The colony was maintained by breeding Tg male mice with C57BL/6J WT females. Female offspring of S23 and S26 showed similar phenotypes, and all the experiments presented in this paper were performed using offspring from S23. The mice were housed under controlled environmental conditions with free access to water and food. Illumination was provided between 0600 and 1800 h. Experimental protocols were approved by the regional ethical committee of Umeå University.

Reagents, antibodies and immunological detection methods

The rabbit polyclonal antibodies against phospho-Smad1 (serine 463/465)/Smad5 (serine 463/465)/Smad8 (serine 426/428), Smad1, phospho-Smad2 (serine 465/467), Smad2, connexin 43, p27^{kip1} (p27; Cdkn1b), Akt, phospho-Akt (serine 473), p70S6 kinase, phospho-p70S6 kinase (threonine 389), mammalian target of rapamycin (mTOR), phospho-mTOR (serine 2448), phospho-p44/42 mitogen activated protein kinase (MAPK) (threonine 202/tyrosine 204), and p44/42 MAPK were obtained from Cell Signaling Technologies (Beverly, MA). Rabbit polyclonal antibody against connexin 37 was purchased from Alpha Diagnostics International (San Antonio, TX). Rabbit polyclonal antibody against Foxo3a (FKHRL1) was from Upstate Biotechnology (New York, NY). The proliferating cell nuclear antigen (PCNA) Staining Kit was purchased from Invitrogen (Sweden). Rabbit polyclonal antibody against progesterone receptor (PR) was purchased from Dako Sweden (Solna, Sweden). Mouse monoclonal antibodies against β -actin, bromodeoxyuridine (BrdU) and FLAG M2-HRP, and pregnant mare serum gonadotropin (PMSG), human chorionic gonadotropin (hCG), and BrdU were purchased from Sigma-Aldrich (Stockholm, Sweden). Western blot analyses were carried out according to the instructions for different antibodies from the suppliers, and visualized using the ECL Plus Western Blotting Detection System (Amersham Biosciences, Uppsala, Sweden).

Quantification of ovarian follicles, histological analyses and immunohistochemistry

Ovaries were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. To count the numbers of ovarian follicles, paraffin-embedded ovaries were serially sectioned at 8 μ m and stained with Hematoxylin for morphological observation. Ovarian follicles at different developmental stages, including type 3b, type 4, type 5, and types 6-7 were counted in all sections of an ovary, based on the standards established by Pedersen and Peters (Pedersen and Peters, 1968). In each section, follicles that contained oocytes with clearly visible nuclei were scored as previously reported (Johnson et al., 2004), and the total number of follicles at any particular developmental stage was calculated as the sum of the number of follicles from all sections of an ovary. Judged from careful morphological analysis, the incidence of counting the same follicle twice or missing a follicle was low. Oocyte diameters were measured with a Zeiss AX10 microscope, and only oocytes in which the nucleoli were clearly visible were measured.

Immunohistochemistry for detection of Foxo3a, PCNA, BrdU, p27 and PR was performed on rehydrated paraffin sections using the ABC Staining System (Santa Cruz Biotechnology, Santa Cruz, CA) according to the manufacturer's instructions. Negative controls omitting primary antibodies were included in each experiment. For the in vivo BrdU incorporation assay, mice were injected with BrdU (100 mg per kg body weight) for 2 hours, and the ovaries were examined by immunohistochemistry using an anti-BrdU antibody as described above.

Preparation of ovarian extract

Ovaries were dissected free of fat and adhering tissues and extracts were prepared on ice by homogenizing in a lysis buffer containing 50 mM Tris-HCl (pH 8.0), 120 mM NaCl, 20 mM NaF, 20 mM β -glycerophosphate, 1 mM EDTA, 6 mM EGTA (pH 8.0), 1% NP-40, 1 mM DTT, 5 mM benzamidine, 1 mM PMSF, 250 μ M sodium orthovanadate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 μ g/ml pepstatin, followed by centrifugation at 18,000 *g* for 20 minutes at 4°C. The supernatants were collected and protein concentrations were measured using the bicinchoninic acid (BCA) protein assay, and equal amounts of proteins were used for the western blot. Results of western blot were normalized against levels of β -actin in the lysates.

Assessment of the estrous cycle

Vaginal smears were used to assess the phases of the estrous cycle. This was performed by washing the vaginal epithelium with 50-100 μ l of autoclaved 0.9% NaCl and transferring part of the washing solution onto a microscopic slide for observation. The estrous phase indicating approaching ovulation

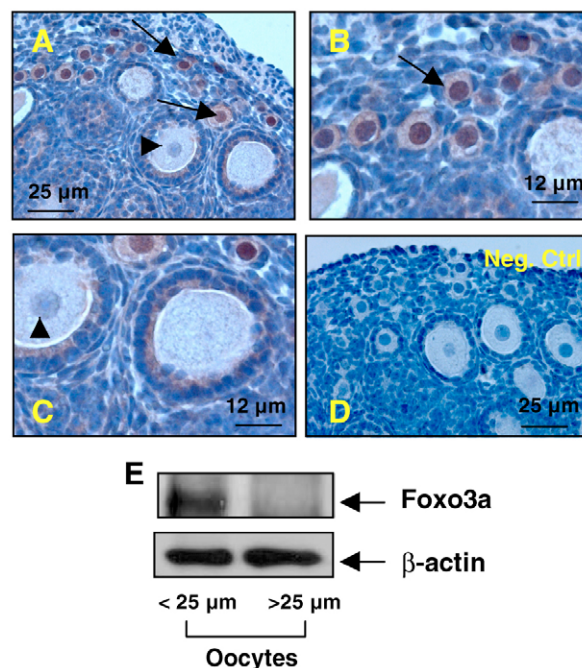


Fig. 1. Expression of Foxo3a in WT mouse oocytes.

(A-D) Immunostaining of Foxo3a in ovarian follicles of postnatal mice. Ovaries from 8-day-old mice were collected and embedded in paraffin, and sections were used for staining of Foxo3a and then counter-stained with Hematoxylin. The immunohistochemistry signals appear as a reddish-brown color and counter-stained background appears as a blue color. (B,C) Images at a higher magnification. (D) Negative control for immunostaining of Foxo3a, in which primary antibody against Foxo3a was not included. (E) Expression of Foxo3a in oocytes that were larger or smaller than 25 μ m. Oocytes were isolated from 17- to 20-day-old female C57/BL6 mice and sorted as described in Materials and methods. Levels of β -actin are shown as an internal control. All experiments were repeated at least three times, and representative results are shown; for each experiment, $n=15-20$.

was indicated by the finding of large, squamous-type epithelial cells without nuclei in the vaginal smear (Rugh, 1990). Under the light conditions used, ovulation was considered to take place at around 0200 h. To obtain preovulatory follicles or newly formed corpora lutea, ovaries were collected 2–4 hours before ovulation, or 36–48 hours after ovulation.

RNA extraction, RT-PCR and real-time PCR

Total RNA from ovaries was extracted using TRIzol (Invitrogen), following the manufacturer's instructions. To avoid contamination with genomic DNA, each total RNA sample was treated with RNase-free DNase I (Roche) and mRNA was purified with the mRNA Purification Kit (Qiagen). The mRNA was reverse transcribed (RT) using Superscript III (Invitrogen) according to the manufacturer's instructions for PCR or real-time PCR. Real-time PCR was run under standard conditions using iQ SYBR Green Supermix (BioRad) in the iCyclerIQ Multicolor Real-Time PCR Detection System (BioRad) according to the manufacturer's instructions. All real-time PCR results were normalized against levels of β -actin mRNA in the same samples. Sequences of primers for RT-PCR or real-time PCR for *Bmp15*, *Gdf9* and β -actin are available upon request.

Synthesis of FSH receptor RNA probe and in situ hybridization

A cDNA fragment (nucleotides 805–1224) of the mouse FSH receptor cDNA (kindly provided by Dr Ilpo Huhtaniemi, Imperial College, London, UK) was subcloned into the pCRII-TOPO vector (Invitrogen) as a template for synthesis of a probe for in situ hybridization. The riboprobe for in situ hybridization was labeled with digoxigenin-labeled UTP using the Dig RNA-labeling kit (Roche Diagnostics Scandinavia, Bromma, Sweden). In situ hybridization was performed on 10 μ m cryostat sections as previously described (Liu et al., 1996; Schaeren-Wiemers and Gerfin-Moser, 1993). Sense strands of the probes were used in parallel as background controls.

Isolation of oocytes from postnatal mouse ovaries

The isolation of oocytes, separation of small oocytes from partially grown oocytes using a cell-dispersing screen with a 25 μ m opening, and the lysis of oocytes were performed as previously described (Reddy et al., 2005).

Statistical analysis

All experiments were repeated at least three times. For comparison of follicle number and oocyte diameter in WT and Tg ovaries, differences between the two groups were calculated by Student's *t*-test; a difference was considered to be significant if $P < 0.01$.

RESULTS

Expression of constitutively active Foxo3a in mouse oocytes causes female infertility

In WT mice, Foxo3a was found to be expressed mainly in the nuclei of oocytes of primordial (type 2) follicles and early 'transient' primary (type 3a) follicles (Fig. 1A,B, arrows). Its expression in oocytes of primary (type 3b) follicles (Fig. 1A,C, arrowheads) and further-developed follicles (not shown) was dramatically downregulated. As confirmed by western blotting, Foxo3a was highly expressed in oocytes that were smaller than 25 μ m, but its expression was below the detection limit in oocytes that were larger than 25 μ m (Fig. 1E). To study the physiological significance of the dramatic downregulation of Foxo3a in oocytes of primary and further-developed follicles, we generated a Tg mouse model to maintain the expression of constitutively active Foxo3a in oocytes of primary and further-developed follicles using the oocyte-specific *Zp3* promoter (Epifano et al., 1995) (for details, see Fig. 2).

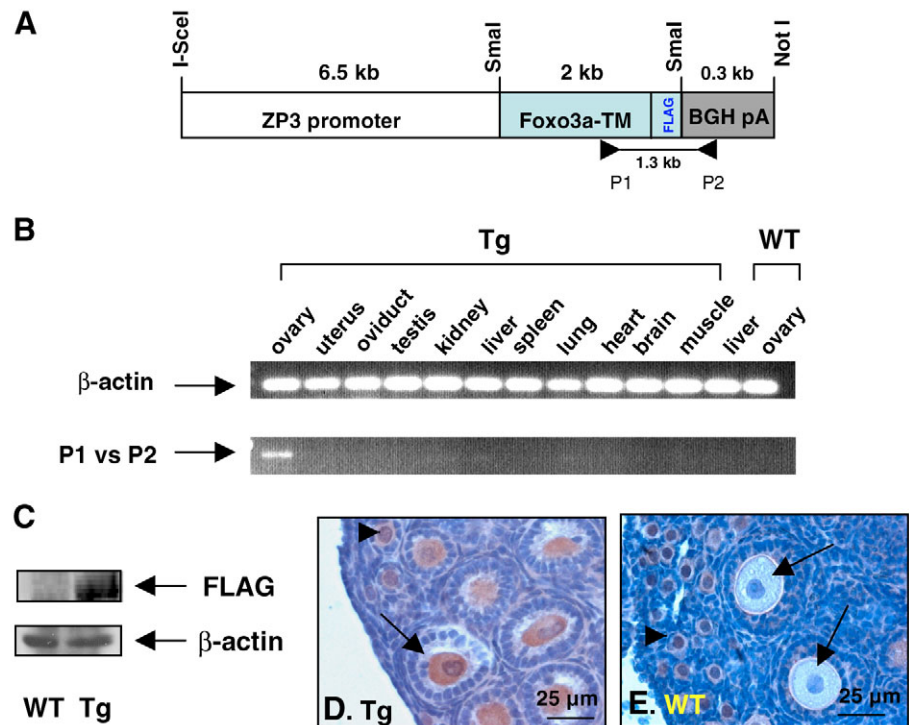
To check the fertility of the *Zp3*-Foxo3a Tg mice, we bred 6-week-old female Tg mice and their WT littermates with stud males. During a period of 6 months, the five breeding pairs of WT control mice gave an average of 47.4 pups per pair. Of the six breeding pairs of Tg mice, however, only two Tg females delivered once, with one pup per delivery, and the other four Tg female mice never became pregnant. The average number of pups during the 6-month period for Tg mice was 0.3 pups per pair. Thus, female *Zp3*-Foxo3a Tg mice are considered to be infertile, or severely subfertile.

Fig. 2. Generation and characterization of *Zp3*-Foxo3a Tg mice.

(A) DNA construct for generation of the Tg mice. The construct consists of a 6.5 kb *Zp3* promoter, a 2 kb constitutively active Foxo3a cDNA (Foxo3a-TM) with a FLAG tag, and a 0.3 kb bovine growth hormone (BGH) poly-A tail. P1 and P2 indicate the positions from which the two genotyping PCR primers were derived. I-SceI, *Sma*I and *Not*I restriction sites are indicated.

(B,C) The expression of constitutively active Foxo3a-FLAG in ovaries of Tg mice was confirmed by RT-PCR where a band of approximately 1.3 kb was detected (B), and by detection of the FLAG tag at the C-terminus of the exogenous Foxo3a protein in ovarian lysates of Tg mice (C). Ovaries from 15- to 17-day-old *Zp3*-Foxo3a Tg mice and WT littermates were homogenized and subjected to western blotting for FLAG tag and β -actin expression.

(D,E) Localization of Foxo3a antigen in ovaries of 8-day-old Tg mice and WT littermates. Immunohistochemistry confirmed Foxo3a expression in the nuclei and cytoplasm of oocytes from primary follicles (D, arrow) and secondary follicles (not shown) of Tg mice, but not in oocytes from similar follicles in WT ovaries (E, arrows). Nuclear expression of Foxo3a in oocytes of primordial follicles, which is believed to be endogenous expression, was also detected in both Tg mice and WT mice (D,E, arrowheads). The experiments were repeated three times and representative results are shown.



Retarded follicular development in Zp3-Foxo3a Tg mice

The severely reduced fertility in female Zp3-Foxo3a Tg mice indicates that there may be defects in their ovarian follicular development. To test this hypothesis, we studied the first wave of synchronized follicular development by morphological analysis of Tg and WT ovaries at postnatal day (PD) 8, 13 and 24.

At PD8, the morphologies of Tg and WT ovaries were grossly similar, containing mostly primordial and primary follicles (Fig. 3D,E). The numbers of primary (type 3b) follicles in Tg ovaries were not significantly different from the corresponding numbers in WT ovaries (Fig. 3A). There were less secondary (type 4) follicles in the

Tg ovaries than in WT ovaries, although the number of type 4 follicles in the 8-day-old ovaries was low (Fig. 3A). There were also a few type 5 follicles with 3-5 layers of granulosa cells in the ovaries of WT mice at PD8, but not in the ovaries of Tg mice of the same age (Fig. 3A). At PD13, in terms of gross morphology, the Tg ovaries appeared to be smaller than the WT ovaries (Fig. 3F,G), which was found to be caused by dramatically greater numbers of type 4 and type 5 follicles in the WT ovaries (Fig. 3B). As shown in Fig. 3H (arrows), in WT mice the first wave of follicular growth had typically reached the type 4 and 5 stages (with 2 or 3 layers of granulosa cells), whereas the majority of follicles found in the Tg mice were still at the stages of types 3b or 4 (Fig. 3I, arrows).

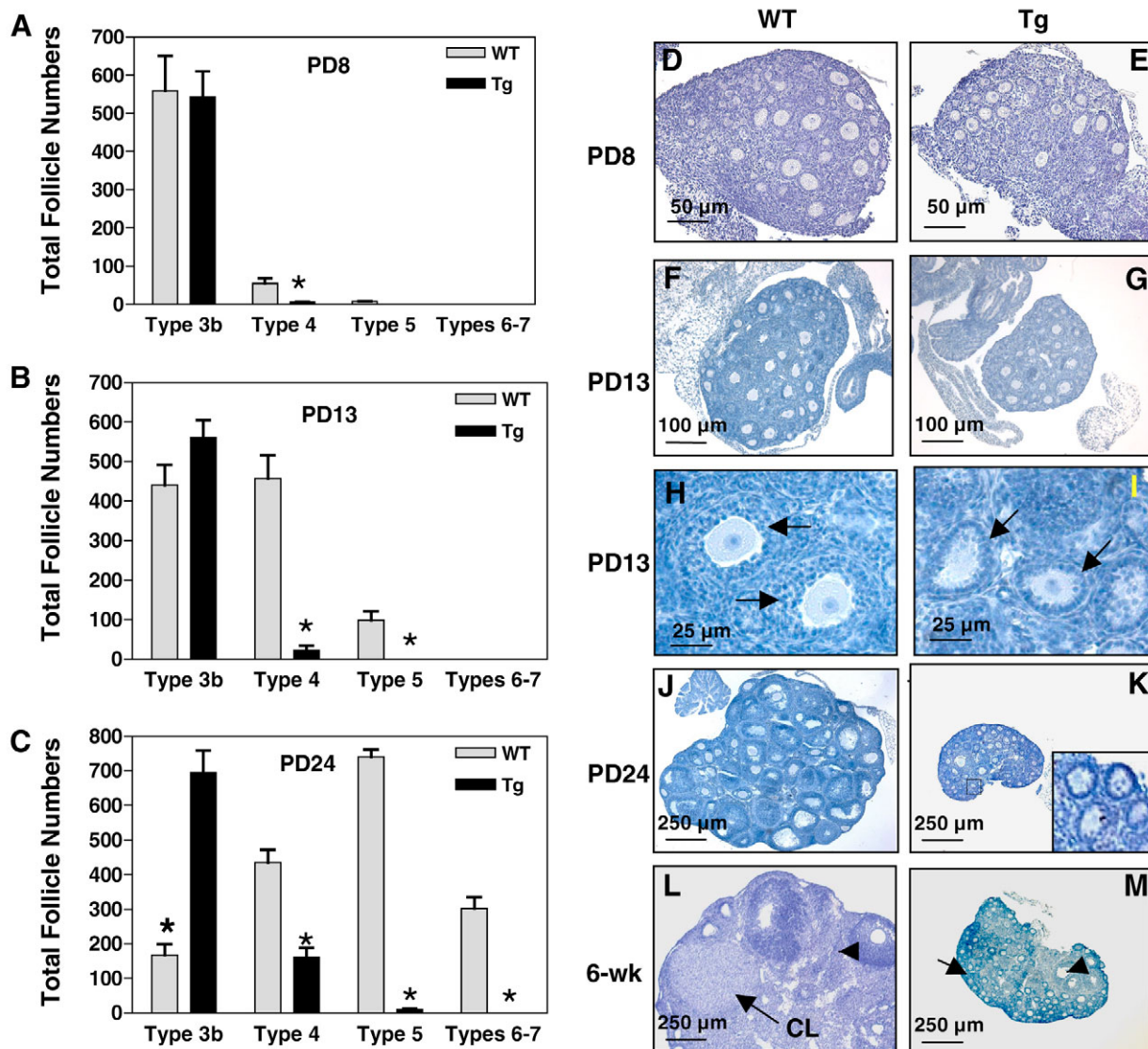


Fig. 3. Histological analysis of ovaries from postnatal Zp3-Foxo3a Tg mice and their WT littermates. Ovaries from 8-, 13- and 24-day-old, and 6-week-old Zp3-Foxo3a Tg mice and their WT littermates were isolated, fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Serial 8 μ m sections were prepared and stained with Hematoxylin for morphological observation. (A-C) Total numbers of follicles (mean \pm s.e.m.; * P <0.01) of type 3b, type 4, type 5 and types 6-7 per ovary in Tg mice (Tg) and WT littermates (WT) at PD8, PD13 and PD24. For each genotype and each age, ovaries from 3-5 mice were used. (D-M) Representative images of ovaries from Zp3-Foxo3a Tg mice (Tg) and WT littermates (WT) at PD8, PD13, PD24 and 6 weeks (6-wk). At PD13, in WT mice the first wave of follicular growth had reached the type 4 and 5 stages (H, arrows), whereas the majority of follicles in Tg ovaries were still at the stages of types 3b or 4 (I, arrows). In 6-week-old WT mice, corpus luteum (CL; L, arrow) and developing follicles (L, arrowhead) were seen, whereas, in Tg mice, most of the follicles were types 3b and 4 (M, arrow), with occasionally a few follicles of type 5 (M, arrowhead).

At PD24, the WT ovaries were dramatically larger than the Tg ovaries (Fig. 3J,K). The WT ovaries mostly had follicles of types 5-7 with antra (Fig. 3C,J), whereas the Tg ovaries still had mostly type 3b and type 4 follicles (Fig. 3C,K). In young adult (6-week-old) WT mice, corpora lutea (CL) could be identified, indicating that ovulation had taken place (Fig. 3L, arrow). In Tg mice of the same age, however, the ovaries were still significantly smaller and contained mostly follicles of types 3b and 4 (Fig. 3M, arrow) and occasionally a few follicles of type 5 (Fig. 3M, arrowhead). Thus, our data show that the expression of constitutively active Foxo3a in mouse oocytes causes retardation of follicular development.

Arrest of granulosa cell proliferation and reduced FSH receptor expression in Zp3-Foxo3a Tg mice

In order to determine the cause of retarded follicular growth, we attempted to determine whether there is less proliferation or, alternatively, more apoptosis of the granulosa cells in the Tg mice. Using 25-day-old mice, we found that the number of apoptotic granulosa cells in Zp3-Foxo3a Tg mice and their WT controls was similar, as measured by 3'-end labelling (data not shown). On the other hand, there was dramatically more proliferation of granulosa cells in WT ovaries than in Tg ovaries, as shown by immunohistochemical staining for PCNA in ovarian sections (Fig. 4A,B), and by *in vivo* BrdU incorporation assays (Fig. 4C,D). In

follicles of Tg mice, very few granulosa cells were PCNA- or BrdU-positive (Fig. 4B,D, arrows) as compared with their WT controls (Fig. 4A,C, arrows), indicating that there was an arrest of granulosa cell proliferation.

By measuring *FSH receptor* mRNA expression by semi-quantitative *in situ* hybridization, we found that granulosa cells of Tg ovaries showed much less expression of *FSH receptor* (Fig. 4F, arrows; 25-day-old mouse) than granulosa cells of WT littermates (Fig. 4E, arrows). This would explain why most of the follicles of Tg mice did not develop beyond the secondary (type 4) follicle stage, and why the Tg ovaries responded poorly to exogenous PMSG and hCG stimulation (data not shown).

Reduced oocyte sizes in Zp3-Foxo3a Tg mice

We measured the sizes of oocytes in type 3b and type 4 follicles of Tg and WT ovaries at PD8 and PD13, and found that the average diameters of oocytes in Tg type 3b follicles ($19.47 \pm 3.14 \mu\text{m}$) and Tg type 4 follicles ($24.08 \pm 6.29 \mu\text{m}$) were significantly less ($P < 0.001$) than the corresponding values for their WT counterparts ($24.27 \pm 3.06 \mu\text{m}$ in type 3b follicles and $34.36 \pm 4.21 \mu\text{m}$ in type 4 follicles) (Fig. 5). The theoretical average volumes of WT type 3b, WT type 4, Tg type 3b and Tg type 4 oocytes as calculated from the average radii were $7481 \mu\text{m}^3$, $21,229 \mu\text{m}^3$, $3863 \mu\text{m}^3$ and $7307 \mu\text{m}^3$, respectively. This indicates that oocyte growth was severely depressed in the Zp3-Foxo3a Tg mice.

Downregulation of Bmp15 and suppression of Smad pathway activation in oocytes of Zp3-Foxo3a Tg mice

We hypothesized that the molecules downstream of Foxo3a in oocytes could be molecules that are secreted from the oocytes and that regulate granulosa cell proliferation directly through paracrine regulation. As the *Zp3* promoter used in this study is known to be active in primary follicles and follicles that are more developed (Epifano et al., 1995; Lan et al., 2004), we measured *Bmp15* and *Gdf9* mRNA levels in ovaries of 6- and 8-day-old Tg and WT

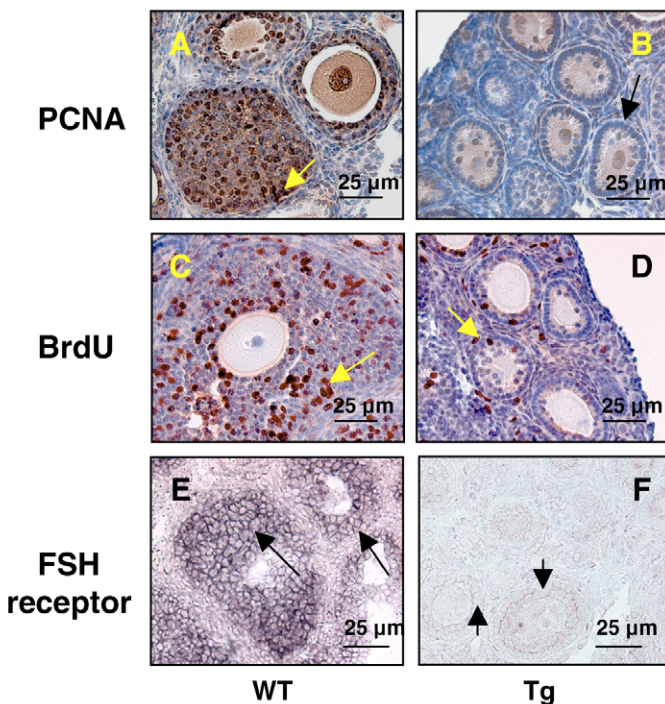


Fig. 4. Arrested granulosa cell proliferation and reduced FSH receptor expression in Zp3-Foxo3a Tg mice. Twenty-five-day-old Tg or WT mice were used for ovarian staining of PCNA and FSH receptor, or for the BrdU incorporation assay. (A,B) Immunostaining of PCNA expression in ovarian sections from WT (A, arrow) and Tg (B, arrow) mice. Signals appear in brown using DAB as substrate. (C,D) Reduced incorporation of BrdU into granulosa cells of Tg mice (D, arrow) as compared with granulosa cells of WT littermates (C, arrow). Signals appear reddish-brown using AEC as substrate. (E,F) Reduced expression of FSH receptor mRNA in ovaries of Tg mice (F, arrows) as compared with that in ovaries of WT mice (E, arrows), as measured by *in situ* hybridization. Signals appear in black.

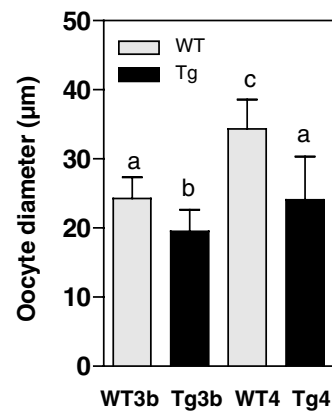


Fig. 5. Expression of constitutively active Foxo3a in mouse oocytes leads to significantly reduced oocyte size. Zp3-Foxo3a Tg mice, or their WT littermates, were sacrificed at PD8 and PD13, and oocyte diameters measured. The mean diameter of oocytes from type 3b follicles was $24.27 \pm 3.06 \mu\text{m}$ for WT mice (WT3b), and $19.47 \pm 3.14 \mu\text{m}$ for Tg mice (Tg3b). Mean oocyte diameter in type 4 follicles was $34.36 \pm 4.21 \mu\text{m}$ for WT mice (WT4) and $24.08 \pm 6.29 \mu\text{m}$ for Tg mice (Tg4). Different lowercase letters (a, b, c) indicate significant differences ($P < 0.001$). For each genotype and each follicle type, 100 oocytes were measured from ovarian sections.

ovaries, where the exogenous Foxo3a transgene starts to be expressed in Tg ovaries although no apparent morphological changes in follicular development are observed. We found that in 6- and 8-day-old mice, ovarian *Gdf9* mRNA levels were similar in WT and Tg ovaries (not shown). The *Bmp15* mRNA levels in Tg ovaries were approximately 14.0% and 16.3% of the levels in WT ovaries at PD6 and PD8, respectively (Fig. 6A,B), indicating that *Bmp15* transcription may be directly regulated by Foxo3a in the oocyte. In 15- to 17-day-old mice, where granulosa cells are undergoing rapid proliferation in WT mice but not in the Tg mice, we found that levels of both *Bmp15* and *Gdf9* mRNA in Tg ovaries were less than 10% of those in WT ovaries (Fig. 6C,D). Even so, we consider the lower

Gdf9 expression in Tg ovaries at PD15-17 to be a consequence, but not the cause, of the retarded oocyte growth and follicular development in the Tg mice, as *Gdf9* mRNA levels at PD6 and PD8 were similar in Tg and WT ovaries.

As Smads are well-characterized downstream molecules of *Bmp15* and *Gdf9* signaling (Juengel and McNatty, 2005; Kaivo-Oja et al., 2003; Moore et al., 2003; Roh et al., 2003), we also studied the activation states of Smads in ovaries of 8-day-old and 15- to 17-day-old Tg and WT mice, using antibodies against phosphorylated Smad1/5/8 (Smad1/5/8-*P*, also known as p-Smad1/5/8) and phosphorylated Smad2 (Smad2-*P*, also known as p-Smad2). We found that although the levels of Smad1/5/8-*P*, Smad1, Smad2-*P* and Smad2 were similar in WT and Tg ovaries at PD8 (Fig. 6E-I, PD8), the activation level of Smad1/5/8 (Fig. 6E, PD15-17, Tg) and the expression level of Smad1 (Fig. 6F, PD15-17, Tg) were suppressed in 15- to 17-day-old Tg ovaries as compared with either WT controls (Fig. 6E,F, PD15-17, WT) or Tg ovaries at PD8 (Fig. 6E,F, PD8, Tg). The level of activated Smad2 in Tg ovaries at PD15-17 (Fig. 6H, PD15-17, Tg) was similar to the Smad2-*P* level at PD8 (Fig. 6H, PD8, Tg) and did not show any elevation as in the WT ovaries at PD15-17 (Fig. 6H, PD15-17, WT). As the level of Smad2 in Tg ovaries at PD15-17 was similar to that in the WT ovaries (Fig. 6I, PD15-17), our data indicate that the lower Smad2-*P* level in the Tg ovaries at PD15-17 was caused by Smad2 activation per se. These data indicate that the downregulation of *Bmp15* in Zp3-Foxo3a Tg mice may result in suppressed activation of the Smad pathways, leading to retardation of granulosa cell proliferation.

Downregulation of connexin 37 and connexin 43 in ovaries of Zp3-Foxo3a Tg mice

We also studied the expression of connexin 37 and connexin 43 (Gja4 and Gja1, respectively – Mouse Genome Informatics) in Tg ovaries, which are important molecules for establishment of oocyte-granulosa and granulosa-granulosa gap junctions (Ackert et al., 2001; Gittens et al., 2003; Kidder and Mhawi, 2002; Simon et al.,

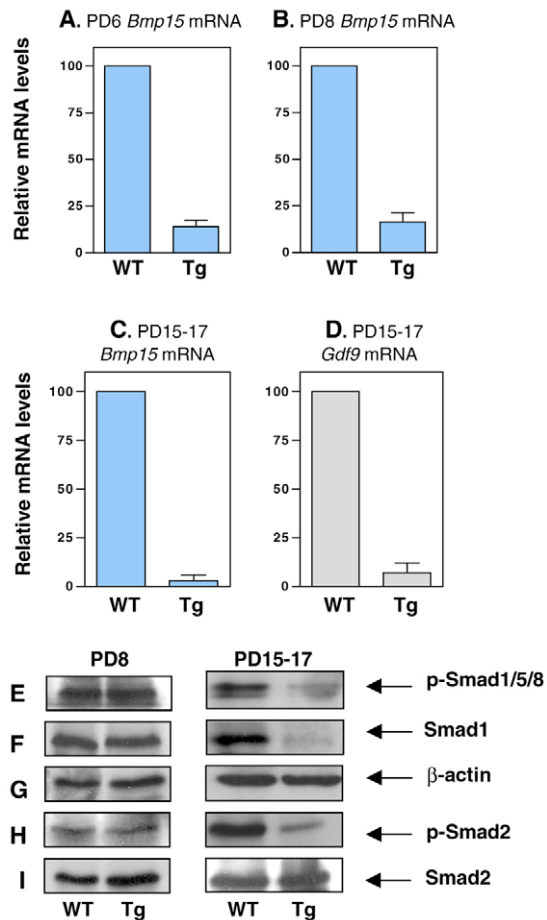


Fig. 6. Downregulation of *Bmp15* expression and suppression of Smad pathway activation in ovaries of Zp3-Foxo3a Tg mice.

Ovaries from 6-, 8- and 15- to 17-day-old Tg or WT mice were collected and used for RNA and protein extraction. (A-C) Levels of *Bmp15* mRNA from Tg and WT ovaries at PD6, PD8 and PD15-17, as measured by quantitative real-time PCR. (D) Levels of *Gdf9* mRNA from Tg and WT ovaries at PD15-17, as measured by quantitative real-time PCR. The relative levels of *Bmp15* or *Gdf9* mRNA were normalized against levels of β -actin mRNA. The mRNA levels in WT mice were defined as 1.0. mRNA levels in Tg ovaries were calculated relative to those of WT mice and are shown as mean \pm s.e.m. (E-I) Western blot analysis for levels of activated Smad1/5/8 (E, p-Smad1/5/8), total Smad1 (F), β -actin (G), activated Smad2 (H, p-Smad2), and total Smad2 (I) in WT and Tg ovaries at PD8 and PD15-17. All experiments were repeated at least three times. For each experiment with PD6 and PD8 mice, $n=10-15$; for experiments with PD15-17 mice, $n=5-8$. Representative results are shown.

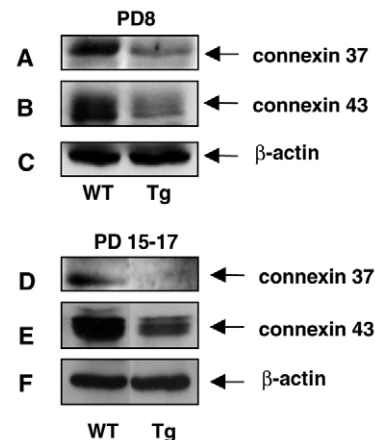


Fig. 7. Reduced expression of connexin 37 and connexin 43 in Zp3-Foxo3a Tg mice. Ovaries from 8- and 15- to 17-day-old Tg mice or WT littermates were collected and used for western blot analysis. (A,D) Levels of connexin 37 in WT and Tg ovaries at PD8 and PD15-17; (B,E) levels of connexin 43 in WT and Tg ovaries at PD8 and PD15-17; (C,F) levels of β -actin, used as a control for protein loading. Experiments were repeated at least three times. For each experiment with PD8 mice, $n=10-15$; for experiments with PD15-17 mice, $n=5-8$. Representative images are shown.

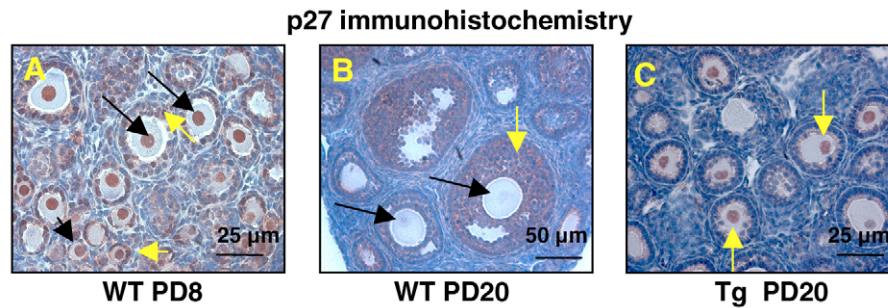


Fig. 8. p27 expression is arrested in oocyte nuclei of Zp3-Foxo3a Tg mice. Ovaries from 8- and 20-day-old WT mice and 20-day-old Tg mice were collected for immunohistochemistry for p27. (A) In 8-day-old WT mice (WT PD8), p27 was expressed in the nuclei of oocytes from primary/secondary (large arrows) and primordial follicles (small arrow), and also in the granulosa cells of these follicles (yellow arrows). (B) In the follicles of 20-day-old WT mice (WT PD20), p27 was only found to be expressed in the proliferating granulosa cells (yellow arrow), but not in oocytes of the developing follicles (black arrows). (C) In the ovaries of 20-day-old Tg mice (Tg PD 20), however, there was still expression of p27 in the nuclei of the oocytes (yellow arrows). The experiments were repeated three times and representative results are shown.

1997). Using western blot analysis, we found that connexin 37 was below the detection limit in 6-day-old WT or Tg ovaries, and that connexin 43 levels were similar in Tg and WT ovaries at PD6 (not shown). In 8-day-old ovaries, however, both connexin 37 and connexin 43 levels were considerably lower in the Tg mice than in WT mice (Fig. 7A-C), indicating that the transcription of both connexin 37 and connexin 43 is negatively regulated by Foxo3a in the oocyte. Markedly lower levels of connexin 37 and connexin 43 were still seen in Tg ovaries at PD15-17 (Fig. 7D-F).

Expression of p27 is arrested in the nuclei of oocytes in Zp3-Foxo3a Tg mice

One important downstream molecule of the PI3K pathway is the cyclin-dependent kinase (Cdk) inhibitor p27, which is a negative regulator of the mammalian cell cycle and cell growth in many physiological and pathological processes (Fero et al., 1996). As shown in Fig. 8, in ovaries of 8-day-old WT mice, p27 was found to be expressed in the nuclei of oocytes in primordial, primary and

secondary follicles (Fig. 8, black arrows), which is in accordance with a previous report (Zhang et al., 1999). In addition, p27 was found to be expressed in granulosa cells of these follicles (Fig. 8A, yellow arrows). In partially grown oocytes, however, as shown in follicles from a 20-day-old WT mouse, the expression of p27 in the nuclei of oocytes was diminished (Fig. 8B, black arrows), whereas its expression was still seen in the granulosa cells (Fig. 8B, yellow arrow). These results indicate that p27 may be a negative regulator of oocyte growth. To determine whether p27 is a possible downstream molecule of Foxo3a in mouse oocytes, we studied the pattern of localization of p27 in oocytes of Zp3-Foxo3a Tg mice. We found that in ovaries of 20-day-old Tg mice, the expression of p27 was sustained in the nuclei of the oocytes (Fig. 8C, yellow arrows), implying that p27 is continuously active in Tg oocytes, which probably functions to inhibit oocyte growth.

Activation of Akt, mTOR, p70S6 kinase and MAPK is not compromised in oocytes of Zp3-Foxo3a Tg mice

Signaling molecules of the PI3K pathway including Akt, and mTOR and p70S6 kinase (Frap1 and Rps6kb1, respectively – Mouse Genome Informatics), and molecules of the MAPK pathway, are universal substances that accelerate cell proliferation and increase cell size (Cantley, 2002; Fingar et al., 2002). We wondered whether these growth-enhancing molecules would be compromised in the oocytes of Tg mice, which might explain their retarded oocyte growth. By measuring phosphorylated protein levels of Akt, mTOR, p70S6 kinase and p42/44 MAPK in isolated oocytes from 15- to 17-day-old WT and Tg mice, we found that the activation states of these molecules were not suppressed in Tg oocytes relative to WT oocytes (Fig. 9), indicating that these basic growth-enhancing pathways are not affected by the expression of constitutively active Foxo3a in the Tg oocytes.

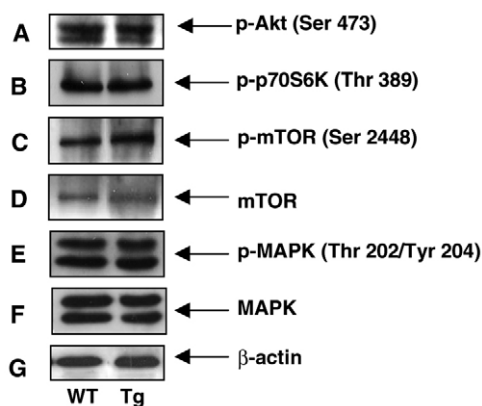


Fig. 9. Activation status of Akt, p70S6 kinase, mTOR and MAPK in oocytes of WT and Tg mice. Oocytes were isolated from 15- to 17-day-old WT or Tg mouse ovaries and lysed. Samples containing 50 μ g of protein were analyzed by western blot. Levels of activated Akt (A), activated p70S6 kinase (B), activated mTOR (C), total mTOR (D), activated MAPK (E), total MAPK (F), and β -actin (G) as an internal loading control, are shown. The experiments were repeated three times; for each experiment $n=15-20$ for each genotype. Representative results are shown.

Anovulation and luteinization of unruptured follicles in adult Zp3-Foxo3a Tg mice

In adult Tg mice, most of the follicles were arrested at early stages of follicular development, as illustrated in Fig. 3M. Irregular estrous cycles were observed in these mice. At the same time that there was a lack of continuous follicular development in the Tg ovaries, a few follicles did however develop further and reached the size of preovulatory follicles (type 8) (Fig. 10A). Even so, these developed follicles showed no sign of cumulus oocyte complex (COC)

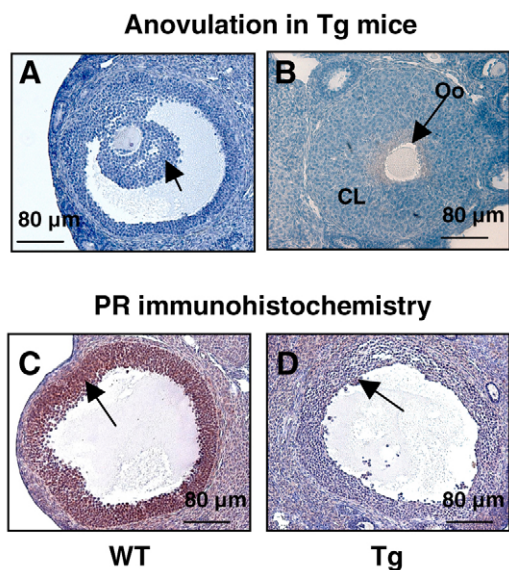


Fig. 10. Defective COC expansion, anovulation and luteinization of unruptured follicles in adult *Zp3-Foxo3a* Tg mice. Adult female mice were checked by vaginal smears for the estrous stage, which indicates approaching ovulation. To obtain preovulatory follicles or newly formed corpus luteum (CL), ovaries were collected 2–4 hours before ovulation, or 36–48 hours after ovulation. (A) A developed follicle of preovulatory size in the Tg mice, with defective cumulus oocyte complex (COC) expansion. Note the tightly packed cumulus cells (arrow). (B) A CL with a trapped oocyte (Oo, arrow) in the Tg mice. In total, 15 adult female Tg mice were analyzed and representative results are shown. (C, D) Immunostaining for progesterone receptor (PR) in preovulatory follicles of WT (C, arrow) and Tg (D, arrow) mice. The experiments were repeated three times and representative results are shown.

expansion; cumulus cells surrounding the oocyte always showed an atypical tight structure (Fig. 10A, arrow). Indeed, CL with trapped oocytes (Fig. 10B) were regularly seen in ovaries of adult Tg mice, indicating that Tg mice have defects in ovulation. In order to address whether the very low number of pups born was indeed caused by defective ovulation or, alternatively, by excess resorption of the embryos, we checked for the presence of ova on the mornings when the female Tg mice were plugged by stud males. Of the five female mice used, none showed any ovulated ova in their oviducts. We also checked for the possible presence of embryos at E9.5; however, none of the four plugged Tg mice carried any embryos. Our data thus confirmed that the almost complete infertility was caused by defects in follicular development and anovulation. Further analysis showed that the anovulation phenotype in *Zp3-Foxo3a* Tg mice may be caused by substantially reduced expression of PR in the large follicles of Tg mice (Fig. 10D, arrow), as compared with the high expression of PR in preovulatory follicles of WT mice (Fig. 10C, arrow).

DISCUSSION

Activation of the oocyte PI3K pathway, including the activation of Akt and suppression of Foxo3a in oocytes, has recently been proposed to influence oocyte growth and early follicular development (Liu, 2006; Liu et al., 2006; Reddy et al., 2005). In the current study, we have shown that Foxo3a is mainly expressed in the nuclei of oocytes in primordial and early primary follicles, and its expression is dramatically downregulated in oocytes of primary and more-developed follicles. Based on our current understanding that

the nuclear localization of Foxo3a indicates an active state (Accili and Arden, 2004; Arden and Biggs, III, 2002), our data suggest that Foxo3a in oocytes may be a factor that restrains primordial follicles from being activated, and that downregulation of Foxo3a expression in oocytes may be a prerequisite for oocyte growth and follicular development.

To test this hypothesis, we generated a Tg mouse model to maintain the expression of constitutively active Foxo3a in oocytes in primary and more-developed follicles using the *Zp3* promoter (Epifano et al., 1995). We found that the constant expression of Foxo3a in mouse oocytes not only led to retardation of oocyte growth and follicular development, but also to anovulation and luteinization of unruptured follicles. These defects subsequently cause infertility in female Tg mice. This work therefore suggests that Foxo3a is an important intra-oocyte signaling molecule that negatively regulates oocyte growth, follicular development and female fertility in mice. Thus, we hypothesize that during follicular activation, the release from transcription inhibition by Foxo3a may be of importance in initiating the expression of *Bmp15*, *connexin 37* and *connexin 43*, which in turn facilitates the growth of oocytes and follicles.

Important studies by Castrillon et al. (Castrillon et al., 2003) have shown that conventional Foxo3a-knockout mice exhibit excessive activation of primordial follicles. Nevertheless, the question remains whether Foxo3a functions in the ovary itself, as raised by Brenkman and Burgering (Brenkman and Burgering, 2003) in their review appraising the work of Castrillon et al., or at what developmental stages Foxo3a exerts its role in repressing follicular development. Based on results from the current study, we suggest that it is the intra-oocyte Foxo3a that plays a decisive role in controlling follicular activation and early development. This study therefore also reinforces the theory that oocytes carry key signals for follicular activation and development (Albertini and Barrett, 2003; Eppig, 2001; Matzuk et al., 2002).

Oocyte-granulosa and granulosa-granulosa gap junctions are essential for normal folliculogenesis (Kidder and Mhawi, 2002). In the connexin multigene family, connexin 37 is crucial for the establishment of oocyte-granulosa gap junctions, which is important for oocyte development. In connexin 37-knockout mice, oocyte growth ceases at a diameter of 52 μm (Carabatsos et al., 2000), and the sole absence of connexin 37 from oocytes is in itself sufficient to compromise both oocyte and follicular development, as has been shown using chimeric ovaries with connexin 37-deficient oocytes and WT granulosa cells (Gittens and Kidder, 2005). Connexin 43 is involved in establishing the gap junctions between granulosa cells, and mouse ovarian follicles lacking connexin 43 are arrested in the early preantral stages (Ackert et al., 2001; Gittens et al., 2003). In the present study, we have provided evidence that both connexin 37 and connexin 43 are negatively regulated by Foxo3a in oocytes. We suggest that the negative regulation of connexin 37 and connexin 43 by the Foxo3a transgene in oocytes is one of the factors that lead to retardation of follicular development in the Tg mice. Connexin 37 may be a downstream molecule of Foxo3a in mouse oocytes. It is probable that in the Tg mice, the retarded oocyte growth is caused by hindered oocyte-granulosa communication due to low connexin 37 expression that is insufficient to establish oocyte-granulosa gap junctions, through which essential signals, nutrients, ions and other requisites for oocyte growth are normally transported from the surrounding granulosa cells. Connexin 43, which is expressed by granulosa cells, is also regulated by oocyte Foxo3a, probably via an indirect pathway. The remarkable reduction in connexin 43 expression observed in Tg ovaries at

PD8 seems to be a reason for the arrest in granulosa cell proliferation, because at this stage the *Zp3* promoter is just becoming active and primary (type 3b) follicles are the dominant follicle types in both WT and Tg ovaries. However, the mechanisms by which Foxo3a in oocytes regulates connexin 37 and connexin 43 expression in follicles are not currently known.

The two TGF- β family members produced by oocytes, Bmp15 and Gdf9, have fundamental roles in the paracrine signaling between oocytes and granulosa cells that controls follicular development (Dong et al., 1996; Elvin et al., 1999b; Elvin et al., 1999a; Otsuka and Shimasaki, 2002; Vitt et al., 2000a; Vitt et al., 2000b). Investigation into the mechanisms by which Bmp15 and Gdf9 influence female fertility remains one of the most active areas of research. Previous studies have indicated that these two molecules activate the Smad pathways in granulosa cells (for reviews, see Hashimoto et al., 2005; Liao et al., 2004; Mazerbourg et al., 2004; McNatty et al., 2005; Moore and Shimasaki, 2005; Pangas and Matzuk, 2004; Shimasaki et al., 2004). Our current data provide novel information that Foxo3a in oocytes may be an upstream regulator of Bmp15. It is possible that intra-oocyte Foxo3a suppresses the activation of ovarian Smad pathways by negatively regulating the production of Bmp15, thereby inhibiting the proliferation and differentiation of surrounding granulosa cells. We propose that intra-oocyte Foxo3a may negatively regulate the transcription of *Bmp15* directly, based on sequence data showing that several Foxo3a-binding sites are present in the *Bmp15* promoter (not shown). Thus, with the occurrence of follicular activation, the emergence of Bmp15 expression in primary follicles (Shimasaki et al., 2004) may be triggered by the downregulation of Foxo3a in oocytes.

At PD6 and PD8, the levels of *Gdf9* mRNA were found to be similar in Tg and WT mice, indicating that *Gdf9* is not directly regulated by Foxo3a in oocytes. The lower *Gdf9* mRNA levels in 15- to 17-day-old Tg mice is probably caused by the retardation of oocyte growth in development-arrested follicles in these mice. This hypothesis is supported by a report that in *Gdf9*-knockout mice, although follicular development is arrested at the primary (type 3b) stage (Dong et al., 1996), the oocytes generally grow larger, leading to a mismatch in oocyte:follicle size (Carabatsos et al., 1998). This phenomenon is opposite to the situation in our Tg mice where smaller oocyte and follicle sizes were seen due to retarded oocyte growth.

It has been reported that female Bmp15-knockout mice are subfertile, with reduced ovulation rate but with minimal histopathological defects in the ovary (Yan et al., 2001). In Bmp15^{-/-} Gdf9^{+/-} double-mutant (DM) mice, defects in COC expansion were observed and the DM oocytes did not support expansion of the oocyte-cumulus cells, indicating that secretion of both Bmp15 and Gdf9 by oocytes is important for supporting the process of cumulus expansion (Su et al., 2004). In the current study, the cumulus expansion defect observed in our Zp3-Foxo3a Tg mice is comparable to what was observed in Bmp15^{-/-} Gdf9^{+/-} DM mice (Su et al., 2004). On the other hand, in our Tg mice, cumulus cells surrounding the oocyte always showed a tight structure, which is the opposite situation to that with the Bmp15^{-/-} Gdf9^{+/-} DM cumulus cells, which are loosely attached and readily fall off the oocyte (Yan et al., 2001). Thus, our Tg mouse model cannot be considered to be identical to Bmp15^{-/-} mice or Bmp15^{-/-} Gdf9^{+/-} DM mice, as a low level of *Bmp15* mRNA was still found to be expressed in oocytes, and the levels of *Gdf9* mRNA were similar in primary/secondary follicles of WT and Tg mice at PD8. It is not clear yet whether or not *Gdf9* levels are lower in the few 'preovulatory' oocytes in the Tg mice, and further studies will be required to investigate how Foxo3a in oocytes may regulate *Gdf9* transcription in preovulatory follicles, which may in turn regulate

cumulus expansion prior to ovulation. We believe that the defect in ovulation in our Tg mice is caused by multiple molecular mechanisms that are regulated by Foxo3a in oocytes.

The p27 protein is a Cdk inhibitor that suppresses cell growth. After being phosphorylated, p27 shuttles from the nucleus to the cytoplasm, whereby its inhibitory effects can be abolished (Cunningham et al., 2004; Shin et al., 2005; Viglietto et al., 2002). Our data from the current study demonstrate that in Zp3-Foxo3a Tg mice, p27 expression is maintained in the nuclei of oocytes, indicating that Foxo3a in oocytes prevents the shuttling of p27 from the nucleus to the cytoplasm, or prevents the downregulation of p27 expression, thereby maintaining the growth-inhibitory function of p27 in oocytes. This notion is supported by previous studies with other cell types showing that Foxo3a can enhance the expression of p27 (Chandramohan et al., 2004; Dijkers et al., 2000). Moreover, our unpublished data also suggest that p27 in primary oocytes may participate in the suppression of primordial follicle activation, because in p27-knockout mice primordial follicles were prematurely activated. The underlying mechanism of how Foxo3a may regulate p27 levels and regulate the localization of p27 in mouse oocytes is being investigated in our laboratory.

Another interesting finding from the current study is that in the Tg mice, expression of the Foxo3a transgene in oocytes resulted in luteinization of unruptured 'preovulatory' follicles, with oocytes being trapped in the CL. In spite of the possible regulation through altered production of Bmp15 and Gdf9, further analyses have shown that the anovulation phenotype in our Tg mice may be caused by the dramatically reduced expression of PR in large follicles. PR is a nuclear receptor transcription factor that is induced in the granulosa cells of preovulatory follicles in response to the surge of luteinizing hormone. PR has been shown to be essential for ovulation, as mice lacking this molecule fail to ovulate and are infertile (Robker et al., 2000). The assumption that the considerably reduced PR levels may cause anovulation in the Tg mice is supported by our finding that cathepsin L, a protease that facilitates ovulation, was also downregulated in granulosa cells of large follicles in the Tg mice (not shown), a phenomenon similar to that observed in PR-knockout mice (Robker et al., 2000). At this stage of the study, however, it is not clear whether Foxo3a produced by oocytes can directly regulate the expression of PR in preovulatory granulosa cells.

Based on the results of the current study and from our previous report that Kitl can induce the phosphorylation/suppression of Foxo3a via the activation of PI3K/Akt in cultured mouse and rat oocytes (Reddy et al., 2005), we suggest that a well-balanced activation of the intra-oocyte PI3K pathway is of importance in controlling the rates of oocyte growth and follicular development (Liu, 2006; Liu et al., 2006). In other words, a deregulated PI3K pathway in the oocyte may lead to disturbed follicular development and impaired fertility, as is the case in the Zp3-Foxo3a Tg mice. In addition, as Foxo3a is a transcription factor, other downstream genes and related regulation mechanisms in oocytes remain to be elucidated. Additional Foxo3a-independent pathways in oocytes, such as the mTOR-p70S6 kinase-mediated cascades and the MAPK pathway, are still just as active in the Tg oocytes, indicating that there exist overlapping signaling mechanisms that account for the rapid oocyte growth during follicular activation and early development.

In summary, our study has revealed the functional roles of intra-oocyte Foxo3a in the regulation of oocyte growth, follicular development and ovulation. The findings from the current study may provide some useful information in the search for oocyte-borne genetic aberrations that lead to defects in follicular development and ovulation in human diseases, such as premature ovarian failure.

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