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Endothelial and steroidogenic cell migration are regulated by WNT4 in the developing mammalian gonad

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SUMMARY

The signalling molecule WNT4 has been associated with sex reversal phenotypes in mammals. Here we show that the role of WNT4 in gonad development is to pattern the sex-specific vasculature and to regulate steroidogenic cell recruitment. Vascular formation and steroid production in the mammalian gonad occur in a sex-specific manner. During testis development, endothelial cells migrate from the mesonephros into the gonad to form a coelomic blood vessel. Leydig cells differentiate and produce steroid hormones a day later. Neither of these events occurs in the XX gonad. We show that WNT4 represses mesonephric endothelial and steroidogenic cell migration in the XX gonad, preventing the formation of a male-specific coelomic blood vessel and the production of steroids. In the XY gonad, *Wnt4* expression is downregulated after sex determination. Transgenic misexpression of *Wnt4* in the embryonic testis did not inhibit coelomic vessel formation but vascular pattern was affected. Leydig cell differentiation was not affected in these transgenic animals and our data implies that *Wnt4* does not regulate steroidogenic cell differentiation but represses the migration of steroidogenic adrenal precursors into the gonad. These studies provide a model for understanding how the same signalling molecule can act on two different cell types to coordinate sex development.

Key words: WNT, Gonad, Endothelial, Mouse

INTRODUCTION

The development of the mammalian gonad is a coordinated process that initiates identically in both sexes and then, after sex is determined by the action of the testis-determining gene *Sry* in the XY gonad, diverges in a sex-specific manner (Swain and Lovell-Badge, 2002). Studies in the mouse have shown that within the XY gonad, *Sry* triggers a series of male-specific processes that ensure the proper development of the testis. These include an increase in proliferation of the coelomic epithelium that surrounds the gonad, formation of a blood vessel at the coelomic surface of the gonad, migration of mesonephric cells into the gonad and formation of testicular cords (Brennan et al., 2002; Buehr et al., 1993; Capel et al., 1999; Martineau et al., 1997; Schmahl et al., 2000). After these events take place, the Leydig cells of the testis begin expressing genes involved in steroid biosynthesis. No comparable events occur within the XX gonad at the same stage of development.

The development of vasculature within the mouse gonad initiates in the genital ridge in both males and females before the action of *Sry* at around 11 days post coitum (dpc) (Brennan et al., 2002). After sex determination, however, the mechanism of vascular formation is different between the sexes. Further development of the vasculature in the ovary occurs through proliferation of cells already present within the organ. In the testis, development of vasculature occurs through the recruitment of additional cells from the mesonephros. This difference gives rise to the sex-specific vascular pattern of the gonad. In the testis, a large artery is formed at the coelomic surface through which the blood flow is rerouted at around 12 dpc. It has been suggested that this male-specific vascular system is required for the export of testosterone from the testis to the rest of the embryo to ensure masculinisation. The ovary has no coelomic blood vessel. Instead, the main ovarian artery is found at the mesonephros-gonad boundary.

The migration of mesonephric cells into the developing gonad is a male-specific event that begins by 11.5 dpc in the mouse and is critical to the development of the testis (Buehr et al., 1993; Martineau et al., 1997; Tilmann and Capel, 1999). Studies using in vitro organ co-culture systems have shown that a population of these migrating cells surround the Sertoli cells within the gonad and have characteristics of peritubular myoid cells (Martineau et al., 1997). Sertoli cells and peritubular myoid cells interact to form testicular cords, an event that is coincident with the activation of male-specific gene expression. A second group of migrating cells were found in locations characteristic of developing vasculature and were positive for endothelial cell markers such as Pecam, Flt-1 and Tie-2 (Brennan et al., 2002; Martineau et al., 1997). Additional migrating cells negative for endothelial markers were found

associated with the endothelium and had characteristics of myoepithelial cells.

Steroidogenic cell differentiation in the gonad is poorly understood. The action of *Sry* in the XY gonad is thought to trigger the differentiation of Sertoli cells and these cells in turn are thought to direct the differentiation of the rest of the testis (Swain and Lovell-Badge, 2002). Differentiated mouse Leydig cells are seen at 12.5 dpc following the appearance of testis cords and male-specific vasculature. Little is known about how Leydig cells arise and how their development is controlled. The signalling molecule Desert hedgehog (DHH), which is produced by Sertoli cells, has been implicated in Leydig cell development (Yao et al., 2002). Embryos mutant for *Dhh* showed a reduced number of Leydig cells in the XY gonad whereas other processes such as mesonephric cell migration were not affected, suggesting a direct role for this factor in the induction of Leydig cell differentiation.

The signalling molecule WNT4 has been associated with female sexual development in the mouse (Vainio et al., 1999). In the developing gonad, the *Wnt4* gene is expressed in both sexes prior to 11.5 dpc. After sex determination, expression persists in the XX gonad but is downregulated in the XY gonad. XX embryos that are mutant for *Wnt4* showed failure of Müllerian duct formation and differentiation of the Wolffian duct into the male ductal system. The mutant embryonic *Wnt4* ovary showed a loss of oocytes and ectopic expression of enzymes involved in steroid hormone biosynthesis. This latter phenotype led Vainio et al. to propose that WNT4 was suppressing Leydig cell development in the XX gonad (Vainio et al., 1999). Our studies show a novel role for WNT4 in the gonad that provides an alternative explanation for the masculinisation phenotype observed in the *Wnt4* mutant animals. We show that WNT4 represses endothelial and steroidogenic cell migration into the developing XX gonad, preventing both the formation of a male-specific coelomic blood vessel and ectopic steroid production. In addition, we show that misexpression of *Wnt4* in the XY gonad does not inhibit Leydig cell differentiation but does affect the pattern of the developing coelomic blood vessel.

MATERIALS AND METHODS

Mice strains

Mutant *Wnt4* mice were obtained from Jackson Labs but were originally created in Andrew McMahon's laboratory (Stark et al., 1994) and kept on a 129/Sv background. The identification of homozygous, heterozygous and wild-type embryos was done as described previously (Stark et al., 1994). ROSA26 mice were obtained from Robin Lovell-Badge but were originally created by Phil Soriano (Friedrich and Soriano, 1991) and kept on a mixed background. The mice expressing green fluorescent protein (GFP) ubiquitously were obtained from Jackson Labs [Stock TgN (GFPU)5Nagy] (Hadjantonakis et al., 1998). All transgenic animals were made by pronuclear injections into eggs derived from CBA × C57BL/6 F1 females mated to F1 males. In most cases transgenic and ROSA26 males were mated to MF1 females to obtain litters for analysis.

Whole-mount immunohistochemistry

The tissues to be analysed were dissected and fixed overnight in 4% paraformaldehyde at 4°C. After rinsing in PBS, the samples were incubated for 3 hours at 4°C in 50 mM ammonium chloride in PBS and then left overnight at 4°C in a solution containing 0.1% hydrogen peroxide, 10% goat serum and 1% triton in PBS. Primary antibody staining was done overnight at 4°C in a PBS solution containing 10% goat serum, 1% triton and the Pecam antibody (Pharmingen, 1/50 dilution). After rinsing five times with a wash solution containing PBS, 10% goat serum and 10% triton, the samples were incubated overnight at 4°C in wash solution containing a secondary antibody conjugated with peroxidase (Pierce, 1/300 dilution). Samples were then rinsed five times in wash solution and revealed with 4-chloro-1-naphtol as a substrate (SIGMA). The Cy5-conjugated secondary antibody in Fig. 2 was used as described (Brennan et al., 2002).

Whole-mount in situ hybridisation

Assays were performed as described previously (Wilkinson and Nieto, 1993). The probe for *Jag1* has been described previously (Brennan et al., 2002). The probe for *Pdgfra* was provided by Christer Betsholtz. The probe for 3β-hydroxysteroid dehydrogenase (3β-HSD) (*Hsd3b* – Mouse Genome Informatics) was derived from a PCR amplified product that comprised the region from 873 to 1473 of the mouse cDNA. (NCBI accession M58567). The *Cyp11a1* and *Sf1* (*Nr5a1* – Mouse Genome Informatics) probe were obtained from Keith Parker. The *Wnt4* probe was obtained from Andrew McMahon (Harvard University).

In vitro organ co-culture assay

In vitro co-culture assays were done as described previously (Martineau et al., 1997). Briefly, mesonephroi and gonads were dissected from different animals and were combined on agar blocks and cultured for 48 to 96 hours. The samples were then washed in PBS, fixed in 2% paraformaldehyde/0.1% glutaraldehyde and stained for β-galactosidase activity as described. The sex of the mesonephroi had no effect on our migration studies as shown previously (Martineau et al., 1997).

BAC constructs

The *Cyp11a1* and *Sf1* BAC clones were obtained from a 129SV mouse library (Research Genetics, USA). The Cyp11a1:LacZ construct was made using the method described by Carvajal et al. (Carvajal et al., 2001) and an improved version described elsewhere (Cox et al., 2002). The βgeo gene with a polyadenylation site derived from SV40 was obtained from Rosa Beddington (National Institute for Medical Research) and was introduced at the first KpnI site in the *Cyp11a1* coding sequence such that a fusion protein was made. A fragment was created that had the βgeo gene flanked by 2.5 kb of upstream *Cyp11a1* sequence (from SpeI to KpnI) and 2.2 kb of downstream *Cyp11a1* sequence (the next KpnI fragment). This fragment was introduced into the shuttle vector for homologous recombination. The modified BACs were characterised by Southern analysis using *Cyp11a1*- and *lacZ*-specific probes.

The Sf1:Wnt4 construct was created using the method described by Swaminathan et al. (Swaminathan et al., 2001). The DY380 cells were obtained from Neal Copeland (Lee et al., 2001). The *Wnt4* cDNA was obtained from Andy McMahon (Harvard University). A BamHI to SphI 700 bp genomic fragment containing the first and second exon of the Sf1 gene was cloned into puc18. A fragment containing the *Wnt4* cDNA and the rabbit β-globin intron and polyadenyation signal sequences (Swain et al., 1998) was inserted at the SacII site of this construct, which is found in the 5′ untranslated region of the Sf1 gene. This construct was used as a source of fragment to introduce into DY380 cells containing the unmodified Sf1 BAC, which were induced at 42C and made electrocompetent. The modified BACs were characterised by Southern analysis using *Sf1*- and *Wnt4*-specific probes.

For making transgenic mice with the modified BAC constructs, DNA was prepared using the Qiagen maxiprep kit (Qiagen, UK) and dialysed against microinjection buffer (10 mM Tris H-Cl pH 7.5, 0.1 mM EDTA pH 8.0 and 100 mM NaCl) and injected as circular DNA at different concentrations.

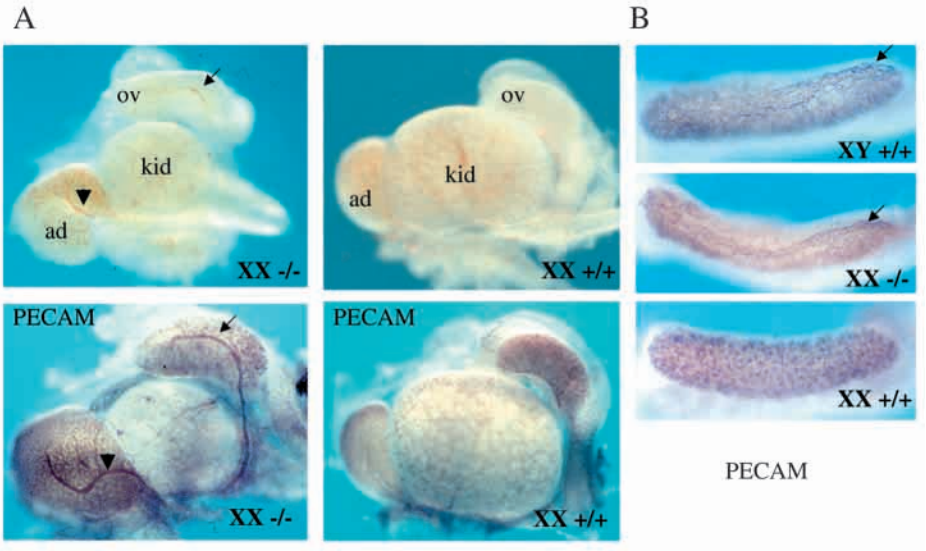

Fig. 1. The coelomic blood vessel forms in the *Wnt4*$^{-/-}$ ovary. (A) Light microscope images and Pecam stained images of kidney (kid), adrenal (ad) and ovary (ov) from 15.5 dpc embryos that were wild-type (+/+) and homozygous (–/–) for the mutant *Wnt4* allele as indicated. (B) Gonads from 12.5 dpc embryos that were wild-type (+/+) or homozygous (–/–) for the mutant *Wnt4* allele stained with a Pecam antibody, which labels the endothelium and germ cells at 12.5 dpc (speckled pattern in XX +/+ sample). In all images anterior is towards the left. Coelomic blood vessels are indicated by arrows and the ectopic vessel in *Wnt4*$^{-/-}$ adrenals is indicated by an arrowhead.

Cyp11a1:*lacZ* transgenic animals were typed by PCR using a *Cyp11a1*-specific primer (GCTCAGTGCTGGTATTGCTG) and a *lacZ*-specific primer (AGATGGGCGCATCGTAACCG). The Sf1:Wnt4 transgenic animals were typed by PCR using primers that were specific to the rabbit β-globin intron and polyadenylation sequences (GGAGACAATGGTTGTCAACAG and GCTAG-AGCTGAGAACTTCAG).

RTPCR on Sf1:Wnt4 transgenic tissues

RTPCR was performed as described previously (Capel et al., 1993). RNA was extracted from various tissues from transgenic animals, the RNA was reverse transcribed and PCR was performed. To identify transgenic-specific transcripts we used primers that spanned the rabbit β-globin intron sequences (GCTAGAGCTGAGAACTTCAG and CAAGGGGCTTCATGATGTCC). HPRT was used as a control for the presence of RNA in the samples (Capel et al., 1993).

RESULTS

Coelomic blood vessel formation in the mutant *Wnt4* XX gonad

Vascular formation in the mammalian gonad has been shown to occur in a sex-specific manner (Brennan et al., 2002). In the male, a large blood vessel forms at the coelomic surface of the testis that is absent from the ovary. Our analysis of gonads of XX mouse embryos that were homozygous for a mutant *Wnt4* allele (–/–) revealed the presence of a large coelomic blood vessel in the ovary, which stained with an antibody to Pecam, a marker of endothelial and germ cells (Fig. 1). A large ectopic vessel was also observed in the adrenal of *Wnt4*$^{-/-}$ embryos from both sexes (arrowhead in Fig. 1).

The ectopic vessel in the *Wnt4*$^{-/-}$ XX gonad was observed as early as 12.5 dpc, the same stage as when this vessel appears in the wild-type male gonad (Fig. 1B). The coelomic vessel in the mutant ovary had a different appearance to that of the male gonad in that it lacked branches, which, in the testis, descend between testis cords and connect to other blood vessels forming a network.

Most vascular markers such as Pecam label endothelial cells in both XX and XY gonads. However, sex-specific markers such as *Jag1* and platelet-derived growth factor receptor alpha (*Pdgfr α*) are normally associated with the coelomic blood vessel in the XY gonad but are not found in the XX gonad (Brennan et al., 2002; Brennan et al., 2003). We performed whole-mount in situ hybridisation for *Jag1* and *Pdfgr α* expression and found both were present near the ectopic vasculature in XX gonads from *Wnt4*$^{-/-}$ embryos, but were not present in gonads from wild-type (+/+) or heterozygous (+/–) XX embryos (Fig. 2). *Jag1* is expressed in both the coelomic vessel region and the interstitium of the developing testis. However, in the *Wnt4*$^{-/-}$ XX gonad *Jag1* expression was only associated with the blood vessel and not other interstitial regions of the gonad.

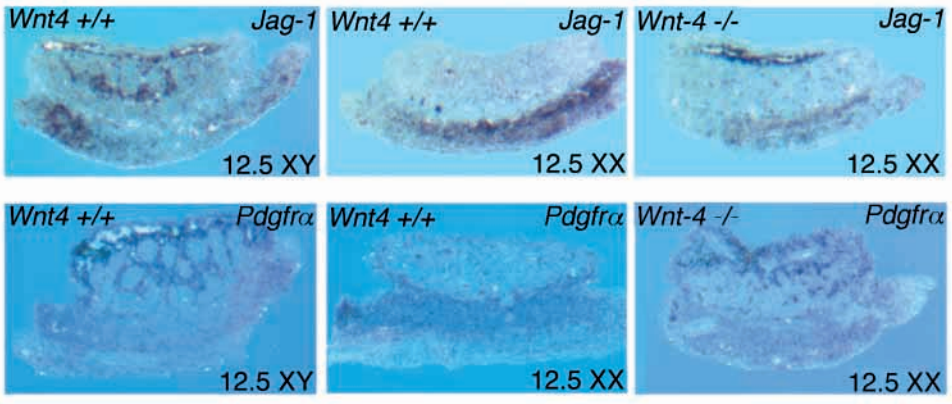

Fig. 2. Genes normally expressed in the coelomic vessel in the testis are found associated with the ectopic coelomic vessel in the *Wnt4*$^{-/-}$ XX gonad. Whole-mount in situ hybridisation for *Jag1* and *Pdgfra* expression was performed on gonads from *Wnt4*$^{-/-}$ or *Wnt4*$^{+/+}$ XX and XY 12.5 dpc embryos as indicated.

Studies have shown that a large proportion of the testis vasculature, including the male-specific coelomic blood vessel, is formed from endothelial cells that migrate from the mesonephros (Brennan et al., 2002). This migration is a male-specific event and does not occur in the XX gonad. To investigate whether the coelomic blood vessel observed in the *Wnt4*$^{-/-}$ ovary was formed by mesonephric cell migration we used an in vitro organ co-culture system. Mesonephroi from 11.5 dpc and 12.5 dpc embryos where the *lacZ* gene was expressed ubiquitously (ROSA26 line) were incubated next to XX and XY gonads derived from 11.5 dpc and 12.5 dpc embryos which were homozygous, heterozygous and wild-type for the mutant *Wnt4* allele. After incubation the samples were stained for β-galactosidase activity and, as shown previously, mesonephric cells expressing the *lacZ* gene were found within the wild-type XY gonad but not within the XX gonad (Fig. 3A). However, when a *Wnt4*$^{-/-}$ XX gonad was cultured apposed to a wild-type mesonephros, *lacZ*-expressing cells were found within the gonad (all 13 *Wnt4*$^{-/-}$ XX gonads that were assayed showed this phenotype, 8 from 11.5 dpc embryos and 5 from 12.5 dpc embryos) (Fig. 3A). These results show that migration of mesonephric cells into the XX gonad is inhibited by the presence of WNT4.

At least three cell types migrate into the XY gonad from the mesonephros. Martineau et al. identified these as peritubular myoid, perivascular and endothelial cells (Martineau et al., 1997). The pattern of the mesonephric cells that migrated into the XX gonad of the *Wnt4*$^{-/-}$ ovary suggested that they were endothelial cells. To confirm this hypothesis we incubated 11.5 dpc XX gonads from *Wnt4*$^{-/-}$ embryos with mesonephroi from 11.5 dpc embryos where the GFP gene was expressed ubiquitously. After incubation these samples were stained for an antibody to Pecam. As expected, GFP-positive cells were found in the mutant ovary and most of these cells also stained for Pecam (Fig. 3B, red). These results show that WNT4 represses endothelial cell migration into the XX gonad from the mesonephros.

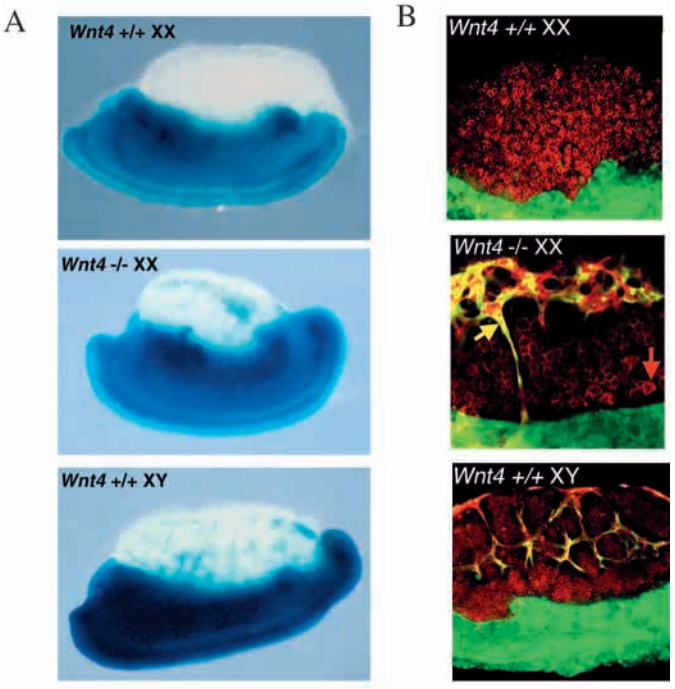

Fig. 3. Mesonephric endothelial cells migrate into *Wnt4*$^{-/-}$ XX gonads. (A) Mesonephroi from 12.5 dpc embryos ubiquitously expressing *lacZ* were incubated with gonads from 12.5 dpc embryos that were *Wnt4*$^{+/+}$ XX and XY or *Wnt4*$^{-/-}$ XX and stained for β-galactosidase activity. (B) Mesonephroi from 11.5 dpc embryos ubiquitously expressing green fluorescent protein (GFP) (green) were incubated with gonads from 11.5 dpc embryos that were *Wnt4*$^{+/+}$ XX and XY or *Wnt4*$^{-/-}$ XX. After incubation the sample was stained with an antibody against Pecam (red). Pecam marks endothelial cells and germ cells (red arrow indicates Pecam-stained germ cells; yellow arrow indicates Pecam-stained endothelial cells).

The *Wnt4* gene is also expressed in the developing mesonephros. We therefore wanted to investigate whether mesonephroi from *Wnt4*$^{-/-}$ embryos had a role in mesonephric migration into the gonad. For this, we bred mice with the mutant *Wnt4* allele with the ROSA26 line. Using mice from this cross, we incubated wild-type 11.5 dpc XX and XY gonads with mesonephroi from 11.5 dpc embryos that were homozygous for the mutant *Wnt4* allele and were also expressing *lacZ* ubiquitously. Analysis of these cultures showed that mesonephric cell migration patterns were normal: migration into the XY gonad was still observed but did not occur exogenously into XX gonads (data not shown; all 16 mutant mesonephroi, 10 were assayed with XX gonads and 6 with XY gonads, showed this phenotype). These results indicate that the repressive role of WNT4 on mesonephric cell migration is driven primarily by WNT4 protein produced in the XX gonad.

WNT4 represses steroidogenic cell migration

Vainio et al. reported the presence of ectopic cells expressing steroidogenic cell markers such as *Cyp17* and 3β-HSD in the mutant *Wnt4* XX gonads (Vainio et al., 1999). These markers are usually found in adrenals and testicular Leydig cells but are not present in the ovary at early stages of gonad development. We performed a detailed analysis of the expression pattern of the gonad and adrenal steroidogenic marker 3β-HSD in *Wnt4*$^{-/-}$ embryos during early stages of gonad development. Our analysis revealed that the ectopic cells expressing this marker were few and tended to cluster around the anterior region of the gonad of both sexes, close to the region where the adrenal was forming at early stages of gonad development in both sexes (Morohashi, 1997) (Fig. 4). As development proceeded the ectopic cells were found in other regions of the gonad. This pattern was also found for other steroidogenic markers such as *Cyp11a1* and *Cyp17*. Heikkila et al. recently extended this study to include a marker specific to steroidogenic adrenal cells, *Cyp21*, which they found ectopically expressed within the *Wnt4*$^{-/-}$ XX gonad (Heikkila et al., 2002). This pattern of expression suggested that the ectopic steroidogenic cells in the *Wnt4*$^{-/-}$ XX gonad had migrated from the mesonephros during development.

To investigate this possibility we used a transgenic line of mice we created with a Bacterial Artificial Chromosome (BAC) construct containing the *Cyp11a1* gene in which the *lacZ* gene was inserted by homologous recombination into the coding region (Cyp11a1:*lacZ*). We analysed *lacZ* expression in this line of mice during gonad and adrenal development and found it to follow the pattern seen for the endogenous *Cyp11a1* gene (data to be published elsewhere). Using in vitro organ co-culture experiments we incubated mesonephroi from embryos

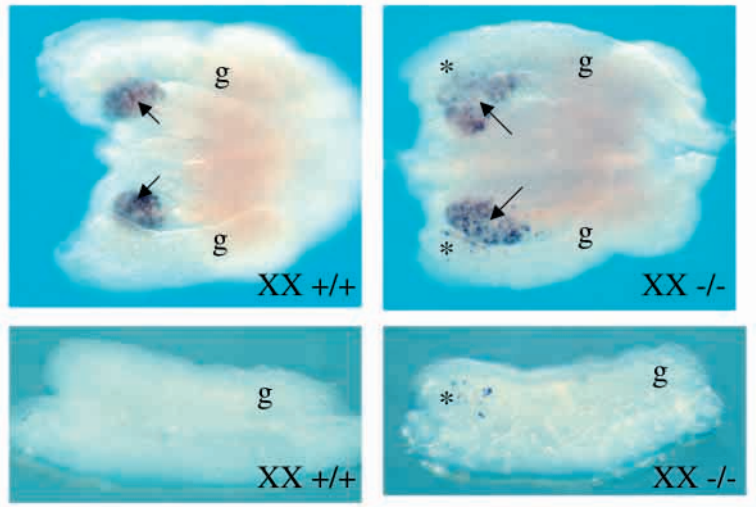

Fig. 4. Ectopic steroidogenic cells are clustered at the anterior region of the *Wnt4*$^{-/-}$ gonad. Whole-mount in situ hybridisation for 3β-hydroxysteroid dehydrogenase (3β-HSD) expression was performed on gonads and mesonephric region from *Wnt4*$^{+/+}$ or *Wnt4*$^{-/-}$ XX 12.5 dpc embryos as indicated. The top panels show the area within the urogenital region where the adrenal forms, which is indicated by the arrows. The asterisks indicate the anterior region of the gonad where the ectopic steroidogenic cells are found in the *Wnt4*$^{-/-}$ embryos. The position of the gonad is marked 'g'. In all images, anterior is leftwards.

from this line with XX wild-type and *Wnt4* mutant gonads from 11.5 dpc and 12.5 dpc embryos. After incubation the samples were stained for β-galactosidase activity and, as expected, no wild-type XX gonads showed the presence of *lacZ*-expressing cells (16 samples were analysed). In contrast, five out of eleven *Wnt4*$^{-/-}$ XX gonads showed the presence of *lacZ*-expressing cells (Fig. 5). These results show that WNT4 represses steroidogenic cell migration from the mesonephros into the XX gonad.

The expression pattern of steroidogenic markers in the *Wnt4*$^{-/-}$ XX gonads suggested that the steroidogenic cells that migrated into the *Wnt4*$^{-/-}$ XX gonad were derived from the anterior region of the mesonephros where the adrenal was forming. To investigate this possibility we performed in vitro organ co-culture experiments with mesonephroi from Cyp11a1:*lacZ* embryos in which the anterior region had either been included or removed. As expected, no *lacZ*-positive cells were found within the XX *Wnt4*$^{-/-}$ gonad when the anterior region of the mesonephros was absent (2 out of 2 samples showed this phenotype) (Fig. 5). Consistent with our previous results, two out of two samples in these experiments showed *lacZ*-expressing cells within the XX *Wnt4*$^{-/-}$ gonad when the anterior portion was included. These studies indicate that adrenal precursor cells can migrate into the XX gonad in *Wnt4* mutant embryos.

The role of *Wnt4* in testicular vascular formation and steroidogenic cell differentiation

Our detailed analysis of the expression of the *Wnt4* gene at early stages of gonad development showed that it is expressed in the early gonad and mesonephros of both sexes before sex determination takes place. After *Sry* expression in the male gonad, *Wnt4* is downregulated in the testis whereas it is upregulated in the ovary. Mesonephric expression continues in both sexes (data not shown). These results are consistent with those of Vainio et al. (Vainio et al., 1999). Our results show that at early stages of gonad development in both sexes the role of WNT4 is to repress the formation of the coelomic blood vessel and prevent the presence of ectopic steroidogenic cells in the gonad. The downregulation of *Wnt4* in the XY gonad after the action of *Sry* suggested that ectopic expression of WNT4 might have a repressive role in vascular formation and steroidogenic cell differentiation in the XY gonad. To investigate this possibility we sought to misexpress WNT4 in the developing testis. For this we used a BAC construct containing the *Sf1* gene. We chose *Sf1* because it is expressed continuously at high levels in the genital ridge throughout early gonadogenesis and in the Sertoli and Leydig cells of the developing testis (Ikeda et al., 1994). We inserted the *Wnt4* cDNA into the 5′ untranslated region of the *Sf1* gene by

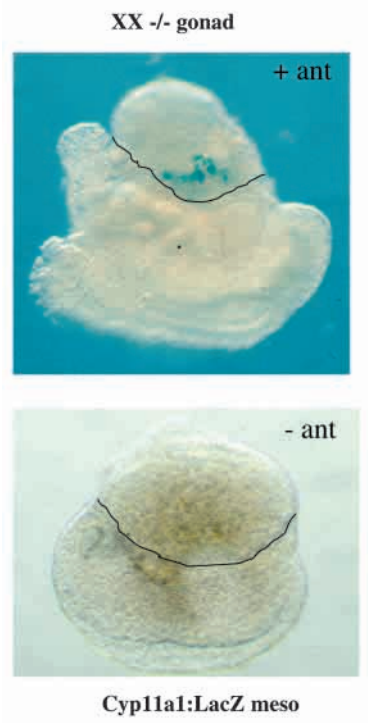

Fig. 5. Steroidogenic cells migrate from the anterior region of the mesonephros into the *Wnt4*$^{-/-}$ XX gonad. Gonads from *Wnt4*$^{-/-}$ XX 11.5-12.5 dpc embryos were incubated with mesonephroi from Cyp11a1:*lacZ* 11.5-12.5 dpc embryos and stained for β-galactosidase activity. (Top) The anterior region of the mesonephros was included in the co-culture, and (bottom) the anterior region of the mesonephros was removed. The lines indicate the boundary between gonad and mesonephros in the co-cultures.

homologous recombination and made transgenic mice that contained this BAC construct (called Sf1:Wnt4).

Expression analysis of the animals containing the Sf1:Wnt4 BAC construct showed that sequences from the transgenic construct were expressed during development in a pattern similar to that of the *Sf1* gene. Whole-mount in situ hybridisation for *Wnt4* expression on testes from transgenic embryos revealed ectopic expression of *Wnt4* in the gonad, which resembled the *Sf1* pattern of expression (Fig. 6) (transgenic embryos from four different integration events were analysed in this way and showed this phenotype). RTPCR analysis showed that transgenic-specific sequences were expressed in the adrenal but not in the kidney (transgenic embryos from nine different integration events were analysed) and in the urogenital ridge but not in limb at 11.5 dpc, the stage when *Wnt4* is downregulated in XY gonads (transgenic embryos from two integration events were analysed) (data not shown).

Contrary to our expectation, all testes from Sf1:Wnt4 transgenic embryos that were generated showed the presence of a coelomic vessel when observed by light microscopy (testes were analysed at different stages ranging from 13.5 dpc to 15.5 dpc and were derived from ten different integration events). All these embryos showed expression of the transgene in the adrenal. However, when stained with an antibody against Pecam six out of seven testes that were analysed from transgenic embryos from different integration events showed that the structure of the coelomic vessel was abnormal (Fig. 7A). In most cases the blood vessels failed to coalesce to form a single prominent vessel as seen in gonads from non-transgenic embryos. This phenotype was similar to the pattern of the coelomic vessel from testis of wild-type younger, 12.5 dpc embryos, which is initially a series of branches that later coalesce to form the prominent coelomic artery (see Fig. 1B and Fig. 7A, part d). However, in the case of the transgenic embryos the vessels did not form one prominent vessel but usually resulted in highly branched vessels that did not run the length of the testis. Although there is some variation in the pattern of the non-transgenic coelomic vessel, the transgenic testes were readily distinguishable at this stage. This shows that WNT4 expression in the developing testis does not inhibit the initiation of coelomic blood vessel formation but does interfere with the pathway and patterning of this principle artery.

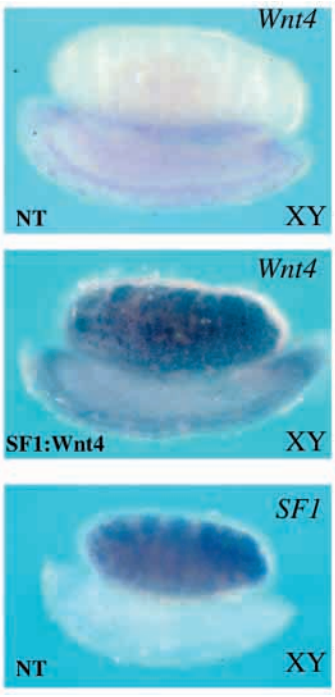

Fig. 6. *Wnt4* is expressed in testis of Sf1:Wnt4 transgenic animals. Whole-mount in situ hybridisation for *Wnt4* expression was performed on 13.5 dpc testis from transgenic and non-transgenic (NT) embryos and for *Sf1* expression on 13.5 dpc testis from a non-transgenic.

To investigate whether Leydig cell differentiation was affected in the Sf1:Wnt4 transgenic embryos we analysed the pattern of *Cyp11a1* expression in the transgenic testes. Whole-mount in situ hybridisation was performed on three transgenic testes from different integration events and all showed a normal number of *Cyp11a1*-expressing cells (Fig. 7B). These results indicate that ectopic WNT4 does not inhibit Leydig cell differentiation.

DISCUSSION

The role of WNT4 in vascular formation in the gonad

Vascular formation during gonad development is a dynamic process that is modulated depending on the sex of the gonad. Our studies show that WNT4 signalling is part of the mechanism that establishes sex-specific vasculature. In the ovary, its main role is to repress endothelial cell migration from the mesonephros into the XX gonad and prevent the formation of a coelomic blood vessel, a testis-specific event. Interestingly, a prominent blood vessel was also observed in the adrenal of *Wnt4*$^{-/-}$ embryos, which was not present in wild-type embryos (see Fig. 1). *Wnt4* is expressed in the adrenal during development (Heikkila et al., 2002). This suggests that during development of the urogenital region one of the roles of WNT4 is to pattern vascular formation by specifying domains where endothelial cell migration is inhibited.

Marker analysis in the *Wnt4*$^{-/-}$ XX gonad showed that male-specific genes associated with the coelomic blood vessel in the normal testis were expressed, suggesting that a male-specific pathway had been initiated in the female gonad. However, in agreement with Vainio et al. (Vainio et al., 1999), we did not find any Sertoli cell-specific markers or any sign of testis cord formation at early stages of gonad development. This suggests that the signalling molecules required for endothelial cell migration are different from those required for cord formation. Endothelial cells have been shown to promote differentiation of organs such as the pancreas and liver (Lammert et al., 2001; Matsumoto et al., 2001). However, in this case endothelial cell migration did not induce differentiation of male somatic cells or morphological development of the testis such as testis cord formation, suggesting that other male-specific factors are required.

Our studies of *Wnt4*$^{-/-}$ XX gonads indicated that WNT4 represses endothelial cell migration and coelomic blood vessel formation during gonad development. These findings suggested that downregulation of *Wnt4* expression seen in the XY gonad after the action of *SRY* was a required step to ensure coelomic vessel formation in the testis. Our misexpression studies showed that WNT4 does not inhibit the initiation of coelomic blood vessel formation in the testis. However, the

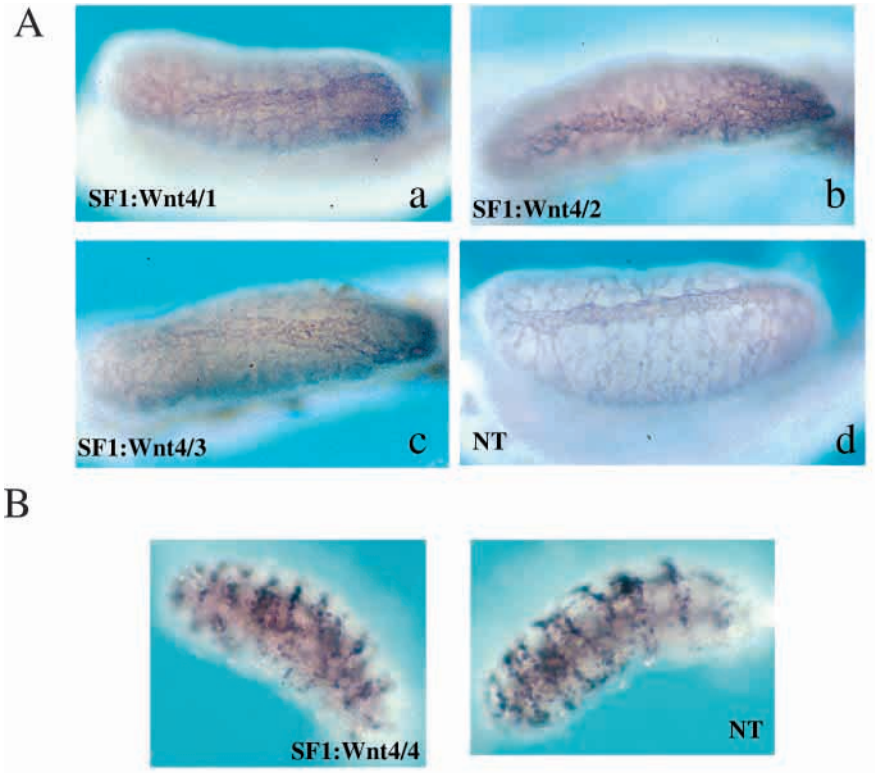

Fig. 7. Vascular and steroidogenic phenotype observed in the Sf1:Wnt4 transgenic XY gonads. (A) Coelomic blood vessel pattern is affected in Sf1:Wnt4 transgenic testis. Testis from transgenic (a-c) and non-transgenic (NT) (d) 13.5 dpc embryos were stained with an antibody to Pecam. (B) Leydig cell differentiation occurs in Sf1:Wnt4 transgenic testis. Whole-mount in situ hybridisation for *Cyp11a1* expression on 13.5 dpc XY gonads from a Sf1:Wnt4 transgenic embryo (left) and a non-transgenic embryo (NT) (right). The transgenic gonads were derived from different integration events (1-4).

pattern of the coelomic blood vessel in the Sf1:Wnt4 transgenic XY animals was found to be disorganised, showing that a high level of WNT4 interfered with vascular patterning in the testis.

Our results suggest that repression by WNT4 is inefficient in the transgenic embryos. One explanation for our results is that two different signalling systems contribute to coelomic blood vessel formation in the testis, only one of which is repressed by WNT4. In the Sf1:Wnt4 transgenic XY embryos, the alternative operative signalling system may partially rescue some coelomic vessel patterning. An alternative and simpler explanation is that the molecular environment in the XY gonad is different to that of the XX gonad and that WNT4 is prevented from acting efficiently in the testis. For example, a testis-specific factor produced as a consequence of *SRY* action in the Sertoli cell lineage could inhibit WNT4 action in the transgenic XY gonad. A reasonable candidate for this factor is the TGFβ-family member, anti-Müllerian hormone (AMH). *Amh* expression in the XY gonad begins around 11.5 dpc (Hacker et al., 1995; Munsterberg and Lovell-Badge, 1991). Moreover, an association between AMH signalling and β-catenin, an element of the canonical WNT signalling pathway, has been previously reported (Allard et al., 2000). After 11.5 dpc, AMH could antagonise the activity of the ectopic WNT4 and limit its disruptive effect on vessel pattern formation to a brief period during testis development. The variability seen in the phenotype of the Sf1:Wnt4 transgenic embryos could then be explained by subtle differences in the timing of expression of the transgene with respect to that of the antagonist.

The role of WNT4 in steroidogenic cell recruitment

Production of steroids during gonad development is highly regulated in a sex-specific manner. WNT4 is part of the signalling pathway that ensures that the XX gonad does not produce sex hormones that masculinise the developing embryo (Vainio et al., 1999). Our results show that the role of WNT4 in this process is to repress the migration of steroidogenic cells from the mesonephros into the XX gonad during early gonad development. Various observations indicated that the migrating steroidogenic cells in the *Wnt4*$^{-/-}$ embryos were adrenal cell precursors. The expression pattern of steroidogenic cell markers, including the adrenal-specific marker *Cyp21*, at early stages of gonad development in *Wnt4*$^{-/-}$ XX embryos showed that the ectopic steroidogenic cells clustered in the area of the gonad that was closest to the developing adrenal (Heikkila et al., 2002). Also, our in vitro co-culture experiments using mesonephroi from Cyp11a1:*lacZ* embryos showed that *lacZ*-positive cells were found in the XX *Wnt4*$^{-/-}$ gonad only when the region of the mesonephros where the adrenal gland normally forms was included.

Our analysis of the Sf1:Wnt4 transgenic embryos showed that WNT4 has a disruptive effect on vascular pattern formation in the testis. However, the presence of WNT4 in the XY gonad had no effect on Leydig cell differentiation. These results are therefore consistent with our view obtained from the *Wnt4* mutant embryos, that WNT4 acts to repress the migration of a few steroidogenic adrenal cells into the gonad. However, in contrast to the proposal of Vainio et al. (Vainio et al., 1999), it is not required to repress the differentiation of Leydig cell precursors already present within the gonad. It is most probable that the latter differentiate in situ in response to signals from the Sertoli cells, which may include DHH (Yao et al., 2002). Perhaps *Wnt4* expression in the ovary can be considered a 'safety factor' to help ensure the adrenal precursors do not enter and give inappropriate expression of steroids in the female.

The data presented here shows that WNT4 represses the molecular pathway that controls the process of migration of at least two different cell types from the mesonephros into the gonad. Our data is consistent with the model that WNT4 is

preventing the action of the cell migration signal produced by the gonad, either by repressing the expression of the gene encoding the signal or by directly inhibiting the diffusion of the (long-range) signal to the mesonephros. Candidate factors for the cell migration signal include vascular endothelial growth factor (VEGFA), endocrine gland vascular endothelial growth factor (EG-VEGF) and fibroblast growth factor 9 (FGF), which are found in the developing gonad (Ferrara, 1999; Colvin et al., 2001; LeCouter et al., 2002). We have analysed the expression of *FGF9*, *EG-VEGF* and the different isoforms encoded by the *VEGFA* gene in the *Wnt4*$^{-/-}$ XX gonad but find no evidence of repression of the expression of these genes by WNT4 action (data not shown). We have obtained recent data that shows that the ovary-specific gene follistatin is not expressed in the XX *Wnt4* mutant gonad (H. Yao and B.C., unpublished). This suggests an unexpected mechanism of action of WNT4 that involves the activation of expression of an antagonist of molecules from the TGFβ superfamily. Further studies will reveal how this pathway is involved in this process. Interestingly, Shu et al. have shown that another member of the WNT family, WNT7b, is implicated in vascular development in the lung (Shu et al., 2002). However, in contrast to the work presented here there is no direct effect of WNT7b on endothelial development. Instead, their results show a defect in the development of vascular smooth muscle cells in mice lacking this factor.

The precise nature of the molecular pathways involved in cell movements within the developing gonad need to be established and this will be the focus of future work. However, our results clarify the role of WNT4 in this process as well as providing a new hypothesis of how cell movements are controlled in a sex-specific manner such that they lead to the appropriate morphogenesis of either an ovary or a testis.

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