DISSERTATION

Roles of TGFβ Signaling in Sexual Fate Decision of Mouse Germ Cells

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ABSTRACT

Germ cells have a capacity to undergo meiosis, a unique form of cell cycle halving the genetic material, and ultimately differentiate into sperms or oocytes. Thus, these haploid cells are crucial for transfer of genetic information from one generation to the next without affecting chromosome number. A fundamental question in reproductive biology relates to how these cells determinate their fate and commit to either spermatogenesis or oogenesis. Interestingly, the decision of their sexual fate is independent of their XX or XY chromosomal constitution. Instead, sexual fate of germ cells is determined by the cues from their environment. In the mouse, exposed to high level of retinoic acid (RA), germ cells in fetal ovaries embark on meiosis, and simultaneously, these germ cells commit to oogenesis, expressing several essential factors for follicular development. In contrast, germ cells in testes do not enter meiosis until after birth. They are protected from meiotic entry by *Cyp26b1*, encoding an enzyme that directly degrades RA. Meanwhile, an essential intrinsic factor Nanos2 is expressed in XY germ cells and promotes male differentiation. It is considered that the precise regulation of RA level in fetal gonads is important for specification of germ cell fate. However, it is still unclear what signals directly induce *Nanos2* expression in fetal testes. Moreover, simultaneous progression of meiosis and oogenesis in XX germ cells had been uncoupled, which prevented us to unveil the mechanism whereby oogenesis is regulated. In my studies, via functional analysis of transforming growth factor beta (TGFβ) signaling pathways: nodal/activin and bone morphogenetic protein (BMP) signalings in fetal gonads, I proved that nodal/activin signaling is responsible for both *Nanos2* induction and meiotic suppression of XY germ cells in testes, while BMP signaling determines sexual fate of XX germ cells in ovaries independently of RA. My thesis is composed of three parts.

In the first part (Chapter I), I described the identification of nodal/activin as a key regulator of the male germ cell fate. A male-specific expression pattern of genes involved in nodal/activin signaling implied an important role of this signaling pathway in testicular differentiation. Indeed, inhibition of nodal/activin signaling *ex vivo* using specific inhibitors drives male germ cells into meiosis and causes failure of *Nanos2* induction. Moreover, I proved that nodal/activin played dual roles in both suppression of meiosis and the induction of *Nanos2*, because the suppression of meiosis by an RA receptor antagonist could not rescue the elimination of *Nanos2* expression caused by the loss of nodal/activin signaling. Nodal and activin-A bind their receptors that subsequently activate the Smad2/3/4 transcriptional machinery and trigger expression of target genes. Because these receptors are ubiquitously expressed, it is possible that nodal/activin-A directly works on germ cells or indirectly acts via activating somatic cells, which may send a secondary signal to promote male germ cell differentiation. Induction of *Nanos2* expression was observed when activin-A was added to purified male germ cells, implying the direct regulation of nodal/activin-A to germ cells. In addition, when Smad4, a mediator of nodal/activin signaling, was specifically deleted from male germ cells, some male germ cells entered meiosis and failed to express *Nanos2*. These phenomena were never observed in control testes, suggesting that the activation of nodal/activin signaling through Smad proteins is required for spermatogenesis. However, only a small part of germ cells entered meiosis, implying a Smad4-independent pathway also contributes to regulate germ cell fate. Moreover, I clarified that the initiation of nodal/activin signaling requires Fgf9 signaling, which is secreted from pre-Sertoli cells under the control of SRY, the sex determinant of mouse testes. Therefore, nodal signaling is specifically initiated in XY germ cells.

In the second part (Chapter II), I investigated how nodal/activin signaling suppresses meiosis and induces *Nanos2* expression, by focusing two factors p38 mitogen-activated protein kinase (MAPK) and OTX2 transcription factor, because (i) these two factors are specifically expressed in male germ cells; (ii) during the formation of anterior and posterior body axis, these two factors are regulated by nodal signaling. Indeed, OTX2 expression level decreased after nodal inhibitor treatment, indicating the regulation of OTX2 expression by nodal signaling in germ cells. To examine the role of OTX2 in male sexual differentiation, I deleted *Otx2* from fetal testes. After the deletion of *Otx2*, activation of *Nanos2* expression was temporally suppressed and recovered in later stage, suggesting the loss of OTX2 was rescued by some other factors. Indeed, I detected the higher expression of *Otx3*, which have the same binding site with OTX2, in mutant testes. Then, using a specific inhibitor I found that the suppression of p38 MAPK *ex vivo* in fetal testes caused ectopic meiotic entry of male germ cells. In addition, I found that the expression level of *Nanos2* decreased after the inhibitor treatment and the downregulation of *Nanos2* was vanished when these testes were exposed to RA receptor antagonist. These results suggested that p38 MAPK was merely permissive but not instructive for the *Nanos2* expression, in which p38 protected male germ cells from entering meiosis by the RA signaling. Unexpectedly, the expression of pp38 persisted even nodal signaling was suppressed, implying that there is an independent pathway to activate p38 signaling. I conclude that nodal/activin signaling regulates male germ cell fate through OTX2-dependent induction of *Nanos2* expression. I also like to propose that both of these two pathways are essential for the suppression of RA signaling, although the activation of p38 is independent of nodal.

In the last part (Chapter III), I report that BMP signaling plays dual roles in sex

determination of XX germ cells. BMP signaling regulates meiotic progression after RA-dependent meiotic initiation and promotes XX germ cell fate independently of RA. To clarify the functions of BMP signaling, Smad4, a co-activator of both nodal/activin and BMP signaling pathways was conditionally deleted before sex determination of germ cells. Germ cells in the mutant ovaries fail to form double strand breaks (DSBs) during meiosis. The expression levels of several genes involved in DSBs were significantly decreased after the disruption of BMP signaling. Interestingly, the disruption of DSBs was not ascribed to the loss of RA signaling, because the expression of *Stra8*, one of the direct targets of RA, is normally initiated. Notably, exogenous addition of RA into *Smad4* mutant ovaries could not rescue the deficiency. Considering that nodal/activin signaling is not activated at this stage in ovaries, the phenotype observed in mutant ovaries was imputed to the loss of BMP signaling.

Furthermore, the disruption of BMP signaling in the fetal ovaries causes XX germ cells to maintain pluripotency genes, slight upregulation of male-specific genes (*Nodal* and *Nanos2*) and fail to activate *Figla* and *Nobox*, which are essential factors for follicular development. Therefore, the loss of BMP signaling resulted in incomplete sex reversal of XX germ cells. Interestingly, the upregulation of nodal signaling was not accompanied with the enhancement of Fgf9 signals, suggesting an indirect role of Fgf9 in the initiation of nodal signaling by the suppression of BMP signaling. Importantly, the initiation of sex reversal of XX germ cells was not caused by meiotic failure, because the suppression of meiotic initiation by the treatment of the RA receptor inhibitor did not lead to sex reversal. I conclude that BMP regulates oogenesis by impeding male pathway, and instead activating genes required for oogenesis. The initiation of sex reversal occurs independently of meiotic entry in the *Smad4* mutant

ovaries, indicating that BMP but not RA regulates XX germ cell fate. Finally, by the treatment of *Smad4* mutant ovaries with the RA receptor inhibitor, I proved that incomplete sex reversal of XX germ cells was ascribed to RA judging by the successful induction of complete sex reversal of XX germ cells in *Smad4* mutant ovaries after the suppression of RA signaling.

Taken together, my study revealed that TGFβ signaling pathways are essential for sex determination of mammalian germ cells. In ovaries, accompanying with meiotic initiation by RA, BMP instructs oogenesis by inducing genes required for the formation of primordial follicle and meiotic progression, and also suppressing the male-specific nodal signaling. BMP might directly act on germ cells or indirectly induce a secondary signal from somatic cells to regulate germ cell fate. In contrast, XY germ cells are protected from RA by an enzyme CYP26B1 that is regulated by Fgf9/Sox9 signaling. Meanwhile, germ cell intrinsic nodal signaling promotes spermatogenesis by inducing *Nanos2* expression. The disruption of RA and BMP signalings in fetal ovaries is sufficient for the upregulation of nodal signaling and for the induction of complete sex reversal of XX germ cells indicating spermatogenesis is a default pathway for germ cells. However, the mechanism whereby nodal signaling is initiated is still unknown. It might be a cell autonomous event in germ cells or be triggered by a signal from somatic cells which is suppressed by BMP signaling in fetal ovaries. These results provide important information to understand the mechanisms guiding sex determination of germ cells. Moreover, TGFβ members are highly conserved among animals, implying similar mechanisms might also exist in other animals. Therefore, the mechanism of sex determination in germ cells might be far more conserved than we previously considered.

Chapter I
Nodal/activin signaling promotes male germ cell fate

INTRODUCTION

Testicular differentiation in the mouse is triggered by transient expression of the Sry gene in pre-Sertoli cells around embryonic day (E) 10.5 (Koopman et al., 1990). The SRY protein upregulates *Sox9* expression by binding to multiple elements within its enhancer, and SOX9 then induces the expression of fibroblast growth factor-9 (FGF9) (Sekido and Lovell-Badge, 2008). FGF9 is indispensable for testis differentiation, and its deletion causes male-to-female sex reversal (Colvin et al., 2001). Fibroblast growth factor (FGF) receptors are expressed in somatic and germ cells, and it has been proposed that FGF9 regulates testicular differentiation by acting on both these cell types (Bowles et al., 2010).

Production of the NANOS2 protein, an essential intrinsic factor in male germ cells, is one of the important events triggered by the FGF signaling pathway to accomplish male sexual differentiation. Ectopic expression of *Nanos2* in female germ cells causes induction of male-type differentiation (Suzuki and Saga, 2008) and elimination of this gene in the testis causes a complete loss of spermatogonia (Tsuda et al., 2003). FGF9 also functions to suppress meiosis, a process observed in female germ cells from E13.5 (Bowles et al., 2010). In embryonic ovaries, retinoic acid (RA) is responsible for the initiation of meiosis (Bowles et al., 2006; Koubova et al., 2006; MacLean et al., 2007). RA induces the expression of the premeiotic marker stimulated

by retinoic acid (Stra)-8, which is required for premeiotic DNA replication and is also indispensable for meiotic prophase (Baltus et al., 2006). In contrast, RA is degraded in the testis by CYP26B1, a P450 enzyme originating in somatic cells, such that male germ cells cannot enter meiosis until after birth. The expression level of *Stra8* at E12.5 is higher in *Cyp26b1*$^{-/-}$ *Fgf9*$^{+/-}$ male germ cells than in *Cyp26b1*$^{-/-}$ *Fgf9*$^{+/+}$ male germ cells, suggesting that FGF9 works to suppress the upregulation of *Stra8* in male germ cells independently of *Cyp26b1* (Bowles et al., 2010). In this study, I addressed this question by investigating the factors acting downstream of FGF9 signaling.

FGF9 also works to suppress *Wnt4* expression in somatic cells, because in *Fgf9*$^{-/-}$ XY gonads the expression of *Wnt4* is initiated ectopically (Kim et al., 2006). *Wnt4* is critical for ovarian development (Bernard and Harley, 2007) and loss of *Wnt4* causes partial female-to-male sex reversal (Vainio et al., 1999; Ottolenghi et al., 2007). Downstream of *Wnt4* signaling, *Follistatin* and *Bmp2* have been considered as the target genes to regulate ovarian organogenesis (Yao et al., 2004; Kashimada et al., 2011). Until now, how FGF9 orchestrates testicular differentiation in both germ and somatic cells is unknown. In this Chapter, I examined this question by looking for factors acting downstream of FGF9 signaling.

Nodal is a member of the transforming growth factor beta (TGFβ) superfamily,

which activates the SMAD2/3/4 transcriptional machinery through binding to heteromeric complexes of type I activin receptor-like kinase (ALK)-4 (ACVR1B-Mouse Genome Informatics) and ALK7 (ACVR1C-Mouse Genome Informatics), and to type II activin receptor (Reissmann et al., 2001; Schier, 2003). Besides Nodal, the TGFβ superfamily includes bone morphogenetic proteins (BMPs), TGFβs, anti-Müllerian hormone, growth and differentiation factors, the distantly related glial cell line-derived neurotrophic factor, and activins. All of these play important roles in gonadal development in both sexes (Munsterberg and Lovell-Badge, 1991; Yi et al., 2001; Nicholls et al., 2009; Moreno et al., 2010; Mendis et al., 2011). For example, TGFβ3 is expressed in both gonocytes and Leydig cells in fetal and neonatal testes, and TGFβ2 has been detected in Leydig cells and some gonocytes. Conditional knockout (KO) of the gene for TGFβRII, the specific receptor of TGFβs, leads to an increased proportion of proliferating and apoptotic gonocytes and to male sterility (Moreno et al., 2010). Moreover, activin β subunit KO mice (*Inhba*$^{-/-}$) show significantly smaller testes at birth with 50% lower Sertoli cell numbers, compared with normal mice (Mendis et al., 2011).

In this chapter, I investigated the roles of Nodal and activin-A (Inhba- Mouse Genome Informatics), derived from germ cells and somatic cells respectively, on the

promotion of the male sexual differentiation pathway and suppression of the female differentiation pathway.

RESULTS

Nodal and *Lefty1/2* are expressed specifically in male germ cells

To explore the upstream factors required for expression of the male-specific factor NANOS2, I first sought to detect the target of FGF9 in male germ cells. Considering that *Fgf9* starts to be expressed from embryonic day (E)11.5 and that *Nanos2* expression peaks at E13.5, I searched for genes that were specifically expressed in germ cells during these stages, using microarrays. The genes involved in Nodal signaling were detected successfully. I confirmed the male-specific expression of these genes using whole mount *in situ* hybridization (Figure I-1A). To test if these genes were expressed in germ cells or somatic cells, I then performed double *in situ* hybridization with *Nanos2* mRNA (a marker of germ cells). *Nodal* was expressed in all male germ cells expressing *Nanos2* (Figure 1-1B upper panel), whereas *Lefty1* and *Lefty2* mRNAs were localized only in a subset (Figure 1-1B middle and lower panels). I also isolated germ cells from E12.5 testes, and the results of RT-qPCR amplification indicated that the mRNAs of *Nodal* and *Lefty1/2* existed only in the germ cells, but not in somatic cells

(Figure I-1C). Although the mRNAs of *Nodal* and *Lefty1/2* were located in germ cells, the expression of the gene encoding another member of the TGFβ superfamily, activin-A, was observed in somatic cells (Figure I-1C), suggesting that Nodal/activin signaling is active in both germ cells and somatic cells. Indeed, this activation was proven by strong signals for phosphorylated-SMAD2 (pSMAD2), the effector of the nodal/activin signaling pathway, in both male germ cells and somatic cells (Figure 1-2A), but not in any cells of female gonads (Figure I-2B).

Inhibition of nodal/activin signaling disrupts sex differentiation of male gonads *ex vivo*

To determine the role of nodal/activin signaling in testicular differentiation more precisely, I treated the isolated testes with SB431542, a specific inhibitor of type I ALK 4/5/7 receptors (Inman et al., 2002). Expression of pSMAD2 in the treated testes was completely abolished after 24 h (Figure I-3A). Nodal regulates its own expression through a positive-feedback loop and activates *Lefty1/2* (Lowe et al., 2001; Hamada et al., 2002). Accordingly, the mRNA levels of *Nodal*, *Lefty1* and *Lefty2* were also significantly decreased (Figure I-3B), further confirming that nodal/activin signaling was almost completely repressed by SB431542.

The expression of *Nanos2* mRNA was only partially suppressed by SB431542 when testes were removed from E12.5 embryos (Figure I-3B), because transcription had started. However, the percentage of NANOS2-positive cells was dramatically reduced when gonads dissected from E11.5 embryos were treated with the inhibitor (Figure I-3C and 3D). I also examined the expression of the protein DNMT3L, which is involved in genomic imprinting (an important process of male sexual differentiation) and is only expressed in male germ cells from E14.5 to E18.5 (Bourc'his et al., 2001; Bourc'his and Bestor, 2004; Sakai et al., 2004). As anticipated, DNMT3L expression was downregulated after inhibitor treatment (Figure I-3E), confirming that the male differentiation program was strongly repressed in the absence of nodal/activin signaling. In addition, the expression levels of *Stra8* and *Rec8*, which encode a meiotic inducer and a meiotic cohesion factor, respectively (Eijpe et al., 2003; Lee et al., 2003), were dramatically upregulated by inhibitor treatment (Figure I-3A). As expected, γH2AX (H2AF-Mouse Genome Informatics) and DMC1, which are required to repair meiotic DNA double-stand breaks, were also detected after inhibitor treatment, suggesting that germ cells without nodal/activin signals initiated meiosis (Figure I-3F). Moreover, when E11.5 testes were treated with an ALK5 inhibitor (Maherali and Hochedlinger, 2009), the expression levels of *Nodal*, *Lefty1/2* and *Nanos2* were also

downregulated accompanied by the enhanced expression of meiotic markers (*Stra8, Dmc1* and *Rec8*) (Figure I-4A). In contrast, the addition of exogenous activin-A promoted *Nanos2* expression and downregulated *Stra8* in our culture system (Figure I-4B), further confirming that nodal/activin-A was sufficient for promoting the male differentiation pathway and for suppressing meiosis.

Nodal/activin signaling is essential for cell survival in male gonads

I observed severe apoptosis in both germ cells and somatic cells after inhibitor treatment (Figure I-4C left panel), implying an irreplaceable role of nodal/activin signaling in cell survival in testes. However, apoptosis was not observed when ovaries were treated with this inhibitor (Figure I-4C right panel). I then determined if ectopic meiosis of male germ cells could account for cell apoptosis after inhibitor treatment. Notably, simultaneous inhibition of both the nodal/activin and RA signaling pathways through the simultaneous addition of SB431542 and the RA receptor antagonist AGN 193109, failed to block this apoptosis, although the initiation of meiosis was suppressed (Figure I-5A, B), suggesting that nodal/activin signaling was responsible for cell survival in testes.

Nodal/activin signaling acts as an inducer of *Nanos2* expression independently of RA

I investigated the mechanism by which nodal/activin signaling regulates *Nanos2* expression. At least two signaling cascades appear to be necessary for *Nanos2* induction. One is responsible for the initiation of *Nanos2* transcription, and the final effector of this cascade must be an inducer (a transcription factor) that binds directly to the *Nanos2* enhancer. It has been suggested that FGF9 signaling is involved in this cascade because *Nanos2* expression was promoted via exogenous FGF9 in a germ cell culture system (Bowles et al., 2010). The other signaling cascade must be responsible for suppressing RA, because *Nanos2* expression was completely inhibited without disrupting FGF9 signaling in *Cyp26b1*-null testes (which show higher RA levels) (Bowles et al., 2010). I have already confirmed the role of nodal/activin-A in impeding meiosis (by antagonizing RA). To test whether nodal/activin directly activated *Nanos2* expression, I examined the expression of *Nanos2* in the absence of both nodal/activin and RA signaling. If nodal/activin signaling simply suppressed RA activity, then inhibition of RA activity should rescue the downregulation of *Nanos2* caused by the loss of such signaling. Because SB431542 treatment caused RA-independent apoptosis (Figure I-5A, B), limiting our analysis of germ cell fate, I used benzyloxycarbonyl-Val-Ala-Asp

(OMe)-uoromethylketone (Z-VAD-FMK) to suppress apoptosis (Slee et al., 1996). Apoptosis was inhibited successfully, and *Nanos2* expression was not rescued by the suppression of RA signaling (Figure I-5C, D). These results suggest that nodal/activin signaling works both as an inducer of *Nanos2* and as a suppressor of RA (which will induce meiosis), and that these two functions act independently.

Nodal/activin signals promote male sexual differentiation directly

Given that pSMAD2 was observed in both germ cells and somatic cells (Figure1-2A), I investigated whether nodal/activin signaling acted on male germ cells directly or indirectly by testing the effects of SB431542 and activin-A on isolated male germ cells. E12.5 male germ cells were purified by immunomagnetic sorting (Pesce and De Felici, 1995) and cultured with SB431542 or activin-A. I confirmed that treatment with SB431542 and activin-A did not affect the expression of *Mvh* (*Ddx4*-Mouse Genome Informatics), which was used for normalization (Figure I-6A). *Nanos2* expression was decreased after 24 h culture with SB431542 (Figure I-6B). Interestingly, this suppression of *Nanos2* expression was accompanied by downregulation of the nodal signaling pathway (Figure I-6B, *Nodal*, *Lefty1*, and *Lefty2*), implying that nodal signals from germ cells contributed to promotion of *Nanos2* expression. The reduction in

Nanos2 expression after inhibitor treatment was less evident, because germ cells were isolated from E12.5 testes in which transcription had commenced. However, I were unable to analyze the effect of SB431542 on germ cells from E11.5 gonads, because these cells cannot proceed to male differentiation because of the loss of somatic signals (Ohta et al., 2012). Instead, I demonstrated that the addition of activin-A to isolated germ cells resulted in increases in both *Nodal* and *Nanos2* expression levels (Figure I-6C).

To confirm our results *in vivo*, I specifically deleted *Smad4*, a mediator of nodal/activin signaling, in male germ cells (Yang et al., 2002). I assumed that if nodal/activin signaling worked directly on germ cells, then loss of *Smad4* in male germ cells should disrupt the male pathway and result in meiotic entry. *Smad4* $^{flox/+}$/*Pou5f1-CreERT2*$^{+/-}$ males were crossed with either *Smad4*$^{flox/+}$/*Pou5f1-CreERT2*$^{+/-}$ or *Pou5f1-CreERT2*$^{lacZ/+}$ females. Tamoxifen was injected at E10.5 and E11.5 to induce Cre conditionally in germ cells, and gonads were harvested at E14.5. The results of X-gal staining indicated that Cre was specifically induced in most germ cells (Figure I-7A). Immunohistochemical analysis of the meiotic marker γH2AX (a marker of the sites of double-strand breaks) indicated that some male germ cells in the mutant mice entered meiosis, compared to none of the germ cells in the control testes (Figure1-7B).

As expected, no NANOS2 expression was observed in the γH2AX-positive germ cells (Figure I-7C). Similar results were observed when *Smad4* was deleted using *Stella-MerCreMer* transgenic mice. Some *Smad4*-null germ cells entered meiosis at E14.5 in the mutant testes (Figure I-7E). These results suggest that nodal/activin signals activate the SMAD complex to promote the male germ cell fate and suppress the initiation of meiosis. However, I cannot exclude the possible existence of a SMAD4-independent pathway, because of the mild phenotype of *Smad4* mutant mice.

Redundant functions of TGFβ superfamily members in the differentiation of male germ cells

As already mentioned, somatic and male germ cells synthesize two different members of the TGFβ superfamily: activin-A and nodal, respectively (Figure I-1C). The observation that both nodal and activin-A have the capacity to activate pSMAD2/3 signaling pathways and that pSMAD2 signals persist even in *Inhba*-KO testes (Archambeault and Yao, 2010; Mendis et al., 2011) led us to consider the possibility that nodal and activin-A might work redundantly in fetal testes. To test this hypothesis, I knocked out the *Nodal* gene conditionally by crossing *Nodal*$^{flox/+}$*/Rosa-CreERT2* $^{+/-}$ (or *Nodal*$^{flox/flox}$*/Rosa-CreERT2* $^{+/-}$) females with *Nodal*$^{flox/flox}$ males. Tamoxifen was injected

at E10.5 and gonads were harvested at E14.5 and E16.5. The results of RT-qPCR indicated that the *Nodal* gene was successfully deleted using Cre recombinase (Figure I-8A). Deletion of the *Nodal* gene resulted in decreased *Nanos2* mRNA levels at E14.5 (Figure I-8A). In addition, fewer NANOS2-positive germ cells were detected in the mutant mice at E14.5 (Figure I-8B). Unexpectedly, deletion of *Nodal* resulted in no significant change in the expression levels of *Lefty1* and *Lefty2*, two of the target genes of Nodal/Activin signaling, and had no effect on pSMAD2 levels (Figure I-9A and B). In addition, the defects observed in the mutant mice at E14.5 were completely rescued at E16.5, as judged by the normal expression levels of NANOS2 and DNMT3L (Figure I-8C). I conclude that, even if the process of differentiation is retarded, male germ cells in *Nodal*-null testes can still complete male sexual differentiation.

FGF9 regulates male sexual differentiation through activation of nodal/activin signaling

Male sexual differentiation in germ cells is believed to be initiated by FGF9, a factor secreted from somatic cells. To investigate the relationship between FGF9 and nodal/activin signaling pathways, I examined the expression levels of *Nodal*, *Lefty1/2* and *Inhba* after treatment with the FGF receptor inhibitor SU5402. As shown in Figure

I-10A, the expression levels of these genes were significantly decreased. Moreover, no pSMAD2 was detected in inhibitor-treated testes, indicating that initiation of the nodal/activin signaling pathway was abolished in the absence of FGF signaling (Figure I-10B). As expected, the expression level of *Nanos2* mRNA was also downregulated (Figure I-10A). In addition, the expression of *Stra8* was augmented in a dose-dependent manner (Figure I-10C). This result differed from that of a previous study in which FGF9 had no effect on the expression of *Stra8* (Bowles et al., 2010). I ascribed this difference to the higher concentration of inhibitor used in our study; the 50% inhibitory concentration of SU5402 is 10–20 μM, and the concentration of 5 μM used in the previous study might thus have been too low. By contrast, the expression levels of SOX9 and FGF9 were unchanged by treatment with either a nodal inhibitor or with activin-A (Souquet et al., 2012) (Figure 1-11), implying that nodal/activin signals act downstream of FGF signaling. Inhibition of FGF together with RA signaling could not promote male differentiation (Figure I-10D). This indicates that FGF signaling plays a dual role in promoting the male pathway and in suppressing meiosis.

I hypothesized that FGF9 might promote male sexual differentiation though activating nodal/activin signaling. To test this possibility, I added an exogenous TGFβ signal (activin-A) together with SU5402 to isolated testes from E12.5 embryos.

Activin-A enhanced *Nanos2* expression in the absence of FGF9 signaling (Figure I-12A and B). Furthermore, additional treatment with SB431542 resulted in the marked abrogation of this enhancement, indicating that activin-A works through the nodal/activin signaling pathway (Figure I-12A and B). Interestingly, when E11.5 fetal testes were treated with SU5402 and activin-A together, the expression of *Nanos2* could not be rescued (Figure I-12C).

Nodal/activin is required for suppressing the female pathway in somatic cells

Because pSMAD2 was also detected in somatic cells, I sought to examine the effect of nodal/activin signaling on Sertoli cells using the expression of the specific marker SOX9. During testicular cord formation, germ cells are surrounded by Sertoli cells in a regular fashion. In contrast, this arrangement was disordered when testes were cultured in the presence of SB431542 (Figure I-11A). Disruption of the testis cords was also observed by detecting laminin protein, which marks the basal lamina surrounding the testis cords (Figure I-118B) (Tilmann and Capel, 1999). This disruption was not associated with the downregulation of SOX9 at either the mRNA or protein level (Figure I-11C), indicating that nodal/activin signaling was dispensable for SOX9 expression but was required for the maintenance of testicular cord formation. To further

understand the cause of disruption of Sertoli cells, I investigated the expression levels of several female-specific genes in somatic cells. These genes included *Wnt4* and *Foxl2*, which are considered to be master genes in the female gonadal differentiation pathway; a double KO mouse for these genes shows female-to-male sex reversal (Ottolenghi et al., 2007). I also assessed the expression levels of *Bmp2* and follistatin, thought to be the targets of WNT4 and FOXL2 (Yao et al., 2004; Kashimada et al., 2011). Gene expression levels were compared before and after either FGF receptor inhibitor (SU5402) treatment or activin receptor inhibitor (SB431542) treatment. Consistent with previous studies, loss of FGF9 signaling caused dramatic upregulation of these genes (Figure I-13A). Interestingly, even though *Wnt4* and *Foxl2* mRNA levels did not change after SB431542 treatment, *Bmp2* and follistatin expression levels were significantly upregulated (Figure I-13A). These results indicate that in somatic cells, FGF9 and nodal/activin signaling acts in a different manner to inhibit the female differentiation pathway. At an earlier stage (E11.5), FGF9 antagonized WNT4 and FOXL2 independent of nodal/activin signaling, and loss of FGF9 signaling could not be rescued by exogenous activin-A (Figure I-12C). However, at a later stage (E12.5), FGF9 was necessary for the initiation/maintenance of nodal/activin signaling, which thwarted the expression of *Bmp2* and follistatin. At this stage of development, the function of FGF9

can be supplanted using exogenous activin-A (Figure I-11A and B).

Overall, these results suggest that FGF9 regulates testicular differentiation though the activation of nodal/activin signaling in both germ cells and somatic cells. In germ cells, nodal/activin-A directly promotes male germ cell fate and suppresses meiosis. In somatic cells, nodal/activin-A is responsible for protecting male somatic cells from entering the female differentiation pathway (Figure I-13B).

DISCUSSION

Suppression of meiotic entry and induction of the male-specific gene *Nanos2* in germ cells are indispensable for establishing the eventual spermatogenetic program in the testis. If germ cells are exposed to a high level of RA, they enter meiosis and lose the opportunity to express male-specific genes regardless of whether they have the potential to do so (Bowles et al., 2006; Koubova et al., 2006). By constrast, in the absence of *Nanos2* expression, male germ cells enter meiosis and are eliminated by apoptosis (Tsuda et al., 2003). Many studies have focused on how germ cells suppress meiosis (Bowles et al., 2006; Koubova et al., 2006; Suzuki and Saga, 2008; Ewen et al., 2010). However, the route by which male-specific genes are induced is ambiguous. Previous studies suggested a role for FGF9 in induction of the male pathway (Bowles et al.,

2010), stimulating the search for downstream factors involved in FGF9 signaling. Here, I showed that nodal/activin signaling functions downstream of FGF9 and that it plays a dual role in male sexual differentiation both in promoting male-specific gene activation and impeding female differentiation.

A recent study showed similar results to our inhibitor experiments and claimed an autocrine role for nodal/activin signaling (Souquet et al., 2012). Although many other studies (Moreno et al., 2010; Mendis et al., 2011; Souquet et al., 2012) have indicated that nodal/activin receptors exist on the cell surface of male germ cells, it is not sufficient to conclude that nodal acts as an autocrine factor on the basis of inhibitor experiments alone, because inhibitor treatment abolishes nodal/activin signaling in both somatic and germ cells, and it is possible that nodal/activin signaling may induce another factor in somatic cells, which could affect male germ cells. The current study demonstrated that nodal/activin-A works directly on germ cells by two different approaches. Firstly, I observed contrary effects of a nodal inhibitor and activin-A on purified male germ cells. Secondly, I proved that germ-cell-specific deletion of *Smad4* caused some male germ cells to enter meiosis, indicating that SMAD complexes in male germ cells are responsible for suppressing meiosis, and eliminating the possibility that nodal/activin-A affects germ cells via somatic cells. Interestingly, in *Smad4* conditional

KO mice, however, only some germ cells entered meiosis. I considered two possible explanations for this. One is the presence of a mechanism whereby nodal/activin-A regulates germ cell fate independently of SMAD4; indeed, TGF-β activates ERK, JNK and P38 mitogen-activated protein kinase pathways independently of the SMAD complex (Derynck and Zhang, 2003). The function of these signaling pathways in male sexual differentiation will be analyzed in future studies. Another possibility involves the stability of the SMAD4 protein. Given that *Smad4* shows an ubiquitous expression pattern during early developmental stages (Luukko et al., 2001), it is possible that SMAD4 protein remains in most germ cells after deletion of the *Smad4* gene, and is only degraded in a small set of germ cells. Unfortunately, no reliable antibodies are available, thus limiting the analysis of SMAD4 protein levels. However, ectopic meiosis and the loss of NANOS2 expression in some germ cells indicates the existence of a SMAD4-dependent pathway, by which nodal/activin signaling directly regulates male germ cell fate. Moreover, if other somatic factors are triggered by nodal/activin-A that affect male germ cell fate, exclusive interruption of nodal/activin signals in somatic cells should give rise to defects in germ cells, whereas conditional deletion of *Smad4* in Sertoli cells was associated with grossly normal spermatogenesis (Archambeault and Yao, 2010), implying the absence or limited function of these factors. Hence, our study

presents the first evidence that nodal/activin signals act directly on germ cells.

Expression of *Stra8*, an essential gene for meiosis, was significantly elevated after disruption of nodal/activin signaling in male germ cells, indicating that the cells entered meiosis. Regarding the regulation of meiotic entry in male germ cells, it is believed that meiosis is suppressed by *Cyp26b1*, encoding an RA-degrading enzyme (Bowles et al., 2006; Koubova et al., 2006). However, neither inhibition of nodal/activin signaling nor deletion of *Nodal* caused downregulation of *Cyp26b1* expression (data not shown), implying that even at low testes levels of RA, disruption of nodal/activin signaling still induced *Stra8* expression. It is unlikely that nodal/activin degrades RA directly in male germ cells. I propose a model in which downstream targets of nodal/activin and RA signaling compete for binding sites in the *Stra8* enhancer (or promoter) to inhibit or initiate its expression.

I confirmed the redundant roles of nodal and activin-A in sex differentiation by analyses of testes in *Nodal* conditional KO mice, in which pSMAD2 persisted in both somatic and germ cells. Notably, *Lefty1* and *Lefty2* expression levels were maintained, even though these genes were dramatically decreased in *Nodal* hypomorphic mutant embryos (Lowe et al., 2001). Both activin-A and nodal proteins are generally considered to act through the same pathway, by activating the SMAD

complex (Heldin et al., 1997). It therefore seems that activin-A, rather than nodal, induces *Lefty1/2* expression in germ cells in *Nodal*$^{-/-}$ mice. However, even at E16.5, *Nanos2* expression was still negatively affected by the loss of nodal at E14.5. I postulate that activin-A takes time to replenish the signals responsible for initiation of *Nanos2* activity. The different recovery rates of *Lefty1/2* and *Nanos2* expression suggest that, unlike *Lefty1/2*, *Nanos2* is not a direct target of nodal/activin signaling.

This study is the first to show that nodal and activin-A work downstream of FGF9 in germ cells and somatic cells, respectively. However, the mechanism by which FGF9 regulates their expression is unknown. Interactions between these two signaling pathways have already been investigated in *Xenopus*, in which FGF is required for activin-mediated induction of mesoderm (Cornell and Kimelman, 1994). A recent study in zebrafish indicated that FGF works downstream of the nodal signaling pathway and had capacity to induce *one-eyed pinhead*, a homolog of the Nodal cofactor Cripto, which makes a positive regulatory loop between FGF and the nodal signaling pathways (Mathieu et al., 2004). However, we could not exclude the possibility that FGF9 indirectly regulates nodal and activin-A.

Another pivotal finding of our study was a mechanism by which Sertoli cells are protected from entering the female pathway. Sertoli cells have the potential to

differentiate into granulosa cells, even in adult mice, and loss of *Dmrt1* in Sertoli cells causes female reprogramming even in adult testes (Matson et al., 2011). Interestingly, *Dmrt1* mutant mice only showed feminization after birth (Raymond et al., 2000; Matson et al., 2011), implying the presence of other factors inhibiting entry to the female pathway in fetal testes. I suggest that nodal/activin A are strong candidates for these factors. The loss of nodal/activin signaling led to upregulation of *Bmp2* and follistatin. It is possible that nodal/activin suppresses female reprogramming in Sertoli cells through inhibiting BMP signaling, because BMP2 induces follistatin expression (Kashimada et al., 2011). Supporting this idea, nodal contributes to inhibition of the BMP pathway in mouse embryonic stem cells through SMAD7 (Galvin et al., 2010). Ectopic activation of *Bmp2* and follistatin may be one cause of dysgenesis of the testicular cords. Either conditional deletion of *Smad4* in Sertoli cells or specific KO of the gene for Activin βA in fetal Leydig cells decreased Sertoli cell proliferation and caused abnormal testicular histology (Archambeault and Yao, 2010). I therefore suggest that nodal/activin-A acts on Sertoli cells to promote cell proliferation and suppress female differentiation.

In summary, the present study revealed the functions of nodal/activin signals acting on both germ cells and somatic cells, thus shedding light on our understanding of testicular differentiation. Nodal/activin is a highly conserved signaling pathway,

suggesting that a similar mechanism might also control testicular differentiation in other mammals.

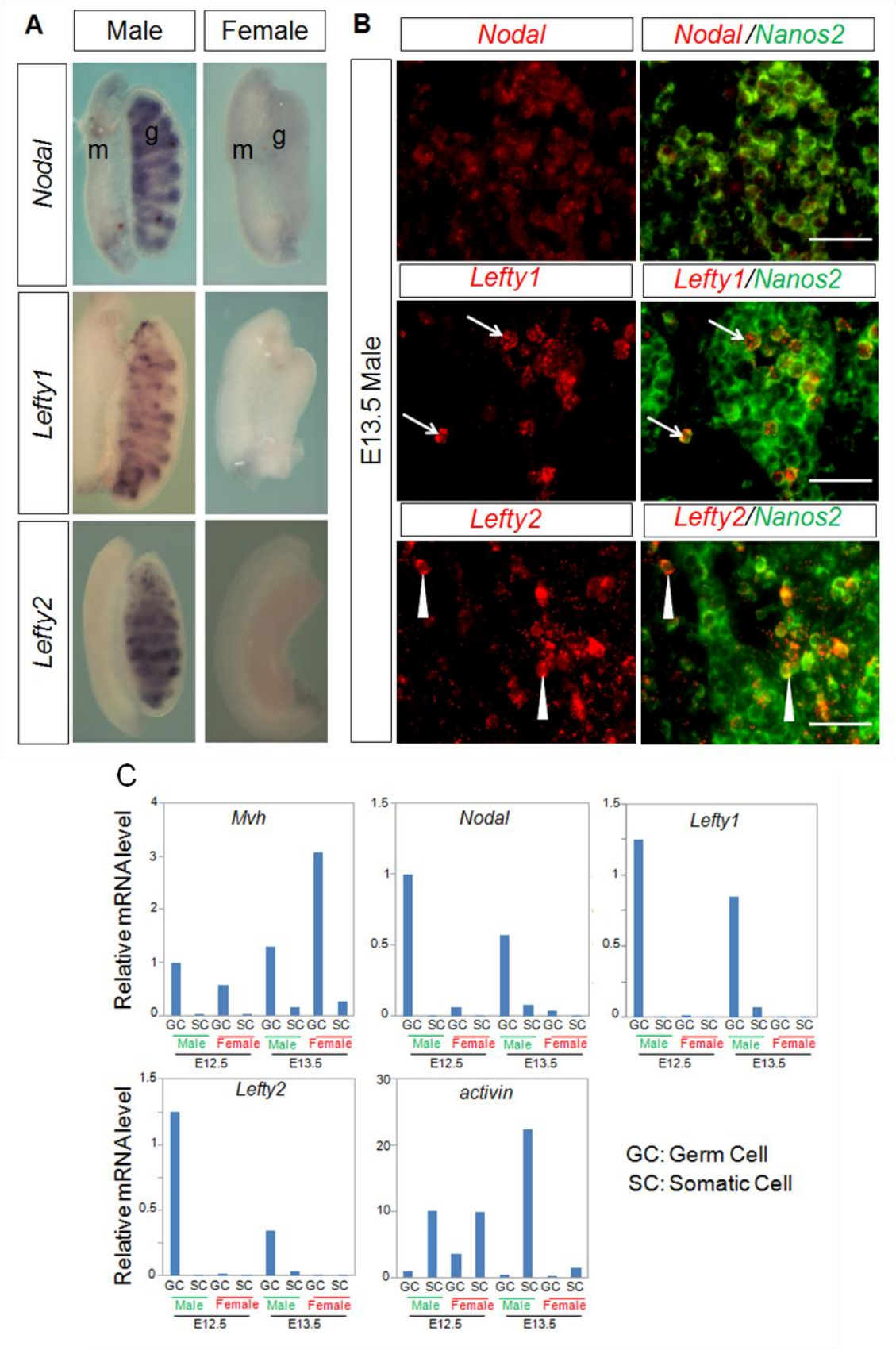

A
Male
Female
Nodal
Lefty1
Lefty2
m
g
m
g
B
Nodal
Nodal/Nanos2
Lefty1
Lefty1/Nanos2
Lefty2
Lefty2/Nanos2
E13.5 Male
C
Mvh
Nodal
Lefty1
Lefty2
activin
Relative mRNA level
GC SC GC SC GC SC GC SC
Male Female Male Female
E12.5 E13.5
GC: Germ Cell
SC: Somatic Cell

Figure I-1. Expression patterns of genes involved in nodal/activin signaling in embryonic gonads.
(A) Whole-mount RNA *in situ* hybridization of embryonic day (E) 13.5 mouse testes and ovaries using probes for the genes indicated. (B) Double-section *in situ* hybridization was performed using *Nanos2* mRNA as a marker of germ cells. *Nodal* mRNA, *Lefty1* mRNA (arrows) and *Lefty2* mRNA (arrowheads) were restricted in male germ cells at E13.5. (C) Germ cells and somatic cells were separated immunomagnetically from embryonic day (E)12.5–13.5 gonads and RT-qPCR was performed to analyze the expression levels of *Mvh*, *Nodal*, *Lefty1/2* and *Inhba*. Key: m, mesonephros; g, gonad. Scale bars: 50 μm.

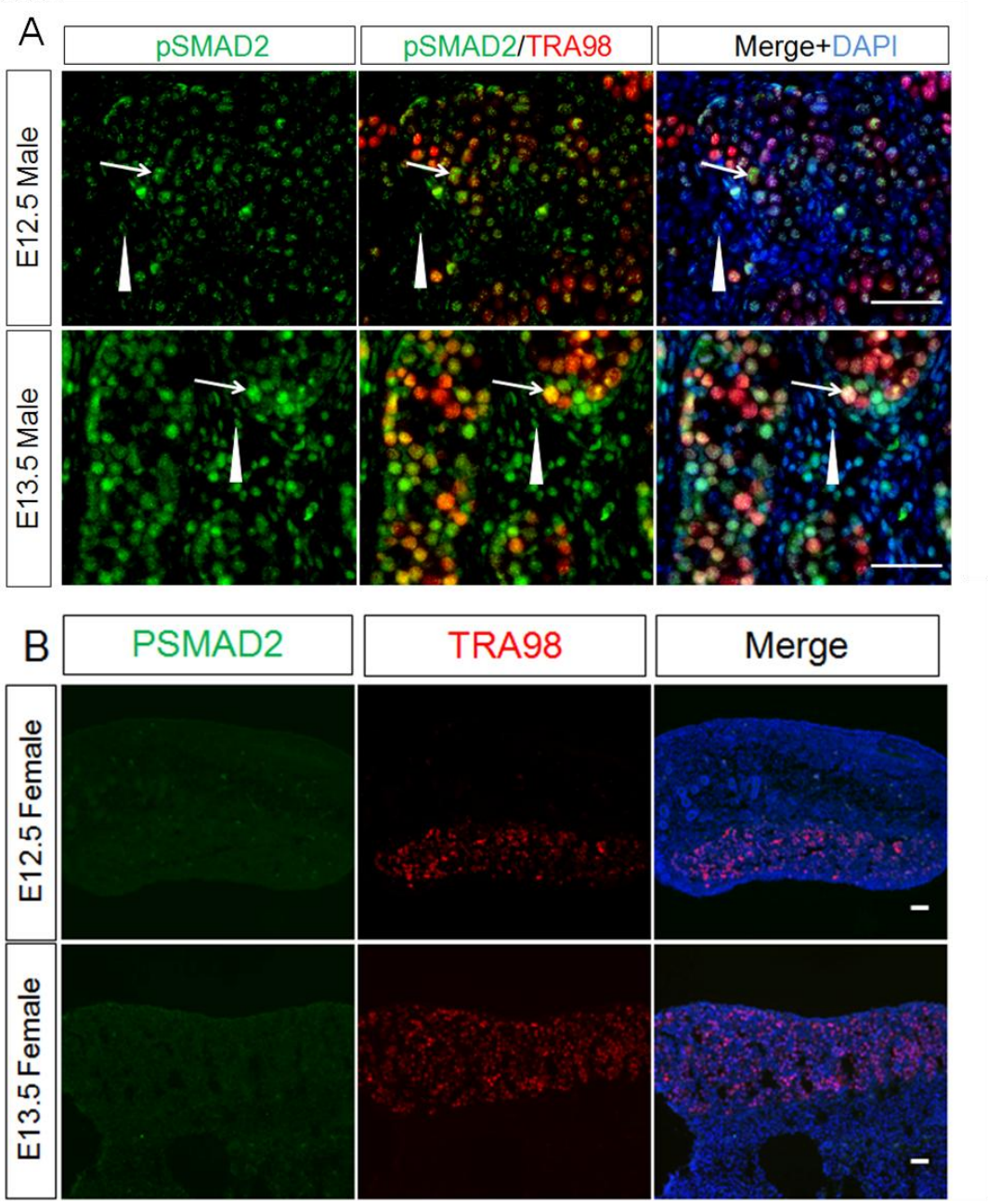


Figure I-2 Localization of pSMAD2 in fetal gonads.
Double immunostaining for pSMAD2 (green) and the germ cell marker TRA98 (red) in the testis (A) and ovaries (B) at E12.5 and E13.5. pSMAD2 expression was exclusively observed in both germ cells (arrows) and somatic cells in fetal testes (arrowheads). Scale bars: 50 μm.

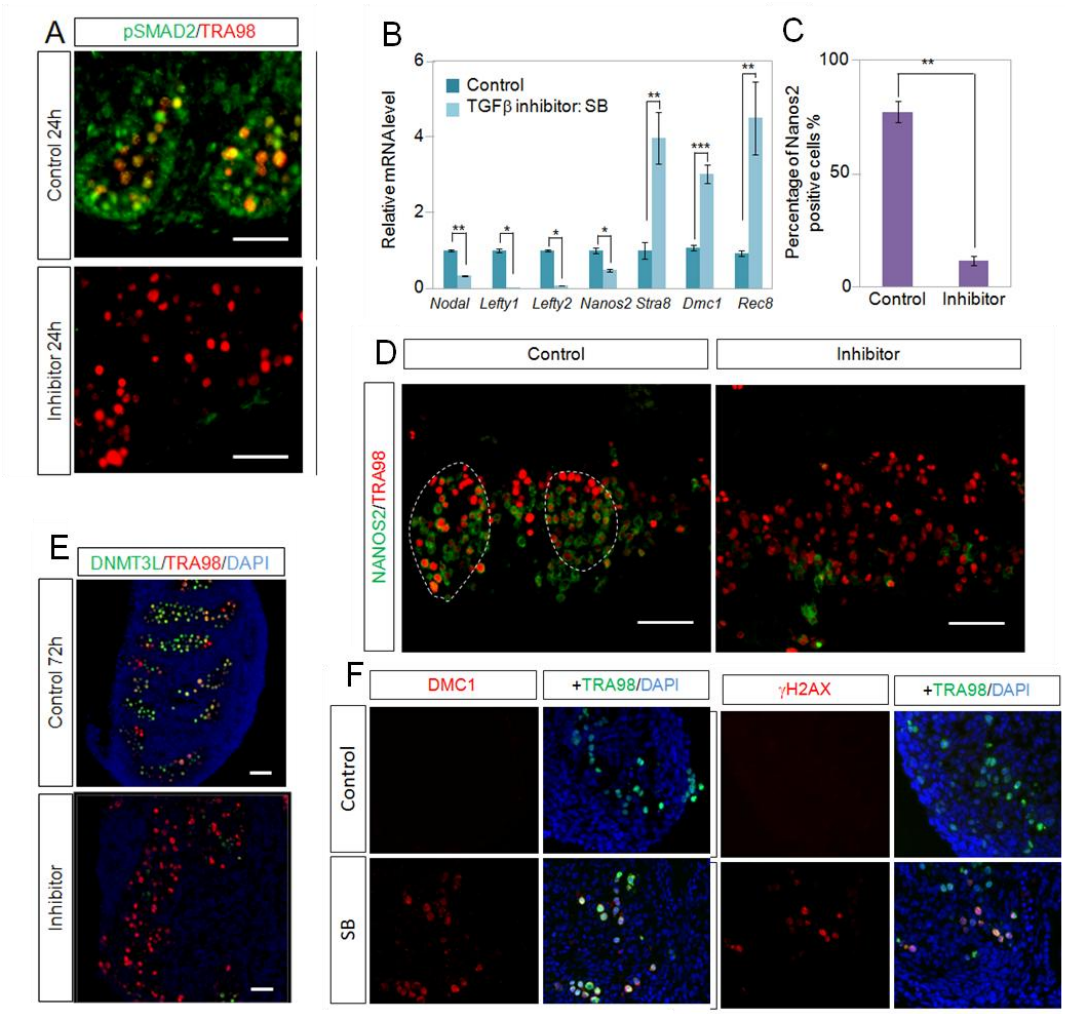


Figure I-3 Nodal/activin is responsible for the initiation of *Nanos2* expression and suppression of meiosis *ex vivo*. Testes from mice at E11.5 (C , D and F) or E12.5 (A, B and E) were cultured with the TGFβ receptor inhibitor SB431542 (40 μM) or DMSO (control vehicle) for 24 h (A and B) or 72 h (C-F). (A) Results of immunostaining for pSMAD2 (green) together with TRA98 (red), indicate that SB431542 suppressed the expressions of both pSMAD2. (B) The indicated genes were analyzed by RT-qPCR (using *Mvh* as a normalization control; $n = 6$). (C) Percentages of NANOS2-positive cells in DMSO and inhibitor-treated testes ($n = 4$). (D and E) Immunostaining of NANOS2 and DNMT3L after culture. (F) Immunostaining with DMC1 and γH2AX indicated that treatment of testes with SB431542 promoted meiosis. Dashed lines outline a testicular cord. Bars in graphs represent mean ±s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bars: 50 μm.

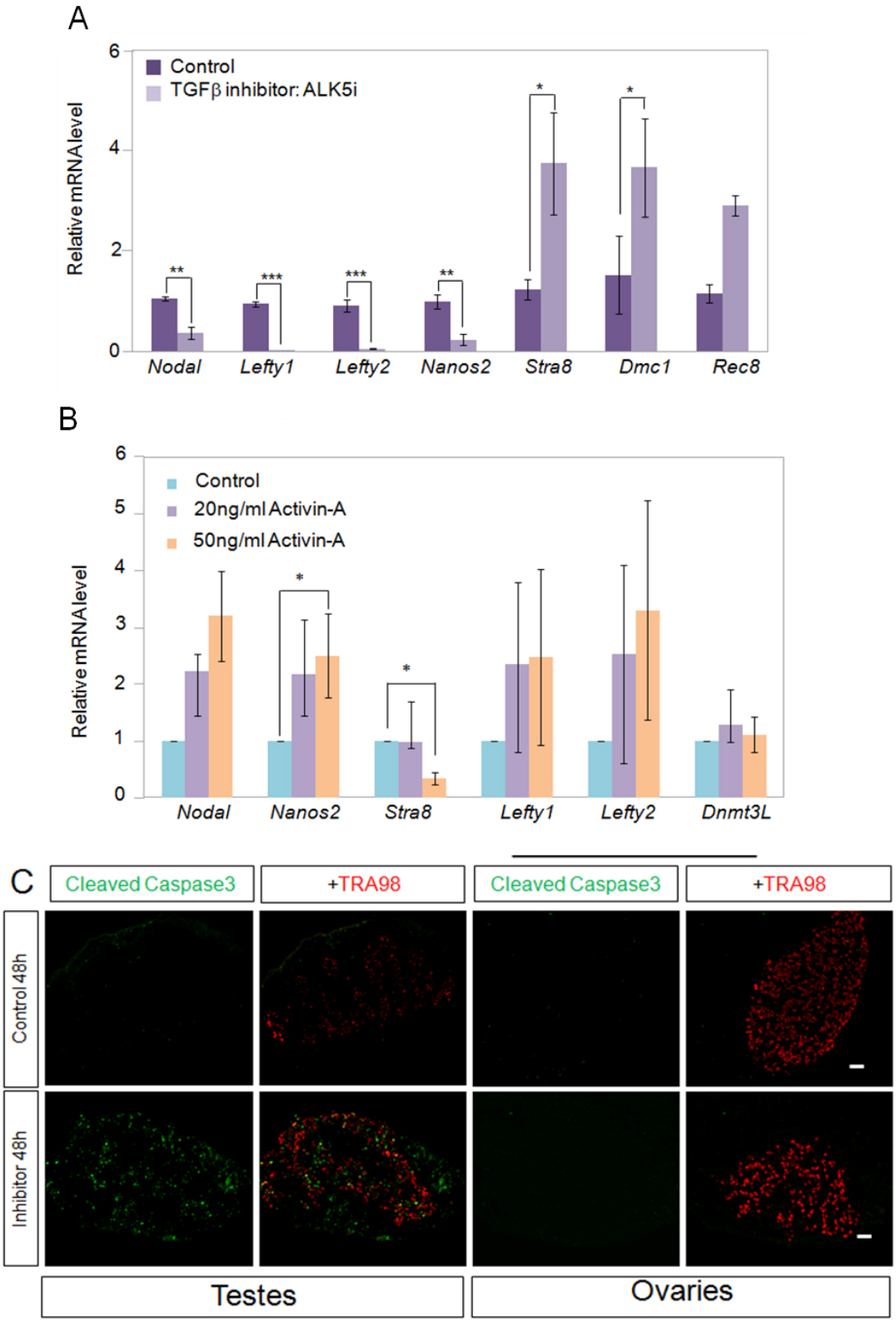

A
Control
TGFβ inhibitor: ALK5i
Relative mRNA level
Nodal
Lefty1
Lefty2
Nanos2
Stra8
Dmc1
Rec8
B
Control
20ng/ml Activin-A
50ng/ml Activin-A
Relative mRNA level
Nodal
Nanos2
Stra8
Lefty1
Lefty2
Dnmt3L
C
Cleaved Caspase3
+TRA98
Cleaved Caspase3
+TRA98
Control 48h
Inhibitor 48h
Testes
Ovaries

Figure I-4 Nodal/activin-A promotes male germ cell differentiation and is essential for cell survival. (A) E11.5 testes were cultured with ALK5 inhibitor (15 μM) or DMSO for 48 h. The expression changes of indicated genes are shown. (B) E12.5 testes were cultured in the presence of Activin-A (20 or 50 ng) for 24 h, and gene expression levels were analyzed by RT-qPCR (using *Mvh* as a normalization control; $n = 3$) (C) Testes or ovaries from mice at E12.5 were dissected and cultured with SB431542 (40 μM) or DMSO for 24 h. Gonads were then immunostained with anti-cleaved caspase 3 (green) and TRA98 antibodies (red). Many male germ cells and somatic cells entered apoptosis (C, left panel). However, no apoptotic cells were observed in inhibitor-treated ovaries (C, right panel). Bars in graphs represent mean ±s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Scale bar : 50 μm.

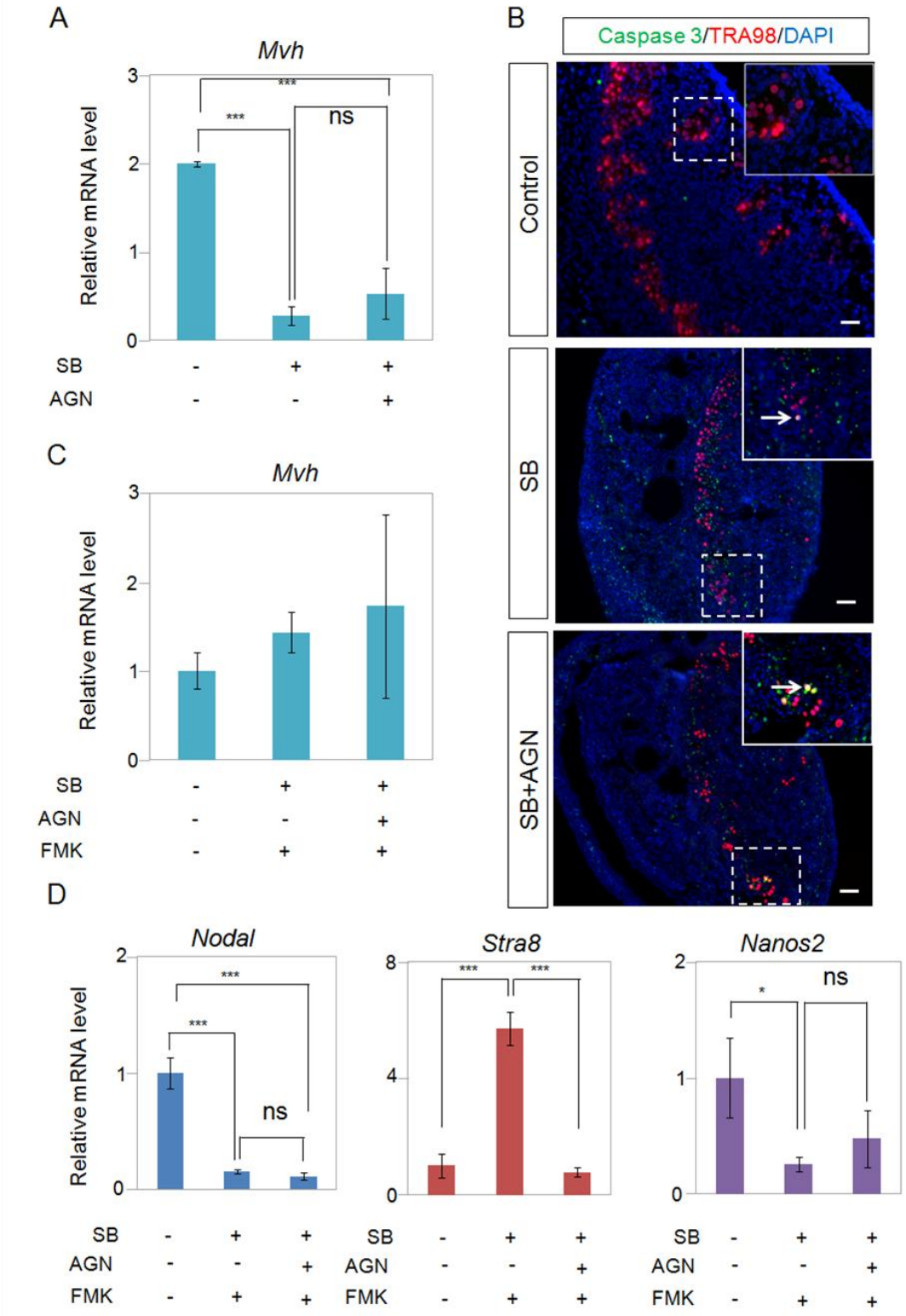

A
Mvh
Relative mRNA level
3
2
1
0
***
***
ns
SB - + +
AGN - - +
B
Caspase 3/TRA98/DAPI
Control
SB
SB+AGN
C
Mvh
Relative mRNA level
3
2
1
0
SB - + +
AGN - - +
FMK - + +
D
Nodal
Relative mRNA level
2
1
0
***
***
ns
SB - + +
AGN - - +
FMK - + +
Stra8
8
4
0
***
***
SB - + +
AGN - - +
FMK - + +
Nanos2
2
1
0
*
ns
SB - + +
AGN - - +
FMK - + +

Figure I-5 RA-independent induction of apoptosis and downregulation of *Nanos2* expression.
(A) RT-qPCR analyses of *Mvh* in E11.5 mouse testes treated with SB431542 (40 μM) or the RA receptor antagonist AGN193109 (5 μM) for 48 h ($n = 3$ using *glyceraldehyde 3-phosphate dehydrogenase (G3pdh)* as a normalization control). (B) Immunostaining for the apoptotic marker cleaved caspase-3 in treated testes. Apoptotic cells are indicated by arrows ($n = 2$). Insets show higher magnification of the boxed areas (C and D) RT-qPCR analyses of *Mvh*, *Nodal*, *Stra8* and *Nanos2* expression in E11.5 testes treated with the indicated drugs for 48 h ($n = 3$ for *Mvh*, using *G3pdh* as a normalization control; for others *Mvh* was used as a normalization control). Bars in graphs represent mean ±s.e.m. *$P < 0.05$, ***$P < 0.001$. ns, not significant. Scale bars: 50 μm.

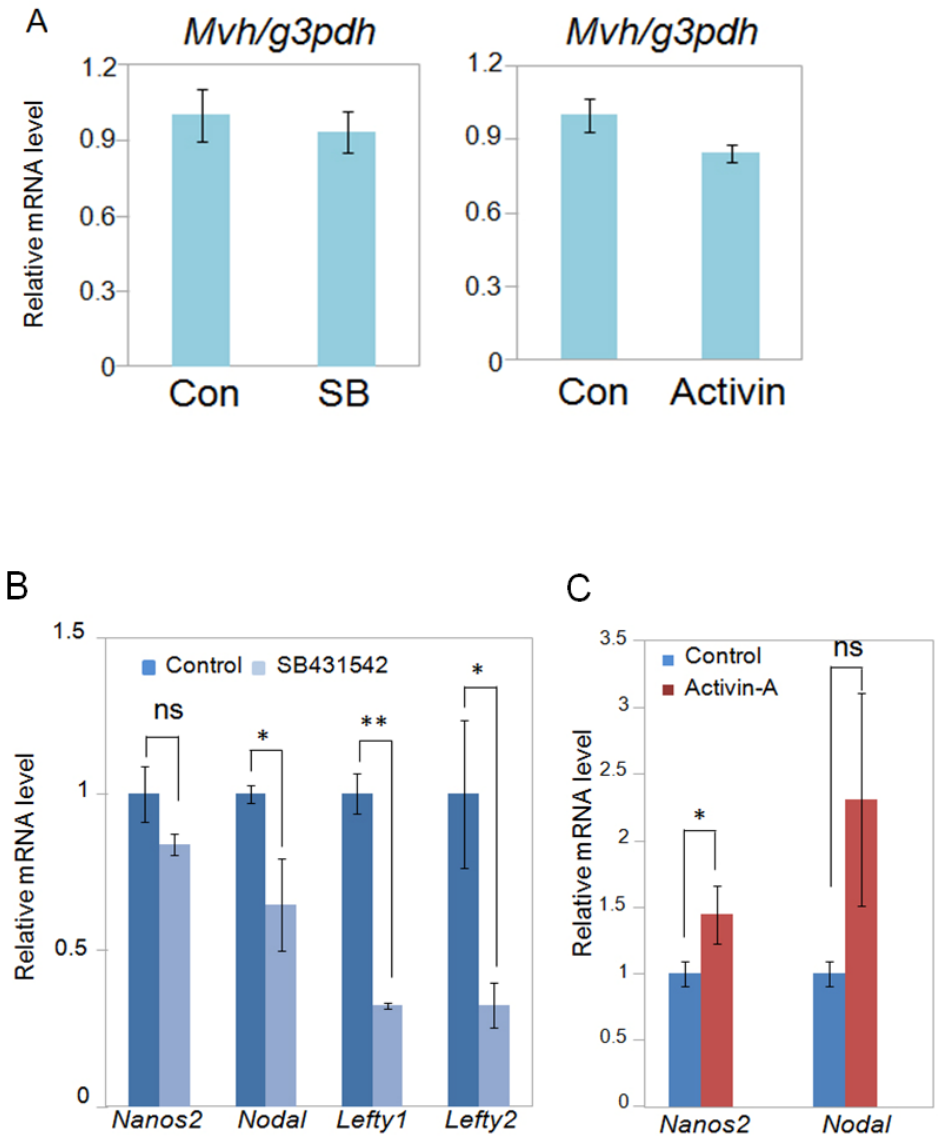


Figure I-6 Nodal and activin act directly on male germ cells. Male germ cells were isolated from E12.5 mouse testes and cultured with SB431542 or Activin-A for 24 h; RT-qPCR was used to assay the expression levels of *Mvh* (A), *Nodal*, *Lefty1/2* and *Nanos2* (B and C). Three independent experiments were performed. Bars in graphs represent mean ±s.e.m. *P < 0.05, **P < 0.01. ns, not significant. Scale bars: 50 μm.

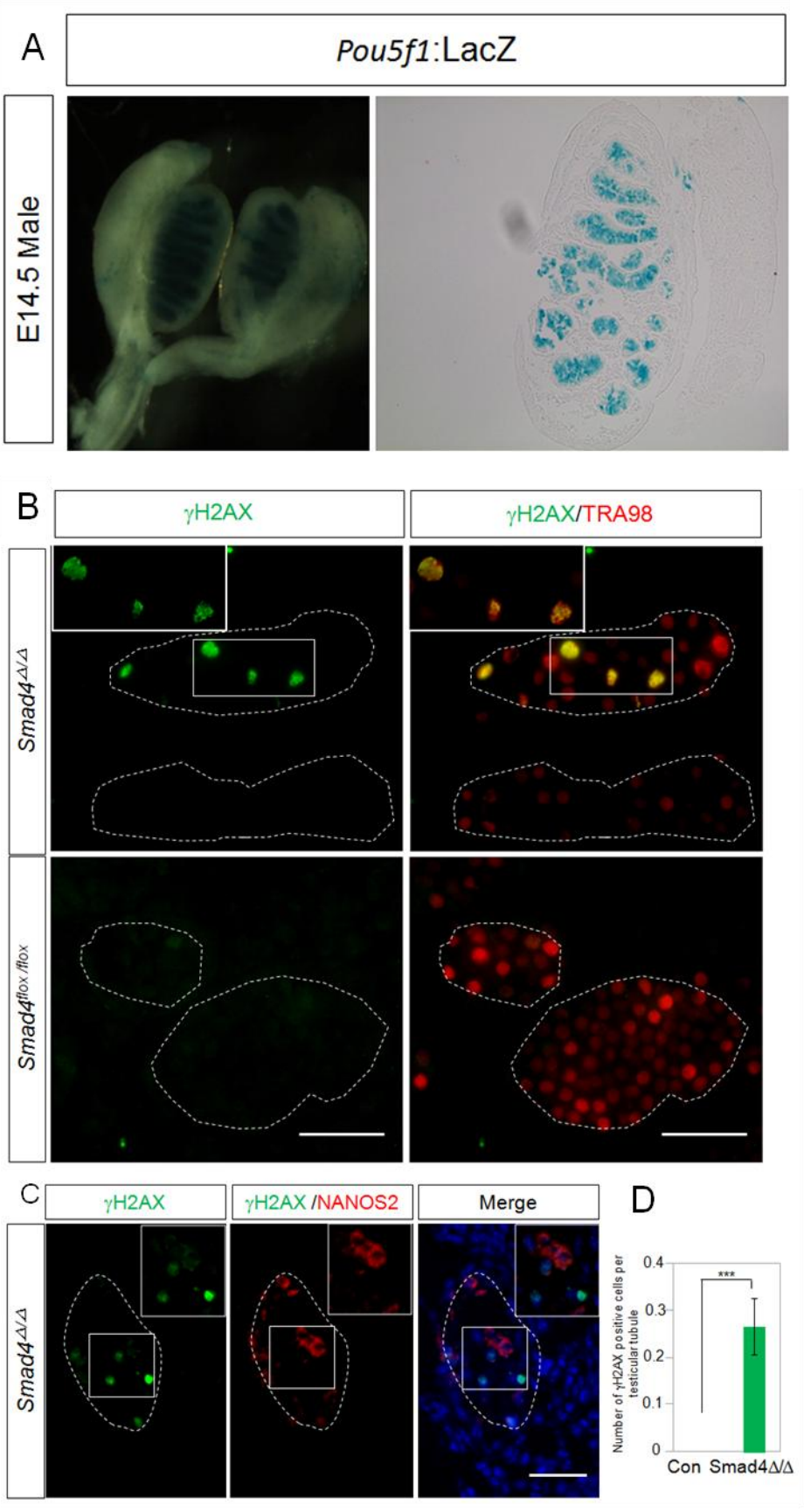

A
Pou5f1:LacZ
E14.5 Male
B
γH2AX
γH2AX/TRA98
Smad4Δ/Δ
Smad4flox/flox
C
γH2AX
γH2AX /NANOS2
Merge
Smad4Δ/Δ
D
Number of γH2AX positive cells per testicular tubule
0.4
0.3
0.2
0.1
0
***
Con
Smad4Δ/Δ

Figure I-7 Analysis of *Smad4* conditional knockout mice.
(A-C) The germ cell-specific line *Pou5f1*-CreERT2 was used to conditionally delete *Smad4*. Tamoxifen was injected at E10.5 and E11.5 ($n = 3$). (A) X-gal staining of *Pou5f1-CreERT2*$^{LacZ/+}$ testes at E14.5. (B and C) Immunohistochemical detection of γH2AX and TRA98 (B) or γH2AX and NANOS2 (C) in *Smad4*-null male germ cells at E14.5. Insets show higher magnification of the boxed areas. (D) Number of γH2AX-positive cells in the testicular tubule at E14.5. The germ cell-specific line *Stella*-MerCreMer was used to delete *Smad4* conditionally. 4-Hydroxyamoxifen was injected at E10.5 and E11.5. After immunostaining, γH2AX-positive cells were counted in three randomly chosen sections for each sample ($n = 3$). A testicular cord is delineated by white dashed lines. Bars in graphs represent mean ±s.e.m. ***P < 0.001.. Scale bars: 50 μm.

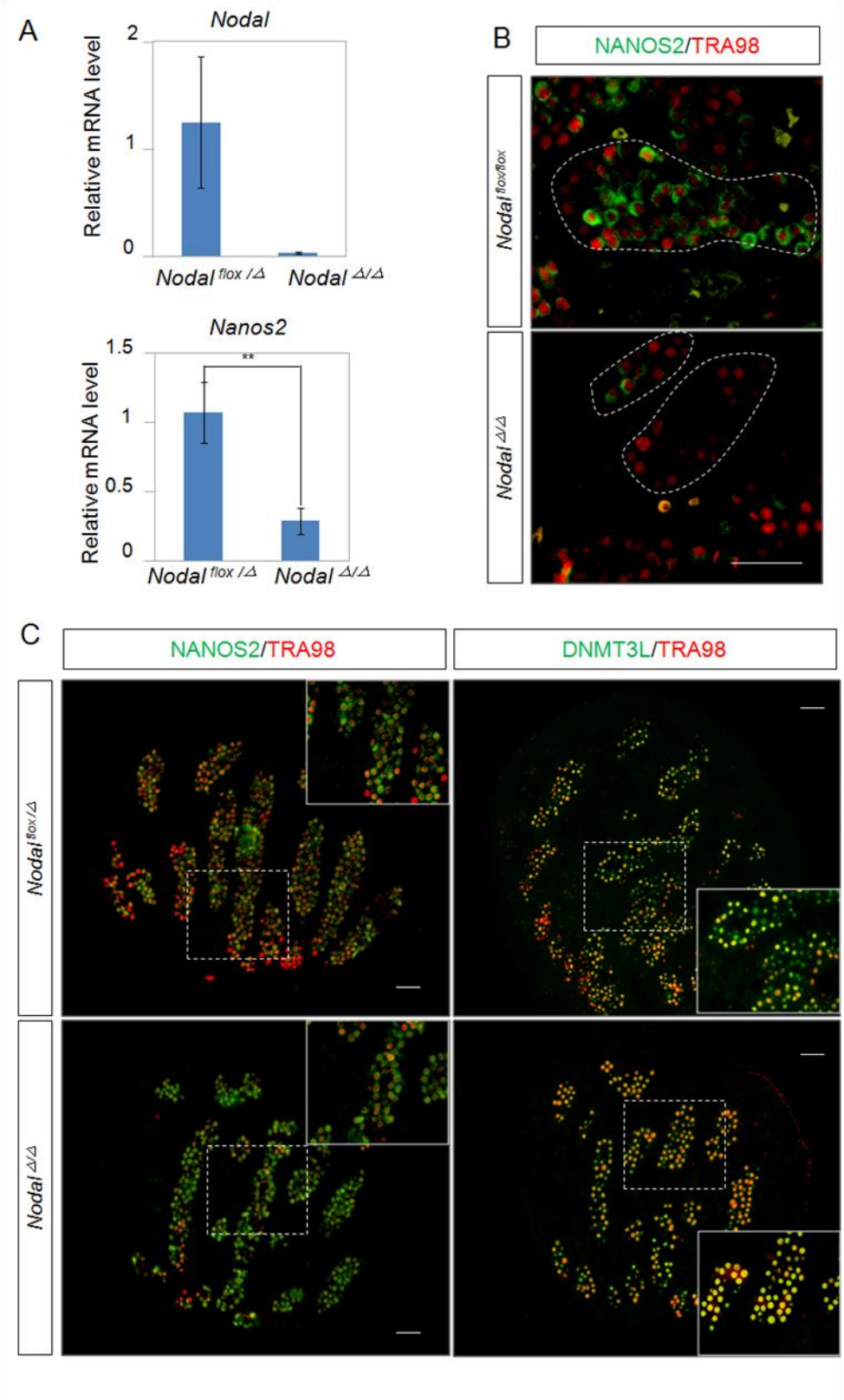

A
Nodal
Relative mRNA level
2
1
0
Nodal flox /Δ
Nodal Δ/Δ
Nanos2
Relative mRNA level
1.5
1
0.5
0
**
Nodal flox /Δ
Nodal Δ/Δ
B
NANOS2/TRA98
Nodal flox/flox
Nodal Δ/Δ
C
NANOS2/TRA98
DNMT3L/TRA98
Nodal flox/Δ
Nodal Δ/Δ

Figure I-8. Mutually redundant roles of Nodal and Activin-A in sex differentiation of male germ cells

(A) RT-qPCR analysis of *Nodal* and *Nanos2* mRNA levels in the testes of $Nodal^{\triangle/\triangle}$ mice (for mutants, $n = 3$; for controls, $n = 4$). Tamoxifen was injected at E10.5 and gonads were harvested at E14.5. (B) Immunostaining for NANOS2 in $Nodal^{\triangle/\triangle}$ and $Nodal^{flox/flox}$ testes. (C) Immunostaining for NANOS2 (left) and DNMT3L (right) in the E16.5 $Nodal^{\triangle/\triangle}$ testis. The expression of these genes indicated that male sexual differentiation was proceeding normally. Insets show higher magnification of the boxed areas. Dashed lines outline a testicular cord. Bars in graphs represent mean ±s.e.m. **P < 0.01. Scale bars: 50 μm..

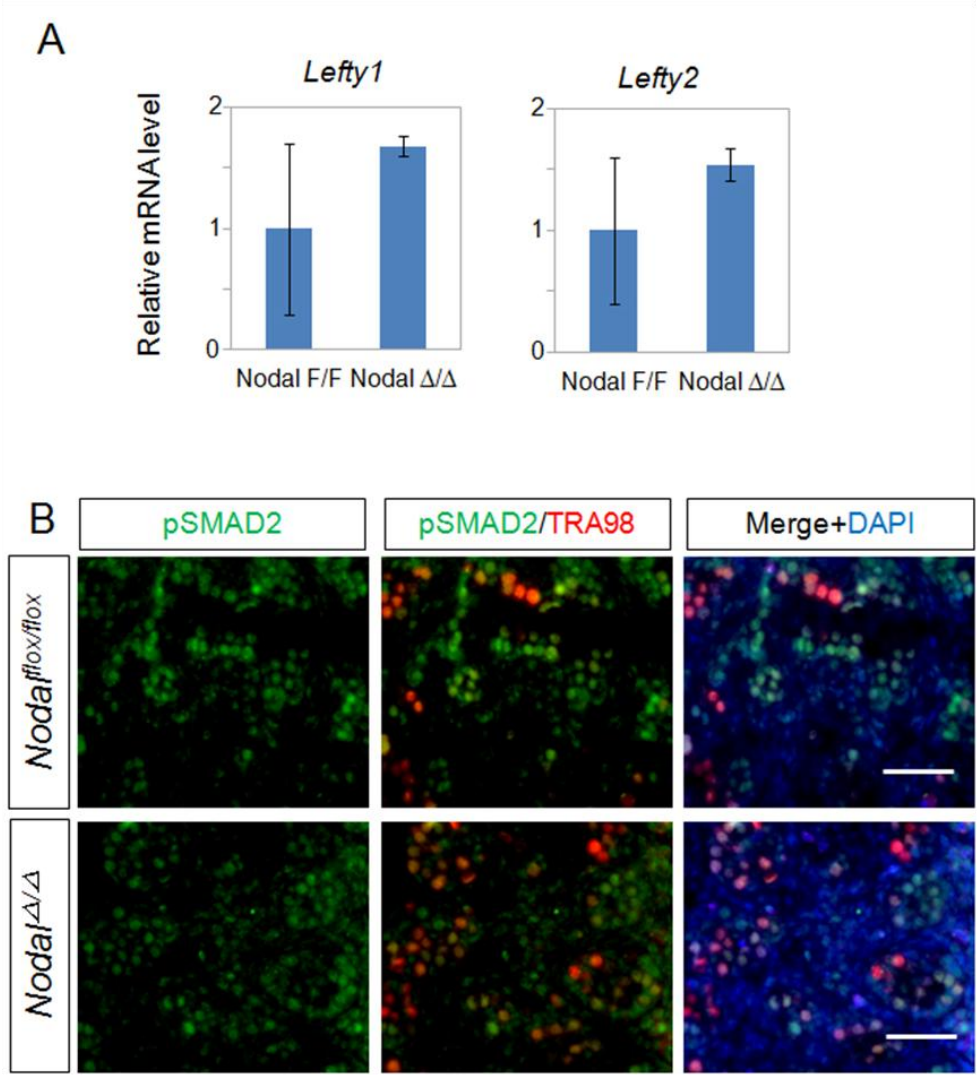
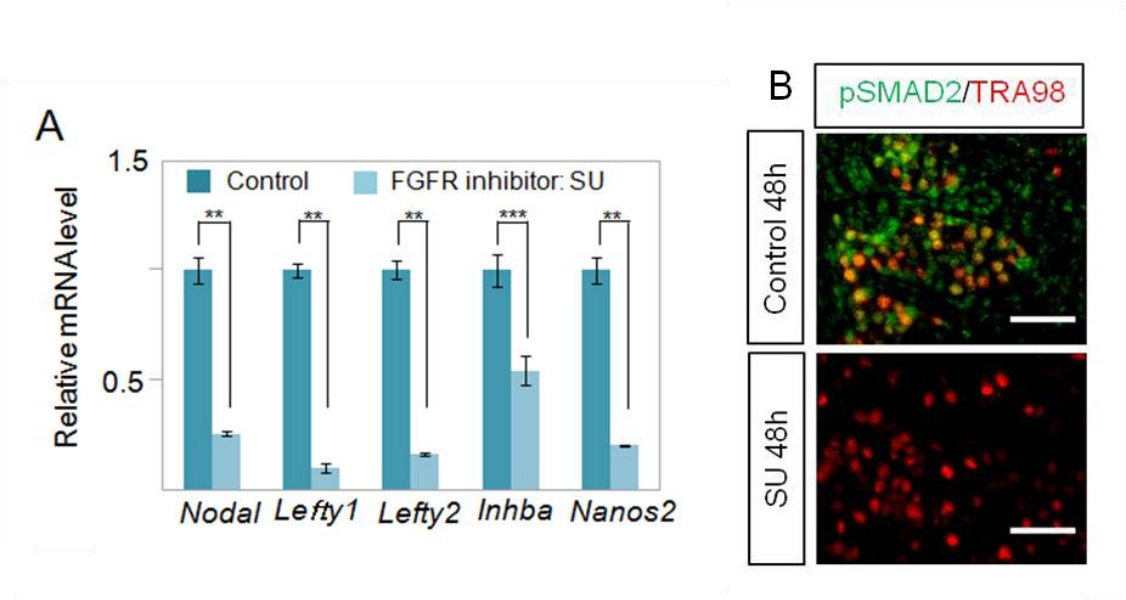
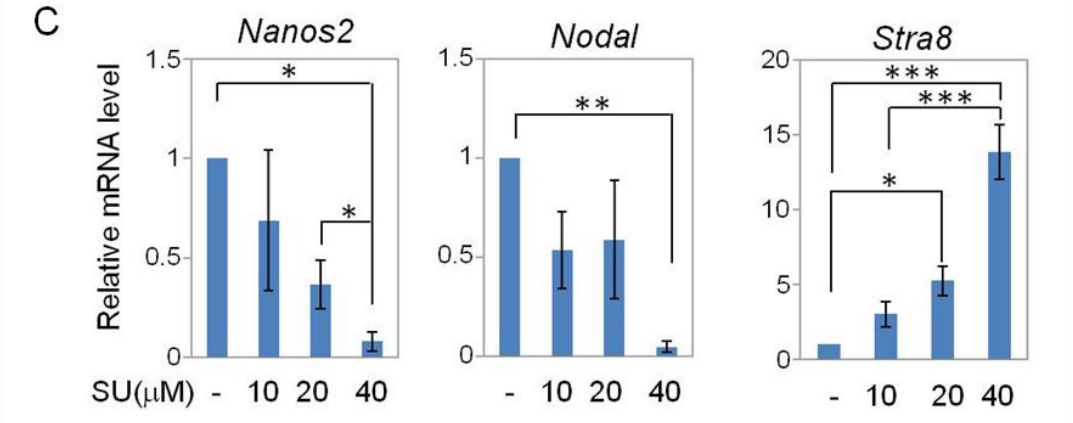


Figure I-9 Expression of Lefty1/2 and pSMAD2 persists in the testes of *Nodal*$^{\Delta/\Delta}$ mice.

(A) Expression of *Lefty1/2* in E14.5 testes from *Nodal*$^{flox/flox}$ and *Nodal*$^{\Delta/\Delta}$ mice was analyzed using RT-qPCR. (B) Immunostaining with anti-TRA98 (red) and anti-pSMAD2 (green) antibodies in E13.5 testes from *Nodal*$^{flox/flox}$ and *Nodal*$^{\Delta/\Delta}$ mice. Bars in graphs represent mean ±s.e.m. Scale bar: 50 μm.


A
Relative mRNA level
1.5
0.5
Control
FGFR inhibitor: SU
**
**
**
***
**
Nodal
Lefty1
Lefty2
Inhba
Nanos2
B
pSMAD2/TRA98
Control 48h
SU 48h


C
Nanos2
Nodal
Stra8
Relative mRNA level
1.5
1
0.5
0
20
15
10
5
*
*
**
***
***
*
SU(μM) - 10 20 40
- 10 20 40
- 10 20 40

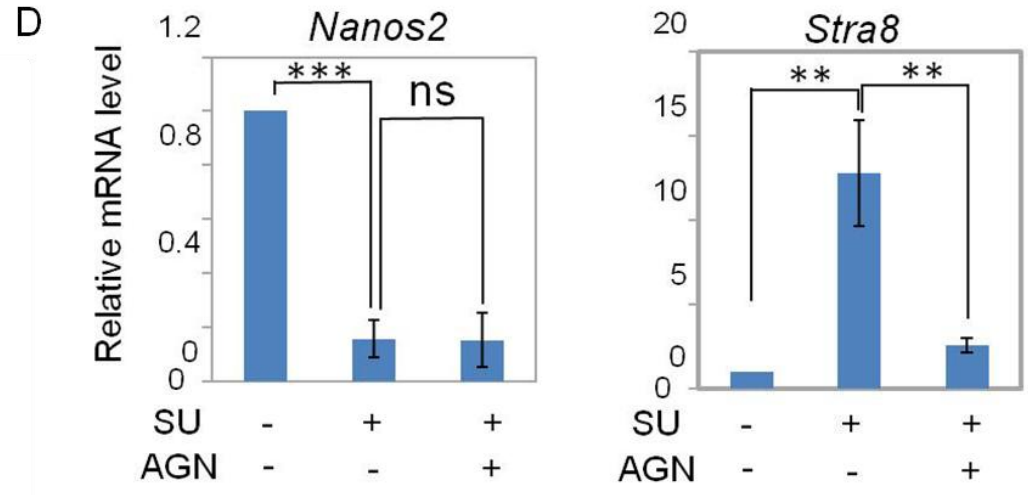

D
Nanos2
Stra8
Relative mRNA level
1.2
0.8
0.4
0
20
15
10
5
0
***
ns
**
**
SU - + +
AGN - - +
SU - + +
AGN - - +

Figure I-10 Nodal signaling acts downstream of FGF signaling.
(A) E11.5 testes were cultured with the FGF receptor inhibitor SU5402 (40 μM) or DMSO (control vehicle) for 48 h and the expression levels of *Lefty1/2*, *Nodal*, *Nanos2* and *Inhba* were examined using real-time RT-qPCR ($n = 3$, using *Mvh* as a normalization control). (B) E11.5 testes were treated with DMSO or SU5402 (40 μM) for 24 h and the localization of pSMAD2 was examined using immunostaining. (C) E11.5 testes were cultured with the FGF receptor inhibitor SU5402 (10, 20 or 40 μM) or DMSO (control vehicle) for 48 h and the expression levels of *Stra8*, *Nodal* and *Nanos2* were examined using real-time RT-PCR ($n = 3$, using *Mvh* as a normalization control). (D) Testes at E11.5 were cultured with DMSO or SU5402 (40 μM; middle panel) or SU5402 together with the RA receptor antagonist AGN 193109 (5 μM) for 48 h. The mRNA levels of *Nanos2* and *Stra8* were then examined using RT-qPCR. Bars in graphs represent mean ±s.e.m. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. ns, not significant. Scale bar: 50 μm.

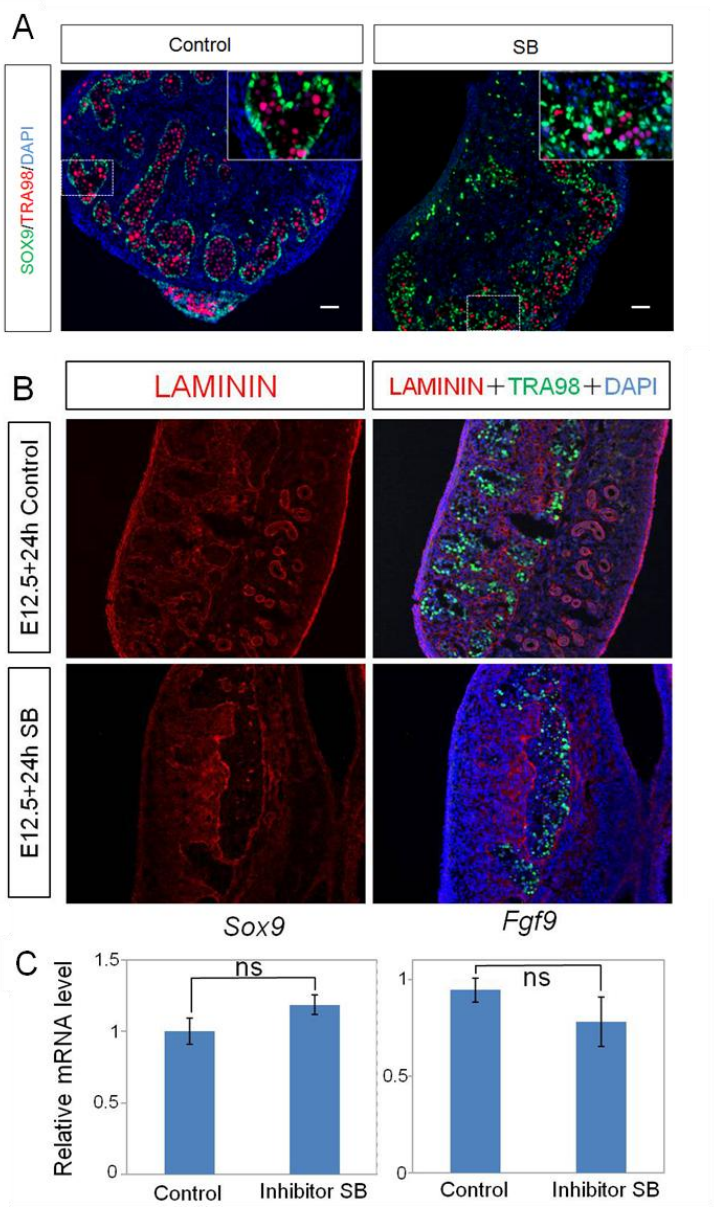


Figure I-11 SB431542 treatment disrupts testicular cords but does not affect Ffg9 and Sox9 expression

(A-C) E12.5 testes were cultured with the TGFβ receptor inhibitor SB431542 (40 μM) or DMSO (control vehicle) for 48 h (C) or 24 h (A and B). (A-B) Immunostaining for SOX9 (A) and LAMININ (B) after inhibitor treatment. (C) The expression levels of *Sox9* and *Fgf9* were investigated using RT-qPCR ($n = 3$). Bars on the graphs represent the mean ±s.e.m. ns, not significant.

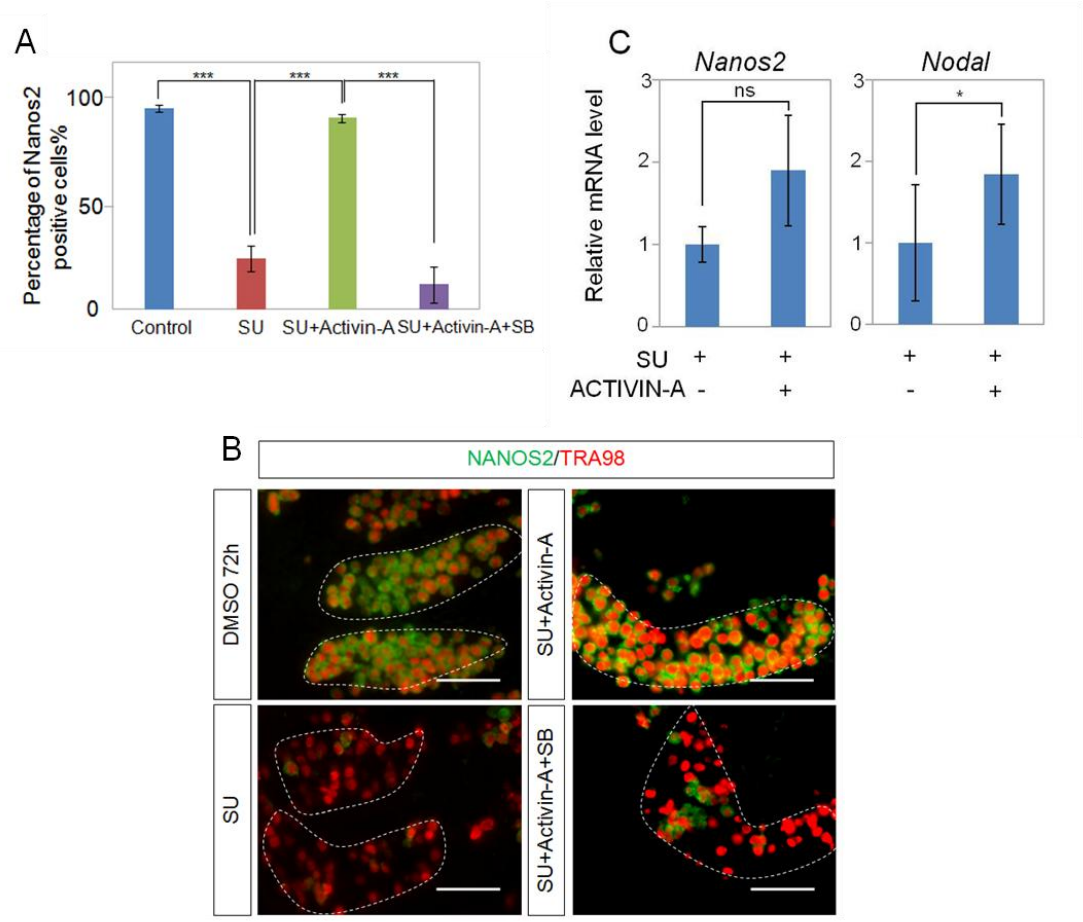


Figure I-12 Interactions between FGF and Nodal/Activin signaling pathways in the differentiation of male germ cells

(A and B) E12.5 testes were cultured for 72 h with SU5402 (40 μM), Activin-A (100 ng/ml), or SB431542 (40 μM) as indicated. (A) Percentage of NANOS2-positive cells after treatment ($n = 3$). (B) Immunostaining with an anti-NANOS2 antibody (green) and the germ cell marker TRA98 (red). (C) E11.5 testes were cultured for 48 h with SU5402 (40 μM), or together with Activin A (100 ng/ml) as indicated. Expression levels of *Nodal* and *Nanos2* were assayed using qRT–PCR ($n = 3$). Dashed lines outline a testicular cord. Bars in graphs represent mean ±s.e.m. *$P < 0.05$. ns, not significant. Scale bars: 50 μm.

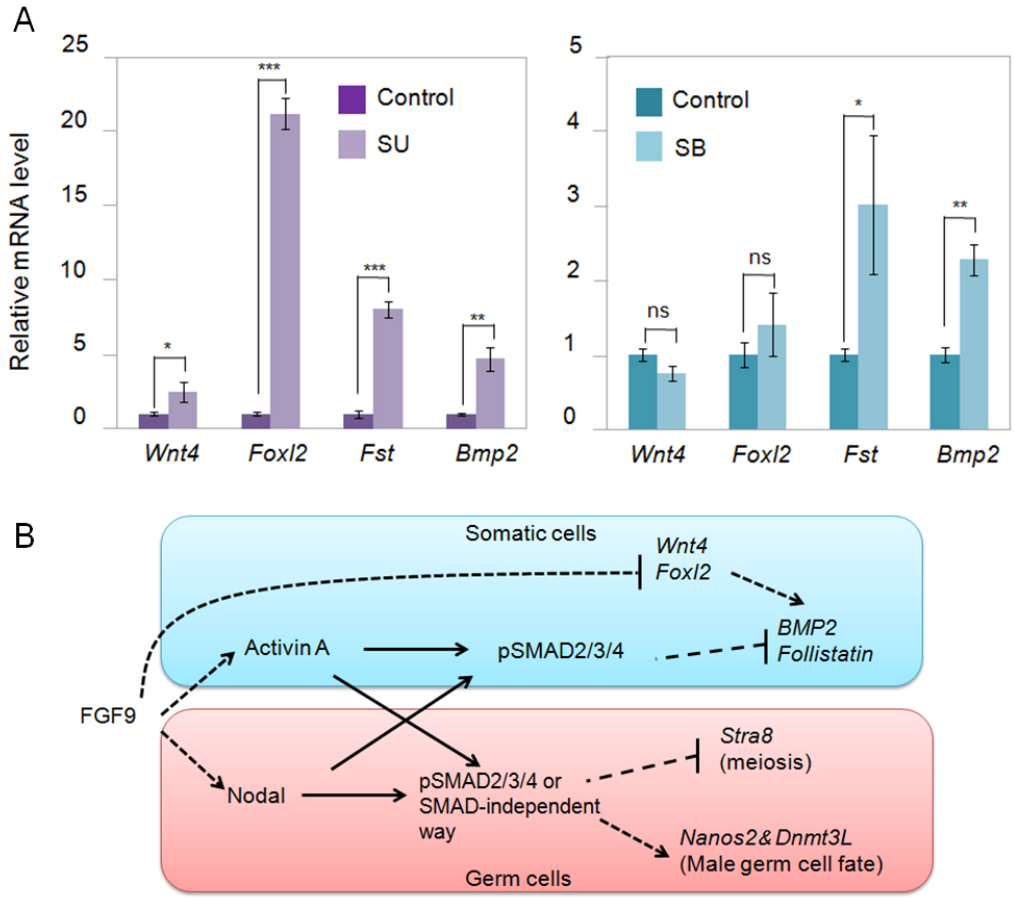


Figure I-13 Nodal/Activin is required for normal differentiation of somatic cells

(A) E11.5 testes were cultured with the FGF receptor inhibitor SU5402 (40 μM) or SB431542 (40 μM) together with FMK (10 μM) for 48 h and the expression levels of indicated genes were examined using RT-qPCR ($n = 3$ using *G3pdh* as a normalization control). (B) Schematic drawing of the model proposed in our study. FGF signals activate the nodal/activin signaling pathway in both somatic cells and germ cells. In germ cells, the nodal signaling pathway then triggers male sexual differentiation including the initiation of expression of the male-specific genes, *Nanos2* and *Dnmt3L*. In addition, it suppresses *Stra8*, which is an essential gatekeeper of meiosis. However, in somatic cells the activin-A thwarts the process of female differentiation by inhibiting *Bmp2* and follistatin. Bars in graphs represent mean ±s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. ns, not significant. Scale bars: 50 μm.

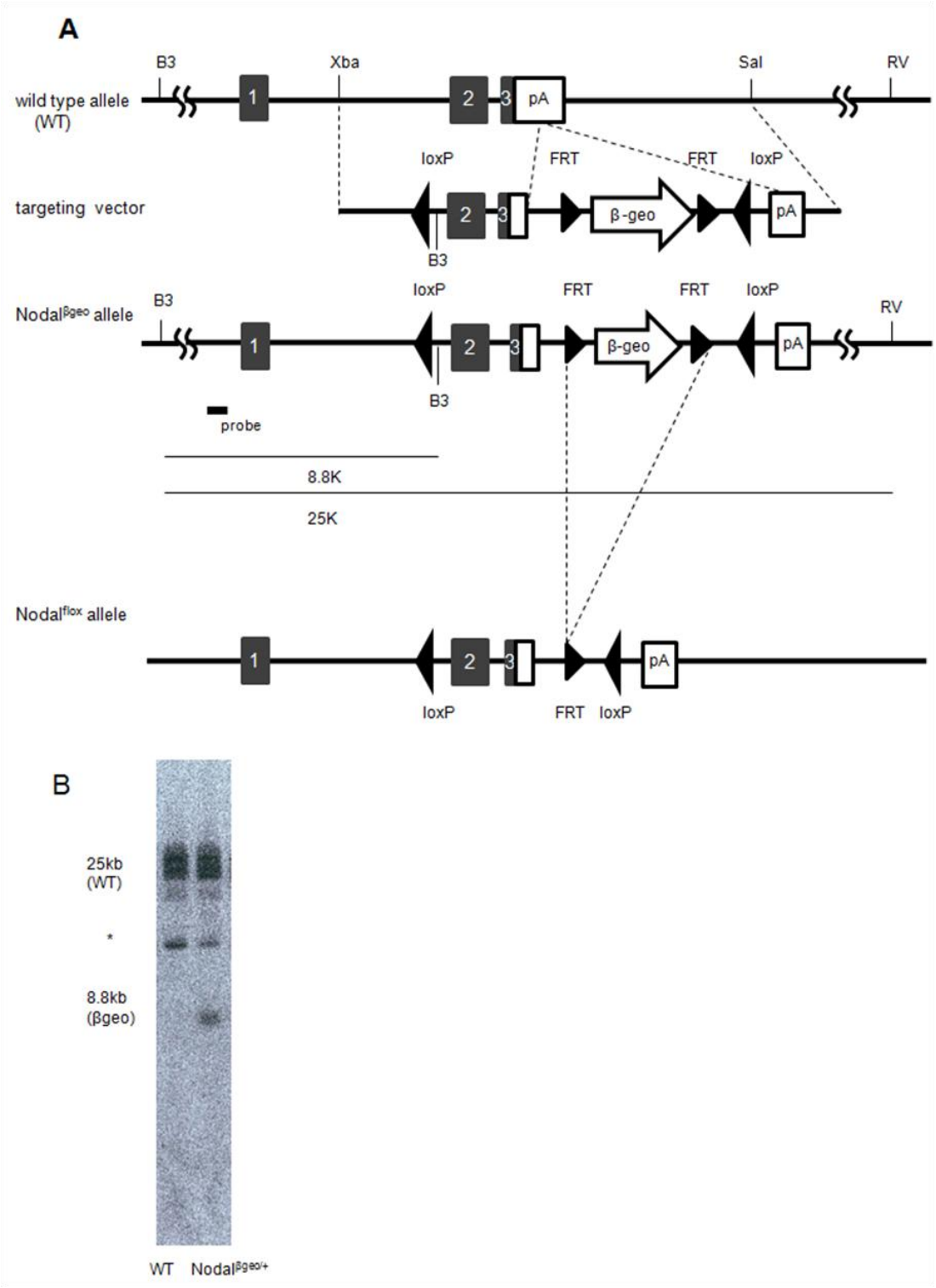

A
B3
Xba
Sal
RV
wild type allele
(WT)
1
2
3
pA
loxP
FRT
FRT
loxP
targeting vector
β-geo
pA
B3
Nodal^βgeo allele
B3
loxP
FRT
FRT
loxP
RV
β-geo
pA
B3
probe
8.8K
25K
Nodal^flox allele
1
2
3
pA
loxP
FRT
loxP
B
25kb
(WT)
*
8.8kb
(βgeo)
WT
Nodal^βgeo/+

Figure I-14 The generation of floxed alleles for Nodal
(A) Targeting strategy. Genomic organization of *Nodal* gene is shown at the top of the panel. Homologous recombination would insert a *loxP* site into the intron 1 and a [*FRT-βgeo-FRT-loxP*] cassette into the 3-untranslated region, generating the $Nodal^{\beta geo}$ allele. B3, BanIII; RV, EcoRV; Sal, SalI; Xba, XbaI. Southern blot analysis of EcoRV and BanIII-digested DNA with the 5'-external probe (probe) detects 8.8 kb fragment and 25 kb fragment in the wild-type Nodal allele, and the $Nodal^{\beta geo}$ allele, respectively. (B) Southern blot analysis of ES clones. Genomic DNA was digested with EcoRV and BanIII and subjected to hybridization with the 5' external probe indicated in A. 8.8 kb fragment is detected in correctly-targeted ES clone. 15 kb fragment shown by asterisks is not derived from Nodal gene.

Chapter II
Functional analysis of factors that might downstream of nodal signaling

INTRODUCTION

In the previous Chapter-I, I have proved valuable functions of nodal/activin signaling in both meiotic suppression and *Nanos2* induction during the development of male germ cells. In order to clarify the precise mechanisms by which nodal/activin acts, I sought to identify the downstream factors of nodal/activin signaling in the fetal testes.

Generally, nodal or acitivin binds a complex of transmembrane receptors (type I and II) that subsequently phosphorylate Smad2/3. The phosphorylated Smad2/3 form a complex with a common Smad4, which translocates into nucleus and activates target genes. Nodal/activin signaling plays many essential roles during vertebrate development and there is no evidence indicating the existence of the universal downstream genes of nodal/activin signaling except the components of this signaling (Schier, 2003). Therefore, the downstream genes of nodal/activin signaling are highly dependent on its context. In this Chapter, I focus on two factors that might be the downstream of nodal/activin signaling, Otx2 and p38 mitogen-activated protein kinase (MAPK) pathways.

Mice have three *Drosophila* Orthodenticle homologue (*Otx*) genes: *Otx1*, *Otx2* and *Otx3*. They are transcription factors containing a bicoid-like homeodomain. *Otx2* null mice fail to specify the rostral neuroectoderm and gastrulation, causing embryonic lethality (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996). Interestingly, function of *Otx2* during the formation of anterior neural plate and gastrulation can be replaced by *Otx1*, implying functional equivalency exists among Otx family (Acampora et al., 1998; Suda et al., 1999). During the formation of anterior-posterior axis, Otx2 works as a downstream target of SMAD2-induced FoXA2 complexes that are formed in the visceral endoderm, is essential for axis rotation (Kimura-Yoshida et al., 2007).

Otx3 has the same binding site with other *Otx* genes, and is involved in postnatal survival, growth and brain development (Ohtoshi et al., 2002; Zhang et al., 2002; Ohtoshi and Behringer, 2004).

Nodal/activin signaling also activates other signaling cascades, including Erk, JNK and p38 mitogen-activated protein kinase (MAPK) pathways (Derynck and Zhang, 2003). P38 MAPK signaling is triggered by various cellular stresses, inflammatory cytokines and growth factors (Zarubin and Han, 2005). Activated p38 MAPK translocates into nucleus and initiates target transcriptional factors (Zervos et al., 1995; Yang et al., 1999; Zhao et al., 1999). During the formation of anterior-posterior axis, nodal is required for the activation of p38 signaling pathway, which in turn strengthens nodal signaling by phosphorylating the Smad2 linker region and increasing the level of Smad2 activation. (Clements et al., 2011). Recently, an interesting study revealed a role of p38 MAPK signaling in initiation/maintenance of the sex-determining gene SRY in somatic cells (Bogani et al., 2009; Gierl et al., 2012; Warr et al., 2012). After sex determination, p38 MAPK signaling is exclusively expressed in germ cells and works as an essential gatekeeper of meiosis (Ewen et al., 2010). However, how p38 suppresses meiosis and the role of p38 signaling in the induction of male-specific genes is unknown.

In this chapter, I showed evidence that Otx2 acts as a downstream gene of nodal/activin and is involved in the expression of *Nanos2* using Otx2 conditional knockout mice. Moreover, I proved that p38 signaling plays a critical role on suppressing RA signaling independently of CYP26B1 and it provides proper environment required for the initiation of male-specific genetic programming.

RESULTS

Smad2 is required for the activation of nodal signaling

To facilitate the analysis of downstream factors of nodal/activin signaling, I firstly tried to block nodal signaling *in vivo*. In the chapter I, I suggested that a Smad4-independent pathway might exist to transmit nodal signals since the deletion of *Smad4* was not sufficient to completely disrupt nodal signaling. Yet, the requirement of Smad2/3 for the activation of *Nanos2* expression in response to nodal/activin-A is unclear. Therefore, *Smad2* and *Smad3* were conditionally deleted to analyze the effect of each protein on the germ cell development. By injection of tamoxifen into *Smad2*$^{flox/flox(+)}$*Smad3*$^{flox/flox(+)}$ *Rosa-CreERT2*$^{+/-}$ mice at E10.5 and E11.5, *Smad2* or *Smad3* was deleted ubiquitously. Fetal testes were harvested from E13.5 or E14.5. No double mutant testis has been harvested from the above mating protocol. However, the deletion of *Smad2* alone was found to be sufficient to impede nodal signaling, causing the downregulation of *Nanos2* expression and the induction of *Stra8* (Figure II-1 A). Unexpectedly, this phenotype was not observed when *Smad3* was deleted (Figure II-1 B). I concluded that Smad2 is sufficient and necessary for transduction of nodal signaling in male germ cells.

Specific expression of OTX2 in male germ cells

To analyze the downstream genes of nodal/activin signaling that trigger *Nanos2* expression, I searched transcription factors whose expression were downregulated after nodal inhibitor SB431542 treatment using microarrays. As a result, I identified OTX2 as a candidate factor. *Otx2* mRNA was specifically detected at E13.5 testes using whole mount *in situ* hybridization (Figure II-2A). In addition, results of immunostaining indicate a germ-cell-specific expression pattern of OTX2 at E12.5-E13.5 testes (Figure

II-2B). Accompanying with the decline of nodal/activin signaling (Spiller et al., 2012b), the expression level of OTX2 gradually decreased from E14.5 (Figure II-2B). No signal was detected in the fetal ovaries (Figure II-2B).

OTX2 is regulated by nodal/activin signaling

To confirm that whether the expression of *Otx2* is controlled by nodal/activin signaling, I investigated OTX2 expression level after the blocking of nodal signaling. As expected, the suppression of nodal/activin signaling either by the inhibitor treatment or the deletion of *Smad2* significantly diminished the OTX2 expression level, suggesting Otx2 is controlled by nodal signaling (Figure II-3).

OTX2 is involved in, but not essential for the initiation of *Nanos2* expression

As a transcription factor, OTX2 might act to directly trigger the expression of genes involved in sexual differentiation of XY germ cells such as: *Nanos2*. Then, I analyzed sexual fate of male germ cells lacking Otx2. Because Otx2 knockout mice die before the formation of gonads (Acampora et al., 1995; Matsuo et al., 1995; Ang et al., 1996), I conditionally deleted *Otx2* from E10.5 by injection of tamaxifen into *Otx2*$^{flox/flox}$*/Rosa-CreERT2*$^{+/-}$ mice. Testes were harvested from E13.5 or E14.5 mice. Complete deletion of either Otx2 mRNA or protein was confirmed by qPCR or immunostaining at E13.5 (Figure II-4 A and B). Notably, I also observed the reduction of *Nanos2* expression in the mutant testes at this stage (Figure II-4 A and B). However, the discrepancy between perfect deletion of Otx2 and incomplete repression of Nanos2 reflect the fact that *Otx2* is not essential for *Nanos2* expression. Indeed, the expression of *Nanos2* was recovered to the same level compared with control testes at E14.5

(Figure II-5 A).

Redundant roles of Otx2/3 in the induction of *Nanos2* expression

It has been reported that OTX1, OTX2 and OTX3 have the same consensus binding sequence, prompting me to consider that OTX1 and OTX3 might work to induce *Nanos2* expression in the absence of OTX2. To confirm this hypothesis, I analyzed the expression level of *Otx1* and *Otx3* in *Otx2* null germ cells. Interestingly, I found the up-regulation of *Otx3* expression in *Otx2* mutant implying redundant functions of Otx2 and Otx3 in the induction of *Nanos2* expression.

Inhibition of p38 Signaling Disruptes Male Differentiation

Phospho-p38 (pp38) MAPK is another possible factor activated by nodal/activin signaling. To analyze the function of p38 signaling pathway during male germ cell development, I investigated the localization of pp38 MAPK using immunostaining. Previous studies indicate that pp38 is essential for the initiation of *sry* expression in somatic cells (Bogani et al., 2009; Gierl et al., 2012; Warr et al., 2012). Consistent with these results, I detected pp38 MAPK in both somatic cells and germ cells at E11.5 (Figure II-6A). However, the localization of pp38 MAPK was exclusively observed in germ cells from E12.5 to E13.5 (Figure II-6B and 6C). Interestingly, the expression level also attenuated from E14.5 with the reduction of nodal/activin signaling (Figure II-6D).

Then I used specific inhibitor (SB203580) to investigate the involvement of p38 signaling in testicular differentiation. Suppression of p38 signaling using SB203580 increased the expression of *Stra8*, an essential gene for meiosis, consistent

with previous studies (Ewen et al., 2010) (Figure II-7A). Moreover, signals of γH2AX, a marker of double strand break during leptonema and zygonema was also detected after the inhibitor treatment, further confirming meiosis was induced (Hunter et al., 2001; Mahadevaiah et al., 2001) (Figure II-7B). Notably, the expression level of two male specific genes: NANOS2 and DNMT3L is significantly reduced after treatment (Figure II-7A, 6C and 6D). These results indicate that male germ cells lacking of p38 signaling failed to lock in the male pathway and entry meiosis.

Relationship between p38 signaling and nodal/activin signaling in male germ cells

The disruption of p38 signaling led to meiotic initiation and failure of *Nanos2* expression, a phenotype reminiscent of the case of suppressing nodal/activin signaling in testes (Figure I-3, I-4, II-1A compared with Figure II-7). Is p38 signaling regulated by nodal/activin signaling? Unexpectedly, pp38 persisted in the *Smad2*-null testes where nodal signaling was dramatically repressed (Figure II-8A), suggesting that nodal/activin signaling is dispensable for the activation of pp38. Moreover, negligible change of the expression level of *Nodal* was observed after p38 inhibitor treatment (Figure II-8B). In addition, signals of phosphorylated SMAD2, an effector of nodal/activin signaling, persisted after treatment, indicating p38 signaling have effect on neither initiation nor maintenance of nodal/activin signaling(Figure II-8C). Therefore, nodal/activin and p38 signaling work independently and might cooperate with each other during testicular differentiation.

P38 signaling inhibits meiosis Independent of NANOS2

The suppression of p38 signaling the dramatic reduced the *Nanos2* expression level and

caused meiotic entry. Because NANOS2 suppresses meiosis (Suzuki and Saga, 2008), p38 signaling might inhibit meiosis through activation of NANOS2 (Figure II-9A). It is also possible that both of these two factors are essential for meiotic suppression (Figure II-9B). To test these possibilities, I treated E12.5 *Nanos2*$^{-/-}$ testes with the inhibitor of p38 signaling and investigated the expression of *Stra8* and *Nanos2* (Figure II-9C and 9D). I reasoned that if p38 and NANOS2 suppresses meiosis independently, the inhibition of two factors (p38 and Nanos2) would cause much more severe phenotype, which could be judged by the augment of *Stra8* expression level, when compared with the suppression of merely one factor (p38 or Nanos2). Indeed, the expression level of *Stra8* in the inhibitor-treated *Nanos2*$^{-/-}$ testes is much higher than that in either the *Nanos2*$^{-/-}$ testes or the inhibitor-treated testes (Figure II-9D, columns 2, 3 and 4). Interestingly, the suppression of p38 resulted in higher expression of Stra8 than the deletion of Nanos2 (Figure II-9D, columns 2 and 3), suggesting at this stage (E12.5) p38 plays a major role in meiotic suppression. To further confirm the expression level of *Stra8*, I used *Nanos2*$^{-/-}$ *Stra8*$^{gfp/+}$ mice where expression of *Stra8* is monitored by GFP. I observed strong GFP signals after the inhibitor treatment but not in the testes without the treatment regardless of whether *Nanos2* was presence or not (Figure II-9E and 9F). These results indicate that p38 signaling suppresses meiosis independent of NANOS2.

P38 signaling inhibited meiosis independent of CYP26B1

Next, I asked how p38 signaling suppresses meiosis in testes. One possible mechanism is to regulate the expression of *Cyp26b1*, a gene encoding an enzyme that degrades RA. If this is the case, the inhibition of p38 signaling would reduce *Cyp26b1* expression level. However, I did not observe any change of *Cyp26b1* expression level after the

inhibitor treatment using either qPCR or *in situ* hybridization (Figure II-10A-C). I concluded that p38 signaling inhibit meiosis independent of CYP26B1.

RA but not STRA8 affects sex differentiation of XY germ cells

RA is sufficient to induce meiosis and suppress *Nanos2* expression. Even CYP26B1 is presence to degrade RA from mesonephros, it is reasonable to consider that RA can be synthesized by germ cells and p38 is responsible for degradation this part of RA. If this was the case, the disruption of RA signaling would rescue the phenotype caused by the loss of p38 signaling. To test this hypothesis, I simultaneously inhibited RA and p38 signaling by addition of RA receptor antagonist (AGN193109) and SB203580, respectively, into E11.5 testes and examined the expression of *Nanos2* after 48 hours. Diminishment of *Stra8* expression indicates successful inhibition of RA signaling (Figure II-11A). Notably, the reduction of *Nanos2* expression level caused by SB203580 was completely recovered when RA receptor antagonist was present (Figure II-11B). From these results, I conclude that p38 signaling protects male germ cells from the disruption of RA signaling and it does not directly trigger *Nanos2* expression.

How p38 signaling permits *Nanos2* expression in the germ cells exposed to RA? It is unlikely that p38 directly degrade RA like CYP26B1. Because pp38 translocate into nucleus, it might suppresses targets of RA which impede *Nanos2* expression. Considering STRA8 is one of the known targets of RA, I speculated that STRA8 inhibits *Nanos2* expression (Figure II-11C). If it were the case, the deletion of *Stra8* in male germ cells could substitute the function of p38 and allow *Nanos2* to be expressed even in the absence of p38 (Figure II-11C). To assess this hypothesis, I cultured testes from E12.5 *Stra8*$^{gfp/gfp}$ mice for 24 hours with SB203580 and

investigated the expression of *Nanos2*. Unexpectedly, the failure of *Nanos2* expression indicate the role of p38 signaling could not be replaced by the deletion of *Stra8*, thus other factors rather than STRA8 perturb male differentiation downstream of RA signaling (Figure II-11D).

It has been reported that RA disturbs mitotic arrest of male germ cells (Trautmann et al., 2008), prompting me to investigate whether p38 signaling is responsible for mitotic arrest. The failure of mitotic quiescence entry might impede *Nanos2* expression in the absence of p38 signaling. To test this possibilities, I examined the expression of Ki-67, a common marker of mitotic cells which detects all active phases of the cell cycle except for G0 state (Gerdes et al., 1984), after P38 inhibitor treatment. Surprisingly, 30% of germ cells entered G1/G0 arrest even in the absence of p38 signaling, which corresponds to the similar proportion of G1/G0 arrest in control germ cells after cultured for 24 hours (Figure II-12A and 12B). Therefore, p38 signaling was not essential for germ cells to enter mitotic arrest.

P38 signaling does not work downstream of Nanos2

Even though the level is low, the pp38 expression remained at E14.5 (Figure II-6D), prompting me to consider that pp38 might also function downstream of Nanos2 to promote male differentiation. To assess the role of p38 signaling as a possible downstream factor of NANOS2, I ectopically expressed *Nanos2* in testes and treated these testes with p38 inhibitor. If p38 also works downstream of Nanos2, suppression of p38 would induce abnormal differentiation of male germ cells. However, I found that Dnmt3L was normally expressed after *Nanos2* was ectopically expressed regardless of absence or presence of p38 signaling (Figure II-13), suggesting that p38

signaling exclusively acts upstream to permit *Nanos2* expression.

DISSUCTION

Induction of *Nanos2* expression

The induction of *Nanos2* is most important event in germ cell development, because Nanos2 is sufficient to promote spermatogenesis even in ovaries (Suzuki and Saga, 2008). Deletion of *Otx2* caused transient reduction of *Nanos2* expression, implying OTX2 might directly control the *Nanos2* transcription. Indeed, I found several binding site of OTX2 on the promoter or enhancer region of *Nanos2* gene (data not shown). However, redundant role of OTX family limits analysis to fully prove my idea. Interestingly, OTX2 along could not activated *Nanos2* enhancer using luciferase assay, implying the presence of the factor that cooperates with Otx2 to induce *Nanos2* expression (data not shown). Using luciferase assay, it has been proved that Lim1 (Lhx1) can directly bind with OTX2 and enhances OTX2-mediate gene expression (Nakano et al., 2000). Interestingly, Lhx1 is involved in the localization of PGCs in the embryonic hindgut before they migrate into genital ridge of PGCs (Tanaka et al., 2010). Future work will be performed to uncover whether these factor orchestrate to induce *Nanos2* expression.

Low level of RA induces meiosis in fetal testes

The inhibition of p38 MAPK results in meiotic entry and the failure of *Nanos2* induction in male germ cells. RA contributes to this phenotype because the suppression

of RA signaling with an RAR inhibitor completely recovered *Nanos2* expression. Where does RA come from? Generally, mesonephoros has been considered as the source of RA which is degraded by an enzyme CYP26B1 in somatic cells (Bowles et al., 2006). Given that *Cyp26b1* is normally expressed in somatic cells (Figure II-10), RA from mesonephoros is not responsible for meiotic entry in the absence of p38 signaling. In addition, the treatment of testes (without mesonephoros) with the p38 inhibitor induced meiosis, confirming that mesonephoros is not the only source of RA (data not shown). One possible explanation for these results is that endogenous RA of male germ cells induces meiosis in the absence of p38 signaling. Consistent with this hypothesis, a recent study suggests that E11.5 XX gonads without mesonephros still enter meiosis in culture condition devoid of any retinoids (Guerquin et al., 2010). However, a RA reporter mouse line in which *lacZ* reporter gene expression is regulated by a RA response element (RARE-LacZ mice), shows no LacZ signals in germ cells, implying that level of endogenous RA is very low. I conclude that p38 signaling is essential for the suppression of RA signaling in male germ cells.

Retinoic acid may also regulate male germ cell fate independently of meiosis and mitosis

It is widely accepted that RA regulates germ cell fate through sex-specific induction of meiosis (Baltus et al., 2006; Bowles et al., 2006; Koubova et al., 2006; MacLean et al., 2007). Moreover, exogenous RA also prevents male germ cell from keeping mitotic arrest and promotes proliferation (Trautmann et al., 2008). Here, I showed evidence that the low level of endogenous RA impedes the differentiation of XY germ cells independently of effect on either mitosis or meiosis (Figure II-11 and II-12). It is

reasonable to conclude that RA might have many other functions in gonadal development other than the regulation of meiosis and mitosis. RA signaling plays an important role in early organogenesis (Duester, 2008), and it has different targets in several tissues and acts to repress or activate these downstream genes. For example, it has been shown that RA is necessary for the caudal expression of Cdx1, a key regulator of the development of the axial skeleton. Moreover, during lung development, RA functions both as inducer of Hoxa5 gene and a suppressor of TGFβ signaling (Wang et al., 2006; Chen et al., 2007). RA also promotes differentiation of embryonic stem cells (ESCs) *in vitro*, whereas it is used to select embryoid bodies (formed from differentiated ESCs)-derived PGCs, implying the multi-function of RA in different type of cells (Geijsen et al., 2004). Regrettably, because targets of RA signaling are highly dependent on the context, I cannot identify the targets during testicular development in this thesis.

P38 signaling suppresses RA to allow male differentiation

Recent studies uncovered an important role of mitogen-activated protein kinase kinase kinase 4 (MAP3K4) in sex determination through showing that loss of function of MAP3K4 caused male to female sex reversal (Bogani et al., 2009). I found that p38 is also essential for sex differentiation of male germ cells. These two functions are consistent with the expression profile of pp38. At early stage, pp38 signals are detected in both germ cells and somatic cells and are observed exclusively in germ cells after E12.5 (Figure II-6).

In male germ cells, p38 signaling does not directly induce *Nanos2* expression, considering that inactivation of *Nanos2* in the absence of p38 signaling could be rescued by the suppression of RA signaling. Therefore, I conclude that p38 allow sexual

differentiation of male germ cells through antagonizing RA signaling. To my knowledge, this is the first report showing an antagonistic role of RA and p38 signaling. Instead, there is a study uncovered the possible relationship between RA signaling and TGFβ, whereby *all-trans* RA reduced phosphorylation of SMAD2/3 in response to TGFβ in the human promyelocytic leukemia cell line (Cao et al., 2003). In testes, neither the addition of exogenous RA nor the loss of function of CYP26B1 suppresses nodal signaling (data not shown). Therefore, RA and nodal/p38 signaling might compete to activate or repress their common targets during sexual differentiation of male germ cells.

In summary, my results together with previous chapter provide a panoramic view of initiation of male sex differentiation. FGF9 secreted from Sertoli cells is required for the activation of *Nodal* expression in germ cells directly or indirectly. Downstream of nodal signaling, OTX2 acts as an inducer to trigger *Nanos2* expression and meanwhile cooperates with p38 signaling that allow *Nanos2* expression through antagonize RA signaling. Endogenous RA from germ cells impedes male differentiation through induction of meiosis and other unknown factors (Figure II-14).

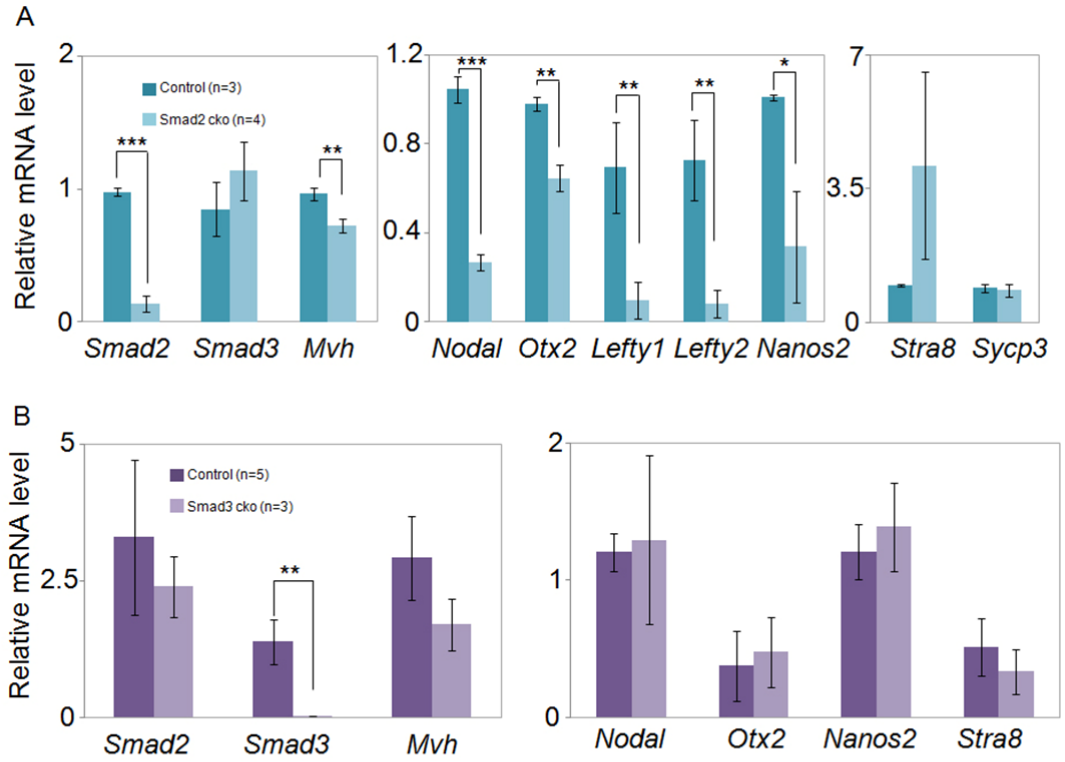


Figure II-1. Smad2 but not Smad3 is required for the activation of nodal signaling. (A) RT-qPCR analysis of the mRNA level of indicated genes in *Smad2* (A) and *Smad3* (B) mutant testes. Tamoxifen was injected at E10.5 and E11.5. Testes were harvested from E13.5 (A) and E14.5 (B) testes. Bars in graphs represent mean ±s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001. Scale bars: 50 μm.

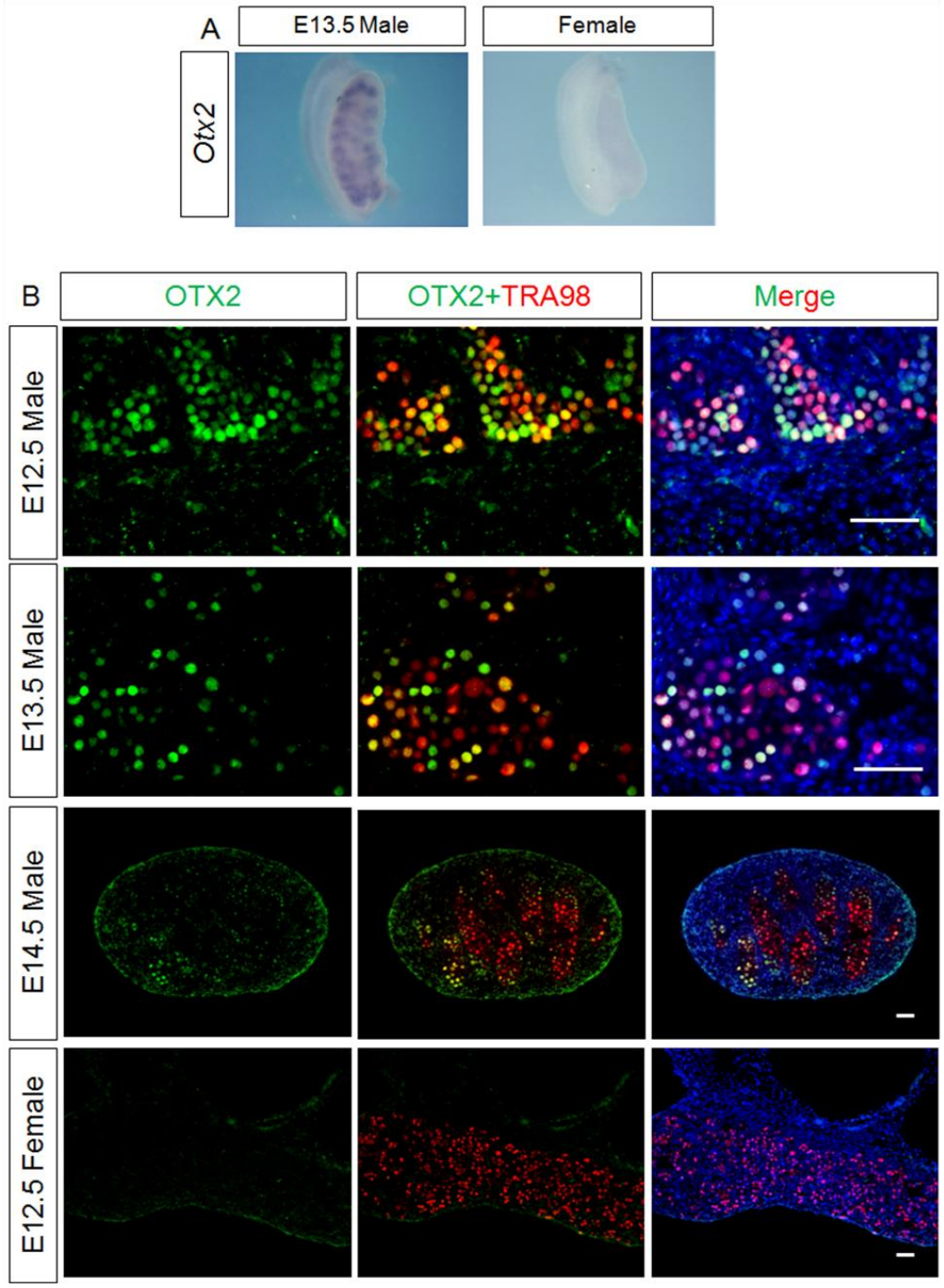


Figure II-2. Germ-cell-specific expression of OTX2 in fetal testes
(A) *In situ hybridization* of *Otx2* probe in the E13.5 testis and ovary. (B) Double immunostaining for OTX2 (green) and the germ cell marker TRA98 (red) in the indicated gonads. Scale bars: 50 μm.

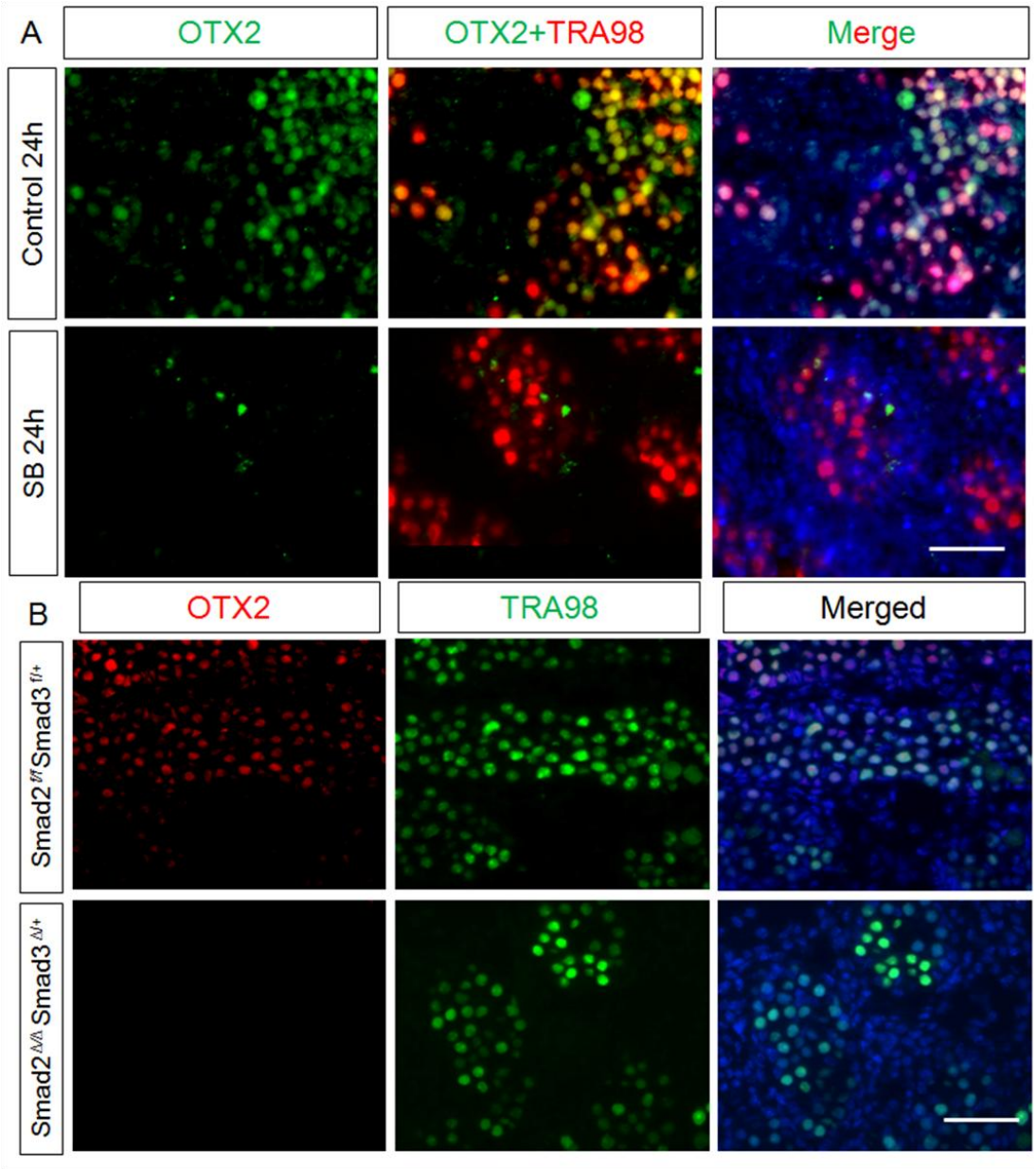


Figure II-3. OTX2 is regulated by nodal/activin signaling

Double immunostaining for OTX2 (green) and the germ cell marker TRA98 (red) in the indicated gonads. (A)Testes from mice at E12.5 were cultured with the SB203580 (40 μM) or DMSO (control vehicle) for 24 h. (B) Testes were harvested from Smad2 mutant mice at E13.5. Scale bars: 50 μm.

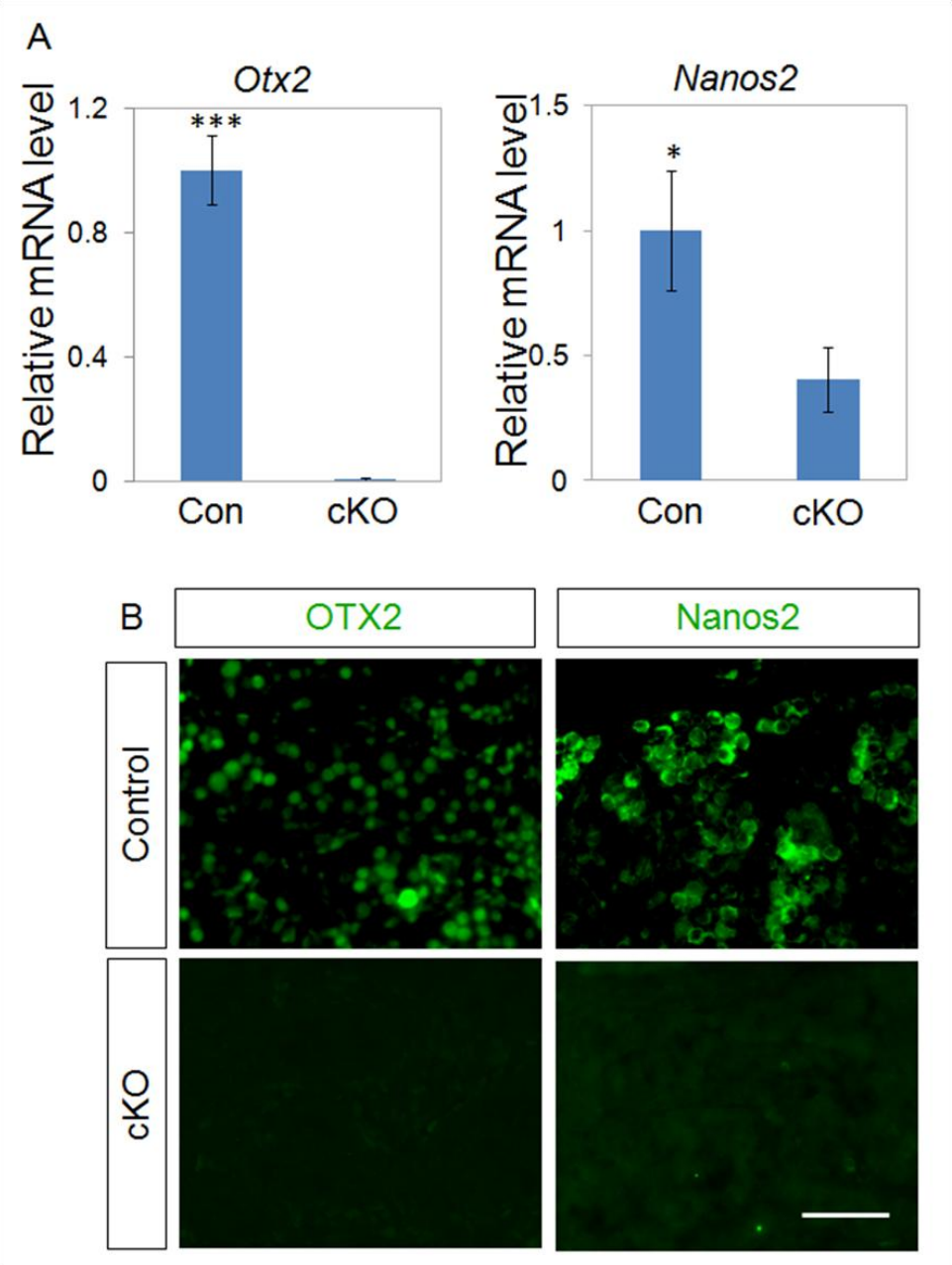


Figure II-4. OTX2 is involved in the induction of Nanos2 expression
(A) RT-qPCR analysis of *Otx2* and *Nanos2* mRNA levels in E13.5 testes of $Otx2^{\triangle/\triangle}$ mice (for mutants, $n = 3$; for controls, $n = 5$). (B) Double immunostaining for OTX2 and TRA98, Nanos2 and TRA98 in E13.5 testes of $Otx2^{\triangle/\triangle}$ mice. Tamoxifen was injected at E10.5 and gonads were harvested at E13.5. Bars in graphs represent mean ±s.e.m. *P < 0.05, ***P < 0.001. Scale bars: 50 μm.

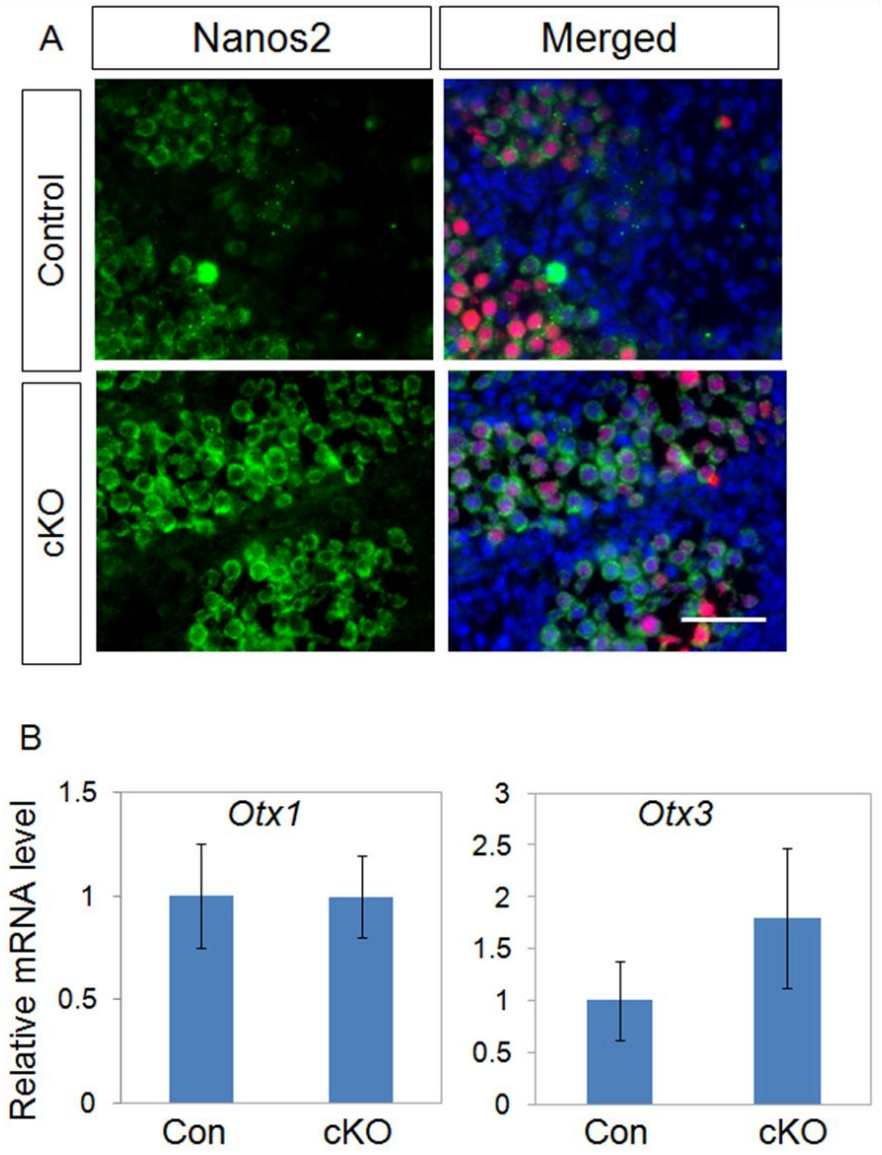


Figure II-5. Redundant function of OTX2 and OTX3

(A) Double immunostaining for Nanos2 and TRA98 in E14.5 testes of *Otx2*$^{\triangle/\triangle}$ mice. (B) RT-qPCR analysis of *Otx1* and *Otx3* mRNA levels in E13.5 testes of *Otx2*$^{\triangle/\triangle}$ mice (for mutants, $n = 3$; for controls, $n = 5$).Tamoxifen was injected at E10.5 and gonads were harvested at either E13.5 or E14.5. Scale bars: 50 μm.

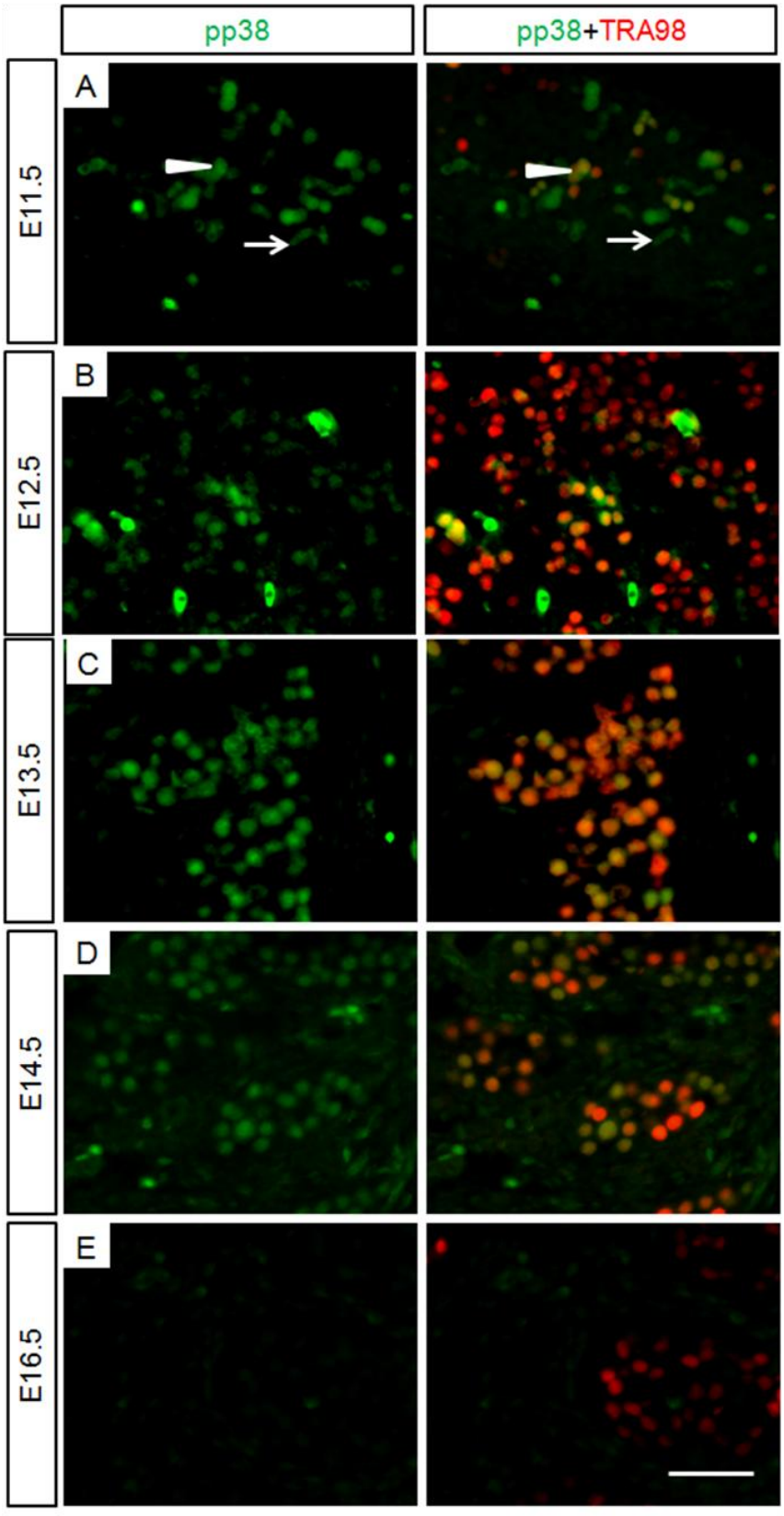

pp38
pp38+TRA98
A
E11.5
B
E12.5
C
E13.5
D
E14.5
E
E16.5

Figure II-6. Expression Patterns of pp38 in Fetal Testes

(A-E) Double immunostaining for pp38 (green) and the germ cell marker TRA98 (red) in the male gonads at E11.5 (A), E12.5 (B), E13.5 (C), E14.5 (D) and E16.5 (E). Pp38 expression was observed in both germ cells (arrowhead) and somatic cells (arrow) at E11.5. At E12.5, the expression of pp38 exclusively showed high expression in germ cells. However, its expression level downregulated from E14.5 and total disappeared at E16.5.

Scale bars = 50 μm.

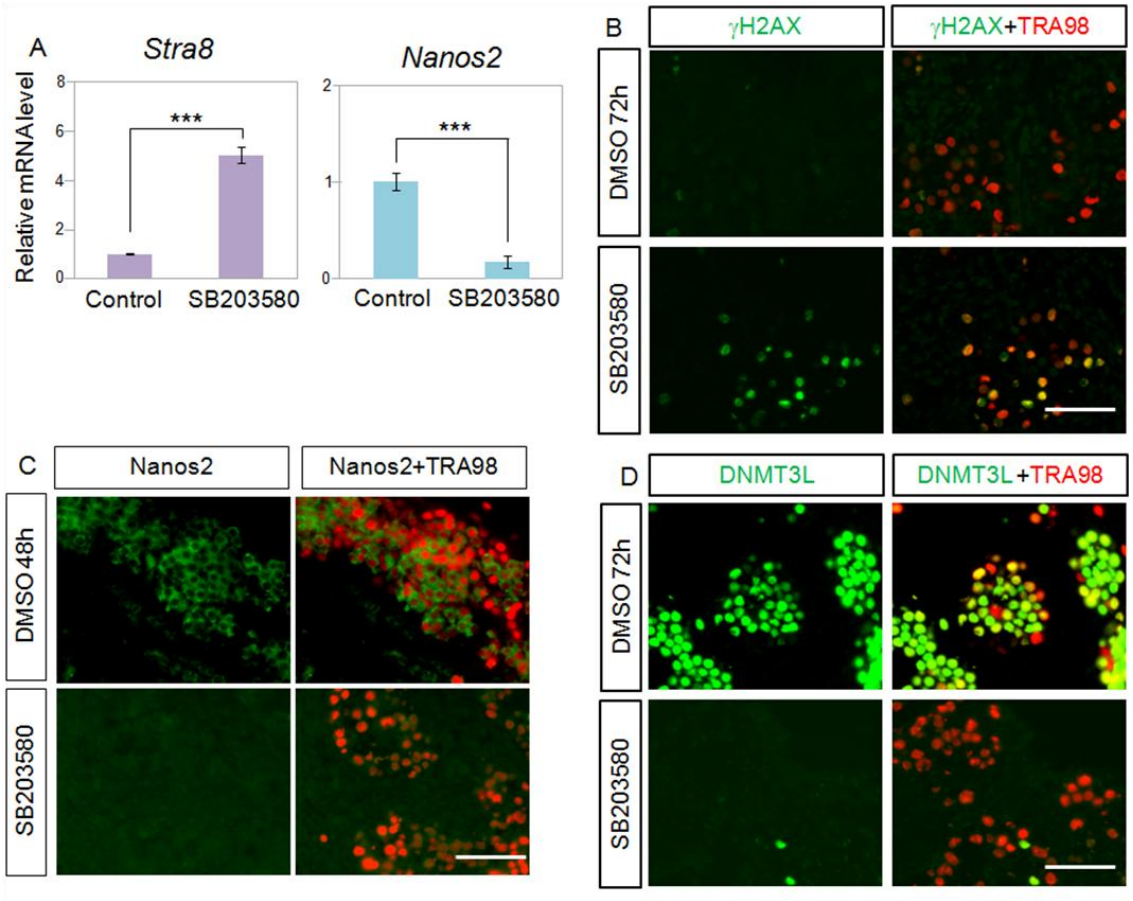


Figure II-7. P38 Signaling is Responsible for Male Sex Differentiation
(A–D) Testes from mice at E12.5 were cultured with the SB203580 (40 μM) or DMSO (control vehicle) for 24 h (A) or 72 h (B-D). (A) qRT-PCR was performed for the analyses of *Nanos2* and *Stra8* (using *Mvh* as the normalization control; n = 3) (B-D) Immunostaining for DNMT3L, NANOS2 and γH2AX after culture. After SB203580 treatment, almost all germ cells entered meiosis, and loss expression of male specific genes. Bars in graphs represent the mean ± SEM. ***P < 0.001. Scale bars = 50 μm.

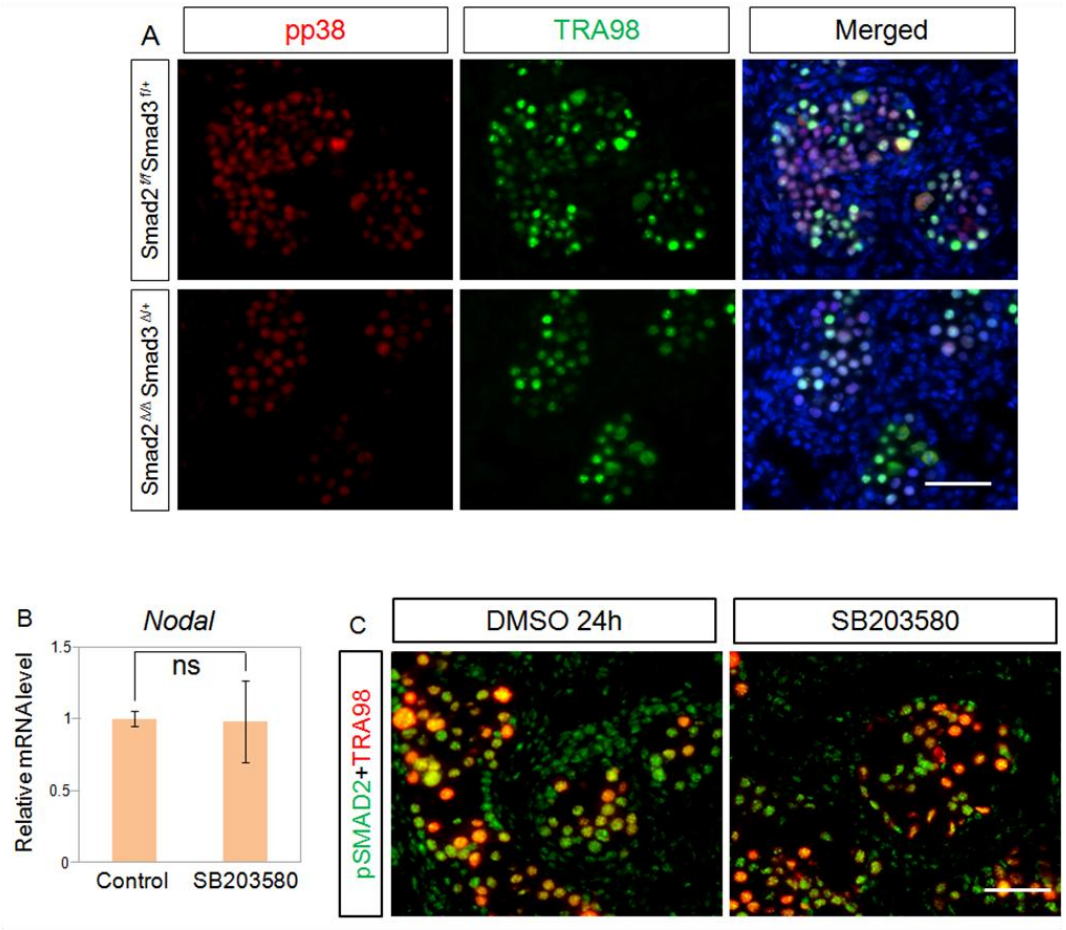


Figure II-8 P38 signaling works independent of nodal/activin-A

(A) Immunochemical analysis of pp38 (red) and TRA98 (green) in the E13.5 *Smad2* mutant testes. (B-C) Male germ cells were isolated from E12.5 testes and cultured with SB203580 or DMSO for 24 h; Expression of Nodal was investigated using qRT-PCR (B). Immunochemical analysis of pSmad2 (green) and TRA98 (red) in the indicated testes (C). Scale bars = 50 μm. Bars in graphs represent the mean ± SEM.

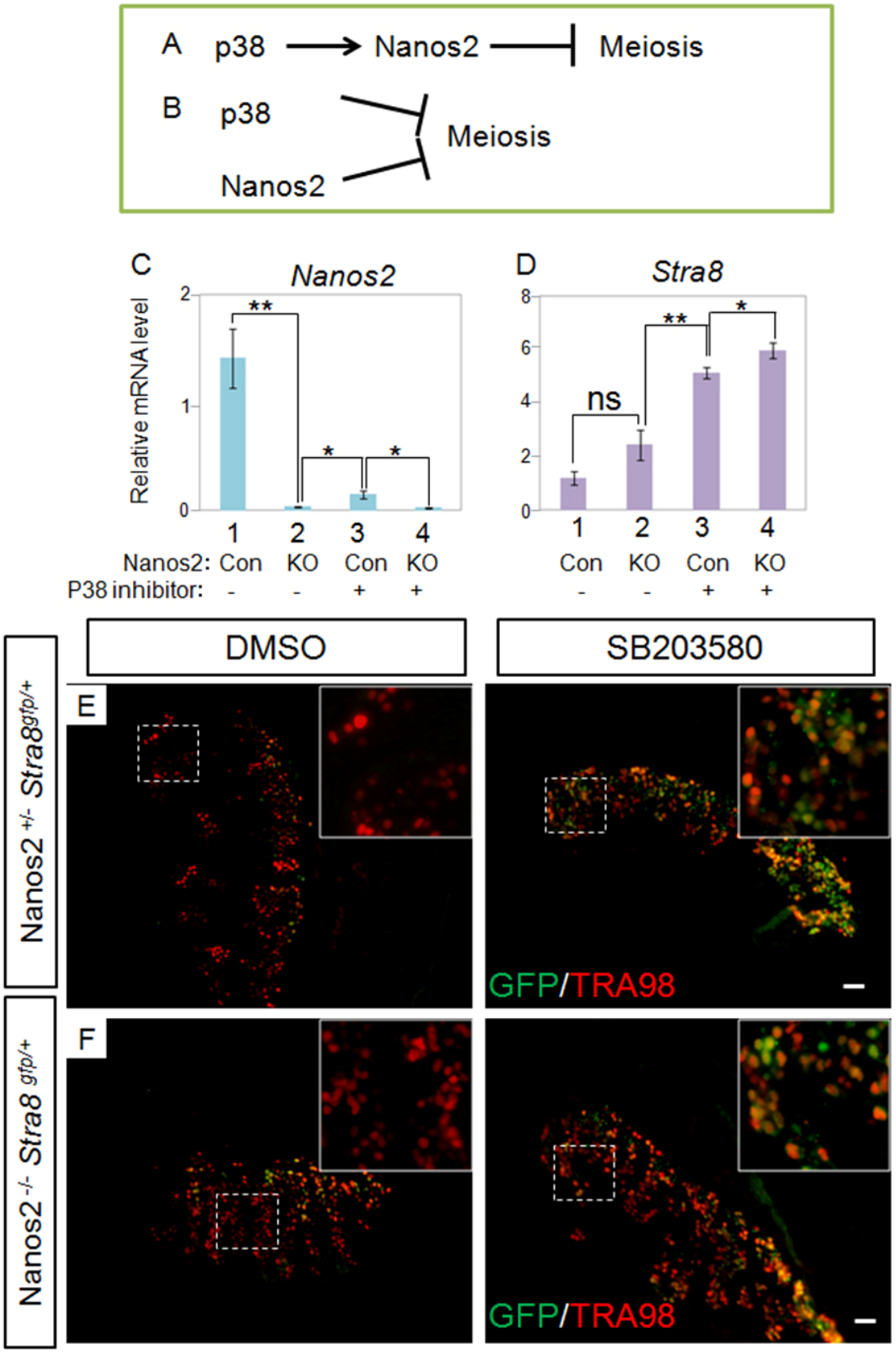

A
p38
Nanos2
Meiosis
B
p38
Nanos2
Meiosis
C
Nanos2
Relative mRNA level
2
1
0
**
*
*
1
2
3
4
Nanos2: Con KO Con KO
P38 inhibitor: - - + +
D
Stra8
8
6
4
2
0
ns
**
*
1
2
3
4
Con KO Con KO
- - + +
DMSO
SB203580
Nanos2 +/- Stra8 gfp/+
E
GFP/TRA98
Nanos2 -/- Stra8 gfp/+
F
GFP/TRA98

Figure II-9. NANOS2-independent Suppression of Meiosis by P38 Signaling

(A-B) Two possible models for the mechanism of meiosis suppression. Testes were harvested at E12.5 and cultured for 24 (C and D) or 48 hours (E and F). (C-D) qRT-PCR analyses of *Nanos2* and *Stra8* (using Mvh as the normalization control; n = 3) in the *Nanos2* knockout testes or control testes (*Nanos2*$^{-/-}$ or *Nanos2*$^{+/-}$) treated with SB203580 (40 μm). (E-F) Immunostaining for the GFP in the indicated testes. GFP represents expression of *Stra8*. After SB203580 treatments, *Nanos2*$^{-/-}$*Stra8*$^{egfp/+}$ testes and *Nanos2*$^{+/-}$*Stra8*$^{egfp/+}$ showed same level of GFP expression, indicating that SB203580 induces *Stra8* expression independent of Nanos2. Bars in graphs represent the mean ± SEM. *P < 0.05, **P < 0.01. ns, not significant. Scale bars = 50 μm.

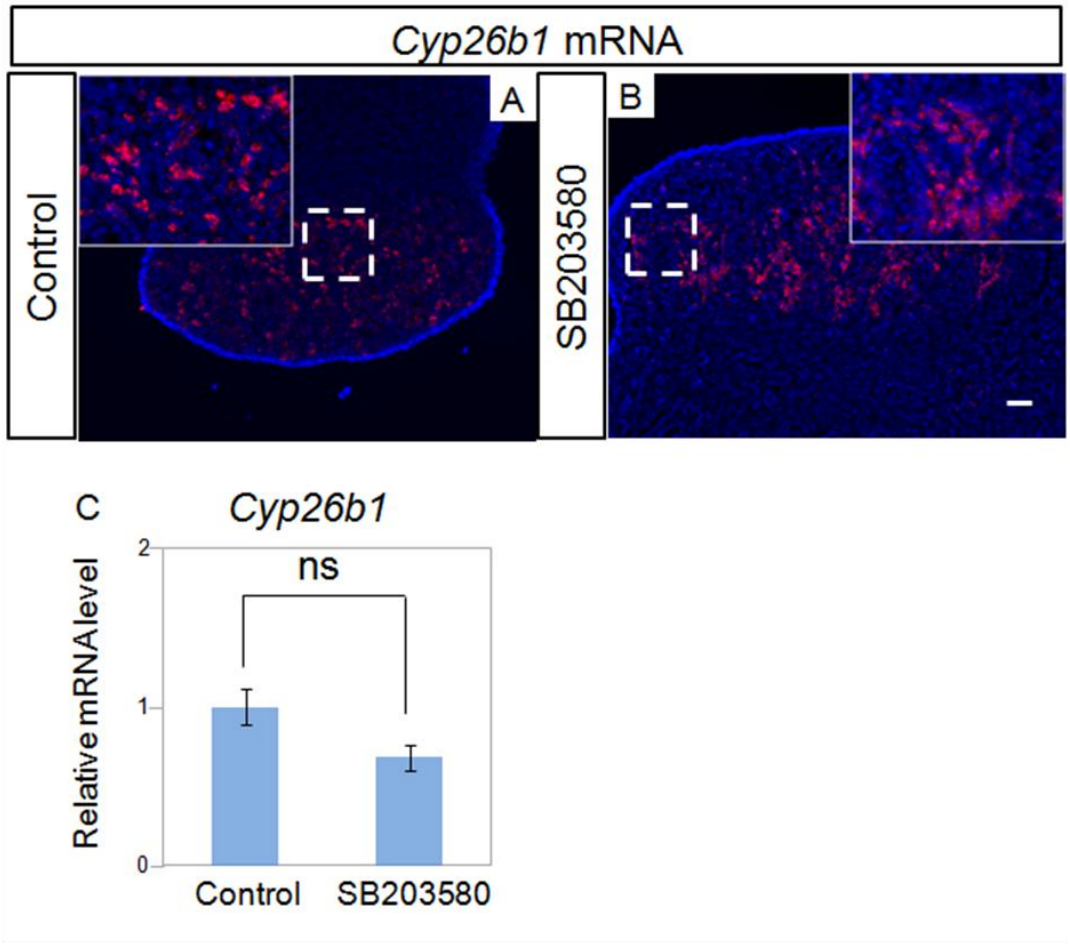


Figure II-10. P38 Signaling works independent of CYP26B1
(A, B) Fetal testes were isolated from E12.5 mice and cultured with SB203580 (40 μm) or DMSO for 24 h; Expression of Cyp26b1 was investigated using *in situ* hybridization (A and B) and qRT-PCR (C). Bars in graphs represent the mean ± SEM. ns, not significant. Scale bars = 50 μm.

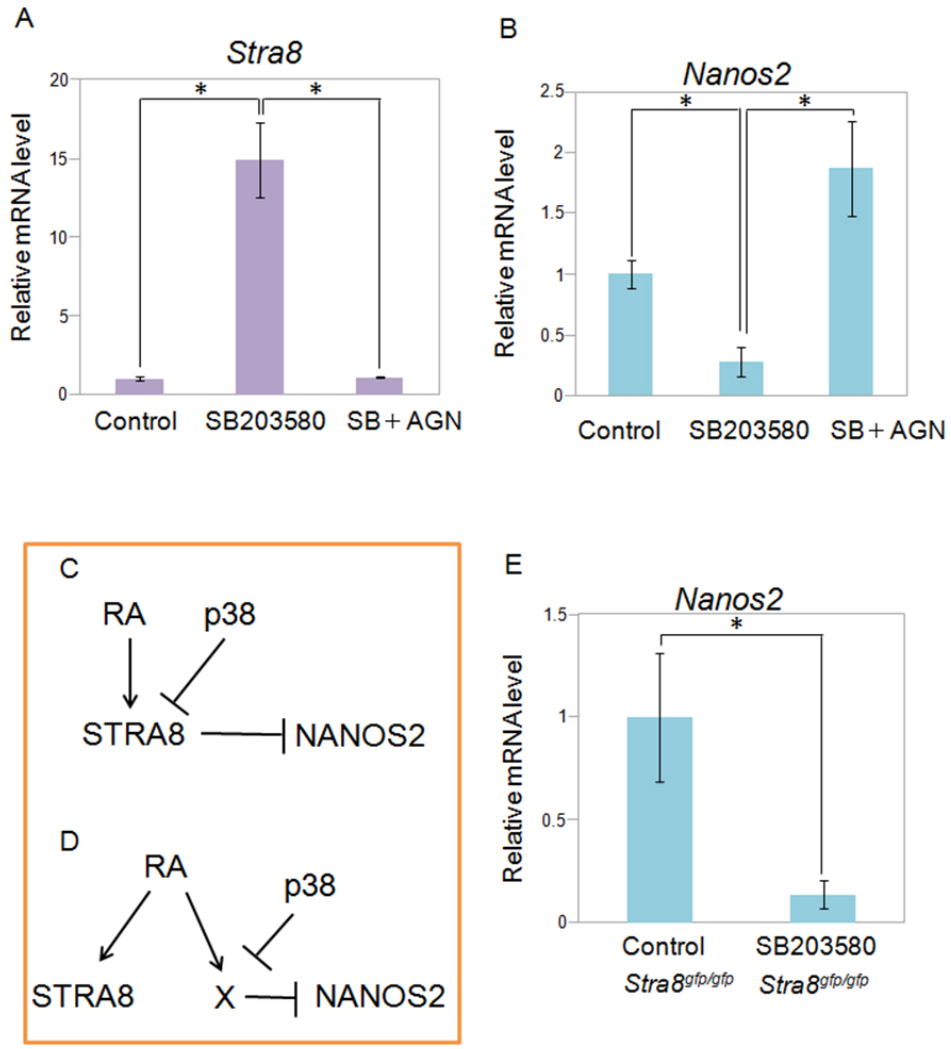
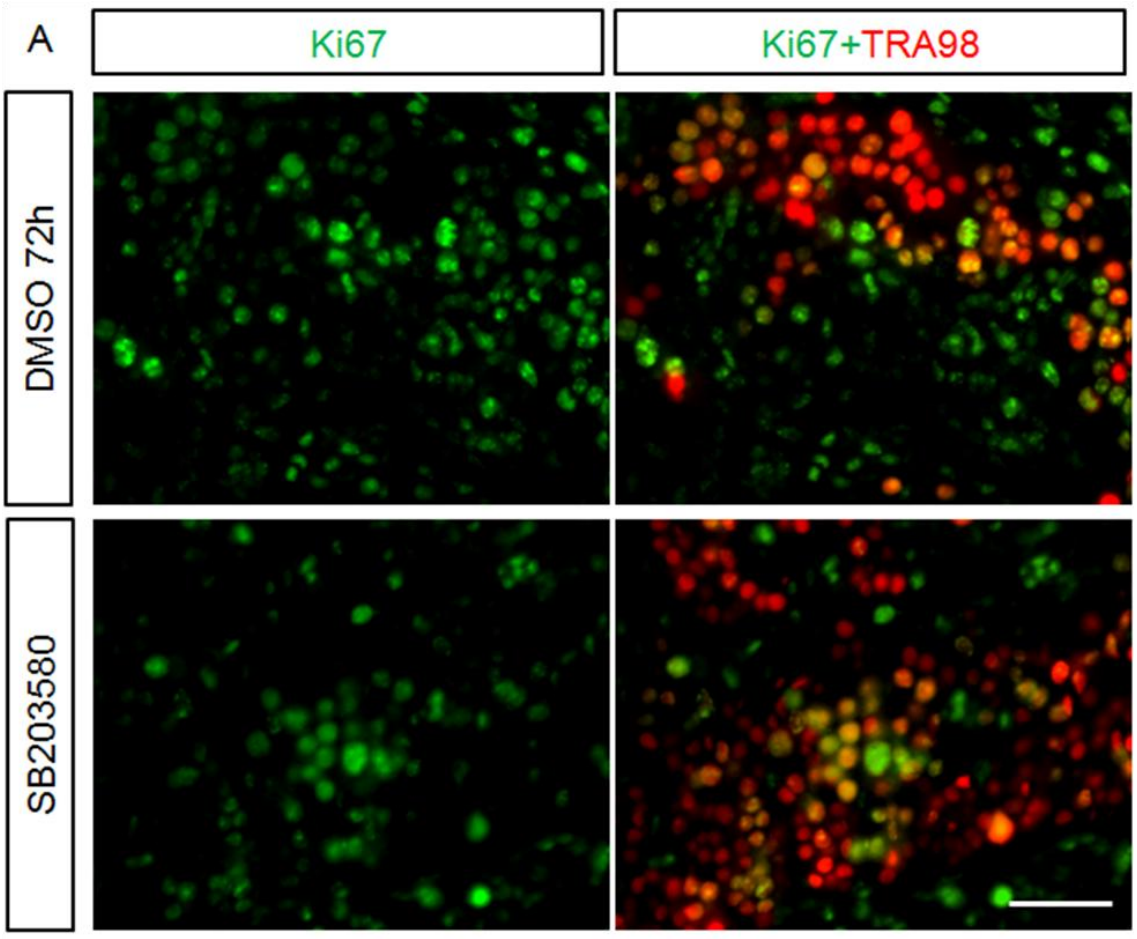


Figure II-11. Antagonistic Role of P38 Signaling and RA

(A-B) E11.5 testes were cultured with p38 inhibitor SB203580 or together with RAR inhibitor AGN (5 μM) for 48 h and the expression levels of *Stra8* and *Nanos2* were examined using qRT-PCR (n = 3; using Mvh as the normalization control). (C-D) Two possible models for the relationship between p38 signaling and RA. P38 signaling permits Nanos2 expression through inhibition of Stra8 directly (C) or independently (D). (E) Stra8-null testes were isolated at E12.5 and cultured with or without SB203580 for 24 hours. Expression of Nanos2 was investigated using qRT-PCR. Bars in graphs represent the mean ± SEM. *$P < 0.05$.


A
Ki67
Ki67+TRA98
DMSO 72h
SB203580

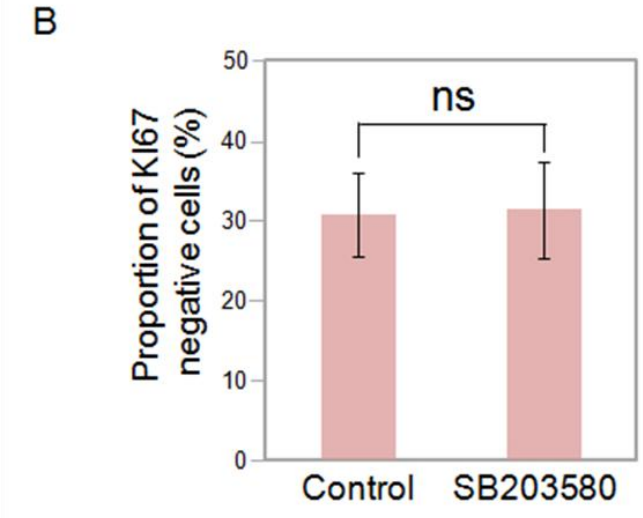

B
ns
Proportion of KI67 negative cells (%)
50
40
30
20
10
0
Control
SB203580

Figure II-12. P38 Signaling is not Involved in Mitotic Arrest of Male Germ Cell

(A-B) E12.5 testes were cultured with p38 inhibitor SB203580 (40 μM) or DMSO for 48 h and immunostaining with an anti-Ki67 antibody (green) and the germ cell marker TRA98 (red). Ki67 positive germ cells are counted (n = 3). Bars in graphs represent the mean ± SEM. ns, not significant. Scale bars = 50 μm.

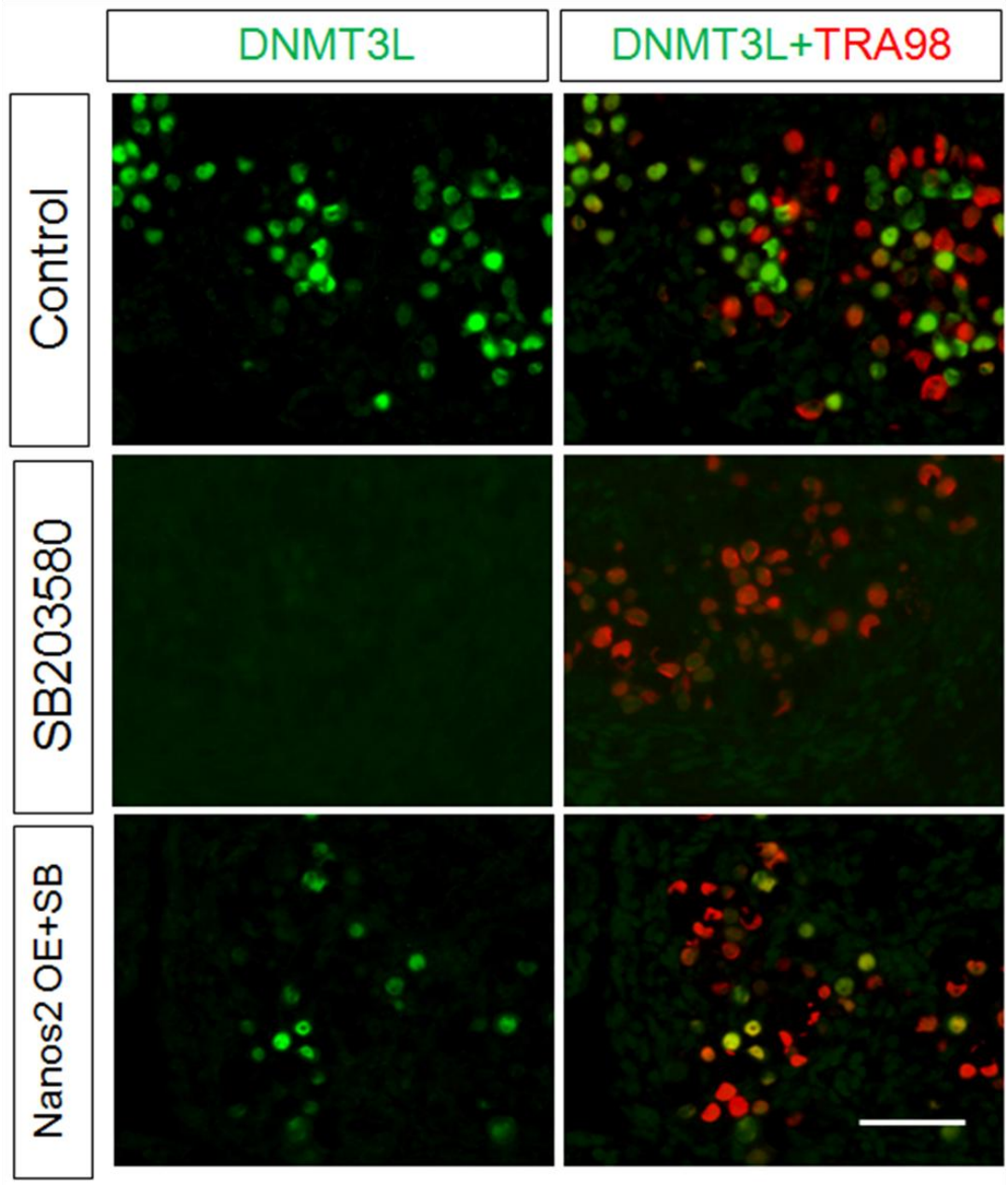


Figure II-13. P38 signaling Acts Upstream of Nanos2

The *CAG-loxP-CAT-lox-Flag-tagged-Nanos2 Pou5f1/Oct4-CreERT2* transgenic female mice were crossed with male mice with same genotype. Tamoxifen was injected at E10.5 to induce Nanos2 expression. E12.5 testes were harvested and cultured with or without p38 inhibitor for 72 hours. Expression of DNMT3L was investigated using anti-DNMT3L antibody. Scale bars = 50 μm.

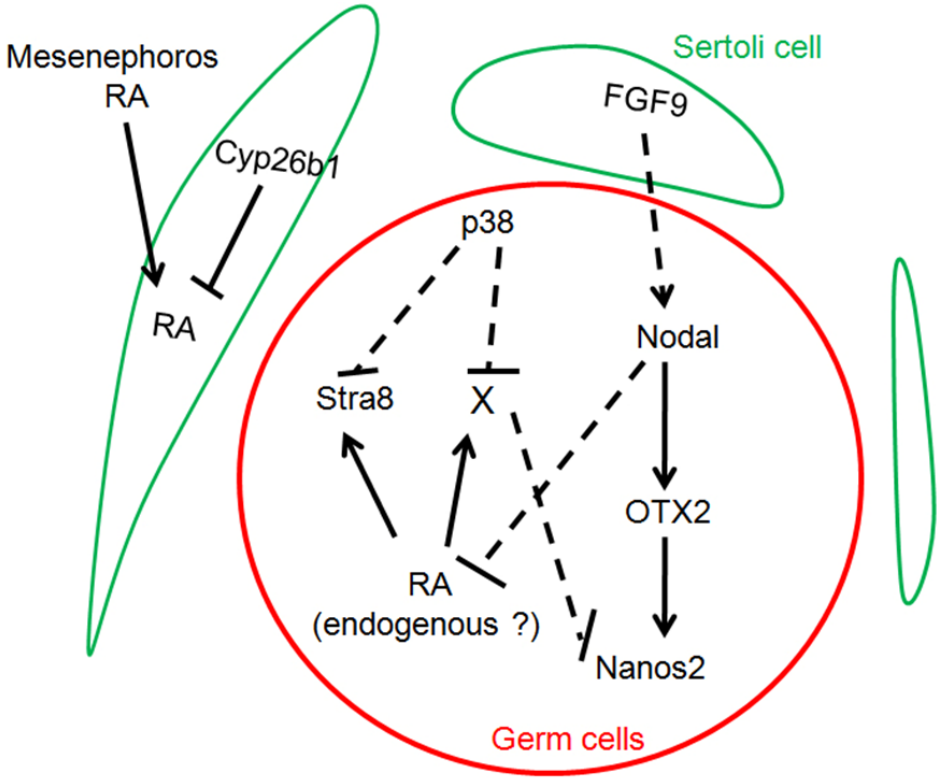


Figure II-14. RA and P38 Work Antagonistically to Determine Male Germ Cell Fate

FGF9 secreted from Sertoli cells is required for the activation of *Nodal* expression in germ cells directly or indirectly. Downstream of nodal signaling, OTX2 acts as an inducer to trigger *Nanos2* expression and meanwhile cooperates with p38 signaling that allow *Nanos2* expression through antagonize RA signaling. RA that might synthesized by germ cells impedes male differentiation through induction of meiosis and other unknown factors

Chapter III

BMP signaling regulates XX germ cell fate independently of RA

INTRODUCTION

R-spondin1 (RSPO1) is a female sex determinant in human and the disruption of this gene leads to complete female-to-male sex reversal (Parma et al., 2006). Mechanistically, R-spondin proteins enhance Wnt signaling by clearing a negative regulator of Wnt signaling (Hao et al., 2012). In mice, the regulation of Wnt signaling by Rspo1 is also critical for ovarian development, however, unlike the case in human, loss of either Rspo1 or Wnt4 results in only partial female-to-male sex reversal (Vainio et al., 1999; Bernard and Harley, 2007; Ottolenghi et al., 2007; Chassot et al., 2008). Both Rspo1 and Wnt4 are expressed and appear to act in somatic cells, because stabilization of β-catenin, the downstream gene of Wnt signaling in somatic cells leads to partial male-to-female sex reversal (Maatouk et al., 2008). Another factor involved in female sex determination is the forkhead transcriptional regulator FOXL2 which suppresses male pathway in somatic cells even in the adult mice (Uhlenhaut et al., 2009). Deletion of *Foxl2* and *Wnt4* in mice causes female-to-male sex reversal, including germ cells (Ottolenghi et al., 2007). These factors provide appropriate environment for oogenesis of XX germ cells.

Once XX germ cells migrate into fetal ovaries, they enter prophase I of meiosis upon stimulation by retinoic acid (RA) from E13.5 and arrest in the diplotene stage until birth. Downstream of RA, the helix–loop–helix (HLH) protein STRA8 (stimulated by retinoic acid gene 8) is responsible for the initiation of meiosis (Baltus et al., 2006; Bowles et al., 2006; Koubova et al., 2006; MacLean et al., 2007). Simultaneously, these germ cells commit to oogenesis and start to express oocyte-specific genes, such as growth and differentiation factor 9 (Gdf9), bone morphogenetic protein 15 (Bmp15) and zona pellucid gene 1-3 (Zp1-3), which stimulates follicular development (Dong et al.,

1996; Elvin et al., 2000; Dean, 2002). Two transcription factors: Figla (Joshi et al., 2007) and Nobox (Rajkovic et al., 2004) play critical roles to regulate oogenesis. FIGLA is a basic helix-loop-helix transcription factor required for the expression of *Zp1-3* (Joshi et al., 2007). NOBOX is a homeobox-contining factor essential for the upregulation of *Gdf9*, *Bmp15* and *Pou5f1* (Rajkovic et al., 2004).

The deletion of *Wnt4*, *Foxl2* or *Rspo1* results in the upregulation of Fgf9/Sox9 signaling pathway, which is specially activated in male somatic cells, suggesting an antagonistic interaction between these factors (Ottolenghi et al., 2005; Kim et al., 2006; Ottolenghi et al., 2007). Fgf9/Sox9 signaling is responsible for the activation of *Cyp26b1*, a gene encoding an enzyme that degrades RA. As a result, retinoic acid (RA) is degraded and oogenesis is disrupted in the *Wnt4*, *Foxl2* or *Rspo1* mutant ovaries. Hence, RA tips the balance in sex determination of mice germ cells. However, it is unclear whether RA is the sufficient factor to determine XX germ cell fate and, if not, what factor regulates XX germ cells fate.

In this chapter, I tried to uncover the role of BMP signaling during sex determination of XX germ cells. BMPs are a major subgroup of transforming growth factor (TGF)-β superfamily. After binding with its receptors on the cell membrane, BMPs stimulate the phosphorylation of the Smad intracellular effector proteins including BMP receptor-activated R-Smads (consist of Smad1, 5 and 8) and the common Smad4. Smad4 heterotrimerizes with two phosphorylated R-Smads and the heterotrimer subsequently translocates to nucleus, binds with DNA and regulates gene expression. The expression of *Bmp2* is regulated by Wnt signaling in somatic cells and shows a female specific expression (Yao et al., 2004).

Here, I identified BMP signaling as a key regulator of sex determination for

XX germ cells. I analyzed XX germ cell fate in the absence of BMP signaling using either *Smad4* conditional knockout mice or specific inhibitor of BMP signaling. Through comparative analyses of the functions between BMP signaling and RA signaling, I conclude that BMP signaling determinates XX germ cell fate independently of RA.

RESULTS

BMP signaling is activated in fetal ovaries

To investigate whether BMP signaling is activated in fetal ovaries, I examined the expression of several BMP receptors in germ cells and somatic cells, respectively. As shown in Figure III-1A, Bmp receptors are ubiquitously expressed in both germ cells and somatic cells of either fetal testes or ovaries. BMP ligands bind with their receptors, activating them to phosphorylate SMAD1/5/8, the intracellular effector proteins, which initiate the transcription of their target genes. Examination of the localization of pSMAD1/5/8 signals indicates that BMP signaling is activated in both germ cells and somatic cells (Figure III-1B).

Loss of BMP signaling caused failure of DSBs

To understand the role of BMP signaling during ovarian development, I conditionally deleted Smad4, a co-activator of both nodal/activin and BMP signaling. Considering that nodal/activin signaling is not activated at this stage in ovaries (Souquet et al., 2012; Spiller et al., 2012a) (Chapter I), the phenotype observed in *Smad4*-deficient ovaries is imputed to the loss of BMP signaling. By injecting tamoxifen at E10.5 and E11.5 in *Smad4*$^{f/f}$ */Rosa26-ERT2Cre* mice, I conditionally deleted *Smad4*. Upon Cre-mediated

recombination, mRNA level of *Smad4* in mutant ovaries was nearly half of that in the control ovaries, implying successful deletion of *Smad4* (Figure III-2A). Initiation of meiosis is one of the most important events during ovarian development. Exposed to RA signaling, XX germ cells undergo premeiotic replication, meiotic chromosome condensation, cohesion, synapsis and recombination. To elucidate the effect of BMP signaling on meiosis, I examined whether DNA double-strand breaks (DSBs), which trigger meiotic recombination, occurred in the *Smad4*-deficient ovaries. After DSBs induction, the histon H2A variant H2AX is phosphorylated to generate γH2AX that is responsible for recruitment of DNA-repair machinery (Hunter et al., 2001; Mahadevaiah et al., 2001). Because ubiquitous loss of *Smad4* led to embryonic lethality at E15.5 when most of germ cells complete DSBs in normal ovaries, the mutant ovaries were harvested from E13.5 and cultured for two days. I found that the proportion of γH2AX positive germ cells was significantly decreased in the *Smad4*-deficient ovaries when compared with the control ovaries (Figure III-2B and 2D). Consistent with this result, the expression level of *Spo11* and *Dmc1*, which encodes a topoisomerase required for the formation and the repair of meiotic DSBs, respectively, was also downregulated (Figure III-2C, 2E and 2F). Moreover, similar results were also observed upon treatment of E11.5 ovaries with BMP signaling inhibitor LDN 193189 for 48h (Figure III-3). These results indicate that Smad4-mediated BMP signaling is required for XX germ cells to induce DSBs.

BMP is required for DSBs independently of RA

Because RA is essential for meiosis, I then asked whether BMP signaling regulated meiosis through a RA dependent or independent manner. In fetal testes, RA is degraded

in the somatic cells by an enzyme encoded by *Cyp26b1* (Bowles et al., 2006; Koubova et al., 2006; MacLean et al., 2007), while in ovaries the expression of *Cyp26b1* is indirectly suppressed by *Wnt4* and *Foxl2*, the master genes of female sex determination (Vainio et al., 1999; Uda et al., 2004; Ottolenghi et al., 2005; Ottolenghi et al., 2007; Uhlenhaut et al., 2009). I investigated whether the ectopic expression of *Cypb26b1* in somatic cells was responsible for the meiotic failure after downregulation of BMP signaling in the mutant ovaries. I found that the expression level of neither *Cyp26b1*, nor, *Fgf9* and *Sox9*, the upstream genes was affected by the reduction of BMP signaling at E13.5 when they were usually up-regulated in testes (Figure III-4A). In addition, neither *Wnt4*/*Foxl2* nor their downstream genes (*Axin2*, *Lef1* and *Fst*) expression was altered (Figure III-4A). Notably, the expression level of *Stra8*, which is directly induced by RA, SYCP3, a component of the synaptonemal complex, and DAZL, an intrinsic factor that enables germ cells to initiate meiosis, was unexpectedly unchanged (Figure III-4A-4C), suggesting that meiotic initiation normally occurred even in the absence of BMP signaling. Interestingly, even mRNA level of *Stra8* was unchanged at E13.5, I observed the persistence of STRA8 protein in the *Smad4*-deficient ovaries at E14.5 when its expression level declined in the control ovaries, implying a negative-feedback loop exists to regulate *Stra8* expression after meiotic initiation (Figure III-4D).

To further confirm my conclusion that BMP signaling works independently of RA, I asked that whether addition of exogenous RA promotes meiotic progression in mutant ovaries. *Smad4* mutant ovaries were harvested and cultured from E12.5 in the presence of 1 μM RA for 48 hours. Results of immunostaining with γH2AX suggested that the exogenous RA could not induce meiotic DSBs in *Smad4*-deficient ovaries (Figure III-5). Taken together, my results indicate that BMP signaling promotes meiosis

independently of RA.

Initiation of sex reversal of XX germ cells in *Smad4* mutant ovaries

After sex determination, germ cells in fetal testes and ovaries commit to spermatogenesis and oogenesis, respectively. From E13.5, XX germ cells gradually lose their PGC property by downregulating pluripotency genes, enter meiosis and initiate gene expression involving in follicular development. On the other hand, male germ cells maintain their pluripotency until at least E14.5, when NANOS2 that expression is under the control of nodal/activin signaling becomes active to promote male germ cell fate (Chapter I). Germ cells in the *Smad4*-mutant ovaries failed to induce DSBs, prompting me to investigate a possibility that these germ cells enter the male pathway.

I expected that these germ cells in the *Smad4*-mutant ovaries maintained their pluripotency because of the failure of meiosis. Indeed, I observed the higher expression levels of several pluripotency related genes: *Sox2*, *Nanog*, *Utf1* and *Oct4* in the *Smad4*-deficient ovaries (Figure III-6A). In the control ovaries, no translational products of these genes can be detected at E14.5, however in the mutant ovaries, these markers localized in a considerable number of germ cells (Figure III-6B and 6C). Results of double staining with pluripotency marker SOX2 and meiotic marker γH2AX indicated that these two markers were expressed in a two distinct cell populations each other (Figure III-6D). Considering the efficiency of Cre-mediated recombination, I evaluated that germ cells failed to delete *Smad4* gene responded to BMP signaling and induced DSBs, otherwise they kept pluripotency.

In fetal testes, maintenance of pluripotency is regulated by nodal signaling (Spiller et al., 2012b). Indeed, I observed upregulation of genes involved in the nodal

signaling pathway such as *Nodal, Lefty1, Lefty2* and *Otx2* in *Smad4*-deficient ovaries (Figure III-7A and 7B). My previous studies proved that the nodal signaling induces *Nanos2* expression. Consistent with these results, the expression level of *Nanos2* and another male germ cell marker: *Tdrd5* was augmented (Yabuta et al., 2011) (Figure III-6C). In contrast, two factors which are responsible for oogenesis: *Figla* and *Nobox* were not activated in *Smad4*-deficient ovaries (Figure III-6D and 6E). These results implied the conversion of XX germ cells into spermatogenetic pathway in the absence of BMP signaling.

Sex determination of XX germ cells is independent of meiosis

Two possible mechanisms can be considered to explain the phenotype observed in the *Smad4* mutant ovaries: (i) the ectopic upregulation of nodal signaling in *Smad4*-deficient ovaries led to meiotic failure; (ii) the obstruction of meiosis altered sexual fate of XX germ, which activated nodal signaling. To validate these two possibilities, I asked whether the disruption of meiosis was sufficient to reverse the sexual fate of XX germ cells. To suppress meiosis, I tried two methods: organ culture with inhibitors and genetic deletion of *Stra8* that is necessary for meiotic initiation. After the treatment of E11.5 ovaries with either BMP signaling inhibitor or RAR antagonist for 48 hours, I examined the expression level of pluripotency markers. The suppression of BMP but not RA signaling gave rise to the persistent expression of puripotency markers by XX germ cells (Figure III-8) indicating meiotic disruption did not contribute to sexual reversal of XX germ cells. Moreover, comparison of gene expression profiles between $Stra8^{-/-}$ and $Stra8^{+/-}$ (or $Stra8^{+/+}$) XX germ cells suggested that $Stra8^{-/-}$ germ cells normally expressed female-specific genes without any signs of

spermatogenesis (Figure III-8B). These results indicate that sex reversal of XX germ cells in *Smad4*-deficient ovaries is imputed to the reduction of BMP signaling but not meiotic failure. To further prove that BMP signaling promotes XX germ cell fate independently of meiosis, I isolated *Smad4* mutant ovaries from E12.5 and cultured for 48 h with the exogenous RA. I observed that SOX2 expression was persisted in many germ cells of mutant ovaries regardless of the presence or absence of the exogenous RA (Figure III-9). I conclude that BMP signaling regulates XX germ cell fate independently of RA.

Complete sex reversal of XX germ cells in the ovaries via simultaneous suppression of RA and BMP signalings

In *Smad4*-deficient ovaries, even though several male-specific genes were induced in XX germ cells, these germ cells could not embark on spermatogenesis, because meiosis has already been initiated by RA (Figure III-4). Subsequently, many germ cells were cleared by apoptosis from E14.5 and all of germ cells were lost when mutant ovaries from E14.5 embryos were cultured for three days (Figure III-10).

Thus, I hypothesized that if RA signaling was suppressed in *Smad4*-null ovaries before meiotic initiation, XX germ cells would enter spermatogenesis by the up-regulation of nodal signaling. To prove this idea, I examined the expression of Nanos2 expression after culture of E12.5 ovaries from *Smad4* mutant mice with RA receptor antagonist AGN 193109 for 48 hours. As expected, the expression level of *Nanos2* was dramatically increased (Figure III-11A and B). Notably, I also detected the expression of DNMT3L, which is involved in genomic imprinting (an important process of male sexual differentiation) and is only expressed in male germ cells from E14.5 to

E18.5 (Bourc'his et al., 2001; Bourc'his and Bestor, 2004; Sakai et al., 2004) after 72 hours of cultivation (Figure III-11C). Most importantly, the commitment to spermatogenesis of these germ cells occurred independently of FGF9/Sox9 signaling pathway, because the adoption of spermatogenesis after cultivation was not accompanied by the upregulation of these factors (Figure III-13). These results indicate that simultaneous suppression of BMP and RA signaling resulted in complete sex reversal of XX germ cells.

DISCUSSION

In this chapter, I proved the significant role of BMP signaling in sex determination of XX germ cells. BMP functions independently of RA to regulate meiotic progression and the sexual fate of XX germ cells.

BMP is essential for DSBs independently of RA

The question whether an ovarian meiosis-inducer and/or a testicular meiosis-inhibitor exist has been debated for a long time. RA has been proved as the inducer of meiosis (Bowles et al., 2006), however, there is evidence suggesting that the testicular meiosis-inhibitor is also present. Germ cells in the *Cyp26b1*-null testes are exposed to high level of RA and enter meiosis, but differ from XX germ cells in wild type ovaries in terms of meiotic progression level. FGF9 is considered as the meiosis-inhibitor, because the expression level of *Stra8* at E12.5 is higher in *Cyp26b1*$^{-/-}$ *Fgf9*$^{+/-}$ male germ cells than in *Cyp26b1*$^{-/-}$ *Fgf9*$^{+/+}$ male germ cells (Bowles et al., 2010). In my study, I showed that, even though meiotic initiation was induced, DSBs were not observed in many XX germ cells in the absence of BMP signaling, suggesting a meiosis-inhibitor

which is suppressed by BMP signaling in normal ovaries was ectopically induced in the absence of BMP signaling. Importantly, I did not observed any changes of *Fgf9* expression level, denying the possibility that FGF9 is the meiosis inhibitor. In contrast, ectopic activation of nodal signaling was observed in mutant ovaries. Because nodal works downstream of Fgf9 signaling, I concluded that nodal is a meiosis-inhibitor in the testes and this inhibitor is inactivated by BMP signaling in ovaries.

Meanwhile, the expression of genes involved in pluripotency was maintained in those germ cells which failed to induce DSBs (Figure III-6D). The disruption of meiosis did not give rise to the persistence of these genes implying that downregulation of pluripotency marker is one checkpoint for the induction of DSBs. In XX germ cells, the expression of these markers (Nanog, Oct4, Utf1 and Sox2) was downregulated soon after germ cells enter meiosis. In XY germ cells, however, the expression was maintained at least to E14.5. It is possible that the maintenance of these markers is essential for suppression of ectopic meiosis in fetal testes. These factors might transcriptionally repress meiosis-related gene expression by directly binding with their promoter or enhancer region. Importantly, previous study suggested that these pluripotency genes were under the control of nodal signaling in fetal testes (Spiller et al., 2012a). I propose here a model that the nodal signaling acts as a meiosis-inhibitor to inactivate meiosis-related genes through the maintenance of pluripotency genes. To fully prove this model, I need to show that the knockdown of these pluripotency markers in the *Smad4* mutant ovaries promotes meiotic progression. In addition, it is interesting to ask whether BMP signaling is required for meiotic progression in adult testes. Several studies indicate that BMP signaling also plays important role in spermatogenesis (Zhao et al., 1996; Pellegrini et al., 2003), implying a common

mechanism might exist during gametogenesis.

Cooperation of BMP and RA signaling in fetal ovaries is sufficient to determine germ cell fate

Mammalian sex determination in germ cells depends on the signalings that they receive from surrounding environment. Until now, several genes have been identified as the master genes of female sex determination such as *Rspo1*, *Wnt4* and *Foxl2*, and the deletion of these genes leads to the different levels of female-to-male sex reversal (Vainio et al., 1999; Kim et al., 2006; Ottolenghi et al., 2007; Chassot et al., 2008). However, in these mutant mice, the sex reversal was accompanied by the reduction of RA level caused by the ectopic upregulation of *Cyp26b1*. In these cases, it is difficult to evaluate the contribution of RA during sex reversal. In *Smad4*-deficient ovaries, sex reversal of XX germ cells was observed independently of the reduction of RA level suggesting these processes can be separable. These results indicate that BMP signaling but not RA signaling is essential for sex determination of XX germ cells. In other words, a female pathway is considered as BMP signaling-induced oogenesis and separable from RA-dependent meiosis decision.

BMP might directly act on germ cells or indirectly induce a secondary signal from somatic cells to regulate germ cell fate. In my preliminary study, sex reversal of germ cells in *Smad4*$^{f/f}$*/Rosa26-ERT2Cre* ovaries were not recapitulated when *Smad4* was deleted specifically from somatic cells in *Smad4*$^{f/f}$*/WT1* (Wilms tumor 1 homolog) *–ERT2Cre*, implying a direct function of BMP in germ cells (data not shown). More evidence will be gained by analysis of mutant mice where *Smad4* would be exclusively obliterated from germ cells.

Even though BMP signaling is the key regulator of XX germ cell fate, the suppression of BMP signaling alone was not sufficient to completely reverse the sexual fate of XX germ cells. This phenomenon might be ascribed to two possible reasons. First, in the *Smad4* mutant ovaries, meiotic initiation of XX germ cells via RA signaling (Figure III-4), suppressed progression of spermatogenetic events. Second, RA disrupts male pathway by inducing unknown factor (which is inhibited by p38 signaling pathway in testes as I describe in Chapter II). In either case, simultaneously suppression of BMP and RA signalings from E12.5 results in complete sex reversal of XX germ cells as I showed in Figure III-11. Notably, the upregulation of Fgf9/Sox9 pathway was not observed when these germ cells adopted spermatogenetic male pathway, suggesting that for the sexual decision of male germ cells, the sex determinant SRY and its downstream factors FGF9/SOX9 merely act to suppress female pathway (BMP and RA signaling) and have no direct role in the initiation of nodal signaling and the induction of *Nanos2* (Figure III-12). There are two possible mechanisms whereby nodal is induced: (1) the loss of BMP signaling caused the upregulation of some factor in somatic cells which induces nodal in germ cells; (2) direct repression of nodal in germ cells by BMP signaling is released in the *Smad4* mutant ovaries. In either case, spermatogenesis can be considered as a default pathway when germ cells fail to initiate oogenesis in the absence of BMP and RA signaling. Especially, the expression levels of all of somatic genes that I have investigated in this study were unchanged in *Smad4* mutant ovaries, implying the direct suppression of nodal by BMP signaling in germ cells. Because nodal that promotes spermatogenesis is an intrinsic factor, I hypothesized that sex determination of XY germ cells is a cell autonomous event. To fully prove this idea, germ cells will be isolated from E11.5 testes and be cultured with RAR antagonist

and BMP inhibitor to examine whether spermatogenesis would be induced in the absence of somatic cells.

It is interesting to explore the sexual fate of germ cells receiving neither BMP nor nodal signaling. However, when I treated *Smad4* mutant ovaries from E12.5 embryos with nodal inhibitor SB431542, most of germ cells died (data not shown), suggesting proper signaling was necessary for germ cell survival. Taken together, I conclude that cooperation of RA and BMP signaling level is essential for sex determination of XX germ cells.

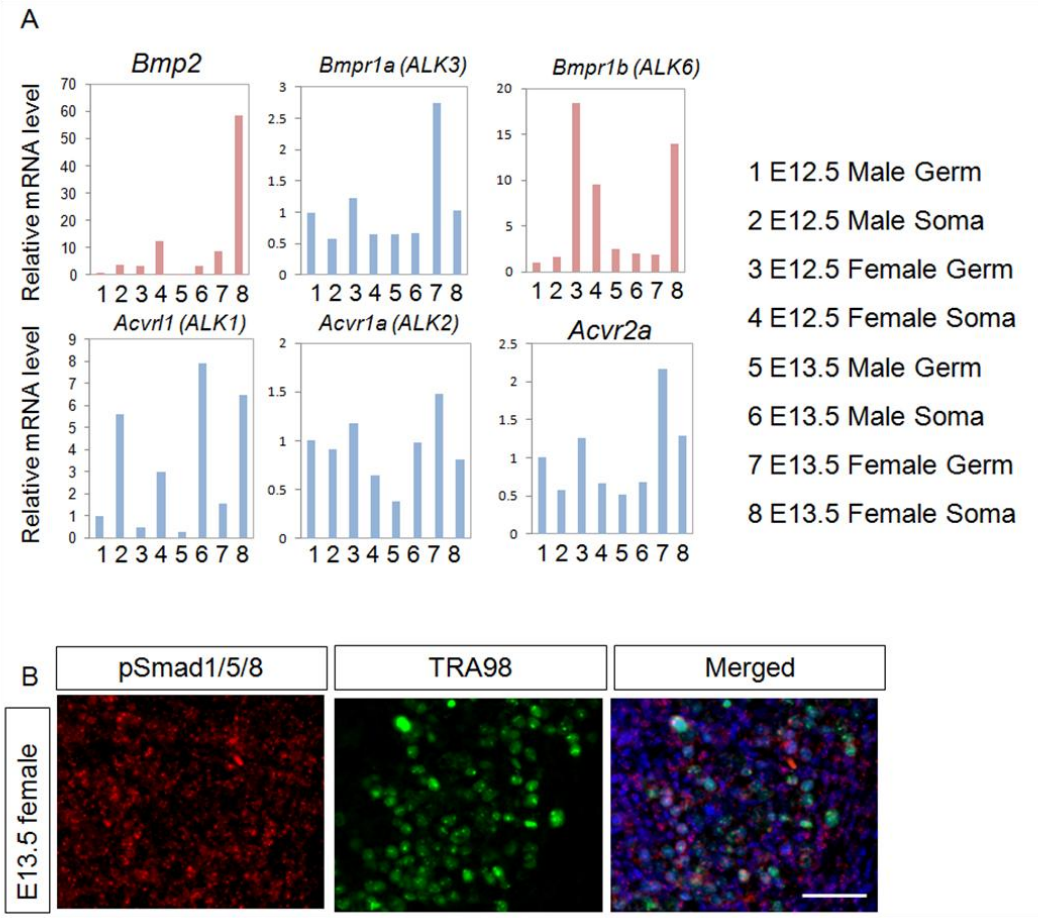


Figure III-1. BMP signaling is activated in both germ cells and somatic cells. (A) RT-qPCR analysis of mRNA levels of BMP signaling-relative genes in isolated germ cells and somatic cells from E12.5 or E13.5 ovaries. (B) Immunohistochemical detection of phosphorylated-SMAD1/5/8 in the E13.5 ovary. Scale bars, 50 μm.

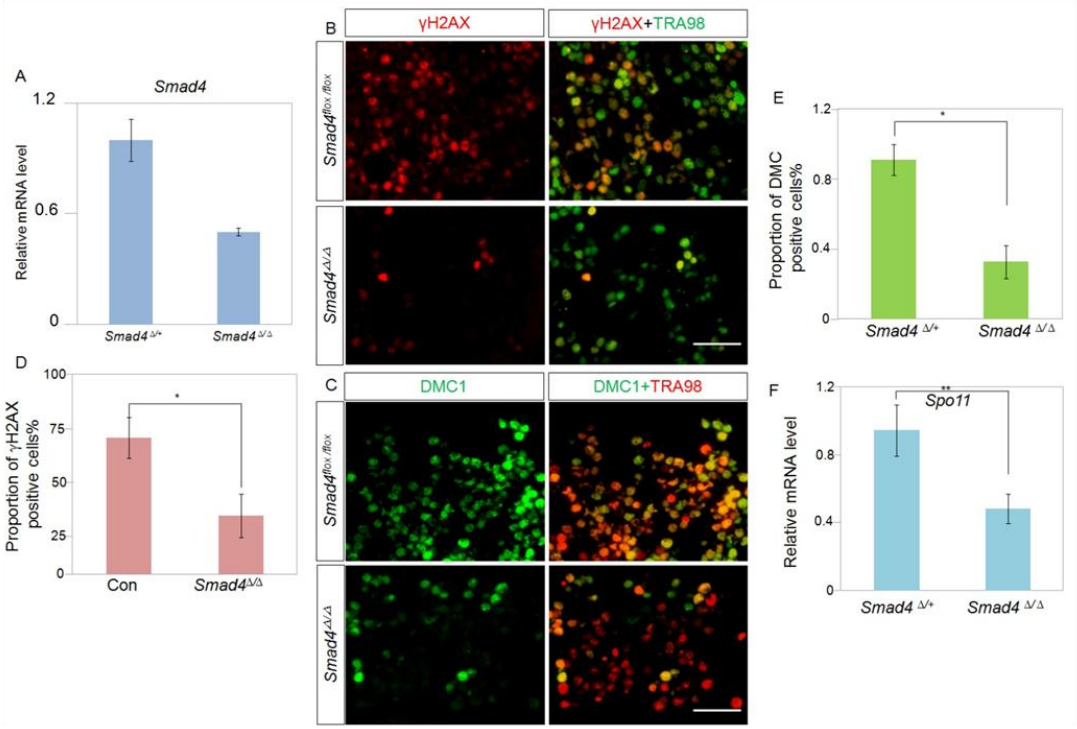


Figure III-2. BMP signaling is required for DSBs in fetal ovaries.
(A) RT-qPCR analysis of mRNA levels of *Smad4* in *Smad4*$^{\Delta/\Delta}$ and *Smad4*$^{\Delta/+}$ ovaries at E13.5. (B-E) The ovaries were dissected from E13.5 embryos and cultured for two days.Immunohistochemical detection of γH2AX (B) or DMC1 (C) with a germ cell marker TRA98 in the ovaries of *Smad4*$^{\Delta/\Delta}$ or *Smad4*$^{flox/flox}$ embryos. (D-E) Proportion of γH2AX-positive (D) and DMC-positive (E) cells in Smad4-null and control ovaries (n=3). (F) Expression level of *Spo11* in the E14.5 *Smad4*$^{\Delta/\Delta}$ and *Smad4*$^{\Delta/+}$ (n=4) ovaries. Error bars represent SD among biological replicates. Scale bars, 50 μm.

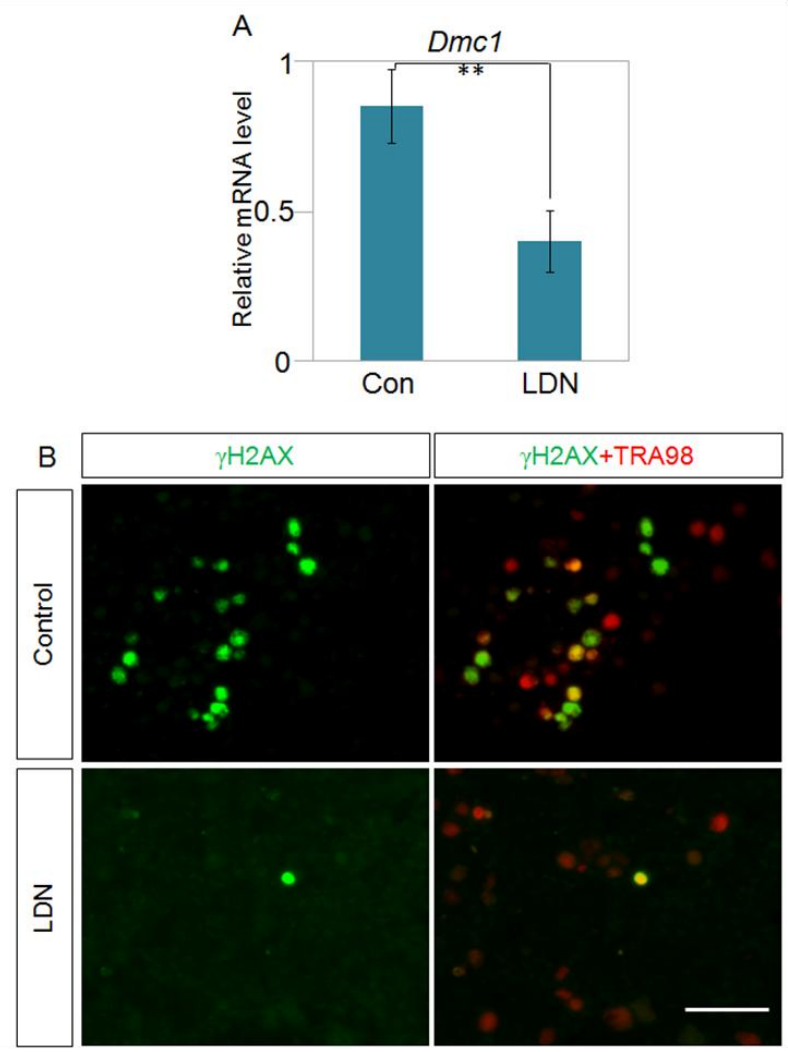


Figure III-3. BMP signaling inhibitor LDN 193189 suppresses DSBs *ex vivo*. Ovaries were harvested from E11.5 embryos and cultured for two days with or without LDN 193189 (1 μM). To facilitate our analysis, an apoptosis inhibitor Z-VAD-FMK (10 μM) was also added in the medium. (A) RT-qPCR analysis of *Dmc1* expression level after LDN 193189 treatment. (B) Immunohistochemical detection of γH2AX and TRA98 after culture. Scale bars, 50 μm.

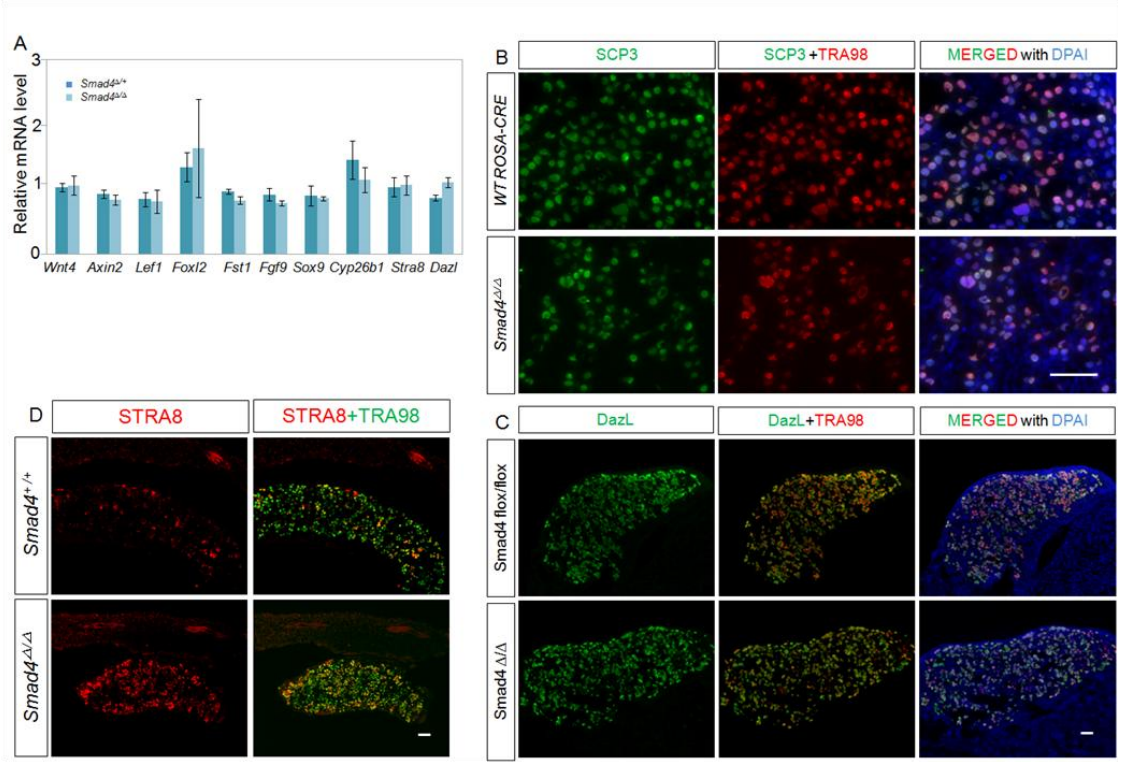


Figure III-4. BMP signaling regulates meiosis independently of RA signaling.

(A) RT-qPCR analysis of indicated genes in *Smad4*$^{\Delta/\Delta}$ (n=4) and *Smad4*$^{\Delta/+}$ (n=3) ovaries at E13.5. (B-C) Immunohistochemical detection of SCP3 (B) and DAZL (C) and TRA98 in *Smad4*$^{\Delta/\Delta}$ and control ovaries. Ovaries were harvested from E13.5 embryos and cultured for two days. (D) Immunohistochemical detection of STRA8 and TRA98 in *Smad4*$^{\Delta/\Delta}$ and wild-type ovaries.

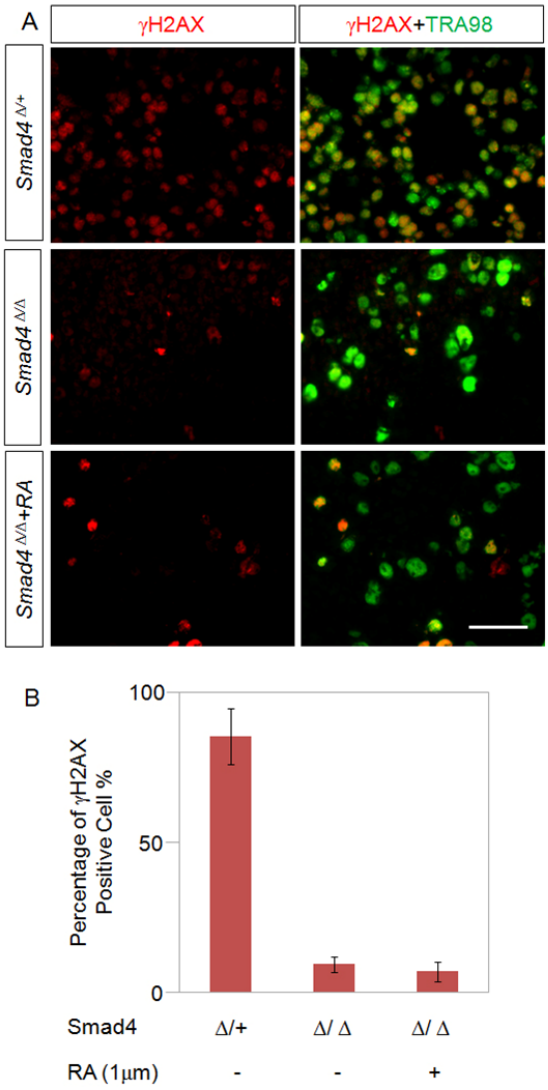


Figure III-5. RA could not rescue *Smad4* conditional knockout ovaries. The ovaries were harvested from E12.5 embryos and cultured for two days with or without 1 μm RA. (A) Immunohistochemical detection of γH2AX and TRA98 in the indicated ovaries. (B) Proportion of γH2AX-positive cells in the indicated ovaries. Error bars represent SD among biological replicates. Scale bars, 50 μm.

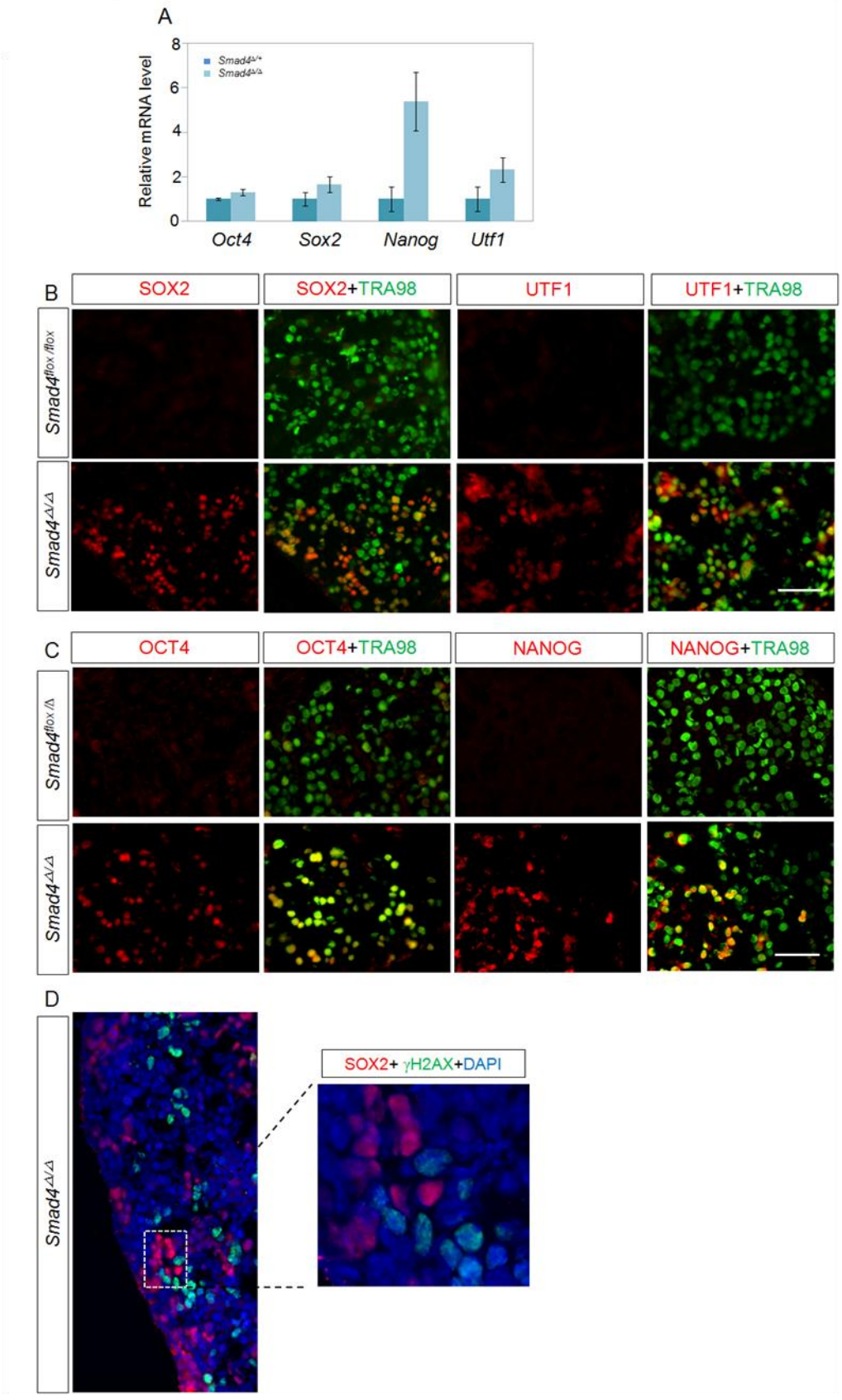

A
Relative mRNA level
8
6
4
2
0
Oct4
Sox2
Nanog
Utf1
B
SOX2
SOX2+TRA98
UTF1
UTF1+TRA98
C
OCT4
OCT4+TRA98
NANOG
NANOG+TRA98
D
SOX2+ γH2AX+DAPI

Figure III-6. Maintenance of pluripotency in XX germ cells in the absence of BMP signaling. (A) RT-qPCR analysis of pluripotency related genes in *Smad4*$^{\Delta/\Delta}$ (n=4) and *Smad4*$^{\Delta/+}$ (n=3) ovaries at E13.5. (B-C) Immunohistochemical detection of SOX2, OCT4, NANOG and UTF1 together with TRA98 in the *Smad4*$^{\Delta/\Delta}$ and *Smad4*$^{\Delta/+}$ ovaries at E14.5. (D) Immunohistochemical detection of SOX2 and γH2AX in the *Smad4*$^{\Delta/\Delta}$ ovaries at E14.5.

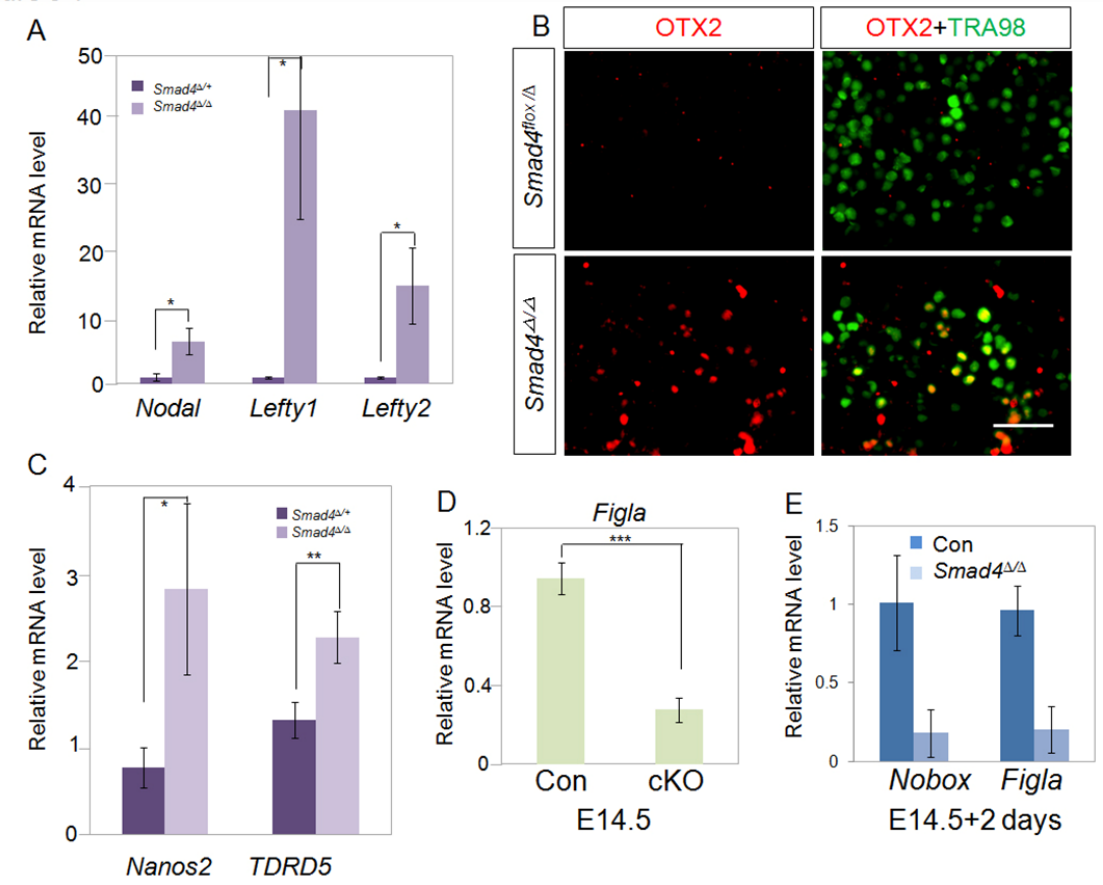


Figure III-7. BMP signaling suppresses the male pathway and promotes oogenesis. (A) RT-qPCR analysis of genes related to the nodal signaling pathway in *Smad4*$^{\Delta/\Delta}$ (n=4) and *Smad4*$^{\Delta/+}$ (n=3) ovaries at E13.5. (B) Immunohistochemical detection of OTX2 together with TRA98 in the *Smad4*$^{\Delta/\Delta}$ and *Smad4*$^{\Delta/+}$ ovaries at E14.5. (C) RT-qPCR analyses of male-specific genes *Nanos2* and *TDRD5* in *Smad4*$^{\Delta/\Delta}$ (n=4)and *Smad4*$^{\Delta/+}$ (n=3) ovaries at E13.5. (D-E) RT-qPCR analyses of oocyte-specific genes *Figla* and *Nobox* in control and mutant ovaries. The ovaries were harvested at E14.5 (D) or further cultured for two days (E). Error bars represent SD among biological replicates. Scale bars, 50 μm.

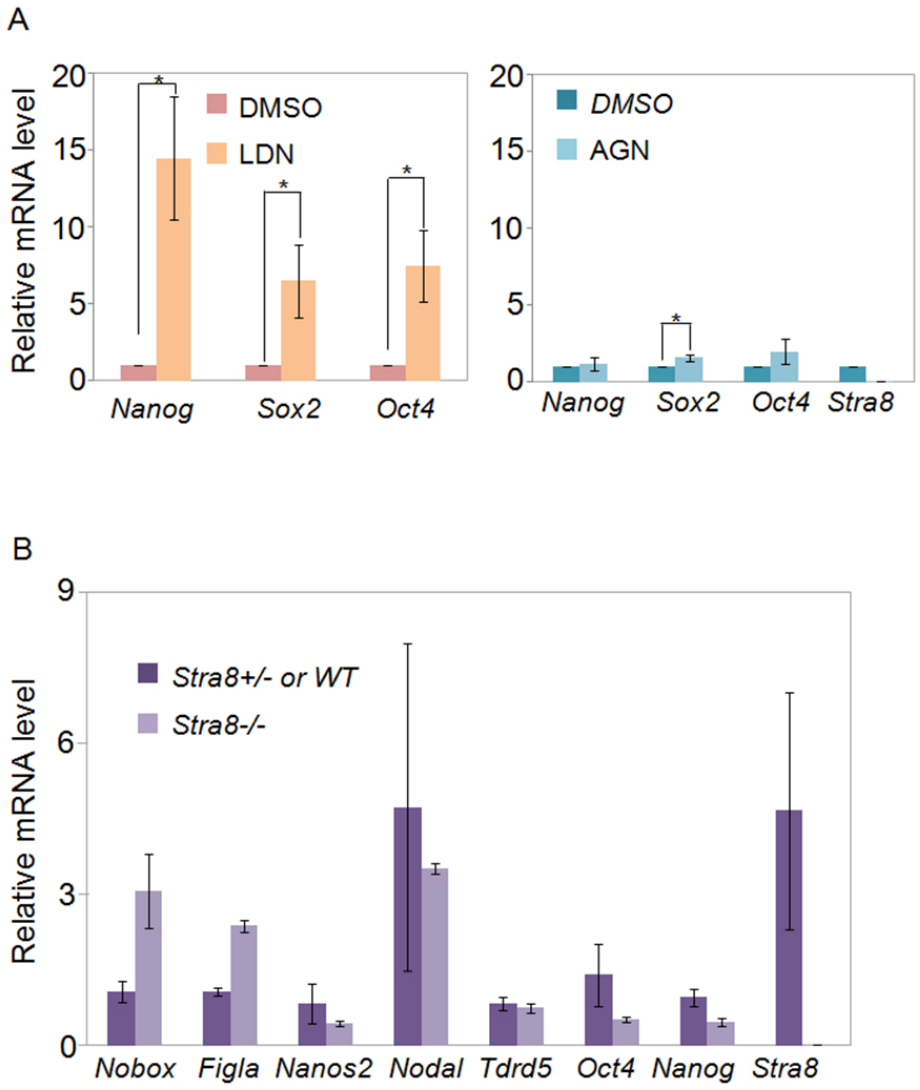


Figure III-8. Sex reversal is not induced in XX germ cells even in the absence of RA signaling or *Stra8*. (A) RT-qPCR analyses of indicated gene expressions after BMP inhibitor LDN 193189 (1 μM) or RAR antagonist AGN 193109 (5 μM) treatment. The ovaries were harvested from E11.5 embryos and cultured for two days with indicated inhibitors. Z-VAD-FMK (10 μM) was used to prevent apoptosis (n=4). (B) RT-qPCR analyses of indicated genes in Stra8-deficient (n=2) or control ovaries (n=4) at E14.5. Error bars represent SD among biological replicates.

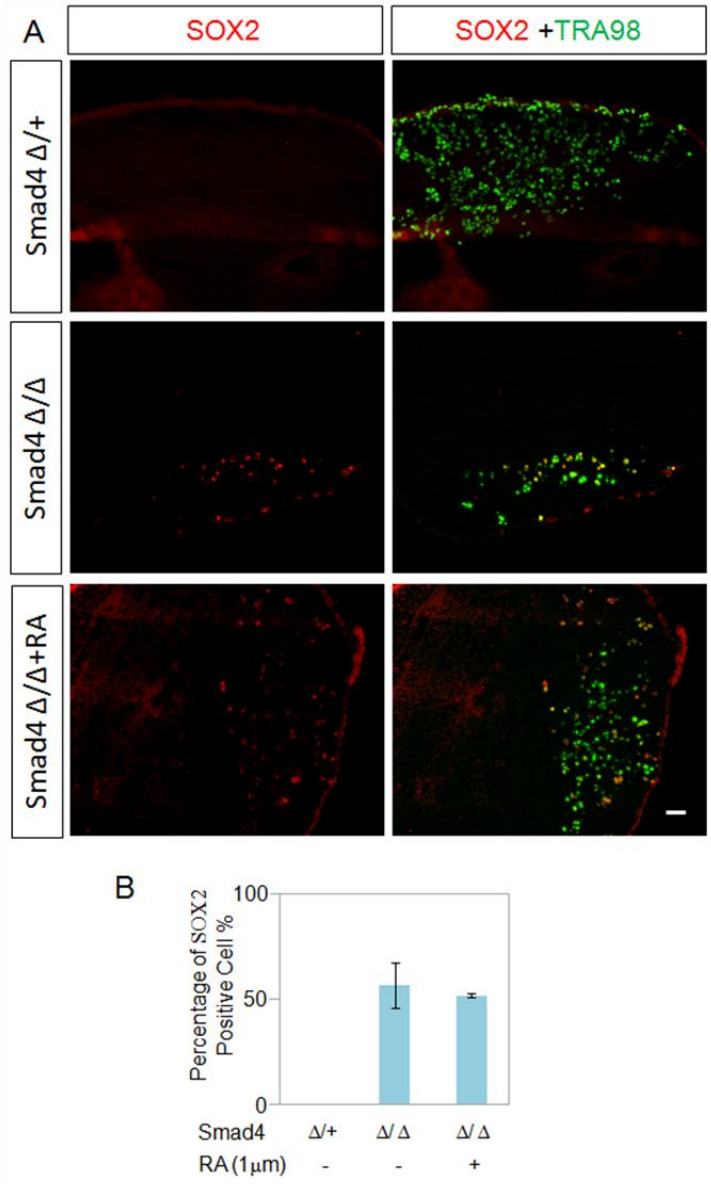


Figure III-9. BMP signaling suppresses male pathway independently of RA. (A-B) The ovaries were harvested from E12.5 embryos and cultured for two days with or without 1 μm RA. (A) Immunohistochemical detection of SOX2 and TRA98 in the indicated ovaries. (B) Proportion of SOX2-positive cells in the indicated ovaries. Error bars represent SD among biological replicates. Scale bars, 50 μm.

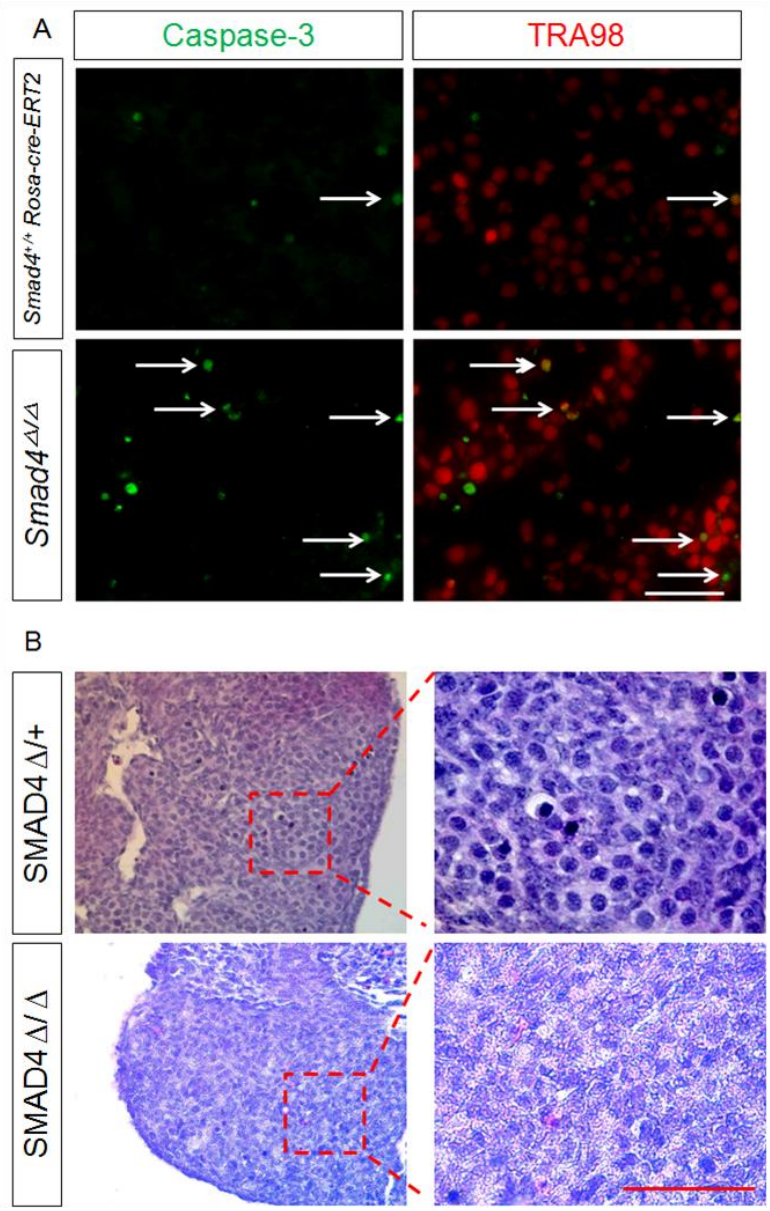


Figure III-10. XX germ cells in *Smad4*$^{\Delta/\Delta}$ ovaries enter apoptosis. (A) Immunohistochemical detection of apoptosis with cleaved-caspase3 and TRA98 in *Smad4*$^{\Delta/\Delta}$ and control ovaries at E14.5. (B) Photographs of hematoxylin and eosin-stained ovarian sections from *Smad4*$^{\Delta/\Delta}$ and *Smad4*$^{\Delta/+}$ ovaries. The ovaries were harvested from E14.5 embryos and cultured for three days. Scale bars, 50 μm.

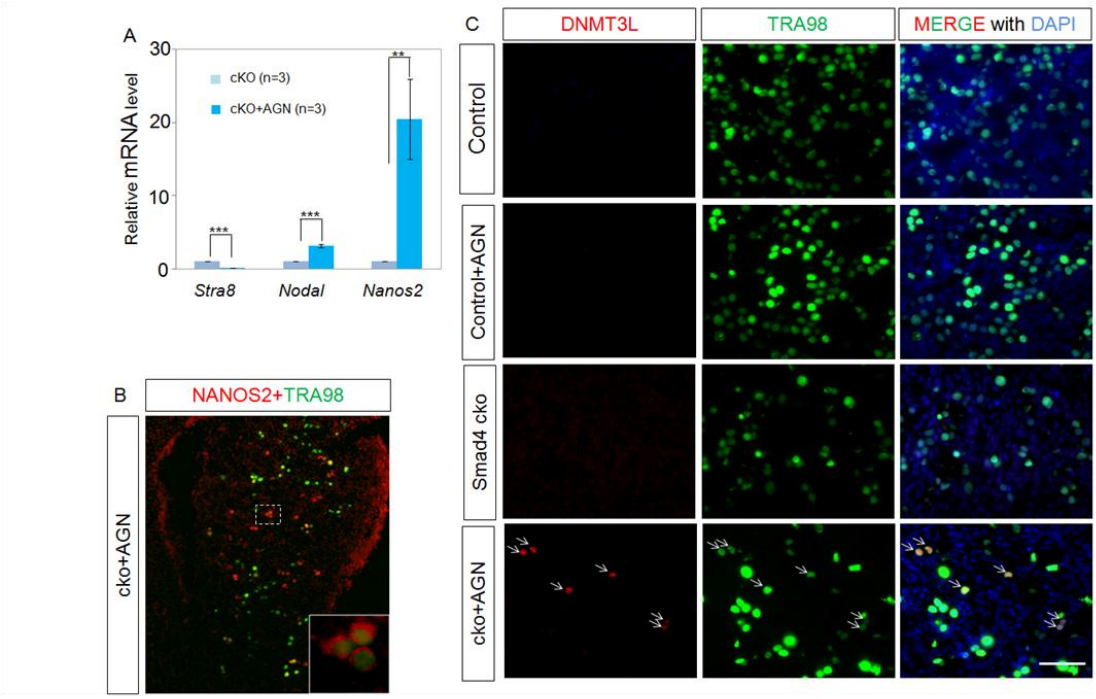


Figure III-11. Cooperation of BMP and RA in the suppression of male-specific genes.

(A) RT-qPCR analyses of *Stra8*, *Nodal* and *Nanos2* expressions after the cultivation of *Smad4* mutant mice with or without RAR antagonist AGN 193109 (5 μM) for 48 hours. Mutant ovaries were harvested from E12.5 and 4-Hydroxytamoxifen (2μM) was added in both cases. (B and C) Immunohistochemical detections of Nanos2 and TRA98 (B), and DNMT3L and TRA98 (C) at 72 hours after the treatment. Error bars represent SD among biological replicates. Scale bars, 50 μm.

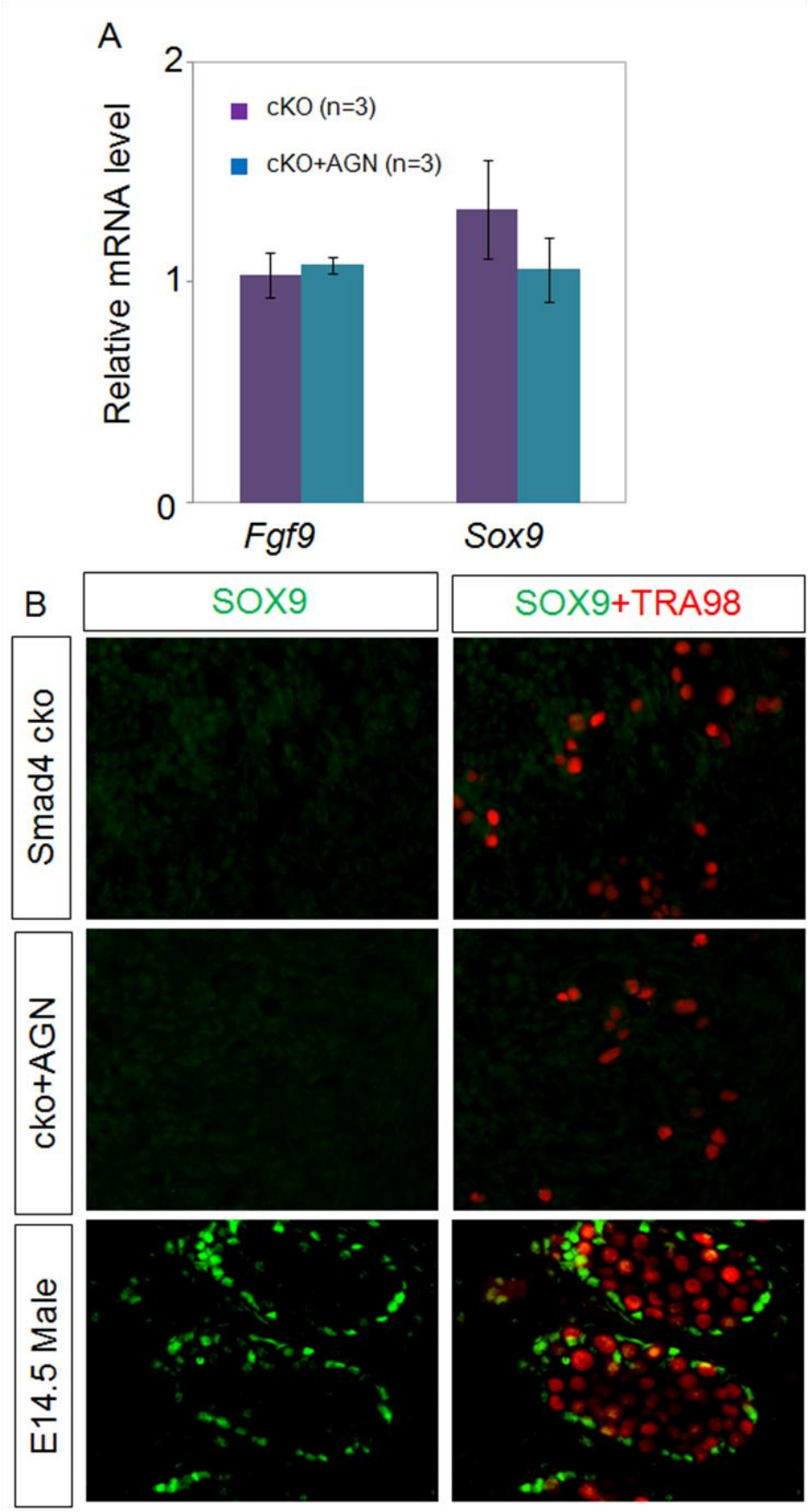

A
Relative mRNA level
2
1
0
cKO (n=3)
cKO+AGN (n=3)
Fgf9
Sox9
B
SOX9
SOX9+TRA98
Smad4 cko
cko+AGN
E14.5 Male

Figure III-12. FGF9/Sox9 signaling is dispensable for spermatogenesis.
(A) RT-qPCR analyses of *Fgf9* and *Sox9* expressions after the cultivation of *Smad4* mutant mice with or without RAR antagonist AGN 193109 (5 μM) for 48 hours. Mutant ovaries were harvested from E12.5 and 4-Hydroxytamoxifen (2μM) was added in both cases. (B) Immunohistochemical detection of SOX9 and TRA98 after 48 hour treatment. E14.5 male testis was used as a positive control. Error bars represent SD among biological replicates. Scale bars, 50 μm.

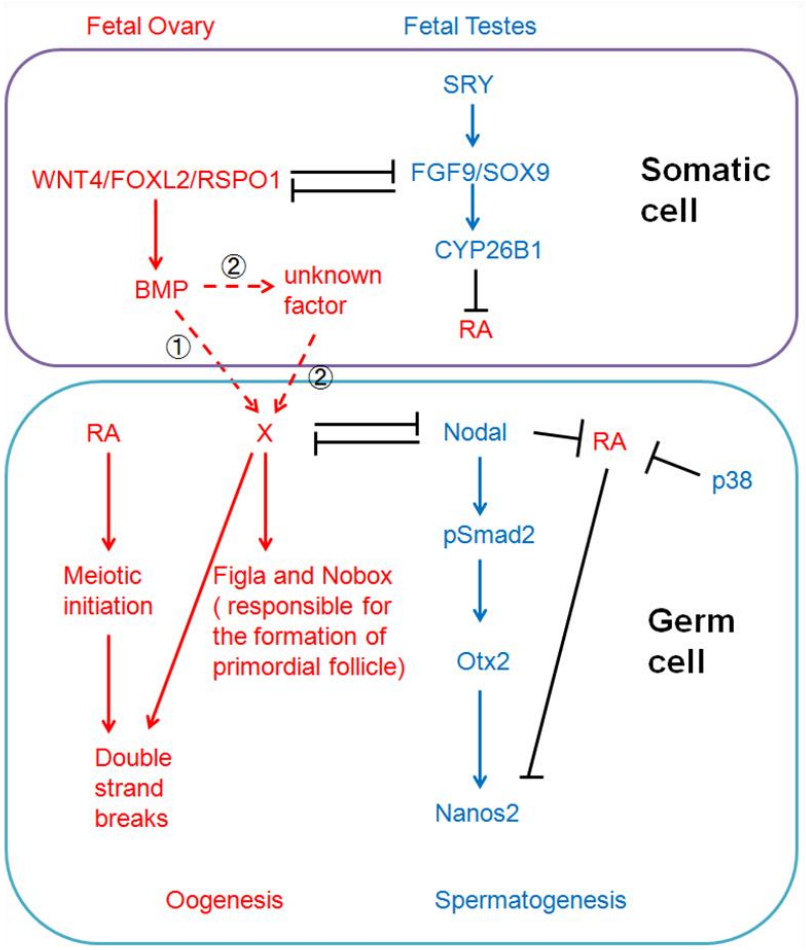


Figure III-12. A model for sex determination of germ cells.
In ovaries, accompanying with meiotic initiation by RA, Wnt signaling activates BMP to instruct oogenesis by inducing genes required for the formation of primordial follicle and meiotic progression, and meanwhile for the suppression of the male-specific nodal signaling. BMP might directly act on germ cells or indirectly induce a secondary signal from somatic cells to regulate germ cell fate. In contrast, XY germ cells are protected from RA by an enzyme CYP26B1 that is regulated by Fgf9/Sox9 signaling. Meanwhile, germ cell intrinsic nodal signaling promotes spermatogenesis by inducing *Nanos2* expression. The disruption of RA and BMP signalings in fetal ovaries is sufficient for upregulation of nodal signaling and for the induction of complete sex reversal of XX germ cells, indicating that spermatogenesis is a default pathway for germ cells.

CONCLUSION

How an individual's sex is determined is one of the great questions of philosophy. The earlier hypothesis of sex determination was straightforward: women were men whose development arrests too early because of the environmental condition such as: temperature and nutrition. This environmental sex determination mechanism remained the major scientific theory until the identification of the X and Y chromosomes. In mammalian, the correlation of the female sex with an XX karyotype and the male sex with an XY karyotype strongly indicates that nuclear inheritance rather than environmental factors determined the sex. In 1990, the first male sex determinant: SRY, which indeed locates on the Y chromosome has been identified in mice, further confirming the genetic sex determination of mammalian. Interestingly, the Sry is not expressed in the male germ cells but in the surrounding somatic cells, implying that sex of germ cells is determined by the microenvironment where they reside. In last two decades, accumulative genetic data demonstrated that Fgf and Wnt signalings were indispensable for the sexual fate determination of male and female germ cells, respectively. However, it is unclear how these two pathways operate germ cells' fate.

In this study I revealed that TGFβ signaling pathways were essential for sex determination of mammalian germ cells. In ovaries, accompanying with meiotic initiation by RA, Wnt signaling activates BMP to instruct oogenesis by inducing genes required for the formation of primordial follicle and meiotic progression, and meanwhile for the suppression of the male-specific nodal signaling. BMP might directly act on germ cells or indirectly induce a secondary signal from somatic cells to regulate germ cell fate. In contrast, XY germ cells are protected from RA by an enzyme CYP26B1 that is regulated by Fgf9/Sox9 signaling. Meanwhile, germ cell intrinsic

nodal signaling promotes spermatogenesis by inducing *Nanos2* expression. The disruption of RA and BMP signalings in fetal ovaries is sufficient for upregulation of nodal signaling and for the induction of complete sex reversal of XX germ cells, indicating that nodal-induced spermatogenesis is a default pathway for germ cells. However, the mechanism whereby nodal signaling is initiated is still unknown. It might be a cell autonomous event in germ cells or be triggered by a signal from somatic cells which is suppressed by BMP signaling in fetal ovaries.

Future work will be focused on the downstream factors of BMP and nodal signaling and how these factors orchestrate to promote germ cells' sex. In view of conservatism of nodal and BMP signaling, I believe that the widespread mechanism might exist to determine an individual's sex across species.

MATERIALS AND METHODS

Mice

ICR strain mice (Clea, Japan) were used in all embryonic gonadal culture experiments. To generate *Nodal* conditional KO mice, a targeting vector was constructed with genomic *Nodal* clones isolated from an E14 embryonic stem (ES) cell genomic DNA library. Two ES clones that have been shown to undergo homologous recombination by Southern blot analysis were used to generate *Nodal*$^{\beta geo/+}$ mice. *Nodal*$^{\beta geo/+}$ mice were crossed with *CAG-Flp* transgenic mice (Kanki et al., 2006) to generate *Nodal*$^{flox/+}$ mice (Figure 1-14). The generation of *Nanos2*$^{-/-}$ mice and *Stra8*$^{gfp/gfp}$ and the methods of genotyping were as described (Tsuda et al., 2003) (Saba and Kato et al., unpublish). The generation of the floxed *Smad2*, *Smad3* and *Smad4* allele has been described previously (Li et al., 2008) (Yang et al., 2002). *Rosa-CreERT2* mice were purchased from Artemis Pharmaceuticals GmbH and the *Pou5f1/Oct4-CreERT2* transgenic mouse strain was established in our laboratory (Geyer et al., 2011). *Stella-MerCreMer* mice were established previously (Hirota et al., 2011). All mouse experiments were carried out with the permission of the Animal Experimentation Committee at the National Institute of Genetics.

Microarray

RNA samples were prepared from XX and XY gonads from E12.5–E15.5 embryos (six–20 gonads each). For each hybridization assay, 500 ng of total RNA was labeled with Cy3 and hybridized to a Whole Mouse Genome Oligo Microarray (G4122F; Agilent) in accordance with the manufacturer's protocol using a Low RNA Input Linear Amplification Kit, One Color (Agilent) and Gene Expression Hybridization Kit

(Agilent). Data have been deposited in Gene Expression Omnibus under accession number GSE37720.

Organ culture

All gonads were cultured in 24-well tissue culture plates with 10% horse serum in DMEM at 37°C on 5-μm nucleopore filters (Hiramatsu et al., 2010). Some gonads were also cultured in medium containing the TGFβ receptor inhibitor SB431542 (Sigma-Aldrich, #S4317), ALK5 inhibitor (Wako, 012-23021), BMP inhibitor LDN 193189 (Stemgent, #04-0074), the FGF inhibitor SU5402 (Calbiochem, #572630), the RA receptor antagonist AGN 193109 (Toronto Research Chemical Inc.), P38 inhibitor SB203580 (Sigma-Aldrich, #S8307), 4-Hydroxytamoxifen (Sigma-Aldrich, #H7904), all-trans-Retinoic Acid (Wako,#182-01111), Z-VAD-FMK (Peptide Institute Inc.) or activin-A (Sigma-Aldrich, #H4666) at the indicated concentrations. For activin-A treatment, gonads were incubated in culture medium containing activin-A for 1 h at 37°C and then located on the filter membrane.

***In situ* hybridization**

In situ hybridization of whole mounts and sections of testes was performed essentially as described previously (Saga et al., 1996). The primer sets used for synthesizing antisense probes for *in situ* hybridization were as follows (5′–3′): *Nodal*: forward TTGAGCCAAGAAGAGGATCTGGTATGG and reverseCTCCACAATCATGTCCTTGTGGTGTTC; *Lefty1*: forward CTGTCGCTGGTTAGCCTCAGGGAA and reverse CTCCCTCCTTCACGCTGACAATCATGG;

Lefty2: forward CCGTCTAGGTCCCAAGAACTTTTCAGG and reverse CAAGGAGGTCATCTCTGAGGCAACAC.

Cyp26b1, forward (5′–3′) CGGAGAATGTGCGCAAGATCCTACT: reverse (5′–3′) CCGGGTCAAACACATTCACGTCCTT.

Otx2: forward (5′–3′) TGTCTTATCTAA AGCAACCGCCTT AC and reverse (5′–3′) CAGCATTGA AGTTAAGCTTCCAAG AG

The probe for *Nanos2* was as described previously (Tsuda et al., 2006).

X-gal staining and immunohistochemistry

X-gal staining was performed according to (Saga et al., 1992). Gonads were fixed in 4% paraformaldehyde, embedded in OCT compound (Tissue-Tek, Sakura) and sectioned (8 μm) using a cryostat. After preincubation with 3% skim milk in PBST (PBS+0.1% Tween 20) for 30 min, the sections were stained with the primary antibodies TRA98 (1:10000; a gift from Y. Nishimune, Osaka University, Osaka, Japan), anti-cleaved-caspase3 (1:200: Cell Signaling Technology), anti-SOX9 (1:250; a gift from Y. Kanai, Tokyo University, Tokyo, Japan), anti-γH2AX (1:5000; Abcam), anti-DMC1 (1:200; Santa Cruz), anti-laminin (1:3000; Sigma), anti-DNMT3L (1:200; a gift from S. Yamanaka, Kyoto University, Kyoto, Japan), anti-NANOS2 (1:200) ((Suzuki et al., 2007), anti-SOX2 (1:200, Santa Cruz), anti-OCT4 (1;200, BD biosciences), anti-NANOG (1:200, Cosmo bio), anti-pp38 (1:100, Cell Signaling Technology), anti-pSMAD1/5/8 (1:400, Cell Signaling Technology) and anti-pSMAD2 (1:400; Cell Signaling Technology). This was followed by staining with donkey anti-rabbit/rat/goat or anti-rat IgG secondary antibodies conjugated with either Alexa

488 or Alexa 594 diluted 1:250 (Invitrogen). For anti-pSMAD2, anti-pSMAD1/5/8, anti-NANOG and anti-pp38, the primary antibodies were diluted using Can Get Signal (Toyobo). The other primary antibodies were diluted in 3% skim milk in PBST. All the secondary antibodies were diluted in PBST.

Reverse transcription real-time quantitative PCR (RT-qPCR)

Total RNA was prepared from fetal testes of *Nodal* mutant embryos and testes of ICR strain embryos at each embryonic stage using RNeasy Mini Kits (Qiagen). Total RNA was then used for cDNA synthesis using PrimeScript RT Reagent Kits with gDNA Erase (Takara). PCR reactions were carried out with SYBR premix Ex Taq (Takara) using a Bio-Rad MiniOpticon Real Time PCR Detection System (Bio-Rad).

The following PCR primer pairs were used for amplification (5′–3′): *Nanos2*: forward ACAGCAGTCAGTCAGTCTC and reverse CCGAGAAGTCATCACCAG; *Nodal*: forward AGCCAAGAAGAGGATCTGGTATGG and reverse GACCTGAGAAGGAATGACGGTGAA; *Lefty1*: forward AGTCCTGGACAAGGCTGATGTG and reverse CGAACACTAGCAGGTGAGTGGA; *Lefty2*: forward ATCGACTCTAGGCTCGTGTCCATC and reverse CACAATTGCCTTGAGCTCCGTAGTC; *Mvh*: forward GTTGAAGTATCTGGACATGATGCAC and reverse CGAGTTGGTGCTACAATAATACACTC; *G3pdh*: forward ACCACAGTCCATGCCATCAC and reverse TCCACCACCCTGTTGCTGTA; *Stra8*: forward CCTAAGGAAGGCAGTTTACTCCCAGTC and reverse GCAGGTTGAAGGATGCTTTGAGC; *Inhba*: forward

CCAAGGAAGGCAGTGACCTGTCAGT and reverse

TCTCCTGGCACTGCTCACAAGCAATC; *Sox9*, forward

AAGACCACCCCGATTACAAGTACCA and reverse

TCAGATCAACTTTGCCAGCTTGCAC; *Dnmt3l*, forward

GCTATGCGGGTGTGGAGCAAC and reverse

TCACCAGGAGGTCAACTTTCG; *Foxl2*, forward

GCCTCAACGAGTGCTTCATCAAGGT and reverse

AGTTGTTGAGGAACCCCGATTGCAG; *follistatin*, forward

CATCCCTTGTAAAGAAACGTGTGAG and reverse

TTATTGGTCTGATCCACCACACAAG; *Wnt4*, forward

ATCGCCTATGGCGTAGCCTTCTCAC and reverse

CCGTGGCACCGTCAAACTTCTCCTT; *Bmp2*, forward

GATACAGGAAGCTTTGGGAAACAGTAG and reverse

CTGTGTTCATCTTGGTGCAAAGACC; *Dmc1*, forward

CCCTCTGTGTGACAGCTCAAC and reverse

GGTCAGCAATGTCCCGAAG; *Rec8*, forward

CTACCTAGCTTGCTTCTTCCCA and reverse

GCCTCTAAAAGGTGTCGAATCTG. *Sox2*, forward

GCGGAGTGGAAACTTTTGTCC and reverse

CGGGAAGCGTGTACTTATCCTT; *Nanog*, forward

CCGCTTGCACTTCATCCTTTG and reverse

CCTCAGCCTCCAGCAGATGC; *Oct4*, forward

TCACCTTGGGGTACACCCAG and reverse

CATGTTCTTAAGGCTGAGCTGC; *Otx1*, forward

AGGGCGGAAGCTATGGTCAGGGATAC and reverse

CGGGCTCCTTGTAATCCAAGCAATCGG; *Otx2*, forward

TATGGACTTGCTGCATCCCTCCGTGGGCTA and reverse

TGGCAGGCCTCACTTTGTTCTGACCTCCAT; *Otx3*, forward

AACAACCTGATGCACTACTCGTCTT and reverse

CAATGCTCGTGGTTTTACTGTTCAGG; *Uft1*, forward

ATGTCCCGGTGACTACGTCTGATG and reverse

AGTCTCGGAGTTTGTCCTTGAGGAA ; *TDRD5*, forward

AGGGGTCATATTCTACAGGATTCCC and reverse

CGCTTTAGCTCAGGATCAACAGTC; *Figla*, forward

GCCCCTCCTCTTCTTTCTTCA and reverse

CAGAGCAGGAAGCCCAGTAAA; *Nobox*, forward

AATGTGGAGCCTGGGAGAGC and reverse

ATGAGGGTGCTGAGAGGGTG; *Cyp26b1*, forward

GAGACTCTTCACGCCCGTCT and reverse

TCGTGAGTGTCTCGGATGCT; *Spo11*, forward

GTAAGTACACTCTGGACAGGTAG and reverse

GATCCCACTGACAAAGCATGACC; *Smad4*, forward

CCTGTTGTGACTGTGGATGGCTATG and reverse

AGACCTTTATATACGCGCTTGGGTAGA; *Bmpr1a*, forward

ATTGGTTCAGCGAACTATTGCCAAA and reverse

TGGCACATTTCAGGAAGTCATAGAGAG; *Bmpr1b*, forward

TGAGAGACTTGATCGAGCAGTCTCA and reverse

GATATCTGCAGCAATGAACCCCAGAA; *Acvr2a*, forward

ATTTGTGCATTTTGGGTGTACAGAC and reverse GTTCTCATGCTTCATTCCAGGTAGA; *Acvrl1*, forward CTGCAACCACAACGTGTCTCTGATG and reverse GTAGTACAGTCGCTGTCCAGGAAGT; *Acvr1a*, forward AATGTCGCTGTGAAGATCTTCTCCT and reverse CTGGCTATGGACAGTACAATCCGAA; *Axin2*, forward AAAACGGATTCAGGTCCTTCAAGAG and reverse CGGAAAATGAGGTAGAGACACTTGG; *Lef1*, forward ACCCTGATGAAGGAAAGCATCCAGA and reverse AGTTGACATCTGACGGGATGTGTGA.

Germ cell isolation and culture

Testes were collected from E12.5 mice and dissociated using 0.25% trypsin and 1 mM EDTA. For each experiment, five to eight pairs of gonads were pooled. Germ cells were isolated using magnetic sorting (MACS, Miltenyi Biotec) with a mouse monoclonal antibody to stage-specific embryonic antigen-1 and anti-IgM magnetic beads (Pesce and De Felici, 1995). Media were used according to a previous study, with modifications (Bowles et al., 2010). The medium for germ cell culture contained KnockOut DMEM (optimized for ES cells, Invitrogen), KnockOut serum (Invitrogen, 10%), penicillin and streptomycin (Invitrogen, 100 × diluted), MEM with non-essential amino acids (Invitrogen, 100×), L-glutamine (2 mM), 0.5 mM pyruvate and β-mercaptoethanol (Invitrogen, 1000×). Germ cells from 10-16 testes were cultured in six-well plates on 0.4 μm polycarbonate membranes (Corning Inc.). All plates were maintained at 37°C under 5% CO_2 in air.

Statistical analysis

For quantitative analyses among multiple samples, statistical significance was assessed using one-way ANOVA followed by Tukey's post-hoc tests for selected pairs of genotypes. For quantitative analyses between two different samples, statistical significance was assessed using Student's *t*-tests. Asterisks in Figures indicate the levels of statistical significance: *P < 0.05; **P < 0.01; ***P < 0.001; ns, not significant.

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