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Expression and Functional Analysis of *Dkk1* during Early Gonadal Development

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Dkk1 · *Rspo1* · Testis development · *Wnt4*

Abstract
WNT signalling plays a central role in mammalian sex determination by promoting ovarian development and repressing aspects of testis development in the early gonad. Dickkopf homolog 1 (DKK1) is a WNT signalling antagonist that plays critical roles in multiple developmental systems by modulating WNT activity. Here, we examined the role of DKK1 in mouse sex determination and early gonadal development. *Dkk1* mRNA was upregulated sex-specifically during testis differentiation, suggesting that DKK1 could repress WNT signalling in the developing testis. However, we observed overtly normal testis development in *Dkk1*-null XY gonads, and found no significant upregulation of *Axin2* or *Sp5* that would indicate increased canonical WNT signalling. Nor did we find significant differences in expression of key markers of testis and ovarian development. We propose that DKK1 may play a protective role that is not unmasked by loss-of-function in the absence of other stressors.

A.N.C. and J.B. contributed equally to this work.

Development of the bipotential mouse gonad into either a testis or an ovary depends on a transcriptional balancing act [Kim and Capel, 2006]. Testis development is triggered by SRY, the Y-chromosomal testis-determining factor, which needs to upregulate genes such as *Sox9* and *Fgf9* early enough and strongly enough to overcome the ovarian program of development. Conversely, the testicular program can be overcome to some extent by the actions of ovarian secreted factors RSPO1 (roof plate-specific spondin 1) and WNT4 (wingless-type MMTV integration site 4), both of which appear to function via activating the canonical β-catenin signalling pathway [reviewed by Tevosian and Manuylov, 2008]. Central to this competitive mode of sexual differentiation are factors that antagonise the opposing pathway to ensure faithful development of one gonadal fate, and avoid ambiguous outcomes such as ovotestes.

WNT signalling has emerged as a core module of the program regulating ovary development. In *Wnt4*-null XX mouse gonads, partial sex reversal is observed: müllerian ducts are absent, wolffian ducts are retained and the coelomic blood vessel, which is characteristic of the testis, forms ectopically [Vainio et al., 1999; Jeays-Ward et al., 2003; Heikkila et al., 2005]. In addition, ovary-associated transcripts *Bmp2, Fst* and *Dax1* are not expressed in *Wnt4*-knockout XX gonads, although expression of

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Rspo1 is not affected [Jordan et al., 2001; Mizusaki et al., 2003; Yao et al., 2004; Chassot et al., 2008]. Curiously, ablation of *Wnt4* delays testis development in XY gonads, perhaps reflecting an early role in the supporting cell lineage [Jeays-Ward et al., 2004]. *Rspo1*-null XX mouse gonads are also partially sex-reversed, with a phenotype similar to that of *Wnt4*-null XX gonads, although reportedly slightly more severe [Chassot et al., 2008; Tomizuka et al., 2008]. RSPO1 appears to be genetically upstream of WNT4, since early female-specific upregulation of *Wnt4* expression is not observed in *Rspo1*-null gonads [Chassot et al., 2008; Tomizuka et al., 2008]. In humans, mutations in *RSPO1* have been shown to underlie cases of XX sex reversal [Parma et al., 2006], affirming the importance of WNT signalling to human ovarian development.

Mechanistically, WNT4 activates β-catenin remotely, by binding a Frizzled (Fz) receptor (one of the family of Fz serpentine receptors) and phosphorylating its co-receptor, low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6), thereby triggering stabilization of cytosolic β-catenin, allowing its accumulation in the cytoplasm and subsequently facilitating translocation to the nucleus [He et al., 2004; Tamai et al., 2004; Davidson et al., 2005; Zeng et al., 2005]. In the nucleus, β-catenin complexes with TCF (T-cell factor)/LEF (lymphoid enhancer factor) transcription co-factors which activate the transcription of target genes. In the absence of nuclear β-catenin, TCF/LEF proteins recruit Groucho proteins and act conversely as transcriptional repressors.

DKK1 is a secreted antagonist of LRP6; DKK1 binds with high affinity to LRP6 and, together with its co-receptor Kremen, induces LRP6 endocytosis, thus effectively removing the LRP6 protein from the cell surface and making cells less able to respond to WNT and RSPO1. Although Kremen was thought to be integral to this process, a recent report has shown that DKK1 can also act in the absence of this protein [Ellwanger et al., 2008]. Loss of DKK1 frees the LRP6 from inhibition, resulting in enhanced WNT signalling activity. RSPO1, which also interacts with the LRP6 co-receptor, activates β-catenin signalling via phosphorylation of LRP6 [Nam et al., 2006; Wei et al., 2007], or by competitive binding to Kremen [Binnerts et al., 2007], thereby preventing the DKK1/Kremen-dependent internalization of LRP6. Whatever is the mechanism of action, evidence for DKK1 repression of WNT/β-catenin signalling is extensive.

Dkk1 displays a sex-specific expression pattern in the developing gonad [Manuylov et al., 2008]. It is expressed initially in the developing gonad of both sexes at 11.5 days post coitum (dpc). *Dkk1* is upregulated in the testis at 12.5 dpc and 13.5 dpc, whereas in the ovary, its expression is repressed by the GATA4-FOG2 transcription complex [Manuylov et al., 2008]. Despite the relatively low expression of *Dkk1* in the developing ovary, β-catenin target genes *Sp5* and *Irx3*, as well as the key ovarian development gene *Foxl2*, were found to be upregulated in *Dkk1*-null XX gonads at 12.5 dpc [Manuylov et al., 2008]. Because of the known function of DKK1 in dampening cellular response to WNT and RSPO1 signalling, we hypothesised that *Dkk1*-null XY gonads might be particularly sensitive to WNT4 and RSPO1 and therefore could become feminised to some extent. We studied *Dkk1*-null XY gonads at 11.5 dpc and 12.5 dpc time points and found no upregulation of canonical β-catenin pathway target genes. In addition, we found no apparent feminisation of the XY gonads in terms of marker gene expression and morphology. These results suggest that *Dkk1* plays a backup or fail-safe role in preventing WNT signalling, rather than providing the primary means of repressing the ovarian pathway in mice.

Materials and Methods

Mouse Strains and Dissection

Expression profiling of *Dkk1* was performed with wild-type embryos collected from timed matings of outbred Swiss Quackenbush (Affymetrix profiling) or CD1 (in situ hybridisation) mice. To assess the role of *Dkk1* in gonad development, $Dkk1^{+/-}$ mice [Mukhopadhyay et al., 2001], maintained on a 129/C57BL6 background, were intercrossed. Noon of the day on which the mating plug was observed was designated 0.5 dpc. For more accurate staging, the tail somite (ts) stage of the embryo was determined by counting the number of somites posterior to the hind limb [Hacker et al., 1995]. Using this method, 11.5 dpc corresponds to ~18 ts, and 12.5 dpc to 28 ts. At 11.5 dpc, embryos were sexed by PCR for the Y-linked gene *Zfy* [Koopman et al., 1991]. From *Dkk1*-null litters, one urogenital ridge (UGR, gonad plus mesonephros) was dissected and fixed for immunofluorescence analysis; the other UGR was used for quantitative RT-PCR. Tail tissue from each embryo was used for genotyping as described [Mukhopadhyay et al., 2001], and all embryos genotyped as $Dkk1^{-/-}$ displayed craniofacial abnormalities consistent with the reported phenotype. All animal work was conducted according to protocols approved by the Animal Ethics and Care Committees of the University of Queensland and the Children's Medical Research Institute and the Children's Hospital at Westmead.

Affymetrix Profiling

Gonad pools at 11.0, 12.5 and 13.0 dpc were used to produce probes for screening Affymetrix mouse 430v2 chips as detailed previously [Holt et al., 2006]. Experiments were run in triplicate and expression was calculated relative to 18S rRNA.

In situ Hybridisation

Whole-mount in situ hybridisation was carried out using standard methods [Hargrave et al., 2006]. The *Dkk1* probe was supplied by Gudmap (Probe ID, 2178) (http://uqgudmap.imb.uq.edu.au/search_probe.phtml).

Whole-Mount Immunofluorescence

Immunofluorescence and image capture was performed as described [Combes et al., 2009]. Rabbit anti-SOX9 antibody has been described previously [Wilhelm et al., 2005] and was used at 1:200. Rat anti-VE-Cadherin (anti-vascular endothelial cadherin, BD Biosciences) was used at 1:200. Secondary antibodies (goat anti-rabbit Alexa Fluor 488 and anti-rat Alexa Fluor 594, Invitrogen) were used at 1:300.

Quantitative Realtime RT-PCR

Each UGR was processed and analysed individually. Total RNA was extracted and DNase-treated using an RNeasy Micro kit (Qiagen) as per manufacturers' instructions. cDNA was generated using a High-Capacity cDNA Archive kit (Applied Biosystems) according to manufacturers' instructions. Relative cDNA levels were analysed by the comparative cycle time (Ct) method of quantitative RT-PCR (qRT-PCR) with reactions including Taqman PCR master mix (Applied Biosystems, ABI) and Taqman gene expression sets. Duplicate (Taqman) or triplicate (SYBR) assays were carried out on an ABI Prism 7000 Sequence Detector System, and the mean relative level of expression and associated standard deviations were calculated. Endogenous control, used to normalize gene expression levels, was *Tbp* (encoding TATA box binding protein). Taqman gene expression sets were as follows: Mm00446973_m1 *(Tbp)*, Mm00443610_m1 *(Axin2)*, Mm00500463_m1 *(Irx3)*, Mm00491634_m1 *(Sp5)*, Mm00437341_m1 *(Wnt4)*, Mm00431729_m1 *(Dax1)*, Mm00507076_m1 *(Rspo1)*, Mm00843544_s1 *(Foxl2)*, Mm00514982_m1 *(Follistatin)*, Mm01340178_m1 *(Bmp2)*, Mm00448840_m1 *(Sox9)*, Mm00442795_m1 *(Fgf9)*, Mm00432820_g1 *(Dhh)*, Mm00558507_m1 *(Cyp26b1)*, Mm03023963_m1 *(Amh)* and Mm00490735_m1 *(Cyp11a1)*.

Statistics

Student's t tests (unpaired, 2-tailed) were used to determine statistical significance of expression differences between XY wild-type and XY null samples.

Results

Dkk1 *Becomes Male-Specific in Its Expression Pattern Shortly after Sex Determination*

Sry, the mammalian testis-determining gene, is expressed from 10.5 to 12.5 dpc in mouse foetal gonads [Koopman et al., 1990]. In a microarray experiment designed to find novel genes expressed in a male- or female-specific manner shortly after SRY triggers testis fate, we found that *Dkk1* is upregulated at 12.0 dpc in the developing testis, shortly after the upregulation of *Sox9* (fig. 1A). By whole-mount in situ hybridisation (fig. 1B), low levels of *Dkk1* expression were observed in the XY gonad at 11.5 dpc (solid arrow head). Expression was also observed in the paramesonephric duct (arrow) and mesonephric tubules (open arrowheads). At 12.5 dpc in XY gonads, *Dkk1* was expressed predominantly under the coelomic epithelium (arrowhead), whilst at 13.5 dpc, *Dkk1* expression expanded to encompass the poles of the testis. *Dkk1* was also expressed in the mesenchyme around the paramesonephric (müllerian) duct in 13.5-dpc XY samples (arrow) and could be related to the male-specific regression of this structure [Orvis and Behringer, 2007]. *Dkk1* transcripts were not detected in 11.5- or 12.5-dpc XX gonads. At 11.5 dpc, mesonephric expression of *Dkk1* in ovaries was similar to that observed in the testes. Differences emerged at 12.5 dpc where *Dkk1* expression was restricted to the dorsal end of the paramesonephric duct (arrows), more severely in XY compared to XX samples. Expression in mesonephric tubules also varied between the sexes (open arrowheads), and persisted in 13.5-dpc XX mesonephroi while it was not detected in XY samples of the same age. A single focal point of *Dkk1* expression was consistently observed in the posterior region of the 13.5-dpc ovary (arrowhead). Others have demonstrated a similar expression pattern of *Dkk1* in wild-type XY and XX gonads by qRT-PCR, and demonstrated that expression of *Dkk1* is first apparent in the central region of the XY coelomic epithelium at 12.5 dpc [Manuylov et al., 2008].

Ablation of Dkk1 *Does Not Affect Expression of Key Marker Genes of Gonadal Development*

In order to establish whether *Dkk1* has an important role during testis development, we examined the effect of loss of *Dkk1* function on the expression of key markers of testis and ovarian development. We found little alteration in expression of such genes at 11.5 dpc (online suppl. fig. 1; for all online supplementary material, see www.karger.com/doi/10.1159/000327709) or 12.5 dpc (fig. 2). At 12.5 dpc, there was no upregulation of targets of the WNT canonical pathway *(Axin2* and *Sp5)* [Jho et al., 2002; Clevers, 2006; Fujimura et al., 2007], suggesting that excessive WNT signalling is not occurring in the absence of *Dkk1* (fig. 2A). Furthermore, there was no upregulation of *Irx3*, a WNT target gene that is highly expressed in the developing ovary [Braun et al., 2003; Jorgensen and Gao, 2005]. In addition, there was no molecular evidence of feminisation of the *Dkk1*-null XY gonad (fig. 2B) or significant difference in the expression of markers of testicular fate (fig. 2C). In summary, ablation of *Dkk1* had no discernible effect on the early expression of key genes known to be associated with sex determination and gonad development.

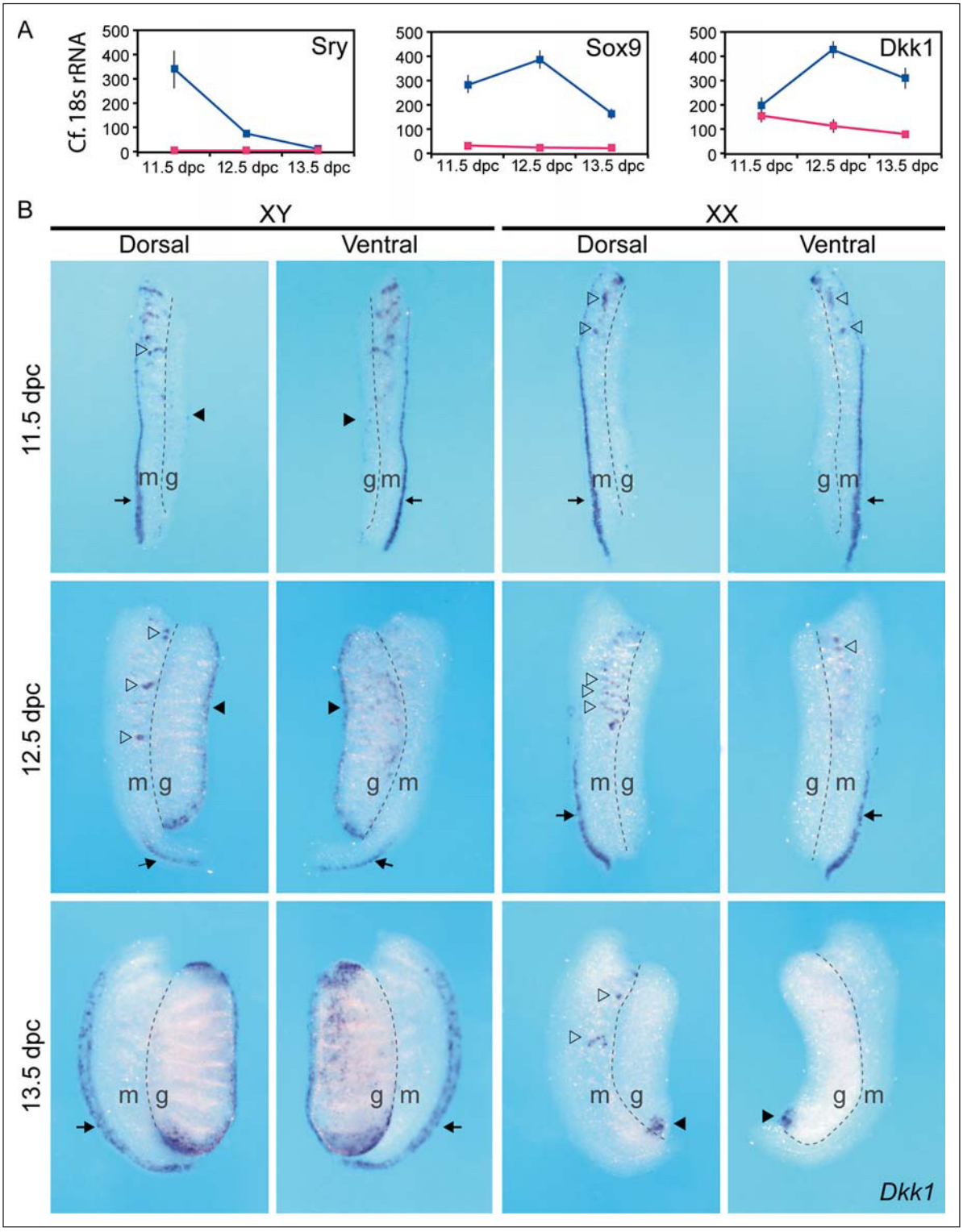

Fig. 1. *Dkk1* is expressed in developing testis shortly after *Sry* and *Sox9* are upregulated. **A** Expression of *Sry* (1450579_x_at), *Sox9* (1434950_at) and *Dkk1* (1420360_at), as detected by Affymetrix microarray screening. **B** Whole-mount in situ hybridisation of *Dkk1* at 11.5, 12.5 and 13.5 dpc in XY and XX urogenital ridges. Both dorsal and ventral views are shown. g = Gonad; m = mesonephros; arrows = paramesonephric duct; open arrowheads = *Dkk1* expression in mesonephric tubules; solid arrowheads = *Dkk1* expression in gonads.

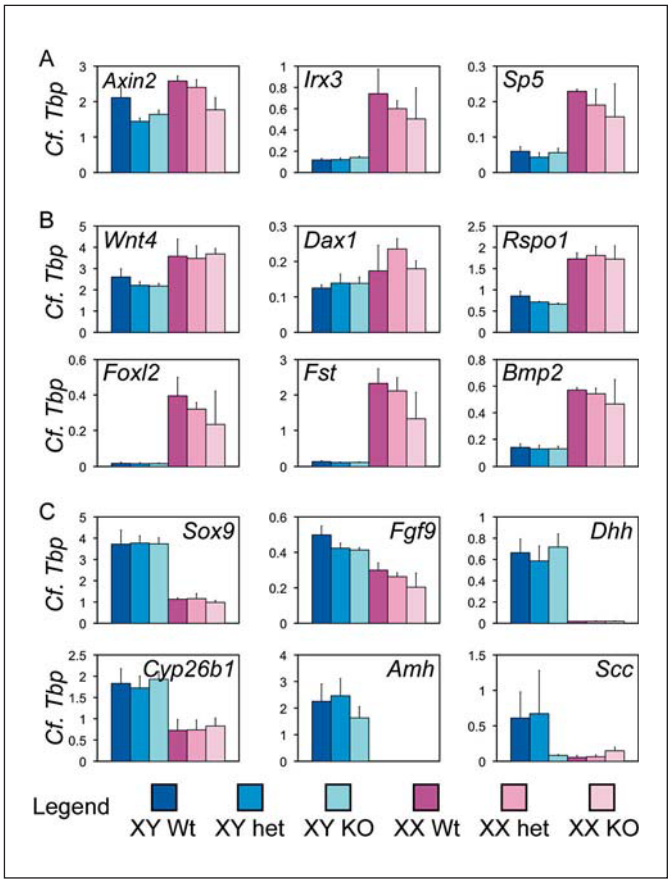

Fig. 2. Testis development in the *Dkk1*-null embryos appears normal with respect to expression of marker genes. **A** qRT-PCR analysis of transcriptional targets of the canonical WNT pathway, **B** markers of ovarian development, and **C** markers of testicular development in 12.5 dpc UGR samples from wild-type (Wt), heterozygous (het) and null (KO) *Dkk1* embryos. Bars indicate the mean ± 1 SEM; n = 5, 3, 5, 2, 4, 3. Endogenous control *Tbp* (encoding TATA box binding protein) was used to normalize gene expression levels.

Ablation of Dkk1 *Has Little Effect on Morphological Development of the Foetal Testis*

In view of the observation that loss of *Dkk1* function has no effect on the core testis transcriptional program, we next investigated whether any developmental delay or disorganisation of testis development could be found in *Dkk1*-null XY gonads. Particular attention was given to the development of the male-specific coelomic blood vessel [Brennan et al., 2002], which assembles under the coelomic epithelium at 12.5 dpc, correlating with the restricted expression of *Dkk1* RNA at this stage. Immunostaining results obtained using antibodies that mark Sertoli cells (anti-SOX9) and germ cells/endothelial cells (anti-PECAM-1) revealed no delay or disruption of testis cord formation (fig. 3, asterisks). Further, endothelial cells that form the coelomic vessel localised to the appropriate area in *Dkk1*-null gonads and displayed a similar level of organisation to staged-matched controls (fig. 3, arrows).

Discussion

WNT signalling is known to be important in mouse and human ovarian development. The finding of a male-specific expression of a WNT antagonist, DKK1, shortly after testis fate is determined [Manuylov et al., 2008; present study], raises the possibility that DKK1 might play a role in modulating WNT signalling activity during gonadal development. As *Wnt4* is expressed at early stages in both male and female gonads, it seemed plausible that the action of DKK1 might be critical in preventing WNT4-related feminisation activity in the XY gonads. However, our data show that ablation of *Dkk1* has no apparent effect on mouse foetal testis development. Although we cannot rule out the possibility of a later phenotype arising, WNT/β-catenin acts early, and if the absence of *Dkk1* had an effect on sex determination, we would expect to see it in the time window studied. Two alternative explanations for these findings are redundancy of function between DKK1 and other WNT antagonists, or that DKK1 protects against ectopic WNT activity.

Although *Dkk1* is a member of a multigene family, evidence for redundancy between DKK1 and proteins of related function in the gonad is not strong. Our microarray results suggest that *Dkk2, Dkk4, Wif* and *Cerberus* are not expressed at significantly higher levels in XY than in XX gonads and there is only a very modest upregulation of *Dkk3* (online suppl. fig. 2). Male-specific expression of other (mechanistically different) antagonists of WNT signalling, *Sfrp1* and *Sfrp2* [Warr et al., 2009], may compensate for the lack of *Dkk1*. Testes are smaller and have fewer cords in compound mutants lacking *Sfrp1* and *Sfrp2* [Warr et al., 2009], leaving open the possibility of redundancy between *Dkk1* and the *Sfrp* genes. However, this possibility remains to be tested by analysing double- or triple-knockout mice.

Alternatively, the primary mechanism for WNT suppression in the testis may occur independent of signal inhibition. In this case, DKK1 could serve a protective role that is only apparent when challenged by ectopic

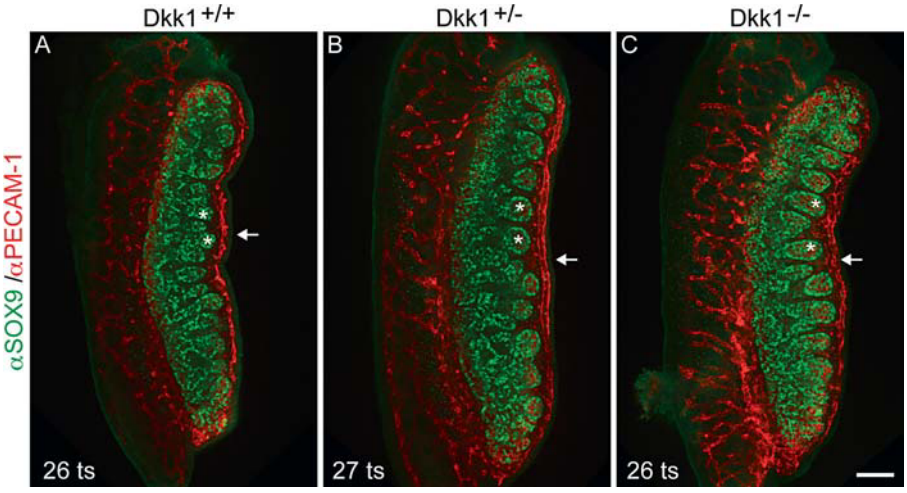

Fig. 3. Testis development in the *Dkk1*-null embryos appears normal morphologically. **A** Immunofluorescent analysis of whole-mount UGRs from wild-type (*Dkk1*$^{+/+}$), **B** *Dkk1*$^{+/-}$, and **C** *Dkk1*$^{-/-}$ embryos (12.5 dpc). SOX9 (green) marks Sertoli cells, PECAM (red) marks germ cells and vasculature. No delay or disruption of testis cord formation is seen (asterisks). Endothelial cells localise to the appropriate area in *Dkk1*-null gonads and display a similar level of organisation to staged-matched controls (arrows). Tail somite (ts) stages for each sample are indicated. Scale bar = 100 μm.

WNT activity. Further studies could test whether *Dkk1*-null gonads are more susceptible to WNT-induced feminisation through culture with chemical agents such as lithium chloride [Bernard et al., 2008; Maatouk et al., 2008], or by studying available WNT4 overexpression mouse models [Jordan et al., 2001]. If DKK1 is found to play such a role, the *Dkk1*-null mouse model may prove useful in future mouse studies as a sensitized background to elucidate the molecular control of the female program of gonadal development underpinned by WNT signalling.

If signal inhibition is not the primary means of suppressing WNT signalling in the developing testis, then what is? There is a strong correlation between expression of the transcription factor SOX9 and cessation of WNT activity in the mouse foetal gonad. Overexpression of *Sox9* is sufficient to suppress the ovarian program and masculinise XX gonads [Vidal et al., 2001], and conditional inactivation of this gene results in immediate upregulation of *Wnt4* and *Foxl2* in XY gonads [Barrionuevo et al., 2006]. However, no evidence has yet emerged for direct suppression of *Wnt4* transcription by SOX9, which is conventionally viewed as a transcriptional activator. Further studies are required to understand the mechanism of SOX9 dominance over WNT activity, although at least part of this action is mediated by the secreted signalling molecule FGF9 [Kim et al., 2006].

Accumulating evidence suggests that gene dosages and expression dynamics vary substantially between mouse and human. Although our data indicate that DKK1 is not critical for mouse testis development, it remains possible that *DKK1* mutations underlie some cases of partial sex reversal or gonadal abnormality in humans, or that *DKK1* may act as a genetic modifier of these conditions.

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