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RESEARCH ARTICLE

Nr5a1 suppression during the murine fetal period optimizes ovarian development by fine-tuning Notch signaling

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ABSTRACT

The nuclear receptor NR5A1 is equally expressed and required for development of the gonadal primordia of both sexes, but, after sex determination, it is upregulated in XY testes and downregulated in XX ovaries. We have recently demonstrated, in mice, that this downregulation is mediated by forkhead box L2 (FOXL2) and hypothesized that adequate suppression of *Nr5a1* is essential for normal ovarian development. Further, analysis of human patients with disorders/differences of sex development suggests that overexpression of *NR5A1* can result in XX (ovo)testicular development. Here, we tested the role of *Nr5a1* by overexpression in fetal gonads using a *Wt1*-BAC (bacterial artificial chromosome) transgene system. Enforced *Nr5a1* expression compromised ovarian development in 46,XX mice, resulting in late-onset infertility, but did not induce (ovo)testis differentiation. The phenotype was similar to that of XX mice lacking Notch signaling. The expression level of *Notch2* was significantly reduced in *Nr5a1* transgenic mice, and the ovarian phenotype was almost completely rescued by *in utero* treatment with a NOTCH2 agonist. We conclude that suppression of *Nr5a1* during the fetal period optimizes ovarian development by fine-tuning Notch signaling.

KEY WORDS: NR5A1, Ad4BP, SF1, Notch signaling, Premature ovarian insufficiency, Sex determination

INTRODUCTION

In mammals, gonadal development, including sexual differentiation, is a unique process in that two completely different organs, testes and ovaries, arise from a common precursor, the bipotential genital ridge. Initially, the morphogenesis of the bipotential genital ridge appears to be similar between male and female embryos, and involves a suite of genes including empty spiracles homeobox 2 (*Emx2*), GATA-binding protein 4 (*Gata4*), two isoforms of Wilms' tumor 1 (*Wt1*; *Wt1*+KTS and *Wt1*–KTS), LIM homeobox 9 (*Lhx9*), and nuclear receptor 5a1 (*Nr5a1*) (She and Yang, 2014). Sex-specific differentiation of the gonads begins with expression of the Y-linked gene sex-determining region Y (*Sry*), the master switch of male determination in mammals (Koopman et al., 1991), and its autosomal target gene *Sox9* (Sekido and Lovell-Badge, 2008). SOX9 is expressed in pre-Sertoli cells and directly upregulates a number of male-specific genes in cooperation with NR5A1, leading to differentiation of Sertoli cells (Wilhelm et al., 2007), which orchestrate testis development. In the absence of *Sry*, female genes such as *Wnt4* and *Foxl2* are upregulated, leading to ovarian development (Wilhelm et al., 2007). During ovarian development, bidirectional communication between germ cells and somatic granulosa cells leads to follicular development. For developmental maturation of ovarian follicles, several genes and molecular pathways are required, such as growth differentiation factor-9 (*Gdf9*), bone morphogenetic protein 15 (*Bmp15*) and the Notch signaling pathway, to mediate the interaction (Suzuki et al., 2015).

NR5A1 (also known as Ad4BP or SF1) is a member of the nuclear receptor superfamily. In mice, *Nr5a1* is expressed from ∼9.5 days postcoitum (dpc), in the bipotential genital ridge and the adrenogenital primordium (Ikeda et al., 2001), and knockout models show complete gonadal agenesis in both XX and XY, suggesting that NR5A1 is essential for genital ridge development in both sexes (Luo et al., 1994; Majdic et al., 2002). In addition to initiating gonadal development, NR5A1 plays crucial roles in testicular development. It is transcriptionally upregulated in the developing mouse testes (Hanley et al., 1999), where it acts as a cofactor of the male sex-determining factor SRY to induce *Sox9* expression (Sekido and Lovell-Badge, 2008). Subsequently, it cooperates with SOX9 to maintain the expression of *Sox9* itself (Sekido and Lovell-Badge, 2008) and upregulate other Sertoli cell-specific genes including *Amh* (De Santa Barbara et al., 1998). Furthermore, NR5A1 is essential for the differentiation of the testicular steroidogenic cells, fetal Leydig cells (Buaas et al., 2012). Consistent with its essential roles in testis differentiation, heterozygous loss-of-function mutations in *NR5A1* cause XY female sex reversal in humans (Achermann et al., 1999; Correa et al., 2004; Hasegawa et al., 2004; Mallet et al., 2004).

In contrast to its transcriptional upregulation in fetal testes, *Nr5a1* expression in mouse fetal ovaries decreases after 12.5 dpc (Ikeda et al., 2001). We have recently demonstrated that this downregulation is mediated by forkhead box L2 (FOXL2), a key ovarian transcription factor expressed mainly in pregranulosa/granulosa cells (Takasawa et al., 2014). FOXL2 directly binds to the proximal promoter of *Nr5a1*, thereby antagonizing the actions of WT1–KTS that upregulates the expression of *Nr5a1* in collaboration with LHX9.

Recently, 46,XX individuals with testicular or ovo-testicular disorders/differences of sex development have been reported carrying mutations in codon 92 of *NR5A1*, including the R92W and R92Q mutations (Baetens et al., 2016; Bashamboo et al., 2016; Igarashi et al., 2017; Miyado et al., 2016; Swartz et al., 2017). The

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variant protein was thought to function in the XX gonads by escaping the suppressive action of NR0B1 (also known as DAX1), a pro-ovary factor (Igarashi et al., 2017; Miyado et al., 2016; Swain et al., 1998). The presence of testicular tissue in the probands further suggested that ectopic activity of NR5A1 may drive testis differentiation in the absence of the *SRY* gene.

These observations raise two key questions. First, it is not clear whether *Nr5a1* can function as a male sex-determining factor, i.e. whether elevated *Nr5a1* expression levels in XX gonads (where the male-determining gene *Sry* is absent) are sufficient to direct the fate of gonads towards testicular development. Second, it is not known whether repression of *Nr5a1* is essential for appropriate ovarian development.

To address these questions, we exploited a bacterial artificial chromosome (BAC) transgene system (Zhao et al., 2014), whereby *Nr5a1* expression is driven by *Wt1* regulatory sequences. In the fetal XX gonads, endogenous *Wt1* is continuously expressed in supporting cells including pre-granulosa cells and coelomic epithelium (Bandiera et al., 2015; Maatouk et al., 2012; Rackley et al., 1993). Hence, by directing transgenic *Nr5a1* expression to *Wt1*-expressing XX gonadal supporting cells, we aimed to investigate the consequence of *Nr5a1* overexpression in the relevant cell types in XX mouse fetal gonads.

Molecular and phenotypic analysis of the two transgenic mouse lines generated demonstrated first that enforced *Nr5a1* expression alone is insufficient to switch the fate of the 46,XX gonads toward testicular formation in mice, and, second, that overexpression of NR5A1 disrupts ovarian follicular development and causes premature ovarian insufficiency. Because the ovarian phenotype resembled that of compromising the Notch signaling pathway (Trombly et al., 2009; Xu and Gridley, 2013; Vanorny et al., 2014), we conducted further experiments to establish that ectopic NR5A1 acts by dysregulating levels of Notch signaling during gonadal development.

RESULTS

Overexpression of *Nr5a1* fails to cause XX sex reversal in mice

Using the piggyBac-enabled *Wt1*-BAC system (Zhao et al., 2014) (Fig. 1A), we successfully generated two transgenic mouse lines (Tg-A and Tg-S) expressing an *Nr5a1*-IRES-*Egfp* transgene (Fig. 1B–P). Immunofluorescence (IF) intensity suggested that the expression levels of NR5A1 and reporter protein EGFP were higher in the Tg-S line than in Tg-A mice. The expression of exogenous NR5A1 and EGFP persisted until after birth (Fig. S1A–I). Quantitative reverse transcriptase PCR (qRT-PCR) also showed that *Nr5a1* mRNA levels were higher in the Tg-S line, although sample size limited the statistical significance (Fig. 1Q). The kidneys and adrenal glands, which express endogenous *Wt1* at high levels, developed no apparent abnormalities in either Tg-A or Tg-S mice, suggesting that the gonadal phenotypes of the transgenic mice were unlikely to be caused by impaired function of those organs.

In both Tg-A and Tg-S lines, XX mice developed female internal and external genitalia (Fig. S2A–F). Morphologically, the gonads in adult XX Tg-S mice were streak-like and elongated (Fig. S2F). In Tg-A mice, fetal ovaries at 15.5 dpc were longer and thinner than those from wild type (Fig. S3A–D). However, at postnatal day (P) 0, the shape of the ovaries in Tg-A mice was similar to that in wild-type mice, indicating that the elongation of the gonads had resolved by that stage. Since a similar phenotype was reported in *Sox4*$^{-/-}$ mice (Zhao et al., 2017), we examined the expression levels of *Sox4* in the XX Tg-A gonads at 15.5 dpc and indeed found significantly reduced expression of *Sox4* (Fig. S3E). In *Sox4*$^{-/-}$ mice, the expression level of *Nr5a1* was not altered (Zhao et al., 2017). Hence, our data suggest that elevated NR5A1 repressed *Sox4*, resulting in abnormal morphology of the fetal ovaries.

In both transgenic lines, histological examination of XX adult gonads revealed the presence of ovarian follicles but detected no seminiferous tubule-like structures (Fig. 2; Fig. S2G–J). Consistently, FOXL2, a marker of ovarian granulosa cells, was expressed in XX adult gonads in both mouse lines (Fig. 2B,C). In contrast, SOX9, a marker of testicular Sertoli cells, was not detected (Fig. 2F,G). Based on these data, we conclude that *Nr5a1* overexpression alone is insufficient to cause XX sex reversal in mice.

Presence of ectopic steroidogenic cells in fetal ovaries of *Nr5a1* trangenic mice

In addition to Sertoli cells, NR5A1 is highly expressed in gonadal steroidogenic cell lineages in both sexes and is essential for their differentiation (Buaas et al., 2012; McClelland et al., 2015; Shima et al., 2013). In males, steroidogenic Leydig cells differentiate during fetal testis development and produce androgen (Griswold and Behringer, 2009). In contrast, the ovarian steroidogenic theca cells do not differentiate until after birth (Suzuki et al., 2015; Wilhelm et al., 2007). We asked whether overexpression of *Nr5a1* is able to induce the ectopic differentiation of steroidogenic cells in fetal XX gonads. Immunofluorescence analysis of the XX fetal gonads of Tg-A and Tg-S mice (Fig. 3A–L) revealed that 3β-hydroxysteroid dehydrogenase (3β-HSD), the enzyme mediating the first step of steroidogenesis and a marker of steroidogenic cells (Peng et al., 2002), was detected in the Tg-S XX gonads (Fig. 3G). However, the plasma testosterone levels at P0 were not elevated in XX Tg-S mice compared to wild type (Fig. 3M). This is consistent with the lack of masculinization in XX Tg-S mice (Fig. S2C,F). On the other hand, plasma estrogen levels at P0 were significantly lower in XX Tg-S mice than in wild type (Fig. 3N). The result could be caused by impaired differentiation of ovarian somatic cells in Tg mice or to other environmental factors, such as reduced maternal estrogen.

Enforced expression of *Nr5a1* leads to increased follicular atresia and impaired fertility

After the neonatal period, no obvious dysgenesis or structural abnormalities were found in Tg-A and Tg-S ovaries. However, at P14, histological analysis revealed an increased number of multiple-oocyte follicles (MOFs), suggesting disruption of ovigerous cord fragmentation (Fig. 4A–D). Further, we found generally reduced numbers of follicles of all types compared to wild type. In particular, the number of antral follicles (tertiary, type 6–8), characterized by a fluid-filled cavity, was significantly affected (Fig. 4E). The numbers of antral follicles appeared to be inversely correlated with *Nr5a1* expression levels, with the Tg-S ovaries containing fewer antral follicles than the Tg-A ovaries (Fig. 4E). To determine whether this was caused by reduced proliferation or increased apoptosis of follicular granulosa cells, we analyzed the expression of Ki-67 (also known as Mki67), a marker of proliferating cells, and cleaved caspase 3, a marker of apoptotic cells, in transgenic ovaries at P28. We observed no obvious changes in the follicles containing Ki-67-positive granulosa cells (red, Fig. 5D–F), but a significant increase in secondary follicles (type 4 and 5), the precursors of tertiary follicles, containing cleaved caspase 3-positive granulosa cells (green) in both Tg-A and Tg-S ovaries (Figs 4F and 5A–C,G–I; the boxed areas in Fig. 5G–I are shown enlarged in Fig. 5J–L). The increase in follicular atresia correlated with *Nr5a1* expression levels,

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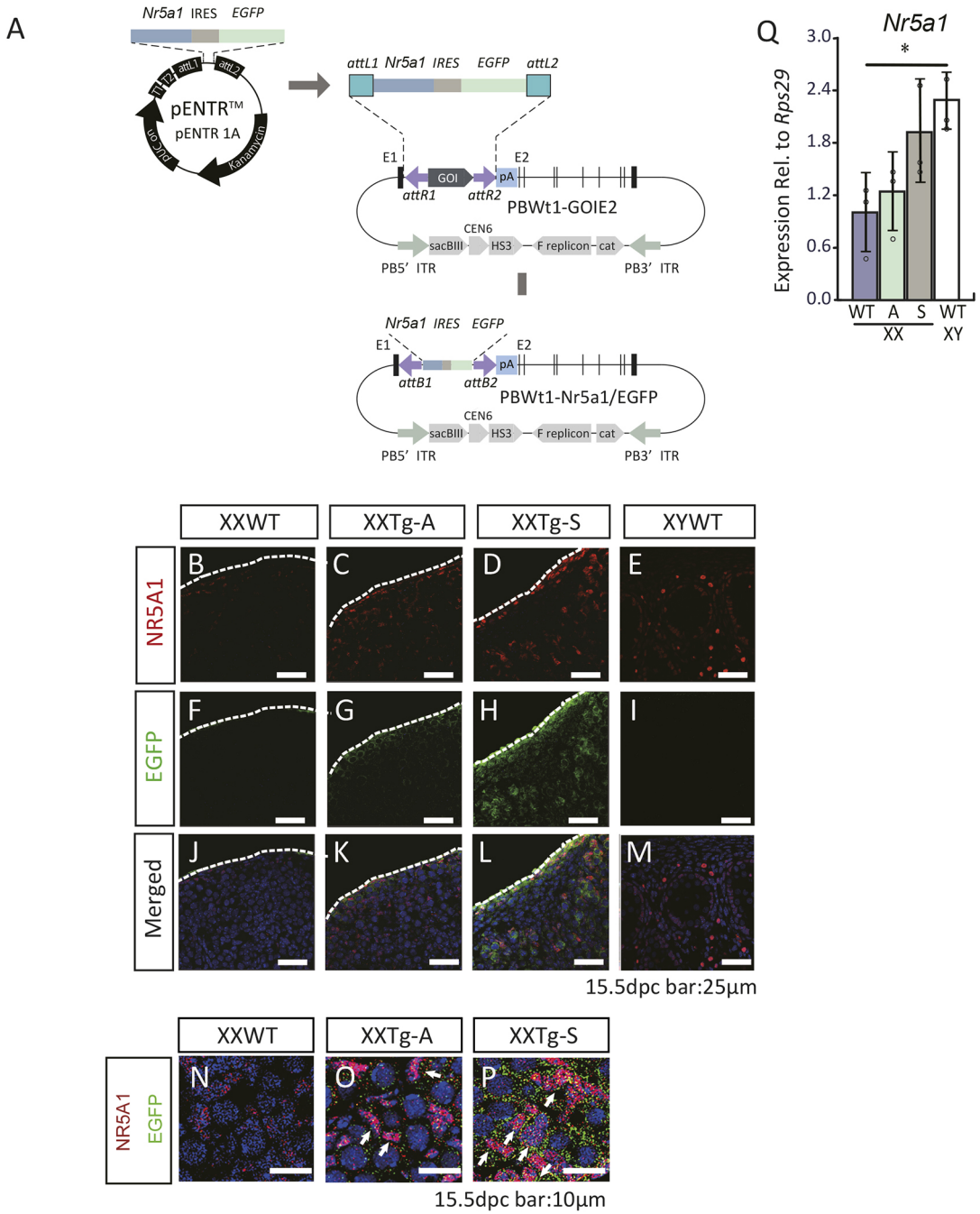

Fig. 1. Generation of transgenic mice with enforced *Nr5a1* expression in XX gonads. (A) Schematic representation of the strategy to generate transgenic mice using the piggyBac-enabled *Wt1*-BAC system. *Nr5a1*-IRES-*Egfp* fragment in the Gateway entry vector was inserted into PBWt1-Dest using LR recombination. (B–P) Transgenic EGFP expression was detected using IF in the XX fetal gonads at 15.5 dpc in both Tg-A and Tg-S lines. Low-magnification (B–M), and high-magnification (N–P) images revealed that EGFP (green) and NR5A1 (red) were expressed in the cytoplasm and nucleus, respectively, of the same cells in XX transgenic mice (arrows) (O,P). By using cryosections and paraffin-embedded samples, at least three sections from each of at least three gonads were examined for each genotype. The images were obtained from the cryosection samples. Scale bars: 25 µm (B–M) and 10 µm (N–P). Dashed lines indicate the edge of the gonad. (Q) qRT-PCR analysis showing that *Nr5a1* was expressed at different levels in the XX fetal gonads of the Tg-A and Tg-S lines at P0. In the XX fetal gonads of the Tg-S line, *Nr5a1* was expressed at levels similar to those in wild-type testes. In the Tg-A line, *Nr5a1* was expressed in the XX fetal gonads at levels between those in wild-type testes and ovaries. Expression levels were normalized to *Rps29*. Mean±s.d., n=3. *P<0.05 (Welch's t-test). A, Tg-A; S, Tg-S; WT, wild type.

with Tg-S ovaries exhibiting higher levels of atresia than Tg-A ovaries (Figs 4F and 5A–C,G–L). No increase in type 6 antral follicles containing cleaved caspase 3-positive cells was observed (Fig. 4F). Together, these results suggest that overexpression of *Nr5a1* caused increased apoptosis of granulosa cells in secondary follicles in a dose-dependent manner, resulting in reduced numbers of antral follicles.

We next assessed the fertility of Tg-A and Tg-S female mice. The fertility of Tg-S female mice was compromised. Of three Tg-S females analyzed, the litter size was reduced from their first mating

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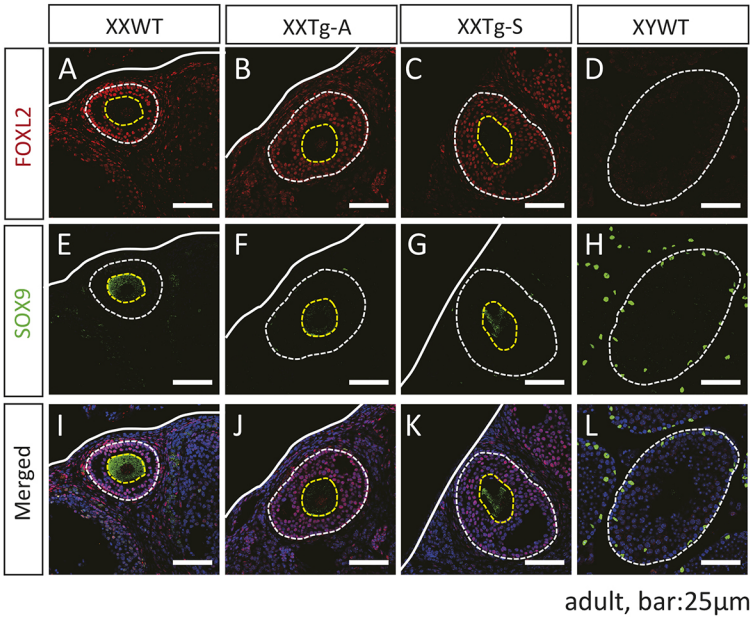

Fig. 2. Transgenic overexpression of *Nr5a1* in XX fetal gonads did not induce male development. (A–L) IF analysis of markers of granulosa (FOXL2, red) or Sertoli cells (SOX9, green) of the gonads in adult mice (6–8 months old). In the XX gonads of the Tg-A and Tg-S lines, only FOXL2-positive granulosa cells were present (B,C), and no SOX9-positive cells were detected (F,G; some nonspecific staining of the oocytes was seen using the anti-SOX9 antibody). In XX gonads, white solid lines indicate the gonadal edges; white and yellow dashed lines indicate the outlines of the ovarian follicles and the oocytes, respectively. Granulosa cells were identified by FOXL2 expression between white and yellow dashed lines. In XY gonads, whited dashed lines indicate testicular tubules. Scale bars: 25 µm.

(Fig. 4G). The reproductive performance of Tg-A females was also reduced. Although Tg-A mice produced four consecutive litters to begin the study, they failed to produce additional litters thereafter (Fig. 4H), suggesting that these females had premature reproductive senescence, analogous to premature ovarian insufficiency in human.

Nr5a1 overexpression represses Notch signaling levels in fetal ovaries

Interactions between germ cells and somatic pregranulosa cells are crucial for the formation of ovarian follicles, and Notch signaling plays a major role in mediating this interaction (Terauchi et al.,

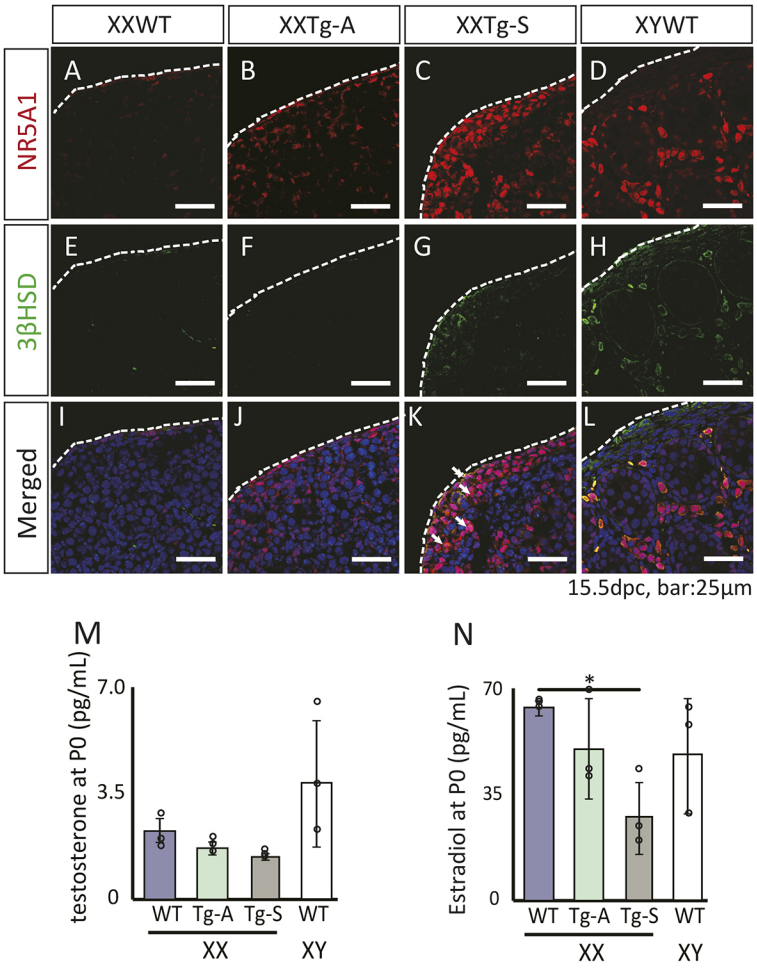

Fig. 3. Formation of ectopic steroidogenic cells in the XX transgenic fetal gonads. (A–L) IF analysis of 3β-HSD, a marker of steroidogenic cells, at 15.5 dpc. In the Tg-S fetal ovaries, 3β-HSD-positive cells were sparsely observed, some of which showed colocalization with NR5A1 (G,K, arrows). Scale bars: 25 µm. (M,N) Plasma testosterone (M) or estradiol (N) levels at P0. No significant increase in the levels of testosterone was observed in XX Tg-A or Tg-S mice compared with wild-type female mice. Mean±s.e.m., n=3. *P<0.05 (Welch's t-test).

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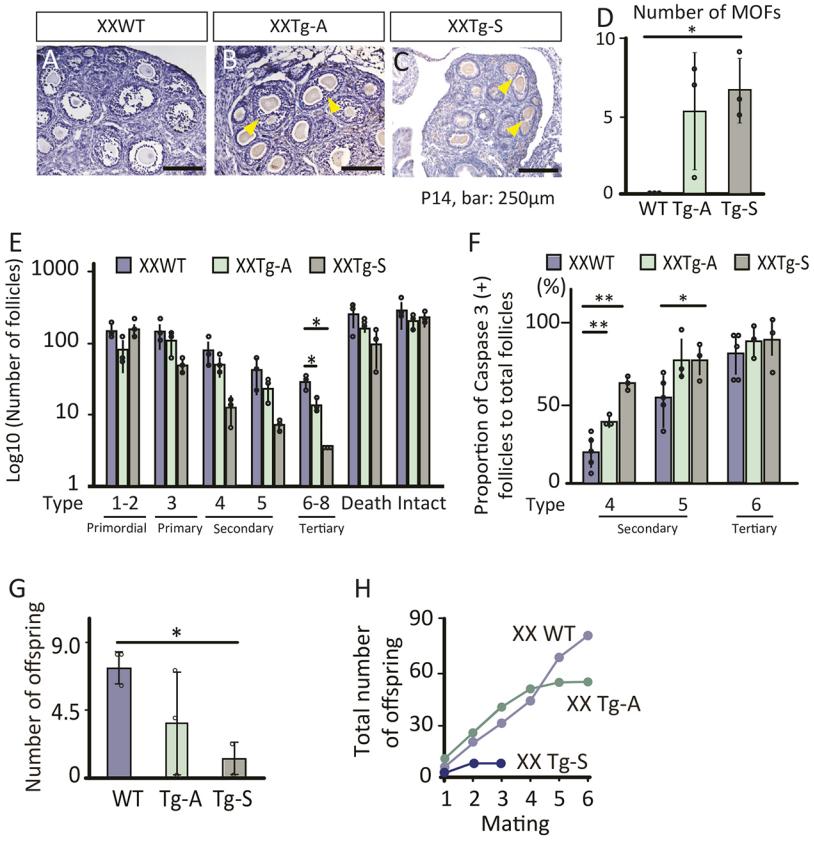

Fig. 4. Impaired ovarian development in *Nr5a1* transgenic mice. (A–C) Histological analysis of ovaries in wild-type, Tg-A and Tg-S females. MOFs were observed in Tg-A and Tg-S ovaries (arrowheads). Scale bars: 250 µm. (D) Numbers of MOFs were counted from serial ovarian sections. Mean ±s.e.m., *n*=3. **P*<0.05 (Welch's test). (E) The numbers of antral follicles (type 6–8) significantly decreased in transgenic mice. The numbers of follicles of each type [1–2 (primordial), 3 (primary), 4–5 (secondary), 6–8 (antral), death and intact] were counted from serial ovarian sections. Mean±s.d., *n*=3. **P*<0.05 (Welch's *t*-test). (F) Quantification of secondary follicles containing cleaved caspase 3-positive cells (Fig. 5A–C). The percentage of cleaved caspase 3-positive follicles was plotted. Mean±s.e.m., *n*=3. **P*<0.05, ***P*<0.01 (Welch's *t*-test). (G) Average litter size of the first mating of wild-type, Tg-A and Tg-S female mice (*n*=3). Mean±s.e.m. **P*<0.05 (Welch's *t*-test). (H) Total number of progeny from wild-type, Tg-S and Tg-A female mice (*n*=3).

2016; Vanorny et al., 2014; Xu and Gridley, 2013). In the developing ovaries, oocytes and other neighboring cells secrete the Notch ligands, including JAG1 and possibly JAG2, which bind to Notch receptors (mainly NOTCH2) present on the surface of pre-granulosa cells, thereby activating Notch signaling (Guruharsha et al., 2012). As a result, pre-granulosa cells proliferate and encapsulate individual germ cells to form primordial follicles, i.e. the resolution of germ cell syncytia (Terauchi et al., 2016). Genetic ablation of either *Jag1* or *Notch2*, two important Notch pathway components, gives rise to MOFs and causes premature reproductive senescence (Vanorny et al., 2014). Because of the similarity in the phenotypes between our *Nr5a1* transgenic mice and *Jag1*- or *Notch2*-deficient mice, we hypothesized that enforced expression of *Nr5a1* may compromise ovarian development by repressing the Notch signaling pathway.

We therefore analyzed mRNA expression levels of a number of genes involved in Notch signaling in 15.5-dpc fetal ovaries using qRT-PCR (Fig. 6A–H). Supporting our hypothesis, we found significant downregulation of several Notch pathway genes, including *Notch2*, *Notch3* and *Dll4*, in Tg-A ovaries compared with wild-type ovaries (Fig. 6B,C,H).

A NOTCH2 agonist rescues the ovarian phenotype in Tg-A mice

To further clarify the contribution of Notch signaling to the ovarian phenotype in *Nr5a1* transgenic mice, we attempted a rescue experiment with a NOTCH2 agonist HMN2-29, a hamster monoclonal antibody (Tanaka et al., 2014). We injected three doses of HMN2-29 or control hamster IgG intraperitoneally into pregnant mice carrying wild-type or Tg-A fetuses at 13.5, 16.5 and 18.5 dpc (Fig. S4A), and analyzed the ovaries postnatally (P14 and P28). No obvious adverse effects of *in utero* administration of the NOTCH2 agonist were observed, as the treated mice appeared grossly normal, with body weight comparable to wild type at P28 (Fig. S4B).

Confirming our hypothesis, we found a reduced number of MOFs in Tg-A mice at P14 upon HMN2-29 treatment (Fig. 7A). Moreover, treatment with the NOTCH2 agonist completely restored the number of antral follicles in XX Tg-A ovaries at P28 to wild-type XX levels, whereas control treatment with hamster IgG did not ameliorate the reduced follicle number (Fig. 7B). Immunofluorescence analysis for markers of cell proliferation (Ki-67, red) or apoptosis (cleaved caspase 3, green) in the XX gonads of the wild-type and Tg-A mice revealed that the complete rescue of antral follicle numbers appeared to be a result of reduced atresia of secondary follicles in the treated ovaries (Fig. 7C–O; the boxed areas in Fig. 7L–O are shown enlarged in Fig. 7P–S). These results suggest that *Nr5a1* fine-tunes Notch signaling levels in fetal ovaries to ensure proper folliculogenesis and normal fertility, and that repression of *Nr5a1* during fetal ovarian development is essential to allow the Notch signaling levels to elevate appropriately.

We further explored molecular mechanisms by which the Notch signaling pathway regulates fetal ovarian development. To this end, we analyzed the expression levels of two master regulators of ovarian development, *Wnt4* and *Foxl2*, in Tg-A ovaries at P0. We found that *Wnt4*, but not *Foxl2*, was significantly downregulated in Tg-A mice compared to wild type (Fig. 7T,U). Importantly, *Wnt4* expression in the Tg-A ovaries was fully restored by the administration of HMN2-29 (Fig. 7U), suggesting that downregulated expression was a result of de-regulated Notch signaling pathway rather than a direct effect of NR5A1 overexpression. Despite the limited statistical significance, possibly due to the limited size of samples, the *Foxl2* expression pattern was similar to that of *Wnt4*, suggesting the possibility that

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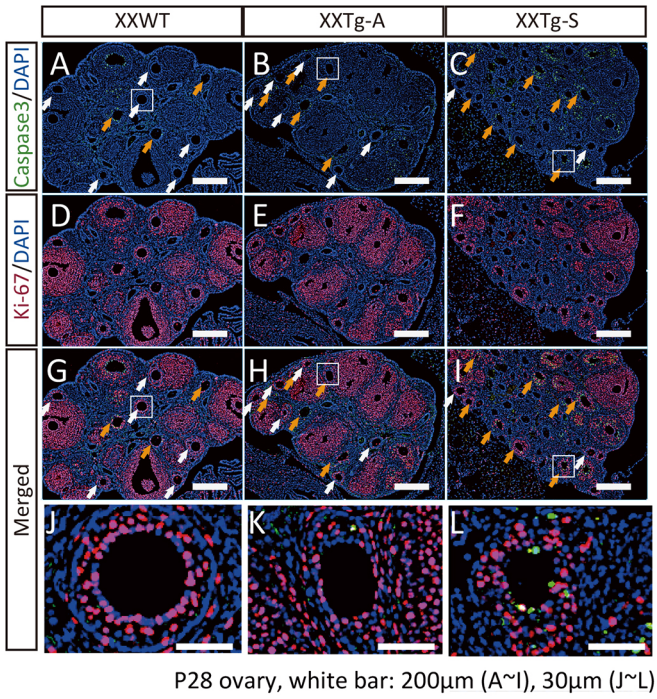

Fig. 5. IF analysis in ovaries of *Nr5a1* transgenic mice. (A–L) IF analysis of markers of cell proliferation (Ki-67, red) or apoptosis (cleaved caspase 3, green) in the XX gonads of wild-type, Tg-A and Tg-S mice. The boxed areas in G–I are shown enlarged in J–L. Orange and white arrows indicate secondary follicles (type 4–5) with or without cleaved caspase 3-positive cells, respectively. Scale bars: 30 µm (J–L) and 200 µm (A–I).

Foxl2 could be also partially regulated by Notch signaling. We note that this regulatory relationship is likely limited to the late-stage fetal ovaries, as expression of the Notch pathway genes starts in fetal ovaries from ∼15.5 dpc (Vanorny et al., 2014).

DISCUSSION

Nr5a1 plays known essential roles in the development of genital ridges in both sexes and fetal testes in male mice. We show in the present study that the downregulation of *Nr5a1* is important for establishing normal Notch signaling in the mouse fetal ovary, and that overexpression of *Nr5a1* compromises ovarian development in mice.

Overexpression of *Nr5a1* alone is insufficient to drive testis determination and differentiation in mice

The *Wt1*-BAC system we used drives robust expression of transgene cargo to fetal testes and ovaries from an early stage of their development (Zhao et al., 2014). Using this system, enforced expression of *Dmrt1* in XX mouse fetal gonads has been shown to drive testicular differentiation (Zhao et al., 2015), indicating the suitability of this system to test whether a given molecule has the ability to cause XX sex reversal. Clearly, forced expression of *Nr5a1* mRNA and protein was unable to stimulate XX sex reversal. Although we cannot exclude the possibility that some transient sex reversal might have occurred earlier than the time points studied, we consider this unlikely because there is no example to date of transient sex reversal that resolves to ovaries. Accordingly, we concluded that overexpression of *Nr5a1* alone is insufficient to drive testis determination and differentiation in mice.

Our results are consistent with the published observation that XX mice carrying heterozygous or homozygous R92W mutation in *Nr5a1* showed no signs of masculinization (Miyado et al., 2016). The R92W variant of NR5A1 is thought to escape the suppressive action of NR0B1, a pro-ovary factor (Igarashi et al., 2017; Miyado et al., 2016; Swain et al., 1998). Our data, together with the R92W observation in mice, are at odds with the presence of testicular or ovo-testicular material in XX humans carrying the R92W variant (Baetens et al., 2016; Bashamboo et al., 2016; Igarashi et al., 2017; Miyado et al., 2016; Swartz et al., 2017). One possible explanation is that NR5A1 may be required at different threshold levels with respect to sex determination in humans and mice. For example, it is

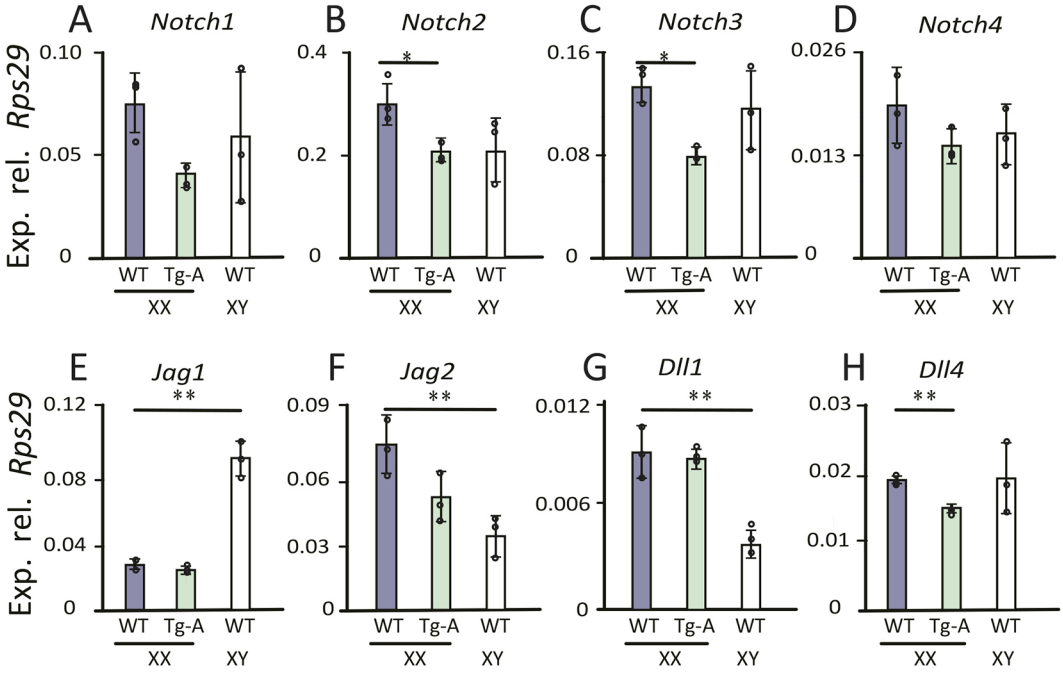

Fig. 6. Expression of the Notch signaling pathway genes in XX Tg-A mice. (A–H) qRT-PCR analysis was performed on total RNA extracted from fetal ovaries of wild-type, Tg-A, and Tg-S mice at 15.5 dpc. Expression levels were normalized to *Rps29*. Mean±s.e.m., n=3. *P<0.05, **P<0.01 (Welch's t-test).

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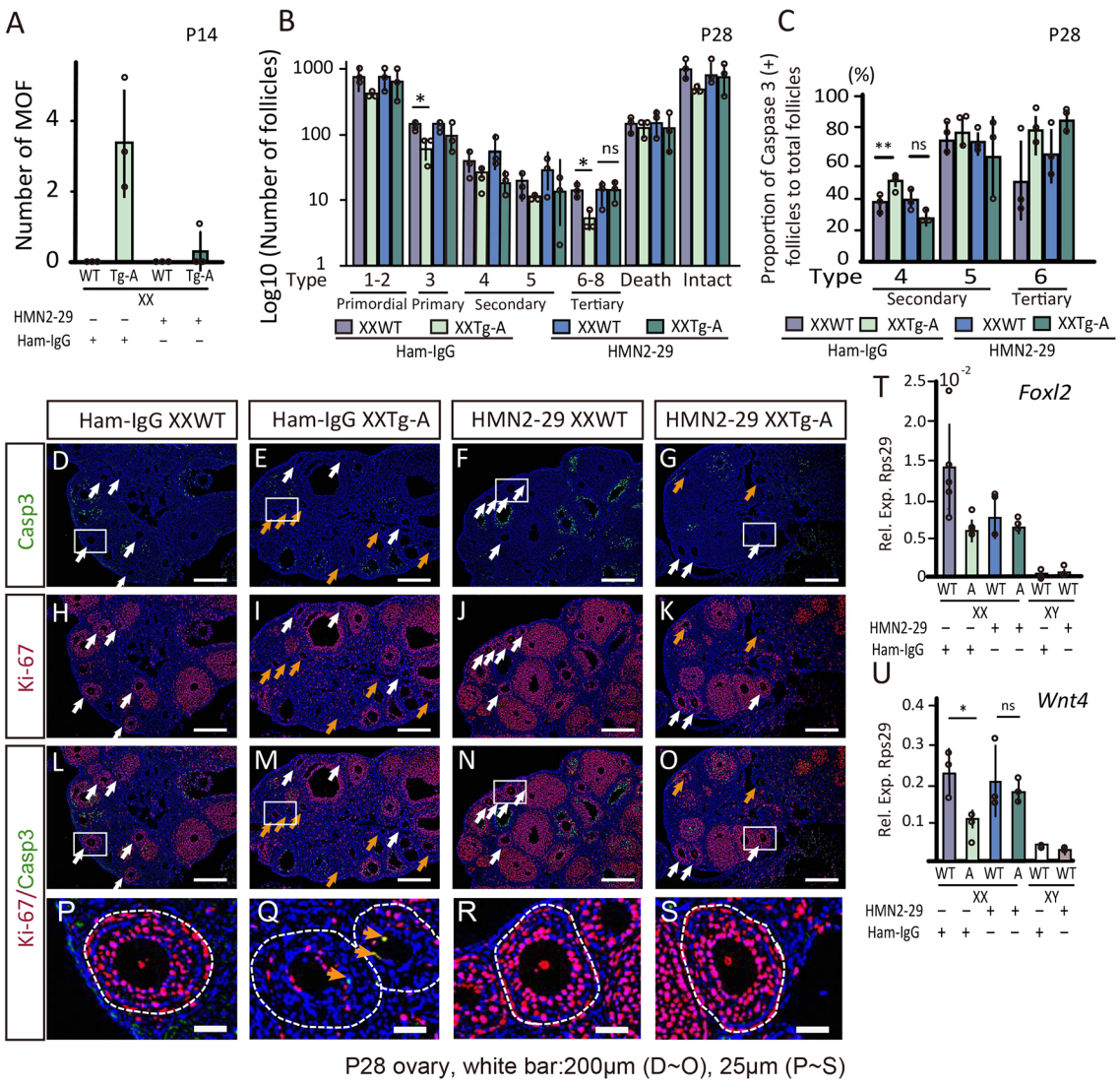

Fig. 7. Administration of a NOTCH2 agonist rescued the ovarian phenotype in Tg-A mice. (A,B) The NOTCH2 agonist HMN2-29 or control hamster IgG was *in utero* administered to wild-type or Tg-A mice, and the ovarian phenotype was analyzed at P14 (A) and P28 (B). HMN2-29 administration almost completely rescued the formation of MOFs in the Tg-A ovaries at P14 (A), and restored the antral follicles to the wild-type level at P28 (B). Mean±s.e.m., n=3. *P<0.05 (Welch's t-test). (C–S) IF analysis, at P28, of markers of cell proliferation (Ki-67, red) or apoptosis (cleaved caspase 3, green) in XX gonads of wild-type and Tg-A mice treated with hamster IgG or HMN2-29. White and orange arrows indicate secondary follicles (type 4–5) with or without cleaved caspase 3-positive granulosa cells, respectively. Scale bars: 25 μm (P–S) and 200 μm (D–O). Quantitation of caspase 3-positive follicles in D–G is shown in C. The boxed areas in L–O are shown enlarged in P–S. Mean±s.e.m., n=3. **P<0.01 (Welch's t-test); ns, not significant. (T,U) qRT-PCR analysis for *Foxl2* (T) and *Wnt4* (U) in P0 gonads of wild-type or Tg-A mice treated with hamster IgG or HMN2-29, respectively. Expression levels were normalized to *Rps29*. Mean±s.e.m., n=3. *P<0.05 (Welch's t-test). ns, not significant.

known that duplication of *NR0B1*, an orphan nuclear receptor gene, causes XY female sex reversal in humans (Zanaria et al., 1994), whereas transgenic overexpression of *Nr0b1* in mice does not normally cause female sex reversal in C57BL mice (Swain et al., 1998). Not only does this demonstrate the principle of different gene dosage requirements in mouse and human sex determination, but also, since a major function of NR0B1 is to antagonize NR5A1 (Ikeda et al., 2001; Ludbrook et al., 2012; Zanaria et al., 1994), it could provide a mechanistic link between different dosage requirements of NR0B1 and NR5A1 between mouse and human.

NR5A1 promotes differentiation of 3β-HSD-positive cells

Our results reveal a novel function of NR5A1 in the negative regulation of the Notch signaling pathway during fetal ovarian development. In addition to its critical functions in ovarian development, Notch signaling also plays a pivotal role in fetal testis development, particularly in the differentiation of fetal Leydig cells. Notch signaling restricts fetal Leydig cell differentiation by promoting and maintaining progenitor cell fate (Tang et al., 2008). Interestingly, NR5A1 has been suggested to promote fetal Leydig cell differentiation by suppressing Notch signaling in this context (Inoue et al., 2016).

Consistent with these reports, we found that overexpression of NR5A1 in the fetal ovaries of Tg-S mice led to the differentiation of 3β-HSD-positive cells, presumably ectopic fetal Leydig cells, at 15.5 dpc. However, the presence of these cells did not lead to an increase in plasma testosterone levels at birth. There may be too few 3β-HSD-positive cells in the transgenic ovaries: this is borne out by

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the fact we did not find significant elevation of *Hsd3b* gene expression in the Tg-S fetal ovaries by qRT-PCR analysis (data not shown). A recent report revealed that fetal Leydig cells do not have the capacity to produce testosterone because they do not express 17β-HSDs, essential for the last step of testosterone synthesis (O'Shaughnessy et al., 2000; Shima et al., 2013). Hence, although some ectopic 3β-HSD-positive presumptive fetal Leydig cell differentiation occurred in the Tg-S mice, the absence of Sertoli cells expressing 17β-HSD (which convert androstenedione to testosterone) means that no testosterone can be produced.

Fetal gonadal development requires optimal levels of *Nr5a1* and Notch signaling

Although exogenous *Nr5a1* continued to be expressed after birth (Fig. S1D–L), it is likely that ectopic expression of *Nr5a1* during fetal life is responsible for the observed ovarian phenotype in the transgenic mice. First, the phenotype could be rescued by injecting the NOTCH2 agonist during the fetal period. It is most likely that this rescue was caused by specific targeted effects of the antibody, based on previous studies. It has been estimated that the half-life of administered monoclonal antibodies in mice is 6–8 days (Vieira and Rajewsky, 1988). In human, the half-life is 2–3 weeks, explaining why, in current clinical practice, monoclonal antibodies are widely used for the therapy of intractable diseases, such as autoimmune and malignant diseases. In the case of HMN29-2, the monoclonal antibody used in the present study, the specificity and pharmacological effects *in vitro* and *in vivo* were established previously using a podocyte culturing system (Tanaka et al., 2014). In the present study, we administered hamster IgG as a negative control, and found that it did not ameliorate the phenotype of the Tg mice. Taken together, the evidence suggests that the effects observed result from sustained and specific recognition of the target Notch in the ovary, and that perturbations of Notch signaling underpin the phenotype of the Tg mice.

Second, in ovarian development, the regulation of Notch signaling by NR5A1 may be context dependent and limited to the fetal stage, since the mRNA levels of *Notch2/3* and *Nr5a1* are known to simultaneously increase after birth (Hinshelwood et al., 2005; Vanorny et al., 2014). Third, one of the major phenotypes of the Tg-A and Tg-S mice is MOFs, which are caused by abnormal nest breakdown in the perinatal period.

Regarding the mechanisms underpinning subfertility in the Tg-A mice, a previous study of a *Jag1* knockout mouse model with reduced Notch signaling revealed a similar subfertility phenotype, although the total number of follicles remained unchanged from wild type (Vanorny et al., 2014). In our Tg-A mice, the number of follicles was similarly unaffected. The phenotypes of Tg-S mice, including apoptosis of follicular cells, would also be caused by the same pathophysiology as Tg-A mice, because the phenotypes of Tg-S mice are similar but more severe. These are in line with the higher expression level of the transgene in Tg-S than that in Tg-A. Further study is required to shed light on the apparent functions of Notch signaling in ovarian development.

Based on our data presented here, and published data, we propose a model of NR5A1 function in fetal gonadal development (Fig. 8). The sexually dimorphic expression pattern of *Nr5a1* in the developing fetal gonads allows Notch signaling activity to be tuned to optimal levels to suit distinct developmental programs. In fetal ovaries, downregulated *Nr5a1* de-represses Notch signaling, thereby allowing appropriate follicular development. On the other hand, elevated levels of NR5A1 in fetal testes represses Notch signaling, allowing fetal Leydig cells to differentiate.

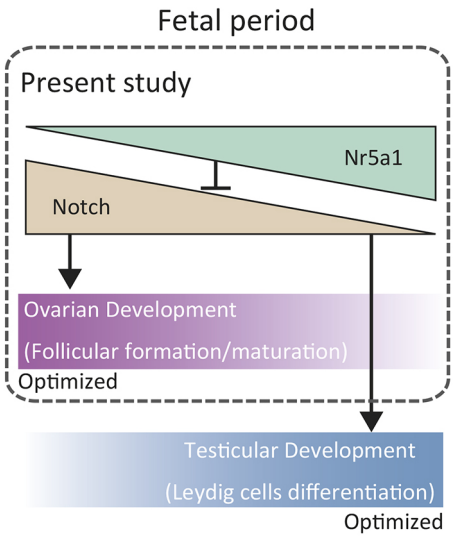

Fig. 8. A model for NR5A1 function in fetal gonadal development in mice. NR5A1 fine-tunes Notch signaling levels to achieve optimal developmental outcomes in both fetal ovaries.

Our model suggests that an optimal level of *Nr5a1* in fetal ovaries is required for proper development of follicles, and that either insufficient or excessive *Nr5a1* expression in fetal ovaries leads to impaired ovarian development. Consistent with this model, *Cited2*-null mice with severely reduced *Nr5a1* expression in fetal ovaries showed impaired expression of several ovarian marker genes (Combes et al., 2010), suggesting that low levels of *Nr5a1* expression are required for ovarian development. Similarly, women carrying loss-of-function mutation in *NR5A1* often develop premature ovarian insufficiency (Lourenço et al., 2009). On the other hand, mild upregulation of *Nr5a1* expression in Tg-A mice was sufficient to impair follicular development and female fertility, even though the increase in expression was very mild compared to wild-type ovaries.

In summary, our study provides novel insight into the molecular pathways regulating fetal ovarian development, about which little is currently understood. We show that insufficiently repressed *Nr5a1* during fetal ovarian development leads to compromised follicular development and fertility issues due to dysregulated Notch signaling. Alterations in *Nr5a1* dosage, either reduced or excessive, result in pathological effects in ovarian development and female fertility, indicating that the precise control of *Nr5a1* at the transcriptional level is essential for optimal ovarian development. Further studies are required to reveal molecular details of the NR5A1–Notch–WNT4 axis in fetal ovarian development. We envisage that the improved understanding of how this pathway regulates ovarian development and female fertility would aid the development of artificial somatic ovarian cells, which in turn may provide a valuable treatment option in reproductive medicine.

MATERIALS AND METHODS

Generating transgenic mice

The mouse transgenesis procedure was based on a protocol described previously (Zhao et al., 2014). Briefly, a sequence containing the mouse *Nr5a1* coding region followed by an internal ribosomal entry site and the sequence encoding enhanced green fluorescent protein (*Nr5a1*-IRES-*Egfp*) was cloned into the PBWt1-Dest vector via Gateway LR recombination (Fig. 1A). Transgenic founder mice were produced by pronuclear injection of the PBWt1-*Nr5a1*-IRES-*Egfp* vector DNA and hyperactive piggyBac

transposase mRNA as described. The XY male founders mated with BDF1 females, and transmitted the transgene through the germ line. For subsequent analyses, we chose two lines, Tg-A and Tg-S. Both lines were maintained by mating F0 or F1 XY transgenic male mice with BDF1 wild-type females. Genotyping and sexing was performed by PCR (primer sequences are listed in Table S1) using genomic DNA prepared from tail or ear biopsies. All animal procedures were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Fertility analysis of transgenic female mice

The fertility of Tg-A and Tg-S female mice, compared with that of wild-type female mice, was assessed after they turned 6 weeks old by continuous mating with ∼30-week-old BDF1 male mice. Three female mice from each line were analyzed. The average number of offspring in the first litter and the aggregated number of offspring from all litters were calculated for each genotype.

Real-time qRT-PCR analysis

RNA was extracted from 15.5 dpc or P0 mice gonads using a Qiagen RNeasy Mini kit according to the manufacturer's instructions. Typically, 0.2 µg total RNA was reverse transcribed using random hexamers (Promega) and SuperScript III reverse transcriptase (Invitrogen). qRT-PCR was conducted using a LightCycler System (Roche Diagnostics, Basel, Switzerland) with the LightCycler DNA Master SYBR Green Kit (Roche) for 45 cycles. Gene expression levels were analyzed using the comparative cycle time (Ct) method. Primers used in these experiments are listed in Table S1. Ribosomal protein S29 (*Rps29*) served as the housekeeping gene for normalization, as it shows minimum variability during fetal gonadal development (Svingen et al., 2009).

Histological analyses

Cryosections

Gonadal samples were fixed overnight in 4% paraformaldehyde (PFA) at 4°C. After washing three times with PBS, samples were incubated overnight in 20% sucrose/PBS at 4°C. Samples were then incubated in 30% sucrose/OCT at 40°C for 30 min and embedded.

Paraffin sections

Gonadal samples were fixed overnight in 4% PFA at 4°C and embedded in paraffin. The blocks were sectioned at 6-µm thickness and were later deparaffinized as previously described (Suzuki et al., 2010).

Hematoxylin and Eosin staining

After staining with 2× Hematoxylin for 4 min, the sections were washed for 12 min under running water and then stained with 1.0% Eosin for 2 min.

IF

Cryosections and paraffin sections were used for IF. For cryosections, 8-µm samples were washed twice with PBS and activated with citric acid solution. The sections were blocked with 5% bovine serum albumin-PBS at room temperature for 1 h, followed by overnight incubation with the primary antibody at 4°C. Next, the sections were washed twice with PBS with Tween 20 (PBS-T), incubated with the secondary antibody at room temperature for 1.5 h, and again washed twice with PBS-T. Finally, the sections were incubated with 4′,6-diamidino-2-phenylindole (DAPI; Dojindo, 1:1000) for 5 min, washed once with PBS-T and mounted in Fluoromount™. Information on antibodies used in this study is provided in Table S2.

Counting numbers of follicles, apoptotic follicles or MOFs

Counting was performed as previously described (Xu and Gridley, 2013). Briefly, serial sections (6-µm thick) from a whole ovary were placed on five slides, each slide containing sequential slices at every 30-µm interval (6 µm×5 slides). The sections analyzed cover the entire ovary, and we thus counted the total number of follicles across the ovary. MOFs were defined as follicles containing more than a single oocyte (Myers et al., 2004; Pedersen and Peters, 1968). Anti-MVH (also known as DDX4) antibody (ab13840, Abcam) and 3,3′-diaminobenzidine (DAB; 25985-50, Nacalai Tesque) staining was used to count the number of follicles and MOFs. The types of follicles were classified according to previous reports (Myers et al., 2004; Pedersen and Peters, 1968), i.e. the 1–2, 3, 4–5 and 6–8 follicular types correspond to 'primordial', 'primary', 'secondary' and 'antral/tertiary' follicles, respectively. We used anti-cleaved caspase 3 (9579, Cell Signaling Technology) to identify apoptotic granulosa cells and calculated the percentage of caspase 3-positive follicles. For quantification, three samples from each mouse were analyzed.

Hormone measurements

Free testosterone and estradiol levels of P0 mice were measured using commercially available ELISA kits (DB52181, IBL International; 582251, Cayman Chemical). Three sets of plasma samples obtained from each of five mice were analyzed according to the manufacturer's protocol.

Preparation and administration of the NOTCH agonist HMN2-29

BDF1 eggs were fertilized *in vitro* with Tg-A sperm and transplanted into the oviducts of ICR mice. The NOTCH2 agonist HMN2-29, a hamster monoclonal antibody, was prepared as described previously (Tanaka et al., 2014). Three doses of 0.5 mg HMN2-29 or control hamster IgG (145-19561, Wako) were intraperitoneally injected into pregnant ICR mice at 13.5, 16.5 and 18.5 dpc.

Statistical analysis

We used the unpaired *t*-test (Welch's test) to demonstrate that a given sample differed statistically from wild-type control (Figs 1 and 3–6) in a direct two-point comparison. We consulted our Institute statistics experts to confirm that this is appropriate.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: R.N., K.K.; Methodology: R.N., K.K., H.S., L.Z., A.T.-H., H.Y., M.T., J.B., P.K.; Software: R.N.; Validation: K.K.; Formal analysis: R.N., K.K., H.S.; Investigation: R.N., K.K.; Resources: L.Z., J.B., P.K., M.K.-A.; Data curation: R.N., K.K., H.S.; Writing - original draft: R.N., K.K.; Writing - review & editing: K.K., L.Z., J.B., P.K.; Visualization: R.N., K.K., H.S.; Supervision: K.K., H.S., Y.K., J.B., P.K., M.K.-A., T.M.; Project administration: K.K.; Funding acquisition: K.K., M.K.-A.

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Supplementary information

Supplementary information available online at
http://jcs.biologists.org/lookup/doi/10.1242/jcs.223768.supplemental

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