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

Quan Wu1, Kohei Kanata2, Rie Saba3, Chu-Xia Deng4, Hiroshi Hamada2 and Yumiko Saga1,3,*

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

1Department of Genetics, SOKENDAI, Yata 1111, Mishima 411-8540, Japan.
2Developmental Genetics Group, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamada-oka, Suita, Osaka 565-0871, Japan.
3Division of Mammalian Development, National Institute of Genetics, Yata 1111, Mishima 411-8540, Japan.
4Genetics of Development and Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA.

*Author for correspondence (ysaga@nig.ac.jp)

Accepted 12 November 2012
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 Nodafllox/− mice. Nodafllox/+ mice were crossed with CAG-Flip transgenic mice (Kanki et al., 2006) to generate Nodafllox/+ 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 (Hirot a 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 M), Z-V AD-FMK (Peptide Institute, 10 μM), 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 TTGAGCCAAAGAGGATCGTGGATGG and reverse CTTCAACATATCGTCTGGTGC; Lefty1: forward CTGTCGCTGGTTAGCCTCAGGGAA and reverse CTCCCTCCTTCAACATGG; Lefty2: forward CGGCTGGACCGTCCTGGTT; Pou5f1: forward AGTACCGAGGTCCAGCAGTGAGAG and reverse GCAGGTTGAAGGATGCTTTGAGC; Nodal: forward AGCCAAGAAGGATCGTGGATGG and reverse GCAGGTTGAAGGATCGTGGATGG; Inhba: forward CCAAGGAAGCAGTACTGGTGAAG and reverse TCTCCCTGACTCGTCACACAA; Gtx9: forward AAAGCAGCCGATGTAATGATTGTT and reverse GCAGTACATTTCTGGCTACATT; Dmnt3l: forward GCTATGCGGGTGTGGAGCAAC and reverse GCAGGTTGAAGGATGCTTTGAGC; Nanos2: forward CCGTCTAGGTCCCAAG and reverse CGGCTGGACCGTCCTGGTT; Foxl2: forward GCCCTCAGAAGTGTTGAGG and reverse AGTGTGTGGAACCCCGATTGCAG; follistatin: forward CATCCTCGTTGAAAGAGACGTTGAGG and reverse TTATTGGTTGCTTCTCCATCAC and reverse CCGGTGCGACGTGGTT; and reverse GCAGGTTGAAGGATGCTTTGAGC; Smad4: forward ACTGCCATCCATGCAC and reverse TGGCGCCCACTGTCCTCAC and reverse GATGCGCCCACTGTCCTCAC and reverse GATGCGCCCACTGTCCTCAC and reverse GATGCGCCCACTGTCCTCAC and reverse GATGCGCCCACTGTCCTCAC and reverse GATGCGCCCACTGTCCTCAC and reverse GATGCGCCCACTGTCCTCAC. The probe for Nosi2 was 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-pSMA D2 (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-pSMA D2, 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 MiniOptic Real-Time PCR Detection System (Bio-Rad).

The following PCR primer pairs were used for amplification (5′-3′):

- Nano2: forward ACACAGGTGACTGCTTCTG and reverse CCGGAAGTCATCACCCAG and reverse CGGCTGGACCGTTCCTGG; Nodal: forward AGTACCGAGGTCCAGCAGTGAGAG and reverse GCAGGTTGAAGGATGCTTTGAGC; Inhba: forward CCAAGGAAGCAGTACTGGTGAAG and reverse TCTCCCTGACTCGTCACACAA; Gtx9: forward AAAGCAGCCGATGTAATGATTGTT and reverse GCAGTACATTTCTGGCTACATT; Dmnt3l: forward GCTATGCGGGTGTGGAGCAAC and reverse GCAGGTTGAAGGATGCTTTGAGC; Nanos2: forward CCGTCTAGGTCCCAAG and reverse CGGCTGGACCGTCCTGGTT; Follistatin: forward CATCCTCGTTGAAAGAGACGTTGAGG and reverse TTATTGGTTGCTTCTCCATCAC and reverse CCGGTGCGACGTGGTT; and reverse GCAGGTTGAAGGATGCTTTGAGC; Smad4: forward ACTGCCATCCATGCAC and reverse TGGCGCCCACTGTCCTCAC and reverse GATGCGCCCACTGTCCTCAC and reverse GATGCGCCCACTGTCCTCAC. The probe for Nosi2 was described previously (Tsuda et al., 2006).

Statistical analysis

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 Figf9 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 TGFβ 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 (Low 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, γH2AX (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
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 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 benzyloxy carbonyl-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 (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. Smad4flac+/Pou5f1-CreERT2+/- males were crossed with either Smad4flac+/+ or Pou5f1-CreERT2+/- 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 were decreased after 24 hours of culture 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-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). 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 γH2AX (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 γH2AX-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 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 (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 Nodalflox/+/Rosa-CreERT2+/– (or Nodalflox/flox/Rosa-CreERT2+/–) females with Nodalflox/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 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) (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.
We hypothesized that FGF9 might promote male sexual differentiation though activating nodal/activin signaling. To test this possibility, we 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 (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) (Tilmann 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).

**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 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...
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 a 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 signaling may induce another factor in somatic cells, which could affect male germ cells.

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 FGF9 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 Dmr1 in Sertoli cells causes female reprogramming even in adult testes (Matson et al., 2011). Interestingly, Dmr1 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 βA 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.

Acknowledgements
We thank Y. Nishimune for generously providing anti-TRA98 antibody, Y. Kanai for the anti-SOX9 antibody and S. Yamanaka for the anti-DNMT3L antibody. We thank H. Kyouko for technical support in performing organ culture. We also thank Y. Furuta for helping us to import Smad4-flox mice.

Funding
This work was supported by the Genome Network Project of the Ministry of Education, Culture, Sports, Science and Technology (MEXT); in part by the Japan Society for the Promotion of Science (JSPS) [KAKENHI Grant Number 21227008 to Y.S.]; and by the Iwatanagi Naoi Foundation [Q.W.J.]. C.-X.D. is supported by the Intramural Program of the National Institutes of Health. Deposited in PMC for release after 12 months.

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

References


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 \([\text{FRT-}\beta\text{-geo-FRT-}
\text{loxP}]\) cassette into the 3’-untranslated region, generating the \(\text{Nodal}^{\beta\text{-geo}}\) 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 an 8.8-kb fragment and a 25-kb fragment in the wild-type Nodal allele and the \(\text{Nodal}^{\beta\text{-geo}}\) 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. 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 μm.
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 TGFβ receptor inhibitor SB431542 (40 μM) 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 μM) 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 μm.
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 TGFβ receptor inhibitor SB431542 (40 μM) or DMSO (control vehicle) for 72 hours. Immunostaining with DMC1 (A) and γH2AX (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 TGFβ receptor inhibitor SB431542 (40 μM; middle panel) or the RA receptor antagonist AGN 193109 (5 μM; lower panel) for 48 hours. After treatment, the expression of NANOS2 was investigated by immunostaining (n=2). Scale bars: 50 μm.
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-CreERT2lacZ/+ 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 ± s.e.m.

Fig. S6. Expression of Lefty1/2 and pSMAD2 persists in the testes of NodalΔ/Δ mice. (A) Expression of Lefty1/2 in E14.5 testes from NodalΔ/Δ and NodalΔ/Δ mice was analyzed using RT-qPCR. (B) Immunostaining with anti-TRA98 (red) and anti-pSMAD2 (green) antibodies in E13.5 testes from NodalΔ/Δ and NodalΔ/Δ mice. Bars in graphs represent mean ± s.e.m. Scale bars: 50 μm.
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 µM) 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 µM) 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 µM) 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 µM; middle panel) or SU5402 together with the RA receptor antagonist AGN 193109 (5 µM) for 48 hours. The mRNA levels of Nanos2 and Stra8 were then examined using RT-qPCR. Bars in the graphs represent the mean±s.e.m. *P<0.05, **P<0.01, ***P<0.001. ns, not significant. Scale bar: 50 µm.
Fig. S8. SB431542 treatment disrupts testicular cords but does not affect Fgf9 and Sox9 expression. (A,B) E12.5 testes were cultured with the TGFβ receptor inhibitor SB431542 (40 μM) 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±s.e.m. (B) Immunostaining with anti-laminin in the testes with or without SB431542 treatment. ns, not significant.