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Nodal/activin signaling promotes male germ cell fate and suppresses female programming in somatic cells

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SUMMARY

Testicular development in the mouse is triggered in somatic cells by the function of *Sry* followed by the activation of fibroblast growth factor 9 (FGF9), which regulates testicular differentiation in both somatic and germ cells. However, the mechanism is unknown. We show here that the nodal/activin signaling pathway is activated in both male germ cells and somatic cells. Disruption of nodal/activin signaling drives male germ cells into meiosis and causes ectopic initiation of female-specific genes in somatic cells. Furthermore, we prove that nodal/activin-A works directly on male germ cells to induce the male-specific gene *Nanos2* independently of FGF9. We conclude that nodal/activin signaling is required for testicular development and propose a model in which nodal/activin-A acts downstream of fibroblast growth factor signaling to promote male germ cell fate and protect somatic cells from initiating female differentiation.

KEY WORDS: Male germ cells, Nodal/activin signaling, *Nanos2*, Sex differentiation, *Nodal* conditional knockout, *Smad4* conditional knockout, Mouse

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). By 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).

FGF9 also works to suppress *Wnt4* expression in somatic cells, because the expression of *Wnt4* is initiated ectopically in *Fgf9*^{−/−} XY gonads (Kim et al., 2006). *Wnt4* is crucial 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 target genes in the regulation of ovarian organogenesis (Yao et al., 2004; Kashimada et al., 2011). However, the means by which FGF9 orchestrates testicular differentiation in both germ and somatic cells is unknown. In this study, we addressed this question by investigating the 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 (Münsterberg 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

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testes at birth with 50% lower Sertoli cell numbers, compared with normal mice (Mendis et al., 2011).

In this study, we investigated the roles of nodal and activin-A (*Inhba*), derived from germ cells and somatic cells respectively, on the promotion of the male sexual differentiation pathway and suppression of the female differentiation pathway.

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*^{geo/+} mice. *Nodal*^{geo/+} mice were crossed with *CAG-Flp* transgenic mice (Kanki et al., 2006) to generate *Nodal*^{lox/+} mice (supplementary material Fig. S1). The generation of the floxed *Smad4* allele has been described previously (Yang et al., 2002). *Rosa-CreERT2* mice were purchased from Artemis Pharmaceuticals 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 E12.5–15.5 embryos (six to 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), the FGF inhibitor SU5402 (Calbiochem, #572630), the RA receptor antagonist AGN 193109 (Toronto Research Chemicals, 5 μM), Z-VAD-FMK (Peptide Institute, 10 μM) 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 hour 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 reverse CTCCACAATCATGCTTGTGGTGTTC; *Lefty1*: forward CTGTGCTGGTTAGCCTCAGGGAA and reverse CTCCTCCTTCA-CGCTGACAATCATGG; *Lefty2*: forward CCGTCTAGGTCCCAAG-AACTTTTCAGG and reverse CAAGGAGGTCATCTCTGAGGCA-ACAC. 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. (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% skimmed milk in PBST (PBS + 0.1% Tween 20) for 30 minutes, the sections were stained with the primary antibodies TRA98 (1:10,000; a gift from Y. Nishimune, Osaka University, Japan), anti-cleaved caspase-3 (1:200; Cell Signaling Technology), anti-SOX9 (1:250; a gift from Y. Kanai, Tokyo University, 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, Japan), anti-NANOS2 (1:200) (Suzuki et al., 2007) 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 (1:250; Invitrogen). For anti-pSMAD2, the primary antibodies were diluted using Can Get Signal (Toyobo). The other primary antibodies were diluted in 3% skimmed 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 CCGA-GAAGTCATCACCAG; *Nodal*: forward AGCCAAGAAGAGGATC-TGGTATGG and reverse GACCTGAGAAGGAATGACGGTGAA; *Lefty1*: forward AGTCTGGACAAGGCTGATGTG and reverse CGAACACTAGCAGGTGAGTGGA; *Lefty2*: forward ATCGACTC-TAGGCTCGTGTCCATC and reverse CACAATTGCCTTGAGC-TCCGTAGTC; *Mvh*: forward GTTGAAGTATCTGGACATGATGCAC and reverse CGAGTTGGTGCTACAATAATACACTC; *G3pdh*: forward ACCACAGTCCATGCCATCAC and reverse TCCACCACCTGT-TTGCTGTA; *Stra8*: forward CCTAAGGAAGGCAGTTTACTCCCAGTC and reverse GCAGGTTGAAGGATGCTTTGAGC; *Inhba*: forward CCAAGGAAGGCAGTGACCTGTGAGT and reverse TCTCTGG-CACTGCTCACAAGCAATC; *Sox9*: forward AAGACCACCCCGATT-CAAGTACCA and reverse TCAGATCAACTTTGCCAGCTTGAC; *Dnmt3l*: forward GCTATGCGGGTGTGGAGCAAC and reverse TCACAGGAGGTCAACTTTCG; *Foxl2*: forward GCCTCAACGA-GTGCTTCATCAAGGT and reverse AGTTGTTGAGGAACCCCG-ATTGCAG; follistatin, forward CATCCCTTGTAAGAAACGTGTGAG and reverse TTATTGGTCTGATCCACCACACAAG; *Wnt4*: forward ATCGCCTATGGCGTAGCCTTCTCAC and reverse CCGTGGCACC GT-CAAACCTTCTCCT; *Bmp2*: forward GATACAGGAAGCTTTGGG-AAACAGTAG and reverse CTGTGTTTCATCTTGGTGCAAAGACC; *Dmc1*: forward CCCTCTGTGTGACAGCTCAAC and reverse GGTCAGCAATGTCCCGAAG; *Rec8*: forward CTACCTAGCTGTG-CTTCTTCCCA and reverse GCCTCTAAAGGTGTGCAATCTG.

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× diluted), L-glutamine (2 mM), 0.5 mM pyruvate and β-mercaptoethanol (Invitrogen, 1000× diluted). Germ cells from 10–16 testes were cultured in six-well plates on 0.4 μm polycarbonate membranes (Corning). All plates were maintained at 37°C under 5% CO₂ 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.

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, we 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, we searched for genes that were specifically expressed in germ cells during these stages, using microarrays. The genes involved in Nodal signaling were detected successfully. We confirmed the male-specific expression of these genes using whole-mount *in situ* hybridization (Fig. 1A). To test whether these genes were expressed in germ cells or somatic cells, we then performed double *in situ* hybridization with *Nanos2* mRNA (a marker of germ cells). *Nodal* was expressed in all male germ cells expressing *Nanos2* (Fig. 1B, upper panel), whereas *Lefty1* and *Lefty2* mRNAs were localized only in a subset (Fig. 1B, middle and lower panels). We 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 (supplementary material Fig. S2A). Although the mRNAs of *Nodal* and *Lefty1/2* were located in germ cells, the expression of the gene encoding another member of the TGF β superfamily, activin-A, was observed in somatic cells (supplementary material Fig. S2A), 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 (Fig. 1C), but not in any cells of female gonads (supplementary material Fig. S2B).

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, we 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 hours (supplementary material Fig. S3A). 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 (Fig. 2A), 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 (Fig. 2A), 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 (Fig. 2B,C). We 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 (supplementary material Fig. S3B), 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 (Fig. 2A). As expected, γ H2AX (H2AFX – Mouse Genome Informatics) and DMC1, which are required to repair meiotic DNA double-strand breaks, were also detected after inhibitor treatment, suggesting that germ cells without nodal/activin signals initiated meiosis (supplementary material Fig. S4A,B). 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*) (Fig. 2D). By contrast, the addition of exogenous activin-A promoted *Nanos2* expression and downregulated *Stra8* in our culture system (Fig. 2E), 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

We observed severe apoptosis in both germ cells and somatic cells after inhibitor treatment (supplementary material Fig. S3C, left panel), implying an irreplaceable role of nodal/activin signaling in

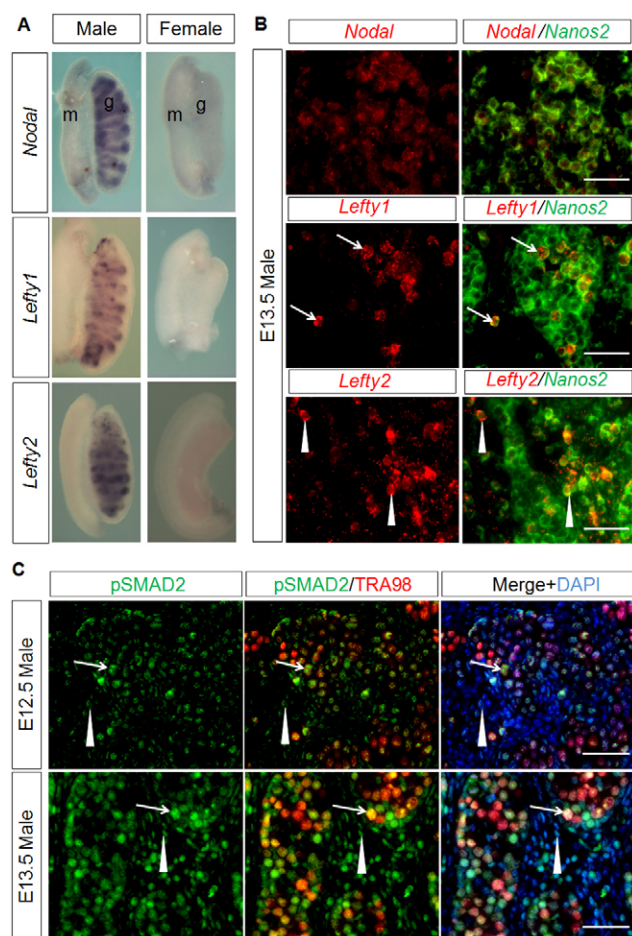


Fig. 1. Expression patterns of genes involved in nodal/activin signaling in embryonic gonads. (A) Whole-mount RNA *in situ* hybridization of E13.5 mouse testes and ovaries using probes for the genes indicated. (B) Double-section *in situ* hybridization was performed using *Nanos2* mRNA as a marker of germ cells. *Nodal* mRNA, *Lefty1* mRNA (arrows) and *Lefty2* mRNA (arrowheads) were restricted in male germ cells at E13.5. (C) Double immunostaining for pSMAD2 (green) and the germ cell marker TRA98 (red) in the testis at E12.5 and E13.5. pSMAD2 expression was observed in both germ cells (arrows) and somatic cells (arrowheads). m, mesonephros; g, gonad. Scale bars: 50 μ m.

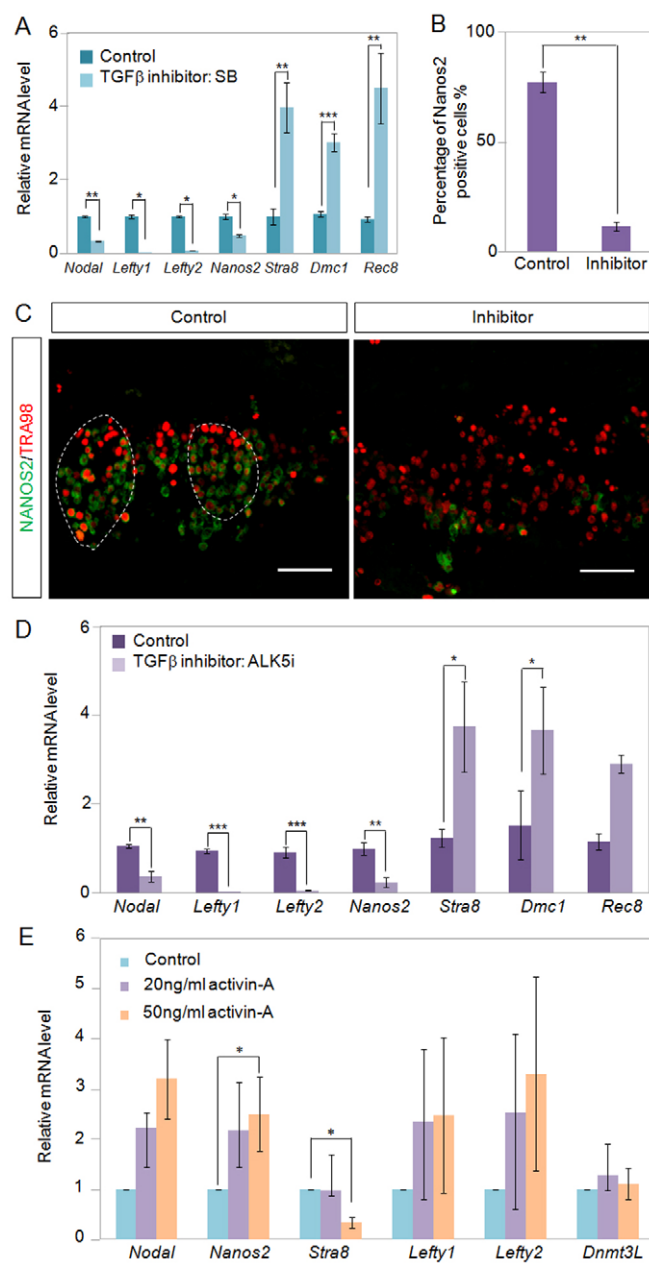


Fig. 2. Nodal/activin is responsible for the initiation of *Nanos2* expression and suppression of meiosis *ex vivo*. (A–C) Testes from mice at E11.5 (B,C) or E12.5 (A) were cultured with the TGFβ receptor inhibitor SB431542 (40 μM) or DMSO (control vehicle) for 24 (A) or 72 (B,C) hours. (A) The indicated genes were analyzed by RT-qPCR (using *Mvh* as a normalization control; *n*=6). (B) Percentages of NANOS2-positive cells in DMSO and inhibitor-treated testes (*n*=4). (C) Immunostaining of NANOS2 after culture. Dashed lines outline a testicular cord. (D) E11.5 testes were cultured with ALK5 inhibitor (15 μM) or DMSO for 48 hours. The expression changes of indicated genes are shown. (E) E12.5 testes were cultured in the presence of activin-A (20 or 50 ng) for 24 hours and gene expression levels were analyzed by RT-qPCR (using *Mvh* as a normalization control; *n*=3). Bars in graphs represent mean±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001. Scale bars: 50 μm.

cell survival in testes. However, apoptosis was not observed when ovaries were treated with this inhibitor (supplementary material Fig. S3C, right panel). We then determined whether ectopic meiosis

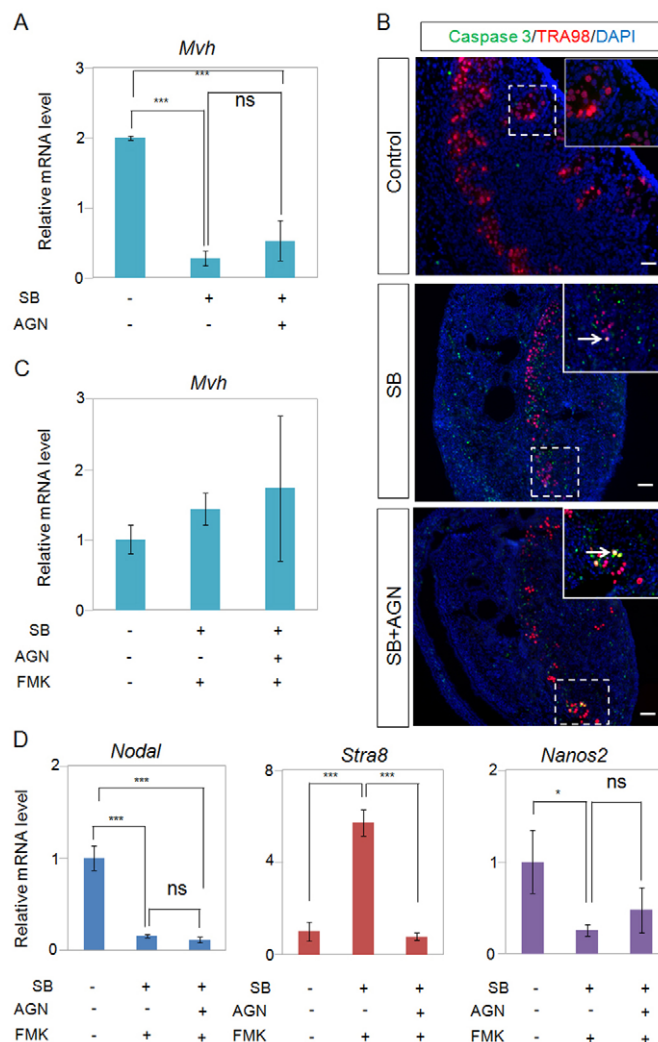


Fig. 3. RA-independent induction of apoptosis and downregulation of *Nanos2* expression. (A) RT-qPCR analyses of *Mvh* in E11.5 mouse testes treated with SB431542 (40 μM) or the RA receptor antagonist AGN193109 (5 μM) for 48 hours [*n*=3 using glyceraldehyde 3-phosphate dehydrogenase (*G3pdh*) as a normalization 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,D) RT-qPCR analyses of *Mvh*, *Nodal*, *Stra8* and *Nanos2* expression in E11.5 testes treated with the indicated drugs for 48 hours (*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.

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 (Fig. 3A,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

We 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). We have already confirmed the role of nodal/activin-A in impeding meiosis (by antagonizing RA). To test whether nodal/activin directly activated *Nanos2* expression, we 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 (Fig. 3A,B), limiting our analysis of germ cell fate, we used benzyloxycarbonyl-Val-Ala-Asp (OMe)-uromethylketone (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 (Fig. 3C,D; supplementary material Fig. S4C). 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 (Fig. 1C), we 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. We confirmed that treatment with SB431542 and activin-A did not affect the expression of *Mvh* (*Ddx4* – Mouse Genome Informatics), which was used for normalization (supplementary material Fig. S5A). *Nanos2* expression was decreased after 24 hours of culture with SB431542 (Fig. 4A). Interestingly, this suppression of *Nanos2* expression was accompanied by downregulation of the nodal signaling pathway (Fig. 4A, *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, we 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, we demonstrated that the addition of activin-A to isolated germ cells resulted in increases in both *Nodal* and *Nanos2* expression levels (Fig. 4B).

To confirm our results *in vivo*, we specifically deleted *Smad4*, a mediator of nodal/activin signaling, in male germ cells (Yang et al., 2002). We 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

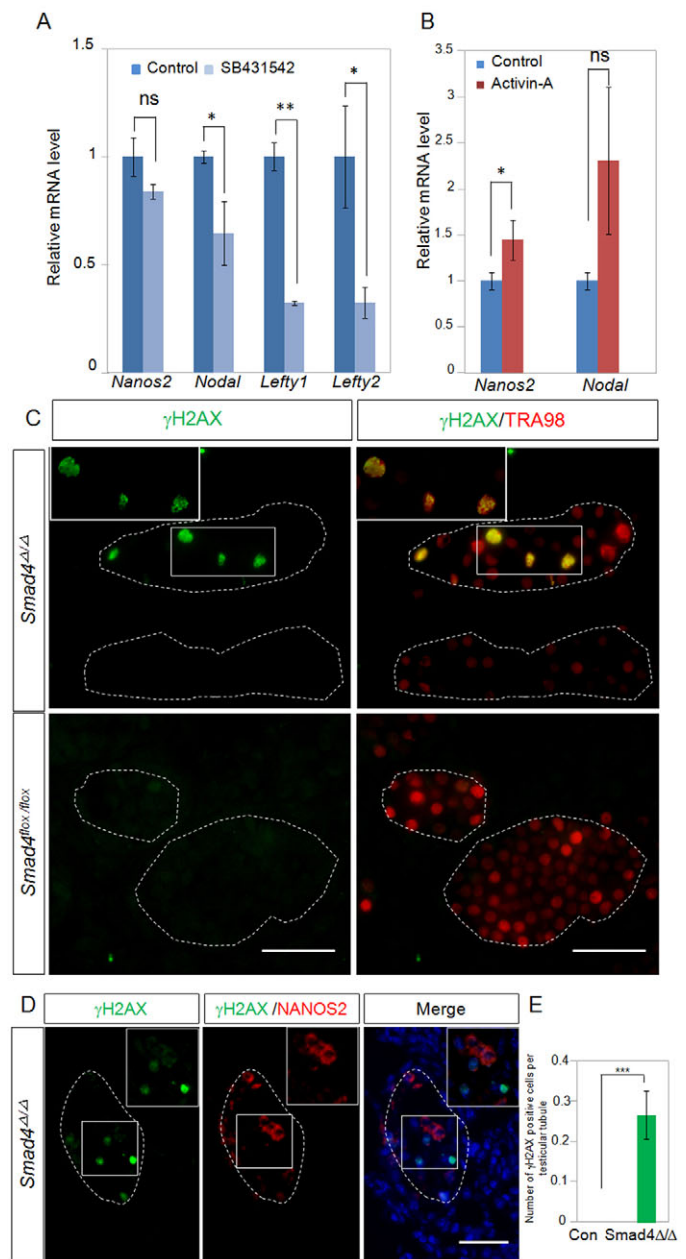


Fig. 4. Nodal and activin act directly on male germ cells.

(A,B) Male germ cells were isolated from E12.5 mouse testes and cultured with SB431542 (A) or activin-A (B) for 24 hours; RT-qPCR was used to assay the expression levels of *Nodal*, *Lefty1/2* and *Nanos2*. Three independent experiments were performed. (C,D) Immunohistochemical detection of γH2AX and TRA98 (C), or γH2AX and NANOS2 (D) in *Smad4*-null male germ cells at E14.5. The germ cell-specific line *Pou5f1*-*CreERT2* was used to conditionally delete *Smad4*. Tamoxifen was injected at E10.5 and E11.5 (*n*=3). Insets show higher magnification of the boxed areas. Dashed lines outline a testicular cord. (E) 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-Hydroxytamoxifen 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). Bars in graphs represent mean±s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001. ns, not significant. Scale bars: 50 μm.

staining indicated that Cre was specifically induced in most germ cells (supplementary material Fig. S5C). Immunohistochemical analysis of the meiotic marker γ H2AX (a marker of the sites of double-strand breaks) indicated that some male germ cells in the mutant mice entered meiosis, compared with none of the germ cells in the control testes (Fig. 4C). As expected, no NANOS2 expression was observed in the γ H2AX-positive germ cells (Fig. 4D). 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 (Fig. 4E). 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, we cannot exclude the possible existence of a SMAD4-independent pathway, because of the mild phenotype of *Smad4* mutant mice.

Redundant functions of TGF β superfamily members in the differentiation of male germ cells

As already mentioned, somatic and male germ cells synthesize two different members of the TGF β superfamily: activin-A and Nodal, respectively (Fig. 1A; supplementary material Fig. S2A). The observations 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, we 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 (Fig. 5A). Deletion of the *Nodal* gene resulted in decreased *Nanos2* mRNA levels at E14.5 (Fig. 5A). In addition, fewer NANOS2-positive germ cells were detected in the mutant mice at E14.5 (Fig. 5B). 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 (supplementary material Fig. S6A,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 (Fig. 5C). We 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, we examined the expression levels of *Nodal*, *Lefty1/2* and *Inhba* after treatment with the FGF receptor inhibitor SU5402. As shown in Fig. 6A, 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 (supplementary material Fig. S7B). As expected, the expression level of *Nanos2* mRNA was also downregulated (Fig. 6A). In addition, the expression of *Stra8* was augmented in a dose-dependent manner (supplementary material Fig. S7A). This result differed from that of a previous study in which FGF9 had no effect on the expression of *Stra8* (Bowles et al., 2010). We ascribed this

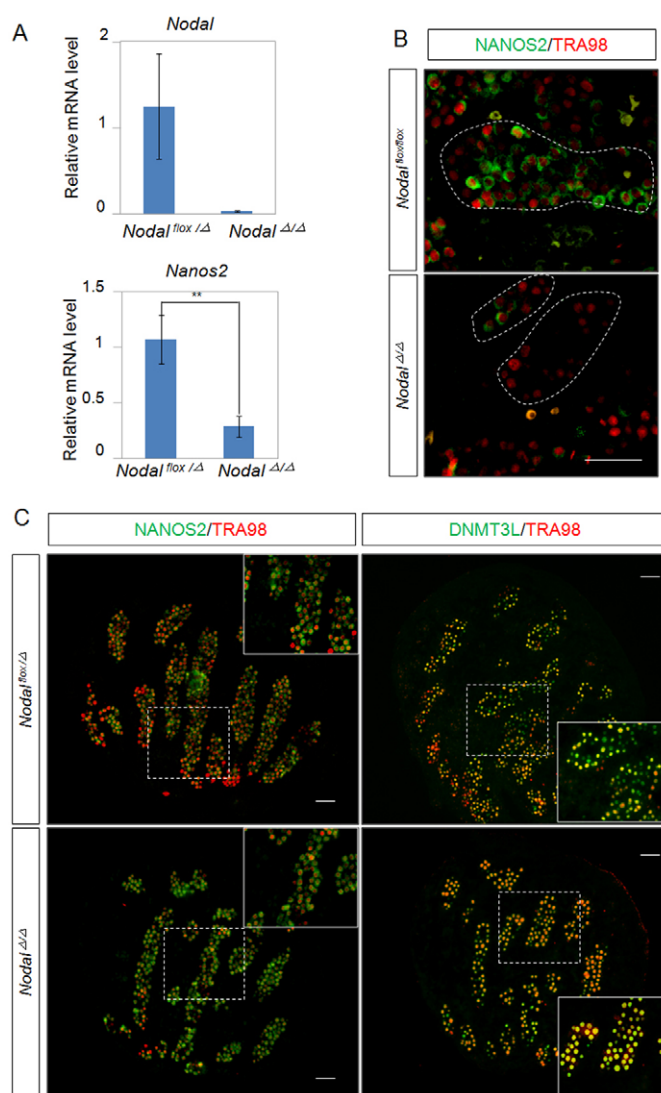


Fig. 5. Mutually redundant roles of Nodal and activin-A in sex differentiation of male germ cells. (A) RT-qPCR analysis of *Nodal* and *Nanos2* mRNA levels in the testes of *Nodal^{Δ/Δ}* mice (for mutants, $n=3$; for controls, $n=4$). Tamoxifen was injected at E10.5 and gonads were harvested at E14.5. (B) Immunostaining for NANOS2 in *Nodal^{Δ/Δ}* and *Nodal^{flox/flox}* testes. (C) Immunostaining for NANOS2 (left) and DNMT3L (right) in the E16.5 *Nodal^{Δ/Δ}* 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 \pm s.e.m. ** $P<0.01$. Scale bars: 50 μ m.

difference to the higher concentration of inhibitor used in our study; the 50% inhibitory concentration of SU5402 is 10–20 μ M, and the concentration of 5 μ M 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) (Fig. 7A; supplementary material Fig. S8A), implying that nodal/activin signals act downstream of FGF signaling. Inhibition of FGF together with RA signaling could not promote male differentiation (supplementary material Fig. S7D). This indicates that FGF signaling plays a dual role in promoting the male pathway and in suppressing meiosis.

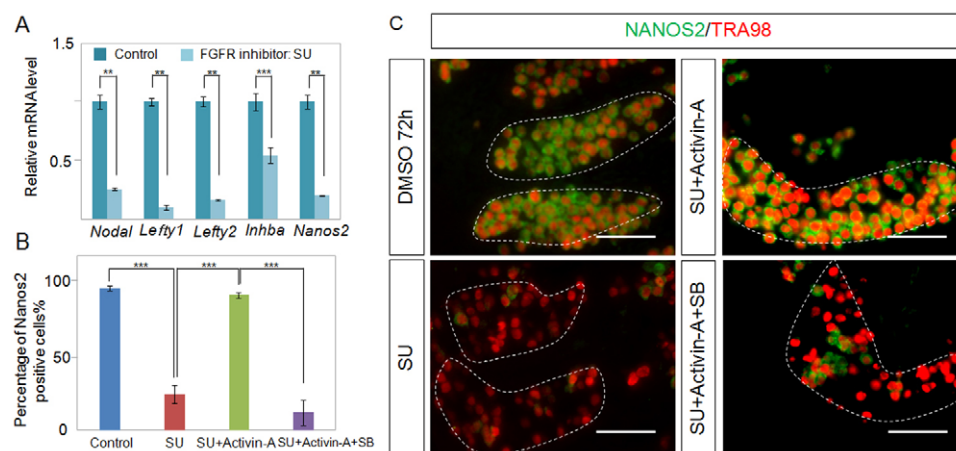


Fig. 6. Interactions between FGF and nodal/activin signaling pathways in the differentiation of male germ cells. (A) E11.5 mouse testes were cultured with the FGF receptor inhibitor SU5402 (40 μ M) or DMSO for 48 hours and the expression levels of *Lefty1/2*, *Nodal*, *Inhba* and *Nanos2* were examined using RT-qPCR ($n=3$; using *Mvh* as normalization control except for *Inhba*, for which *G3pdh* was used for normalization). (B,C) E12.5 testes were cultured for 72 hours with SU5402 (40 μ M), activin-A (100 ng/ml), or SB431542 (40 μ M) as indicated. (B) Percentage of NANOS2-positive cells after treatment ($n=3$). (C) Immunostaining with an anti-NANOS2 antibody (green) and the germ cell marker TRA98 (red). Dashed lines outline a testicular cord. Bars in graphs represent mean \pm s.e.m. ** $P<0.01$, *** $P<0.001$. Scale bars: 50 μ m.

We hypothesized that FGF9 might promote male sexual differentiation through activating nodal/activin signaling. To test this possibility, we added an exogenous TGF β signal (activin-A) together with SU5402 to isolated testes from E12.5 embryos. Activin-A enhanced *Nanos2* expression in the absence of FGF9 signaling (Fig. 6B,C). Furthermore, additional treatment with SB431542 resulted in the marked abrogation of this enhancement, indicating that activin-A works through the nodal/activin signaling pathway (Fig. 6B,C). Interestingly, when E11.5 fetal testes were treated with SU5402 and activin-A together, the expression of *Nanos2* could not be rescued (supplementary material Fig. S7C).

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

Because pSMAD2 was also detected in somatic cells, we 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. By contrast, this arrangement was disordered when testes were cultured in the presence of SB431542 (Fig. 7A). Disruption of the testis cords was also observed by detecting laminin protein, which marks the basal lamina surrounding the testis cords (supplementary material Fig. S8B) (Tilman and Capel, 1999). This disruption was not associated with the downregulation of SOX9 at either the mRNA or protein level (Fig. 7A; supplementary material Fig. S8A), 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, we 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). We 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 (Fig. 7B). Interestingly, even though *Wnt4* and *Foxl2* mRNA levels did not change after SB431542 treatment, *Bmp2* and follistatin expression levels were significantly upregulated (Fig. 7B). These results indicate that FGF9 and nodal/activin signaling inhibited the female differentiation pathway in somatic cells in a different manner. 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 (supplementary material Fig. S7C). 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 (Fig. 6B,C).

Overall, these results suggest that FGF9 regulates testicular differentiation through 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 (Fig. 7C).

DISCUSSION

Suppression of meiotic entry and induction of the male-specific gene *Nanos2* in germ cells are indispensable for establishing the eventual spermatogenic 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 contrast, 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

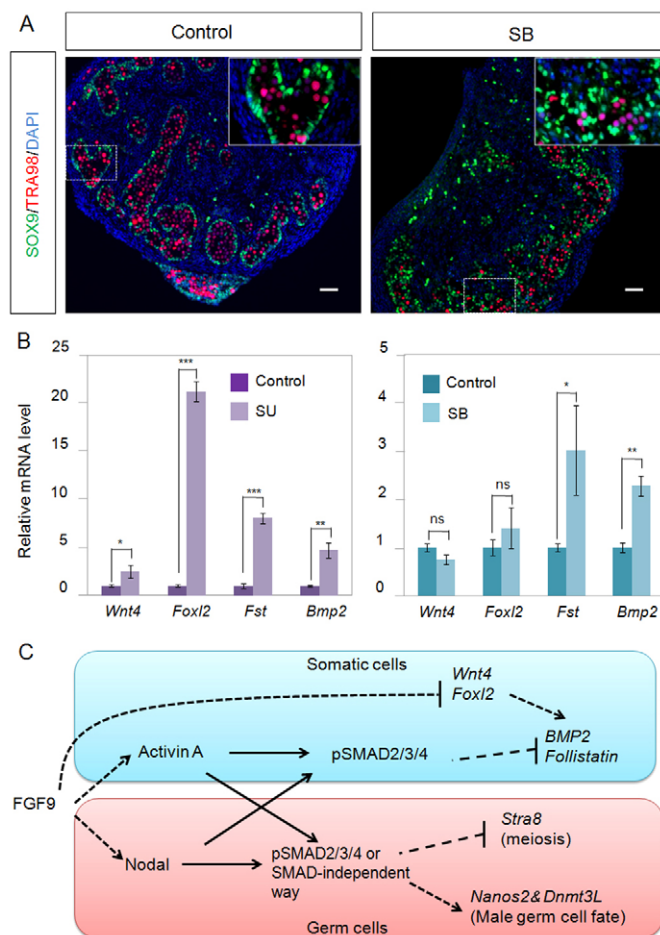


Fig. 7. Nodal/activin is required for normal differentiation of somatic cells. (A) Immunostaining for SOX9 (green) and TRA98 (red)

after inhibitor treatment. Testes from mice at E12.5 were dissected and cultured with the TGF β receptor inhibitor SB431542 (40 μ M) or DMSO for 48 hours. Insets show higher magnification of the boxed areas.

(B) E11.5 testes were cultured with the FGF receptor inhibitor SU5402 (40 μ M) or SB431542 (40 μ M) together with FMK (10 μ M) for 48 hours and the expression levels of indicated genes were examined using RT-qPCR ($n=3$ using *G3pdh* as a normalization control).

(C) 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 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 pathway thwarts the process of female differentiation by inhibiting *Bmp2* and *follistatin*. Bars in graphs represent mean \pm s.e.m. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. ns, not significant. Scale bars: 50 μ m.

involved in FGF9 signaling. Here, we 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, we observed contrary effects of a nodal inhibitor and activin-A on purified male germ cells. Secondly, we 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. We 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. We 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.

We 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. We 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 co-factor Cripto, which makes a positive regulatory loop between FGF and the nodal signaling pathways (Mathieu et al., 2004).

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. We 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 might 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 β A in fetal Leydig cells decreased Sertoli cell proliferation and caused abnormal testicular histology (Archambeault and Yao, 2010). We 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.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.087882/-/DC1>

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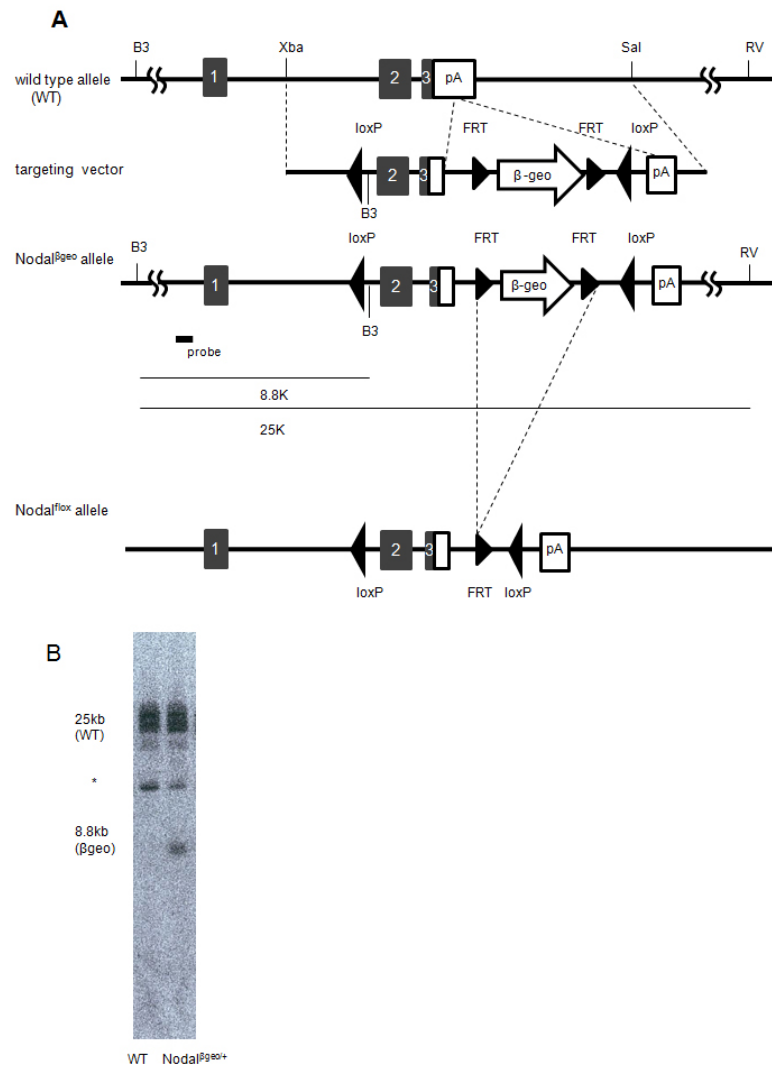


Fig. S1. Generation of floxed alleles for *Nodal*. (A) Targeting strategy. The genomic organization of the *Nodal* gene is shown at the top of the panel. Homologous recombination would insert a *loxP* site into the intron 1 and a [*FRT*- β geo-*FRT*-*loxP*] cassette into the 3'-untranslated region, generating the *Nodal* ^{β geo} allele. B3, *Ban*III; RV, *Eco*RV; Sal, *Sal*I; Xba, *Xba*I. Southern blot analysis of *Eco*RV- and *Ban*III-digested DNA with the 5'-external probe (probe) detects an 8.8-kb fragment and a 25-kb fragment in the wild-type *Nodal* allele and the *Nodal* ^{β geo} allele, respectively. (B) Southern blot analysis of ES clones. Genomic DNA was digested with *Eco*RV and *Ban*III and subjected to hybridization with the 5' external probe indicated in A. An 8.8-kb fragment was detected in correctly targeted ES clones. The 15-kb fragment shown by the asterisk was not derived from the *Nodal* gene.

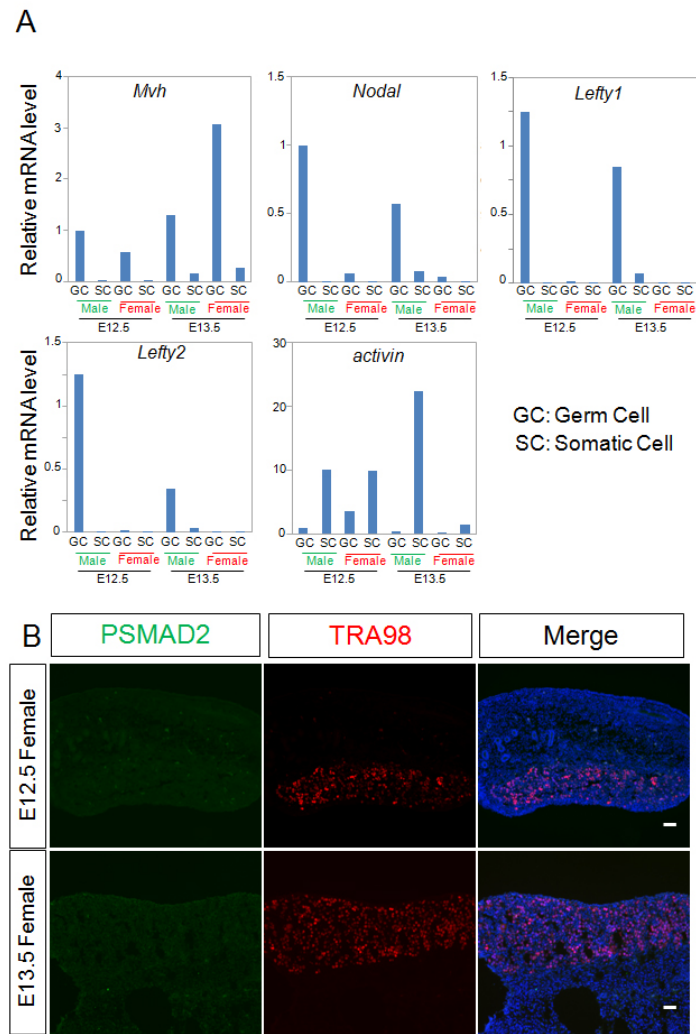


Fig. S2. Nodal/activin signaling is activated in male germ cells but not in fetal ovaries. (A) Germ cells and somatic cells were separated immunomagnetically from E12.5-13.5 gonads and RT-qPCR was performed to analyze the expression levels of *Mvh*, *Nodal*, *Lefty1/2* and *Inhba*. (B) Immunostaining for pSMAD2 (green) in ovaries at E12.5. TRA98 (red) was used as a marker of germ cells. DAPI (blue) was used to detect nuclei. pSMAD2 was not observed in either germ cells or somatic cells. Scale bar: 50 μ m.

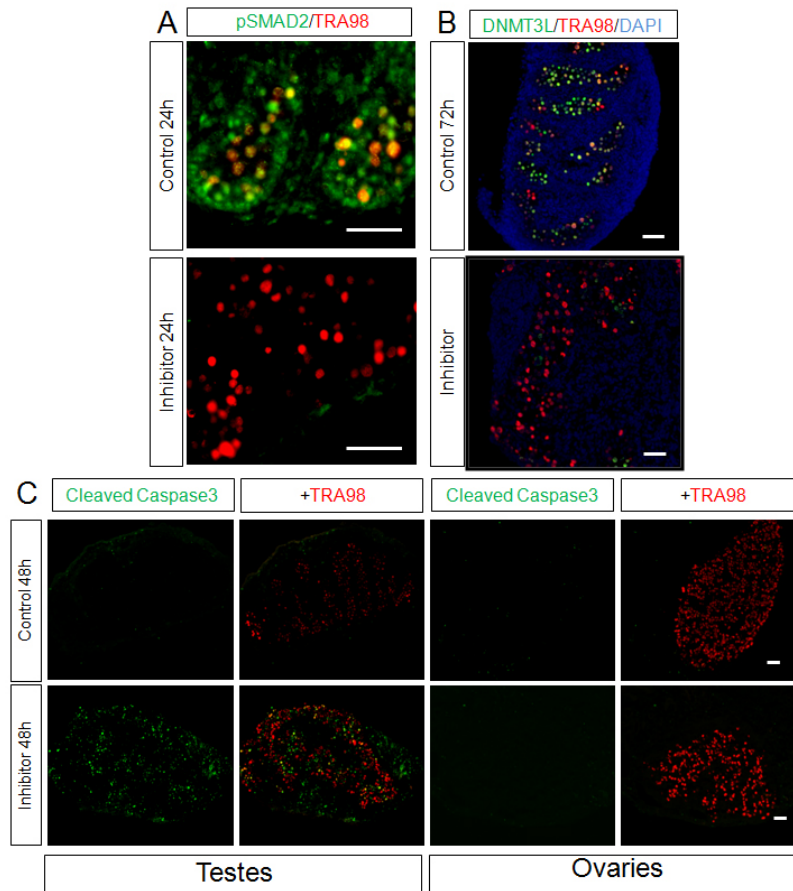


Fig. S3. Treatment with SB431542 suppresses the expression levels of pSMAD2 and DNMT3L and induces apoptotic cell death in the fetal testis. (A,B) Testes from mice at E12.5 were cultured with the TGF β receptor inhibitor SB431542 (40 μ M) or DMSO (control vehicle) for 24 (A) or 72 (B) hours. Results of immunostaining for pSMAD2 (green in A) and DNMT3L (green in B) together with TRA98 (red), indicate that SB431542 suppressed the expressions of both pSMAD2 and DNMT3L. (C) Testes or ovaries from mice at E12.5 were dissected and cultured with SB431542 (40 μ M) or DMSO for 24 hours. Gonads were then immunostained with anti-cleaved caspase 3 (green) and TRA98 (red) antibodies. 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). Scale bars: 50 μ m.

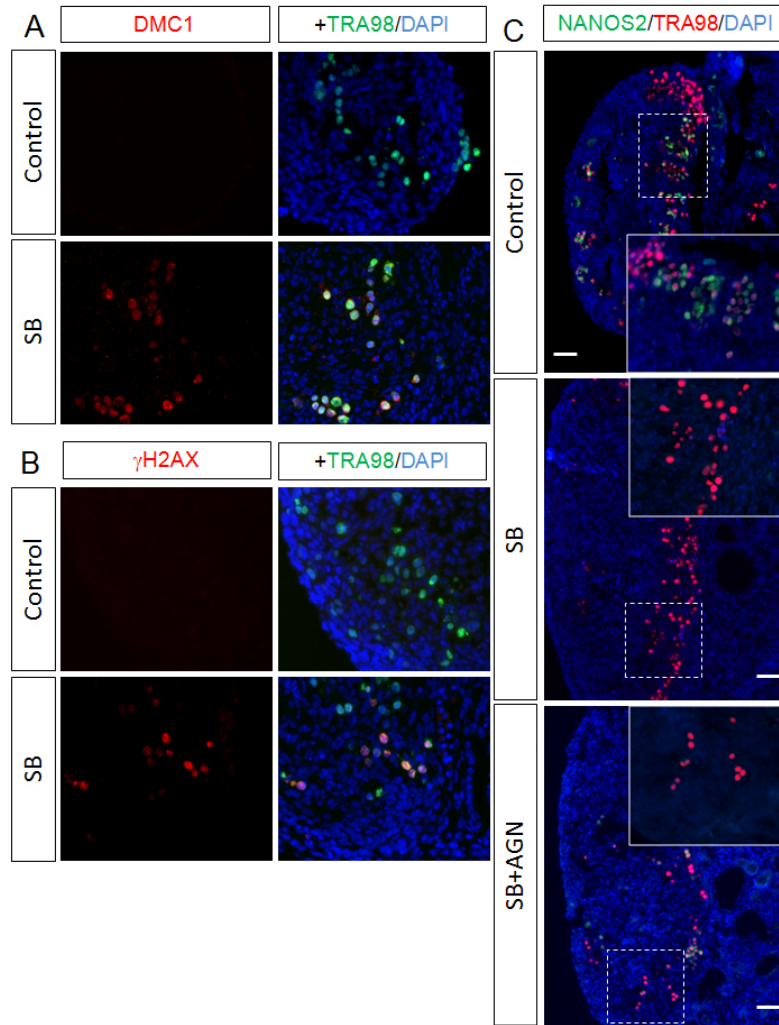


Fig. S4. SB431542 promotes meiosis and inhibits NANOS2 expression independently of RA signaling. (A,B) Testes from mice at E11.5 were cultured with the TGF β receptor inhibitor SB431542 (40 μ M) or DMSO (control vehicle) for 72 hours. Immunostaining with DMC1 (A) and γ H2AX (B) indicated that treatment of testes with SB431542 promoted meiosis. (C) Immunostaining with NANOS2 protein in testes treated with SB431542 alone or together with RA receptor antagonist AGN 193109. Testes from mice at E12.5 were cultured with DMSO (control vehicle; upper panel), the TGF β receptor inhibitor SB431542 (40 μ M; middle panel) or the RA receptor antagonist AGN 193109 (5 μ M; lower panel) for 48 hours. After treatment, the expression of NANOS2 was investigated by immunostaining ($n=2$). Scale bars: 50 μ m.

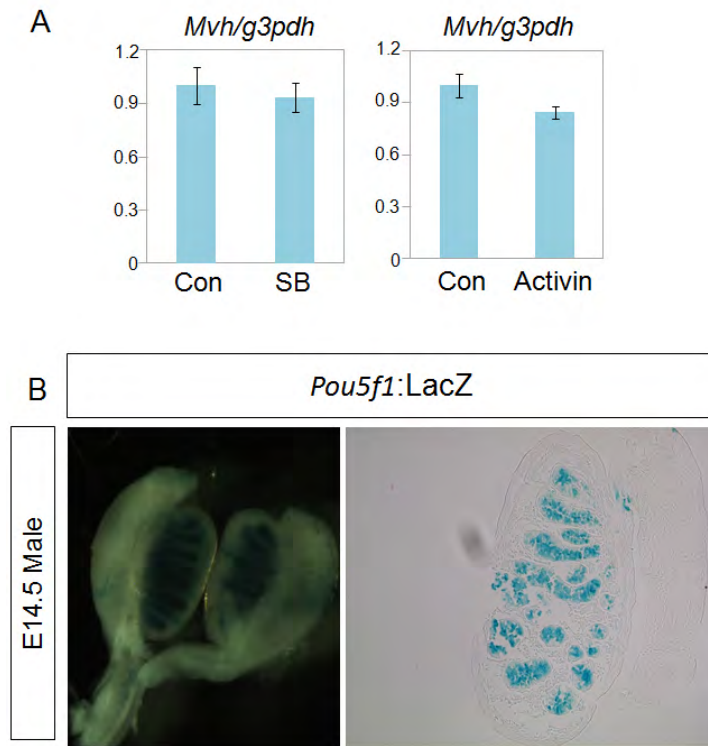


Fig. S5. Nodal/activin signaling directly acts on male germ cells. (A) Male germ cells were isolated from E12.5 testes and cultured with SB431542 or activin-A for 24 hours; RT-qPCR was used to assay the expression levels of *Mvh*. (B) X-gal staining of *Pou5f1-CreERT2^{lacZ/+}* testes at E14.5. Tamoxifen was injected at E10.5 and E11.5 ($n=3$; only one sample is shown). Bars in graphs represent mean \pm s.e.m.

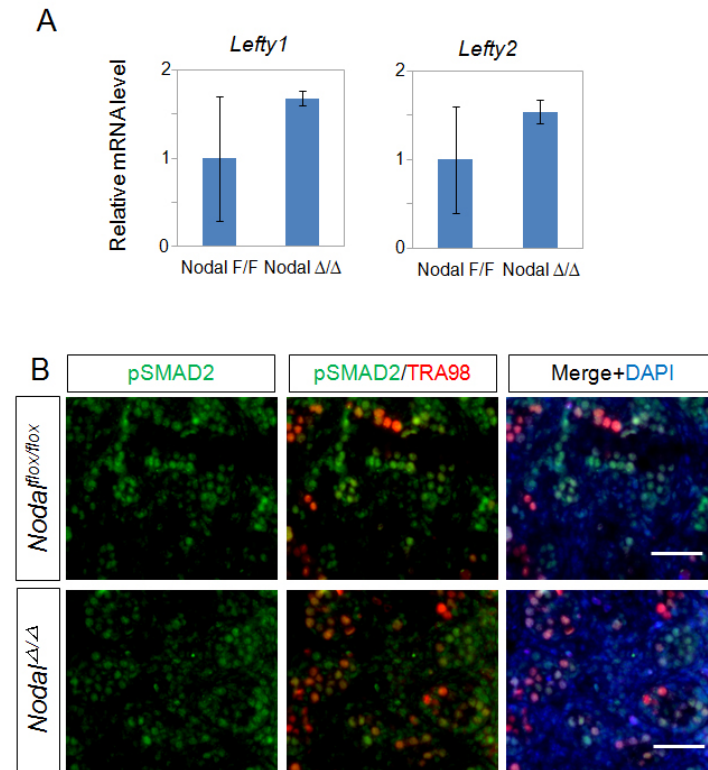


Fig. S6. Expression of Lefty1/2 and pSMAD2 persists in the testes of *Nodal^{Δ/Δ}* mice. (A) Expression of *Lefty1/2* in E14.5 testes from *Nodal^{flox/flox}* and *Nodal^{Δ/Δ}* mice was analyzed using RT-qPCR. (B) Immunostaining with anti-TRA98 (red) and anti-pSMAD2 (green) antibodies in E13.5 testes from *Nodal^{flox/flox}* and *Nodal^{Δ/Δ}* mice. Bars in graphs represent mean \pm s.e.m. Scale bars: 50 μ m.

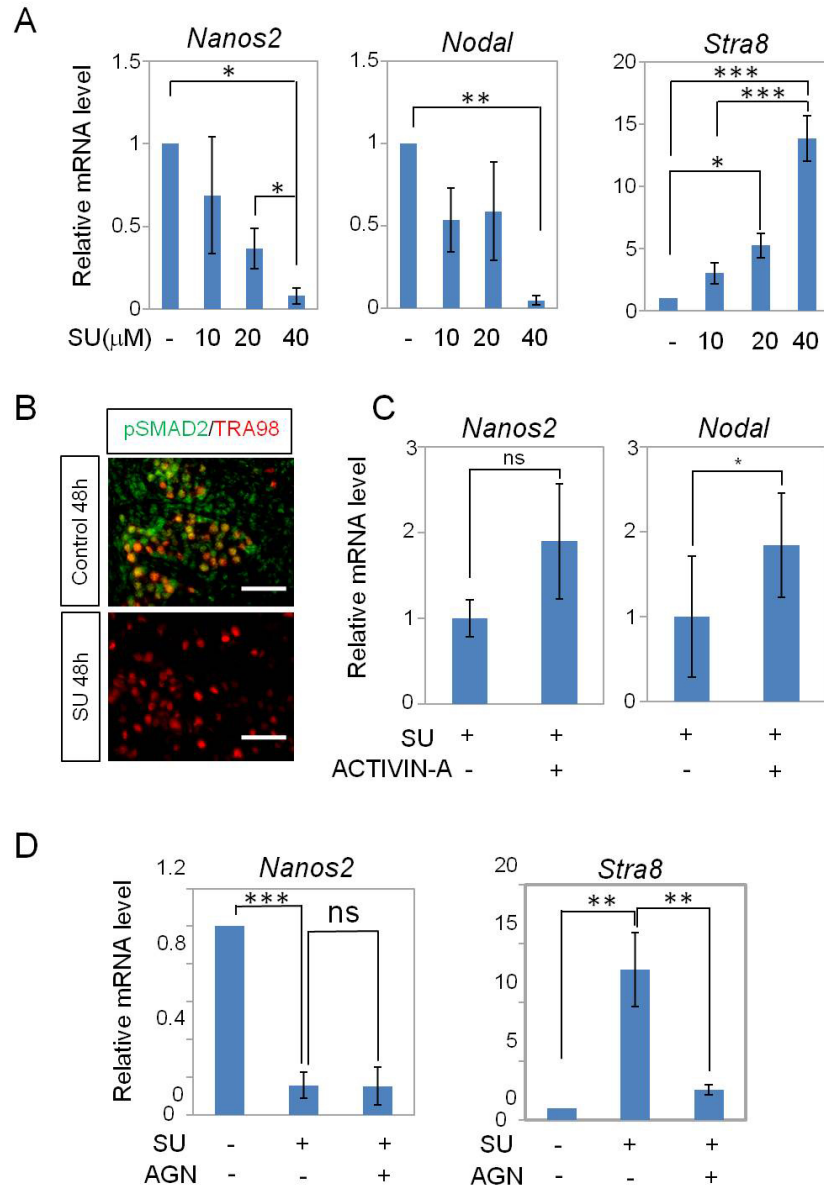


Fig. S7. Nodal signaling acts downstream of FGF signaling. (A) E11.5 testes were cultured with the FGF receptor inhibitor SU5402 (10, 20 or 40 μ M) or DMSO (control vehicle) for 48 hours and the expression levels of *Lefty1/2*, *Nodal* and *Nanos2* were examined using real-time RT-qPCR ($n=3$, using *Mvh* as a normalization control). (B) E11.5 testes were treated with DMSO or SU5402 (40 μ M) for 24 hours and the localization of pSMAD2 was examined using immunostaining. (C) E11.5 testes were cultured for 48 hours with SU5402 alone (40 μ M) or together with activin-A (100 ng/ml), as indicated. Expression levels of *Nodal* and *Nanos2* were assayed using RT-qPCR ($n=3$). (D) Testes at E11.5 were cultured with DMSO or SU5402 (40 μ M; middle panel) or SU5402 together with the RA receptor antagonist AGN 193109 (5 μ M) for 48 hours. The mRNA levels of *Nanos2* and *Stra8* were then examined using RT-qPCR. Bars in the graphs represent the mean \pm s.e.m. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. ns, not significant. Scale bar: 50 μ m.

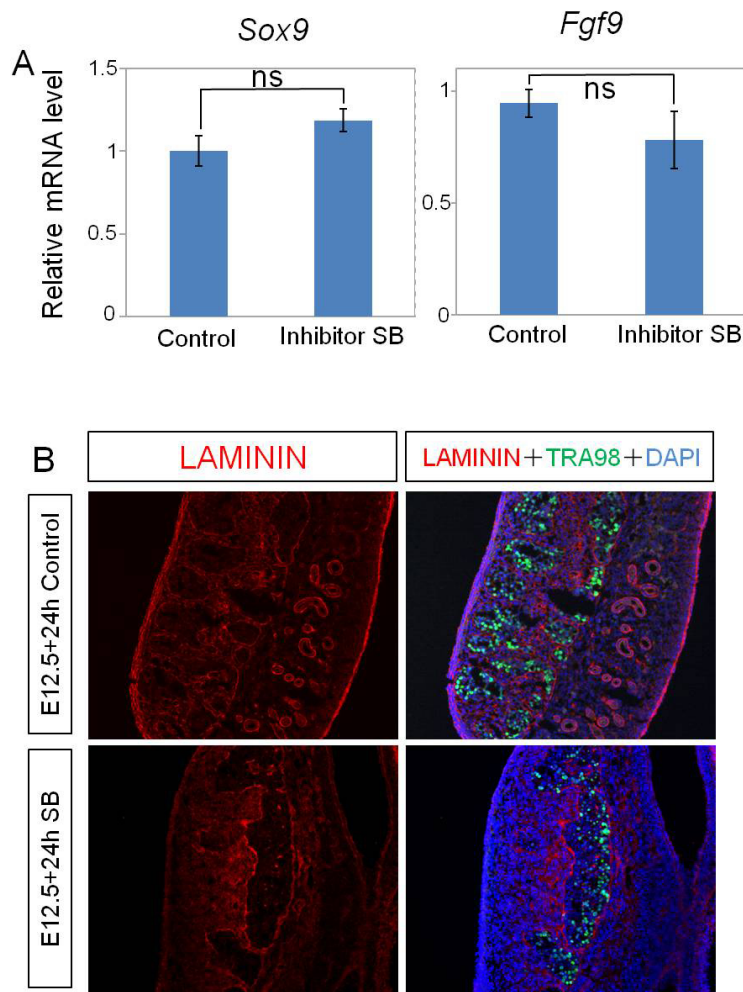


Fig. S8. SB431542 treatment disrupts testicular cords but does not affect *Fgf9* and *Sox9* expression. (A,B) E12.5 testes were cultured with the TGF β receptor inhibitor SB431542 (40 μ M) or DMSO (control vehicle) for 48 (A) or 24 (B) hours. (A) The expression levels of *Sox9* and *Fgf9* were investigated using RT-qPCR ($n=3$). Bars on the graphs represent the mean \pm s.e.m. (B) Immunostaining with anti-laminin in the testes with or without SB431542 treatment. ns, not significant.