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Sexually Dimorphic Regulation of Inhibin Beta B in Establishing Gonadal Vasculature in Mice[1]

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Abstract

Sexually dimorphic differentiation of gonads is accomplished through balanced interactions between positive and negative regulators. One of the earliest features of gonadal differentiation is the divergent patterning of the vasculature. A male-specific coelomic vessel develops on the anterior to posterior of the XY gonad, whereas this vessel is absent in XX gonads. It is postulated that the testis-determining gene *Sry* controls formation of the coelomic vessel, but the exact molecular mechanism remains unknown. Here we reveal a novel role for inhibin beta B in establishing sex-specific gonad vasculature. In the testis, inhibin beta B contributes to proper formation of the coelomic vessel, a male-specific artery critical for testis development and, later in development, hormone transportation. On the other hand, in the ovary, inhibin beta B is repressed by WNT4 and its downstream target follistatin, leading to the absence of the coelomic vessel. When either *Wnt4* or follistatin was inactivated, the coelomic vessel appeared ectopically in the XX ovary. However, when inhibin beta B was also removed in either the *Wnt4*-null or follistatin-null background, normal ovarian development was restored and no coelomic vessel was found. Our results indicate that the sex-specific formation of the coelomic vessel is established by positive components in the testis as well as an antagonizing pathway from the ovary. Inhibin beta B is strategically positioned at the intersection of these opposing pathways.

Keywords

activin; embryo; follistatin; ovary; testis

INTRODUCTION

In eutherian mammals, sex determination of the gonad is a dominant process controlled by *Sry* (sex-determining region of the Y chromosome; see review in [1]). *Sry* acts through *Sox9* (Sry-related HMG-box gene 9 [2–4]) to initiate specification of different somatic cell lineages, organization of the testis structure, and production of male hormones. In the absence of *Sry*, or when *Sry* is mutated [5–7], the gonadal primordium follows the alleged “default” pathway and develops into an ovary. However, in some human and animal cases, testes arise in XX individuals without the presence of *Sry*, leading to an alternative hypothesis that the testis pathway is default and normally suppressed in the ovary by an antitestis “Z” factor [8]. In the testis, SRY inhibits the Z factor and therefore allows the progression of the testis pathway. This putative Z factor has yet to be identified, and current evidence strongly favors the idea that SRY acts as a positive regulator responsible for triggering the entire spectrum of testis organization.

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Signaling components downstream of SRY have gradually emerged and molecular connections between these components and cellular events in testis organogenesis have been established based on testis phenotypes in gene knockout models [9–17]. A useful approach to identify novel players in the process of gonadal development is to examine XX embryos with mutations that lead to the appearance of testis structure in ovaries. In conjunction with others, we have discovered that null mutations of either *Wnt4* or follistatin (*Fst*) result in the ectopic appearance of the testis-specific coelomic vessel in XX gonads [18–20]. The coelomic vessel, a testis-specific artery [21], plays a critical role in patterning of testis cords and later in hormone transportation. In the XY gonad, the coelomic vessel forms immediately after the expression of *Sry*, whereas in the XX gonad, WNT4 and FST inhibit formation of the coelomic vessel. When this inhibition is removed, because of the absence of *Wnt4* or *Fst*, the coelomic vessel arises ectopically in the XX gonad [20,22]. Epistatic analysis shows that *Fst* expression requires the presence of *Wnt4* [20]. Aberrant expression of *Wnt4* in humans and mice affects normal testis differentiation [22–24]. Furthermore, similar phenotypes have been observed in a human XX patient with a mutation in the *WNT4* gene [25] and in *Wnt4* knockout mice [18], indicating a conserved role of *Wnt4* in female differentiation [18]. This evidence clearly demonstrates that WNT4 and FST antagonize formation of the testis-specific coelomic vessel in the XX gonad. However, the molecular mechanisms for this suppression remain unknown.

To understand how the testis vasculature is suppressed in the XX gonad and simultaneously established in the XY gonad, we began searching for potential targets of WNT4 and FST. In this report, we identify a connection of inhibin beta B *(Inhbb)* to the WNT4/FST pathway in the sexually dimorphic establishment of vasculature in mouse gonads. We demonstrate genetically that testis-specific vasculature is prevented from forming in the embryonic ovary through the actions of WNT4 and FST. Additionally, activin B, a homodimer of INHBB, is able to induce ectopic testis vasculature formation in the embryonic ovary and acts with other factors to establish defined vasculature in the normal course of testis development.

MATERIALS AND METHODS

Animals

Wnt4$^{-/-}$ mice were obtained from the Jackson Laboratory (strain 129-*Wnt4*^{tm1Amc}). Double knockout mice were generated by first crossing *Wnt4*+/− [18] or *Fst*+/− [26] to *Inhbb*+/− [27] to yield double heterozygotes. The heterozygotes were then mated to produce double knockouts. Genotypes were determined by PCR. Timed matings were produced by housing female mice with males overnight and checking for vaginal plugs the next morning (Embryonic Day [E]0.5 =noon of the day when a vaginal plug was found). The sex of each embryo was determined by PCR for the *Sry* gene. All procedures described within were reviewed and approved by the Institutional Animal Care and Use Committee, and were performed in accordance with the *Guiding Principles for the Care and Use of Laboratory Animals*.

Immunocytochemistry

Samples were fixed overnight in 4% paraformaldehyde in PBS at 4°C and then processed for either the whole mount procedure or frozen sectioning [28]. For whole mount immunocytochemistry, samples were washed in PBS for 10 min and blocked in blocking solution (10% heat-inactivated donkey serum and 0.1% Triton X-100 in PBS) for 1 h at room temperature. A rabbit polyclonal antibody against anti-Müllerian hormone (AMH; 1:200; Santa Cruz) and a rat polyclonal antibody against PECAM1 (1:500; Pharmingen) were added to the blocking solution and samples were incubated overnight at 4°C. Samples were then washed three times for 10 min each in washing solution (1% heat inactivated donkey serum and 0.1% Triton X-100 in PBS) followed by incubation in the blocking solution with the secondary antibodies (FITC-conjugated donkey anti-rabbit and Cy3-conjugated donkey anti-rat

antibodies, 1:200; Jackson Immunochemicals). Samples were washed three times for 10 min each in washing solution and mounted for confocal microscopy. For section immunocytochemistry, frozen samples were sectioned to 10 μm thick, dried on poly-lysine-treated slides, and followed the same staining procedure as the whole mount samples.

Whole Mount In Situ Hybridization

Samples were fixed overnight in 4% paraformaldehyde in PBS at 4°C and processed according to the standard nonradioisotopic procedure using a digoxigenin-labeled RNA probe for *Inhbb* [29].

Organ Culture

Genital ridges (gonad + mesonephros) from E11.5 embryos were obtained for organ culture. To determine the sex of E11.5 embryos, we used a staining method [30] to detect the presence of XX-specific Barr bodies in the amnion of individual embryos. Genital ridges were cultured for 48 h on a 1.5% agar block in Dulbecco's minimal Eagle medium (DMEM) supplemented with 10% fetal calf serum (Hyclone) and 50 ug/ml ampicillin at 37° C with 5% CO_2/95% air. Affi-Gel Blue Gel (100–200 mesh wet; Bio-Rad, #153-7302) was soaked in human activin B (200 ng/μl in PBS; R & D) or PBS (control) for 1 h at 37°C before being added to the top of the gonadal explant. Approximately 15 beads were added to the top of each gonadal explant.

Quantitative Real-Time PCR

Gonads without mesonephroi attached were isolated from embryos at E12.5. Wild-type XY gonads were distinguished from XX gonads by morphological characteristics (formation of testis cords and vasculature). Fifteen to twenty pairs of gonads of each sex were pooled and total RNA was isolated using the Trizol method. A ribosomal RNA, 36B4, was used for normalization. PCR primers include *Inhbb*-forward, 5′-TATGTCCTGGA-GAAGGGCAGC-3′; *Inhbb*-reverse, 5′-GCGATGTCTGCTATCGCCCAG-3′; 36B4-forward, 5′-CGACCTGGAAGTCCAACTAC-3′; and 36B4-reverse, 5′-ATCTGCTGCATCTGCTTG-3′. Complementary DNA was diluted 1:10, then 10 μl was added to 12.5 μl SYBR green PCR mixture (Applied Biosytems), 1.5 μl water, and 1.25 pmol primer mix. PCR reactions were carried out using the ABI Prism 7000 Sequence Detection System (Applied Biosystems). To control for overall gene expression, the average cycle threshold for 36B4 was subtracted from the average Ct value for *Inhbb* to generate ΔCt. Fold change was then calculated as 2 to the −ΔΔCt power ($2^{-\Delta\Delta Ct}$). Finally, relative abundance was presented as ($2^{-\Delta\Delta Ct}$ XY)/($2^{-\Delta\Delta Ct}$ XX). Student *t*-tests ($P < 0.01$ is considered significant) were used to analyze statistical differences between XY and XX samples. Each sample was analyzed in triplicates and experiments were repeated at least three times using at least two different pools of RNA to synthesize cDNA.

RESULTS

Inhibin Beta B, but not Beta A, is Expressed in XX Gonads

Activins consist of either homodimers or heterodimers of inhibin beta A (INHBA) and beta B (INHBB) subunits (activin A =beta A:beta A, activin B =beta B:beta B, and activin AB = beta A:beta B). We examined the expression patterns of these two inhibin subunits in mouse gonads at E12.5, when the development of dimorphic vasculature in gonads occurs. By section in situ hybridization we found that XY gonads expressed both *Inhba* and *Inhbb* (Fig. 1, A and B), whereas the XX gonad expressed *Inhbb* but not *Inhba* (Fig. 1, C and D). Absence of staining with the sense probes showed the specificity of the probes (Fig. 1, E and F; only XY gonads are shown here). The presence of *Inhbb* in the XX gonad indicated that *Inhbb* could be a target of WNT4 and FST. We therefore examined expression of *Inhbb* at E11.5, when the testis-

specific coelomic vessel begins to form. *Inhbb* expression was similar in XY and XX gonads at E11.5 (Fig. 2, A and B; n = 4). At E12.5, *Inhbb* expression in XX gonads was reduced, but not completely eliminated (Figs. 1D and 2, B and F), while XY gonads continue to express a high level of *Inhbb* in testis cords (Figs. 1B and 2E). The staining patterns of *Inhbb* expression were similar in samples analyzed by section (Fig. 1) and whole mount in situ hybridization (Fig. 2), with the exception that the staining in E12.5 XX gonads was low in whole mount samples (compare Fig. 1D with Fig. 2F). To further confirm these in situ hybridization results, we measured the relative abundance of *Inhbb* expression between E12.5 XY and XX gonads by quantitative real-time PCR (Fig. 1G; n =3). *Inhbb* mRNA was present in both E12.5 XY and XX gonads and relative *Inhbb* mRNA abundance in the XY gonad is, on average, 4.68-fold higher than that in the XX gonad ($P < 0.001$).

Epistatic Connections Between *Inhbb, Wnt4,* and *Fst*

The presence of *Inhbb* in the XX gonad suggests that *Inhbb* could be a target of WNT4 and FST, two factors known to play critical roles in early ovarian development [18,20,22]. We therefore examined whether the expression pattern of *Inhbb* was altered in the absence of *Wnt4* or *Fst*. In *Wnt4*−/− XX gonads, *Inhbb* expression at E11.5 and E12.5 was elevated (Fig. 2, C and G, n =4) compared to that in the wild type (Fig. 2, B and F). However, this altered pattern was not observed in the *Fst*−/− XX gonad (Fig. 2, D and H). These results demonstrate a unique molecular relationship between *Inhbb* and *Wnt4*, in which WNT4, but not FST, reduces *Inhbb* expression at the transcriptional level in XX gonads. We also examined *Inhba* expression and found that *Inhba* was not present in *Wnt4*−/− or *Fst*−/− XX gonads (not shown), further indicating that *Inhba* is not a target of WNT4 and FST.

Inhbb Is Responsible for Formation of the Coelomic Vessel in *Wnt4*- and *Fst*-Null XX Gonads

The upregulation of *Inhbb* in *Wnt4*−/− XX gonads at E11.5 and E12.5 (Fig. 2, C and G) suggests that *Inhbb* may be responsible for phenotypes appearing at similar stages in *Wnt4*−/− XX gonads, specifically the ectopic formation of the coelomic vessel. We therefore generated the *Wnt4*−/−;*Inhbb*−/− double-null embryos to test whether removal of *Inhbb* can prevent formation of the coelomic vessel and restore the normal ovarian phenotypes. E12.5 gonads were immunostained with an anti-PECAM1 antibody, which demarcates the vasculature and primordial germ cells [11] (Fig. 3, red staining), and an anti-AMH antibody, which highlights Sertoli cells in testis cords (Fig. 3, green staining). In the wild-type XY and *Wnt4*−/− XX gonad (Fig. 3, A and B), the coelomic vessel formed along the anterior-posterior axis of the coelomic surface, whereas this vessel was not found in the wild-type XX gonad (Fig. 3D). Absence of *Inhbb* in the *Wnt4*-null background reversed the vessel phenotype to wild-type (Fig. 3E), indicating that the upregulation of *Inhbb* in the *Wnt4*−/−XX gonad is responsible for the ectopic formation of the coelomic vessel.

If WNT4 prevents coelomic vessel formation by decreasing *Inhbb* expression, what causes the coelomic vessel to form in the *Fst*−/− XX gonad, in which *Wnt4* remains expressed normally [20]? As shown in Figure 1, D and G, *Inhbb* expression (or activin B production) was still present in normal XX gonads. One function of FST could be to prevent this residual expression of *Inhbb* from inducing the coelomic vessel. To test this hypothesis, we generated *Fst*−/−;*Inhbb* −/− embryos and found that, similarly to the *Wnt4*−/−;*Inhbb*−/− XX gonad, the vessel phenotype in the *Fst*-null background was rescued by inactivating *Inhbb* (Fig. 3, C and F). XX gonads that lost only *Inhbb* (*Wnt4*+/+;*Inhbb*−/− or *Fst*+/+;*Inhbb*−/−) developed normally without any apparent defects (data not shown).

Activin B Induces Ectopic Formation of the Testis-Specific Vessel in Cultured XX Gonads

To further demonstrate the ability of activin B (homodimer of INHBB) to induce formation of the coelomic vessel, we conducted an in vitro gain-of-function experiment by culturing E11.5

XX gonads with human activin B (which shares >98% homology with mouse activin B based on NCBI BLAST analysis). After 48 h of culture, the coelomic vessel had formed in the activin-B treated, but not in the control, XX gonad (Fig. 4, A and B). This ectopic coelomic vessel was located in the cortical domain of the XX gonad, with elaborate branching resembling that present in the XY gonad. Culturing XX gonads in the presence of activin B caused the development of the coelomic vessel, thereby recapitulating the *Wnt4*−/−and *Fst*−/− ovarian phenotypes.

Vasculature Defects in *Inhbb*−/− XY Gonads

One remaining question is whether *Inhbb* or activin B has a functional role in normal coelomic vessel formation in the XY gonad. Under normal circumstances, *Inhbb* is expressed in the XY gonad at E11.5 and E12.5, coinciding with the time that the coelomic vessel forms (Fig. 2). We found that in the *Inhbb*−/− XY gonad, the coelomic vessel still formed, but with varying degrees of abnormality. Compared to the vessel in the wild type at the same stage (E12.5), the calibers of the coelomic vessels in most *Inhbb*−/− XY gonads were ~50% of that in wild-type XY gonad and were occasionally discontinuous (Fig. 4, C and D). In some cases, the branching of the coelomic vessel was also decreased.

DISCUSSION

Activins, members of the transforming growth factor (TGF) beta family of proteins, are involved in the patterning and organogenesis of vertebrate embryos [31,32], as well as the endocrine regulation of various tissues in adults, especially the reproductive system [33,34]. In the adult, activins act to stimulate secretion of FSH from the anterior pituitary and simultaneously modulate germ cell and somatic cell development in gonads. Functions of activins in the adult reproductive system have been examined extensively. Here we reveal a unique role of activin in the sexually dimorphic differentiation of embryonic gonads.

The presence of *Inhbb* in the XX gonad is particularly intriguing because *Fst*, a gene that encodes an activin inhibitor, is expressed in XX but not in XY gonads [19,20], suggesting an ovary-specific inhibition of activin activity. FST binds certain members of the TGF beta family of proteins and prevents them from activating their receptors [35]. FST has the highest affinity for activins, and the inhibitory effects of FST on activins have been established in vivo and in vitro (see reviews [36,37]). *Fst* is expressed in the XX gonad starting at E11.5 [19,20], similarly to *Wnt4*, another XX-specific gene. Genetic analysis has revealed that WNT4 stimulates the expression of *Fst*, and null mutations of either *Wnt4* or *Fst* result in a similar phenotype in the XX gonad: ectopic appearance of the testis-specific coelomic vessel [20,22]. These findings indicate that in the XX gonad, WNT4, and FST form a signaling cascade that suppresses formation of the testis-specific coelomic vessel [20].

Absence of the coelomic phenotypes in *Inhbb*−/−;*Wnt4*−/− and *Inhbb*−/−;*Fst*−/− XX gonads provides the first genetic evidence that *Inhbb* is responsible for the formation of the coelomic vessel in *Wnt4*−/− and *Fst*−/− ovaries. We found that, under normal circumstances, *Inhbb* expression is low in the XX gonad because of the inhibitory effect of WNT4 on mRNA transcription, and when *Wnt4* is absent, *Inhbb* transcription is upregulated (Fig. 2). On the other hand, *Inhbb* expression is not significantly altered in *Fst*−/− XX gonads as compared to *Wnt4* −/− XX gonads, indicating a different mechanism of FST on activin functions. No *Inhba* is expressed in the XX gonads of these knockouts, indicating that activin B is the only protein product produced. We therefore propose that in the *Wnt4*−/− ovary, where both *Wnt4* and *Fst* are absent [20], activin B acts at full strength to induce coelomic vessel formation. In the *Fst*−/− XX gonad, although WNT4 is present and able to reduce *Inhbb* expression (Fig. 2), the residual activin B is still capable of inducing the coelomic vessel without the interference of FST, as shown in the *Fst*−/− ovaries [20]. This notion is further supported by a minor difference

in vessel phenotypes between *Wnt4*- and *Fst*-null ovaries. In the *Wnt4*−/− XX gonad (high *Inhbb* expression; Figs. 2C and 3B), the coelomic vessel was bigger and more elaborate than that in the *Fst*−/− XX gonads (low *Inhbb* expression; Figs. 2D and 3C).

Our findings are also consistent with the idea that multiple/redundant pathways regulate coelomic vessel formation in the testis. It is known that the XY gonad produces unidentified chemoattractants to recruit endothelial cells from the mesonephros to form the coelomic vessel. Many TGF beta family proteins, such as AMH, bone morphogenetic proteins [38], and activin A (data not shown) are able to induce mesonephric cell migration and ectopic coelomic vessel formation in the XX gonad in culture. Activin was also shown to be able to induce tubulogenesis of endothelial cells in vitro [39]. Among these factors, AMH and activin A have the most physiological relevance because of their similar expression time frame to activin B in testis development. Similar to our finding on *Inhbb*−/− testis, neither *Amh*−/− [38] nor *Inhba*−/− (unpublished data) male embryos had overt vasculature defects in testis. Furthermore, overexpression of *Wnt4* in XY gonads did not prohibit formation of the coelomic vessel, despite observed disorganization of the vessel [22,24], further indicating the presence of a compensatory mechanism to maintain coelomic vessel formation. Overexpression of *Fst* results in varying degrees of male infertility, but the development of the coelomic vessel was not examined [40].

WNT4 was originally considered as a candidate for the Z factor based on its ability to suppress Leydig cell differentiation [18]. However, further analysis revealed that the ectopic steroidogenic cells in the *Wnt4*-null ovary were adrenal-derived, and most importantly, they also appeared in the *Wnt4*-null testis [41]. These observations indicate that WNT4 is probably not a suppressor of Leydig cell differentiation. The classic definition of the Z factor is that it can antagonize SRY functions and, at the same time, activate the female pathways [8]. Although WNT4 and its downstream target FST do antagonize formation of the testis-specific vasculature (this study, [20,42]), they cannot override the SRY pathway or trigger the ovarian pathway. In embryonic testes in which *Wnt4* or *Fst* was ectopically expressed, primary testis development was not affected [22,24]. Based on these findings, we conclude that *Wnt4* and *Fst* are not candidate genes for the Z factor.

In summary, our findings place *Inhbb* (or its product activin B) in a unique position among the cellular pathways of gonadal development. One function of activin B is to contribute to coelomic vessel formation in the testis in conjunction with other factor(s). This function is suppressed in the XX gonad through the activity of WNT4 and one of its downstream targets, FST. Conversely, in the XY gonad, testis determining genes including SRY and/or SOX9 (see review [1]) are thought to suppress *Wnt4* expression, allowing activin B to work with other factors, such as other TGF beta proteins, to facilitate coelomic vessel formation (Fig. 4E). It remains uncertain whether the SRY pathway directly regulates *Inhbb*. If this is the case, regulators downstream of SRY (*Sry* is not present in the XX individual) must be active in the *Wnt4*−/− XX gonad to trigger *Inhbb* expression. However, none of the key players in the SRY pathway, such as *Sox9*, desert hedgehog [18], or *Amh*, were present in the *Wnt4* and *Fst*−/−XX gonad, indicating that *Inhbb* expression may not require the activation of the SRY pathway (Fig. 4E). These observations further suggest a novel concept that activin B could act through a neutral or default mechanism to induce coelomic vessel formation in gonadal differentiation.

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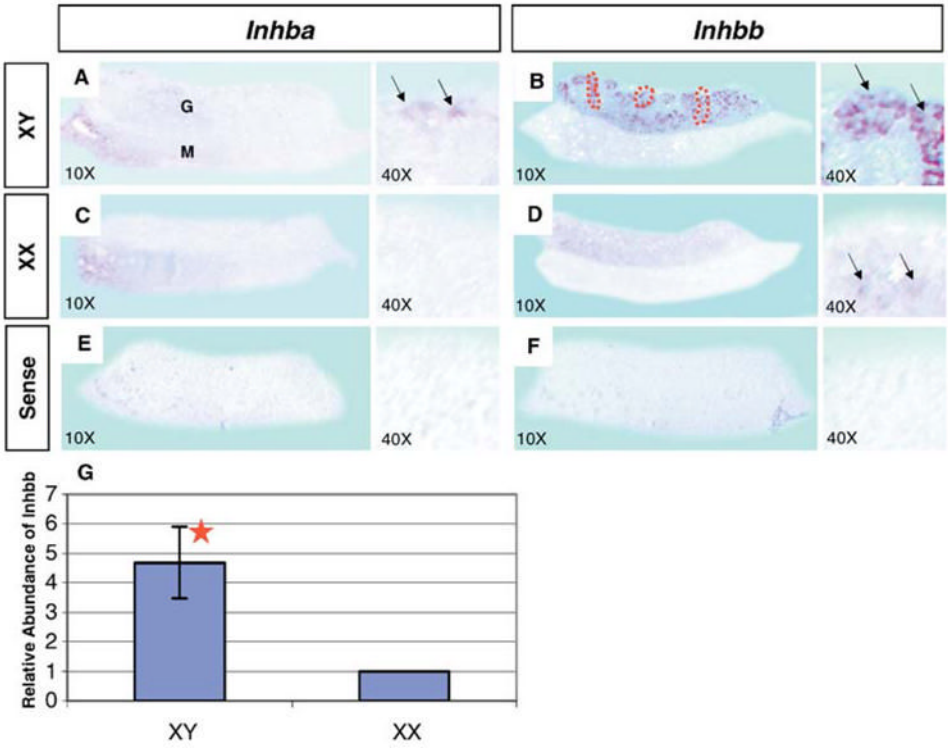

FIG. 1.

Section in situ hybridization of *Inhba* in XY (**A**) and XX (**C**) gonads and *Inhbb* in XY (**B**) and XX (**D**) gonads at E12.5. Positive staining appears as purple deposits and is indicated by black arrows. Negative controls using sense *Inhba* or *Inhbb* probes are shown in **E** and **F**. Magnifications ×10 and ×40 (two magnifications are shown for each sample). Real-time PCR analysis of *Inhbb* expression in XY and XX gonads is shown in **G**. The Y axis represents the relative fold differences between XY and XX gonads. The star indicates a statistical difference ($P < 0.001$). The red dotted outlines indicate testis cords. G, Gonad; M, mesonephros.

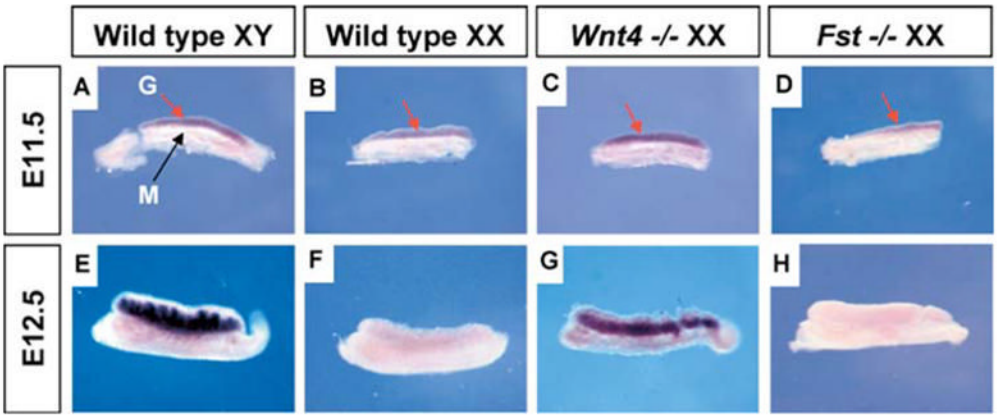

FIG. 2.

Whole mount in situ hybridization for *Inhbb* in gonads from E11.5 and 12.5 wild-type XY (**A** and **E**), wild-type XX (**B** and **F**), *Wnt4*−/− XX (**C** and **G**), and *Fst*−/− XX (**D** and **H**). G, Gonad; M, mesonephros; red arrow, gonad at E11.5. Original magnification ×6.

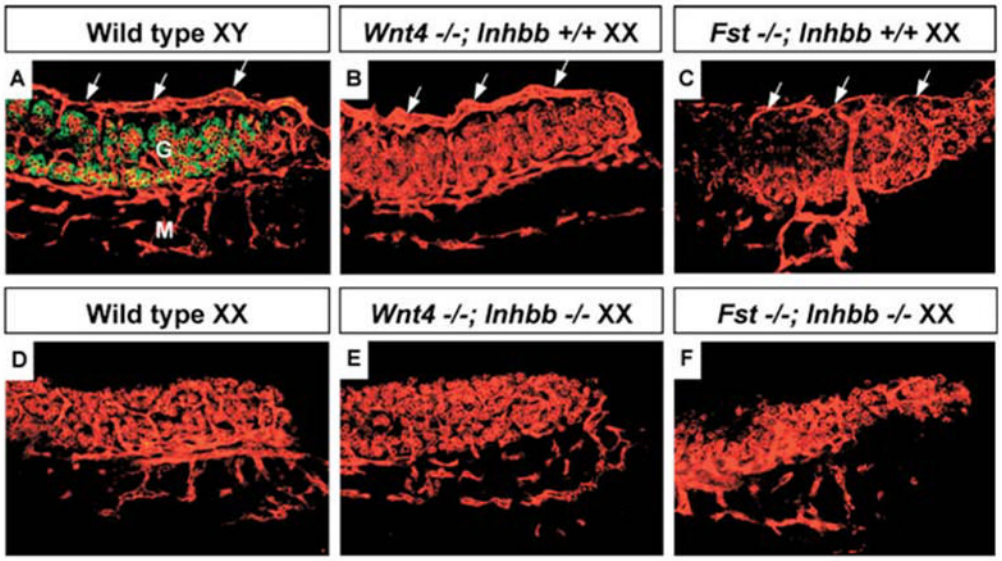

FIG. 3.

Inhbb is responsible for ectopic formation of the coelomic vessel in *Wnt4* and *Fst*−/− XX gonads. The coelomic vessel (white arrows) and vasculature was delineated by an anti-PECAM1 antibody (red), which also marks primordial germ cells (red cells in gonads). Sertoli cells were also highlighted by an anti-AMH antibody (green). **A**) Wild-type XY. **B**) *Wnt4*−/−; *Inhbb*+/+ XX. **C**) *Fst*−/−;*Inhbb*+/+ XX. **D**) Wild-type XX. **E**) *Wnt4*−/−;*Inhbb*−/− XX. **F**) *Fst* −/−;*Inhbb*−/− XX. G, Gonad; M, mesonephros. n = 4. Original magnification ×25.

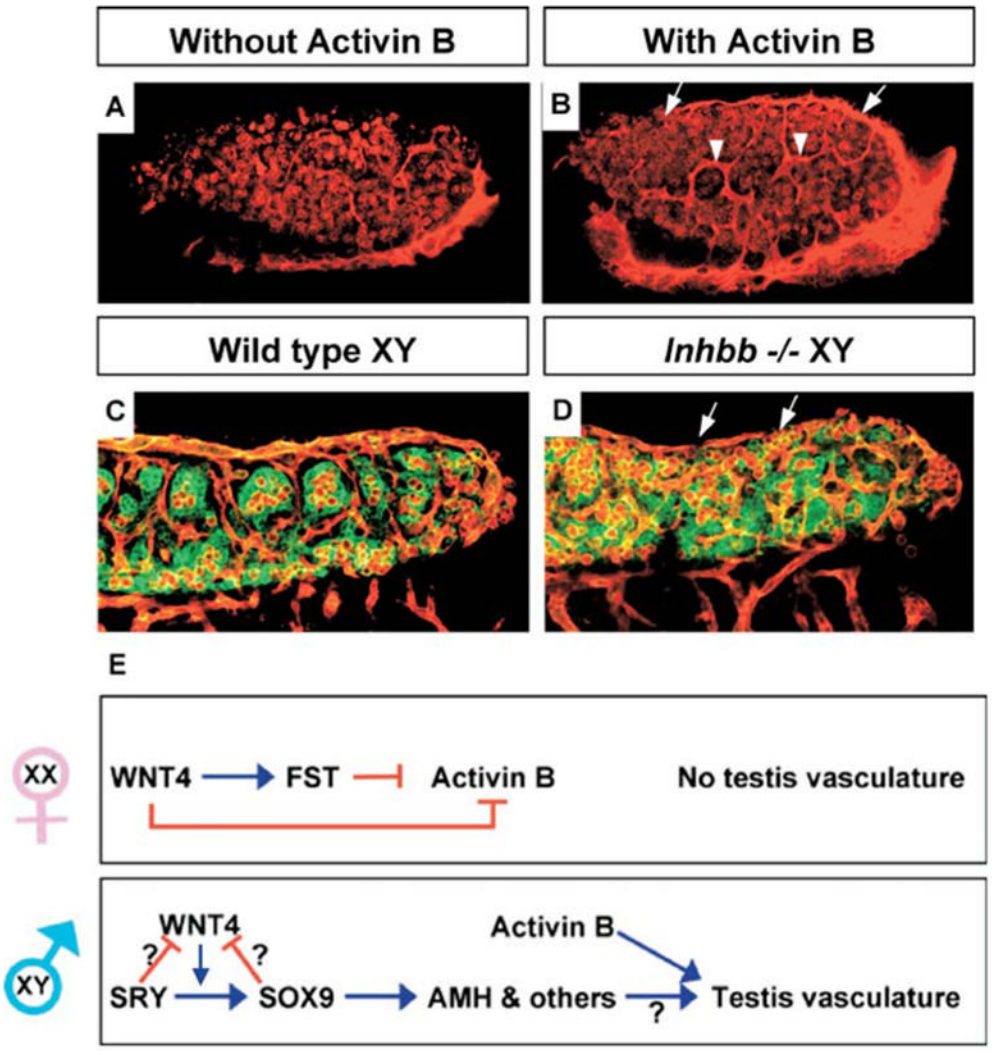

FIG. 4.

Inhbb, or its product activin B, contributes to formation of the coelomic vessel. **A** and **B**) When XX gonads were cultured with human activin B, the coelomic vessel was induced as marked by an anti-PECAM antibody (white arrows). Branching of the coelomic vessel similar to that in the XY gonad was also observed (white arrowheads). Treatment of activin B to the XX gonad did not induce Sertoli cell differentiation, because no AMH immuno-staining (green) was found. **C** and **D**) The coelomic vessel in E12.5 *Inhbb*−/− XY gonads had a smaller caliber and occasional discontinuity compared to the wild type (white arrows). Sertoli cell differentiation (green AMH staining) appeared normal in the *Inhbb*−/− XY gonad. **E**) The proposed model: *Inhbb* (or its product activin B) is strategically positioned where the WNT4/FST signaling cascade and the SRY pathway intersect during mammalian gonad differentiation. Our results also suggest that activin B contributes to coelomic vessel formation independent of the SRY pathway. Original magnification **A** and **B**, ×10; **C** and **D**, ×25.