Disrupted gonadogenesis and male-to-female sex reversal in *Pod1* knockout mice

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Summary

Congenital defects in genital and/or gonadal development occur in 1 in 1000 humans, but the molecular basis for these defects in most cases remains undefined. We show that the basic helix-loop-helix transcription factor Pod1 (capsulin/epicardin/Tcf21) is essential for normal development of the testes and ovaries, and hence for sexual differentiation. The gonads of *Pod1* knockout (KO) mice were markedly hypoplastic, and the urogenital tracts of both XX and XY mice remained indistinguishable throughout embryogenesis. Within *Pod1* KO gonads, the number of cells expressing the cholesterol side-chain cleavage enzyme (*Scc*) was increased markedly. Biochemical and genetic approaches demonstrated that Pod1 transcriptionally represses steroidogenic factor 1 (Sf1/Nr5a1/Ad4BP), an orphan nuclear receptor that regulates the expression of multiple genes (including *Scc*) that mediate sexual differentiation. Our results establish that *Pod1* is essential for gonadal development, and place it in a transcriptional network that orchestrates cell fate decisions in gonadal progenitors.

Key words: Pod1, Tcf21, Sf1, Gonadogenesis, Testis development, Sex reversal, Leydig cell

Introduction

Mammalian sexual differentiation is a complex process that begins with the establishment of genetic sex (XX or XY) at the time of fertilization. In mice, the bipotential gonads arise from the coelomic epithelium of the urogenital ridges and initially are indistinguishable in males and females. Between 10.5 and 12.5 days post coitum (dpc), a gene on the Y chromosome, designated *Sry*, initiates the male developmental pathway (Gubbay et al., 1990; Koopman et al., 1990; Sinclair et al., 1990). By 12.5 dpc, the XY gonads form testicular cords, which contain fetal Sertoli cells and primordial germ cells, surrounded by peritubular myoid cells, steroidogenic Leydig cell precursors, and developing blood vessels in the interstitial region. In the absence of *Sry*, an ovary develops that contains granulosa and steroidogenic thecal cells. In contrast to the testis, morphogenesis of the ovary occurs postnatally and depends upon the presence of viable XX germ cells (McLaren, 1991).

Further reproductive development of the internal and external genitalia is determined by the presence or absence of a functioning testis. The internal genitalia derive from either the Müllerian (paramesonephric) or Wölffian (mesonephric) ducts, which initially are present in both XX and XY embryos. Sertoli and Leydig cells produce three hormones that mediate male sex differentiation. Sertoli cells produce anti-Müllerian hormone (AMH), which causes regression of the Müllerian ducts. Leydig cells produce testosterone, which induces the formation from the Wölffian ducts of seminal vesicles, epididymis, vas deferens, and the peptide hormone insulin-like 3 (Insl3), which is essential for normal testes descent. In the absence of testicular hormones, the Wölffian ducts regress and the Müllerian ducts form the oviducts, Fallopian tubes, uterus and upper vagina in the female developmental pathway (Byskov and Hoyer, 1994).

Although *Sry* unequivocally initiates the male developmental pathway, most of the mechanisms that mediate testes development remain to be defined. Gene knockout studies have established essential roles in early gonadal development for several transcription factors, including Wilms tumor suppressor 1 (*WT1*) (Kreidberg et al., 1993), lim-containing homeodomain protein (*Lhx9*) (Birk et al., 2000), and the orphan nuclear receptor steroidogenic factor 1 (*Sf1*) (Parker et al., 1996), but the precise mechanisms by which these genes contribute to gonadogenesis remain undefined. Even less is known about the transcriptional pathways downstream of these genes that establish specific gonadal cell lineages, and this remains a key area for ongoing investigations.

Transcription factors with the basic helix-loop-helix (bHLH) motif play crucial roles in cell fate determination and differentiation in a variety of tissues, including the gonads. For example, the bHLH factor *Hand1* is required for gonadal

development in *C. elegans* (Mathies et al., 2003), whereas the bHLH gene *FIG alpha* (*Figla*) is required for formation of the primordial follicles in the mammalian ovary (Soyal et al., 2000). To date, however, no bHLH factors have been implicated in mammalian testes development or prenatal sex differentiation.

We previously identified a bHLH protein named Pod1 and generated a null *Pod1* allele through homologous recombination in embryonic stem cells (Quaggin et al., 1999; Quaggin et al., 1998). *Pod1* KO mice displayed defects in kidney, facial muscle, and splenic development, and died at birth from respiratory failure due to an absence of alveoli (Lu et al., 2000; Lu et al., 2002; Quaggin et al., 1999). We subsequently noted that the external genitalia were feminized in XY *Pod1* KO pups (data not shown), prompting us to examine the role of *Pod1* in gonadal development. We show here that the absence of *Pod1* in the urogenital ridges leads to ectopic expression of Sf1, aberrantly committing a population of urogenital progenitor cells to a steroidogenic cell fate in both XX and XY gonads, and disrupting normal processes of gonadal development.

Materials and methods

Targeted disruption of *Pod1* and collection of fetal tissues
Generation of the *Pod1* targeting vector and *Pod1* KO mice has been described in detail (Quaggin et al., 1999). *lacZ* and *neomycin* cassettes replace the first exon that encodes the entire bHLH domain generating a null *Pod1* allele. For timed matings, noon of the day when a vaginal plug was detected was counted as 0.5 dpc. Tail or head DNA was purified from embryos at 11.0-18.5 dpc, or from pups from postnatal day 0 (P0) onwards. The *Pod1* genotype was determined by Southern blot analysis or polymerase chain reaction (PCR), as described (Quaggin et al., 1999). Genetic sex was determined by PCR using *Zfy* primers, which generated a 180-bp fragment in XY samples (Gubbay et al., 1992), and *Rapsyn* primers (Colvin et al., 2001), which generated a 589-bp fragment in all samples.

X-gal staining
Whole genital ridges containing the mesonephros and gonads from embryos at 11.5 and 12.5 dpc were dissected in phosphate-buffered saline (PBS) and transferred to *lacZ* fixative for 30 minutes at room temperature, as described (Partanen et al., 1996). Samples were then rinsed in wash buffer and incubated in *lacZ* stain at 37°C for 20-50 minutes, and post-fixed in 10% formalin for 2 hours. The gonads from XX and XY embryos at 18.5 dpc were dissected and fixed in *lacZ* fixative for 1 hour at room temperature. After rinsing in *lacZ* wash buffer, the gonad was immersed in 30% sucrose overnight at 4°C, and embedded in OCT. Ten micrometer thick sections were cut with a microtome blade on a Leica CM-3050 cryostat. Samples were rinsed in wash buffer, then incubated in *lacZ* stain for 1-4 hours, post-fixed in 10% formalin and counterstained with nuclear Fast Red.

Whole-mount double-label immunohistochemistry
Embryonic genital ridges were dissected and fixed overnight in 4% paraformaldehyde at 4°C. Samples were then washed in PBS and blocked in a solution of 3% BSA, 1% heat-inactivated goat serum, and 0.1% Triton X-100 in PBS for 2-3 hours at room temperature before staining with antibodies. The primary antibodies used were anti-CD31/PECAM (Pharmingen, Ontario, Canada; 1:300 dilution), anti-laminin (1:300 dilution), anti-Sf1 (1:500 dilution), anti-β-galactosidase (Promega, Madison, WI, USA; 1:200 dilution) and anti-GFP (Molecular Probes, Eugene, OR; 1:2000 dilution). Samples were incubated in the primary antibodies and rocked at 4°C overnight. After washing four times for at least 1 hour in PBT, samples were incubated

with secondary antibodies for 1 hour. The secondary antibodies used were Cy3-conjugated goat anti-rat IgG (Jackson Laboratories, Ontario, Canada; 1:500 dilution) to detect anti-CD31, Cy3-conjugated donkey anti-mouse IgG (Jackson Laboratories; 1:200 dilution) to detect anti-β-galactosidase and FITC-conjugated goat anti rabbit IgG (Jackson Laboratories; 1:500) to detect anti-laminin, anti-GFP or anti-Sf1. Samples were finally washed four times for 1 hour in PBT and mounted in DABCO (Sigma) for subsequent confocal microscopy with a Zeiss LSM 410 laser scanning confocal microscope.

Migration assays
XY gonads from 12.5 dpc ICR (albino outbred strain; JAX Laboratories, Bar Harbor, ME, USA) or *Pod1*⁻/⁻ mice were assembled with mesonephroi from 11.5 dpc GFP-positive mice and co-cultured on an agar block for 46 to 72 hours, as described (Martineau et al., 1997). A total of 20 experiments were performed with 10 mice of each genotype. The GFP-positive mice were generated in the laboratory of Dr A. Nagy and express enhanced green fluorescent protein ubiquitously (gift of A. Nagy, Samuel Lunenfeld Research Institute). Organ cultures were collected and fixed, and wholemounts were immunostained for GFP and CD31, and visualized by confocal microscopy as described above.

In situ hybridization
In situ hybridization was performed on paraformaldehyde-fixed/OCT-embedded sections, as described (Conlon and Rossant, 1992). Whole-mount in situ hybridization was performed, as previously described (Wilkinson and Nieto, 1993). Probes used for in situ hybridization were the murine *Scc* probe, a 0.5-kb *Eco*RI-*Bam*H1 fragment (Martineau et al., 1997), *Sox9* (mouse, p*Sox9*, 0.5-kb *Sma*I fragment) (Wright et al., 1995), mouse *11 beta-hydroxylase* (Domalik et al., 1991) (564 bp fragment), *Dhh* (Yao et al., 2002), *Dmc1* (a gift from David Page, MIT; which contains bases 602-1245 of the *Dmc1* gene, GenBank NM 010059), *Wnt4* (a gift from Andy McMahon at Harvard; includes the entire coding region), *follistatin* (a gift from Martin Matzuk at Baylor; consists of a 846 bp fragment of the 3′ UTR of the *follistatin* cDNA). Digoxigenin-labeled probes were prepared according to the Boehringer-Mannheim-Roche protocol.

Analysis of apoptosis
Apoptosis analysis was performed on paraffin sections using TUNEL labeling methods. Embryonic genital ridges were dissected and fixed in 10% formaldehyde at 4°C and embedded in paraffin wax. After de-waxing, samples were rehydrated and digested, before being pre-incubated with One-Phor-ALL-buffer (Amersham Pharmacia Biotech, Canada) for 10 minutes and incubated with TdT solution mix, which includes 1×one-Phor-ALL-buffer; 6 nM Biotin-16-dUTP (Roche Applied Science, Canada), 1 μM dATP; 0.2 U TdT enzyme and 0.01% Triton X-100, for 2 hours at 37°C. Samples were then incubated in ABC solution (VectaStain Kit; Vector Laboratories, Burlingame, CA 94010) and further developed with DAB (Peroxidase Substrate Kit DAB, Vector Laboratories) color staining. Samples were counterstained with Hematoxylin, dehydrated, mounted and photographed.

Results

The internal and external genitalia of *Pod1* KO mice develop abnormally

As described previously, *Pod1* KO mice die shortly after birth because of severe lung defects (Quaggin et al., 1999). Although XX and XY *Pod1* KO mice were born in the expected 50:50 ratio, the external genitalia were feminized in XY pups such that all pups were indistinguishable externally (data not shown). To determine the basis for this sex reversal, we

Fig. 1. Both male and female *Pod1* KO mice have abnormal reproductive tracts. (A-F) Urogenital tracts of 18.5 dpc mice: (A,C,D) XY; (B,E,F) XX. In wild-type XY embryos, the testes (t) are rounded and have descended to the level of the bladder (b). In *Pod1* KO XY embryos, the gonads are much smaller than controls and are adjacent to the kidneys (A). The gonads in *Pod1* KO mice are irregular in shape with an uneven surface (D). In addition, the reproductive duct (green arrowheads) in the XY mutant has not developed into a normal epididymis (e), compared with in the wild-type mouse (C), and the internal genitalia rather resemble those of wild-type and *Pod1* KO females. The ovaries (ov) in a wild-type embryo at 18.5 dpc have descended to just below the kidney (B). In XX *Pod1* KO mice, the right gonad is sometimes observed in this location, but the left gonad never descends properly (B). *Pod1* KO ovaries (F) are similar in size to the wild-type structures (E), but are irregularly shaped and display a morphology similar to the *Pod1* KO testes. Of note, kidneys and bladders in both XY and XX *Pod1* KO mice are also greatly reduced in size relative to controls. t, testis; ov, ovary; a, adrenal gland; k, kidney; b, bladder; e, epididymis. Semi-thin sections demonstrate that the wild-type testis at 18.5 dpc (G) exhibits well-organized testicular cords with Sertoli cells (sc) surrounding germ cells (g), peritubular myoid cells (pm), and extensive interstitial tissue, including Leydig cells (le) and vessels. The *Pod1* KO testis (H)

appears to have degenerated with a number of apoptotic cells (arrows, in H). At 18.5 dpc, most germ cells from control ovaries are in meiotic prophase (g, in I), but in the mutant XX gonad, they still appear as primordial germ cells similar to those in the testes (g in J). TUNEL labeling is shown in XY (K,L) and XX (M,N) gonads from 16.5 dpc embryos. In control gonads (K,M), no apoptotic cells are found in the sections, but in the gonads of Pod1 XY (L) and XX (N) mutants, a marked increase in the number of apoptotic cells (arrows) is observed.
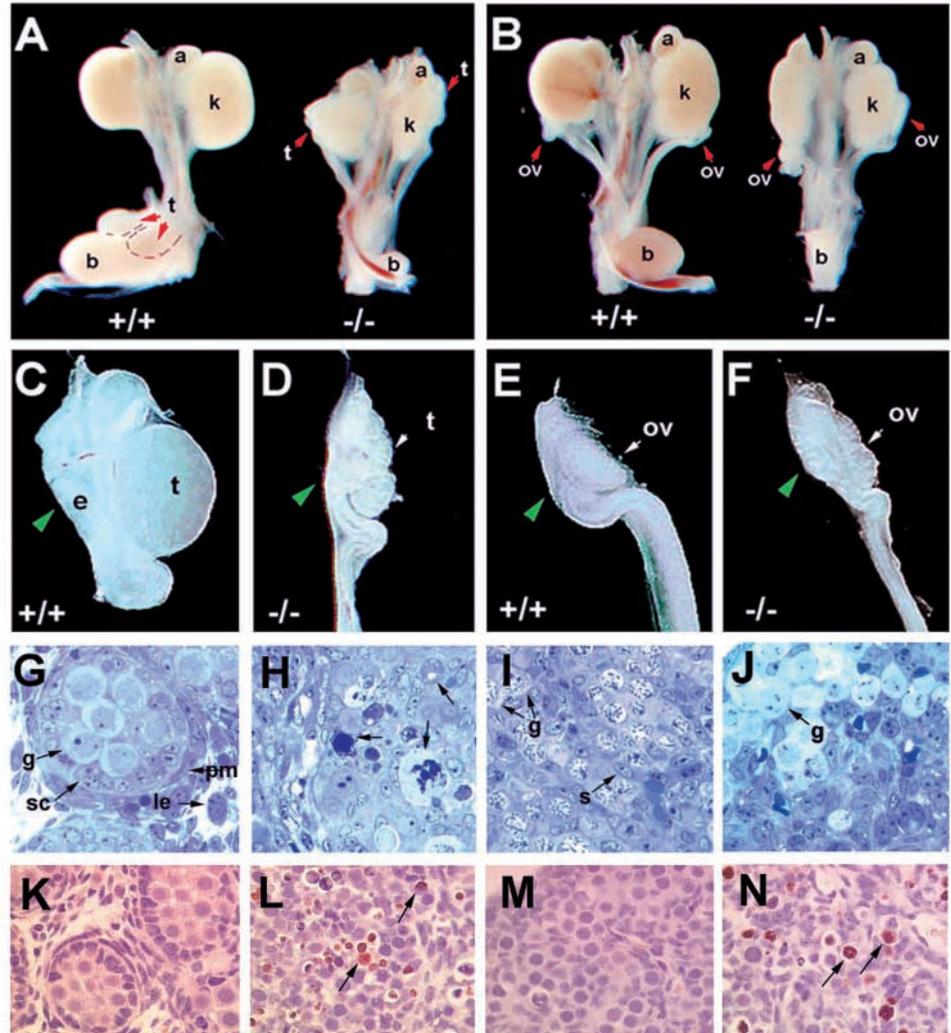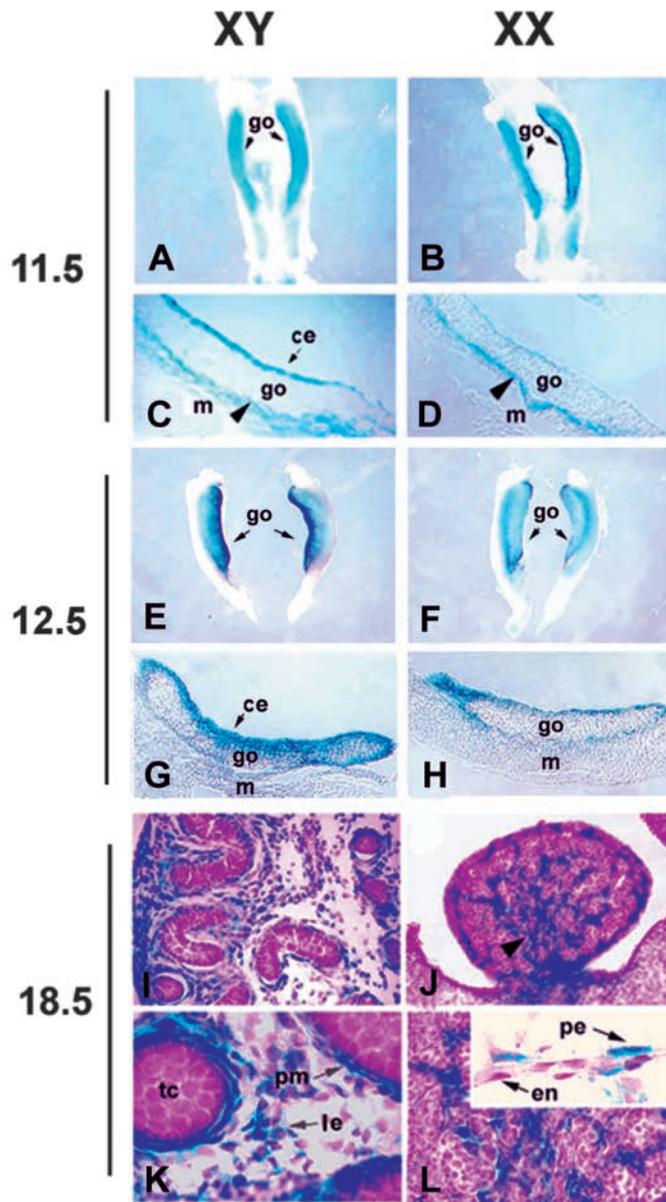
examined the urogenital tracts of *Pod1* KO and wild-type fetuses at 18.5 dpc. As previously reported (Quaggin et al., 1999), kidneys and bladders in both XY and XX mutants were greatly reduced in size relative to controls (Fig. 1A,B). The wild-type testes at 18.5 dpc were rounded and had descended to the level of the bladder (Fig. 1A), and the epididymis was easily identified (Fig. 1C). By contrast, *Pod1* KO testes were dramatically smaller, had an irregular shape and an uneven surface (Fig. 1D), and remained adjacent to the kidneys, frequently connected directly to the adrenal gland (Fig. 1A). The internal genitalia were poorly developed and appeared structurally similar to the corresponding structures in wild-type XX embryos (Fig. 1D,E). At 18.5 dpc, the ovaries from *Pod1*^{+/+} and *Pod1*^{+/−} embryos had descended to a location just below the kidneys (Fig. 1B). In XX *Pod1* KO embryos, the location of the ovaries was variable. The right ovary was found either adjacent to or just below the kidney, whereas the left ovary was always observed adjacent to the kidney, usually still connected to the adrenal gland (Fig. 1B). The *Pod1* KO ovaries were the

same size as wild-type ovaries (Fig. 1E) but were irregular in shape (Fig. 1F). Thus, the gross morphologies of XX and XY *Pod1* KO gonads were indistinguishable at this stage.

Histological analyses of *Pod1* KO testes at 18.5 dpc confirmed the lack of organized testicular cords (Fig. 1H) and revealed numerous cells with features suggestive of apoptosis. Whereas most germ cells in wild-type ovaries at 18.5 dpc were in meiotic prophase (Fig. 1I), those in *Pod1* KO ovaries resembled the germ cells seen in the testes (Fig. 1J). TUNEL staining of 16.5 dpc XY and XX mutant gonads confirmed a marked increase in the number of cells undergoing apoptosis (Fig. 1K-N).

Pod1 is expressed in both XX and XY gonads during embryogenesis

The dramatic gonadal phenotype in male and female *Pod1* KO mice could reflect either intrinsic defects in the gonads or secondary effects due to lack of *Pod1* expression in other sites. To investigate *Pod1* expression, we took advantage of a *lacZ*

lacZ-expressing cells were concentrated in the ovarian medulla and in the interstitial spaces between forming follicles. Similar *lacZ* expression patterns were observed in the adult testis and ovary, and in situ hybridization for *Pod1* transcripts confirmed the expression patterns observed with the *lacZ* reporter (data not shown).
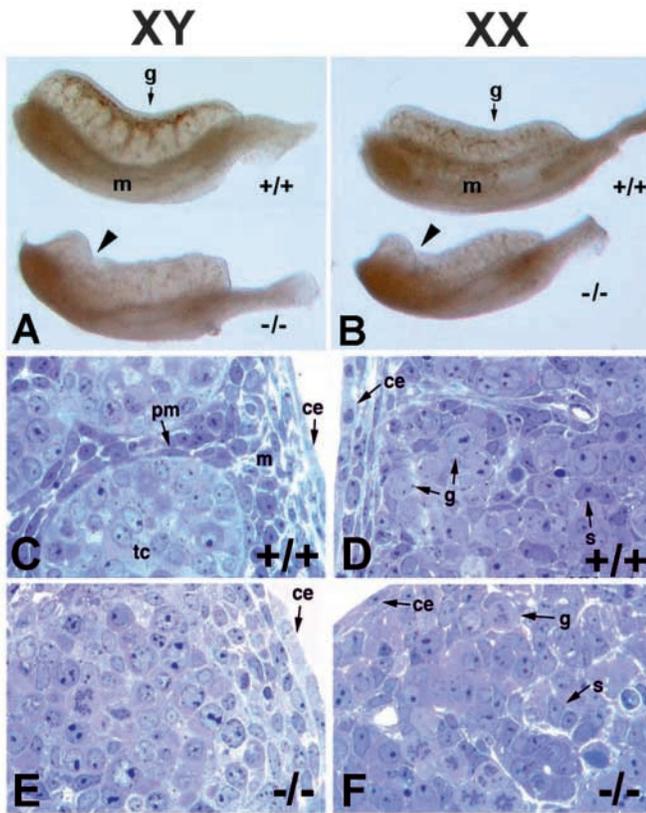
Early gonad development is disrupted in *Pod1* KO mice

To pinpoint when the *Pod1* KO gonads first exhibit abnormalities, we examined earlier stages of gonad development. Gonads are distinguishable from the mesonephros by approximately 10.5 dpc. In males, the Sertoli cells cluster around the primordial germ cells at ~12.0 dpc to initiate testicular cord formation, and by 12.5 dpc, the testes can be grossly distinguished from the ovaries because they are larger and exhibit a male-specific vascular pattern.

At 11.0 dpc, differences were already observed between *Pod1* KO and wild-type gonads. Both XY and XX *Pod1* KO gonads were slightly shortened in length and had an irregular surface (data not shown). At 12.5 dpc, testes from *Pod1* KO embryos lacked the features of normal testes noted above, and instead resembled *Pod1* KO ovaries (Fig. 3A,B). Both XY and XX *Pod1* KO gonads displayed morphological abnormalities, including a large invagination of the surface epithelium near the anterior end of the gonad (Fig. 3A,B).

Microscopic examination of sections from wild-type testes at 12.5 dpc revealed testicular cords, peritubular myoid cells, and extensive mesenchyme in the interstitium between cords (Fig. 3C), whereas no such histological organization was observed in *Pod1* KO testes (Fig. 3E). In genetic females, both wild-type and *Pod1* KO ovaries exhibited little morphological differentiation and showed a similar arrangement of germ and somatic cells. The *Pod1* KO ovary, however, lacked a distinct mesenchymal zone (Fig. 3D,F).

To further assess testicular cord formation in *Pod1* KO testes, we examined expression of laminin and CD31/PECAM. Laminin is a component of the basal lamina deposited by Sertoli cells that delineates testicular cords, whereas PECAM

reporter gene incorporated into the KO allele in *Pod1*$^{+/-}$ mice. At 11.5 dpc, *lacZ* staining was observed in both XY and XX urogenital ridges (Fig. 2A,B). Sections taken from the stained urogenital ridges demonstrated that *Pod1* expression in both sexes localized primarily to the coelomic epithelium of the gonad, and to the boundary region between the gonad and mesonephros (Fig. 2C,D). At 12.5 dpc, *lacZ* expression persisted in both XY and XX gonads (Fig. 2E,F), with somewhat higher expression seen in XY gonads (Fig. 2G,H), mostly concentrated in the coelomic epithelium.

At 18.5 dpc, *lacZ* staining again was apparent in the developing testes and ovaries of *Pod1*$^{+/-}$ embryos (Fig. 2I,J). In the testes, *lacZ* was expressed strongly in peritubular myoid cells immediately surrounding the testis cords, in presumed Leydig cells in the interstitial region, and in pericytes surrounding capillaries (Fig. 2K, and inset in 2L). Expression was also detected throughout the developing ovaries (Fig. 2J,L). Although the specific cell type(s) have not been defined,

**Fig. 3.** Abnormal gonadal morphology in both male and female *Pod1* mutants. (A) At 12.5 dpc, gonads from XY *Pod1* KO mice (bottom) were smaller than in the wild-type testes (top). (B) Gonads from a *Pod1* KO XX embryo at 12.5 dpc also were reduced in size compared with the control. In both XY and XX *Pod1* KO embryos, an abnormal invagination of the surface was observed at the anterior region of the gonad (g) (arrowheads). g, gonad; m, mesonephros. (C-F) Semi-thin sections from 13.5 dpc gonads were stained with Toluidine Blue. At 13.5 dpc, the wild-type XY gonad (C) showed organized testicular cords (tc), peritubular myoid cells (pm) and extensive mesenchyme (m). However, no organized testicular cords, peritubular myoid cells or mesenchyme were seen in *Pod1* KO XY gonads (E). At 13.5 dpc, *Pod1* KO XX gonads (F) showed a similar arrangement of germ (g) and somatic (s) cells as the control (D), but lacked the small but distinct mesenchymal zone near the coelomic epithelium. tc, testicular cord; pm, peritubular myoid cell; m, mesenchyme; ce, coelomic epithelium; g, germ cell; s, somatic cell.

Fig. 4. Structural and vascular defects in *Pod1* KO mice. Confocal images are shown of 12.5 dpc gonads double-labeled with antibodies to laminin (green) and CD31/PECAM (red), which labels both germ cells (round) and vascular cells (elongated). In wild-type males at 12.5 dpc (A,C), the green laminin staining delineated numerous testicular cords (tc, arrows). The wild-type testes also displayed the characteristic coelomic vessel, which forms right beneath the coelomic epithelium (C, white arrowheads). In the *Pod1* KO testes (B,D), no testicular cords were present and the coelomic vessel was also absent. The coelomic epithelium (blue arrowheads) was highly disorganized and invaginated in many locations. In wild-type females (E,G), no clear morphological organization was observed at this stage of development. However, compared with the control, the *Pod1* KO XX gonad (F,H) exhibited a very disorganized surface epithelium, similar to that in gonads from the XY mutant. Of note, germ cell numbers did not appear to be affected in either XY or XX *Pod1* KO mice.

is a membrane protein specific to germ cells and vascular cells. Wild-type testes at 12.5 dpc displayed numerous cords that were clearly outlined by the laminin staining (Fig. 4A). The coelomic epithelium was a well-organized, single layer containing cylindrical epithelial cells above an intact basal lamina (Fig. 4C). The characteristic male-specific coelomic vessel was clearly visible in the mesenchyme just beneath the coelomic epithelium (Fig. 4C). By contrast, the *Pod1* KO testes at 12.5 dpc were disorganized and lacked distinct testicular cords (Fig. 4B). The coelomic epithelium was highly irregular and contained numerous invaginations. The basal lamina was disrupted in regions, and germ cells were directly adjacent to the coelomic epithelium near the basal lamina. No coelomic vessel was observed (Fig. 4D) but, unlike the wild-type testes, numerous vascular circuits extended through the interior of the

gonad and contacted the coelomic epithelium. The germ cells, which migrate from the base of the allantois through the gut mesentery and enter the gonads between 10.5 and 11.5 dpc, were present in comparable numbers in both wild-type and *Pod1* KO testes at 12.5 dpc, as determined by PECAM labeling (Fig. 4C,D).
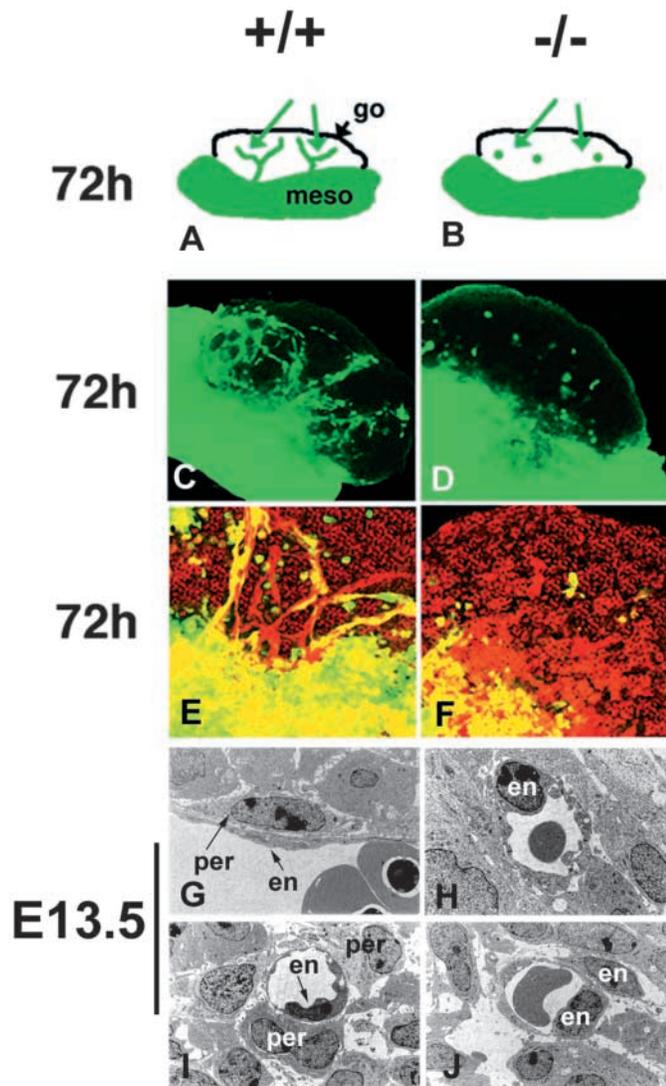
Fig. 5. Vascular defects in *Pod1* KO gonads. Migration assays with GFP-expressing (green) wild-type mesonephros, and wild-type (A,C,E) or mutant (B,D,F) gonads at 72 hours, demonstrated a marked decrease in migration of endothelial cells (red) into *Pod1* KO gonads. Yellow denotes double-staining of GFP and CD31/PECAM-positive cells, confirming the presence of mesonephric-derived vascular networks in wild-type but not *Pod1* KO gonads. At 13.5 dpc, pericytes (per) were observed in close association with endothelial (en) cells of wild-type gonadal capillaries (G,I), but were never seen around *Pod1* KO capillaries (H,J).

At 12.5 dpc, germ and somatic cells were intermingled throughout the wild-type ovaries (Fig. 4E,G), which lacked a distinct histological organization. However, obvious differences were observed in the *Pod1* KO XX gonads, which closely resembled the *Pod1* KO XY gonads (Fig. 4F,H) and displayed an irregular coelomic epithelium with one large invagination and a similar vascular pattern.

Vascular development is abnormal in *Pod1* KO gonads because of an intrinsic defect in the gonad

Endothelial cell migration from the mesonephros into the developing gonad is an early event in testis development

(Brennan et al., 2002). We therefore used co-culture migration assays to determine whether defective endothelial cell migration contributed to the abnormal development of the coelomic vessel and other vessels in the mutant testes. GFP-expressing wild-type mesonephroi were combined with wild-type or *Pod1* KO 12.5 dpc XY gonads, and co-cultured for 48 to 72 hours (Fig. 5A,B). By 48 hours, a GFP-positive vascular network was clearly observed in wild-type gonads but was absent from *Pod1* KO gonads. Although this result is interesting and may explain the defects in vascular patterning observed in the mutant gonads, it is not clear whether Pod1 is normally required within the gonad for endothelial cell migration to occur, or if other morphological defects in the mutant gonads are responsible for the block in migration.

Because *Pod1* also is expressed in pericytes that surround developing capillaries, we used electron microscopy to determine whether pericytes were affected in mutant *Pod1* gonads. At 13.5 dpc (Fig. 5G-J), pericytes were clearly visible around developing capillaries in the wild-type gonads, but were absent in *Pod1* KO gonads. These results suggest that the absence of *Pod1* impedes the differentiation of pericytes, which in turn may be associated with the impaired vasculogenesis seen in the *Pod1* KO gonads.

Sertoli cells can still differentiate in *Pod1* KO testes

Sertoli cells are the first somatic lineage to arise in the testes and are believed to play a crucial role in its subsequent differentiation and organization. To determine whether Sertoli cell differentiation was disrupted in *Pod1* KO testes, we examined the expression patterns of two Sertoli cell-specific markers, *Sox9* and desert hedgehog (*Dhh*). At 12.5 dpc, both *Sox9* and *Dhh* were highly expressed in the wild-type testes (Fig. 6A,C), but were absent in wild-type ovaries (Fig. 6B,D). Both *Sox9* and *Dhh* were expressed in the *Pod1* KO testes, although transcript levels were decreased, particularly in the anterior domain (Fig. 6A,C). Real-time PCR data analysis showed no difference in *Sry* expression between *Pod1* KO and *Pod1*^{+/−} XY gonads (data not shown). These results suggest that *Pod1* is not required for Sertoli cell differentiation (as these cells express Sertoli cell-specific markers), but rather that it may be required for the maintenance or expansion of the Sertoli cell population. As expected, neither *Sox9* nor *Dhh* were expressed in Pod1 KO ovaries. By 18.5 dpc, no *Sox9* or *Dhh* expression was observed in XY *Pod1* KO gonads (not shown), although this may reflect the degeneration of the mutant gonads rather than a disruption of later Sertoli cell development.

The steroidogenic cell population is expanded in *Pod1* KO gonads

Leydig cells are first observed in the embryonic testes shortly after Sertoli cells arise (e.g. at ~12.5 dpc). The cholesterol side-chain cleavage enzyme (*Scc*) is an early marker for Leydig cell differentiation and catalyzes the initial reaction in the steroidogenic pathway (Morohashi and Omura, 1996; Rice et al., 1990). *Scc* is also a marker for the steroidogenic cells of the adrenal glands, which arise adjacent to the gonads. We therefore examined *Scc* expression to assess steroidogenic cell differentiation and development in *Pod1* KO mice. At 12.5 dpc, *Scc* was expressed in scattered cells throughout the wild-type XY testes, as well as in the adrenal primordium of both XY and XX wild-type embryos (Fig. 6E,F). At the same stage, *Pod1* KO
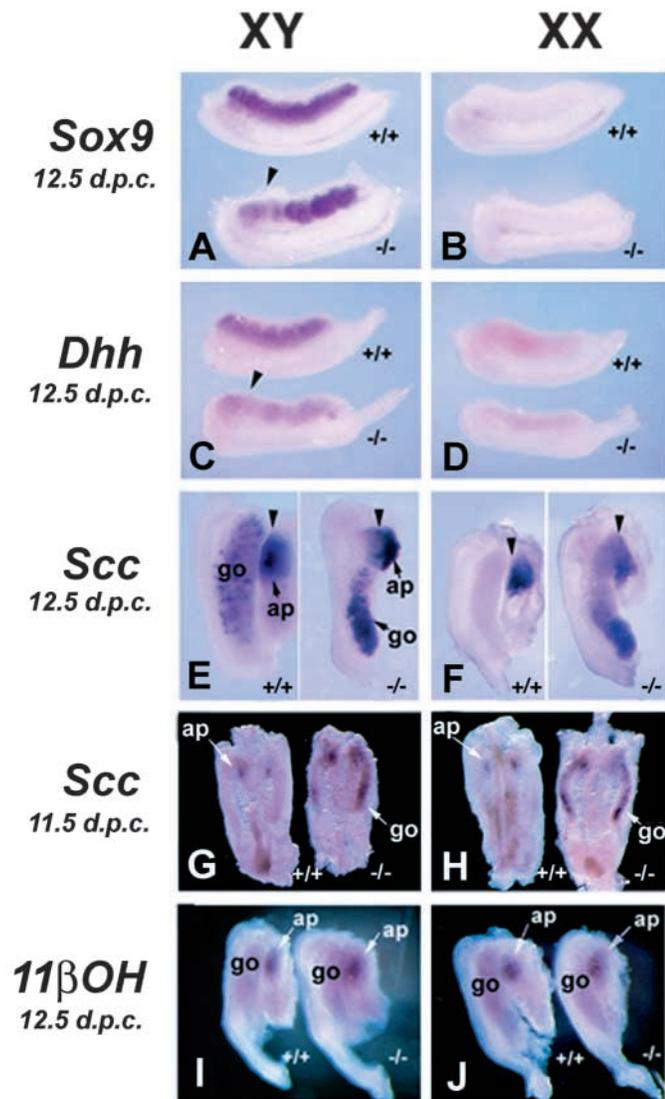
Fig. 6. Disruption of male-specific markers in *Pod1* KO gonads. Whole-mount in situ hybridization for *Sox9* (A,B) and *Dhh* (C,D) was performed with gonads from 12.5 dpc embryos. *Sox9* and *Dhh* were expressed by Sertoli cells in wild-type testes (A,C, top), but were absent in wild-type XX gonads (B,D, top). In *Pod1* XY mutants (A,C, bottom), expression of both *Sox9* and *Dhh* was reduced relative to controls, especially in the anterior domain of the gonad (arrowheads). Neither *Sox9* nor *Dhh* was expressed in gonads from XX mutants (B,D, bottom). *Scc* , a marker of Leydig and adrenocortical cells, was expressed in the wild-type XY gonad (E), and in the adrenal primordial cells (ap) of both XY (E) and XX (F) wild-type embryos. *Pod1* mutant XY and XX gonads (E,F) had greatly increased *Scc* expression, particularly at the posterior end of the gonad. Of note, the anterior ends of the *Pod1* KO gonads did not separate from the adrenal primordium (E,F; arrowheads), although a distinct boundary between the gonads and adrenal glands was present in the controls (E,F, arrowheads). (G,H) At 11.5 dpc, *Scc* expression was restricted to adrenal primordia (ap) of wild-type XX and XY embryos, but was seen throughout the gonads (go) within the urogenital ridges of *Pod1* mutant XX and XY gonads. (I,J) The adrenal-specific steroidogenic marker 11β-hydroxylase was expressed appropriately in the adrenal primordia (ap) of both wild-type and *Pod1* KO mice at 12.5 dpc. ap, adrenal primordia; go, gonad.

demonstrating that the steroidogenic cell lineage is not only expanded but also differentiates prematurely in *Pod1* mutants.
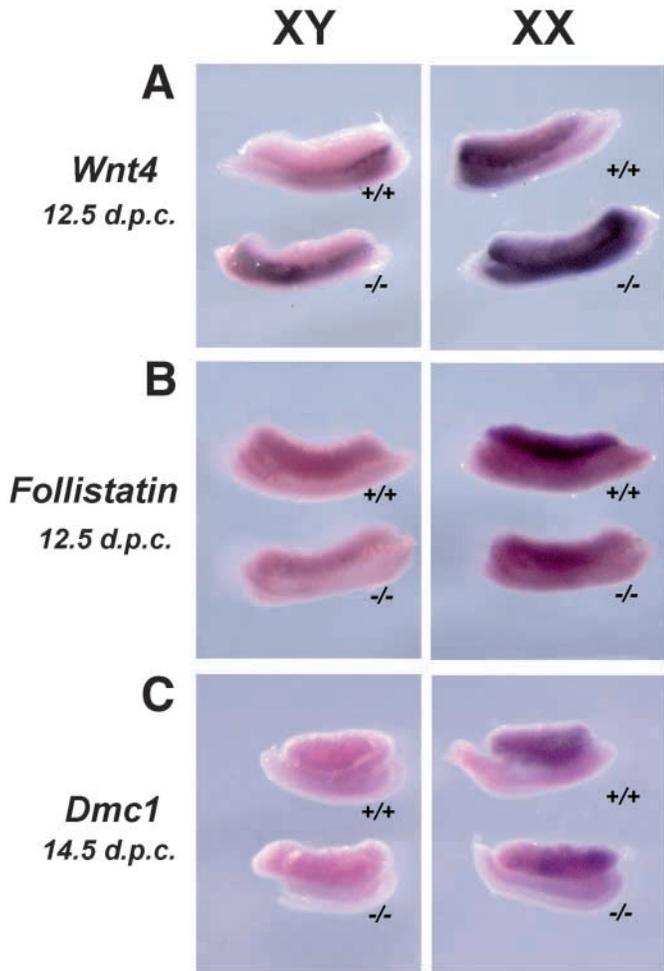
To determine whether other cytochrome P450 steroidogenic enzymes also were aberrantly expressed, we performed in situ analysis for the adrenal-specific enzyme, steroid 11β-hydroxylase (11β-OH). In contrast to *Scc*, this enzyme was appropriately restricted to the adrenal primordia in both wild-type and *Pod1* KO embryos, suggesting that the absence of *Pod1* does not cause a general dysregulation of steroid hydroxylase expression (Fig. 6I,J). Furthermore, these results show that although no clear morphologic boundary can be seen between the adrenal primordial and the gonad in *Pod1* KO embryos, these tissues are distinguishable at the molecular level.

XX markers are expressed in Pod1 KO gonads

To determine how early ovarian development is affected in XX *Pod1* mutant gonads, early markers of XX gonad formation were examined (Fig. 7). We performed in situ analysis for *Wnt4* and *follistatin*, both markers of XX somatic cells, and *Dmc1*, a marker for XX germ cells entering meiosis. *Wnt4* is normally expressed within the gonads of XX but not XY mice at 12.5 dpc. *Wnt4* was expressed in both mutant and wild-type XX gonads at this stage. Of note, expression of *Wnt4* in the mesonephros was increased in both XX and XY mutants, as compared with controls, but the significance of this is not clear. *Follistatin* (Menke and Page, 2002) was also expressed in both mutant and wild-type XX gonads, but at a somewhat reduced level in mutants. Finally, *Dmc1* (Menke et al., 2003) expression was also observed in *Pod1* mutant XX gonads. These results demonstrate that both somatic cells and germ cells initiate aspects of normal ovarian development, but this is clearly disrupted by 18.5 dpc, as no meiotic germ cells are observed in *Pod1*^{−/−} XX gonads at this stage.

The domain of Sf1 expression is expanded in gonads and mesonephroi of *Pod1* KO mice

Steroidogenic factor 1 (Sf1) is an orphan nuclear receptor that

gonads had dramatically higher levels of *Scc* expression relative to controls, particularly within the posterior portion of the gonad (Fig. 6E,F). Although the levels of *Scc* expression were similar in wild-type and *Pod1* KO adrenals, the developing adrenal gland in the *Pod1* KO mice did not separate cleanly from the anterior portion of the gonad. The region of the mutant gonad that maintained contact with the adrenal primordium was the same region in which decreased levels of *Sox9* and *Dhh* were observed.

Although *Scc* also is required for steroid hormone synthesis in the postnatal ovary, mouse ovaries normally do not express *Scc* in utero (Fig. 6F). *Pod1* KO ovaries had marked upregulation of *Scc* relative to wild-type ovaries (Fig. 6F). As in XY *Pod1* KO mice, this expression concentrated at the posterior region of the *Pod1* KO ovary, whereas the anterior end of the gonad again appeared to be fused with the adrenal primordium (Fig. 6F).

At 11.5 dpc, *Scc* expression is restricted to the adrenal primordia of wild-type XX and XY embryos, but is precociously expressed throughout the gonads within the urogenital ridges of *Pod1* KO XX and XY mice (Fig. 6G,H),

Fig. 7. Early female-specific markers are expressed in *Pod1* KO XX gonads. Whole-mount in situ hybridization for *Wnt4* (A) and *follistatin* (B) is shown in XY and XX gonads from 12.5 dpc embryos. Expression of *Dmc1*, a marker of meiotic germ cells (C), is shown in 14.5 dpc embryos. *Wnt4* is expressed in control XX gonads and mesonephroi, and was increased in mesonephroi of XX mutants (–/–). *Follistatin* is also expressed in XX mutant gonads, although the expression is somewhat decreased as compared with controls. *Dmc1* expression is similar in XX mutants and controls.

plays key roles in steroidogenesis and reproduction. Of note, *Sf1* KO mice have adrenal and gonadal agenesis, establishing its essential roles in development of the primary steroidogenic tissues (Parker, 1998; Parker et al., 1996; Parker and Schimmer, 1997). Sf1 is first expressed in the urogenital ridge at ~9 dpc and this expression continues in the early gonads. As sex differentiation occurs, Sf1 expression in the ovary decreases at ~12.5-13.5 dpc, whereas expression persists in both Sertoli cells and Leydig cells in the testes. Sf1 has been proposed to be an essential regulator of *Scc*, prompting us to examine whether the dysregulation of *Scc* might reflect abnormal expression of Sf1. In wild-type testes at E11.5, Sf1-positive cells were scattered throughout the interior of the gonad (Fig. 7A), and no Sf1-positive cells were observed in either the coelomic epithelium or the boundary area between the gonad and the mesonephros. In both male and female *Pod1* KO embryos, Sf1 expression was increased in both the coelomic

epithelium and the boundary region between the gonad and mesonephros (Fig. 8A, and data not shown), where *Pod1* is normally expressed (Fig. 8A). Furthermore, the number of Sf1-positive cells throughout the gonad was increased considerably, very likely in the same cells that aberrantly expressed *Scc*.

To determine whether Sf1 is ectopically expressed in the same cells that normally express *Pod1*, immunohistochemistry was performed using antibodies to β-galactosidase and Sf1. At 12.5 dpc, Pod1-expressing cells do not express Sf1 in heterozygotes. However, co-expression of both Sf1 and β-galactosidase can be seen in mutant gonads using confocal microscopy and immunostaining for β-galactosidase and Sf1 (Fig. 8B).

Discussion

Previous studies have defined essential roles of Pod1 in lung, kidney, facial muscle, and splenic development. We now extend these analyses to the gonads, showing that Pod1 deficiency markedly impaired gonadal development and sex differentiation. We further provide a potential mechanism – the dysregulated expression of Sf1 – to explain the gonadal abnormalities seen in the *Pod1* KO mice.

We show here for the first time that *Pod1* is expressed in the indifferent gonad at 11.5 dpc, subsequently localizing to the interstitial region as the testes form discrete compartments. By 18.5 dpc, the *Pod1*-directed *lacZ* reporter in testes was expressed in peritubular myoid cells, fetal Leydig cells, and pericytes surrounding blood vessels, whereas *lacZ*-expressing cells in the ovaries were found in the medulla and the interstitial spaces between the primordial follicles. We also noted *lacZ* expression directed by *Pod1* regulatory sequences in the coelomic epithelium of the gonad and mesonephric stromal cells at the boundary between the gonad and mesonephros. Thus, the gonadal abnormalities seen in *Pod1* KO mice, which are apparent by the indifferent gonad stage at 11.5 dpc, may reflect intrinsic defects in cells that arise directly in the indifferent gonad. However, *Pod1* is also expressed in regions from which progenitor cells migrate into the gonads to generate several somatic lineages in the interstitial region of the testes (Karl and Capel, 1998; Martineau et al., 1997). Although further studies with cell-specific KO of *Pod1* are needed, it is likely that both intragonadal and extragonadal expression of *Pod1* is required for normal development of the gonads.

One striking defect in *Pod1* KO testes is the absence of the characteristic coelomic vessel. Furthermore, vascular abnormalities were observed throughout both XX and XY gonads. Migration assays showed that endothelial cell migration from wild-type GFP-expressing mesonephroi into XY KO gonads was markedly decreased compared with wild-type gonads. This observation most likely explains the absence of the male-specific coelomic vessel and its branches in the testes, as these structures are known to derive from migrating endothelial cells (Brennan et al., 2002). Gonadal pericytes, which are intimately associated with endothelial cells, also express *Pod1*. Defects in pericyte differentiation have previously been described in *Pod1* KO mice (Cui et al., 2003), and pericytes are absent in *Pod1* KO gonads. Disrupted pericyte development in *Pod1* KO mice may contribute to the observed vascular defects, as previous studies have shown that
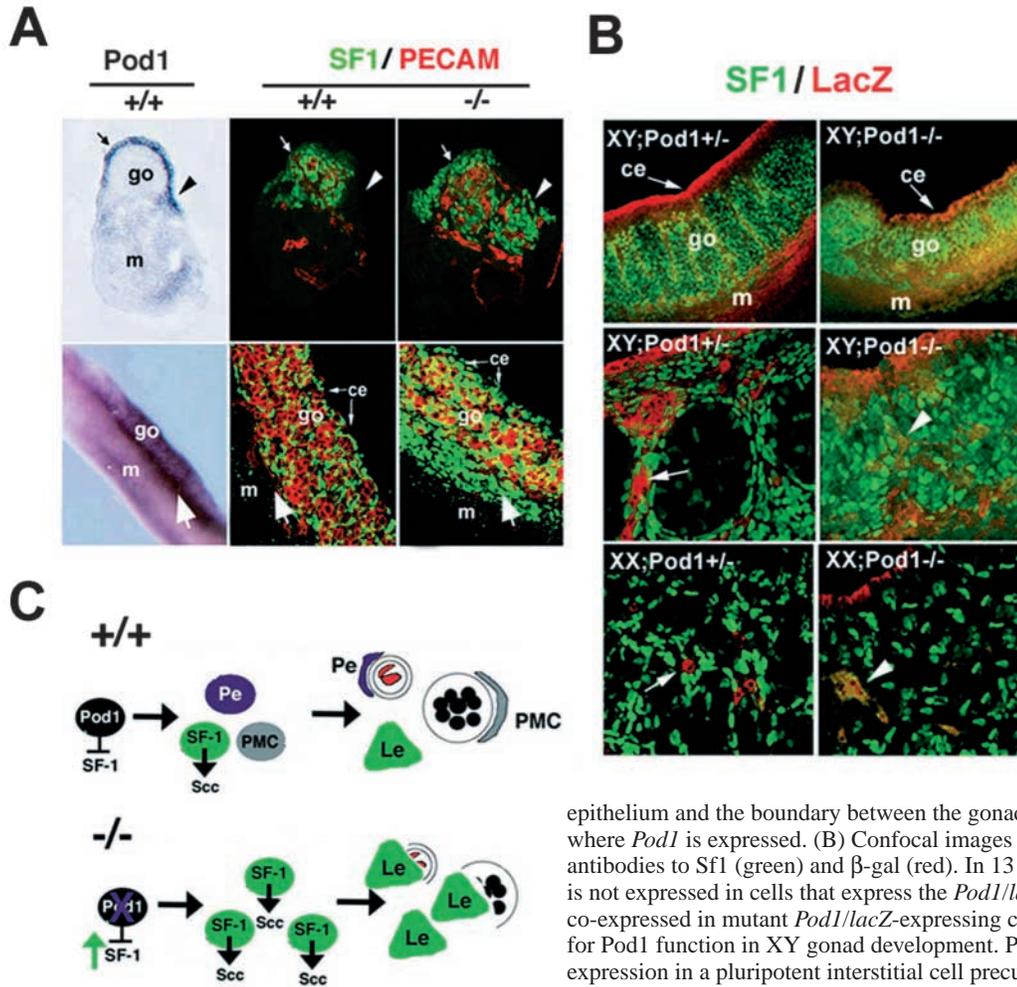
Fig. 8. Expansion of Sf1 expression in gonads and mesonephroi of *Pod1* KO mice. (A) In situ hybridization analyses and immunohistochemistry of transverse sections and whole-mount genital ridges of XY gonads (go) at 11.5 dpc are shown. Gonads were labeled with a riboprobe against *Pod1*, or double-labeled with antibodies to Sf1 (green) and PECAM (red). *Pod1* expression was concentrated in the coelomic epithelium (arrows) and the boundary of the gonad and mesonephros (arrowheads). Sf1-positive cells were evenly scattered in the interior of the control XY gonads. In the *Pod1* KO XY gonads, Sf1 expression was increased in both the coelomic epithelium (arrow) and the interior of the gonad. A large population of Sf1-positive cells was seen at the boundary region between the gonad and the mesonephros (arrowhead). Note that the coelomic epithelium and the boundary between the gonad and the mesonephros are domains where *Pod1* is expressed. (B) Confocal images of 13 dpc gonads double-labeled with antibodies to Sf1 (green) and β-gal (red). In 13 dpc XX and XY control gonads, Sf1 is not expressed in cells that express the *Pod1*/*lacZ* reporter gene. By contrast, Sf1 is co-expressed in mutant *Pod1*/*lacZ*-expressing cells (arrowheads, yellow). (C) Model for Pod1 function in XY gonad development. Pod1 is proposed to repress Sf1 expression in a pluripotent interstitial cell precursor (in the mesonephros and/or coelomic epithelium, ce), thereby permitting differentiation of several interstitial cell lineages, including fetal Leydig cells (Le), peritubular myoid cells (PMC) and pericytes (Pe). Loss of Pod1 leads to ectopic expression of Sf1, and prematurely commits the progenitor cells to a steroidogenic cell lineage.

genetic or physical ablation of pericytes leads to defects in vascular remodeling (Benjamin et al., 1998; Hellstrom et al., 1999; Lindahl et al., 1997).

To further define the basis for the abnormal urogenital development in *Pod1* KO mice, we examined the expression of markers of different gonadal cell lineages. Two markers of Sertoli cells, *Sox9* and *Dhh*, were still expressed in *Pod1* KO testes (albeit at reduced levels), demonstrating that the initial stages of Sertoli cell development can proceed in the absence of *Pod1*. Similarly, early female somatic markers are expressed in XX but not XY mutants, which illustrates that initial stages of the female developmental pathway can also occur without *Pod1*. By contrast, we observed striking changes in the expression of the steroidogenic enzyme *Scc* in both XX and XY *Pod1* KO embryos. Although *Scc* is not expressed in wild-type ovaries during embryogenesis, *Scc* was strongly expressed in the gonads of both XX and XY *Pod1* KO embryos. Because the adrenal primordia did not clearly separate from the gonads in *Pod1* KO embryos, we wondered whether the cells expressing *Scc* in the *Pod1* KO gonads might be ectopically located adrenal cells, as reportedly occurs in *Wnt4* KO mice (Heikkila et al., 2002). However, the adrenal-specific

steroidogenic enzyme 11β-OH was not expressed in the corresponding region of either XX or XY *Pod1* KO gonads, suggesting that the *Scc*-expressing cells in *Pod1* KO gonads are not ectopic adrenal cells. Collectively, our data demonstrate that the absence of *Pod1* permits ectopic expression of *Scc* in the genital ridge, rather than expanding the field of adrenocortical cells into the genital ridge.

How might the absence of *Pod1* be associated with the dysregulated expression of *Scc*? Previous transfection studies have shown that *Scc* expression is activated by the orphan nuclear receptor Sf1 (Clemens et al., 1994), which plays key roles in steroidogenesis and development of the adrenal glands and gonads (Luo et al., 1999). In *Pod1* KO embryos, Sf1 expression was increased both in the coelomic epithelium and at the boundary between the gonad and mesonephros, two regions where *Pod1* is normally expressed. Furthermore, Sf1 was co-expressed with the β-galactosidase reporter that replaced the first exon of the *Pod1* gene, demonstrating ectopic expression of Sf1 in *Pod1*-expressing cells. Together, these findings support the model that Pod1 normally represses Sf1 expression in these sites in a cell autonomous manner, and thus concomitantly prevents the ectopic expression of *Scc*.

Consistent with this model, Pod1 repressed *Sf1* promoter activity in mouse Y1 and MA-10 steroidogenic cell lines in a dose-dependent manner (data not shown). Moreover, mutation of the E box at –82 to –77 markedly decreased *Sf1* promoter activity, as previously described (Tamura et al., 2001). Although previous studies have shown that Pod1 can bind to the E-box in the smooth muscle α-actin and p21 promoters (Funato et al., 2003; Hidai et al., 1998; Lu et al., 1998), the E-box in the *Sf1* promoter does not contain the Pod1 consensus sequence determined through binding-site selection (P. Igarashi, unpublished). Furthermore, Pod1 was unable to bind to the *Sf1* E-box element in EMSAs, even in the presence of the cofactor E12 (data not shown). Instead, we found that Pod1 can inhibit the binding of Usf1 to the *Sf1* E-box in a dose-dependent manner (data not shown), thus preventing the action of this known activator of *Sf1* expression (Daggett et al., 2000) although co-immunoprecipitation results failed to show that Pod1 directly interacts with the Usf1 protein. Collectively, our results suggest that Pod1 represses *Sf1* expression in an indirect manner.

Although our results do not prove that the ectopic expression of Sf1 causes the impaired gonadogenesis in *Pod1* KO mice, they do demonstrate a striking association between *Pod1* deficiency and dysregulated Sf1 expression. Based on this association, we propose that Pod1 normally represses *Sf1* expression in a pluripotent interstitial progenitor cell population, and that dysregulated expression of Sf1 in *Pod1* KO mice commits them to differentiate prematurely or excessively towards the steroidogenic cell lineage (Fig. 8C). Similar ectopic expression of Sf1 in embryonic stem cells forced them to differentiate towards a steroidogenic cell fate, and to express *Scc* (Crawford et al., 1997). In the XY gonad, we further propose that expansion of the Leydig cell population is associated with the loss of peritubular myoid cells and pericytes, thereby disrupting the organization of testicular structure and vasculature. Although the different cell types in the embryonic ovary are less well defined, a similar increase in the steroidogenic lineage was observed, suggesting that *Pod1* plays similar roles in both sexes during the early stages of gonad formation. Regardless of the underlying mechanism, our studies show that *Pod1* is essential for testis and ovary development, and establish a novel transcriptional pathway for allocating the somatic cell lineages within the gonad.

Despite the expanded domain of *Sf1* and *Scc* expression, neither XX nor XY pups underwent virilization of internal or external genitalia, and the testes failed to descend. These findings suggest that the biosynthesis of all three mediators of male sex differentiation is impaired in XY *Pod1* KO mice. As noted above, ectopic Sf1 expression in embryonic stem cells induced the expression of *Scc* but did not induce the full complement of steroidogenic enzymes (Crawford et al., 1997). Studies of KO mice and of patients with impaired sex differentiation suggest that multiple genes interact to direct the complex developmental events in gonadogenesis and sex differentiation (Parker et al., 1999); thus, the combined activation of several genes may be needed to induce the biosynthesis of the hormones that mediate male sex differentiation. Alternatively, it remains possible that the impaired virilization in *Pod1* KO mice results from degeneration of Leydig cells and/or the vascular defects

described above. Nonetheless, our data are consistent with analyses of humans with autosomal dominant and recessive inactivating mutations in SF1 (Achermann et al., 1999; Achermann et al., 2002), suggesting that precise regulation of Sf1 is required for normal gonadal development.

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