Runx3 transcription factor regulates ovarian functions and ovulation in female mice

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Abstract. We previously demonstrated that the Runx3 transcription factor is expressed in the hypothalami, pituitaries, and ovaries of mice, and that Runx3 knockout (Runx3−/−) mice are anovulatory and their uteri are atrophic. Runx3 mRNA expression was detected in the granulosa cells of ovarian follicles, and in the anteroventral periventricular nucleus (AVPV) and arcuate nucleus (ARC). In the present study, we examined the effects of Runx3 knockout on the gene expression of enzymes associated with steroidogenesis. We found decreased Cyp11a1 mRNA expression in Runx3−/− mouse ovaries compared with that in wild-type (wt) mouse ovaries at the age of 8 weeks. In situ hybridization analysis showed that the percentages of Cyp11a1 mRNA-expressing theca cells in follicles of Runx3−/− mice were decreased compared with those of wt mice. In accord with the alterations in Runx3−/− mouse ovaries, Kiss1 mRNA levels in ARC were increased, whereas mRNA levels of kisspeptin in AVPV were decreased, and gonadotropin-releasing hormone in the preoptic area and follicle-stimulating hormone β subunit gene were increased in Runx3−/− mice. Following an ovarian transplantation experiment between Runx3−/− mice and wt mice, corpora lutea were observed when ovaries from Runx3−/− mice were transplanted into wt mice, but not when those from wt mice were transplanted into Runx3−/− mice, suggesting that Runx3 in the hypothalamo-pituitary system may drive gonadotropin release to induce ovulation in the ovary. These findings indicate that Runx3 plays a crucial role in the hypothalamo-pituitary-gonadal axis.

Key words: Mouse, Ovary, Ovulation, Steroidogenesis

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Ovaries obtained from Runx3 knockout (Runx3−/−) mice with the BALB/c genetic background were generated as previously described [18]. All animal care and experimentation was approved by the Animal Care and Use Committee, Okayama University, and was conducted in accordance with the Policy on the Care and Use of Laboratory Animals, Okayama University. Runx3−/− mice were mated, and the offspring were genotyped as described in a previous study [20]. Ovaries from 3-week-old wt mice were removed and dissected free of connective tissue. The ovaries obtained from Runx3−/− mice to wt or Runx3−/− mice, and then ovulation in grafted ovaries was examined by histological observation of grafts.

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 [18]. All animal care and experimentation was approved by the Animal Care and Use Committee, Okayama University, and was conducted in accordance with the Policy on the Care and Use of Laboratory Animals, Okayama University. Runx3−/− mice were mated, and the offspring were genotyped as previously described [12, 19].

Ovarian granulosa cell isolation

Granulosa cell isolation was performed according to a method described in a previous study [20]. Ovaries from 3-week-old wt mice were removed and dissected free of connective tissue. The ovaries were incubated in M199 medium (Sigma-Aldrich, St. Louis, MO, USA) 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 (5 min at 500 × g 4°C) cells were collected.

Brain sample and anterior pituitary sample collection

Whole brains of 8-week-old wt mice at the diestrous stage and Runx3−/− mice were rapidly removed from the skull and frozen in liquid nitrogen. Two parts, one containing the POA and A VPV and the other containing the ARC, were dissected from the frozen brain. The dissected brain sample was further coronally cut 1 mm behind the optic chiasma, dorsally at the upper portion of the third bodies. The dissected brain sample was further coronally cut at the posterior border of the mammillary bodies. The dissected sample was 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 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 (Cyp11a1 and Cyp19a1). Following hybridization for 16 h, the sections were incubated with alkaline phosphatase (AP)-conjugated anti-DIG-antibody (Roche Diagnostics) in blocking solution overnight at 4°C. Hybridization signals were detected in AP buffer containing 35 µg/ml nitro-blue tetrazolium chloride (Wako Pure Chemical, Osaka, Japan) at 37°C for 10 min and 0.2% glycine in PBS for 20 min, and then acetylated 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 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 (Runx3) or 45°C (Cyp11a1 and Cyp19a1). Following hybridization for 16 h, the sections were incubated with alkaline phosphatase (AP)-conjugated anti-DIG-antibody (Roche Diagnostics) in blocking solution overnight at 4°C. Hybridization signals were detected in AP buffer containing 35 µg/ml nitro-blue tetrazolium chloride (Wako Pure Chemical, Osaka, Japan) and 17.5 µg/ml 5-bromo-4-chloro-3′-indoly phosphate p-toluidine salt (Wako Pure Chemical). 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.

Riboprobes

Mouse Runx3, Cyp11a1, and Cyp19a1 riboprobes were generated according to a previously described method [21]. DNA fragments encoding part of mouse Runx3 (NM_019732; 1770–2071), Cyp11a1 (NM_019779.3; 630–1055), and Cyp19a1 (NM_007810.3; 286–789) were obtained by reverse transcription-polymerase chain reaction (RT-PCR) using the following primers: mouse Runx3: 5′-CTC CAG CCC GAG ACT ACA AG-3′ and 5′-AGG GAG GGA GAG AAGATGC CA-3′; mouse Cyp11a1: 5′-CTT TTG AGT CCA ATC TCA GCA GTG-3′ and 5′-GTA CCT TCA AGT TGT GTG CCA-3′; mouse Cyp19a1: 5′-GAG AGT TCA TGA GAG TCT GG-3′ and 5′-CCT TGA CGG ATC GTT CAT AC-3′. The cDNA fragments were subcloned into the pGEM-3z(+) vector. Each plasmid DNA was linearized using restriction enzyme (EcoRI/HindIII) sites of pGEM-3z(+) 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−/− mice were embedded in O.C.T. Compound (Sakura Finetek Japan, Tokyo, Japan), frozen with liquid nitrogen, and sectioned on 10-µm thickness using a cryostat. The dried 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.

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

Total RNA was extracted from tissues using TRIzol Reagent (Bioline, London, UK), 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 performed 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 light.

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: an 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). Melting curve analysis was conducted to confirm the absence of primer dimers. 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.

**Ovarian transplantation**

Ovary collection and transplantations were performed simultaneously. 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 Runx3−/− and wt mice. Ovarian transplantation experiments were carried out as follows: wt ovaries were grafted to wt mice (designated as wt wt); Runx3−/− ovaries were grafted to wt mice (Runx3−/− wt); wt ovaries were grafted to Runx3−/− mice (wt Runx3−/−); Runx3−/− ovaries were grafted to Runx3−/− mice (Runx3−/− Runx3−/−). Nine- to ten-week-old wt and Runx3−/− mice received ovarian grafts from mice of the same age. In the transplantation of wt Runx3−/− mice, ovaries from 4-week-old wt mice were grafted to 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.

**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**

**Expression of Runx3 mRNA in granulosa cells and hypothalamus in female mice**

Granulosa cells were isolated from the ovaries of 3-week-old mice, and were confirmed by detection of the expression of Fshr and Cyp19a1 (Fig. 1A). Runx3 mRNA was detected in the isolated granulosa cells by RT-PCR. 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 as well as in granulosa cells (Fig. 1B). Runx3 mRNA expression in ovaries was verified by in situ hybridization (Fig. 1C, E). Runx3 mRNA hybridization signals were detected in the granulosa cells of antral follicles. No signals were detected with the sense probe (Fig. 1D, F).

**Expression of genes involved in regulation of steroidogenesis in the ovaries of wt and Runx3−/− mice**

To determine whether Runx3 deletion can affect 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. 2).

**In situ hybridization analysis of Cyp11a1 and Cyp19a1 mRNA expression in wt and Runx3−/− mouse ovaries**

Cyp11a1 and Cyp19a1 mRNA expressions in 8-week-old mice were analyzed using DIG-labeled riboprobes. In all in situ hybridization studies, no signals were detected when sense riboprobes were used as the control analysis.

Cyp11a1 mRNA: In wt mice, Cyp11a1 mRNA signals were detected in the interstitial cells and theca interna cells of secondary and antral follicles. 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. 3A, C). Intense signals were detected in the corpora lutea (Fig. 3A). 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. 3B, D, G). No signals were detected with the sense probe (Fig. 3E, F).

Cyp19a1 mRNA: In wt mice, Cyp19a1 mRNA signals were detected in the granulosa cells of antral follicles in both wt and Runx3−/− mice (Fig. 4A, B). Cyp19a1 mRNA expressions in granulosa cells did not differ between wt and Runx3−/− mice (Fig. 4C, D). These results were consistent with the results obtained from quantitative real-time PCR analyses (Fig. 2). No signals were detected with the sense probe (Fig. 4E, F).

**Expression of Gnrh1, Kiss1, Cga, Fshb, and Lhb mRNA in wt and Runx3−/− mice**

To clarify alterations of the hypotalamo-pituitary system in Runx3−/− mice, mRNA levels of GnRH, kisspeptin, FSH, and LH genes were analyzed by real-time PCR. Gnrh1 mRNA levels were significantly higher than those in wt mice (Fig. 5A). Kiss1 mRNA levels in AVPV in Runx3−/− mice were significantly lower than those in wt mice (Fig. 5B), whereas Kiss1 mRNA levels in ARC were significantly higher than those in wt mice (Fig. 5B). 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−/− mice (data not shown). Fshb mRNA levels in anterior pituitaries were significantly higher in Runx3−/− mice than those in wt.
Table 1. Primers used for RT-PCR and real-time PCR

| Gene   | 5’ - sequence - 3’ |  |  | Tm (°C) |  |
|--------|-------------------|---|---|---------|---|
| Runx3  | FP AAGTGGCCGCTGATGGTGGAGCG |  |  | 65      | 369 |
|        | RP CAGTGACCTTGGTGGCTCGGT |  |  | 60      | 350 |
| Fshr   | FP GGAGGCGCGAAAAACTCTGGA |  |  | 60      | 338 |
|        | RP CCGGGGGCACAGTGAAGTTTGGT |  |  | 60      | 426 |
| Nhcg   | FP TGGATTGTTCCAGGCAGACAT |  |  | 55      | 391 |
|        | RP TTGGAGTCTACTGGAGCAATG |  |  | 55      | 562 |
| Cyp11a1| FP CTTTTGAGTCCATCACGCAGTGG |  |  | 55      | 504 |
|        | RP GACGTCGGAGCTCTCTGCTT |  |  | 100     | 60  |
| Cyp17a1| FP CTGCTCATCCTGGCCTATTTTCTT |  |  | 101     | 57  |
|        | RP TTGTGACAGAGTGGATTCCACAT |  |  | 121     | 120 |
| Cyp19a1| FP GAGAAGTTCAAGAGTGAAGTGGG |  |  | 120     | 250 |
|        | RP GGCCCGTCAGAGCTTTCA |  |  | 120     | 55  |
| Hsd3b1 | FP AACCCGACGCTTTGAGTGGTGAAGCAACTA |  |  | 101     | 101 |
|        | RP AAGCAGCAGCAGTGGCTCT |  |  | 69      | 69  |
|        | RP GTGATGGTGAAGTGGGCAGACATG |  |  | 154     | 154 |
|        | RP CCAATCTGGCAGCTTGCAGCTTCTT |  |  | 62      | 62  |
|        | RP TTTGGGAGTCTACTGAGGCAATGGAGCAACTA |  |  | 70      | 70  |
|        | RP GGGCCGATACGGCATCAGCTTCTC |  |  | 67      | 67  |
|        | RP GGTGTCAGAGTGGCAGAGGA |  |  | 58      | 58  |

For RT-PCR

| Gene   | 5’ - sequence - 3’ |  |  | Tm (°C) |  |
|--------|-------------------|---|---|---------|---|
| Runx3  | FP AAGTGGCCGCTGATGGTGGAGCG |  |  | 65      | 369 |
|        | RP CAGTGACCTTGGTGGCTCGGT |  |  | 60      | 350 |
| Fshr   | FP GGAGGCGCGAAAAACTCTGGA |  |  | 60      | 338 |
|        | RP CCGGGGGCACAGTGAAGTTTGGT |  |  | 60      | 426 |
| Nhcg   | FP TGGATTGTTCCAGGCAGACAT |  |  | 55      | 391 |
|        | RP TTGGAGTCTACTGGAGCAATG |  |  | 55      | 562 |
| Cyp11a1| FP CTTTTGAGTCCATCACGCAGTGG |  |  | 55      | 504 |
|        | RP GACGTCGGAGCTCTCTGCTT |  |  | 100     | 60  |
| Cyp17a1| FP CTGCTCATCCTGGCCTATTTTCTT |  |  | 101     | 57  |
|        | RP TTGTGACAGAGTGGATTCCACAT |  |  | 121     | 120 |
| Cyp19a1| FP GAGAAGTTCAAGAGTGAAGTGGG |  |  | 120     | 250 |
|        | RP GGCCCGTCAGAGCTTTCA |  |  | 120     | 55  |

For real-time PCR

| Gene | 5’ - sequence - 3’ |  |  | Tm (°C) |  |
|------|-------------------|---|---|---------|---|
| Runx3 | FP AAGTGGCCGCTGATGGTGGAGCG |  |  | 65      | 369 |
|        | RP CAGTGACCTTGGTGGCTCGGT |  |  | 60      | 350 |
| Fshr  | FP GGAGGCGCGAAAAACTCTGGA |  |  | 60      | 338 |
|        | RP CCGGGGGCACAGTGAAGTTTGGT |  |  | 60      | 426 |
| Nhcg  | FP TGGATTGTTCCAGGCAGACAT |  |  | 55      | 391 |
|        | RP TTGGAGTCTACTGGAGCAATG |  |  | 55      | 562 |
| Cyp11a1| FP CTTTTGAGTCCATCACGCAGTGG |  |  | 55      | 504 |
|        | RP GACGTCGGAGCTCTCTGCTT |  |  | 100     | 60  |
| Cyp17a1| FP CTGCTCATCCTGGCCTATTTTCTT |  |  | 101     | 57  |
|        | RP TTGTGACAGAGTGGATTCCACAT |  |  | 121     | 120 |
| Cyp19a1| FP GAGAAGTTCAAGAGTGAAGTGGG |  |  | 120     | 250 |
|        | RP GGCCCGTCAGAGCTTTCA |  |  | 120     | 55  |

FP: forward primer, RP: reverse primer.

Discussion

The present study showed that Runx3 mRNA was expressed in the granulosa cells of ovarian follicles, and in the AVPV and ARC areas of female mice, and that Runx3 deletion decreased Cyp11a1 mRNA expression in mouse ovaries. Furthermore, Gnrh1 and Kiss1 mRNA expressions were affected in Runx3−/− mice. The significant decrease in Cyp11a1 mRNA levels in Runx3−/− mouse ovaries may result in decreased androgen synthesis, leading to the diminished production of estrogen in granulosa cells. Atrophic uteri in Runx3−/− mice also suggested a decrease in estrogen production in ovaries [13]. The increase in estrogen levels during the proestrous day triggers a preovulatory GnRH/LH surge. Therefore, it is probable that anovulation of Runx3−/− mice was caused by a decrease in estrogen production from granulosa cells, or by alterations in the preovulatory GnRH/LH surge-generating system in the hypothalamus of Runx3−/− mice. In the present study, ovulation was induced in the ovaries of Runx3−/− mice when they were transplanted into wt mice. These results indicate that Runx3−/− mouse ovaries are able to respond to gonadotropins and then to release oocytes, and that dysfunction of the hypothalamo-pituitary system in Runx3−/− mice is closely associated with anovulation.

Cyp11a1 encodes the cholesterol side chain cleavage enzyme.
Runx3 IN MOUSE OVARIES

483

...SSC), 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 is in agreement with a previous study [22]. We found decreased expression of Cyp11a1 mRNA in Runx3−/− mouse ovaries, and a decrease in the percentage of identifiable Cyp11a1 mRNA-expressing cells in the theca cell layers of Runx3−/− mouse ovaries, possibly resulting from the decrease in Cyp11a1 mRNA expression in theca cells.

The decreased expression of Cyp11a1 mRNA may affect the whole process of ovarian steroidogenesis, although Hsd3b1 and Cyp19a1 mRNA levels in Runx3−/− mouse ovaries did not change. Estrogen is a final product of the steroidogenesis of granulosa cells, and is closely related to the progress of folliculogenesis, onset of ovulation, and uterine growth and functions [14]. Defects in the female reproductive system observed in Runx3−/− mice, such as retarded folliculogenesis, anovulation, and atrophied uteri [12], may be attributable to estrogen deficiency. Therefore, Runx3 is one of the key transcription factors involved in the regulation of ovarian functions.

In the present study, Runx3 mRNA expression was detected in granulosa cells, but not in theca cells. Cyp11a1 expression in the theca cells is regulated not only by LH [23], but also by growth factors produced in granulosa cells [24]. Therefore, the decreased Cyp11a1 mRNA expression in theca cells may result from a disorder of LH...
secretion induced by Runx3 deletion, and/or alterations in granulosa cell functions relating to the control of theca cell functions. Inhibin and activin, both produced in granulosa cells, stimulate and inhibit androgen synthesis in theca cells, respectively [25, 26]. Interestingly, a recent report suggests that Inhbb, which is one of the inhibin subunit genes, is involved in the regulation of Cyp11a1 expression in mouse ovaries [27]. IGF1, produced in granulosa cells, regulates androgen production through stimulation of Cyp11a1 and Hsd3b1 expression [23, 28, 29]. Considering these findings, it is probable that the decreased Cyp11a1 mRNA expression in Runx3−/− mouse ovaries is partly caused by altered production of growth factors in granulosa cells. Studies on such theca-granulosa cell interaction in Runx3−/− mouse ovaries are needed.

In a previous study, Runx3 mRNA expression was detected in the mouse hypothalamus [12]. The present study more clearly demonstrated Runx3 mRNA expression in the AVPV and ARC of the female mouse hypothalamus, suggesting that Runx3 may play roles in neuronal system regulation in both AVPV and ARC areas, although the identity of the Runx3 mRNA-expressing cells has not been determined. Concurrent increase in Gnrh1 and Fshb mRNA levels in Runx3−/− mice indicates that FSH production was increased by enhanced GnRH release. GnRH release is well known to be regulated by kisspeptin neurons in the AVPV and ARC. ARC kisspeptin neurons are involved in regulation of the negative feedback system of FSH and LH secretion [16, 17, 30, 31]. Therefore, if the negative feedback system of gonadotropin secretion in ARC functions properly, low estrogen level may stimulate Kiss1 mRNA expression in the ARC, leading to the stimulation of GnRH release. In the present study, we observed elevation of Kiss1 mRNA levels in the ARC in Runx3−/− mice. Considering these observations, it is concluded that Runx3 in the ARC may not be involved in the negative feedback system of FSH and LH secretion.
system of Kiss1 mRNA expression in the ARC.

Ovarian transplantation into wt or Runx3<sup>−/−</sup> mice was performed in order to determine whether the hypothalamo-pituitary system in Runx3<sup>−/−</sup> mice is impaired in regulation of ovulation, or whether Runx3<sup>−/−</sup> mouse ovaries loses the ability to ovulate. Wt mice bearing Runx3<sup>−/−</sup> ovarian grafts exhibited regular estrous cycles like wt mice bearing wt ovarian grafts, whereas Runx3<sup>−/−</sup> mice bearing wt ovarian grafts exhibited irregular estrous cycles. With regard to formation of corpora lutea in ovarian grafts, ovarian grafts contained many corpora lutea in wt mice bearing Runx3<sup>−/−</sup> ovarian grafts, but not in Runx3<sup>−/−</sup> mice bearing wt ovarian grafts. These findings demonstrate that corpora lutea were observed in the ovaries of Runx3<sup>−/−</sup> mice when the ovaries were located in the hormonal milieu of wt mice, and hence ovulation was induced. In contrast, the hypothalamo-pituitary...
system of Runx3−/− mice failed to generate the GnRH/LH surge that induces ovulation, because corpora lutea were not detected in the ovaries of wt mice when they were transplanted into Runx3−/− mice. Thus, these findings suggest that the ovaries in Runx3−/− mice had the ability to produce E2 and undergo ovulation, and that anovulation in Runx3−/− mice was caused by Runx3 deletion in the hypothalamo-pituitary system leading to a probable alteration of gonadotropin secretion. This is consistent with our recent finding that gonadotropin treatment in 3-week-old Runx3−/− mice induced ovulation, suggesting that the ovaries of Runx3−/− mice could respond to gonadotropins and ovulate [12]. In Runx3−/− mice, antral follicles were present in ovaries and the mRNA levels of Gnrh1 in hypothalami and Fshb in anterior pituitaries were increased, suggesting that Runx3 in the hypothalmo-pituitary system is involved in LH surge release. Although expression of Kiss1 mRNA in the AVPV and Lhb mRNA in the anterior pituitary were observed in Runx3−/− mice, it remains unclear whether positive feedback and LH secretion are normal occurrences in these mice. The role of Runx3 in the hypothalamo-pituitary system remains to be studied.

In conclusion, Runx3 plays a pivotal role in the hypothalmo-pituitary-gonadal axis. Within the hypothalmo-pituitary system, Runx3 is involved in gonadotropin release, which induces steroidogenesis, and subsequently follicular maturation and ovulation in the ovary.

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