RESEARCH ARTICLE

Follistatin Operates Downstream of *Wnt4* in Mammalian Ovary Organogenesis

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Wnt4^{-/-} **XX gonads display features normally associated with testis differentiation, suggesting that WNT4 actively represses elements of the male pathway during ovarian development. Here, we show that** *follistatin* **(***Fst***), which encodes a TGFβ superfamily binding protein, is a downstream component of** *Wnt4* **signaling.** *Fst* **inhibits formation of the XY-specific coelomic vessel in XX gonads. In addition, germ cells in the ovarian cortex are almost completely lost in both** *Wnt4* **and** *Fst* **null gonads before birth. Thus, we propose that WNT4 acts through FST to regulate vascular boundaries and maintain germ cell survival in the ovary.** *Developmental Dynamics 230:210–215, 2004.*
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INTRODUCTION

The formation of a testis or an ovary from the bipotential gonad is the primary step in sex determination in mammals. Testis determination is controlled by the expression of the Y-chromosome gene *Sry*, which initiates the differentiation of Sertoli cells and the architectural arrangement of somatic cells in the gonad to form a testis (reviewed in Lovell-Badge, 1992; Capel, 1996). When *Sry* is expressed in the gonad of an XX embryo, a testis forms (Koopman et al., 1991). Conversely, when *Sry* is absent or carries a critical mutation, an ovary develops (Gubbay et al., 1992; Hawkins et al., 1992). These results suggested that ovarian fate behaves as a default pathway, arising in the absence of prior specification of the testis pathway. However, occasional XX individuals (in human and other mammalian species) develop as males in the absence of the *Sry* gene. The most parsimonious explanation for this finding is that the ovarian pathway operates by repressing the male pathway, such that loss of this repressor, called "Z," leads to the activation of the complete male developmental program (McElreavey et al., 1993).

Few ovary-specific genes have been discovered, although several genes have been identified that are essential for early gonadal development in both sexes (Kriedberg et al., 1993; Luo et al., 1994; Miyamoto et al., 1997; Katoh-Fukui et al., 1998; Birk et al., 2000; Hammes et al., 2001). Only two genes that are specifically up-regulated in the ovary have been investigated so far, *Dax1* and *Wnt4*.

DAX1 was initially a candidate ovary-determining gene based on the identification of human *DAX1* within the region of the X chromosome known to cause male to female sex reversal when duplicated in XY individuals (Bardoni et al., 1994; Zanaria et al., 1994). However, genetic evidence in the mouse has indicated that *Dax1* is not essential for ovary development and may instead play a role in the testis pathway (Yu et al., 1998; Meeks et al., 2003a–c).

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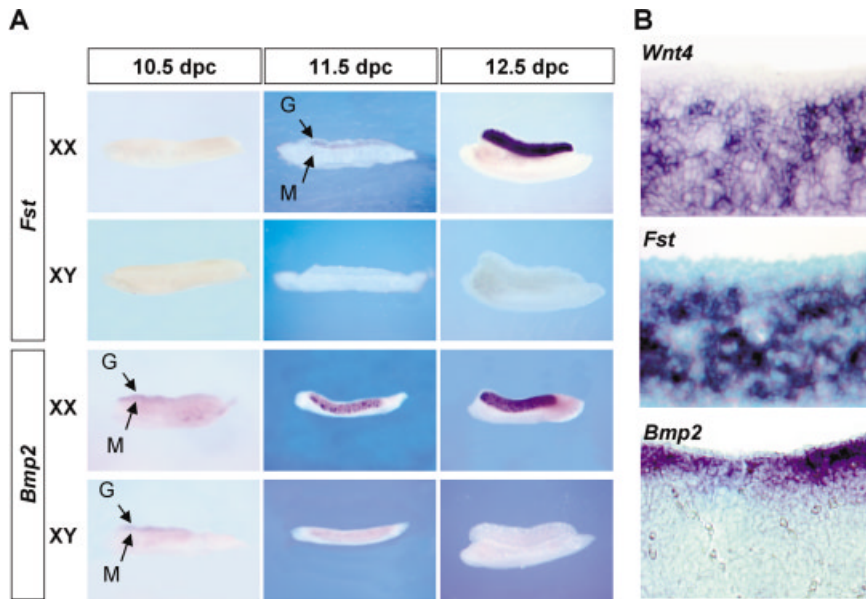
Fig. 1. Expression of *Fst*, *Bmp2*, and *Wnt4* in mouse gonads. **A:** Temporal expression of *Fst* and *Bmp2* in XX and XY gonads from 10.5 to 12.5 days post coitum (dpc) embryos by whole-mount in situ hybridization. The gonad (G, arrow) is a thin layer of cells on the top of the mesonephros (M, arrow) at early stages. In the whole-mount stains for *Bmp2*, the gonad is turned slightly toward the viewer. **B:** Spatial expression of *Wnt4*, *Fst*, and *Bmp2* in a 12.5 dpc XX gonad by section in situ hybridization. The dark purple stain represents specific binding of the RNA antisense probes in all in situ hybridizations.

Fig. 2. The epistatic relationship of *Wnt4*, *Bmp2*, and *Fst* in ovary development. **A:** Whole-mount in situ hybridization for *Bmp2* (12.5 days post coitum (dpc)) and *Fst* (14.5 dpc) in wild-type (WT) and *Wnt4*−/− XX gonads. **B:** Whole-mount in situ hybridization for *Bmp2* (12.5 dpc) and *Wnt4* (14.5 dpc) in wild-type and *Fst*−/− XX gonads.

Duplication of part of human chromosome 1p, including the *WNT4* gene, was also shown to lead to 46,XY male-to-female sex reversal, suggesting that, like *DAX1*, *WNT4* might also play a dose-sensitive role in mammalian sex determination (Jordan et al., 2001). The idea that *Wnt4* could be an anti-testis gene was originally put forward as a result of the discovery that loss of *Wnt4* leads to a partial female to male sex reversal in mice. In *Wnt4*−/− XX gonads, male-like steroid-secreting cells differentiate early. By the time of birth, germ cells are absent, and the XX tissue has reverted to cord-like structures (Vainio et al., 1999). Recent findings in *Wnt4*−/− mice indicated that the ectopic steroidogenic cells found in early *Wnt4*−/− XX gonads might be adrenal precursor cells that were misapportioned to the gonad at the stage when the adrenal and gonadal primordia separated (Heikkila et al., 2002). Further investigation of the phenotype of *Wnt4*−/− mutants revealed that the coelomic vessel that is normally specific to testis development forms ectopically in XX gonads. The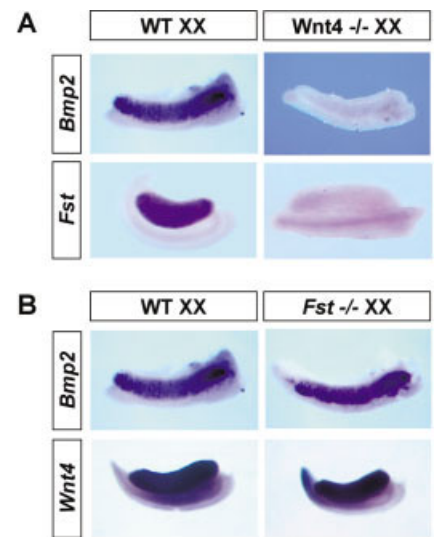 ectopic formation of this vessel in XX gonads occurs through the normal male mechanism of migration of endothelial cells from the mesonephros into the gonad (Jeays-Ward et al., 2003). These data indicate that a normal function of *Wnt4* is to repress aspects of the male pathway by blocking the migration of endothelial cells into XX gonads.

How WNT4 controls this process in XX gonads is not known. Here, we show that *Wnt4* controls the expression of *follistatin* (*Fst*), which encodes an activin binding protein known to regulate the hypothalamic–pituitary–gonadal axis (Phillips and de Kretser, 1998). *Fst* acts downstream of *Wnt4* to inhibit the formation of the coelomic vessel and to maintain germ cell survival in the cortical domain of the ovary.

RESULTS AND DISCUSSION

Follistatin and *Bmp2* Are Expressed Downstream of *Wnt4* During Ovary Development

We performed a detailed investigation of the spatial and temporal expression patterns of two genes relative to *Wnt4*, *follistatin* (*Fst*; Menke and Page, 2002; Menke et al., 2003), and *bone morphogenetic protein 2* (*Bmp2*; identified in our in situ hybridization screen of BMP family genes). Both genes are expressed specifically in the embryonic ovary. *Fst* first appears in the 11.5 days post coitum (dpc) XX gonad, reaches its peak at 12.5 dpc (Fig. 1A), and gradually decreases after 14.5 dpc (data not shown). *Fst* and *Wnt4* are expressed in a similar pattern, in cells several layers beneath the coelomic epithelium of the XX gonad (Fig. 1B). *Bmp2* is expressed in gonads of both sexes at 10.5 dpc, becomes XX-specific at 11.5 dpc, and is gradually extinguished after 14.5 dpc. In contrast to the expression domain for *Wnt4* and *Fst*, *Bmp2* is expressed in cells just beneath the coelomic epithelial layer of XX gonads (Fig. 1B). The expression patterns of *Wnt4*, *Fst*, and *Bmp2* are not substantially altered in *W/Wᵛ* gonads devoid of germ cells (data not shown), indicating that all three genes are expressed in somatic cell lineages and do not depend on the presence of germ cells in the early ovary.

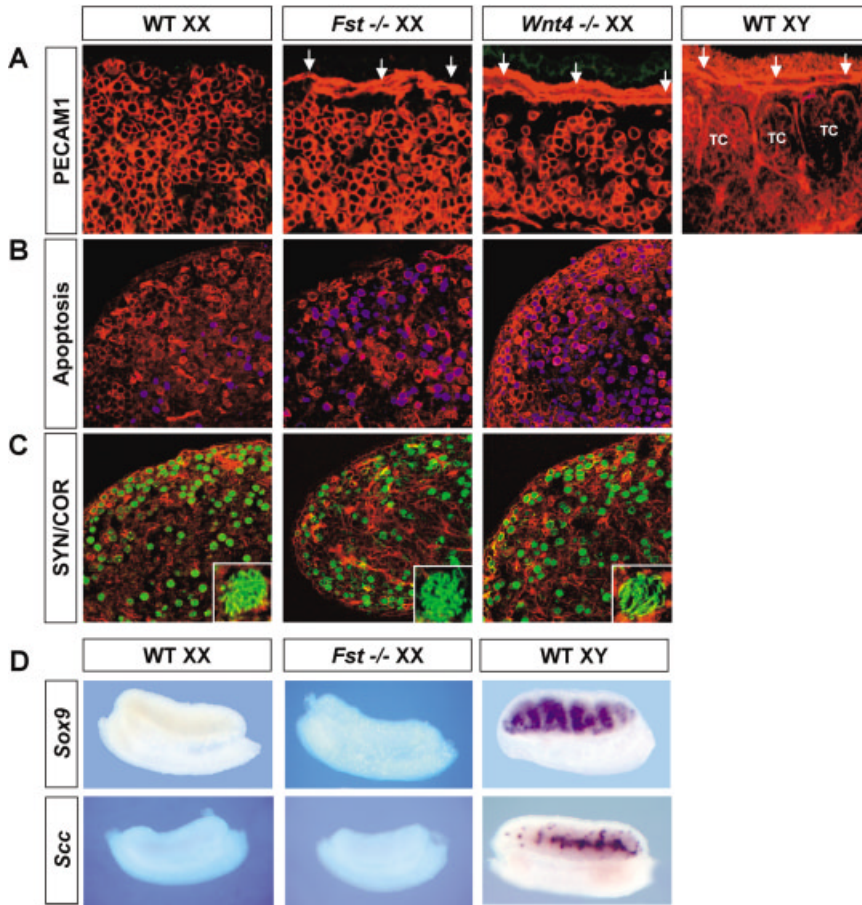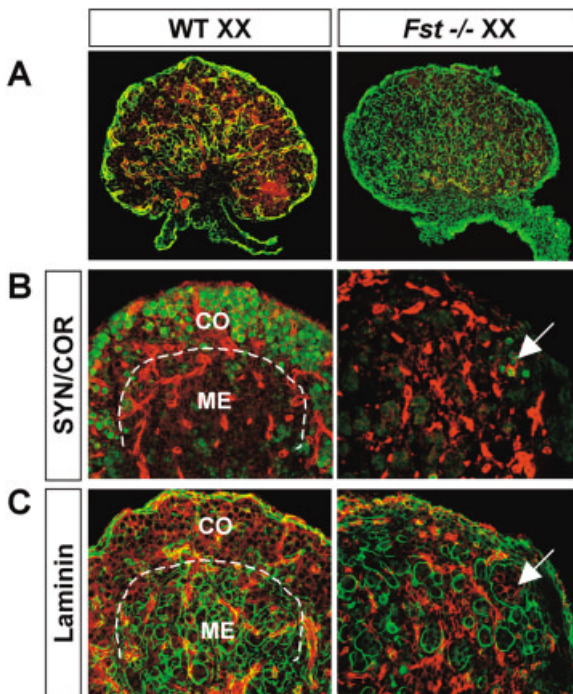To investigate the epistatic relationship of these genes in the ovar-

Fig. 3.

ian pathway, we analyzed the expression of *Bmp2* and *Fst* in *Wnt4⁻/⁻* XX gonads. Neither *Bmp2* nor *Fst* is expressed at any stage between 12.5 and 14.5 dpc in *Wnt4⁻/⁻* XX gonads (Fig. 2A), indicating that both genes are downstream of *Wnt4* and could lie in pathways regulating the phenotypes observed in *Wnt4⁻/⁻* mutants. To determine whether loss of *Fst* affects the expression of *Bmp2* and *Wnt4*, we investigated the expression profiles of these genes in *Fst⁻/⁻* XX gonads (Matzuk et al., 1995). Expression of both *Bmp2* and *Wnt4* is similar to patterns in wild-type gonads (Fig. 2B), confirming that *Fst* is activated downstream of *Wnt4*. It is

Fig. 3. The coelomic vessel forms in *Fst⁻/⁻*, and *Wnt4⁻/⁻* XX gonads; meiotic germ cells undergo apoptosis. **A:** Immunostaining for PECAM-1 in 12.5 days post coitum (dpc) wild-type (WT) XX, *Fst⁻/⁻* XX, *Wnt4⁻/⁻* XX, and wild-type XY gonads. PECAM-1 (red) is a membrane marker for endothelial cells of the vasculature and for primordial germ cells. Arrows indicate formation of the coelomic vessel on the surface of *Fst⁻/⁻* XX, *Wnt4⁻/⁻* XX, and wild-type XY gonads (TC = testis cord). **B:** Double staining for PECAM-1 (red) and apoptosis (blue) by TUNEL assay in 16.5 dpc wild-type, *Fst⁻/⁻*, and *Wnt4⁻/⁻* XX gonads. **C:** Double immunostaining for PECAM-1 (red) and SYN/COR (green) of adjacent 8-μm sections in B. Pictures in the insets are higher magnification (100×) of individual germ cells from the sections. Alignment of meiotic chromosomes can be seen clearly by SYN/COR staining. **D:** Whole-mount in situ hybridization for *Sox9* and *Scc* in 12.5 dpc wild-type XX, *Fst⁻/⁻* XX, and wild-type XY gonads.

Fig. 4. The structure of the ovarian cortex is lost in 18.5 days post coitum (dpc) *Fst⁻/⁻* XX gonads. **A:** A lower magnification view with double immunostaining for PECAM-1 (red) and laminin (green). Note that the size of the XX gonad is similar in wild-type (WT) and *Fst⁻/⁻*. Distinct cortex and medulla tissue boundaries can be seen at higher magnification in wild-type, but not in *Fst⁻/⁻* XX gonads (B,C). **B:** Double immunostaining for PECAM-1 (red) and SYN/COR (green): In the wild-type XX gonad, germ cells with SYN/COR staining were located in the cortical region (CO) of the gonad. In the *Fst⁻/⁻* XX gonad, >99% of germ cells were lost with rare germ cells enclosed inside cord structures (arrows in B,C). **C:** Double immunostaining for PECAM-1 and laminin1 in adjacent 8-μm sections: In the medullary region (ME) of the wild-type XX gonad, cord-like structures are delineated by laminin1 (a dotted line separates the cortical and medullary regions of the XX gonads). The cord-like structure extended throughout the cortical domain in the *Fst⁻/⁻* XX gonad.

Fig. 4.

worth noting that, although *Bmp2* expression is absent in *Wnt4*⁻/⁻ mutants (Fig. 2A), it is undisturbed in *Fst*⁻/⁻ mutants (Fig. 2B), indicating a branch point in the *Wnt4* pathway upstream of *Fst*. Unfortunately, *Bmp2*⁻/⁻ mutants could not be investigated, because they do not survive to mid-gestation (Zhang and Bradley, 1996).

Inactivation of *Follistatin* Leads to Formation of the Coelomic Vessel in the XX Gonad

Examination of gonads from *Fst*⁻/⁻ mice revealed the presence of the coelomic vessel on the surface of XX gonads at 12.5 dpc, a phenotype identical to that observed in *Wnt4*⁻/⁻ XX gonads at the same stage (Fig. 3A, arrows). The coelomic vessels in *Fst*⁻/⁻ and *Wnt4*⁻/⁻ XX gonads are located in the same domain as in normal XY gonads, just beneath the coelomic epithelium. In both cases, the vessel lacks the branches that descend between testis cords in XY gonads (Fig. 3A). No defects in testis differentiation in XY gonads from *Fst*⁻/⁻ embryos were identified (data not shown).

Because formation of the coelomic vessel is normally an event downstream of *Sry* and Sertoli cell differentiation, we investigated whether Sertoli differentiation is activated in *Fst*⁻/⁻ XX gonads. We found that *Sox9* (Fig. 3D) and anti-Müllerian hormone (*Amh*, data not shown), two Sertoli-specific genes, are not expressed in *Fst*⁻/⁻ XX gonads during early developmental stages, similar to results previously reported in *Wnt4*⁻/⁻ XX gonads at these stages (Vainio et al., 1999). These results indicate that formation of the coelomic vessel does not require Sertoli cell differentiation nor does it require *Sry*, because this gene is not present in XX animals. Expression of Sertoli-specific genes such as *desert hedgehog* and *Amh* is activated after birth in *Wnt4*⁻/⁻ XX gonads, possibly as the indirect result of germ cell loss (Vainio et al., 1999). We were not able to examine these patterns in *Fst*⁻/⁻ XX gonads because of the perinatal lethality of *Fst*⁻/⁻ embryos (Matzuk et al., 1995).

Another defect reported in *Wnt4*⁻/⁻ XX gonads was the incomplete sep-

aration of adrenal cells from the gonad primordium. Adrenal-specific steroid producing cells were found in the anterior portion of *Wnt4*⁻/⁻ XY and XX gonads (Heikkila et al., 2002). We examined the expression of P450 side-chain cleavage (*Scc*), a marker for steroidogenic cells, in *Fst*⁻/⁻ XX gonads to determine whether *Fst*⁻/⁻ gonads share similar defects in steroidogenic cell sorting as occur in *Wnt4*⁻/⁻ XX gonads. No *Scc* expression was found in *Fst*⁻/⁻ XX gonads at any stage examined (Fig. 3D). This result indicates that *Fst* regulates only some of the pathways downstream of *Wnt4*.

Germ Cells Are Lost From the Ovarian Cortex in Both *Wnt4*⁻/⁻ and *Fst*⁻/⁻ Mutants

Germ cells are lost in *Wnt4*⁻/⁻ XX gonads by the time of birth (Vainio et al., 1999); therefore, we compared the fate of germ cells in wild-type, *Wnt4*⁻/⁻, and *Fst*⁻/⁻ XX gonads. We found that numbers of germ cells in *Wnt4*⁻/⁻ and *Fst*⁻/⁻ XX gonads are similar to numbers in wild-type XX gonads from 11.5 to 15.5 dpc (data not shown). However, at 16.5 dpc, more than 90% of germ cells in *Wnt4*⁻/⁻ and *Fst*⁻/⁻ XX gonads undergo apoptosis in contrast to ~30% in wild-type gonads (Fig. 3B). In wild-type XX gonads, apoptosis of germ cells is restricted to the medullary region; apoptosis is not seen in the coelomic domain where germ cells survive and accumulate. In contrast, in *Wnt4*⁻/⁻ and *Fst*⁻/⁻ XX gonads, germ cells undergo apoptosis throughout the gonad, in both the medulla and the coelomic domains.

To investigate whether germ cells in *Wnt4* and *Fst* XX mutants enter meiosis normally, we assayed the appearance of components of the synaptonemal complex of meiotic chromosomes during the zygotene–pachytene transition using an antibody against SYN/COR (Dobson et al., 1994). In wild-type XX gonads, germ cells enter meiosis at 13.5 dpc and progress from leptotene to diplotene and meiotic arrest by 18.5 dpc. We found that the timing of the zygotene–pachytene transition and the number of germ cells positive for SYN/COR were similar among wild-

type, *Wnt4*⁻/⁻, and *Fst*⁻/⁻ XX gonads (Fig. 3C). The chromosomal localization of SYN/COR was also normal in germ cells of *Wnt4*⁻/⁻ and *Fst*⁻/⁻ XX gonads at 16.5 dpc (Fig. 3C, insets). Thus, *Wnt4* and *Fst* are not essential for the initial phases of meiosis but are required for survival of meiotic germ cells in the coelomic domain at pachytene and diplotene stages.

The massive germ cell apoptosis in *Fst*⁻/⁻ XX gonads from 16.5 dpc onward leads to an almost complete loss of germ cells near birth, similar to data reported for *Wnt4*⁻/⁻ XX gonads (Vainio et al., 1999). Immediately before birth (18.5–19.5 dpc), meiotic germ cells are located in the coelomic domain (cortex) of wild-type XX gonads (Fig. 4A,B). In wild-type gonads, a sharp division between the medulla and cortical regions is demarcated by the deposition of a laminin-rich boundary and the distinct tissue organizational differences between the two regions of the ovary (Fig. 4B). When *Fst* is absent, more than 99% of meiotic germ cells are lost and the boundary between cortical and medullary regions disappears (Fig. 4C). Cord-like structures fill the cortical region, and the few remaining meiotic germ cells are enclosed inside (Fig. 4B,C, arrows). This finding is similar to the phenotype previously described for *Wnt4* XX mutants after birth (Vanio et al., 1999).

On the basis of the similar phenotypes between *Wnt4*⁻/⁻ and *Fst*⁻/⁻ XX gonads and the absence of *Fst* expression in *Wnt4*⁻/⁻ XX gonads, we propose that FST acts downstream of WNT4 to inhibit the migration of endothelial cells from the mesonephros and the formation of the coelomic vessel. Formation of the male-specific coelomic vessel in the ovary of these two knockouts is especially intriguing because key genes in the male pathway such as *Sox9* are not expressed (Vidal et al., 2001). This observation suggests that, even though endothelial cell migration and formation of the coelomic vessel is normally XY-specific, it does not require *Sry* or the differentiation of Sertoli cells. Instead, formation of the coelomic vessel is actively inhibited in XX gonads through WNT4/FST signaling. These findings seem to be

consistent with the "Z" theory that ovary-determining genes act as inhibitors of the testis pathway in the XX gonad (McElreavey et al., 1993). However, the theoretical ovary determining gene, "Z," is classically defined as a master inhibitor of the initial step of the testis cascade (Vaiman and Pailhoux, 2000). We found that WNT4/FST signaling inhibits a specific downstream aspect of testis development, the formation of the coelomic vessel.

Fst also plays a critical role in the survival of female germ cells. In the case of Wnt4 mutant ovaries, the loss of germ cells might have resulted from the ectopic production of steroids in the XX gonad. However, steroid-producing cells are not present in Fst$^{-/-}$ XX gonads; thus, ectopic steroid production cannot explain germ cell loss in this case. Because of the specific expression of Bmp2 in the cortical domain, it is a candidate for a gene promoting germ cell survival in this region. In Fst$^{-/-}$ XX gonads, expression of Bmp2 is normal by in situ analysis; however, disregulation at the protein level remains an open possibility.

One appealing hypothesis is that formation of the coelomic vessel alters the cellular and signaling environment of the ovarian cortex. The coelomic vessel forms in the cortical domain of Wnt4$^{-/-}$ and Fst$^{-/-}$ XX gonads, just beneath the coelomic epithelium, before the stage when germ cells normally begin to accumulate there. This cortical domain may provide a protective niche where meiotic germ cells escape from the extensive apoptosis that occurs in the medullary region of wild-type XX gonads. The ovary is not devoid of vasculature, but the major vessels are normally found in the medulla of the organ at this stage (Brennan and Capel, 2002). The formation of the coelomic vessel could introduce new signals into this protected domain that antagonize the survival of germ cells. Whether the coelomic vessel is directly involved in causing germ cell loss is not clear. It is possible that Fst controls pathways that affect germ cell survival independent of coelomic vessel formation.

Regulation of Fst expression by WNT3A was reported in human embryonic carcinoma cells (Willert et al., 2002). Our result provides the first physiological link between WNT4 and FST in an animal model. FST is known to modulate the activity of TGF-β family proteins such as activins and BMPs by direct binding (Chang et al., 2002). Recent work on hair follicle development revealed an intricate network among follistatin, activins, and BMPs (Nakamura et al., 2003). Of interest, TGF-β family proteins such as AMH and BMPs induce the formation of the coelomic vessel and cause loss of germ cells in the XX gonad in organ culture experiments (Ross et al., 2003; H.H-C. Yao and B. Capel, unpublished data). Furthermore, we and others had found that activins and many members of the BMP family are expressed in both embryonic XX and XY gonads (Feijen et al., 1994). These results suggest that activins and BMPs could be potential targets of FST in normal ovary development. This intricate control of a very specific aspect of vascular development and its association with altered organ structure and germ cell survival suggest that we have only begun to understand the role of vasculature in organogenesis and development.

In summary, we have identified a pathway downstream of WNT4 signaling that operates through FST to inhibit vascular development in the ovary. This signaling cascade is unique in that it possesses both anti-testis (inhibition of the coelomic vessel) and pro-ovary functions (survival of meiotic germ cells), and constitutes the first multigene signaling pathway defined in early development of the ovary.

EXPERIMENTAL PROCEDURES

Animals

Wnt4$^{-/-}$ mice were obtained from The Jackson Laboratory (strain 129-Wnt4^{tm1Amc}). Fst$^{-/-}$ mice were generated and genotyped as previously described (Matzuk et al., 1995). Timed matings were produced by housing female mice with males overnight and checking for vaginal plugs the next morning (0.5 dpc = noon of the day when a vaginal plug was found). The sex of each embryo was determined by Giemsa staining for X chromatin Barr bodies in cells of the amniotic sac (Palmer and Burgoyne, 1991).

In Situ Hybridization

Samples were fixed overnight in 4% paraformaldehyde in PBS at 4°C and processed according to Henrique et al. (1995). A digoxigenin-labeled RNA probe was detected by using an alkaline phosphatase conjugated anti-digoxigenin antibody.

Immunocytochemistry

For immunostaining against laminin, samples were fixed overnight in 4% paraformaldehyde in PBS at 4°C. For double immunostaining against PECAM-1 and SYN/COR, samples were fixed 1 hr in 1% paraformaldehyde at 4°C. Samples were then processed and cut into 8-μm frozen sections as described (Karl and Capel, 1998). Primary antibody incubations were carried out overnight at 4°C in blocking solution (1:200 dilution of rabbit anti-laminin1 antibody, provided by Harold Erickson; 1:500 dilution of rat anti–PECAM-1 antibody, Pharmingen; 1:800 dilution of a rabbit polyclonal antibody against SYN1/COR1, provided by Peter Moens). Secondary antibody incubations were performed overnight at 4°C with a 1:500 dilution of fluorescently conjugated secondary antibodies (fluorescein isothiocyanate (FITC)- or Cy5-conjugated goat anti-rabbit antibody and Cy3-conjugated goat anti-rat antibody, Jackson Immunochemicals). Sections were washed three times for 5 min each in washing solution and mounted on glass slides in DABCO. Images were collected on a Zeiss LSM confocal microscope and processed using Adobe Photoshop.

TUNEL Assay

Frozen sections were obtained and incubated in the reaction mix with 0.5 μl of TdT Enzyme from Sigma (35 U/μl) and 50 μl of FITC-dUTP/dNTP mix from Boehringer Mannheim for 1 hr at 37°C in a humid chamber. For control, only the nucleotide mix was added for reaction. Our standard

immunocytochemistry protocol was followed to double stain the section with PECAM-1.

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