Roles of transcription factor Runx3

in the regulation of ovarian functions in female mice

2016, September

Fumiya Ojima

Graduate School of Natural Science and Technology

(Doctor’s Course)

OKAYAMA UNIVERSITY
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Chapter 1

General Introduction
General introduction

The runt domain transcription factors, the polyomavirus enhancer binding protein 2/core binding factors (PEBP2/CBF), are heterodimeric transcriptional regulators composed of α and β subunits [1-3]. The α subunit is required for binding to the consensus DNA sequence as well as for dimerization with the β subunit. The gene encoding the α subunit is homologous to the Drosophila gene runt, and three runt-related genes, Runx1, Runx2, and Runx3, have been identified in rats and mice. These Runx proteins interact with Smad2 or Smad3, and play an important role in transforming growth factor-β (TGF-β) superfamily signaling [4]. Although the runt domain of mouse Runx1, Runx2, and Runx3 is tightly conserved, the C-terminal portion varies in amino acid sequence among Runx family member transcription factors (Fig. 1), hence it is probable that each protein exerts distinct roles in gene transcription regulation [5].

Runx1 is involved in the regulations of hematopoiesis [6] and neurogenesis [7], and Runx2 in the regulation of bone development [8, 9]. Runx3 is involved in the regulation of T-cell development [10, 11] and neurogenesis [12, 13]. Runx3 functions as a tumor suppressor in a variety of cancers, including gastric and esophageal ones through the attenuation of cell growth and the induction of apoptosis [14]. Runx3 also suppresses gastric epithelial cell growth by inducing cell cycle regulator p21WAF1/Cip1 expression and induces apoptosis of gastric epithelial cells by up-regulating proapoptotic gene Bim [15-17].

In recent years, it has been reported that members of Runx family are involved in the regulation of ovarian functions. Runx1 and Runx2 play crucial roles in periovulatory process of rat ovaries [18-21]. The expression of Runx1 and Runx2 mRNAs was highly up-regulated in periovulatory granulosa cells and newly formed corpora
lutea after LH surge. The LH-induced Runx1 and Runx2 expression was involved in the expression of several periovulatory genes [20, 22-24]. Runx2 regulates gene expression of β subunit of follicle stimulating hormone (FSH) in gonadotropes [25]. Interestingly, Runx2 suppresses Runx1 expression in granulosa cells [26].

Sakuma et al. (2008) and Tsuchiya et al (2012) demonstrated that female Runx3 knockout (Runx3−/−) mice were anovulatory and their uteri were atrophic. The ovaries in adult Runx3−/− mice were smaller than those in wild-type (wt) mice. Corpora lutea were not observed in Runx3−/− mice, indicating lack of ovulation in those mice. The numbers of primary, secondary, and antral follicles in Runx3−/− mice were significantly lower than those in wt mice at 8 weeks of age [27]. These results showed that Runx3 is an important factor in the regulation of follicular development and ovulation, and no ovulation in Runx3−/− mice is attributed to dysfunction of regulatory mechanism of gonadotropin secretion. In addition, the atrophic uteri observed in Runx3−/− mice suggested the decrease in estrogen and progesterone (P4) production in their ovaries [28]. These findings clearly showed that Runx3 regulates ovarian functions as well as Runx1 and Runx2.

Folliculogenesis in the ovary is regulated by the members of TGF-β superfamily [29-32] and Smad2/3 [33, 34]. The Smad family mediates signal transduction of the TGF-β family members [35, 36]. Because Runx3 plays important roles in TGF-β signaling, and the deletion of Smad3 severely affects the follicular development [36, 37], Runx3 may be involved in the regulation of folliculogenesis through TGF-β signaling.

The later stage of follicular development including the transition of preantral follicles to antral follicles is regulated by FSH [38-41]. Luteinizing hormone (LH), another gonadotropin, regulates ovulation and luteinization [42].

Estrogen is also involved in follicular development [43, 44]. Cholesterol, the starting material of all steroid hormones, is transported to the mitochondria by
steroidogenic acute regulatory protein (StAR), and converted to pregnenolone by the cholesterol side chain cleavage enzyme (SCC). Pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase (3βHSD). Progesterone is converted to 17α-hydroxyprogesterone by 17α-hydroxylase, to androstenedione or testosterone in theca cells. Estradiol is converted from testosterone by aromatase in granulosa cells [45-48]. This steroidogenesis in follicular cells is regulated by FSH and LH [49-51]. In Runx3−/− mouse ovaries, production of both estrogen and progestin appeared to be decreased, because the uteri of Runx3−/− mice are atrophic. However, it is not clear whether Runx3 deletion can affect steroidogenesis in mouse ovaries, and how Runx3 regulates steroidogenesis.

Ovulation is triggered by LH surge at the evening of proestrous day, and the LH surge is generated by a positive feedback action of E2 through a stimulatory action of kisspeptin neurons in the anteroventral periventricular nucleus (AVPV) [52]. Kisspeptin neurons in the arcuate nucleus (ARC) are involved in the negative feedback regulation of LH and FSH secretions [53-56]. Since Runx3−/− mice did not ovulate, it is highly probable that regulatory system of gonadotropin secretion did not work properly. However, it is not clear how Runx3 deletion can affect the hypothalamo-pituitary system by which gonadotropin secretion and ovulation are regulated.

Taken together, these findings suggest that Runx3 play a role in the regulation of ovarian function. In comparison to Runx1 and Runx2, however, the role of Runx3 in female mouse reproduction remains unclear. Therefore, the purpose of the present study was to clarify the role of Runx3 in the regulation of ovarian functions in female mice. In the first part of the present study, Runx3 mRNA-expressing cells in mouse ovaries were identified by in situ hybridization, because Runx3 expressing cells were not identified in mouse ovaries. The roles of Runx3 in regulation of folliculogenesis, steroidogenesis, and ovulation were studied using Runx3−/− mice, because it was valuable that investigation
of the effect of Runx3 deletion in regulation of ovarian functions and little is known about the alterations of ovarian functions in Runx3−/− mice although Runx3−/− mice were anovulatory and infertile. In the second part, therefore, ovaries of immature mice were studied because ovulation started at around 6 weeks of age and no corpora lutea; hence, it is easy to analyze follicular functions in the absence of corpora lutea. In the third part, to further elucidate the influence of Runx3 deletion on steroidogenesis and ovulation, ovaries at 8 weeks of age were investigated following previous reports [27, 28]. In the last part, mouse ovarian genes that were regulated by Runx3 were investigated.

Runx3−/− mice used in the present study were generously awarded from Dr. Fukamachi, Tokyo Medical and Dental University, and were originally generated by the method of gene targeting [57].
Runx1 (mouse 451 AA)

Runx2 (mouse 513 AA) (% identity to Runx1)

Runx3 (mouse 409 AA) (% identity to Runx1)
**Fig. 1.** Schematic representation of the structure of mouse Runx proteins. runt: Runt domain; Q: glutamine (Q)-residue stretch; A: alanine (A)-residue stretch; NLS: nuclear localizing signal; NMTS: nuclear matrix targeting signal; TA: *trans*-activation domain; ID: auto-inhibitory domain; VWRPY: VWRPY-motif. Numerals within parentheses below the Runx2 and Runx3 schemes indicate the percentages of amino acid (AA) identity of the subdomains in comparison with those of Runx1.
Chapter 2

*Runx3* mRNA expression in hypothalamo-pituitary-ovarian axis
Introduction

Three runt-related genes, *Runx1*, *Runx2*, and *Runx3*, have been identified in mammals. Runx members interact with Smad2 or Smad3, and play a key role in TGF-β superfamily signaling [4]. *Runx1* and *Runx2* are expressed in granulosa cells of mature follicles and luteinizing granulosa cells, and are involved in periovulatory process of ovaries [18-21]. *Runx3* deletion results in the lack of folliculogenesis and ovulation. In addition, *Runx3* mRNA and *Runx3* protein are detected in mouse ovaries [27, 28]. These reports suggested that *Runx3* is a key regulator of ovarian functions in mouse ovaries as well as *Runx1* and *Runx2*. However, *Runx3* localization in mouse ovaries is not clear. In this chapter, the localization of *Runx3* mRNA in mouse ovaries was studied by a combination of RT-PCR analysis and *in situ* hybridization analysis, because *Runx3* may be involved in cell-specific regulations of ovarian functions in mouse ovaries.

*Runx3*−/− mice were anovulatory [27], and was considered to lose the ability to trigger the ovulation. The dysfunction of hypothalamo-pituitary system in the regulation of ovulation may be induced by *Runx3* deletion in female mice. *Runx3* mRNA expression had been already reported [27]. However, *Runx3*-expressing neurons in the hypothalamus have been not studied. Therefore, *Runx3* mRNA expression in mouse hypothalamic AVPV and ARC was examined, because those areas were regulatory centers for gonadotropin secretion and ovulation [58].
Materials and Methods

Animals

Male and female BALB/c mice were used in this study. Runx3 knockout (Runx3<sup>−/−</sup>) mice with the BALB/c genetic background were generated as previously described [57]. 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 the Laboratory Animals, Okayama University. Runx3<sup>+/−</sup> mice were mated, and offsprings were genotyped as previously described [59].

Ovaries from wt and Runx3<sup>−/−</sup> mice were embedded in O.C.T.Compound (Sakura Finetek Japan, Tokyo, Japan), and frozen with liquid nitrogen.

Whole brains were rapidly removed from the skull and frozen with liquid nitrogen. Two parts, one containing the POA and AVPV and the other containing the ARC, were dissected from the frozen brain. Briefly, an anterior coronal cut was approximately 2 mm anteriorly from the anterior part of optic chiasma, and posterior coronal cut was performed at the posterior border of the mammillary bodies. The dissected block was further coronally cut 1 mm behind the optic chiasma, and dorsally at the upper portion of the third ventricle (approximately 2 mm from the ventral surface of the block), and laterally at the hypothalamic fissure.

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, Shiga, Japan) according to 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 sec, annealing temperature for 30 sec, and 72 °C for 30 sec; and 10 min at 72°C. A 10-µl aliquot of each reaction was electrophoresed on a 2% agarose gel, and the gel was stained with ethidium bromide, and photographed under ultraviolet rays.

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 10 sec, 40 cycles 95°C for 5 sec, and 60°C for 31 sec, followed by a melting-curve analysis (95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, 60°C for 15 sec). A melting-curve analysis was conducted to confirm the absence of primer dimmers. The primers used in this study are summarized in Table 1. Standard curves were generated by serial dilution of total cDNA, and the amount of each target mRNA level was normalized against the amount of ribosomal protein L19 (Rpl19) mRNA levels.

Riboprobes

Mouse Runx3 riboprobes were generated according to the method previously described [60]. DNA fragments encoding part of mouse Runx3 (NM_019732; 1770-2071) were obtained by reverse transcription-polymerase chain reaction (RT-PCR) using the following primers: mouse Runx3 5΄- CTC CAG CCC GAG ACT ACA AG -3΄ and 5΄- AGG GAG GGA GAG AAA GTC CA -3΄. The cDNA fragments were subcloned into the pGEM-3Zf(+) vector. Each plasmid DNA was linearized using restriction enzymes (EcoRI/HindIII) site of pGEM-3Zf(+) and RNA probes were synthesized using a T7 and SP6 polymerase system (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The probe was labeled with digoxigenin (DIG) (Roche Diagnostics, Mannheim, Germany).
**In situ hybridization analysis**

Ovaries from wt and Runx3<sup>−/−</sup> mice were embedded in O.C.T. Compound (Sakura Finetek 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 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 subjected to hybridization solution containing DIG-labeled anti-sense or sense riboprobes (50 ng/slide) in pre-hybridization solution overnight at 53°C. Following the hybridization for 16 hours, 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 Industries, Osaka, Japan) and 17.5 μg/ml 5-bromo-4-chloro-3′-indoylphosphate p-toluidine salt (Wako Pure Chemical Industries).

**Statistical analysis**

The differences in means between the two groups were analyzed using Student’s t-test (Kaleida Graph, Synergy Software, Reading, PA, USA). The differences were considered significant at P< 0.05.
Results

RT-PCR analyses of Runx3 mRNA in granulosa cells and hypothalami in female mice

Granulosa cells

Granulosa cells were isolated from the ovaries of 3-week-old mice, and were confirmed by detection of expression of Fshr and Cyp19a1 (Fig. 2A). Runx3 mRNA was detected in the isolated granulosa cells using RT-PCR.

Hypothalamus

Runx3 mRNA expression in the AVPV and ARC of 8-week-old wt female mice at diestrus was analyzed by real-time PCR. Runx3 mRNA was detected in both areas (Fig. 2B).

In situ hybridization analysis of Runx3 mRNA in mouse ovaries

Runx3 mRNA localization in mouse ovaries was investigated by in situ hybridization, since Runx3 mRNA expression was detected in granulosa cells in the RT-PCR analysis above. Runx3 mRNA hybridization signals were detected in the granulosa cells of primary follicles, secondary follicles, and antral follicles but not in corpora lutea, which are in agreement with the results from the RT-PCR analysis (Fig. 2C, E). No signals were detected with the sense probe (Fig. 2D, F).
Discussion

It is known that Runx1 and Runx2 expressions are induced by LH and are detected in luteinizing granulosa cells [20, 21]. The localization of Runx3 expression in the ovaries had not been studied although the expression of Runx3 mRNA and Runx3 proteins were observed in mouse ovaries [27, 28]. The present study demonstrated Runx3 mRNA expression in granulosa cells by RT-PCR and in situ hybridization analyses. Runx3−/− mice show retardation of follicular development [27]. These findings suggest that Runx3 is a key regulator in early stage of folliculogenesis in granulosa cells and Runx3 may be involved in the regulations of follicular functions in mouse ovaries as well as Runx1 and Runx2. Runx1 and Runx2 were thought to regulate the luteinization or ovulation [20, 23, 26]. Runx3 may regulate folliculogenesis and ovulation through granulosa cell functions. Importantly, Runx3 mRNA was detected in granulosa cells from the primary follicles to mature Graafian follicles. Runx3 may be involved in the regulation of follicle functions from the early stage of folliculogenesis.

Runx1 and Runx2 expressions in luteinizing granulosa cells [20, 21] and Runx3 mRNA expression in granulosa cells of follicles suggest that the members of Runx family, Runx1, Runx2, and Runx3, are involved in regulation of granulosa cell functions throughout folliculogenesis. Runx3 may be a key regulator of follicular functions from primary follicles to antral follicles and Runx1 and Runx2 are also key regulators of follicular functions of later stage of follicles. Runx3 may be involved in regulation of the other Runx family members because Runx2 regulates Runx1 expression in granulosa cells [26]. The timing of expression of Runx3 in ovaries was different from that of Runx1 and Runx2. Therefore, Runx3 may play different roles from Runx1 and Runx2 in the regulation of granulosa cell functions in mouse ovaries.

The expression of Runx3 mRNA was observed in various mouse organs,
including ovaries and hypothalami. In addition, gonadotropin treatment induced superovulation in the ovaries of Runx3−/− mice [27], indicating the possibility that Runx3 expressed in hypothalamic areas may be involved in the regulation of gonadotropin secretion. LH surge required for ovulation was generated by positive feedback action of E2 through a stimulatory action of kisspeptin neurons existing in AVPV, and kisspeptin neurons in ARC are involved in the negative feedback regulation of LH and FSH secretions [53-56]. Runx3 mRNA expression in hypothalami was clearly demonstrated using RT-PCR. Runx3 mRNA was expressed in the hypothalamic areas containing AVPV and ARC of the female hypothalamus, suggesting that Runx3 may play roles in neuronal system regulation in both AVPV and ARC [61]. In conclusion, Runx3 mRNA was expressed in granulosa cells in ovaries and AVPV and ARC in hypothalami, suggesting that Runx3 expressed in both organs was involved in the regulation of gonadotropin secretion, and probably ovarian function.
Summary

The members of Runx family (Runx1, Runx2, and Runx3) are known to be involved in the regulation of follicular functions. Runx1 and Runx2 are expressed in luteinizing granulosa cells in ovaries. However, localization of Runx3 in mouse ovaries had been not clear although Runx3 protein expression was detected in mouse ovaries. Here, it was demonstrated that Runx3 mRNA was expressed in granulosa cells of primary follicle, secondary follicles, and antral follicles. Runx3 was expressed in granulosa cells as well as Runx1 and Runx2, but may play different roles from Runx1 and Runx2 in the regulation of granulosa cell functions in mouse ovaries, because the timing of expression of each gene was different. The members of Runx family are involved in regulation of granulosa cell functions throughout folliculogenesis. Runx3 mRNA expression was detected in the hypothalamic AVPV and ARC areas in the female mouse brain. Female Runx3−/− mice were anovulatory. Therefore, Runx3 expressed in mouse granulosa cells, AVPV and ARC is probably involved in the regulation of ovarian function.
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**Table 1.** Primers used for RT-PCR and real-time PCR.
Fig. 2. Expression of Runx3 mRNA in granulosa cells (GC) and hypothalami of female mice. GC were isolated from the ovaries of 3-week-old mice. The expressions of Runx3, Fshr, Lhgr, Cyp11a1, Hsd3b1, Cyp17a1, and Cyp19a1 mRNA were analyzed in the GC and whole ovaries of 3-week-old mice (A). Expression of Runx3 mRNA levels in 3-week-old wt mouse GC and in the POA-AVPV and ARC of 8-week-old wt mouse hypothalami (B). In situ hybridization analysis revealed Runx3 mRNA signals in the GC of 8-week-old mouse ovaries (C, E). No signals were detected when a sense probe was used (D, F). Bar = 300 µm (C, D), 50 µm (E, F). The figure was reproduced from Ojima et al. (2016).
Chapter 3

*Runx3* deletion affects expressions of key regulators of follicular functions and ovulation in hypothalamo-pituitary-ovary axis of immature female mice
Introduction

Recent studies showed the retardation of folliculogenesis, decline in steroidogenesis, and alterations of hypothalamo-pituitary system in adult Runx3−/− mice [27, 28]. Furthermore, we had revealed Runx3 mRNA expression in mouse granulosa cells [61]. The proper regulations of folliculogenesis and steroidogenesis in immature mouse ovaries were necessary for the following maturation of female reproductive functions, and some of growth factors and hormones produced in granulosa cells were involved in regulations of folliculogenesis [62-66]. Estrogen production in granulosa cells is necessary for follicular development [67-69]. However, it is not clear whether Runx3 deletion affects steroidogenesis in immature mouse ovaries. Therefore, the purpose of the present study was to clarify roles of Runx3 on steroidogenesis in ovarian follicles. As a first part of the present study we examined mRNA levels of Fshr (FSH receptor), Lhcgr (LH receptor), and the key regulators of steroidogenesis in ovarian granulosa cells: Star (steroidogenic acute regulatory protein), Cyp11a1 (p450sc, cholesterol side chain cleavage enzyme), Hsd3b1 (hydroxy-δ-5-steroid dehydrogenase), Cyp17a1 (p450c17, steroid 17α hydroxylase), and Cyp19a1 (p450arom, aromatase) mRNA levels in Runx3−/− mouse ovaries and granulosa cells. In particular, we studied E2 production of Runx3−/− mouse granulosa cells because Runx3 mRNA was expressed in mouse granulosa cells [61].

Among several studies regarding ovarian cancer development, controversial results have been obtained on Runx3 expression in ovarian cancer cells and roles of Runx3 as the tumor suppressor [70-72]. These findings altogether, however, suggest that Runx3 is involved in the regulation of proliferation of granulosa cells. Therefore, another purpose of the present study was to clarify the role of Runx3 on folliculogenesis. As the second part of the present study we examined gene expression of members of the
TGF-β superfamily, anti-Müllarian hormone (AMH), insulin-like growth factor 1 (IGF1), and subunits of activin and inhibin, in Runx3−/− mouse ovaries because it is well known that the members of the TGF-β superfamily are involved in the regulation of folliculogenesis [30].
Materials and Methods

Animals

Male and female BALB/c mice were used in this study. Runx3 knockout (Runx3<sup>−/−</sup>) mice with the BALB/c genetic background were generated as previously described [57]. 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 the Laboratory Animals, Okayama University. Runx3<sup>+/−</sup> mice were mated, and offspring were genotyped as previously described [59].

Ovaries obtained from Runx3<sup>−/−</sup> and wt mice 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 two sections, and each follicle was followed through consecutive sections to ensure that it was counted only once.

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

Total RNA was extracted from tissues or isolated granulosa cells using TRIzol Reagent (Bioline), and reverse-transcribed using the Prime Script RT-PCR System (Takara Bio) according to the manufacturer’s instructions. Random hexamers were used for the RT reactions.

PCR was carried out using Blend Taq (Toyobo) and a Gene Amp PCR System 9700 thermal cycler (Applied Biosystems). The PCR conditions were as follows: 2 min at 94°C; an appropriate number of cycles of 94°C for 30 sec, annealing temperature for 30 sec, and 72 °C for 30 sec; and 10 min at 72°C. A 10-μl aliquot of each reaction was electrophoresed on a 2% agarose gel, and the gel was stained with ethidium bromide, and photographed under ultraviolet rays.
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 sec, 40 cycles 95°C for 5 sec, and 60°C for 31 sec, followed by a melting-curve analysis (95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, 60°C for 15 sec). A melting-curve analysis was conducted to confirm the absence of primer dimmers. The primers used in this study are summarized in Table 2. Standard curves were generated by serial dilution of total cDNA, and the amount of each target mRNA level was normalized against the amount of ribosomal protein L19 (Rpl19) mRNA levels.

Riboprobe

Mouse Cyp11a1 riboprobes were generated according to the previously described method [60]. DNA fragments encoding part of mouse Cyp11a1 (NM_019779.3; 630-1055) were obtained by RT-PCR using the following primers: mouse Cyp11a1 5'- CCT TTG AGT CCA TCA GCA GTG -3' and 5'- GTA CCT TCA AGT TGT GTG CCA -3'. The cDNA fragments were subcloned into the pGEM-3Zf(+) vector. Each plasmid DNA was linearized using restriction enzymes (EcoRI/HindIII) site of pGEM-3Zf(+) and RNA probes were synthesized using a T7 and SP6 polymerase system (Promega) according to the manufacturer’s instructions. The probe was labeled with digoxigenin (DIG) (Roche Diagnostics).

In situ hybridization analysis

Ovaries from wt and Runx3−/− mice were embedded in O.C.T.Compound (Sakura Finetek 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) at 37°C for 10 min, 0.2% glycine in PBS for 20 min, and 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 subjected to hybridization solution containing DIG-labeled anti-sense or sense riboprobes (50 ng/slide) in pre-hybridization solution overnight at 45°C. Following the hybridization for 16 hours, 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 Industries) and 17.5 µg/ml 5-bromo-4-chloro-3′-indoylphosphate p-toluidine salt (Wako Pure Chemical Industries).

To evaluate the effect of Runx3 deletion on Cyp11a1 mRNA expressions, follicles in medial sections of serial ovarian sections were selected from five wt mice and three Runx3−/− mice, and both identifiable Cyp11a1 mRNA-expressing theca cells and all theca cells were counted by light microscopy. Data are expressed as the percentage of the number of Cyp11a1 mRNA-expressing theca cells against total number of theca cells.

**Ovarian granulosa cell isolation and culture**

Granulosa cell isolation and culture was performed according to the method used in previous studies [73]. Ovaries from 3-week-old wt and Runx3−/− mice were removed and dissected free of connective tissue. Ovaries were 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 granulosa cells but not oocytes to pass through. After centrifugation, cells were collected to analyze for Runx3.
mRNA expression in granulosa cells. For further analysis, after centrifugation, the cells were cultured in McCoy 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% CO₂. The cells were seeded at a density of 5×10⁴ cells/well in 24-well plates. After 24 hours, the medium was changed to serum-free McCoy 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 hr before further analysis. The levels of estradiol and progesterone in the media were determined by a chemiluminescent immunoassay using Architect estradiol and progesterone kits (Abbott Co., Tokyo, Japan).

Analysis of steroid production

For steroid assay, 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). Granulosa cells were cultured without (control) and with ovine FSH (Sigma-Aldrich, 30 ng/ml) [74]. After 48 hours, the culture supernatant was collected and stored at −20°C until assay.

Statistical Analysis

Data were shown as the mean ± S.E. Differences among groups were analyzed by analysis of variance followed by Tukey’s test. The differences in means between the 2 groups were analyzed by a Student’s t-test. The differences were considered significant when P<0.05.
Results

Morphology of ovaries in Runx3−/− mice

Morphological changes of ovaries of 2-month-old Runx3−/− mice had been already reported [27]. The numbers of primary, secondary, and antral follicles in 2-month-old Runx3−/− mice were significantly lower than those in wt mice, and Runx3−/− mouse ovaries were devoid of corpora lutea, indicating no ovulation. In the present study, Runx3−/− mouse ovaries from 2 weeks to 8 weeks of age were studied to clarify effects of Runx3 deletion on follicle development (Fig. 3A). At the age of 2 weeks, all four types of follicles including primordial follicles were found in the ovary and their numbers did not differ between wt and Runx3−/− mice. At the age of 3 weeks, the numbers of primary and antral follicles per an ovary were significantly fewer in Runx3−/− mice than those in wt mice, while the number of secondary follicles per an ovary was more in Runx3−/− mice than that in wt mice (Fig. 3B). At the age of 8 weeks, 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, which was in consistent with our previous study [27].

Expression of growth factor genes involved in regulation of follicular functions in ovaries of wt and Runx3−/− mice

To clarify whether Runx3 deletion affect follicular functions in 3-week-old mice, we analyzed Amh, Igf1, Inha, Inhba, and Inhbb mRNA levels in wt and Runx3−/− mouse ovaries using quantitative real-time PCR. Inha, Inhba, and Inhbb mRNA levels were lower in Runx3−/− mouse ovaries than in wt ones, while mRNA levels of Amh and Igf1 did not differ significantly from those in wt mice (Fig. 4).
Expression of genes involved in regulations of steroidogenesis in the ovaries of wt and Runx3−/− mice

To determine whether Runx3 deletion can affect steroidogenesis in 3-week-age mouse ovaries, we analyzed the mRNA of Fshr, Lhcgr, Star, Cyp11a1, Hsd3b1, Cyp17a1, and Cyp19a1 mRNA levels in wt and Runx3−/− mouse ovaries using real-time PCR. Levels of all mRNAs examined were decreased in Runx3−/− mouse ovaries, although significant differences in Star and Cyp17a1 mRNA levels were not detected owing to large variation in wt mouse ovaries (Fig. 5).

In situ hybridization analysis of Cyp11a1 mRNA expression in wt and Runx3−/− mouse ovaries

Cyp11a1 mRNA signals were detected in some of the interstitial cells and also some of the theca interna cells of ovaries of 3-week-old mice (Fig. 6A, B, C, D). In Runx3−/− mice, Cyp11a1 mRNA signals were detected in interstitial cells and theca interna cells, but the number of Cyp11a1 mRNA-containing cells was lower than that in wt mice (Fig. 6G). No signals were detected with the sense probe (Fig. 6E, F). These results are consistent with the result of quantitative real-time PCR analyses shown in Fig. 5.

Expression of genes involved in steroidogenesis in freshly prepared granulosa cells from wt and Runx3−/− mouse ovaries

To clarify the role of Runx3 in the steroidogenesis in granulosa cells, the mRNA levels of Fshr, Cyp11a1, Hsd3b1, and Cyp19a1 mRNA in the freshly prepared granulosa cells from wt and Runx3−/− mice were analyzed by quantitative real-time PCR.

In granulosa cells isolated from Runx3−/− mouse ovaries, the expression of Cyp19a1 mRNA was significantly decreased compared with that in wt mice (Fig. 7). On
the other hand, the expressions of Fshr, Cyp11a1, and Hsd3b1 mRNA in granulosa cells did not differ between Runx3−/− and wt mice.

*FSH treatment on E2 and P4 production in freshly prepared granulosa cells from wt and Runx3−/− mouse ovaries*

It is well known that steroidogenesis in granulosa cells is regulated by gonadotropins. Granulosa cells of wt and Runx3−/− mice were cultured with or without FSH (30 ng/ml) for 48 hours, and effects of FSH on the production of E2 and P4, and expression of gonadotropin receptor genes and steroidogenic enzyme gene in granulosa cells of Runx3−/− and wt mice were studied.

The basal estrogen levels in Runx3−/− mouse granulosa cells were lower than those in wt mice (Fig. 8A). In contrast, the basal progesterone levels in Runx3−/− mice did not differ significantly from those in wt mice (Fig. 8B). However, FSH treatment significantly increased estrogen production in Runx3−/− mouse granulosa cells, but not in wt granulosa cells. There was no difference in FSH-induced progesterone production between wt and Runx3−/− mice (Fig. 8B).

In Runx3−/− mouse granulosa cells, the basal expression of Cyp19a1 mRNA was significantly decreased compared with wt mice (Fig. 8C). FSH treatment increased Cyp19a1 mRNA levels in both wt and Runx3−/− granulosa cells. Conversely, FSH treatment did not significantly affect the levels of Fshr, Cyp11a1, and Hsd3b1 mRNA expression in both wt and Runx3−/− mice (Fig. 8C).
The development of primordial follicles to preantral follicles proceeds under the control of intra-ovarian regulatory system [30], and that of preantral follicles to antral follicles is regulated by FSH [38-41]. In this chapter, we evaluated folliculogenesis during the prepubertal period in the Runx3−/− mouse ovaries. Effect of Runx3 deletion on folliculogenesis became evident at 3 weeks of age, and Runx3−/− mouse ovaries contained fewer primary and antral follicles, and more secondary follicles than wt mouse ovaries. The alterations of folliculogenesis in Runx3−/− mice became apparent from the early phase of follicle development, and the early folliculogenesis was gonadotropin-independently regulated. Therefore, the change in the number of secondary follicles observed in 3-week-old Runx3−/− mouse ovaries were probably caused by alterations of intra-ovarian regulatory system controlled by Runx3 within granulosa cells. Runx3 may be at least involved in the initial recruitment of follicles from the resting pools [75].

Lots of evidence demonstrated that folliculogenesis is regulated by TGF-β/BMP family members produced in oocytes, granulosa cells, and theca cells [30, 76]. GDF-9, produced in oocytes, stimulates the early stage of follicular development [77], and is involved in the regulation of early folliculogenesis [78, 79]. BMP-15, produced in oocytes, stimulates proliferation of granulosa cells [80]. BMP-7, produced in theca cells, stimulates primordial-primary follicle transition and granulosa cell proliferation [81]. AMH, produced in granulosa cells, regulates the primordial follicle recruitment [66]. Thus, several TGF-β/BMP family growth factors affect folliculogenesis, and intracellular TGF-β/BMP signaling needs Smad proteins. Smad3 deletion caused a slower growth of primordial follicles to the antral follicles [37]. The retarded growth of follicles observed in Smad−/− ovaries seem to be similar to the changes in folliculogenesis observed in
Runx3−/− mouse ovaries at the age of 3 weeks. Runx3 has been reported to act as an important factor in TGF-β/BMP signaling pathways [15, 82]. Considering these previous and present findings, it is probable that Runx3 plays roles in the regulation of folliculogenesis by interacting with TGF-β/BMP system.

Activins stimulate the proliferation of primordial germ cells and granulosa cells of early immature follicles [63, 83-87]. Activins also stimulate FSHR expression in granulosa cells [88-90] and synergize with FSH to regulate differentiation of granulosa cells during late folliculogenesis [91-93]. Inhibins, which generally antagonize activin actions [32], are involved in inhibitory regulation of primordial follicle recruitment and early follicle development [91, 94]. We demonstrated that Inha, Inhba, and Inhbb mRNA expressions were decreased in Runx3−/− mouse ovaries, suggesting that syntheses of activins or inhibins were decreased in them. A recent study of Inhbb knockdown mice showed that Inhbb is involved in the regulation of granulosa cell proliferation in mouse ovaries [95]. These changes in folliculogenesis in Inhbb knockdown mice were similar to those in Runx3−/− mouse ovaries. Therefore, the retarded folliculogenesis in Runx3−/− mouse ovaries was probably due to the decreased expression of subunits of activins and/or inhibins, which are involved in the regulation of early folliculogenesis.

Cyp11a1 mRNA was expressed in theca interna cells, parts of granulosa cells of preovulatory follicles, and corpora lutea [96]. The present in situ hybridization analysis demonstrated the decrease in the number of Cyp11a1 mRNA expressing cells in theca cells of Runx3−/− mice. Cyp19a1 mRNA was expressed in granulosa cells [97-99]. 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 [43, 44]. Therefore, it is probable that decreased production of E2 affected the late stage of folliculogenesis.

Cyp11a1 expression in theca cells is regulated by LH [51], and also 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 [32, 100-102]. Thus, the theca cell functions are regulated by growth factors produced in granulosa cells as well as LH. Therefore, it is highly probable that Runx3-regulated growth factors in granulosa cells control theca cell functions in a paracrine manner, because Runx3 was not expressed in theca cells. The decreased Cyp11a1 expression in theca cells of Runx3−/− mouse ovaries may lead to a decrease in androgen production, resulting in a decrease in estrogen production, because androgens are converted to estrogen by aromatase encoded by Cyp19a1 in granulosa cells.

Cyp19a1 mRNA levels were lower in Runx3−/− mouse ovaries than in those of wt mice. FSH treatment increased Cyp19a1 mRNA levels in cultured granulosa cells of both wt and Runx3−/− mouse ovaries, but the FSH-induced increase in Runx3−/− mice was lower than in wt mice. On the other hand, Fshr mRNA expression was not altered by FSH treatment in both granulosa cells of wt and Runx3−/− mice, although FSHR expression was not determined. The reduction of FSH response on Cyp19a1 mRNA expression suggests that Runx3 is involved in the regulation of Cyp19a1 expression, although it is not clear whether Runx3 directly regulates Cyp19a1 transcription. Therefore, it is probable that Runx3 exerts an essential role in FSH-induced Cyp19a1 mRNA expression. Further study, however, is needed to clarify molecular mechanism of Runx3 action on Cyp19a1 transcription.

FSH responsiveness of granulosa cells is partly regulated by activins. In immature follicles activins stimulate FSHR expression [90] and enhance FSH responsiveness of granulosa cells, while in mature follicles activins suppress steroidogenesis and prevent their terminal differentiation, resulting in induction of atresia [32, 103]. Activins increase FSH-induced aromatase activity in rat granulosa cells [86, 104, 105]. In Runx3−/− mouse ovaries, activin production was decreased,
leading to the diminished response to FSH treatment. Therefore, it is highly probable that Runx3 exerts roles in the mechanism regulating FSH-induced E2 production in granulosa cells. Conversely, with regard to P4 production, the effects of FSH treatment on *Cyp11a1* and *Hsd3b1* expression and P4 secretion in granulosa cells of *Runx3*<sup>−/−</sup> mice did not differ from those of wt mice. As *Runx3* mRNA was not detected in corpora lutea, Runx3 is probably not involved in regulation of P4 production in lutein cells.
**Summary**

*Runx3* mRNA was expressed in granulosa cells, suggesting that Runx3 regulate granulosa cell functions. *Runx3* deletion induced retardation of follicular development at the age of 8 weeks. However, it is not clear whether *Runx3* deletion affects folliculogenesis and steroidogenesis. Here, in 3-week-old *Runx3*<sup>−/−</sup> mouse ovaries, the numbers of primary and antral follicles were decreased and the number of secondary follicles was increased. *Inha, Inhba*, and *Inhbb* mRNA levels were decreased in *Runx3*<sup>−/−</sup> mouse ovaries. These findings suggest that Runx3 expressed in granulosa cells may involved in intra-ovarian regulations of follicular development. *Cyp11a1* mRNA expression in theca cells was decreased, and *Cyp19a1* mRNA expression was decreased and E2 production was diminished in granulosa cells. In addition, FSH responsiveness of *Runx3*<sup>−/−</sup> mouse granulosa cells was decreased. These findings suggest that Runx3 is involved in intra-ovarian regulations of steroidogenesis, hence, *Runx3* deletion resulted in retardation of follicular development. Furthermore, Runx3 may regulate growth factors expressed in granulosa cells which control functions of theca cells, because *Cyp11a1* mRNA expression was decreased in *Runx3*<sup>−/−</sup> mouse theca cells. Therefore, Runx3 is involved in regulation of folliculogenesis and steroidogenesis in mouse ovaries.
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Table 2. Primers used for RT-PCR and real-time PCR.
Fig. 3. Morphological observations of Runx3−/− mouse ovaries. (A) Ovaries were collected from wt and Runx3−/− mice at the age of 2 weeks, 3 weeks, 5 weeks, 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 according to the procedure described in Materials and Methods. * P < 0.05, ** P < 0.01, significantly different from wt mice.
Fig. 4. Expression of growth factor genes in the whole ovary of wt and Runx3<sup>−/−</sup> 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<sup>−/−</sup> mice was normalized against that of Rpl19 mRNA. Each group consists of five mice. Data are expressed as the mean ± SEM of triplicate wells. * \( P < 0.05 \), *** \( P < 0.001 \), significantly different from wt mice.
Fig. 5. Expression of gonadotropin receptor, steroidogenic acute regulatory protein (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, Lhcg, Star, Cyp11a1, Hsd3b1, Cyp17a1, and Cyp19a1 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. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, significantly different from wt mice.
**Fig. 6.** *In situ* hybridization analysis of *Cyp11a1* mRNAs in ovaries from wt and *Runx3*<sup>−/−</sup> mice at 3 weeks of age. Ovarian sections were obtained from wt mice (A, C, E) and *Runx3*<sup>−/−</sup> mice (B, D, F). DIG-labeled anti-sense riboprobes for *Cyp11a1* and sense probes were used. *Cyp11a1* mRNA signals were detected in theca cells (C, D). No signals were detected when a sense probe was used for hybridization (E, F). Quantitative analysis of *Cyp11a1* mRNA-positive theca cells in ovaries from wt and *Runx3*<sup>−/−</sup> mice was performed, and the percentage of *Cyp11a1* mRNA-positive cells in theca cells was calculated (G). *** *P* < 0.001, significantly different from wt mice. Bar = 300 µm (A, B), 50 µm (C-F).
**Fig. 7.** Expression of gonadotropin receptor and steroidogenic enzymes in the freshly prepared granulosa cells of wt and *Runx3−/−* mice. Real-time PCR were performed for quantitative analyses of *Fshr*, *Cyp11a1*, *Hsd3b1*, and *Cyp19a1* mRNA expression. The amount of each mRNA of wt and *Runx3−/−* mice was normalized against that of *Rpl19* mRNA. Each group consists of nine mice. Data are expressed as the mean ± SEM of triplicate wells. *P* < 0.05, significantly different from wt mice.
**Fig. 8.** Effect of Runx3 deletion on steroidogenesis of cultured granulosa cells. Granulosa cells were treated with 100 nM androstenedione without (control) and with FSH (30 ng/ml). After 48 hour-culture, the levels of estradiol and progesterone in the medium were determined by enzyme labeled immunoassay. Data are expressed as the mean ± SEM of tetracate wells. **P < 0.01, ***P < 0.001, significantly different from control of wt mice or control of Runx3<sup>−/−</sup> mice or FSH of wt mice.
Chapter 4

*Runx3* deletion affects expressions of key regulators of follicular functions and ovulation in hypothalamo-pituitary-ovary axis of adult female mice
Introduction

Female Runx3−/− mice are anovulatory, and infertile because of the atrophic uteri. Runx3 is clearly involved in regulation of female reproductive functions. At 8 weeks of age, retardation of folliculogenesis and no corpora lutea were observed in Runx3−/− mouse ovaries [27]. In addition, Runx3−/− mouse uteri were atrophic [28]. These changes in Runx3−/− mice suggest decrease in estrogen production in ovaries. However, it is not clear whether Runx3 deletion affects steroidogenesis in ovaries or not. In Chapter 2, we showed Runx3 mRNA expression in granulosa cells [61]. The purpose of the present study was to clarify the role of Runx3 in regulation of folliculogenesis and ovulation. Since ovarian steroidogenesis occurred in theca cells and granulosa cells, the mRNA levels of Fshr, Lhcgr, and the key regulators of steroidogenesis in ovarian granulosa cells: Star, Cyp11a1, Hsd3b1, Cyp17a1, and Cyp19a1 mRNA levels in wt and Runx3−/− mouse ovaries were examined following the procedures described in Chapter 3.

Gonadotropin secretion is regulated by gonadotropin-releasing hormone (GnRH) and kisspeptin. Kisspeptin neurons are located in AVPV and ARC [53-56]. Consequently, it may be reasonable to examine the role of Runx3 in regulations of GnRH neurons and kisspeptin neurons. Therefore, another purpose of the present study was to clarify the involvement of Runx3 on the regulation of hypothalamic GnRH and kisspeptin system in female mice. To investigate effect of Runx3 on GnRH and kisspeptin gene expressions, we analyzed mRNA levels of GnRH and kisspeptin gene in POA-AVPV and ARC area. Furthermore, mRNA levels of GnRH and kisspeptin in 3-week-old Runx3−/− mice were studied.

In addition, to analyze contributions of the hypothalamo-pituitary system to the anovulation of Runx3−/− mice, we performed transplantation of ovaries obtained from Runx3−/− mice to wt or Runx3−/− mice, and then ovulation in grafted ovaries was
examined by histological observation of grafts because the recent study showed that Runx3−/− mouse ovaries at immature age could ovulate when gonadotropin treatment was given [27].
**Materials and Methods**

*Animals*

Male and female BALB/c mice were used in this study. *Runx3* knockout (*Runx3*−/−) mice with the BALB/c genetic background were generated as previously described [57]. 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 the Laboratory Animals, Okayama University. *Runx3*+/− mice were mated, and offsprings were genotyped as previously described [59].

Ovaries from wt and *Runx3*−/− mice were embedded in O.C.T.Compound (Sakura Finetek Japan, Tokyo, Japan), frozen with liquid nitrogen.

Whole brains were rapidly removed from the skull and frozen in liquid nitrogen. Two parts, one containing the POA and AVPV and the other containing the ARC, were dissected from the frozen brain. Briefly, an anterior coronal cut was approximately 2 mm anteriorly from the anterior part of optic chiasma, and posterior coronal cut was performed at the posterior border of the mammillary bodies. The dissected block was further coronally cut 1 mm behind the optic chiasma, and dorsally at the upper portion of the third ventricle (approximately 2 mm from the ventral surface of the block), and laterally at the hypothalamic fissure.

Anterior pituitaries of 8-week-old wt at the diestrous stage and *Runx3*−/− mice and 3-week-old wt and *Runx3*−/− mice were rapidly removed and frozen in liquid nitrogen.

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

Total RNA was extracted from tissues using TRIsure Reagent (Bioline), and reverse-transcribed using the Prime Script RT-PCR System (Takara Bio) according to
the manufacturer’s instructions. Random hexamers were used for the RT reactions.

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

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 10 sec, 40 cycles 95°C for 5 sec, and 60°C for 31 sec, followed by a melting-curve analysis (95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, 60°C for 15 sec). A melting-curve analysis was conducted to confirm the absence of primer dimmers. The primers used in this study are summarized in Table 3. Standard curves were generated by serial dilution of total cDNA, and the amount of each target mRNA level was normalized against the amount of ribosomal protein L19 (Rpl19) mRNA levels.

Riboprobes

Mouse Cyp11a1 riboprobes were generated according to the previously described method [60]. DNA fragments encoding part of mouse Cyp11a1 (NM_019779.3; 630-1055) were obtained by RT-PCR using the following primers: mouse Cyp11a1 5’- CCT TTG AGT CCA TCA GCA GTG -3’ and 5’- GTA CCT TCA AGT TGT GTG CCA -3’. The cDNA fragments were subcloned into the pGEM-3Zf(+) vector. Each plasmid DNA was linearized using restriction enzymes (EcoRI/HindIII) site of pGEM-3Zf(+) and RNA probes were synthesized using a T7 and SP6 polymerase system (Promega) according to the manufacturer’s instructions. The probe was labeled with digoxigenin (DIG) (Roche
In situ hybridization analysis

Ovaries from wt and Runx3<sup>−/−</sup> mice were embedded in O.C.T. Compound (Sakura Finetek 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) at 37°C for 10 min, 0.2% glycine in PBS for 20 min, and 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 subjected to hybridization solution containing DIG-labeled anti-sense or sense riboprobes (50 ng/slide) in pre-hybridization solution overnight at 45°C. Following the hybridization for 16 hours, 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 Industries) and 17.5 µg/ml 5-bromo-4-chloro-3’-indoylphosphate p-toluidine salt (Wako Pure Chemical Industries). To evaluate the effect of Runx3 deletion on Cyp11a1 mRNA expressions, follicles in medial sections of serial ovarian sections were selected from five wt mice and three Runx3<sup>−/−</sup> mice, and both identifiable Cyp11a1 mRNA-expressing theca cells and all theca cells were counted by light microscopy. Data are expressed as the percentage of the number of Cyp11a1 mRNA-expressing theca cells against total number of theca cells.

Ovarian transplantation
Ovary collection and transplantations were performed simultaneously. \textit{Runx3}^{−/−} and wt mice were ovariectomized under light anesthesia, and their ovaries were pulled out from a dorsal incision and were removed from the bursa surrounding the ovaries. Collected ovaries were maintained in M199 medium containing 25 mM HEPES and 0.1% bovine serum albumin (BSA, Sigma-Aldrich) at room temperature until transplantation. After ovariectomy, one ovary was immediately placed subcutaneously on the left ventral side of \textit{Runx3}^{−/−} and wt mice. Ovarian transplantation experiments were carried out as follows: wt ovaries were grafted to wt mice (designated as wt-wt); \textit{Runx3}^{−/−} ovaries were grafted to wt mice (\emph{Runx3}^{−/−}-wt); wt ovaries were grafted to \textit{Runx3}^{−/−} mice (wt-\emph{Runx3}^{−/−}); \textit{Runx3}^{−/−} ovaries were grafted to \textit{Runx3}^{−/−} mice (\emph{Runx3}^{−/−}-\emph{Runx3}^{−/−}). Nine- to ten-week-old wt and \textit{Runx3}^{−/−} mice received ovarian grafts from mice of the same age. In the transplantation of wt-\emph{Runx3}^{−/−} mice, ovaries from 4-week-old wt mice were grafted to \textit{Runx3}^{−/−} mice, since ovulation did not occur at the age of 4 weeks. To evaluate the number of estrous cycles, vaginal smears were checked for 17 consecutive days during pre-transplantation and post-transplantation periods. The estrous cycle was classified into the following four phases: proestrus, estrus, metoestrus, and diestrus. Ovarian grafts were collected 17 days after transplantation, and processed for histological observation.

\textbf{Statistical analysis}

The differences in means between the two groups were analyzed using Student’s $t$-test. The differences were considered significant at $P<0.05$. 

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Results

Expression of genes involved in regulation of steroidogenesis in the ovaries of wt and Runx3\(^{-/-}\) mice

To determine whether Runx3 deletion affects steroidogenesis in 8-week-old mouse ovaries, we analyzed the mRNA levels of Fshr, Lhcgr, Star, Cyp11a1, Hsd3b1, Cyp17a1, and Cyp19a1 in wt and Runx3\(^{-/-}\) mouse ovaries using real-time PCR. Wt mice at the diestrous stage, and Runx3\(^{-/-}\) mice showing acyclic state and diestrous vaginal smear, were selected for the analysis. Cyp11a1 mRNA levels in Runx3\(^{-/-}\) mouse ovaries were lower than those in wt mice. There were no differences in the mRNA levels of Fshr, Lhcgr, Star, Hsd3b1, Cyp17a1, and Cyp19a1 between wt and Runx3\(^{-/-}\) mice (Fig. 9).

In situ hybridization analysis of Cyp11a1 mRNA expression in wt and Runx3\(^{-/-}\) mouse ovaries

To analyze changes of gene expressions of steroidogenesis regulators in Runx3\(^{-/-}\) mouse ovaries, Cyp11a1 mRNA expression in 8-week-old mice was analyzed using DIG-labeled riboprobes. In all in situ hybridization studies, no signals were detected when sense riboprobes were used as the control analysis. In wt mice, Cyp11a1 mRNA signals were detected in the interstitial cells and theca interna cells of secondary and antral follicles (Fig. 10A). In preovulatory antral follicles, granulosa cells that were located near the follicular basement membrane expressed Cyp11a1 mRNA signals, but the cells surrounding an oocyte did not (Fig. 10C). Intense signals were detected in the corpora lutea (Fig. 10A). In Runx3\(^{-/-}\) mice, Cyp11a1 mRNA signals were detected in interstitial cells and theca interna cells (Fig. 10B, D), but the number of Cyp11a1 mRNA-containing cells was lower than that in wt mice (Fig. 10G). No signals were detected with the sense probe (Fig. 10E, F).
Expression of Gnrh1 and Kiss1 mRNA in wt and Runx3<sup>−/−</sup> mice

To clarify alteration of the ovulation systems in Runx3<sup>−/−</sup> mouse hypothalami, mRNA levels of GnRH and kisspeptin genes were analyzed by real-time PCR. Gnrh1 mRNA levels were significantly higher than those in wt mice (Fig. 11A). Kiss1 mRNA levels in AVPV in Runx3<sup>−/−</sup> mice were significantly lower than those in wt mice (Fig. 11B), whereas Kiss1 mRNA levels in ARC were significantly higher than those in wt mice (Fig. 11B). In addition, estrogen receptor α (Esr1) mRNA levels were determined by real-time PCR, and did not differ between the POA-AVPV and ARC of wt and Runx3<sup>−/−</sup> mice (Fig. 11C). At the age of 3 weeks, Gnrh1 mRNA levels were not different between wt and Runx3<sup>−/−</sup> mice (Fig. 12A). Kiss1 mRNA levels in AVPV in Runx3<sup>−/−</sup> mice were significantly lower those in wt mice (Fig. 12B). On the other hand, Kiss1 mRNA levels in ARC were not different between wt and Runx3<sup>−/−</sup> mice (Fig. 12B). In addition, Esr1 mRNA levels were determined by real-time PCR, and were not different in POA-AVPV and ARC between wt and Runx3<sup>−/−</sup> mice (Fig. 12C).

Expression of Cga, Fshb, and Lhb mRNA in wt and Runx3<sup>−/−</sup> mice

To clarify alteration of the anterior pituitary functions in Runx3<sup>−/−</sup> mice, mRNA levels of FSH and LH genes were analyzed by real-time PCR. Fshb mRNA levels in anterior pituitaries were significantly higher in Runx3<sup>−/−</sup> mice than those in wt mice. In contrast, Lhb and Cga mRNA levels in anterior pituitaries did not differ between Runx3<sup>−/−</sup> and wt mice (Fig. 13). At the age of 3 weeks, Cga, Fshb, and Lhb mRNA levels in Runx3<sup>−/−</sup> mouse anterior pituitaries were lower than in wt mouse ones (Fig. 14).

Evaluation of contribution of the hypothalamic-pituitary system or ovaries to the anovulatory status of Runx3<sup>−/−</sup> mice assessed by ovarian transplantation
To study the contribution of the hypothalamo-pituitary system or ovaries to the anovulatory status of Runx3−/− mice, we carried out reciprocal ovarian transplantation between Runx3+/− mice and wt mice, and monitored vaginal smears for 17 consecutive days before and after the transplantation. The experimental grafts were conducted as follows: wt mice (designated as wt-wt); Runx3−/− ovaries were grafted to wt mice (Runx3+/−-wt); wt ovaries were grafted to Runx3−/− mice (wt- Runx3+/−); and Runx3−/− ovaries were grafted to Runx3−/− mice (Runx3+/−-Runx3−/−). Before the ovarian transplantation, wt mice exhibit regular cyclic estrous cycles, whereas Runx3−/− mice had irregular or acyclic estrous cycles (Table 4). After the ovarian transplantation, there was no difference in the number of estrous cycles during the observation period (17 days) between Runx3+/−-wt mice and wt-wt mice. In contrast, in wt- Runx3+/− mice and Runx3+/− Runx3−/− mice, the number of the cycles was significantly lower than that in wt-wt mice (Table 4).

The presence of corpora lutea in ovarian grafts was assessed as an indication of ovulation. Corpora lutea were detected in ovarian grafts collected from wt-wt mice (Fig. 15A, E), but were not detected in ovarian grafts collected from Runx3+/−- Runx3−/− mice (Fig. 15B, F). In spite of the lack of corpora lutea in ovaries of Runx3+/− mice, numerous antral follicles and corpora lutea were detected in ovarian grafts collected from Runx3+/−-wt mice (Fig. 15C, G). However, although there were no corpora lutea in the ovarian grafts from wt- Runx3−/− mice, antral follicles were observed (Fig. 15D, H).
Discussion

The present study showed that Runx3 deletion decreased Cyp11a1 mRNA expression in mouse ovaries, suggesting decreased estrogen production in Runx3−/− mouse ovaries because decreased Cyp11a1 expression may bring about a decrease in cholesterol side chain cleavage enzyme (SCC) expression, resulting in diminished production of androgens, which may lead to the diminished estrogen production in granulosa cells. Atrophic uteri in Runx3−/− mice also suggested decline in estrogen production [28]. Furthermore, alteration of mRNA levels of Gnrh1 and Kiss1 in Runx3−/− mouse hypothalami and Fshb mRNA in Runx3−/− mouse anterior pituitaries were shown in the present study. Therefore, it is probable that anovulatory in Runx3−/− mice was caused by a decreased estrogen production in granulosa cells or by alterations in GnRH/LH surge-generating system in Runx3−/− mouse hypothalami. In this chapter, we demonstrated that ovulation was induced in the Runx3−/− ovaries when they were transplanted into wt mice. These results indicate that Runx3−/− mouse ovaries have an ability to respond to gonadotropins and ovulation, and then to ovulate, and that dysfunction of the hypothalamo-pituitary system in Runx3−/− mice is closely associated with anovulation.

Cyp11a1 encodes SCC, which is a key enzyme catalyzing the rate limiting step in the synthesis of steroid hormones in ovaries. In situ hybridization analysis showed that Cyp11a1 mRNA was expressed in theca interna cells, some of the granulosa cells in preovulatory follicles, and corpora lutea, which are in agreement with a previous study [96]. We demonstrated that Cyp11a1 mRNA expression in Runx3−/− mouse ovaries was decreased. Furthermore, the percentage of identifiable Cyp11a1 mRNA-expressing cells in the theca cell layers of Runx3−/− mouse ovaries was decreased. It is highly probable that the decrease in Cyp11a1 mRNA expression in theca cells was one of the reasons for
a decreased estrogen production. Estrogen is a key factor in follicular development, onset of ovulation and uteri growth and functions [68]. Defects in the female reproductive system observed in Runx3−/− mice, such as retarded folliculogenesis, anovulation [27], and atrophied uteri [28], may be attributable to estrogen deficiency. Therefore, it is apparent that Runx3 is a key regulator of ovarian functions because Runx3 regulated aromatase expression in granulosa cells.

In this chapter, we demonstrated increased mRNA levels of Gnrh1 and Fshb in Runx3−/− mice, suggesting that the increased FSH production was caused by the increased GnRH production. GnRH release is regulated by kisspeptin neurons in the AVPV and ARC, and the kisspeptin neurons in ARC are involved in negative feedback system of FSH and LH [53-56]. In addition, we observed elevation of Kiss1 mRNA levels in the ARC in Runx3−/− mice. It may be caused by low estrogen production in ovaries through negative feedback system of hypothalamo-pituitary-ovarian axis. The elevation of Kiss1 mRNA levels in the ARC may probably stimulate Gnrh1 and Fshb mRNA expressions. Therefore, Runx3 expressed in ARC neurons may not be involved in the negative feedback system of Kiss1 mRNA expression in ARC.

In 3-week-old Runx3−/− mice, anterior pituitary Cga, Fshb, and Lhb mRNA levels were decreased in Runx3−/− mice, indicating that FSH and LH syntheses were decreased, although hypothalamic Gnrh1 mRNA levels were not decreased. Decreased Kiss1 mRNA levels in AVPV of Runx3−/− mice may not be associated with decreased FSH and LH production, because kisspeptin neurons in ARC is thought to control the basal secretion of FSH and LH [53, 55] and Kiss1 mRNA levels in the ARC of Runx3−/− mice was not different from those of wt mice. Esr1 mRNA levels in AVPV and ARC did not differ between wt and Runx3−/− mice. Currently, it is not clear whether Runx3 is directly involved in gonadotropin subunit gene expressions and kisspeptin gene expression in AVPV. However, these results suggest that the decreased FSH and LH secretion in
Runx3−/− mice affected the FSH-dependent folliculogenesis and LH-induced androgen production in theca cells. Thus, it is probable that Runx3 regulates ovarian functions through hypothalamo-pituitary systems as well as the intra-ovarian systems.

Ovarian transplantation into wt or Runx3−/− mice demonstrated that corpora lutea were observed in the ovaries of Runx3−/− mice when the ovaries were located in the hormonal milieu of wt mice, and hence ovulation was induced, corresponding to ovarian hyperstimulation experiment [27]. In contrast, the hypothalamo-pituitary system of Runx3−/− mice failed to generate the GnRH/LH surge that induces ovulation because no corpora lutea in the ovaries of wt mice when they were transplanted into Runx3−/− mice. Thus, these findings indicate that the ovaries of Runx3−/− mice were able to ovulate, suggesting that the alteration of hypothalamo-pituitary system by Runx3 deletion cause anovulation in Runx3−/− mice. We demonstrated that the negative feedback system in gonadotropin secretion probably work properly because the elevation of Gnrh1 and Fshb mRNA levels was detected in Runx3+/− mice. On the other hand, ovulation, probably LH surge release, was not induced in Runx3−/− mice. Therefore, the regulatory mechanism of LH surge release, probably the positive feedback system of gonadotropin secretion, was affected in Runx3−/− mice. It is highly probable that Runx3 is involved in the regulatory mechanism of LH surge release, although it remains unclear whether positive feedback system and LH secretion work properly in Runx3−/− mice.
Summary

Adult female Runx3−/− mice show anovulatory and atrophic uteri. In 8-week-old Runx3−/− mice, Cyp11a1 mRNA expression in theca cells was decreased as well as in 3-week-old Runx3−/− mice, suggesting that E2 production was decreased in Runx3−/− mouse ovaries. Furthermore, mRNA levels of Kiss1 in AVPV and Gnrh1 in POA in hypothalami and Fshb in anterior pituitaries were increased, suggesting the negative feedback systems worked properly under low estrogen production in Runx3−/− mice. While, ovarian transplantation demonstrated that Runx3−/− mouse ovaries had an ability to ovulate. Furthermore, it was demonstrated that decrease in Kiss1 mRNA levels in AVPV was detected in 3-week-old and 8-week-old Runx3−/− mice. In 3-week-old Runx3−/− mice, Cga, Fshb, and Lhb mRNA levels were decreased in anterior pituitaries. These findings suggest Runx3 deletion affect the regulation of hypothalamo-pituitary system. Runx3 mRNA expression was also detected in AVPV. These findings suggest that Runx3 may be involved in regulation of ovulation in mouse hypothalamo-pituitary axis.
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<thead>
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<th>gene</th>
<th>Primer List for RT-PCR and real-time PCR</th>
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<td>Runx3</td>
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<tr>
<td>FP</td>
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<tr>
<td>RP</td>
<td>CAGTGACCTTGATGGCTCGGT</td>
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<td>Tm (°C)</td>
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<tr>
<td>Product (bp)</td>
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<td>For real-time PCR</td>
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<td></td>
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<td>GnRH1</td>
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<td>RP CTCTCTGCATACCCGCGATTTCTT</td>
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<td>Esr1</td>
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<td>Lhb</td>
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Table 3. Primers used for RT-PCR and real-time PCR.
### Pre-transplantation

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<th>Genotype</th>
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<td>wt</td>
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<td>3.14 ± 0.34</td>
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<tr>
<td>Runx3⁺⁻</td>
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<td>0.14 ± 0.14 *</td>
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### Post-transplantation

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<th>Experimental group</th>
<th>Donor</th>
<th>Recipient</th>
<th>No of recipients</th>
<th>No of recipients showing successful transplantation (%)</th>
<th>No of estrous cycles during of the period (17 days)</th>
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<td>wt</td>
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<td>5/5 (100%)</td>
<td>2.20 ± 0.20</td>
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<td>Runx3⁺⁻</td>
<td>wt</td>
<td>8</td>
<td>6/8 (75%)</td>
<td>2.17 ± 0.31</td>
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<tr>
<td></td>
<td>wt</td>
<td>Runx3⁺⁻</td>
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<td>5/5 (100%)</td>
<td>0.40 ± 0.24 *</td>
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<td>Runx3⁺⁻</td>
<td>Runx3⁺⁻</td>
<td>3</td>
<td>3/3 (100%)</td>
<td>0 *</td>
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</table>
Table 4. The number of estrous cycles in wt and Runx3−/− mice. Estrous cycles were assessed by vaginal smears for 17 consecutive days. The vaginal cell specimens were observed according to a procedure described in Material and Methods. Results are expressed as mean ± SEM. *P < 0.001, significantly different from wt mice.

Effect of ovarian transplantation between wt and Runx3−/− mice on the occurrence of estrous cycles. Ovarian transplantation was performed using 9- to 10-week-old wt mice and Runx3−/− mice according to the protocol described in Material and Methods. Estrous cycles were assessed by vaginal smears for 17 consecutive days. The vaginal cell specimens were observed according to a procedure described in Material and Methods. Results are expressed as mean ± SEM. *P < 0.001, significantly different from wt recipients transplanted with ovaries from wt donors. The Table was reproduced from Ojima et al. (2016).
Fig. 9. Expression of gonadotropin receptor, steroidogenic acute regulatory protein (StAR), and steroidogenic enzymes in the whole ovary of wt and Runx3<sup>−/−</sup> mice at the age of 8 weeks. Real-time PCR was performed for quantitative analysis of Fshr, Lhcgr, Star, Cyp11a1, Hsd3b1, Cyp17a1, and Cyp19a1 mRNA expression. The amount of each mRNA in wt and Runx3<sup>−/−</sup> 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, significantly different from wt mice. The figure was reproduced from Ojima et al. (2016).
**Fig. 10.** *In situ* hybridization analysis of *Cyp11a1* mRNAs in ovaries from wt and *Runx3*−/− mice at 8 weeks of age. Ovarian sections were obtained from wt mice (A, C, E) and *Runx3*−/− mice (B, D, F). DIG-labeled anti-sense riboprobes for *Cyp11a1* and sense probes were used. *Cyp11a1* mRNA signals were detected in theca cells (C, D). No signals were detected when a sense probe was used for hybridization (E, F). Quantitative analysis of *Cyp11a1* mRNA-positive theca cells in ovaries from wt and *Runx3*−/− mice was performed, and the percentage of *Cyp11a1* mRNA-positive cells in theca cells was calculated (G). * P < 0.05, significantly different from wt mice. Bar = 300 µm (A, B), 50 µm (C-F). The figure was reproduced from Ojima et al. (2016).
**Fig. 11.** *Gnrh1, Kiss1, and Esr1* mRNA levels in wt and *Runx3*−/− mice at 8 weeks of age. *P* < 0.05, **P** < 0.01, significantly different from wt mice. The figure was reproduced from Ojima et al. (2016).
**Fig. 12.** *Gnrh1, Kiss1,* and *Esr1* mRNA levels in wt and *Runx3<sup>−/−</sup>* mice at 3 weeks of age.

*P < 0.05*, significantly different from wt mice. The figure was reproduced from Ojima et al. (2016).
**Fig. 13.** *Cga, Fshb*, and *Lhb* mRNA levels in wt and *Runx3*/*−−* mice at 8 weeks of age. *P* < 0.05, significantly different from wt mice. The figure was reproduced from Ojima et al. (2016).
**Fig. 14.** *Cga, Fshb*, and *Lhb* mRNA levels in wt and *Runx3*<sup>−/−</sup> mice at 3 weeks of age. *P* < 0.05, **P** < 0.01, ***P*** < 0.001, significantly different from wt mice.
Fig. 15. Histological evaluation of ovarian transplantation. Ovarian transplantation was performed using 9- to 10-week-old $\text{Runx3}^{-/-}$ and wt mice according to the procedure described in the Material and Methods. Ovarian grafts were collected from wt-wt (A, E), $\text{Runx3}^{-/-}$-$\text{Runx3}^{-/-}$ (B, F), $\text{Runx3}^{-/-}$-wt (C, G), wt-$\text{Runx3}^{-/-}$ (D, H) mice 17 days after transplantation, and processed for histological observation. The boxed areas depicted in A, B, C, D correspond to the areas shown in E, F, G, H, respectively. CL: corpora lutea. Bar = 300 µm (A-D) and 20 µm (E-H). The figure was reproduced from Ojima et al. (2016).
Chapter 5

Deletion and overexpression of Runx3 affect gene expressions of growth factors in granulosa cells
Introduction

Several evidence indicated the involvement of Runx3 on the regulation of steroidogenesis in mouse ovaries [61]. We demonstrated the decrease in Cyp11a1 mRNA expression in Runx3−/− mouse theca cells, although Runx3 mRNA expression was not detected in theca cells, but in granulosa cells [61], suggesting that Runx3 may regulate growth factors expressed in granulosa cells, which control functions of theca cells. Activins and inhibins, belonging to TGF-β family growth factors, are well known to be regulators of follicular functions [30, 32]. In Chapter 3, we demonstrated the decrease in mRNA levels of activin and inhibit genes in Runx3−/− mouse ovaries and the decrease in FSH responsiveness in Runx3−/− mouse ovaries. Activins regulates FSH responsiveness of granulosa cells [90]. However, it is not clear whether Runx3 was involved in activin and inhibit genes expression. Therefore, the purpose of the present study was to know the role of Runx3 on regulations of activin and inhibit gene expressions. In this chapter, we examined mRNA levels of activin and inhibit genes in Runx3−/− mouse granulosa cells to clarify whether Runx3 involved in regulation of activins genes expression. Interestingly, a recent study showed that Inhbb deletion in granulosa cells affect Cyp11a1 expression [95]. In addition, we demonstrated the decrease in Cyp11a1 and Inhbb mRNA levels in Runx3−/− mouse ovaries. To clarify whether Runx3 involved in Inhbb mRNA expression in granulosa cells, we examined the mRNA level of Inhbb in granulosa tumor cell line (OV3121) with/without Runx3 overexpression. Furthermore, effects of Runx3 overexpression on Runx1 and Runx2 expression were studied, because Runx2 regulates Runx1 expression in rat ovarian follicles [26].
Materials and Methods

Animals

Male and female BALB/c mice were used in this study. Runx3 knockout (Runx3−/−) mice with the BALB/c genetic background were generated as previously described [57]. 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 the Laboratory Animals, Okayama University. Runx3+/− mice were mated, and offsprings were genotyped as previously described [59].

Ovaries from wt and Runx3−/− mice were embedded in O.C.T.Compound (Sakura Finetek Japan), and frozen with liquid nitrogen.

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

Total RNA was extracted from granulosa cells or OV3121 cells using TRIsure Reagent (Bioline), and reverse-transcribed using the Prime Script RT-PCR System (Takara Bio) according to the manufacturer’s instructions. Random hexamers were used for the RT reactions.

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

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 10 sec, 40 cycles 95°C
for 5 sec, and 60°C for 31 sec, followed by a melting-curve analysis (95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec, 60°C for 15 sec). A melting-curve analysis was conducted to confirm the absence of primer dimmers. The primers used in this study are summarized in Table 5. Standard curves were generated by serial dilution of total cDNA, and the amount of each target mRNA level was normalized against the amount of ribosomal protein L19 (Rpl19) mRNA levels.

Riboprobes

Mouse *Inha*, *Inhba*, and *Inhbb* riboprobes were generated according to the previously described method [60]. DNA fragments encoding part of mouse *Inha* (NM_010564.4: 380-821), *Inhba* (NM_008380.1: 296-689), and *Inhbb* (NM_008381.3: 2054-2361) were obtained by RT-PCR using the following primers: mouse *Inha* 5’-TGC ACA GGA CCT CTG AAC CAG -3’ and 5’- ACC AGG AAA GGA GTG GTC TCA -3’; mouse *Inhba* 5’- ACC CTT CCG AAG GAT GGA CCT AA -3’ and 5’- TGG TGA CTT TGG TTC T -3’; mouse *Inhbb* 5’- CTA GAG TGT GAT GGG CGG AC -3’ and 5’- ACA TCC CGC TTG ACA ATG TT -3’. The cDNA fragments were subcloned into the pGEM-3Zf(+) vector. Each plasmid DNA was linearized using restriction enzymes (EcoRI/HindIII (*Inha* and *Inhbb*); EcoRI/BamHI (*Inhba*)) site of pGEM-3Zf(+) and RNA probes were synthesized using a T7 and SP6 polymerase system (Promega) according to the manufacturer’s instructions. The probe was labeled with digoxigenin (DIG) (Roche Diagnostics).

In situ hybridization analysis

Ovaries from wt and *Runx3*−/− mice were embedded in O.C.T.Compound (Sakura Finetek 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)
at 37°C for 10 min, 0.2% glycine in PBS for 20 min, and 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 subjected to hybridization solution containing DIG-labeled anti-sense or sense riboprobes (50 ng/slide) in pre-hybridization solution overnight at 45°C. Following the hybridization for 16 hours, 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 Industries) and 17.5 µg/ml 5-bromo-4-chloro-3′-indoylphosphate p-toluidine salt (Wako Pure Chemical Industries).

**OV3121 cell culture**

OV3121 cells were cultured in RPMI1640 medium (GE Healthcare Life Sciences) with 10% inactivated FBS and seeded at a density of 5×10^5 cells/well in 12-well plates. After 1 day culture, Medium was changed to a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium without phenol red (DMEM/F12; Sigma-Aldrich) with 1% ITS-G Supplement (Wako Pure Chemical Industries), and cells were transfected using Polyethylenimine (GE Healthcare Life Sciences) and Opti-MEM (Invitrogen, Carlsbad, CA, USA) medium according to the manufacturer's directions with 1000 ng Runx3 expression vector (pcDNA3-mRunx3 or pcDNA3 for control) described below. Forty-eight hours later, the cells were collected to analyze of gene expressions.

**Plasmid construction**

Mouse (m) Runx3 expression vector (pcDNA3-mRunx3) was prepared as follows.
Nucleotide fragments for the expression vector of pcDNA3-mRunx3 was prepared by digestion with Hind III and Eco RI of Runx3 cDNAs amplified by RT-PCR with KOD plus Neo (TOYOBO) using the primer pairs and subcloned into the Hind III and Eco RI sites of pcDNA3 expression vector (Invitrogen). The primers for mRunx3 were 1) 5′-CCC AAG CTT GGG AAA CAG CAG CCA ACC AAG -3′ and 2) 5′- CGG AAT TCC GCA CAG CTA GAG AGG ACA TTG -3′. The sequence of pcDNA3-mRunx3 was analyzed by using an ABI PRISM Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and an automatic sequencer ABI PRISM 310 Genetic Analyzer (Applied Biosystems).

**Statistical analysis**

The differences in means between the two groups were analyzed using Student’s $t$-test. The differences were considered significant at $P < 0.05$. 

Results

Expression of Inha, Inhba, and Inhbb mRNA in wt and Runx3−/− mouse granulosa cells

To clarify whether Runx3 deletion affect activin and inhibin genes expressions in granulosa cells, mRNA levels of Inha, Inhba, and Inhbb were examined by quantitative real-time PCR. No significant differences in those mRNA levels were detected (Fig. 16).

In situ hybridization analysis of Inha, Inhba, and Inhbb mRNA expressions in wt and Runx3−/− mouse ovaries

To study Inha, Inhba, and Inhbb mRNA expressions, in ovaries of Runx3−/− mice at the age of 3 weeks in situ hybridization study was performed. In all in situ hybridization studies no signals were detected when sense riboprobes were used as the control analysis (Fig. 17B, H, 18B, H, 19B, H). Inha, Inhba, and Inhbb mRNA signals were detected between wt and Runx3−/− mice (Fig.17A, C·F, 18A, C·F. 19A, C·F). Inhbb mRNA signals were detected in granulosa cells of primary, secondary, and antral follicles in wt mice. Conversely, in Runx3−/− mice, Inhbb mRNA signals were detected mainly in large follicles and scarcely detected in small follicles (Fig. 19I-L).

Inhbb mRNA expression in OV3121 cells with Runx3 overexpression

To clarify whether Runx3 expression affects Inhbb mRNA expression, we studied the effect of Runx3 overexpression in OV3121 cells by real-time PCR. OV3121 cells expressed Inhbb mRNA although Runx3 mRNA expression was quite low (Fig. 20A). OV3121 cells were transiently transfected with Runx3-expression vector plasmid. Expression of Runx3 in the OV3121 cells was verified by detection of Runx3 mRNA expressions by RT-PCR analysis. Inhbb mRNA expression was increased in OV3121 cells by Runx3 mRNA expression (Fig. 20B). Gene expressions of Runx1 and Runx2, and
some other genes were studied, and no significant differences in those mRNA levels were detected (Fig. 20B).

Analysis of Runx3-responsive element in 5′-flanking region of Inhbb

The Runt domain is known to function in the sequence-specific DNA binding to the core DNA sequence, TGTGGT [106]. Within Inhbb gene 5′-flanking region up to -2000 bp (transcription initiation site designated as +1), it was demonstrated that Runt domain consensus DNA sequence TGTGGT was present in -1018/-1013 region by searching transcription factor binding sites using TFBIND (http://tfbind.hgc.jp/).
**Discussion**

Ovarian functions and folliculogenesis are regulated by pituitary hormones, ovarian hormones, and growth factors produced within ovaries. The present study clearly indicates that Runx3 participates in the regulations of ovarian functions and folliculogenesis. It is very likely that Runx3 is one of key regulators of female reproductive functions. The present study showed a candidate gene of Runx3 target genes in mouse ovaries.

We examined mRNA levels of activins and inhibins genes in granulosa cells isolated from wt and Runx3\(^{−/−}\) mouse ovaries, and no significant differences in those mRNA levels were detected although the decrease in *Inha*, *Inhba*, and *Inhbb* mRNA levels had been detected in Runx3\(^{−/−}\) mouse ovaries. Hence, it is probable that the decrease in those mRNA levels in Runx3\(^{−/−}\) mouse ovaries may be partly due to the decrease in the number of granulosa cells that express *Inha*, *Inhba*, and *Inhbb* mRNAs. We demonstrated the decrease in *Inhbb* mRNA expression in Runx3\(^{−/−}\) mouse granulosa cells of small follicles corresponding with retarded early stage of folliculogenesis in Runx3\(^{−/−}\) mice, which was shown in Chapter 3, suggesting that *Inhbb* expression was reduced particularly in the primary and secondary follicles in Runx3\(^{−/−}\) mice. These findings suggested the decrease in expression of activins and/or inhibins in Runx3\(^{−/−}\) mouse granulosa cells. *Inhbb* mRNA expressions in large follicles in Runx3\(^{−/−}\) mouse ovaries may be stimulated by another regulatory factors because activins are known to be regulated by growth factors produced in follicles [107]. From these findings, it is probable that Runx3 may regulate follicular functions through growth factors produced in granulosa cells, and part of the growth factors may contain *Inhbb* transcript-derived protein.

OV3121 cells are granulosa cell-derived tumor cell line, but are devoid of Runx3 mRNA expression. Runx3 was overexpressed with acute transfection of
Runx3 expression vector. Runx3 mRNA expression was successfully detected by RT-PCR. In the OV3121 cells with Runx3 overexpression Inhbb mRNA expression was elevated compared with the control, suggesting that Runx3 stimulated Inhbb mRNA expression. Thus, Inhbb may be one of Runx3 target genes in mouse ovaries.

Runt domain functions in the sequence-specific DNA binding to the core DNA sequence, TGTGGT [106], and Runt domain consensus DNA sequence was present in -1018/-1013 region of upstream Inhbb gene 5´-flanking region, suggesting that Runx3 may be involved in regulation of Inhbb expression in granulosa cells through this possible Runx3-binding site. However, further study is needed to characterize this putative site.

Runx2 regulates Runx1 expression in granulosa cells [26]. However, the mRNA levels of Runx1 and Runx2 were not affected in OV3121 cells with Runx3 overexpression, suggesting that Runx3 may not be involved in regulation of the other Runx family expression in granulosa cells.
Summary

Ovarian functions and folliculogenesis are regulated by pituitary hormones, ovarian hormones, and growth factors produced within ovaries. The mRNA levels of activins and inhibins genes in granulosa cells isolated from wt and Runx3−/− mouse ovaries were studied by real-time PCR analysis. Cyp11a1 mRNA expression was decreased in Runx3−/− mouse ovarian follicles, suggesting that function of theca cells may be regulated by Runx3. It is probable that Runx3-regulated growth factors produced in granulosa cells may regulate steroidogenesis in theca cells because Runx3 mRNA was expressed in granulosa cells. Activins and inhibins produced in granulosa cells are known to regulate steroidogenesis in theca cell. The mRNA levels of activins and inhibins gene, Inha, Inhba, and Inhbb, were decreased in ovaries, and Inhbb mRNA expression was scarcely detected in granulosa cells of small follicles in Runx3−/− mouse ovaries. In addition, Inhbb mRNA expression was increased in OV3121 cells with Runx3 overexpression. Runt domain consensus DNA sequence was present in -1018/-1013 region of upstream Inhbb gene 5′-flanking region. These findings suggest that Runx3 may regulate follicular functions through growth factors produced in granulosa cells, and part of the growth factors may contain Inhbb transcript-derived protein. While, Runx3 overexpression did not affect the mRNA levels of Runx1 and Runx2, suggesting that Runx3 may not be involved in regulation of the other Runx family members expressions.
Table 5. Primer List for RT-PCR and real-time PCR

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<th>5' - sequence - 3'</th>
<th>Tm (°C)</th>
<th>Product (bp)</th>
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<td>ACCAGGAAAGGATGGTCTCA</td>
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<tr>
<td>Inhba</td>
<td>ACCCTTCCGAAGGGATGCCCTAA</td>
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<td></td>
<td>TGGTGACTTTGGTCCTGGTTCT</td>
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<td>ACATCCCGCTTGACAAATGTT</td>
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<td>Rpl19</td>
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For real-time PCR

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<td>GGATGGAGGAGGAAAGGTATGG</td>
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<td>TGCGGCAATACATAAGTAGA</td>
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<td>Inhba</td>
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<td>TGTCCTGGTTCTGGTTAGGCC</td>
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<tr>
<td>Inhbb</td>
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<td></td>
<td>133</td>
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<tr>
<td></td>
<td>CTGATCGGCGTGAAGCGAAGA</td>
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<td>Rpl19</td>
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<td>58</td>
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<tr>
<td></td>
<td>GTGTCACAGGCTTGCGAGGATGA</td>
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Table 5. Primers used for RT-PCR and real-time PCR.
**Fig. 16.** Expression of growth factor genes in freshly prepared granulosa cells of wt and Runx3<sup>−/−</sup> mice. 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<sup>−/−</sup> mice was normalized against that of Rpl19 mRNA. Each group consists of nine mice. Data are expressed as the mean ± SEM of triplicate wells.
**Fig. 17.** *In situ* hybridization analysis of *Inha* mRNA in ovaries of wt and *Runx3*<sup>−/−</sup> female mice. Ovary sections were obtained from 3-week-old wt (A–F) and *Runx3*<sup>−/−</sup> (G–L) mice. DIG-labeled *Inha* anti-sense (A, C–G, I–L) and sense (B, H) RNA probe were used for analysis. *Inha* mRNA signals in wt and *Runx3*<sup>−/−</sup> mouse ovaries were detected in granulosa cells of primary (C, I), secondary (D, J), preantral (E, K), and antral (F, L) follicles. No signals were detected when the sense probe was used for hybridization (B, H). Bar = 300 µm (A, B, G, H), 50 µm (C–F, I–L).
Figure 18. *In situ* hybridization analysis of *Inhba* mRNA in ovaries of wt and *Runx3*−/− female mice. Ovary sections were obtained from 3-week-old wt (A–F) and *Runx3*−/− (G–L) mice. DIG-labeled *Inhba* anti-sense (A, C–G, I–L) and sense RNA probes (B, H) were used for analysis. *Inhba* mRNA signals in wt and *Runx3*−/− mouse ovaries were detected in granulosa cells of primary (C, I), secondary (D, J), preantral (E, K), and antral (F, L) follicles. No signals were detected when the sense probe was used for hybridization (B, H). Bar = 300 µm (A, B, G, H), 50 µm (C–F, I, J–L).
Figure 19. *In situ* hybridization analysis of *Inhbb* mRNA in ovaries of wt and *Runx3*<sup>−/-</sup> female mice. Ovary sections were obtained from 3-week-old wt (A–F) and *Runx3*<sup>−/-</sup> (G–L) mice. DIG-labeled *Inhbb* anti-sense (A, C–G, I–L) and sense RNA probes (B, H) were used for analysis. In wt mouse ovaries *Inhbb* mRNA signals were detected in granulosa cells of primary (C), secondary (D), preantral (E), and antral (F) follicles. In *Runx3*<sup>−/-</sup> ovaries *Inhbb* mRNA signals were detected in antral follicles (L), but not detected in granulosa cells of primary (I), secondary (J), and preantral (K) follicles. No signals were detected when the sense probe was used for hybridization (B, H). Bar = 300 µm (A, B, G, H), 50 µm (C–F, I–L).
A

Inhbb
Runx3
Rpl19

B

Runx1

Runx2

Inha

Inhba

Inhbb

Relative mRNA levels

Relative mRNA levels

Relative mRNA levels

Relative mRNA levels

pcDNA3 pcDNA3-mRunx3 pcDNA3 pcDNA3-mRunx3
**Figure 20.** Effect of Runx3 on mRNA levels of *Runx1*, *Runx2*, *Inha*, *Inhba*, and *Inhbb* in OV3121 cells. The expression of *Inhbb* and *Runx3* mRNA were analyzed in OV3121 cells. OV3121 cells were transfected with Runx3 expression plasmid vector (pcDNA3-mRunx3) for 48 hours. The expression of *Runx3* mRNA was analyzed after transfection (A). Real-time PCR was performed for quantitative analysis of *Runx1*, *Runx2*, *Inha*, *Inhba*, and *Inhbb* mRNA expression. The amount of each mRNA in pcDNA3 and pcDNA3-mRunx3 was normalized against that of *Rpl19* mRNA (B). Data are expressed as the mean ± SEM of triplicate wells. * P < 0.05, significantly different from transfection with pcDNA3.
Chapter 6

General discussion
Transcription factors of Runx family members (Runx1, Runx2, and Runx3) are involved in female reproductive functions. Runx1 and Runx2 are well known to be expressed in luteinizing granulosa cells and regulate ovarian functions through the respective target genes expression [20, 23, 26]. Runx3 is known to act as a key regulator in ovulation in mouse ovaries because retardation of follicular development and anovulatory resulting in infertile in Runx3−/− mice [27]. Moreover, Tsuchiya et al (2012) demonstrated the atrophic uteri in Runx3−/− mice, suggesting decline in function of steroidogenesis in Runx3−/− mouse ovaries [28]. Nevertheless, little is known about Runx3 in regulation of female reproductive functions.

It seems essential to clarify the localization of Runx3 in mouse ovaries, the role of Runx3 in regulation of folliculogenesis, steroidogenesis, and ovulation and the candidate gene regulated by Runx3, although Runx3 mRNA and Runx3 protein are expressed in mouse ovaries [27, 28]. In the present study following studies have been described. In Chapter 2, Runx3 mRNA expressing cells were identified in ovaries and hypothalami by RT-PCR and in situ hybridization analyses. In ovaries, Runx3 mRNA was expressed in granulosa cells, suggesting that Runx3 was expressed in granulosa cells in mouse ovaries. In hypothalami Runx3 mRNA was expressed in AVPV and ARC, suggesting that Runx3 may play roles in respective organs in hypothalamo-pituitary-ovarian axis. In Chapter 3, effects of Runx3 deletion on folliculogenesis and steroidogenesis in immature mice were studied. Follicular development was retarded in 3-week-old Runx3−/− mouse ovaries. The mRNA levels of steroidogenic enzyme genes were decreased in Runx3−/− mouse ovaries. E2 production in Runx3−/− mouse granulosa cells was decreased. These findings suggest that Runx3 was involved in regulation of folliculogenesis and steroidogenesis. In Chapter 4, effects of Runx3 deletion on steroidogenesis and ovulation in adult mice were studied. The mRNA levels of steroidogenic enzyme genes were decreased in Runx3−/− mouse ovaries. The
mRNA levels of kisspeptin and GnRH genes in hypothalami and gonadotropin genes in anterior pituitaries were altered in Runx3−/− mice. Furthermore, ovarian transplantation demonstrated that Runx3−/− mouse ovaries have an ability to ovulate. These findings suggest that Runx3 is involved in steroidogenesis and ovulation in hypothalamo-pituitary system and intra-ovarian system, respectively. In Chapter 5, the candidate target gene regulated by Runx3 in granulosa cells was provided. Runx3 overexpression affected Inhbb mRNA expression in granulosa tumor cell line. Therefore, the present study suggests that Runx3 is involved in regulation of Inhbb mRNA expression in granulosa cells.

Runx3 expression in hypothalamo-pituitary-ovarian axis

Recent studies including the present study have shown that the runt-related transcription factor family takes part in the regulation of ovarian functions in rats and mice. Runx1 and Runx2 are involved in the regulation of the LH-induced ovulatory process and luteinization in the rat ovary [19-21]. We reported that Runx3 knockout in mice resulted in alterations in follicular development, and no ovulation [27]. The present study shows that Runx3 mRNA was expressed in granulosa cells of mouse ovaries by in situ hybridization analysis, and in the AVPV and ARC areas of female mouse hypothalami by RT-PCR analysis [61]. These findings suggest that ovarian Runx3 is involved in regulation of follicular functions through intra-ovarian systems, which differed from those operated by Runx1 and Runx2, and that hypothalamic Runx3 is involved in regulation of ovulation in hypothalamo-pituitary systems.

Runx3 in ovarian steroidogenesis

Cyp11a1 encodes SCC, which is a key enzyme in the synthesis of steroid hormones in ovaries. In 3-week-old Runx3−/− mice, Cyp11a1 mRNA expression in theca
cells and Cyp19a1 mRNA expression in granulosa cells were decreased. E2 production was diminished in Runx3−/− mouse granulosa cells. In 8-week-old Runx3−/− mice, Cyp11a1 mRNA expression in theca cells was decreased, suggesting the decrease in E2 production as well as in 3-week-old Runx3−/− mice, which well accounted for the atrophic uteri in 8-week-old Runx3−/− mice [28]. Cyp11a1 encodes SCC, which is a key enzyme of steroidogenesis in mouse ovaries. The decrease in Cyp11a1 mRNA level and the number of Cyp11a1 mRNA-expressing theca cells were consistently detected across immature and adult Runx3−/− mice. Therefore, Runx3 deletion affected Cyp11a1 mRNA expression in theca cells, resulting in low androgen production. It is highly probable that E2 production was diminished in the follicles in Runx3−/− mice, because androgens are substrates of aromatase, and the supply of androgen to granulosa cells may be reduced. In addition, Gnrh1 mRNA levels in POA and Kiss1 mRNA levels in ARC in Runx3−/− mice were increased and Fshb mRNA in anterior pituitaries in Runx3−/− mice were also increased, suggesting proper function of negative feedback system induced by low estrogen level in Runx3−/− mice. Therefore, these results indicate the hypoactivity of steroidogenesis in Runx3−/− mouse ovaries, suggesting that Runx3 probably participates in regulation of steroidogenesis in ovarian follicles.

Runx3 in ovarian folliculogenesis

The members of the TGF-β superfamily expressed in granulosa cells are involved in the regulation of folliculogenesis [108], although estrogen produced in granulosa cells is necessary for follicular development [67-69]. Folliculogenesis is also regulated by FSH [90]. In 8-week-old Runx3−/− mouse ovaries, follicular development is retarded [27]. The present study demonstrated that the retardation of folliculogenesis is observed in 3-week-old Runx3−/− mouse ovaries. Activin and inhibin gene mRNA expressions were decreased, and Inhbb mRNA expression tends to decrease in granulosa
cells of small follicles in Runx3<sup>−/−</sup> mouse ovaries at the age of 3 weeks, suggesting that the retardation of the early stage of folliculogenesis was caused by decrease in activins and inhibin in Runx3<sup>−/−</sup> mouse ovaries. In 3-week-old Runx3<sup>−/−</sup> mouse anterior pituitaries, mRNA expressions of gonadotropin β subunit genes were decreased corresponding with the decrease in the number of antral follicles. In addition, FSH responsiveness in Runx3<sup>−/−</sup> mouse granulosa cells was decreased. While, in 8-week-old Runx3<sup>−/−</sup> mouse anterior pituitaries, however, Fshb mRNA expression was increased, suggesting that retardations of folliculogenesis in Runx3<sup>−/−</sup> mouse ovaries may be caused by alteration in intra-ovarian estrogen production and decrease in FSH responsiveness. These findings suggest that Runx3 plays a role in follicular development through involvement in activins and inhibins expression because TGF-β family factors regulates folliculogenesis and Runx3 involved in TGF-β signaling [14, 29, 30, 108].

Runx3 in regulation of ovulation

Sakuma et al (2008) demonstrated that female Runx3<sup>−/−</sup> mice are anovulatory and infertile, and on the contrary, Runx3<sup>−/−</sup> mice are able to ovulate by superovulation treatment [27]. In the present study, Runx3<sup>−/−</sup> mouse ovaries contained corpora lutea when transplanted into wt mice, supporting that Runx3<sup>−/−</sup> mouse ovaries have an ability to ovulate. In adult Runx3<sup>−/−</sup> mice, mRNA levels of Kiss1 in ARC and Gnrh1 in POA in hypothalami and Fshb in anterior pituitaries were increased, suggesting that negative feedback caused by low estrogen level properly worked in Runx3<sup>−/−</sup> mouse hypothalamo-pituitaries system. No corpora lutea in wt mouse ovaries when transplanted into Runx3<sup>−/−</sup> mice, and decrease in Kiss1 mRNA levels in AVPV is consistently detected among 3-week-old and 8-week-old Runx3<sup>−/−</sup> mice, suggesting that positive feedback systems may be altered in Runx3<sup>−/−</sup> mice. Still it is not clear whether
Runx3−/− mouse hypothalamo-pituitary systems were able to properly respond to E2 although Esr1 mRNA level was not different from those of wt mice. From these findings, we hypothesized that Runx3 takes parts in regulation of AVPV-residing kisspeptin-neuron functions. Further analyses on identification of Runx3-expressing neurons in the hypothalamus, and the functioning of positive feedback mechanism to estrogen shall be studied.

**Runx3 in regulation of growth factors expressions in granulosa cells**

Runx3 is well known to be closely associated with in TGF-β signaling pathway, and TGF-β family factors regulate follicular functions [15, 29, 109]. The present study demonstrated Runx3 mRNA expression in granulosa cells. Interestingly, Cyp11a1 mRNA expression in theca cells was decreased in Runx3−/− mouse ovaries, suggesting that the theca cell functions were regulated by Runx3-regulated growth factors produced in granulosa cells. Inha, Inhba, and Inhbb mRNA levels were decreased in Runx3−/− mouse ovaries. Especially, Inhbb mRNA in small follicles were diminished in Runx3−/− mouse ovaries by in situ hybridization analysis. A recent study reports that Inhbb knockdown affects Cyp11a1 expression [95]. The decrease in Cyp11a1 mRNA expression in Runx3−/− mouse ovaries were similar to Inhbb knockdown mice.

Granulosa cell-derived tumor cell OV3121 cells were devoid of Runx3 expression. OV3121 cells were transfected with Runx3-expression vector. Runx3 mRNA was successfully expressed in OV3121 cells. In OV3121 cells with Runx3 overexpression, Inhbb mRNA level was increased, suggesting that Runx3 is involved in regulation of Inhbb mRNA expression in granulosa cells. This was supported by the presence of putative Runx3-responsive element in 5′-flanking region of Inhbb.

The present study clearly indicates that Runx3 is one of key regulators of female
reproductive functions including folliculogenesis, steroidogenesis, and ovulation. Runx3 deletion severely affected gene expression of steroidogenic enzymes, in particular, *Cyp11a1* and *Cyp19a1*, resulting in the low production of estrogen in granulosa cells. The deficiency of estrogen production may lead to the retarded folliculogenesis and anovulation. On the other hand, *Runx3* was expressed in the AVPV, in which GnRH neurons and kisspeptin neurons are located. It is highly probable that Runx3 exerts roles in the regulation of positive feedback system of gonadotropin secretion. Runx3 may be involved in the regulation of AVPV-kisspeptin-expressing neurons in female mice.

*Runx3* mRNA expression was detected in granulosa cells of primary follicles, secondary follicles, and antral follicles, which differed from Runx1 and Runx2 expressions. Runx family transcription factors differentially regulate ovarian functions ovulation. Importantly, Runx3 in the AVPV area of mouse hypothalami may control LH-surge generation that induces ovulation. Thus, Runx3 seems to exert actions in various systems in the hypothalamo-pituitary-ovary system. From these findings, it is concluded that Runx3 is one of key regulators in female reproductive functions, and is an essential factor for ovarian functions and ovulation.
General summary

Transcription factors of Runx family (Runx1, Runx2, and Runx3) are involved in female reproductive functions. Runx1 and Runx2 are well known to be expressed in luteinizing granulosa cells and regulate ovarian functions through the expression of respective target genes. However, little is known about the role of Runx3 in the regulation of female reproductive functions although Runx3 knockout mice show anovulatory. The purpose of the present study was to clarify the mechanism by which ovarian functions are regulated by Runx3 in mice.

Identification of Runx3 mRNA expressing cells in ovaries and hypothalami in mice

In ovaries, the expression of Runx3 mRNA was detected in granulosa cells. In hypothalami by in situ hybridization, on the other hand, Runx3 mRNA was detected in AVPV and ARC. These results suggest that Runx3 may play roles in respective organs in hypothalamo-pituitary-ovarian axis.

Effects of Runx3 knockout on folliculogenesis and steroidogenesis in immature mice

Follicular development was retarded in ovaries of 3-week-old Runx3−/− mice. In Runx3−/− mice, the mRNA levels of steroidogenic enzyme genes were decreased and E2 production was decreased. These findings suggest that Runx3 is involved in the regulation of folliculogenesis and steroidogenesis in ovaries.

Effects of Runx3 knockout on steroidogenesis and ovulation in adult mice

The mRNA levels of steroidogenic enzyme genes were decreased in ovaries of Runx3−/− mice. The mRNA levels of kisspeptin and GnRH genes in hypothalami and those of gonadotropin genes in anterior pituitaries were altered in Runx3−/− mice. Furthermore, ovarian transplantation demonstrated that ovaries of Runx3−/− mice have an ability to ovulate. These findings suggest that Runx3 is involved in steroidogenesis and ovulation in intra-ovarian system and hypothalamo-pituitary system, respectively.
The candidate target gene of Runx3 in granulosa cells

Runx3 overexpression has been shown to affect Inhbb mRNA expression in granulosa tumor cell line, suggesting that Runx3 is involved in regulation of Inhbb mRNA expression in granulosa cells.

From these findings, it is concluded that Runx3 is one of key regulators in female reproductive system, and is essential for ovarian functions and ovulation.
Acknowledgements

I would like to express my hearty thank to Prof. Sumio Takahashi for his valuable comments and discussions through this work. I would like to express my hearty thank to Prof. Sakae Takeuchi for his valuable comments encouragement in this work. I wish to thank Prof. Tatsuya Sakamoto for his valuable comments encouragement in this work. I thank Dr. Hiroshi Fukamachi, Tokyo Medical and Dental University, Prof. Kosei Ito, Nagasaki University, and Prof. Yoshiaki Ito, Institute of Molecular and Cell Biology, Singapore, for their valuable comments encouragement in this work. I thank Prof. Fumio Otsuka, Okayama University, for his valuable comments encouragement on this work. I also thank assistant Prof. Maho Ogoshi and associate Prof. Hirotaka Sakamoto for their valuable comments encouragement on this work. I gratefully acknowledge the work of past and present members of Endocrine Research laboratory.
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