Runx3 regulates folliculogenesis and steroidogenesis in granulosa cells of immature mice

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Abstract

We previously demonstrated that female *Runx3* knockout (*Runx3*$^{-/-}$) mice were anovulatory and their uteri were atrophic, and that *Runx3* mRNA was expressed in granulosa cells. To clarify how Runx3 regulates folliculogenesis and ovulation, we examined the effects of *Runx3* knockout on the gene expression of growth factors associated with folliculogenesis and enzymes associated with steroidogenesis. In *Runx3*$^{-/-}$ mouse ovaries, the numbers of primary and antral follicles were lower than those in wild type (wt) mice at 3 weeks of age, indicating that the loss of *Runx3* affects folliculogenesis. The expression of genes encoding activin and inhibin subunits (*Inha, Inhba*, and *Inhbb*) was also decreased in ovaries from the *Runx3*$^{-/-}$ mice compared with that in wt mice. Moreover, the expression of the genes *Cyp11a1* and *Cyp19a1* encoding steroidogenic enzymes was also decreased. In cultured granulosa cells from 3-week-old mouse ovaries, *Cyp19a1* mRNA levels were lower in *Runx3*$^{-/-}$ mice than those in wt mice. Follicle stimulating hormone (FSH) treatment increased *Cyp19a1* mRNA levels in both wt and *Runx3*$^{-/-}$ granulosa cells in culture, but the mRNA level in *Runx3*$^{-/-}$ granulosa cells was lower than in wt ones, indicating that granulosa cells could not fully function in the absence of *Runx3*. At 3 weeks of age, gonadotropin α subunit, FSHβ subunit and Luteinizing hormone (LH)β subunit mRNA levels were decreased in *Runx3*$^{-/-}$ mice. These findings suggest that Runx3 plays a key role in female reproduction by regulating folliculogenesis and steroidogenesis in granulosa cells.

Keywords　Ovary・Follicle・Estrogen・Mouse・Runx3

Introduction

The mammalian Runt domain transcription factor family consists of Runx1, Runx2, and Runx3 (Ito 1999; Otto et al. 2003; Cameron and Neil 2004). Runx1 and Runx2 play crucial roles in the periovulatory process of rat ovaries (Jo et al. 2004; Hernandez-Gonzalez et al. 2006; Jo and Curry 2006; Park et al. 2008; Liu et al. 2009; Liu et al. 2010; Park et al. 2010; Park et al. 2012). In addition, Runx2 regulates follicle stimulating hormone (FSH) β gene expression in gonadotropes (Breen et al. 2010). Recently, we found that female *Runx3*$^{-/-}$ mice were anovulatory and their uteri were atrophic. The numbers of primary, secondary, and antral follicles in ovaries were also significantly lower in adult *Runx3*$^{-/-}$ mice than in wt mice, and corpora lutea were not observed in *Runx3*$^{-/-}$ mice, indicating a lack of ovulation in the mouse (Sakuma et al. 2008). In adult mouse ovaries, *Runx3* mRNA expression was detected in granulosa cells by *in situ* hybridization, and was also detected in isolated granulosa cells by RT-PCR analysis (Ojima et al. 2016). These findings lead us to the hypothesis that *Runx3* in granulosa cells is involved in the development of ovarian follicles. However, it is not clear how Runx3 regulates folliculogenesis and ovarian functions in mice.

Ovarian folliculogenesis is divided into two stages: the early stage is the gonadotropin-independent growth stage, and the later stage is the gonadotropin-dependent growth one. In the early stage, primordial follicles undergo initial recruitment from their pool to initiate their growth into primary and secondary follicles. This initial recruitment is regulated by intraovarian growth factors, estrogens, and unknown factors other than FSH. In the later stage, cyclic rises of circulating FSH during the estrous cycle recruit antral follicles to initiate the growth into preovulatory follicles and finally the ovulation induced by LH surge (Dierich et al. 1998; Kumar et al. 1997; Lei et al. 2001; Orisaka et al. 2009). Thus, folliculogenesis consists of two distinct stages of follicle recruitment (initial and cyclic ones;

McGee and Hsueh 2000). It is still not clear how Runx3 regulates the early stage of folliculogenesis (the initial recruitment) in mouse ovaries. The cyclic recruitment of follicles starts at the onset of puberty. Therefore, it is necessary to analyze the development of follicles distinctly in two periods: before and after the onset of puberty. We previously investigated follicular development of 8-week-old *Runx3*$^{-/-}$ and wt mouse ovaries (Ojima et al. 2016). The present study primarily focuses upon follicular development during the prepubertal period to clarify the roles of Runx3 in folliculogenesis in the initial recruitment stage.

TGF-β superfamily growth factors, acting as the intra-ovarian growth factors, are involved in the regulation of folliculogenesis (Drummond 2005; Knight and Glister 2006; Richards and Pangas 2010; Knight et al. 2012). As signal transduction of the TGF-β superfamily members is mediated by members of the Smad family (Massague and Wotton 2000; Wrana and Attisano 2000), the deletion of Smad3 severely affects the follicular development (Xu et al. 2002; Tomic et al. 2004; Kaivo-oja et al. 2006). Therefore, we examined the gene expression of members of the TGF-β superfamily, anti-Müllarian hormone (AMH) and subunits of activin and inhibin in *Runx3*$^{-/-}$ mouse ovaries.

Estrogen is another intraovarian factor involved in the regulation of follicular development (Krege et al. 1998; Lubahn et al. 1993). Estrogen production in follicular cells is regulated by FSH and LH (Adashi and Hsueh 1982; Fitzpatrick and Richards 1991). In *Runx3*$^{-/-}$ mouse ovaries, mRNA expression of the gene encoding the cholesterol side-chain cleavage enzyme (SCC) gene *Cyp11a1* in theca cells is decreased in mature female mice (Sakuma et al. 2008; Tsuchiya et al. 2012; Ojima et al. 2016). Aromatase encoded by *Cyp19a1* is a key enzyme of estrogen synthesis in granulosa cells, in which *Runx3* is also expressed. However, the role of Runx3 in *Cyp19a1* expression in granulosa cells is still unclear. Therefore, the expression of genes involved in steroidogenesis including *Cyp19a1* was studied using

granulosa cells collected from the ovaries of 3-week-old mice.

The present study focuses upon the involvement of Runx3 in the early stage of folliculogenesis (the initial recruitment of follicles). We investigated the effects of *Runx3* knockout on the development of follicles, and the involvement of the gene expression of TGFβ superfamily growth factors and enzymes involved in steroidogenesis in follicles. This work provides new insights into the regulatory mechanism of Runx3 in the early stage of folliculogenesis.

Materials and methods

Animals

Male and female BALB/c mice were used in the present study. *Runx3* knockout (*Runx3*$^{-/-}$) mice with the BALB/c genetic background were generated as previously described (Li et al., 2002). All animal care and experiments were approved by the Animal Care and Use Committee, Okayama University, and were conducted in accordance with the Policy on the Care and Use of Laboratory Animals, Okayama University. *Runx3*$^{+/-}$ mice were mated, and their offsprings were genotyped as previously described (Yamamoto et al. 2006).

Ovaries obtained from *Runx3*$^{-/-}$ and wt mice (n=5, each group) were fixed in Bouin's fixative, dehydrated, and embedded in paraffin. Sections (7 μm thick) were cut and stained with hematoxylin and eosin. The number of primordial follicles was counted in every other section, and each follicle was followed through consecutive sections to ensure that it was counted only once.

Riboprobes

Mouse *Cyp11a1* and *Cyp19a1* riboprobes were generated in accordance with a previously described method (Murakami et al. 2005). DNA fragments encoding part of mouse *Cyp11a1* (NM_019779.3; 630-1055) and *Cyp19a1* (NM_007810.3; 286-789) were obtained by reverse-transcription polymerase chain reaction (RT-PCR) using the following primers described in Table 1. The cDNA fragments were subcloned into the pGEM-3Zf(+) vector. Each plasmid DNA was linearized using restriction enzyme (*Eco*RI/*Hind*III) sites of pGEM-3Zf(+) and RNA probes were synthesized using a T7 and SP6 polymerase system (Promega, Madison, WI, USA), in accordance with the manufacturer's instructions. The probe was labeled with digoxigenin (DIG) (Roche Diagnostics, Mannheim, Germany).

In situ hybridization analysis

Ovaries from wt and *Runx3*$^{-/-}$ mice were embedded in O.C.T. Compound (Sakura Finetek Japan, Tokyo, Japan), frozen with liquid nitrogen, and sectioned at 10-μm thickness by a cryostat. The dried sections were treated with 0.5 μg/ml proteinase K (Nacalai Tesque, Kyoto, Japan) at 37°C for 10 min, 0.2% glycine in PBS for 20 min, and subjected to acetylation treatment with 0.15 M acetic anhydride in 0.1 M triethanolamine for 10 min at room temperature. The sections were then treated with pre-hybridization solution containing 4×SSPE, 1×Denhardt's solution, 10% dextran sulfate, 50% deionized formamide, and yeast tRNA (50 μg/slide) at room temperature for 30 min. After the pre-hybridization, the sections were exposed to hybridization solution containing DIG-labeled antisense or sense riboprobes (50 ng/slide) in pre-hybridization solution overnight at 45°C (*Cyp11a1* and *Cyp19a1*). Following the hybridization for 16 h, the sections were incubated with alkaline phosphatase (AP)-conjugated anti-DIG antibody (Roche Diagnostics) in blocking solution overnight at 4°C. Hybridization signals were detected in AP buffer containing 35 μg/ml nitro-blue

tetrazolium chloride (Wako Pure Chemical, Osaka, Japan) and 17.5 μg/ml 5-bromo-4-chloro-3′-indoylphosphate p-toluidine salt (Wako Pure Chemical).

Ovarian granulosa cell isolation and culture

Granulosa cell isolation and culture were performed in accordance with methods used in previous studies (Otsuka and Shimasaki 2002; Miyoshi et al. 2007; Ojima et al. 2016). Ovaries from 3-week-old wt and Runx3$^{-/-}$ mice were collected and dissected free of connective tissue. They were then incubated in M199 medium containing 25 mM HEPES and 0.1% BSA, and were punctured with a 27-gauge needle. Mixtures of granulosa cells and oocytes were filtered through cell strainers (40-μm nylon mesh, BD Falcon, Bedford, MA, USA) that allowed the passage of granulosa cells but not oocytes to pass through. After centrifugation (5 min at 500 × *g*, 4°C), cells were cultured in McCoy's 5A medium (Sigma-Aldrich, St. Louis, MO, USA) containing 10% dextran-coated charcoal-treated fetal bovine serum (DC-FBS, v/v; Life Technologies, Grand Islands, NY, USA) at 37°C in an atmosphere of 5% CO_2. The cells were seeded at a density of 5×10^4 cells per well in 24-well plates. After 24 h, the medium was changed to serum-free McCoy's 5A medium containing 0.1% BSA supplemented with insulin–transferrin–selenium (ITS; Life Technologies) and culture supplement (100 μg/l hydrocortisone, 400 ng/l triiodothyronine, 10 ng/l glucagon, 200 ng/l parathormone) for 24 h before further analysis. The cell types of dissociated follicle cells were confirmed by detection of the expression of granulosa cell-related genes, in accordance with previous studies (Ojima et al., 2016). *Fshr* and *Cyp19a1* mRNA was detected in the dissociated cells from wt mouse ovaries and *Runx3*$^{-/-}$ mouse ovaries. Hence, we concluded that the dissociated cells included granulosa cells and were appropriate for analysis of the effects of FSH treatment on such cells.

RNA extraction and reverse transcription (RT) - polymerase chain reaction (PCR)

Total RNA was extracted from tissues or isolated granulosa cells using TRIsure Reagent (Bioline, London, UK), and reverse-transcribed using the Prime Script RT-PCR System (Takara Bio), in accordance with the manufacturer's instructions. Random hexamers were used for the RT reactions.

PCR was carried out using Blend Taq (Toyobo, Tokyo, Japan) and a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems, Branchburg, NJ, USA). The PCR conditions were as follows: 2 min at 94°C; an appropriate number of cycles of 94°C for 30 s, annealing temperature for 30 s, and 72 °C for 30 s; and then 10 min at 72°C. A 10-μl of aliquot of each reaction was electrophoresed on a 2% agarose gel, which was then stained with ethidium bromide, and photographed under ultraviolet radiation.

Real-time PCR was performed using SYBR Premix Ex Taq (Perfect Real Time; Takara Bio) with an ABI PRISM 7300 Sequence Detection System (Applied Biosystems). The PCR program was as follows: after initial denaturing at 95°C for 10 s, 40 cycles 95^{o}C for 5 s and 60^{o}C for 31 s, followed by a melting-curve analysis (95^{o}C for 15 s, 60^{o}C for 1 min, 95^{o}C for 15 s, and 60^{o}C for 15 s). This latter analysis was conducted to confirm the absence of primer dimmers. Standard curves were generated by serial dilution of total cDNA, and the amount of each target mRNA level was normalized against the amount of mRNA of ribosomal protein L19 (*Rpl19*).

Analysis of steroid production

Estradiol and progesterone levels were measured by a chemiluminescent immunoassay using Architect estradiol and progesterone kits (Abbott Co., Ltd., Tokyo,

Japan), in accordance with the methods used in previous studies (Nakamura et al. 2013). For steroid assays, granulosa cells were plated into 96-well plates and grown in serum-free McCoy's 5A medium containing 100 nM androstenedione (a substrate for aromatase, Sigma-Aldrich). Granulosa cells were cultured without (control) and with ovine FSH (Sigma-Aldrich, 30 ng/ml) (Miyoshi et al., 2007). After 48 h, the culture supernatant was collected and stored at −20°C until assaying. Each group consisted of four wells, and each experiment was performed independently in triplicate.

Statistical analysis

Data are shown as the mean ± S.E. Differences among groups were analyzed by analysis of variance followed by Tukey's test, and the differences in means between the two groups were analyzed by Student's t-test (Kaleida Graph; Synergy Software, Reading, PA, USA). Differences were considered significant at $P<0.05$.

Results

Morphological changes of ovaries in *Runx3*$^{-/-}$ mice

We previously demonstrated delayed folliculogenesis in ovaries of *Runx3*$^{-/-}$ mice at 8 weeks of age (Sakuma et al. 2008). It is however difficult to investigate the initial recruitment of follicles using ovaries of postpubertal mice since the cyclic recruitment of follicles starts after the onset of puberty (McGee and Hsueh 2000). Therefore, in the present study the ovaries of prepubertal mice (2 and 3 weeks of age) were observed (Fig. 1a). At 2 weeks of age, all four types of follicle including primordial follicles were found in the ovary and their numbers did not differ between wt and *Runx3*$^{-/-}$ mice (Fig. 1b). At 3 weeks of age,

the numbers of primary and antral follicles per ovary were significantly lower in *Runx3*$^{-/-}$ mice than those in wt mice, while the number of secondary follicles per ovary were higher in *Runx3*$^{-/-}$ mice than that in wt mice. These changes of the number of follicles during the prepubertal period may be ascribed to the delayed growth of primordial follicles into primary follicles, and of secondary follicles into antral follicles, indicating that the initial recruitment was affected by *Runx3* knockout. At 8 weeks of age, the ovaries of *Runx3*$^{-/-}$ mice were smaller than those of wt mice, possibly because the former did not contain corpora lutea, while the latter contained many corpora lutea. These changes observed in the ovaries of 8-week-old mice were consistent with the findings in our previous study (Sakuma et al. 2008).

Expression of growth factor genes involved in regulation of follicular development and functions in ovaries of wt and *Runx3*$^{-/-}$ mice

The morphological analysis of folliculogenesis in the present study demonstrated that folliculogenesis in the initial recruitment was adversely affected in *Runx3*$^{-/-}$ mouse ovaries, which was assumed to be due to changes in intra-ovarian growth factors, mainly those in the TGF-β superfamily growth factors. Therefore, we analyzed *Amh, Igf1*, *Inha*, *Inhba*, and *Inhbb* mRNA levels in wt and *Runx3*$^{-/-}$ mouse ovaries using quantitative real-time PCR to determine whether *Runx3* knockout can affect gene expression of the TGF-β superfamily growth factors involved in folliculogenesis and other ovarian functions in 3-week-old mice. *Inha*, *Inhba*, and *Inhbb* mRNA levels were lower in *Runx3*$^{-/-}$ mouse ovaries than in wt ones; no significant differences in *Amh* and *Igf1* mRNA levels were detected (Fig. 2). These results suggest that Runx3 may be involved in the regulation of *Inha*, *Inhba*, and *Inhbb* expression, and that the decrease in the synthesis of inhibin and activin subunits may lead to the decrease in inhibin or activin activity in *Runx3*$^{-/-}$ mouse ovaries.

Expression of genes involved in regulation of steroidogenesis in the ovaries of wt and *Runx3*$^{-/-}$ mice

Estrogen synthesis is one of the primary roles of ovarian follicles, and estrogen is another intraovarian factor for folliculogenesis. To determine whether *Runx3* is involved in the regulation of steroidogenesis in mouse ovaries, we analyzed the mRNA levels of *Fshr* (FSH receptor), *Lhcgr* (LH receptor), and the key regulators of steroidogenesis in ovarian granulosa cells, namely, *Star* (steroidogenic acute regulatory protein), *Cyp11a1* (p450scc, cholesterol side-chain cleavage enzyme), *Cyp17a1* (p450c17, steroid 17 α hydroxylase), and *Cyp19a1* (p450arom, aromatase), in wt and *Runx3*$^{-/-}$ mouse ovaries using quantitative real-time PCR. mRNA levels of *Fshr*, *Lhcgr, Cyp11a1*, and *Cyp19a1* mRNA levels were lower in *Runx3*$^{-/-}$ mouse ovaries than in wt ones, while those of *Star* and *Cyp17a1* also appeared to be decreased albeit without reaching statistical significance because of large variations in wt mouse ovaries (Fig. 3). These findings suggest that the expression of aromatase, an enzyme involved in estrogen synthesis, was diminished in *Runx3*$^{-/-}$ mouse ovaries, leading to the decreased synthesis of estrogen.

***In situ* hybridization analysis of *Cyp11a1* and *Cyp19a1* mRNA expression in wt and *Runx3*$^{-/-}$ mouse ovaries**

Real-time PCR analysis of *Runx3*$^{-/-}$ mouse ovaries showed decreased *Cyp11a1* and *Cyp19a1* mRNA expression. However, it is not clear how *Runx3* knockout affected *Cyp11a1* and *Cyp19a1* mRNA-expressing cells in mouse ovaries. Therefore, these cells were analyzed in 3-week-old mice by *in situ* hybridization using DIG-labeled riboprobes (*Cyp11a1,* Fig. 4a–f; *Cyp19a1*, Fig. 4g–l). In all *in situ* hybridization studies, no signals were detected when

sense riboprobes were used for the control analysis (Fig. 4e, f, k, l).

Cyp11a1 mRNA In both wt and *Runx3*$^{-/-}$ mouse ovaries, *Cyp11a1* mRNA signals were detected in some of the interstitial cells and in some of the theca interna cells of secondary and antral follicles (Fig. 4a–d). However, in *Runx3*$^{-/-}$ mouse ovaries, *Cyp11a1* mRNA signals in interstitial cells and theca interna cells were lower than those in wt mice (wt, a, c; *Runx3*$^{-/-}$, b, d).

Cyp19a1 mRNA In wt mouse ovaries, *Cyp19a1* mRNA signals were detected in granulosa cells of primary, secondary and antral follicles (Fig. 4g–j). In *Runx3*$^{-/-}$ mouse ovaries, *Cyp19a1* mRNA signals were lower than those in wt mice (wt, g, i; *Runx3*$^{-/-}$, h, j). Taken together, these findings correspond well to the decreased *Cyp11a1* and *Cyp19a1* mRNA expression in *Runx3*$^{-/-}$ mouse ovaries.

Effects of FSH treatment on E2 and P4 production and genes involved in steroidogenesis in freshly prepared granulosa cells of wt and *Runx3*$^{-/-}$ mice

Steroidogenesis in granulosa cells is regulated by gonadotropin. We thus next cultured granulosa cells of wt and *Runx3*$^{-/-}$ mice with or without FSH (30 ng/ml) for 48 h, and examined the effects of FSH on the production of E2 and P4 and expression of gonadotropin receptor genes and steroidogenic enzyme genes in these cells.

The expression of *Cyp19a1* mRNA in granulosa cells of *Runx3*$^{-/-}$ mouse ovaries was significantly lower than that in wt mouse granulosa cells (Fig. 5). In contrast, the expressions of *Fshr*, and *Cyp11a1* mRNA in granulosa cells did not differ between *Runx3*$^{-/-}$ and wt mice (Fig. 5). FSH treatment increased *Cyp19a1* mRNA levels in both wt and *Runx3*$^{-/-}$ granulosa cells, but the increase in *Runx3*$^{-/-}$ granulosa cells was smaller than that in wt ones. Conversely, FSH treatment did not significantly affect the levels of *Fshr* and *Cyp11a1* mRNA

expression in both wt and *Runx3*$^{-/-}$ mice (Fig. 5).

E2 levels were significantly lower in granulosa cells from *Runx3*$^{-/-}$ mice than those from wt mice without FSH treatment (control) (Fig. 5). FSH treatment significantly increased E2 levels in *Runx3*$^{-/-}$ granulosa cells, but not in wt granulosa cells (Fig. 5), while FSH treatment significantly increased P4 production in both *Runx3*$^{-/-}$ granulosa cells and wt granulosa cells. There was no difference in the FSH-induced response in progesterone production between wt and *Runx3*$^{-/-}$ mice (Fig. 5). These findings clearly indicated that E2 synthesis in *Runx3*$^{-/-}$ mouse ovaries was decreased due to the decreased *Cyp19a1* expression, and the FSH response in granulosa cells in terms of *Cyp19a1* expression was diminished in *Runx3*$^{-/-}$ mouse ovaries.

Expression of *Cga, Fshb, and Lhb* mRNA in wt and *Runx3*$^{-/-}$ mice

To clarify the alteration of gonadotropin gene expression in 3-week-old mice, we analyzed the mRNA levels of *Cga*, *Fshb*, and *Lhb*. All of these levels in *Runx3*$^{-/-}$ mouse pituitaries were lower than those in wt mouse ones (Fig. 6). These findings suggest that FSH and LH production decreased in *Runx3*$^{-/-}$ mouse pituitaries.

Discussion

Recent studies as well as the present one have shown that the Runt-related transcription factor family participates in the regulation of ovarian functions in rats and mice. Runx1 and Runx2 are involved in regulating the LH-induced ovulatory process and luteinization in the rat ovary. We previously demonstrated the expression of *Runx3* mRNA in granulosa cells of ovarian follicles as well as alterations in follicular development and a lack

of ovulation in *Runx3*$^{-/-}$ mice, and we suggested that Runx3 acted in the ovulation-regulatory system in the hypothalamo-pituitary, probably in the regulation of *Kiss1* mRNA expression in the anteroventral periventricular nucleus (AVPV) (Sakuma et al. 2008; Ojima et al. 2016). The present study indicates that Runx3 in granulosa cells is involved in the regulation of folliculogenesis and steroidogenesis in mouse ovaries.

We previously demonstrated that ovaries were markedly reduced in size in 8-week-old *Runx3*$^{-/-}$ mice, with a significantly decreased number of antral follicles and the absence of corpora lutea (Sakuma et al. 2008). In the present study, we found that the transition of primordial follicles to primary follicles and that of preantral follicles to antral follicles seemed to be retarded in *Runx3*$^{-/-}$ mouse ovaries. Early folliculogenesis from primordial follicles to preantral follicles proceeds under the control of the intraovarian regulatory system (Knight and Glister 2006; Richards and Pangas 2010), and that of preantral follicles to antral follicles is regulated by FSH (Kumar et al. 1997; Dierich et al. 1998; Lei et al. 2001; Orisaka et al. 2009). As another possibility, atresia during folliculogenesis may be accelerated by *Runx3* deletion. In fact, we previously found the increase in atretic follicles in 8-week-old *Runx3*$^{-/-}$ mouse ovaries. Although atretic follicles were not examined in the ovaries of 2-3-week-old *Runx3*$^{-/-}$ mice, it is probable that the atresia of antral follicles in *Runx3*$^{-/-}$ mouse ovaries during the peripubertal period was increased like that in 8-week-old *Runx3*$^{-/-}$ ones. Therefore, considering the possibility of accelerated atresia we concluded in the present study that the changes in the early stage of folliculogenesis observed in 3-week-old *Runx3*$^{-/-}$ mouse ovaries were probably caused by alterations of the intraovarian regulatory system, which may be controlled by Runx3 within granulosa cells.

Many reports have demonstrated that folliculogenesis is regulated by TGF-β/BMP family members and insulin-like growth factor 1 (IGF1) produced within follicles (Knight and

Glister 2006; Knight et al. 2012; Otsuka 2013). The retarded early folliculogenesis observed in *Runx3*$^{-/-}$ mouse ovaries at the age of 3 weeks was possibly due to defects in the activities of these growth factor actions. Therefore, we examined anti-Müllarian hormone (AMH), subunits of activins and inhibins, and IGF1. *Inha*, *Inhba,* and *Inhbb* mRNA expression was decreased in *Runx3*$^{-/-}$ mouse ovaries, suggesting that the synthesis of activins or inhibins was decreased in them. When *Inha*, *Inhba,* and *Inhbb* mRNA levels were determined in granulosa cells isolated from wt and *Runx3*$^{-/-}$ mouse ovaries by real-time PCR, no significant differences in these levels were detected (data not shown). Hence, it is probable that the decreases in *Inha*, *Inhba*, and *Inhbb* mRNA levels in *Runx3*$^{-/-}$ mouse ovaries were partly due to the decrease in the number of granulosa cells expressing *Inha*, *Inhba*, and *Inhbb* mRNAs. Low *Inha*, *Inhba*, and *Inhbb* mRNA levels probably led to decreased production of activin B, activin AB, or inhibin B in *Runx3*$^{-/-}$ mouse follicles.

Activins stimulate the proliferation of primordial germ cells and granulosa cells of early immature follicles (Li et al. 1995; Miró and Hillier 1996; Yokota et al. 1997; Liu et al. 1999; El-Hefnawy and Zeleznik 2001; Bristol-Gould et al. 2006). They also stimulate FSHR expression in granulosa cells (Hasegawa et al. 1988; Xiao et al. 1992; Nakamura et al. 1993) and synergize with FSH to regulate the differentiation of granulosa cells during late folliculogenesis (Woodruff et al. 1990; Findlay 1993; Kishi et al. 1998). Inhibins, which generally antagonize the activities of activins (Knight et al. 2012), are involved in the inhibition of primordial follicle recruitment and early follicle development (Woodruff et al. 1990; Myers et al. 2009). In addition, a recent study of *Inhbb* knockdown mice showed that *Inhbb* is involved in the regulation of granulosa cell proliferation in mouse ovaries (M'Baye et al. 2015). These changes in folliculogenesis in *Inhbb* knockdown mice were similar to those in *Runx3*$^{-/-}$ mouse ovaries. Therefore, retarded folliculogenesis in *Runx3*$^{-/-}$ mouse ovaries

was probably due to the decreased expression of subunits of activins and/or inhibins that are involved in the regulation of early folliculogenesis. However, the role of Runx3 in the regulation of activin and/or inhibin gene expression remains to be studied.

Smad proteins function as integrators of TGF-β/BMPs signaling pathways (Miyazono et al. 2001). For example, *Smad3* knockdown was shown to induce the slower growth of primordial follicles into antral follicles (Tomic et al. 2004). The retarded growth of follicles observed in *Smad3*$^{-/-}$ mouse ovaries appears to be similar to the changes in folliculogenesis observed in *Runx3*$^{-/-}$ mouse ovaries. Considering the possible interaction of Runx3 with Smad3 in signal transduction pathways of TGF-β/BMP (Hanai et al., 1999; Ito and Miyazono, 2003), the present study supports previous findings suggesting that Runx3 is involved in the regulation of folliculogenesis as a factor involved in TGF-β/BMP signal transduction.

The decrease in the number of antral follicles developing into preovulatory follicles was the most significant change induced by the loss of *Runx3* in mouse ovaries, which led to the depletion of healthy preovulatory follicles. Follicular development is closely connected to steroidogenesis. Estrogen is one of the most important regulators of follicular development and is the key signal to the hypothalamic–pituitary system for the generation of LH surge and ovulation (Krege et al. 1998; Hegele-Hartung et al. 2004). The capacity for estrogen production in granulosa cells emerges in the late preantral stage. The expression of *Cyp11a1* and *Cyp19a1,* genes encoding key enzyme genes in steroidogenesis, was found to be diminished in *Runx3*$^{-/-}$ ovaries, probably resulting in low estrogen production, defects in follicular development, anovulation and atrophy of uteri. Thus, it is highly probable that Runx3 is involved in the regulation of steroidogenesis in mouse ovaries, in particular estrogen synthesis. *Cyp11a1* mRNA expression was detected in theca interna cells, some

granulosa cells of preovulatory follicles, and corpora lutea (Zlotkin et al. 1986), and C*yp19a1* mRNA expression was detected in granulosa cells (Zhou et al. 1997; Turner et al. 2002; Guigon et al. 2003). The present *in situ* hybridization analysis demonstrated a decrease in the number of *Cyp11a1* mRNA-expressing cells in theca cells of *Runx3*$^{-/-}$ mice in accordance with our previous study (Ojima et al. 2016), as well as diminished expression of C*yp19a1* mRNA in granulosa cells. In fact, *in vitro* analysis of granulosa cells revealed that E2 production decreased in granulosa cells of *Runx3*$^{-/-}$ mice. Estrogen is required for the development of antral follicles to preovulatory follicles (Lubahn et al. 1993; Krege et al. 1998). Therefore, it is probable that the decreased production of E2 affected the late stage of folliculogenesis.

Cyp11a1 expression in theca cells is regulated by LH (Magoffin and Weitsman 1993), and by growth factors and hormones produced in granulosa cells. Inhibin and activin, both produced in granulosa cells, stimulate and inhibit androgen synthesis in theca cells, respectively (Hillier 1991; Hillier et al. 1991; Knight et al. 2012; Hoang et al. 2013). *Inhbb* is involved in the regulation of *Cyp11a1* expression in mouse ovaries (M'Baye et al. 2015). Thus, the functions of theca cells are regulated by growth factors produced by granulosa cells as well as LH. Therefore, it is highly probable that *Runx3*-regulated growth factors in granulosa cells control the functions of theca cells in a paracrine manner because *Runx3* was not expressed in theca cells. Decreased *Cyp11a1* expression in theca cells of *Runx3*$^{-/-}$ mouse ovaries may decrease androgen production, leading to a decrease in estrogen production, because androgens are converted to estrogens by aromatase encoded by *Cyp19a1* in granulosa cells.

Cyp19a1 mRNA expression in granulosa cells was shown to be regulated by FSH. *Cyp19a1* mRNA levels were lower in *Runx3*$^{-/-}$ mouse ovaries than in those of wt ones. FSH

treatment increased *Cyp19a1* mRNA levels in cultured granulosa cells of both wt and *Runx3*$^{-/-}$ mouse ovaries. Although the FSH-induced increase in *Cyp19a1* mRNA levels in *Runx3*$^{-/-}$ mice was lower than that in wt mice, granulosa cells of *Runx3*$^{-/-}$ mouse ovaries appeared to respond normally to FSH treatment. *Fshr* mRNA expression was not altered by FSH treatment in granulosa cells of both wt and *Runx3*$^{-/-}$ mice, although the expression of FSHR was not determined. From these results, it is probable that Runx3 plays an essential role in *Cyp19a1* mRNA expression in granulosa cells. However, it is not clear whether Runx3 directly regulates *Cyp19a1* transcription.

As *Runx3*$^{-/-}$ mouse ovaries contained less antral follicles, the granulosa cells isolated from *Runx3*$^{-/-}$ mouse ovaries contained more immature granulosa cells than those from wt mouse ovaries, leading to less *Cyp19a1* mRNA levels. Therefore, lower *Cyp19a1* expression in *Runx3*$^{-/-}$ mouse ovaries may result in the decrease of estrogen production, and then may affect folliculogenesis.

With regard to P4 production, the effects of FSH treatment on *Cyp11a1* expression and P4 secretion in granulosa cells of *Runx3*$^{-/-}$ mice did not differ from those of wt mice. As *Runx3* mRNA expression was not detected in corpora lutea, Runx3 is probably not involved in P4 production in lutein cells.

Pituitary *Cga, Fshb*, and *Lhb* mRNA levels were decreased in *Runx3*$^{-/-}$ mice, indicating that the levels of FSH and LH synthesis was decreased. These results suggest that decreased FSH and LH secretion may affect the FSH-dependent folliculogenesis and LH-induced androgen production in theca cells. Thus, it is probable that Runx3 regulates ovarian functions through hypothalamic–pituitary systems as well as the intraovarian systems in mice at 3 weeks old, as well as at 8 weeks old as shown in our previous study (Ojima et al. 2016).

In conclusion, we found decreased mRNA levels of genes associated with folliculogenesis, steroidogenesis, and gonadotropin release in *Runx3*$^{-/-}$ mice. We also showed that the expression of genes encoding subunits of activin and inhibin was decreased in *Runx3*$^{-/-}$ mouse ovaries. The present study suggests that Runx3 plays important roles in intraovarian regulatory systems in the early stage of folliculogenesis through TGFβ family growth factors and in the regulation of estrogen synthesis through aromatase expression.

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REFERENCES

Adashi EY, Hsueh AJ (1982) Estrogens augment the stimulation of ovarian aromatase activity by follicle-stimulating hormone in cultured rat granulosa cells. J Biol Chem 257:6077-6083

Breen KM, Thackray VG, Coss D, Mellon PL (2010) Runt-related transcription factors impair activin induction of the follicle-stimulating hormone β-subunit gene. Endocrinology 151:2669-2680

Bristol-Gould SK, Kreeger PK, Selkirk CG, Kilen SM, Cook RW, Kipp JL, Shea LD, Mayo KE, Woodruff TK (2006) Postnatal regulation of germ cells by activin: the establishment of the initial follicle pool. Dev Biol 298:132-148

Cameron ER, Neil JC (2004) The Runx genes: lineage-specific oncogenes and tumor suppressors. Oncogene 23:4308-4314

Dierich A, Sairam MR, Monaco L, Fimia GM, Gansmuller A, LeMeur M, Sassone-Corsi P

(1998) Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc Natl Acad Sci USA 95:13612-13617

Drummond AE (2005) TGFbeta signalling in the development of ovarian function. Cell Tissue Res 322:107-115

El-Hefnawy T, Zeleznik AJ (2001) Synergism between FSH and activin in the regulation of proliferating cell nuclear antigen (PCNA) and cyclin D2 expression in rat granulosa cells. Endocrinology 142:4357-4362

Findlay JK (1993) An update on the roles of inhibin, activin, and follistatin as local regulators of folliculogenesis. Biol Reprod 48:15-23

Fitzpatrick SL, Richards JS (1991) Regulation of cytochrome P450 aromatase messenger ribonucleic acid and activity by steroids and gonadotropins in rat granulosa cells. Endocrinology 129:1452-1462

Guigon CJ, Mazaud S, Forest MG, Brailly-Tabard S, Coudouel N, Magre S (2003) Unaltered development of the initial follicular waves and normal pubertal onset in female rats after neonatal deletion of the follicular reserve. Endocrinology 144:3651-3662

Hanai J, Chen LF, Kanno T, Ohtani-Fujita N, Kim WY, Guo WH, Imamura T, Ishidou Y, Fukuchi M, Shi MJ, Stavnezer J, Kawabata M, Miyazono K, Ito Y (1999) Interaction and functional cooperation of PEBP2/CBF with Smads. Synergistic induction of the immunoglobulin germline Calpha promoter. J Biol Chem 274:31577-31582

Hasegawa Y, Miyamoto K, Abe Y, Nakamura T, Sugino H, Eto Y, Shibai H, Igarashi M (1988) Induction of follicle stimulating hormone receptor by erythroid differentiation factor on rat granulosa cell. Biochem Biophys Res Commun 156:668-674

Hegele-Hartung C, Siebel P, Peters O, Kosemund D, Müller G, Hillisch A, Walter A,

Kraetzschmar J, Fritzemeier KH (2004) Impact of isotype-selective estrogen receptor agonists on ovarian function. Proc Natl Acad Sci USA 101:5129-5134

Hernandez-Gonzalez I, Gonzalez-Robayna I, Shimada M, Wayne CM, Ochsner SA, White L, Richards JS (2006) Gene expression profiles of cumulus cell oocyte complexes during ovulation reveal cumulus cells express neuronal and immune-related genes: does this expand their role in the ovulation process? Mol Endocrinol 20:1300-1321

Hillier SG (1991) Regulatory functions for inhibin and activin in human ovaries. J Endocrinol 131:171-175

Hillier SG, Yong EL, Illingworth PJ, Baird DT, Schwall RH, Mason AJ (1991) Effect of recombinant activin on androgen synthesis in cultured human thecal cells. J Clin Endocrinol Metab 72:1206-1211

Hoang YD, McTavish KJ, Chang RJ, Shimasaki S (2013) Paracrine regulation of theca androgen production by granulosa cells in the ovary. Fertil Steril 100:561-567

Ito Y (1999) Molecular basis of tissue-specific gene expression mediated by the runt domain transcription factor PEBP2/CBF. Genes Cells 4:685-696

Ito Y, Miyazono K (2003) RUNX transcription factors as key targets of TGF-beta superfamily signaling. Curr Opin Genet Dev 13:43-47

Jo M, Curry TE (2006) Luteinizing hormone-induced RUNX1 regulates the expression of genes in granulosa cells of rat periovulatory follicles. Mol Endocrinol 20:2156-2172

Jo M, Gieske MC, Payne CE, Wheeler-Price SE, Gieske JB, Ignatius IV, Curry TE, Ko C (2004) Development and application of a rat ovarian gene expression database. Endocrinology 145:5384-5396

Kaivo-oja N, Jeffery LA, Ritvos O, Mottershead DG (2006) Smad signalling in the ovary. Reprod Biol Endocrinol 4:21

Kishi H, Minegishi T, Tano M, Kameda T, Ibuki Y, Miyamoto K (1998) The effect of activin and FSH on the differentiation of rat granulosa cells. FEBS Lett 422:274-278

Knight PG, Glister C (2006) TGF-β superfamily members and ovarian follicle development. Reproduction 132:191-206

Knight PG, Satchell L, Glister C (2012) Intra-ovarian roles of activins and inhibins. Mol Cell Endocrinol 359:53-65

Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA, Smithies O (1998) Generation and reproductive phenotypes of mice lacking estrogen receptor beta. Proc Natl Acad Sci USA 95:15677-15682

Kumar TR, Wang Y, Lu N, Matzuk MM (1997) Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 15:201-204

Lei ZM, Mishra S, Zou W, Xu B, Foltz M, Li X, Rao CV (2001) Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene. Mol Endocrinol 15:184-200

Li QL, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, Lee KY, Nomura S, Lee CW, Han SB, Kim HM, Kim WJ, Yamamoto H, Yamashita N, Yano T, Ikeda T, Itohara S, Inazawa J, Abe T, Hagiwara A, Yamagishi H, Ooe A, Kaneda A, Sugimura T, Ushijima T, Bae SC, Ito Y (2002) Causal relationship between the loss of RUNX3 expression and gastric cancer. Cell 109:113-124

Li R, Phillips DM, Mather JP (1995) Activin promotes ovarian follicle development in vitro. Endocrinology 136:849-856

Liu J, Park ES, Curry TE, Jo M (2010) Periovulatory expression of hyaluronan and proteoglycan link protein 1 (Hapln1) in the rat ovary: hormonal regulation and potential function. Mol Endocrinol 24:1203-1217

Liu J, Park ES, Jo M (2009) Runt-related transcription factor 1 regulates luteinized hormone-induced prostaglandin-endoperoxide synthase 2 expression in rat periovulatory granulosa cells. Endocrinology 150:3291-3300

Liu X, Andoh K, Abe Y, Kobayashi J, Yamada K, Mizunuma H, Ibuki Y (1999) A comparative study on transforming growth factor-β and activin A for preantral follicles from adult, immature, and diethylstilbestrol-primed immature mice. Endocrinology 140:2480-2485

Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O (1993) Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci USA 90:11162-11166

M'baye M, Hua G, Khan HA, Yang L (2015) RNAi-mediated knockdown of INHBB increases apoptosis and inhibits steroidogenesis in mouse granulosa cells. J Reprod Dev

Magoffin DA, Weitsman SR (1993) Effect of insulin-like growth factor-I on cholesterol side-chain cleavage cytochrome P450 messenger ribonucleic acid expression in ovarian theca-interstitial cells stimulated to differentiate in vitro. Mol Cell Endocrinol 96:45-51

Massagué J, Wotton D (2000) Transcriptional control by the TGF-β/Smad signaling system. EMBO J 19:1745-1754

McGee EA, Hsueh AJ (2000) Initial and cyclic recruitment of ovarian follicles. Endocr Rev 21:200-214

Miró F, Hillier SG (1996) Modulation of granulosa cell deoxyribonucleic acid synthesis and differentiation by activin. Endocrinology 137:464-468

Miyazono K, Kusanagi K, Inoue H (2001) Divergence and convergence of TGF-β/BMP

signaling. J Cell Physiol 187:265-276

Miyoshi T, Otsuka F, Inagaki K, Otani H, Takeda M, Suzuki J, Goto J, Ogura T, Makino H (2007) Differential regulation of steroidogenesis by bone morphogenetic proteins in granulosa cells: involvement of extracellularly regulated kinase signaling and oocyte actions in follicle-stimulating hormone-induced estrogen production. Endocrinology 148:337-345

Murakami Y, Otsuki M, Kusumoto K, Takeuchi S, Takahashi S (2005) Estrogen inhibits interleukin-18 mRNA expression in the mouse uterus. J Reprod Dev 51:639-647

Myers M, Middlebrook BS, Matzuk MM, Pangas SA (2009) Loss of inhibin α uncouples oocyte-granulosa cell dynamics and disrupts postnatal folliculogenesis. Dev Biol 334:458-467

Nakamura E, Otsuka F, Inagaki K, Tsukamoto N, Ogura-Ochi K, Miyoshi T, Toma K, Takeda M, Makino H (2013) Involvement of bone morphogenetic protein activity in somatostatin actions on ovarian steroidogenesis. J Steroid Biochem Mol Biol 134:67-74

Nakamura M, Minegishi T, Hasegawa Y, Nakamura K, Igarashi S, Ito I, Shinozaki H, Miyamoto K, Eto Y, Ibuki Y (1993) Effect of an activin A on follicle-stimulating hormone (FSH) receptor messenger ribonucleic acid levels and FSH receptor expressions in cultured rat granulosa cells. Endocrinology 133:538-544

Ojima F, Saito Y, Tsuchiya Y, Kayo D, Taniuchi S, Ogoshi M, Fukamachi H, Takeuchi S, Takahashi S (2016) Runx3 transcription factor regulates ovarian functions and ovulation in female mice. J Reprod Dev

Orisaka M, Tajima K, Tsang BK, Kotsuji F (2009) Oocyte-granulosa-theca cell interactions during preantral follicular development. J Ovarian Res 2:9

Otsuka F (2013) Multifunctional bone morphogenetic protein system in endocrinology. Acta Med Okayama 67:75-86

Otsuka F, Shimasaki S (2002) A negative feedback system between oocyte bone morphogenetic protein 15 and granulosa cell kit ligand: its role in regulating granulosa cell mitosis. Proc Natl Acad Sci USA 99:8060-8065

Otto F, Lübbert M, Stock M (2003) Upstream and downstream targets of RUNX proteins. J Cell Biochem 89:9-18

Park ES, Choi S, Muse KN, Curry TE, Jo M (2008) Response gene to complement 32 expression is induced by the luteinizing hormone (LH) surge and regulated by LH-induced mediators in the rodent ovary. Endocrinology 149:3025-3036

Park ES, Lind AK, Dahm-Kähler P, Brännström M, Carletti MZ, Christenson LK, Curry TE, Jo M (2010) RUNX2 transcription factor regulates gene expression in luteinizing granulosa cells of rat ovaries. Mol Endocrinol 24:846-858

Park ES, Park J, Franceschi RT, Jo M (2012) The role for runt related transcription factor 2 (RUNX2) as a transcriptional repressor in luteinizing granulosa cells. Mol Cell Endocrinol 362:165-175

Richards JS, Pangas SA (2010) The ovary: basic biology and clinical implications. J Clin Invest 120:963-972

Sakuma A, Fukamachi H, Ito K, Ito Y, Takeuchi S, Takahashi S (2008) Loss of Runx3 affects ovulation and estrogen-induced endometrial cell proliferation in female mice. Mol Reprod Dev 75:1653-1661

Tomic D, Miller KP, Kenny HA, Woodruff TK, Hoyer P, Flaws JA (2004) Ovarian follicle development requires Smad3. Mol Endocrinol 18:2224-2240

Tsuchiya Y, Saito Y, Taniuchi S, Sakuma A, Maekawa T, Fukamachi H, Takeuchi S,

Takahashi S (2012) Runx3 expression and its roles in mouse endometrial cells. J Reprod Dev 58:592-598

Turner KJ, Macpherson S, Millar MR, McNeilly AS, Williams K, Cranfield M, Groome NP, Sharpe RM, Fraser HM, Saunders PT (2002) Development and validation of a new monoclonal antibody to mammalian aromatase. J Endocrinol 172:21-30

Woodruff TK, Lyon RJ, Hansen SE, Rice GC, Mather JP (1990) Inhibin and activin locally regulate rat ovarian folliculogenesis. Endocrinology 127:3196-3205

Wrana JL, Attisano L (2000) The Smad pathway. Cytokine Growth Factor Rev 11:5-13

Xiao S, Robertson DM, Findlay JK (1992) Effects of activin and follicle-stimulating hormone (FSH)-suppressing protein/follistatin on FSH receptors and differentiation of cultured rat granulosa cells. Endocrinology 131:1009-1016

Xu J, Oakley J, McGee EA (2002) Stage-specific expression of Smad2 and Smad3 during folliculogenesis. Biol Reprod 66:1571-1578

Yamamoto H, Ito K, Kawai M, Murakami Y, Bessho K, Ito Y (2006) Runx3 expression during mouse tongue and palate development. Anat Rec A Discov Mol Cell Evol Biol 288:695-699

Yokota H, Yamada K, Liu X, Kobayashi J, Abe Y, Mizunuma H, Ibuki Y (1997) Paradoxical action of activin A on folliculogenesis in immature and adult mice. Endocrinology 138:4572-4576

Zhou J, Kumar TR, Matzuk MM, Bondy C (1997) Insulin-like growth factor I regulates gonadotropin responsiveness in the murine ovary. Mol Endocrinol 11:1924-1933

Zlotkin T, Farkash Y, Orly J (1986) Cell-specific expression of immunoreactive cholesterol side-chain cleavage cytochrome P-450 during follicular development in the rat ovary. Endocrinology 119:2809-2820

Figure Legends

Fig. 1 Morphological observations of *Runx3*$^{-/-}$ mouse ovaries. (a) Ovaries were collected from wt and *Runx3*$^{-/-}$ mice at the age of 2, 3, 5, and 8 weeks. Ovaries of both wt and *Runx3*$^{-/-}$ mice examined at all ages contained primordial follicles, primary follicles, secondary follicles, and antral follicles. However, corpora lutea were not detected in wt and *Runx3*$^{-/-}$ mice at 2–5 weeks of age, and in 8-week-old *Runx3*$^{-/-}$ mice. Bar = 300 μm. (b) Effect of *Runx3* deletion on ovarian folliculogenesis. Ovaries were collected from wt mice and *Runx3*$^{-/-}$ mice at the age of 2 and 3 weeks, and processed for histological observation. The numbers of follicles were counted in accordance with the procedure described in Materials and methods. * $P<0.05$, ** $P<0.01$, significantly different from wt mice.

Fig. 2 Expression of growth factor genes in the whole ovary of wt and *Runx3*$^{-/-}$ mice at the age of 3 weeks. Real-time PCR was performed for quantitative analysis of *Amh*, *Igf1*, *Inha*, *Inhba*, and *Inhbb* mRNA expression. The amount of each mRNA in wt and *Runx3*$^{-/-}$ mice was normalized against that of *Rpl19* mRNA. Each group consisted of five mice. Data are expressed as the mean ± SEM of triplicate wells. * $P<0.05$, *** $P<0.001$, significantly different from wt mice.

Fig. 3 Expression of gonadotropin receptor, StAR and steroidogenic enzymes in the whole ovary of wt and *Runx3*$^{-/-}$ mice at the age of 3 weeks. Real-time PCR was performed for quantitative analysis of *Fshr*, *Lhcgr*, *Star*, *Cyp11a1*, *Cyp17a1*, and *Cyp19a1* mRNA expression. The amount of each mRNA in wt and *Runx3*$^{-/-}$ mice was normalized against that of *Rpl19* mRNA. Each group consists of five mice. Data are expressed as the mean ± SEM of triplicate wells. ** $P<0.01$, *** $P<0.001$, significantly different from wt mice.

Fig. 4 *In situ* hybridization analysis of *Cyp11a1* and *Cyp19a1* mRNAs in ovaries from wt and *Runx3*$^{-/-}$ mice at 3 weeks of age. Ovarian sections were obtained from wt and *Runx3*$^{-/-}$ mice. DIG-labeled antisense riboprobes for *Cyp11a1* and *Cyp19a1* and sense probes were used (*Cyp11a1*, a–f; *Cyp19a1*, g–l). *Cyp11a1* mRNA signals were detected in theca cells (wt, a, c; *Runx3*$^{-/-}$, b, d), and *Cyp19a1* mRNA signals were detected in granulosa cells (wt, g, i; *Runx3*$^{-/-}$, h, j). No signals were detected when the sense probe was used for hybridization (e, f, k, l). Bar = 300 μm (low magnification), 50 μm (high magnification).

Fig. 5 Effect of FSH on E2 and P4 production, and gonadotropin receptor and steroidogenic enzyme expression in the cultured granulosa cells of wt and *Runx3*$^{-/-}$ mice. Granulosa cells were treated with 100 nM androstenedione without (control) and with FSH (30 ng/ml) for 48 h. E2 and P4 levels in the medium were determined by enzyme labeled immunoassay. Data are expressed as the mean ± SEM of four wells. Real-time PCR was performed for the quantitative analysis of *Fshr*, *Cyp19a1*, and *Cyp11a1* mRNA expression. The amount of each mRNA of wt and *Runx3*$^{-/-}$ mice was normalized against that of *Rpl19* mRNA. Each group consisted of four wells, and each experiment was performed independently in triplicate. Data are expressed as the mean ± SEM of four wells. *** $P<0.001$, significantly different from control groups. ## $P<0.01$, ### $P<0.001$, significantly different from wt of the same treatment +++ $P<0.001$, significantly different from control of wt mice.

Fig. 6 Pituitary *Cga*, *Fshb*, and *Lhb* mRNA levels in wt and *Runx3*$^{-/-}$ mice at 3 weeks of age. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, significantly different from wt mice.

Table 1. Primer List for RT-PCR and real-time PCR

For RT-PCR

gene		5' - sequence - 3'	Tm (˚C)	Product (bp)
Cyp11a1	FP	CCTTTGAGTCCATCAGCAGTG	60	426
	RP	GTACCTTCAAGTTGTGTGCCA		
Cyp19a1	FP	GAGAGTTCATGAGAGTCTGG	55	504
	RP	CCTTGACGGATCGTTCATAC		
For real-time PCR				
Fshr	FP	AGCAAGTTTGGCTGTTATGAGG		159
	RP	GTTCTGGACTGAATGATTTAGAGG		
Lhcgr	FP	CCTTGTGGGTGTCAGCAGTTAC		75
	RP	TTGTGACAGAGTGGATTCCACAT		
Star	FP	GACGTCGGAGCTCTCTGCTT		100
	RP	GCCTTCTGCATAGCCACCTC		
Cyp11a1	FP	ACATGGCCAAGATGGTACAGTTG		121
	RP	ACGAAGCACCAGGTCATTCAC		
Cyp17a1	FP	GATCTAAGAAGCGCTCAGGCA		69
	RP	GGGCACTGCATCACGATAAA		
Cyp19a1	FP	AACCCGAGCCTTTGGAGAA		57
	RP	GGCCCGTCAGAGCTTTCA		
Cga	FP	CCACCTCCTCCCTACCAGACT		62
	RP	TGTAACCGTAAAGAGGCAGTGTGT		
Fshb	FP	TGGATTGTTCCAGGCAGACA		70
	RP	GGTGGCAATACCTTGGGAAA		
Lhb	FP	CCCTTGGTCTCCCATTTCTG		67
	RP	TGCTGGTGAGATGCCAGTTG		
Amh	FP	TGCTAGTCCTACATCTGGCTGA		120
	RP	GTCCAGGGTATAGCACTAACAGG		
Igf1	FP	AAAGCAGCCCGCTCTATCC		57
	RP	CTTCTGAGTCTTGGGCATGTCA		
Inha	FP	CTCCCAGGCTATCCTTTTCC		112
	RP	TGGCCGGAATACATAAGTGA		
Inhba	FP	GATCATCACCTTTGCCGAGT		143
	RP	TGGTCCTGGTTCTGTTAGCC		
Inhbb	FP	CACCTTCGCTTCAGTCTCCA		133
	RP	CTGATCGGGGTGAAGCGAAA		
Rpl19	FP	CCCGTCAGCAGATCAGGAA		58
	RP	GTCACAGGCTTGCGGATGA		

a

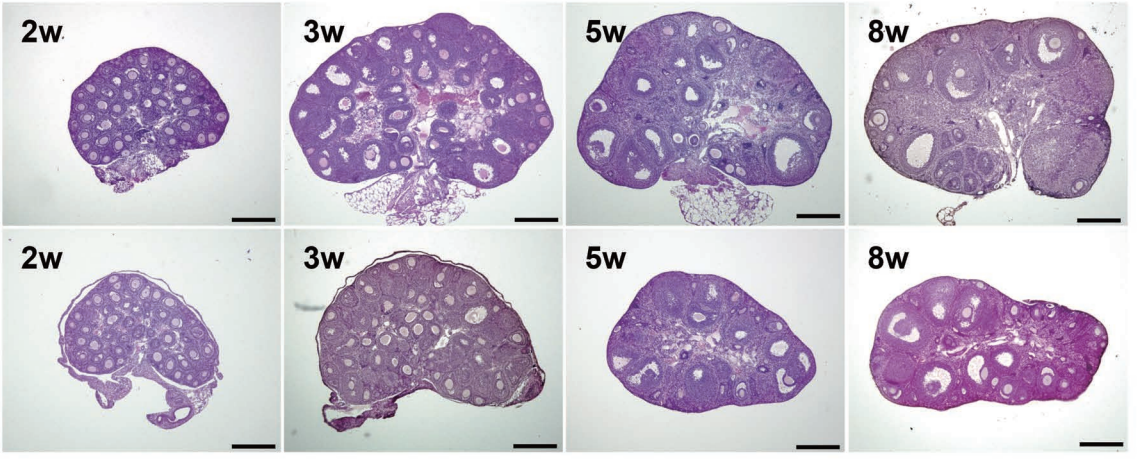

Fig. 1a

b

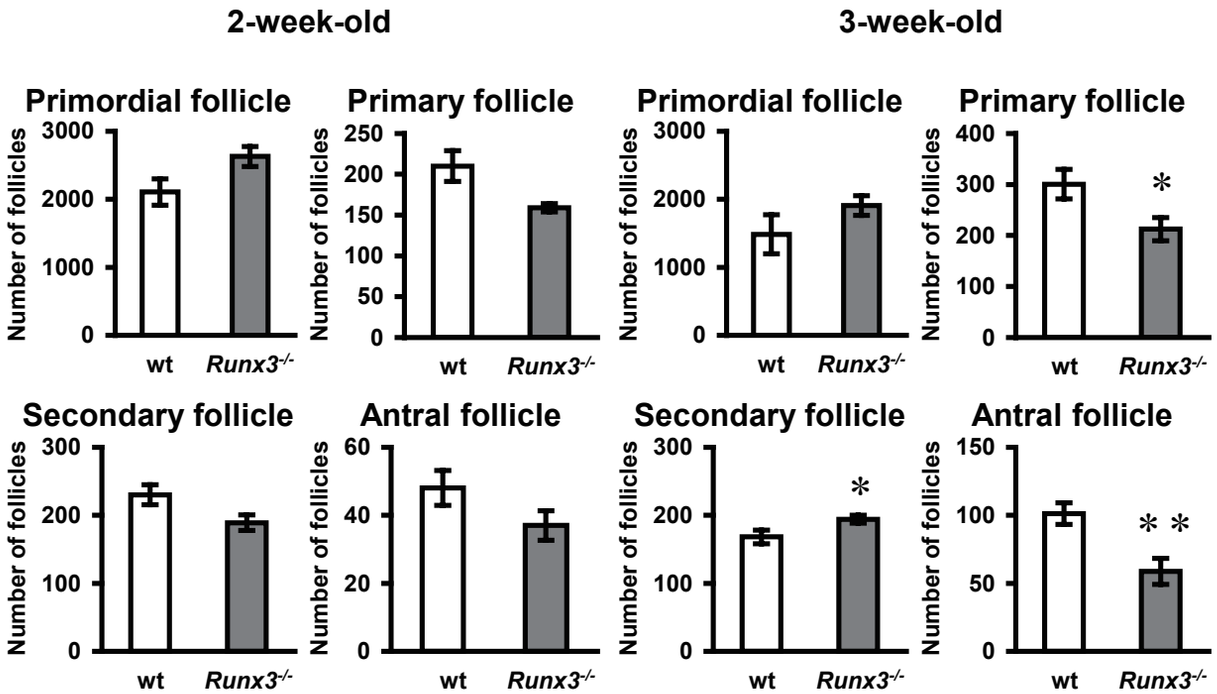
2-week-old
3-week-old
Primordial follicle
Primary follicle
Primordial follicle
Primary follicle
Number of follicles
3000
2000
1000
0
250
200
150
100
50
400
300
*
wt
Runx3-/-
Secondary follicle
Antral follicle
Secondary follicle
Antral follicle
60
40
20
150
* *

Fig. 1b

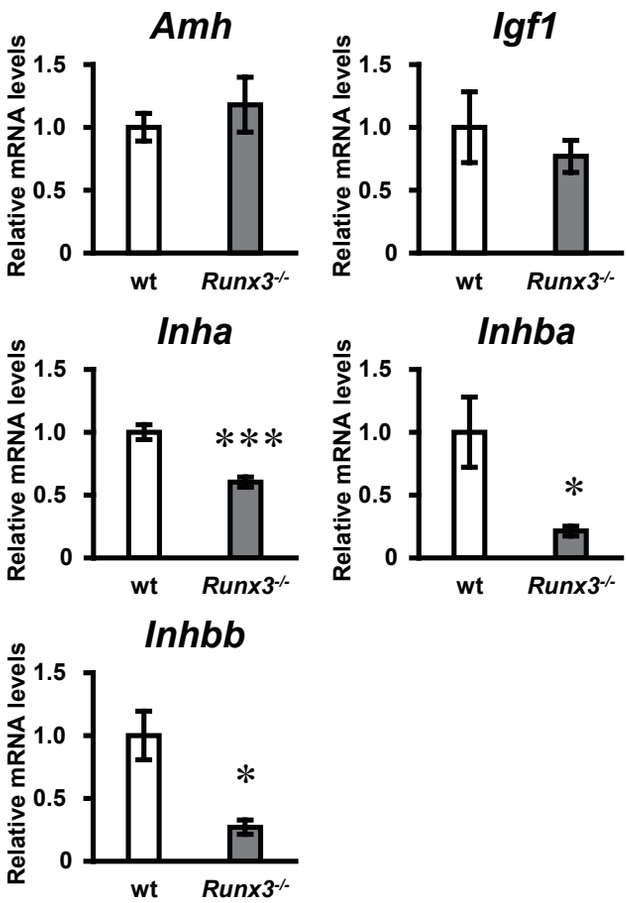
Amh
Igf1
Inha
Inhba
Inhbb
Relative mRNA levels
1.5
1.0
0.5
0
wt
Runx3-/-
***
*
*

Fig. 2

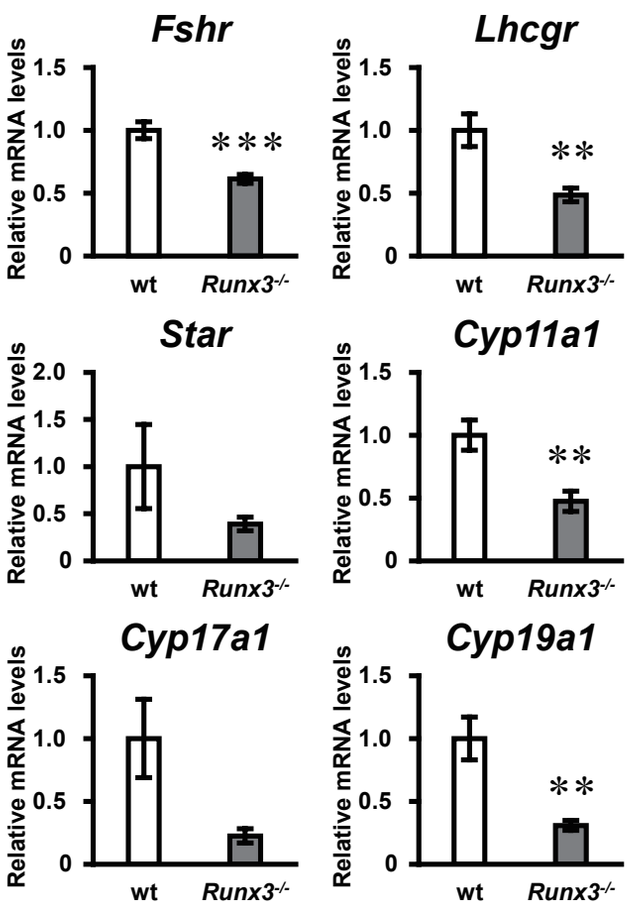
Fshr
Lhcgr
Star
Cyp11a1
Cyp17a1
Cyp19a1
Relative mRNA levels
1.5
1.0
0.5
0
2.0
***
**
wt
Runx3-/-

Fig. 3

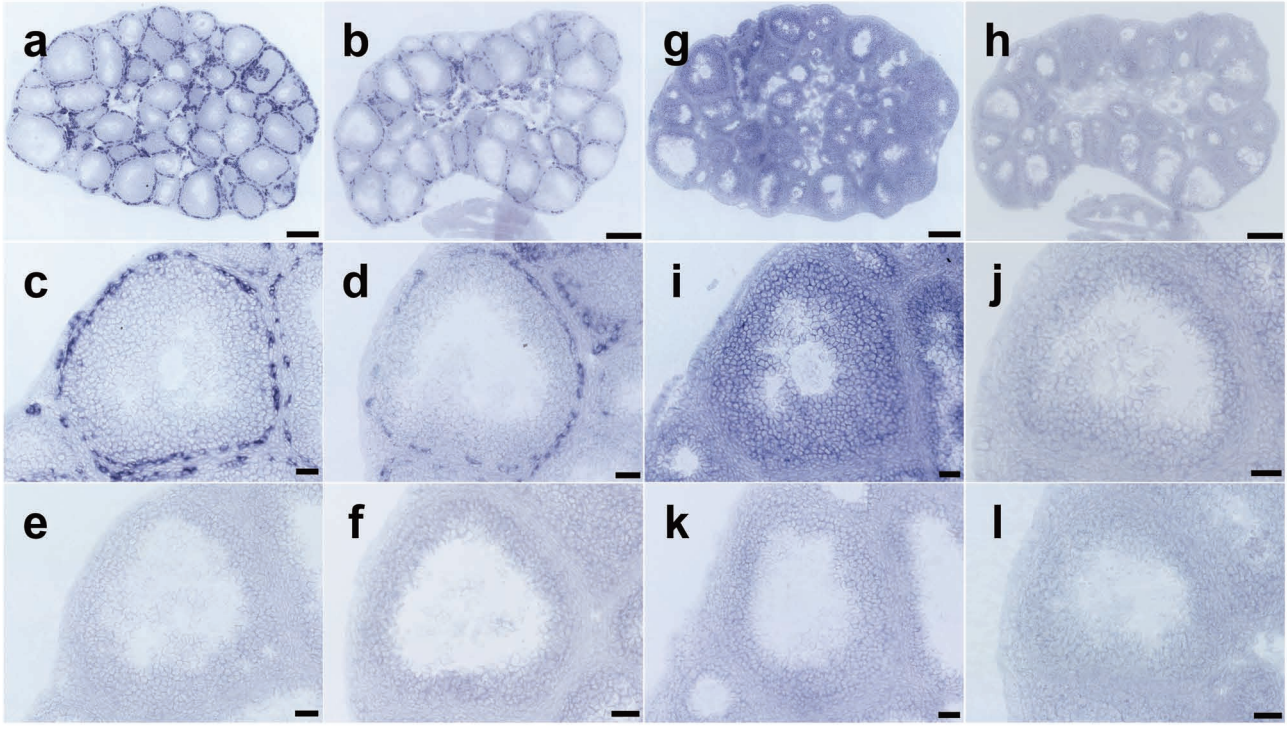

Fig. 4

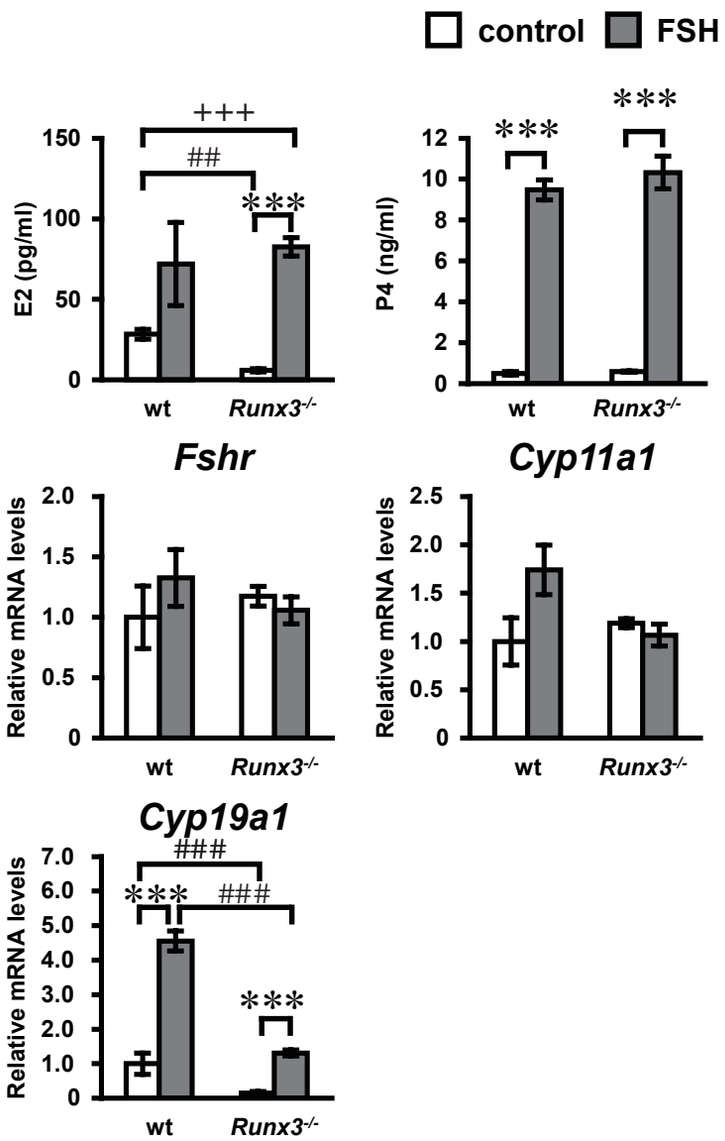
control
FSH
E2 (pg/ml)
150
100
50
0
+++
##
***
wt
Runx3-/-
P4 (ng/ml)
12
10
8
6
4
2
0
***
***
wt
Runx3-/-
Fshr
Relative mRNA levels
2.0
1.5
1.0
0.5
0
wt
Runx3-/-
Cyp11a1
Relative mRNA levels
2.5
2.0
1.5
1.0
0.5
0
wt
Runx3-/-
Cyp19a1
Relative mRNA levels
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
###
***
###
***
wt
Runx3-/-

Fig. 5

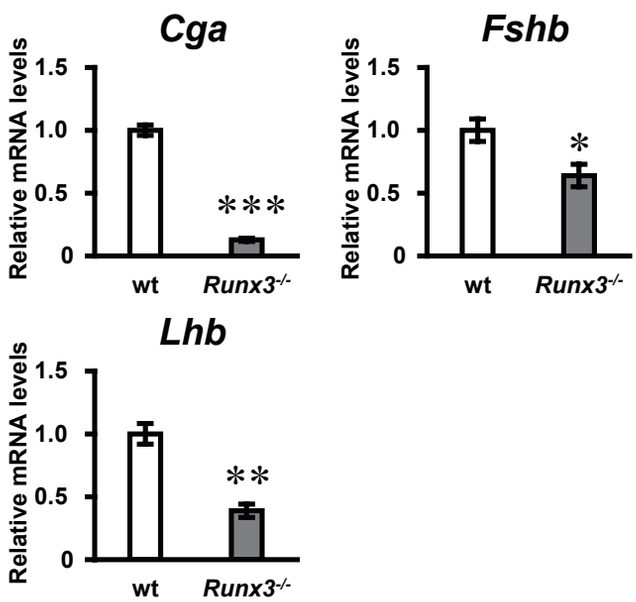
Cga
Relative mRNA levels
1.5
1.0
0.5
0
***
wt
Runx3-/-
Fshb
Relative mRNA levels
1.5
1.0
0.5
0
*
wt
Runx3-/-
Lhb
Relative mRNA levels
1.5
1.0
0.5
0
**
wt
Runx3-/-

Fig. 6