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

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Running title: NR5A1 suppresses Notch signaling during ovarian development

24 **ABSTRACT**

25

26 The nuclear receptor NR5A1 (also known as Ad4BP, or SF1) is essential for the initial steps
27 of mammalian gonadal development. The *Nr5a1* gene is equally expressed in XX and XY
28 gonadal primordia, but after sex determination, is up-regulated in XY and down-regulated in
29 XX gonads. We recently reported a case of 46, XX disorder of sex development (DSD) in
30 which ectopically expressed NR5A1 in XX gonads led to an ovo-testicular phenotype,
31 suggesting that excess NR5A1 can direct the development of immature XX gonads towards
32 testicular formation. However, a direct causal relationship has not been demonstrated in an
33 animal model. Here, using a Wt1-BAC (bacterial artificial chromosome) transgene system,
34 we generated two lines of mice overexpressing *Nr5a1* in the fetal gonads at different levels.
35 One of these lines (Tg-S), highly expressing *Nr5a1*, revealed that enforced *Nr5a1* expression
36 alone is insufficient to switch the fate of the 46,XX gonads toward testicular formation in
37 mice. In the other line (Tg-A) expressing *Nr5a1* at lower level, ovarian development was
38 compromised, with multi-oocyte follicles, reduced number of matured follicles, and impaired
39 expression of *Wnt4*, resulting in late onset infertility at 20 weeks after birth. The phenotype
40 was similar to that of genetically modified mice with impaired Notch signaling. Indeed, the
41 expression level of *Notch2* and *3* was significantly reduced in Tg-A mice, and the ovarian
42 phenotype in Tg-A mice was almost completely rescued by in utero treatment with a *Notch2*
43 agonist HMN2-29. We conclude that suppression of *Nr5a1* during the fetal period optimizes
44 ovarian development by fine tuning of Notch signaling levels.

45 (257 words)

46

47 **AUTHOR SUMMARY**

48 Sexual development is a process of differentiation from undifferentiated bipotential gonads,
49 and insight into sexual differentiation will bring important new knowledge to our

50 understanding of organogenesis. The nuclear receptor NR5A1 which is essential for
51 mammalian gonadal development, is equally expressed in both gonadal primordia, but after
52 sex determination, is up-regulated in XY and down-regulated in XX gonads. We have
53 recently demonstrated that this down-regulation is mediated by ovarian transcription factor,
54 Forkhead box L2 (FOXL2). This finding raised two key questions, whether Nr5a1 can
55 function as a male sex-determining factor, and whether the repression is essential for
56 appropriate ovarian development. By generating two lines Tg mice in XX gonads with
57 different enforced expression levels of Nr5a1, our present study revealed that alterations in
58 Nr5a1 dosage, either reduced or excessive, result in pathological effects in ovarian
59 development and female fertility, indicating that the precise control of Nr5a1 at the
60 transcriptional level is essential for optimal ovarian development. We envisage that the
61 improved understanding of how this pathway regulates ovarian development and female
62 fertility would aid the development of artificial somatic ovarian cells, which in turn may
63 provide a valuable treatment option in reproductive medicine.

64

65 **ABBREVIATIONS**

66 BAC: bacterial artificial chromosome

67 dpc: days post coitum

68 FOXL2: Forkhead box L2

69 HSD: hydroxysteroid dehydrogenase

70 IF: Immunofluorescence

71 MOFs: multiple oocyte follicles

72 PFA: paraformaldehyde

73 qRT-PCR: Quantitative real-time PCR

74 Rps29: Ribosomal protein S29

75 SRY: sex-determining region Y

76

77

1 INTRODUCTION

2

3 NR5A1, also known as Ad4BP or SF1, is a member of the nuclear receptor superfamily. In
4 mice, *Nr5a1* is expressed from about 9.5 days *post coitum* (dpc), in the anlagen of the gonads
5 and the adreno-genital primordium [1], and knockout models show complete gonadal agenesis
6 in both XX and XY, suggesting that NR5A1 is essential for genital ridge development in both
7 sexes [2] [3]. In addition to initiating gonadal development, NR5A1 plays crucial roles in
8 testicular development. It is transcriptionally up-regulated in the developing mouse testes [4],
9 where it acts as a cofactor of the male sex-determining factor SRY (sex-determining region
10 Y) to induce *Sox9* expression [5]. Subsequently, it cooperates with SOX9 to maintain the
11 expression of *Sox9* itself [5] and up-regulate other Sertoli cell-specific genes, including *AMH*
12 (De Santa Barbara et al., 1998). Furthermore, NR5A1 is essential for the differentiation of the
13 testicular steroidogenic cells, fetal Leydig cells [6]. Consistent with its essential roles in testis
14 differentiation, heterozygous loss-of-function mutations in *NR5A1* cause XY female sex
15 reversal in humans [7-10].

16

17 In contrast to its transcriptional up-regulation in fetal testes, *Nr5a1* expression in mouse fetal
18 ovaries decreases after 12.5 dpc [1]. We have recently demonstrated that this down-regulation
19 is mediated by Forkhead box L2 (FOXL2), a key ovarian transcription factor expressed
20 mainly in pregranulosa/granulosa cells [11]. FOXL2 directly binds to the proximal promoter
21 of *Nr5a1*, thereby antagonizing the actions of WT1-KTS. Given the highly conserved
22 sequence for FOXL2 binding, this regulatory mechanism may be maintained in other
23 eutherian mammals [11]. Based on these observations, we hypothesized that adequate
24 suppression of *Nr5a1* may be essential for normal ovarian development. However, the
25 biological relevance of *Nr5a1* suppression during fetal ovarian development has not been
26 clarified.

1
2 Recently, we and others have reported 46,XX testicular or ovo-testicular DSD individuals
3 carrying mutations in codon 92 of *NR5A1*, including the R92W and R92Q mutations [12-16].
4 The variant protein was thought to function in the XX gonads by escaping the suppressive
5 action of NR0B1 (DAX-1), a pro-ovary factor [13, 16, 17]. The presence of testicular tissue in
6 the probands further suggested that ectopic activity of NR5A1 may drive testis differentiation
7 in the absence of the *SRY* gene.

8
9 These observations raised two key questions. Firstly, it is not clear whether *Nr5a1* can
10 function as a male sex-determining factor, i.e., whether elevated *Nr5a1* expression levels in
11 XX gonads where the male determining gene *Sry* is absent, are sufficient to direct the fate of
12 gonads towards testicular development. Secondly, it is not known whether repression of
13 *Nr5a1* is essential for appropriate ovarian development.

14
15 To address these questions, we exploited a BAC (bacterial artificial chromosome) transgene
16 system [18] whereby *Nr5a1* expression is driven by *Wtl* regulatory sequences. In the fetal XX
17 gonads, endogenous *Wtl* is expressed in supporting cells including pre-granulosa cells and
18 coelomic epithelium [19-21]. Hence, by directing transgenic *Nr5a1* expression to *Wtl*-
19 expressing XX gonadal supporting cells, we aimed to investigate the consequence of *Nr5a1*
20 overexpression in the relevant cell types in XX mouse fetal gonads. Molecular and phenotypic
21 analysis of the two transgenic mouse lines generated demonstrated, firstly, that enforced
22 *Nr5a1* expression alone is insufficient to switch the fate of the 46,XX gonads toward
23 testicular formation in mice and, secondly, that overexpression of NR5A1 disrupts ovarian
24 follicular development and causes premature ovarian insufficiency by dysregulating levels of
25 Notch signaling, which is known to be important for ovarian development and function.

26

1 RESULTS

2

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

4 Using the piggyBac-enabled *Wtl*-BAC system [18] (Fig. 1A), we successfully generated two
5 transgenic mouse lines (Tg-A and Tg-S) expressing an *Nr5a1*-IRES-*Egfp* transgene at
6 different levels (Fig. 1B–M). Using quantitative reverse transcriptase PCR (qRT-PCR) we
7 found that XX Tg-S fetal gonads expressed *Nr5a1* at almost the same level as that in wild
8 type testes, whereas the XX Tg-A gonads expressed *Nr5a1* at levels between those in wild
9 type testes and ovaries (Fig. 1N). We confirmed histologically that the kidneys and adrenal
10 glands, expressing endogenous *Wtl* at high levels, developed no apparent abnormalities in
11 either Tg-A or Tg-S mice, suggesting that the gonadal phenotypes of the transgenic mice were
12 unlikely to be caused by the impaired function of those organs.

13

14 In both Tg-A and Tg-S lines, XX mice developed female internal and external genitalia (Fig.
15 2A–F). Morphologically, the gonads in adult XX Tg-S mice were streak-like and elongated
16 (Fig. 2F,J). In Tg-A mice, fetal ovaries were longer and thinner than wild type (Fig. S1A–D),
17 However, the elongation of the gonads resolved by P0. Since a similar phenotype was
18 reported in *Sox4*^{-/-} mice [22], we examined the expression levels of *Sox4* in the XX Tg-A
19 gonads at 15.5 dpc and indeed found significantly reduced expression of *Sox4* (Fig. S1E),
20 suggesting that elevated NR5A1 repressed *Sox4* resulting in abnormal morphology of the fetal
21 ovaries.

22

23 In both transgenic lines, histological examination of XX adult gonads revealed the presence of
24 ovarian follicles but detected no seminiferous tubule-like structures (Fig. 2G–J). Consistently,
25 FOXL2, a marker of ovarian granulosa cells, was expressed in XX adult gonads in both
26 mouse lines (Fig. 2L, M). In contrast, SOX9, a marker of testicular Sertoli cells, was not

1 detected (Fig. 2P, Q). Based on these data, we conclude that *Nr5a1* overexpression alone is
2 insufficient to cause XX sex reversal in mice.

3

4 **Presence of ectopic steroidogenic cells in fetal ovaries of *Nr5a1*-transgenic mice**

5 In addition to Sertoli cells, NR5A1 is highly expressed in gonadal steroidogenic cell lineages
6 in both sexes and is essential for their differentiation (Buaas Swain, Development 2012) [23,
7 24]. In the males, steroidogenic Leydig cells differentiate during fetal testis development and
8 produce androgen [25]. In contrast, the ovarian steroidogenic theca cells do not differentiate
9 until after birth [25-27]. We asked whether overexpression of *Nr5a1* is able to induce the
10 ectopic differentiation of steroidogenic cells in fetal XX gonads. Immunofluorescence
11 analysis of the XX fetal gonads of Tg-A and Tg-S mice (Fig. 3A–L) revealed that 3 β -
12 hydroxysteroid dehydrogenase (3 β -HSD), the enzyme mediating the first step of
13 steroidogenesis and a marker of steroidogenic cells [28], was detected in the Tg-S XX gonads
14 (Fig 3G). However, the plasma testosterone and estradiol levels at P0 were not elevated in XX
15 Tg-S mice compared to wild type (Fig. 3M, N). This is consistent with the lack of
16 masculinization in XX Tg-S mice (Fig. 2C,F).

17

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

19 Histological examination of Tg-A and Tg-S ovaries revealed increased number of multiple
20 oocyte follicles (MOFs) (Fig. 4A–D), suggesting a disruption of ovigerous cord fragmentation.
21 Further, we found that the numbers of antral follicles (type 6–8) decreased significantly in
22 both transgenic lines compared to wild type (Fig. 4E). The numbers of antral follicles
23 appeared to be inversely correlated with *Nr5a1* expression levels, with the Tg-S ovaries
24 containing fewer antral follicles than the Tg-A ovaries (Fig. 4E). To determine whether this
25 was caused by reduced proliferation or increased apoptosis of follicular granulosa cells, we
26 analysed the expression of Ki-67, a maker of proliferating cells, and cleaved Caspase 3, a

1 marker of apoptotic cells, in the transgenic ovaries at P28. We observed no obvious changes
2 in the follicles containing Ki-67-positive granulosa cells (Fig. 4J-L), but a significant increase
3 in secondary follicles (type 4 and 5) containing cleaved Caspase 3-positive granulosa cells in
4 both Tg-A and Tg-S ovaries (Fig. 4F, G-I, M-O). The increase in follicular atresia correlated
5 with *Nr5a1* expression levels, with Tg-S ovaries exhibiting higher levels of atresia than Tg-A
6 ovaries (Fig. 4F,G-I,M-O). No increase in type 6 antral follicles containing cleaved Caspase
7 3-positive cells was observed (Fig. 4F). Together, these results suggest that overexpression of
8 *Nr5a1* caused increased apoptosis of granulosa cells in secondary follicles in a dose-
9 dependent manner, resulting in reduced numbers of antral follicles.

10

11 We next assessed the fertility of Tg-A and Tg-S female mice. Tg-S female mice were almost
12 infertile. Of three Tg-S females analyzed, the litter size was reduced from their first mating
13 (Fig. 4P). The reproductive performance of Tg-A females was also reduced. Although Tg-A
14 mice produced four consecutive litters to begin the study, they failed to produce additional
15 litters thereafter (Fig. 4Q), suggesting that these females underwent premature ovarian
16 insufficiency.

17

18 ***Nr5a1* overexpression represses Notch signaling levels in fetal ovaries**

19 Interactions between germ cells and somatic pregranulosa cells are crucial for the formation
20 of ovarian follicles, and Notch signaling plays a major role in mediating this interaction [29-
21 31]. In the developing ovaries, oocytes and other neighbouring cells secrete the Notch ligands,
22 including JAG1 and possibly JAG2, which bind to Notch receptors (mainly NOTCH2)
23 present on the surface of pre-granulosa cells, thereby activating Notch signaling [32]. As a
24 result, pre-granulosa cells proliferate and encapsulate individual germ cells to form primordial
25 follicles, i.e., the resolution of germ cell syncytia [30]. Genetic ablation of either *Jag1* or
26 *Notch2*, two important Notch pathway components, gives rise to MOFs and causes premature

1 reproductive senescence [29]. Because of the similarity in the phenotypes between our *Nr5a1*
2 transgenic mice and *Jag1*- or *Notch2*-deficient mice, we hypothesized that enforced
3 expression of *Nr5a1* may compromise ovarian development by repressing the Notch signaling
4 pathway.

5
6 We therefore analysed mRNA expression levels of a number of genes involved in Notch
7 signaling in 15.5-dpc fetal ovaries using qRT-PCR (Fig. 5 A~H). Supporting our hypothesis,
8 we found significant down-regulation of several Notch pathway genes, including *Notch2*,
9 *Notch3* and *Dll4*, in Tg-A ovaries compared with wild-type ovaries (Fig. 5B, C, H).

10

11 **A Notch2 agonist rescues the ovarian phenotype in Tg-A mice**

12 To further clarify the contribution of Notch signaling to the ovarian phenotype in *Nr5a1*
13 transgenic mice, we attempted a rescue experiment with a Notch2 agonist HMN2-29, a
14 hamster monoclonal antibody [33]. We injected three doses of HMN2-29 or control hamster
15 IgG intraperitoneally into pregnant mice carrying wild-type or Tg-A fetuses at 13.5, 16.5 and
16 18.5 dpc (Fig. S2A), and analysed the ovaries postnatally (P14 and P28). No obvious adverse
17 effects of *in utero* administration of the Notch2 agonist were observed, as the treated mice
18 appeared grossly normal with body weight comparable to wild type at P28 (Fig. S2B).

19

20 Confirming our hypothesis, we found reduced number of MOFs in Tg-A mice at P14 upon
21 HMN2-29 treatment (Fig. 6A). Moreover, treatment with the NOTCH2 agonist completely
22 rescued the numbers of antral follicles in the Tg-A ovaries at P28 (Fig. 6B). The complete
23 rescue of antral follicle numbers appeared to be a result of reduced atresia of secondary
24 follicles in the treated ovaries (Fig. 6C-O). These results suggest that *Nr5a1* fine-tunes Notch
25 signaling levels in fetal ovaries to ensure proper folliculogenesis and normal fertility, and that

1 repression of *Nr5a1* during fetal ovarian development is essential to allow the Notch signaling
2 levels to elevate appropriately.

3

4 We further explored molecular mechanisms by which the Notch signaling pathway regulates
5 fetal ovarian development. To this end, we analysed expression levels of two master
6 regulators of ovarian development, *Wnt4* and *Foxl2*, in the Tg-A ovaries at P0. We found that
7 *Wnt4*, but not *Foxl2*, was significantly down-regulated in Tg-A mice compared to wild type
8 (Fig. 6P, Q). Importantly, *Wnt4* expression in the Tg-A ovaries were fully restored by the
9 administration of HMN2-29 (Fig. 6Q), suggesting that down-regulated *Wnt4* expression was a
10 result of de-regulated Notch signaling pathway rather than a direct effect of NR5A1
11 overexpression. We note that this regulatory relationship is likely limited to the late-stage
12 fetal ovaries, as expression of the Notch pathway genes starts in fetal ovaries from about 15.5
13 dpc [29].

14

1 **DISCUSSION**

2

3 *Nr5a1* plays known essential roles in the development of genital ridges in both sexes and fetal
4 testes in the male. We show in the present study that it also plays an important role in fetal
5 ovarian development by fine-tuning the levels of Notch signaling.

6

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

9 Of the two transgenic lines generated, the Tg-S line, expressed *Nr5a1* in the XX gonads at
10 levels comparable to that in wild-type testes. Nevertheless, XX mice of the Tg-S line
11 developed female internal and external genitalia. We presume that NR5A1 may play different
12 roles with respect to sex determination in humans and mice, and wild-type NR5A1 may not
13 possess the ability to drive testis differentiation on its own in mice. Consistently, XX mice
14 carrying heterozygous or homozygous R92W mutation in *Nr5a1* showed no signs of
15 masculinization [13], supporting that NR5A1 (and the R92W mutant) may function
16 differently in humans and mice. The differences between humans and mice regarding the
17 molecular mechanisms of sex determination and gonadal development have been documented
18 in several cases. For example, duplication of *DAX-1* (*NR0B1*), an orphan nuclear receptor
19 gene, causes XY female sex reversal in humans [34], whereas transgenic overexpression of
20 *Dax1* in mice does not normally cause female sex reversal [17]. Since a major function of
21 DAX1 is to antagonize NR5A1 [1, 34-36], it is conceivable that NR5A1 also plays a species-
22 specific role in sex determination.

23

24 **NR5A1 promotes fetal Leydig cell differentiation by restricting Notch signaling**

25 Our results reveal a novel function of NR5A1 in the negative regulation of the Notch
26 signaling pathway during fetal ovarian development. In addition to its critical functions in

1 ovarian development, Notch signaling also plays a pivotal role in fetal testis development,
2 particularly in the differentiation of fetal Leydig cells. Notch signaling restricts fetal Leydig
3 cell differentiation by promoting and maintaining the progenitor cell fate [37]. Interestingly,
4 NR5A1 has been suggested to promote fetal Leydig cell differentiation by suppressing Notch
5 signaling in this context [38].

6
7 Consistent with these reports, we found that overexpression of NR5A1 in the fetal ovaries of
8 Tg-S mice led to the differentiation of 3 β -HSD positive cells, presumably ectopic fetal Leydig
9 cells, at 15.5 dpc. However, the presence of these cells did not lead to an increase in plasma
10 testosterone levels at birth. There may be too few 3 β -HSD positive cells in the transgenic
11 ovaries: this is borne out by the fact we did not find significant elevation of *Hsd3b* gene
12 expression in the Tg-S fetal ovaries by qRT-PCR analysis (data not shown). A recent report
13 revealed that fetal Leydig cells do not have the capacity to produce testosterone because they
14 do not express 17 β -hydroxysteroid dehydrogenases (17 β -HSD), essential for the last step of
15 testosterone synthesis [24, 39]. Hence, although some ectopic 3 β -HSD-positive presumptive
16 fetal Leydig cell differentiation occurred in the Tg-S mice, the absence of Sertoli cells
17 expressing 17 β -HSD (which convert androstenedione to testosterone) means that no
18 testosterone can be produced.

19

20

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

22 Based on our and published data, we propose a model of NR5A1 function in fetal gonadal
23 development (Fig. 7). The sexually dimorphic expression pattern of *Nr5a1* in the developing
24 fetal gonads allows the Notch signaling activity to be tuned to optimal levels to suit distinct
25 developmental programs. In fetal ovaries, downregulated *Nr5a1* de-represses Notch signaling,
26 thereby allowing appropriate follicular development. On the other hand, elevated levels of

1 NR5A1 in fetal testes represses Notch signaling, allowing fetal Leydig cells to differentiate.
2 In ovarian development, the regulation of Notch signaling by NR5A1 may be context-
3 dependent and limited to the fetal stage, since the mRNA levels of *Notch2/3* and *Nr5a1* are
4 known to simultaneously increase after birth [29, 40].

5
6 Our model suggests that an optimal level of *Nr5a1* in fetal ovaries is required for proper
7 development of follicles, and that either insufficient or excessive *Nr5a1* expression in fetal
8 ovaries leads to impaired ovarian development. Consistent with this model, *Cited2*-null mice
9 with severely reduced *Nr5a1* expression in fetal ovaries showed impaired expression of
10 several ovarian marker genes [41], suggesting that low levels of *Nr5a1* expression are
11 required for ovarian development. Similarly, women carrying loss-of-function mutation in
12 *NR5A1* often develop premature ovarian insufficiency [42]. On the other hand, mild
13 upregulation of *Nr5a1* expression in Tg-A mice was sufficient to impair follicular
14 development and female fertility, even though the increase in expression was very mild
15 compared to wild-type ovaries.

16
17 In summary, our study provides novel insight into the molecular pathways regulating fetal
18 ovarian development, about which little is currently understood. We show that insufficiently
19 repressed *Nr5a1* during fetal ovarian development leads to compromised follicular
20 development and fertility issues due to dysregulated Notch signaling. Alterations in *Nr5a1*
21 dosage, either reduced or excessive, result in pathological effects in ovarian development and
22 female fertility, indicating that the precise control of *Nr5a1* at the transcriptional level is
23 essential for optimal ovarian development. Further studies are required to reveal molecular
24 details of the NR5A1-Notch-WNT4 axis in fetal ovarian development. We envisage that the
25 improved understanding of how this pathway regulates ovarian development and female

1 fertility would aid the development of artificial somatic ovarian cells, which in turn may
2 provide a valuable treatment option in reproductive medicine.

3

4 **MATERIALS AND METHODS**

5

6 **Generating transgenic mice**

7 The mouse transgenesis procedure was based on a protocol described previously [18]. Briefly,
8 a sequence containing the mouse *Nr5a1* coding region followed by an internal ribosomal
9 entry site and the sequence encoding enhanced green fluorescent protein (*Nr5a1-IRES-Egfp*)
10 was cloned into the PBWt1-Dest vector via Gateway LR recombination (Fig. 1A). Transgenic
11 founder mice were produced by pronuclear injection of the PBWt1-*Nr5a1-IRES-Egfp* vector
12 DNA and hyperactive piggyBac transposase mRNA as described. The XY male founders
13 mated with BDF1 females, and transmitted the transgene through the germ line. For
14 subsequent analyses, we chose two lines, Tg-A and Tg-S. Both lines were maintained by
15 mating F0 or F1 XY transgenic male mice with BDF1 wild type females. Genotyping and
16 sexing was performed by PCR (primer sequences provided in Supplementary Table S1) using
17 genomic DNA prepared from tail or ear biopsies. All animal procedures were approved by the
18 Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

19

20 **Fertility analysis of transgenic female mice**

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

26

1 **Real-time RT-PCR analysis**

2 RNA was extracted from 15.5 dpc or P0 mice gonads using QIAGEN RNeasy Mini kit
3 according to manufacturer's instructions. Typically, 0.2 µg of total RNA was reverse
4 transcribed using random hexamers (Promega) and SuperScript III reverse transcriptase
5 (Invitrogen). Quantitative real-time PCR (qRT-PCR) was conducted using a Light Cycler
6 system (Roche Diagnostics, Basel, Switzerland) with the Light Cycler DNA master SYBR
7 Green kit (Roche) for 45 cycles. Gene expression levels were analysed using the comparative
8 cycle time (Ct) method. Primers used in these experiments are listed in Supplementary Table
9 S1. *Rps29* (Ribosomal protein S29) served as the housekeeping gene for normalization, as it
10 shows minimum variability during fetal gonadal development [36].

11

12 **Histological analyses**

13 *Cryosections*: Gonadal samples were fixed overnight in 4% paraformaldehyde (PFA) at 4 °C.
14 After washing three times with PBS, samples were incubated overnight in 20% sucrose/PBS
15 at 4 °C. Samples were then incubated in 3/4 30% sucrose/OCT at 40 °C for 30 min and
16 embedded.

17 *Paraffin-sections*: Gonadal samples were fixed overnight in 4% PFA at 4 °C and embedded in
18 paraffin. The blocks were sectioned at 6-µm thickness and were later deparaffinised as
19 previously described [43].

20

21 **Hematoxylin and Eosin (HE) stain**: After staining with 2× Haematoxylin for 4 min, the
22 sections were washed for 12 min under running water and then stained with 1.0% Eosin for 2
23 min.

24

25 **Immunofluorescence (IF)**: We employed cryosections and paraffin sections for IF. For cryo-
26 sections, 8 µm of samples were washed twice with PBS and activated with citric acid solution.

1 The sections were blocked with 5% BSA-PBS at room temperature for 1 h, followed by
2 overnight incubation with primary antibody at 4 °C. Next, the sections were washed twice
3 with PBS-T, incubated with secondary antibody at room temperature for 1.5 h, and again
4 washed twice with PBS-T. Finally, the sections were incubated with DAPI (1:1000; Dojindo)
5 for 5 min, washed once with PBS-T, and mounted in Fluoromount™. The information of
6 antibodies used in this study was indicated in Supplementary Table S2.

7

8 **Counting numbers of follicles, apoptotic follicles, or multi-oocyte follicles (MOFs):**

9 Counting was performed as previously described [31]. Briefly, serial sections (6- μ m thick)
10 from a whole ovary were placed on 5 slides, each slide containing sequential slices at every
11 30 μ m interval (6 μ m * 5 slides). Multi-oocytic follicles were defined as follicles containing
12 more than a single oocyte. Anti-MVH antibody (ab13840, Abcam) and DAB staining (25985-
13 50, Nacalai Tesque Inc.) was used to count the number of follicles and MOFs. The types of
14 follicles were classified according to previous reports [44, 45]. We used anti-Cleaved Caspase
15 3 (Cell signaling technology, Cat No. 9579) to identify apoptotic granulosa cells and
16 calculated the percentage of Caspase 3-positive follicles.

17

18 **Hormone measurements**

19 Free testosterone and estradiol levels of P0 mice were measured using commercially available
20 ELISA kits (IBL International (DB52181) and Cayman Chemical (582251)). Three sets of
21 plasma samples obtained from each of 5 mice were analyzed according to the manufacturer's
22 protocol.

23

24 **Preparation and administration of the Notch agonist HMN2-29**

25 BDF1 eggs were fertilized *in vitro* with Tg-A sperm and transplanted into the oviducts of ICR
26 mice. The Notch2 agonist HMN2-29, a hamster monoclonal antibody, was prepared as

1 described previously [33]. Three doses of 0.5 mg of HMN2-29 or control hamster IgG (Wako,
2 Cat No. 145-19561) were intraperitoneally injected into pregnant ICR mice at 13.5, 16.5 and
3 18.5 dpc.

4

5 **Statistical analysis**

6 We used the unpaired *t*-test (Welch's test) to determine statistically significant differences
7 between the test and the control group.

8

9

1 **Disclosure statement**

2 The author declares no conflict of interest associated with this research.

3

4 **Data availability.**

5 Authors can confirm that all relevant data are included in the paper and/ or its supplementary
6 information files

7

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11 study design, data collection and analysis, decision to publish, or preparation of the
12 manuscript.

13

14 **Author Contributions**

15 KK, RN, HS, YK, MKA, TM contributed to the conception and the design of the present
16 study. Acquisition of data, analysis and interpretation of data were performed by RN, HS and
17 KK. The manuscript was drafted by KK, TM and RN, and critically revised by LZ, JB and PK.
18 HS, LZ, JB, PK, MT, MKA and HY provided the materials for the present study, including
19 Piggy BAC construct and Notch agonist. All experiments were performed by RN, HS, LZ,
20 KK and AHT. All authors approved the final version to be published

21

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FIGURE LEGENDS

Figure 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 *Wtl*-BAC system. *Nr5a1*-IRES-*Egfp* fragment in the Gateway entry vector was inserted into PBWt1-Dest using LR recombination. (B-M) Transgenic EGFP expression was detected using immunofluorescence in the XX fetal gonads at 15.5 dpc in both Tg-A and Tg-S lines. EGFP and NR5A1 were co-localized in XX transgenic gonads (K, L). (N) 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 that 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 \pm SD, n = 3.

Figure 2: Transgenic overexpression of *Nr5a1* in XX fetal gonads did not induce male development.

(A–C) External genitalia of wild-type, Tg-A and Tg-S XX mice at 6–8 months old. XX mice in both transgenic lines developed female-type external genitalia. (D–F) Reproductive tracts of wild-type, Tg-A, and Tg-S XX mice at 6–8 months old. In the XX Tg-S mice, the uterine horn and ovaries were elongated compared to wild type (F). (G–J) Histological analysis of gonadal sections at 6-8 months using HE stain. XX gonads of both transgenic lines contained ovarian follicles but no seminiferous tubule-like structures. (K–V) Immunofluorescence analysis for markers of granulosa (FOXL2, in red) or Sertoli cells (SOX9, in green) in the fetal gonads in adult mice. In the XX gonads of the Tg-A and Tg-S lines, only FOXL2-

1 positive granulosa cells were present (L,M), and no SOX9-positive cells were detected (P, Q;
2 some non-specific staining of the oocytes was seen using the anti-SOX9 antibody).

3

4 **Figure 3: Formation of ectopic steroidogenic cells in the XX transgenic fetal gonads.**

5 (A–L) Immunofluorescence analysis for 3 β -HSD at 15.5dpc, a marker of steroidogenic cells.

6 In the Tg-S fetal ovaries, 3 β -HSD positive cells were sparsely observed, some of which

7 showed co-localization with NR5A1 (G,K). (M, N) The plasma testosterone (M) or estradiol

8 (N) levels at P0. No significant increase in levels of testosterone or estradiol was observed in

9 XX Tg-A or Tg-S mice compared with wild-type female mice. Mean \pm SEM, n = 3. * p < 0.05

10 (Welch's t -test).

11

12 **Figure 4: Impaired ovarian development in *Nr5a1* transgenic mice.**

13 (A–C) Histological analysis of ovaries in the wild-type, Tg-A, and Tg-S females. Multi-

14 oocyte follicles (MOFs) were observed in Tg-A and Tg-S ovaries (arrowhead). (D) Numbers

15 of MOFs were counted from serial ovarian sections. Mean \pm SEM, n = 3. * p < 0.05 (Welch's

16 test). (E) The numbers of antral follicles (type 6–8) significantly decreased in transgenic mice.

17 The numbers of follicles of each type [1-2 (primordial), 3 (primary), 4-5 (secondary), 6-8

18 (antral), death and intact] were counted from serial ovarian sections. Mean \pm SD, n = 3. * p <

19 0.05 (Welch's t -test). (F) Quantitation of secondary follicles containing cleaved Caspase 3-

20 positive cells (G-I). The percentage of cleaved Caspase 3-positive follicles was plotted. Mean

21 \pm SEM, n = 3. * p < 0.05, ** p < 0.01 (Welch's t -test). (G–O) Immunofluorescence analysis for

22 markers of cell proliferation (Ki-67: red) or apoptosis (cleaved Caspase 3: green) in the XX

23 gonads of the wild-type, Tg-A and Tg-S mice. Yellow and white arrows indicate secondary

24 follicles (type 4–5) with or without cleaved Caspase 3-positive cells, respectively. (P)

25 Average litter size of the first mating of wild-type, Tg-A, and Tg-S female mice (n = 3). Mean

1 \pm SEM. * p < 0.05 (Welch's t -test). (Q) Total number of progeny from wild-type, Tg-S and
2 Tg-A female mice (n = 3).

3

4 **Figure 5: Expression of the Notch signaling pathway genes in XX Tg-A mice.**

5 (A-H) qRT-PCR analysis was performed on total RNA extracted from fetal ovaries of wild-
6 type, Tg-A, and Tg-S mice at 15.5 dpc. Expression levels were normalized to *Rps29*. Mean \pm
7 SEM, n = 3. * p < 0.05, ** p < 0.01 (Welch's t -test).

8

9 **Figure 6: Administration of a Notch2 agonist rescued the ovarian phenotype in Tg-A** 10 **mice.**

11 (A,B) The Notch2 agonist HMN2-29 or control hamster IgG was *in utero* administered to
12 wild-type or Tg-A mice, and the ovarian phenotype was analyzed at P14 (A) and P28 (B).
13 HMN2-29 administration almost completely rescued the formation of MOFs in the Tg-A
14 ovaries at P14 (A), and restored the antral follicles to the wild-type level at P28 (B). Mean \pm
15 SEM, n = 3. * p < 0.05, ** p < 0.01 (Welch's t -test). (C-O) Immunofluorescence analysis at
16 P28 for markers of cell proliferation (Ki-67: red) or apoptosis (cleaved Caspase 3: green) in
17 XX gonads of wild-type and Tg-A mice treated with hamster IgG or HMN2-29. White and
18 yellow arrows indicate secondary follicles (type 4–5) with or without cleaved Caspase
19 Caspase 3-positive granulosa cells, respectively. Quantitation of Caspase 3-positive follicles
20 in (D-G) is shown in (C). Mean \pm SEM, n = 3. ** p < 0.01 (Welch's t -test); ns, not significant.
21 (P,Q) qRT-PCR analysis for *Foxl2* (P) and *Wnt4* (Q) in P0 gonads of wild-type or Tg-A mice
22 treated with hamster IgG or HMN2-29, respectively. Expression levels were normalized to
23 *Rps29*. Mean \pm SEM, n = 3. * p < 0.05, ** p < 0.01 (Welch's t -test); ns, not significant.

24

- 1 **Figure 7: A model for NR5A1 function in fetal gonadal development in mice.** NR5A1
- 2 fine-tunes Notch signaling levels to achieve optimal developmental outcomes in both fetal
- 3 ovaries and testes.
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Supplementary Figure S1: Overexpression of *Nr5a1* affected the morphology of fetal ovaries in the Tg-A mice.

(A,B) Bright-field images of 15.5 dpc-ovaries from Tg-A and wild-type mice. (C,D) Scatter plot measuring the length or width of the fetal ovaries. $*p < 0.05$ (Welch's *t*-test). (E) qRT-PCR analysis showing that *Sox4* was significantly down-regulated in Tg-A fetal ovaries compared with wild type ovaries at 15.5 dpc. Expression levels were normalized to *Rps29*. Mean \pm SEM, $n = 3$. $*p < 0.05$ (Welch's *t*-test).

Supplementary Figure S2: *In utero* administration of the Notch2 agonist did not affect body weight.

(A) Schematics showing the experimental design. The Notch2 agonist HMN2-29 was administered *in utero* at 13.5, 16.5 and 18.5 dpc via i.p. injection. Hamster IgG was used as the mock control. (B) *In utero* treatment of HMN2-29 did not cause a significant change in mouse body weight at P28. ns, not significant (Welch's *t*-test).

Supplementary Table S1

The list of primer sets used in this study

Supplementary Table S2

The details of antibodies used in this study

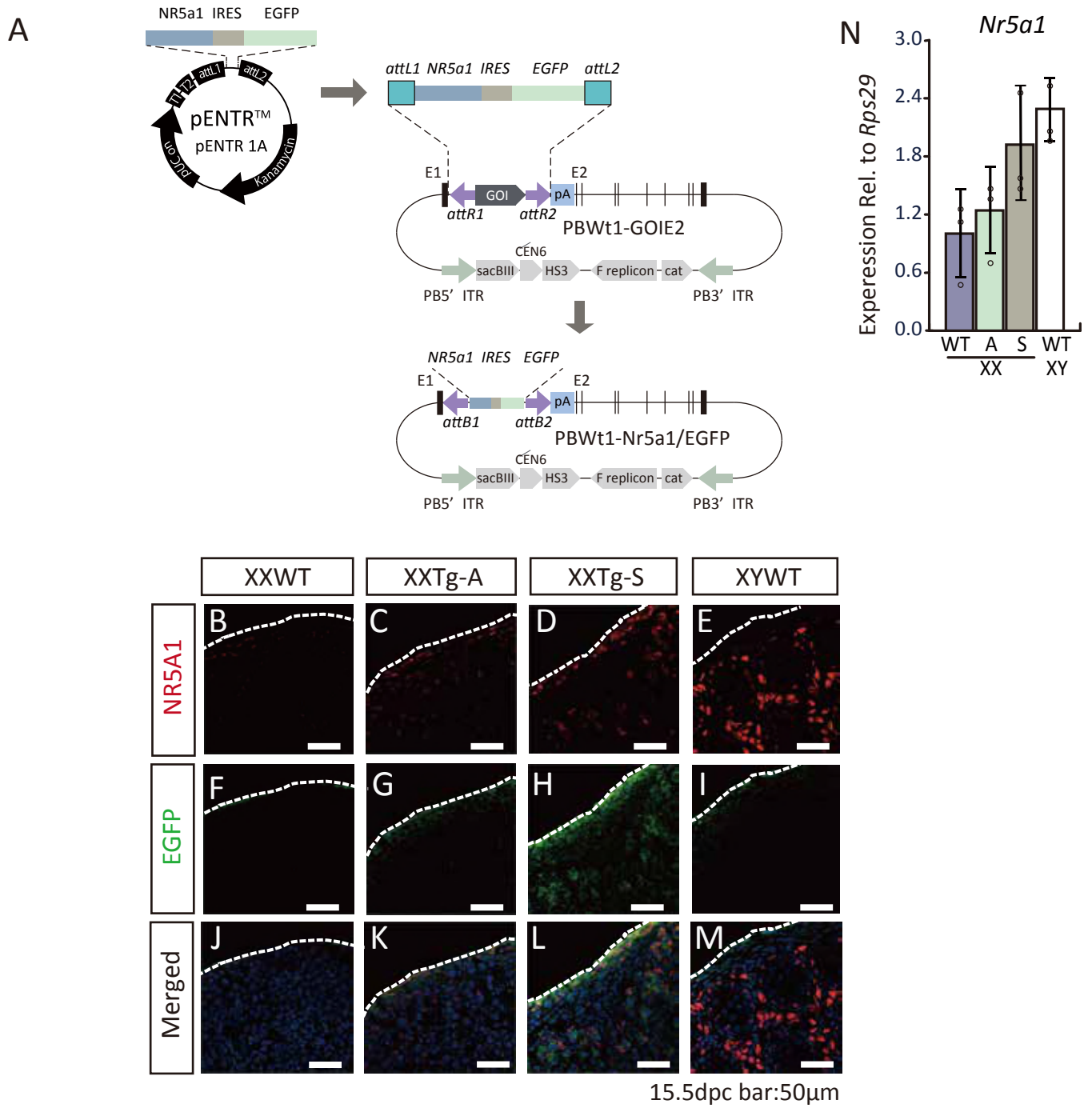


Figure 1

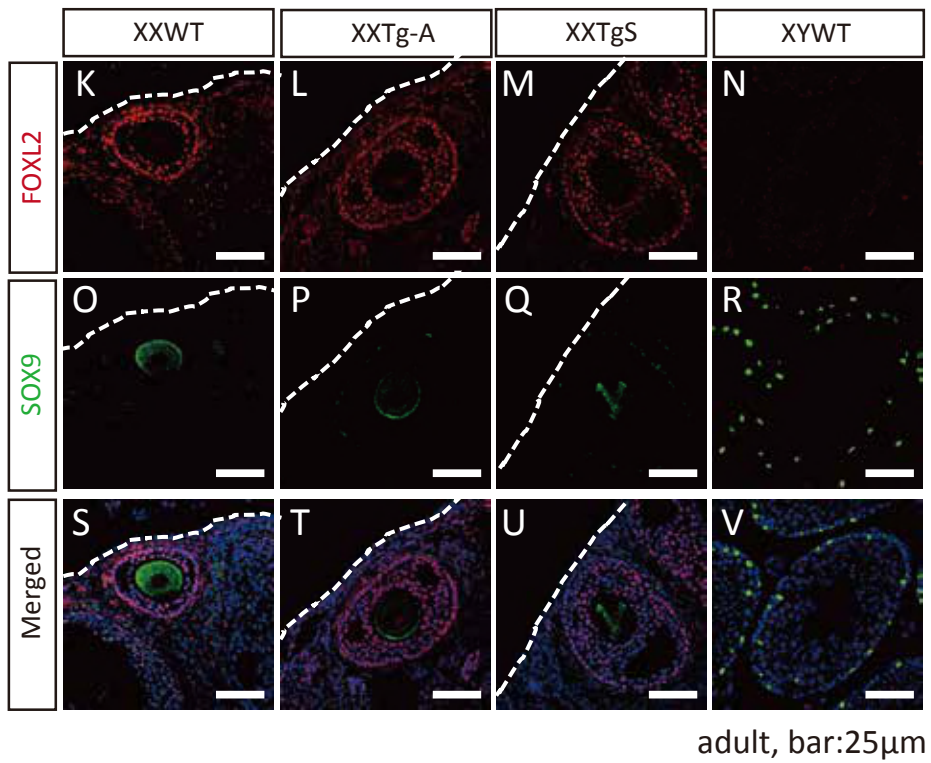
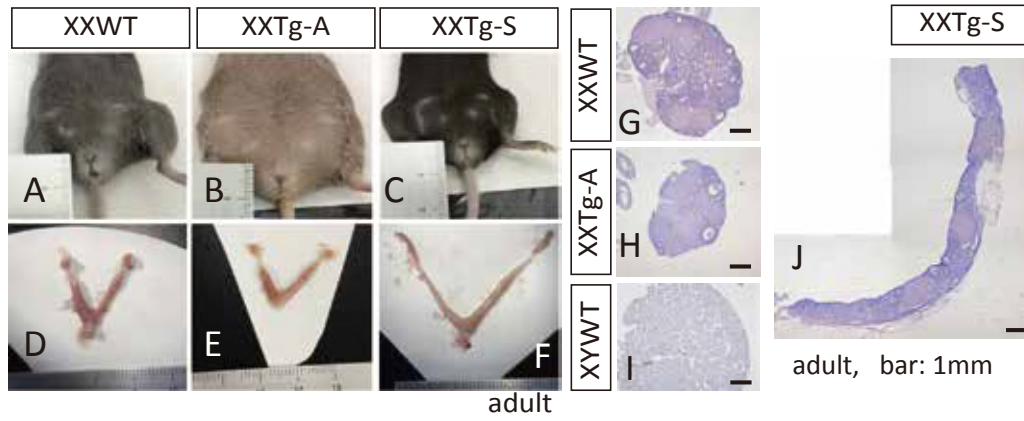


Figure 2

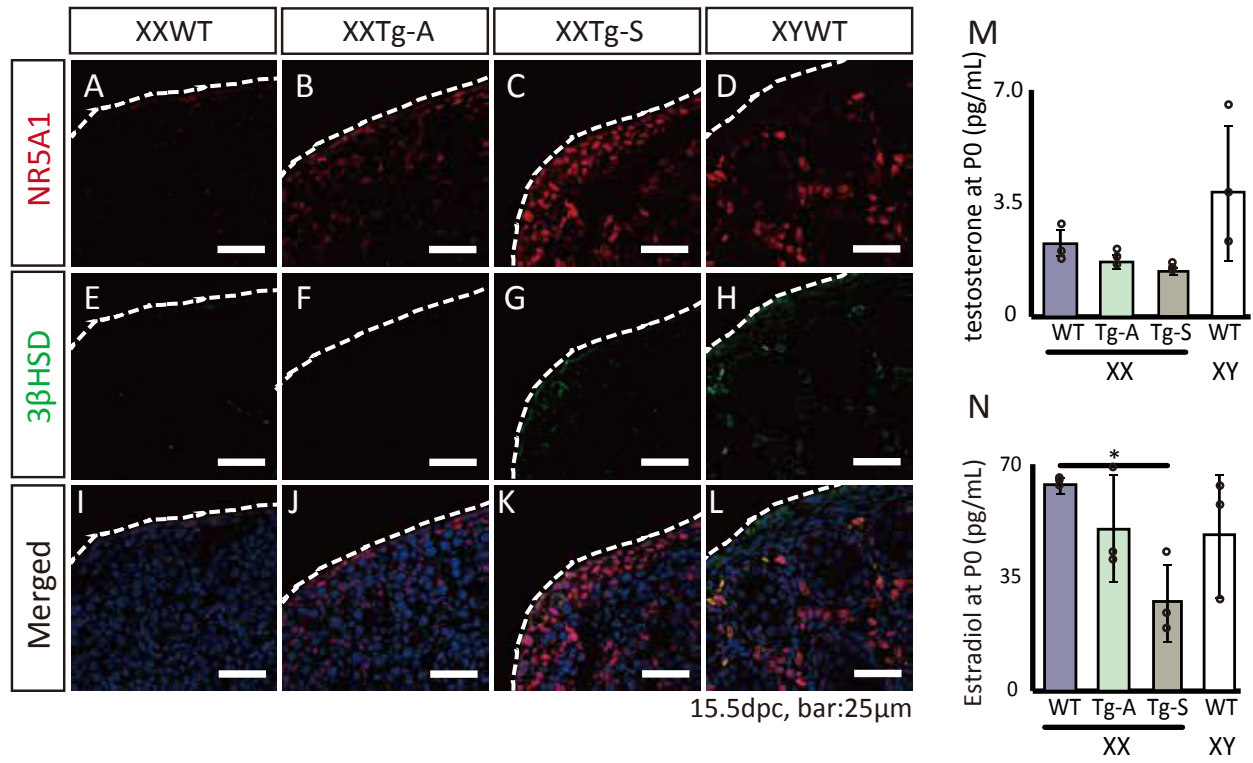


Figure 3

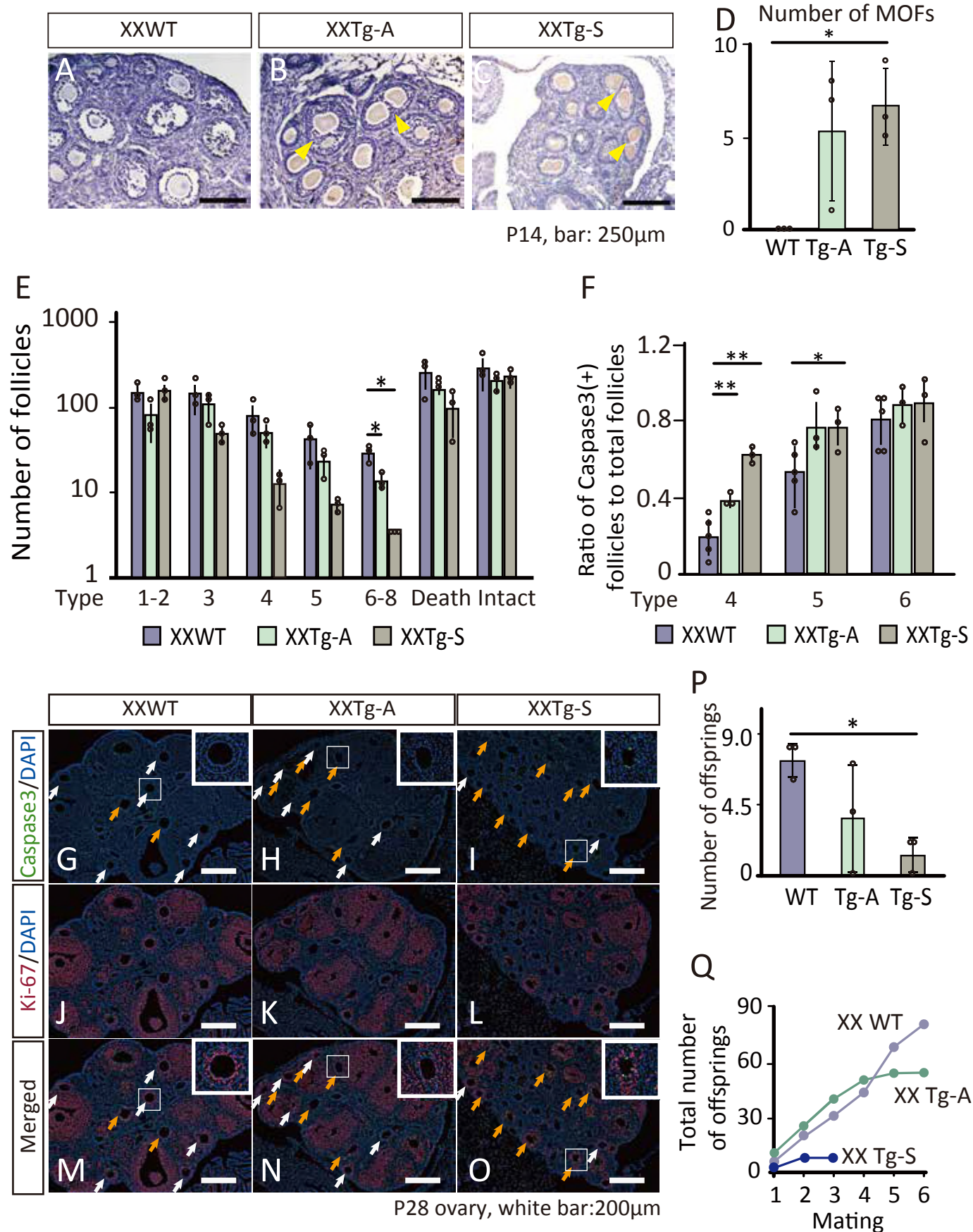


Figure 4

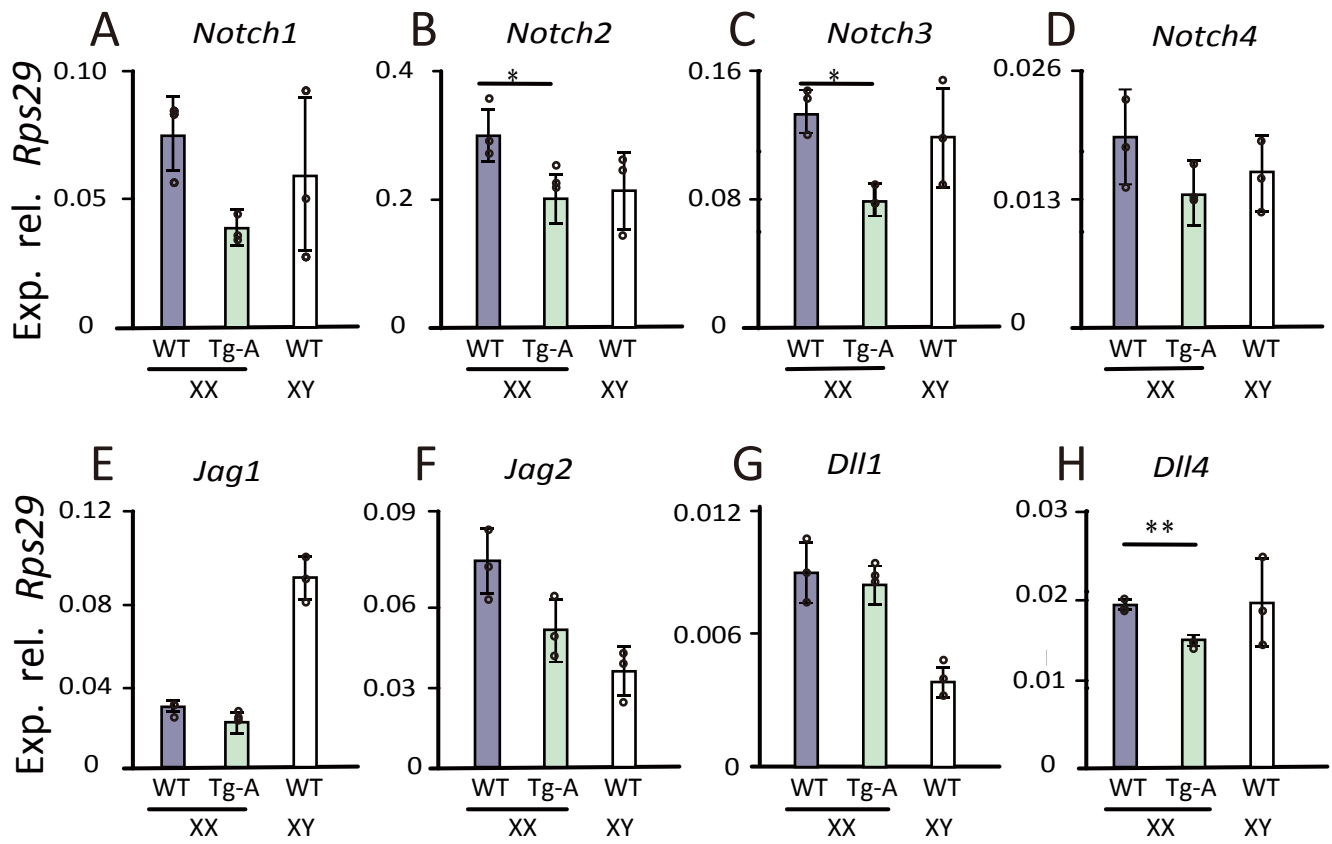


Figure 5

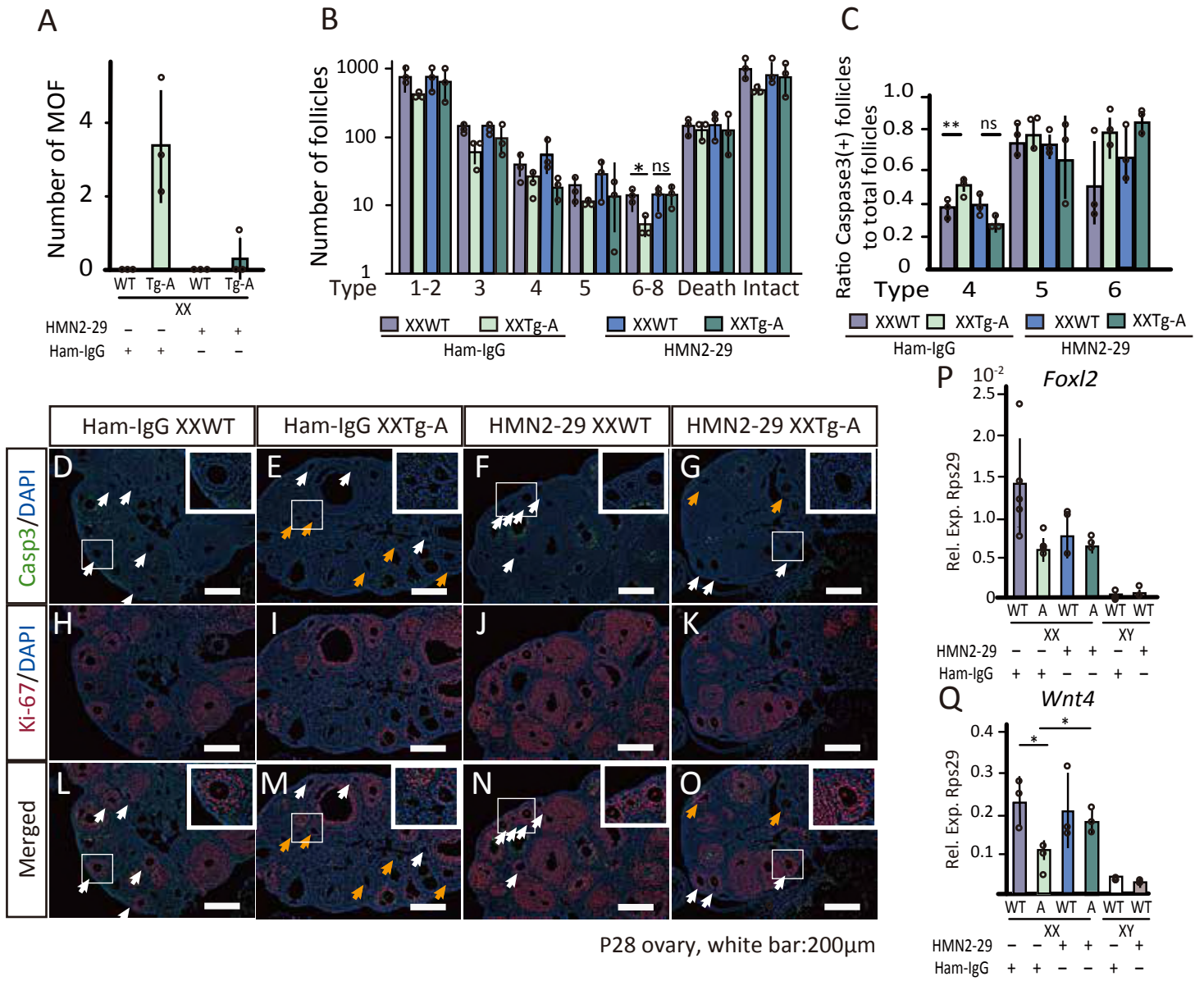


Figure 6

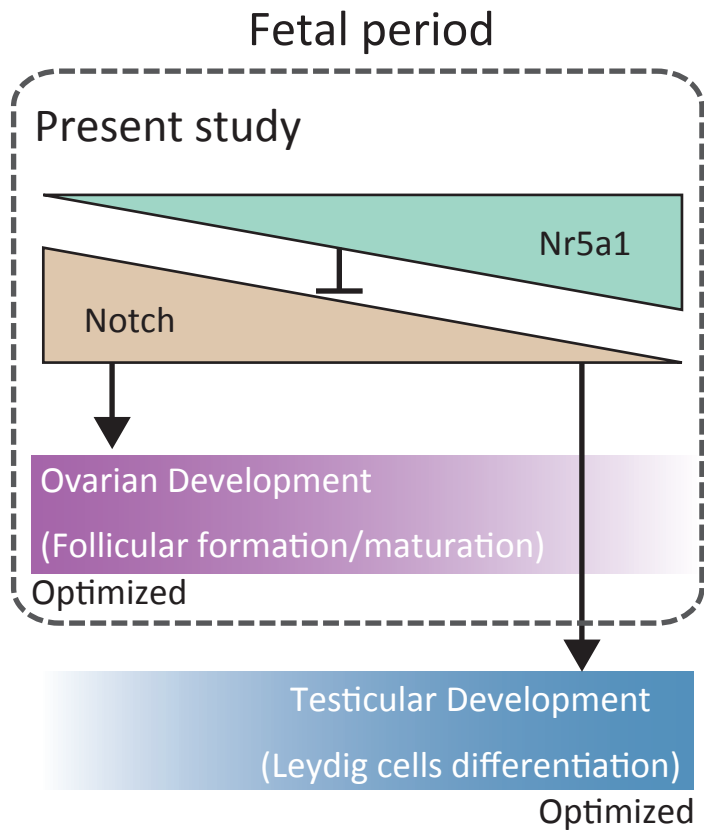


Figure 7