A cell-autonomous role for WT1 in regulating Sry in vivo

Stephen T. Bradford1, Dagmar Wilhelm1, Roberto Bandiera2,3, Valerie Vidal2,3, Andreas Schedl2,3,* and Peter Koopman1

1Division of Molecular Genetics and Development, Institute for Molecular Biosciences, The University of Queensland, Brisbane QLD 4072, Australia, 2INSERM, U636, F-06108 Nice, France and 3Université de Nice-Sophia Antipolis, F-06108 Nice, France

Received May 27, 2009; Revised and Accepted June 15, 2009

Human patients with Frasier syndrome express reduced levels of the +KTS isoforms of the developmental regulator WT1 and exhibit complete XY gonadal dysgenesis and male-to-female sex reversal. Mice with a targeted mutation that blocks production of these isoforms show a reduction in Sry mRNA in the gonad, but the molecular and cellular basis of this reduction has not been established. Using immunofluorescence analysis, we found a significantly lower level of SRY protein per cell in XY Wt1(+KTS)-null mouse gonads. We also found a reduced number of SRY-expressing cells, correlating with a decrease in cell proliferation at and near the coelomic epithelium at 11.5 dpc. No reduction in somatic cell numbers was seen in XX Wt1(+KTS)-null gonads, indicating that the effect of WT1 on cell proliferation is mediated by Sry. Sertoli cell differentiation was blocked in XY Wt1(+KTS)-null mouse gonads, as indicated by the loss of SOX9 and Fgf9 expression, but the addition of recombinant FGF9 to ex vivo gonad cultures rescued the mutant phenotype, as indicated by the induction of the Sertoli-cell specific marker anti-Müllerian hormone. Our data suggest that WT1(+KTS) is involved in the cell-autonomous regulation of Sry expression, which in turn influences cell proliferation and Sertoli cell differentiation via FGF9. Thus, sex reversal in Wt1(+KTS)-null mice and Frasier syndrome patients results from a failure of Sertoli cells both to fully differentiate and to reach sufficient numbers to direct testis development.

INTRODUCTION

The gonad is a unique organ system in which a single primordium can be induced to form one of two distinct organs, a testis or an ovary. The gonads arise as linear ridges on the ventromedial surfaces of the mesonephroi at about 10 days post coitum (dpc) in the mouse. At 10.5 dpc a genetic switch is activated in XY embryos that set the gonad on the pathway of testis differentiation, which in turn virilises the embryo. This switch is a single gene on the Y chromosome, Sry (sex determining region of the Y chromosome). The importance of SRY for testis development was confirmed by the discovery of mutations in this gene in two sex-reversed XY women but not their fathers (1,2). Subsequently, the generation of male Sry XX transgenic mice demonstrated that SRY is not only necessary, but also sufficient for testis differentiation (3).

SRY is expressed in the supporting cell lineage within the developing XY genital ridge of the mouse from about 10.5–12.5 dpc (4–7). A number of genes have been identified as potential regulators of SRY expression. These include several genes identified by mutation analysis in mice to be required for the early formation of the bipotential genital ridges: Emx2 (8), Lhx9 (9), Sf1 (10), Gata4 (11) and Wt1 (12,13).

The present study centres on the role of one of these genes, Wt1, in regulating early events in testis determination. Mice carrying homozygous mutations of Wt1 lack kidneys and gonads due to apoptosis of the corresponding primordia (13). Wt1 encodes a developmental regulator harbouring four C-terminal zinc fingers, with two main isoforms defined by the presence or absence of the amino acids lysine, threonine and serine (KTS) between the third and fourth zinc finger, denoted WT1(+KTS) and WT1(−KTS), respectively (14). The −KTS isoforms bind preferentially to DNA (15),
whereas the +KTS isoforms have a higher binding affinity for RNA (15,16). WT1 has been shown to be important for the regulation of numerous genes involved in urogenital development including anti-Müllerian hormone (AMH) receptor 2 (Amhr2), Sox9, Sfl and Wnt4 (17–19).

A number of observations have implicated WT1 in the regulation of Sry expression. Co-transfection assays demonstrated that the −KTS isoform of WT1 activates the putative human SRY promoter (20) and transfection experiments using NT2D1 cells, a human cell line that expresses SRY endogenously, showed that WT1 (−KTS) was able to up-regulate endogenous human SRY (21). However, controversially, WT1 (+KTS) has been demonstrated to act synergistically with GATA4 to up-regulate transcription of the mouse and pig SRY promoters in vitro (22). Recently, isoform-specific Wt1−null mice have been generated (12). Mice lacking the −KTS isoforms exhibit increased apoptosis in the gonad and reduced gonad size in both XY and XX animals similar to the complete Wt1 knockout (13). However, Sox9 and Amhr were expressed in the mutant XY gonad, suggesting that the pathway directing testis differentiation is still activated (12). In contrast, mice lacking WT1 (+KTS) show male-to-female sex reversal and express only ~25% of the normal amount of Sry mRNA (12). Analysis of gonads of patients with Frasier syndrome, a human condition caused by heterozygous splice site mutations resulting in a reduction of WT1 (+KTS) and characterized by complete gonadal dysgenesis, streak gonads and male-to-female sex reversal, is also associated with reduced overall levels of SRY expression (23). However, it is unclear whether WT1 (+KTS) play a role in SRY regulation or whether gonads in Frasier patients and the Wt1 (+KTS)−/− mouse model are simply deficient in cells expressing SRY.

In this study, we address these issues through in vivo genetic analysis using Wt1 (+KTS)−/− mice. Immunofluorescence and quantitative image analysis revealed reduced levels of SRY protein per cell, a male-specific reduction in the proliferation of somatic gonadal cells and a failure of Sertoli cell differentiation in the mutant gonads. When Wt1 (+KTS)−/− gonads are cultured in the presence of recombinant FGF9, Sertoli cell differentiation was rescued. Our findings support a cell-autonomous role for WT1 (+KTS) in the regulation of SRY and as a consequence, both direct and indirect effects on differentiation and proliferation of pre-Sertoli cells mediated by FGF9.

**RESULTS**

**SRY protein is present and has normal nuclear localization in developing testes of Wt1 (+KTS)−/− embryos**

Previous studies have shown a significant reduction of Sry mRNA in Wt1 (+KTS)-null mice, corresponding with complete male-to-female sex reversal (12). To characterize these mutant mice in detail at the level of protein expression, we performed immunofluorescence analysis on sections of 11.5 dpc mouse embryos using an anti-mouse SRY antibody (7,24). SRY immunofluorescence was detected in cells of both wild-type and mutant genital ridges and did not overlap with immunofluorescence for the germ cell marker OCT4 or the proliferation marker Ki67 (Fig. 1). These results demonstrate that SRY is expressed in both cases in somatic, non-proliferative cells of XY gonads at 11.5 dpc. The lack of proliferation in SRY-positive cells supports previous studies in which pre-Sertoli cells were found to cease dividing temporarily between 18 and 23 tail somite (ts) (27). SRY protein was found to be nuclear in both wild-type and mutant gonads (Fig. 1), indicating no obvious effect of the loss of WT1 (+KTS) on the subcellular distribution of SRY.

**Reduced SRY protein per cell in the developing testis of Wt1 (+KTS)−/− embryos**

Given that SRY is translated and localized to the nucleus in Wt1 (+KTS)−/− mice, we next investigated whether SRY protein was expressed at a similar level per cell in wild-type and mutant gonads. We performed immunofluorescence analysis on tissue sections of wild-type and mutant XY embryos in parallel and imaged them under identical conditions. The intensity of SRY immunofluorescence was clearly lower in mutant gonadal cells when compared with wild-type cells (Fig. 2A). To quantify this difference, we used the Volocity software, which measures the average pixel intensity in digital images of individual SRY-positive cells, as a proxy for the amount of SRY protein within that cell. The mean of these values across a number of sections indicates the mean level of SRY protein per cell. This quantification confirmed an average of 50% reduction in the level of SRY protein per cell in mutant compared with wild-type gonads (P < 0.001; Fig. 2B). No difference was found in the intensity of fluorescence of OCT4 positive cells between wild-type and mutant gonads when quantified (data not shown).

**Reduced number of SRY-expressing cells in the developing testis of Wt1 (+KTS)−/− embryos**

Having established that there is less SRY protein per cell in the mutant gonads, we next examined the number of cells expressing SRY in wild-type and mutant genital ridges. Whole mount immunofluorescence of 11.5 dpc (18–22 ts) XY gonads showed a clear reduction in the number of SRY expressing cells in the mutant compared with the wild-type, as shown by a representative optical section through the middle of the gonads (Fig. 3A). We generated Z-stacks of confocal images through the full depth of the gonad and counted the SRY-positive cells; mutant gonads contained only ~20% of the number of SRY-expressing cells counted in wild-type gonads (P < 0.001; Fig. 3B). A reduced number of SRY positive cells could be caused by a general developmental delay of Wt1 (+KTS)−/− animals. However, a comparison of the number of ts of more than 20 litters at 11.5 dpc did not reveal any differences between wild-type and mutant embryos, suggesting that the overall embryonic development in Wt1 (+KTS)−/− animals is not significantly delayed (data not shown).

To determine whether the reduction in the number of SRY-positive cells had also affected the size and shape of the mutant gonad, we estimated the relative volume of wild-type and mutant gonads using Z-stack images to create three-dimensional reconstructions of the regions with SRY-positive cells within the gonads. At this stage wild-type gonads developed into a thick, crescent organ, whereas Wt1 (+KTS)−/− gonads maintained an undifferentiated streak gonad shape
The volumes of the reconstructions were measured using 3dmod software, revealing that the mutant gonads were significantly smaller than the wild-type gonads (mutant volume ~40% of wild-type, $P < 0.01$; Fig. 3D).

**Wild-type and $Wt1(+/KTS)^{−/−}$ XX gonads contain similar numbers of somatic cells**

The reduction in the number of SRY-positive cells in the mutant XY genital ridges compared with their wild-type counterparts might be explained by two possible scenarios.

Loss of $WT1(+KTS)$ might cause a sex-independent (i.e. non-SRY-related) reduction in proliferation within the bipotential genital ridge, resulting in fewer cells of the supporting cell lineage in both XX and XY embryos. Alternatively, the reduced number of SRY-positive cells might be a consequence of the reduction in SRY levels, based on the observation that one of the early effects of $Sry$ expression is an increase in the proliferation of cells at or near the male coelomic epithelium and that these cells then contribute to the Sertoli cell lineage of the testis (27). In this second scenario, the reduction in the number of supporting cells would be male-specific.
To distinguish between these two possibilities, we performed whole-mount immunofluorescence on 11.5 dpc XX wild-type and mutant gonads, using an anti-SF1 antibody to identify the supporting cell lineage. No clear reduction was visible in the number of SF1-expressing cells in mutant genital ridges compared with the wild-type (Fig. 4A), and quantification revealed no significant difference between mutant and wild-type (Fig. 4B). Similarly, there was no significant reduction in the volume of the region of SF1-positive cells as assessed by 3dmod software (Fig. 4C). This lack of a decrease in somatic cells in the mutant XX gonads indicates that the reduction of supporting lineage cells, and consequently the gonadal volume, in XY Wt1(þKTS)2/2 embryos is a male-specific phenomenon that must by definition occur downstream of Sry.

Absence of male-specific coelomic epithelium proliferation in Wt1(+KTS)−/− gonads

Gonadal volume is known to increase rapidly in XY embryos at 11.5 dpc due to male-specific proliferation of cells at the coelomic epithelium, which amplifies the pre-Sertoli cell lineage of the testis (26,27). Therefore, the SRY-dependent reduction in the number of supporting cells that we observed in Wt1(+KTS)-mutant gonads may be due to impaired proliferation at the coelomic epithelium. To test this possibility, we performed whole mount immunofluorescence on wild-type and mutant gonads, using an anti-Ki67 antibody as a marker of proliferation. The number of Ki67-positive proliferating cells at or near the coelomic epithelium (defined as the outermost epithelial layers in gonad sections) was significantly reduced in the mutant compared with the wild-type (Fig. 3A; mutant /C3/C3/C3 P, 0.05; Fig. 5A), whereas the relative number of Ki67-positive cells in the mesenchyme was similar in all genotypes (Fig. 5B). Therefore, the reduction in the number of SRY-expressing cells observed in the XY mutant gonad is evidently due to a reduction in male-specific, Sry-dependent proliferation within the coelomic epithelium.

Figure 3. Reduced number of SRY-expressing cells and gonadal volume in XY Wt1(+KTS)−/− embryos. (A) Whole mount immunofluorescence of 11.5 dpc male wild-type and Wt1(+KTS)−/− mice. The number of SRY-positive nuclei (green) is higher, and there are more proliferative cells expressing Ki67 (red) in the coelomic epithelium in wild-type compared with mutant gonads. The upper dotted line denoted the border of the coelomic epithelium and mesenchyme, the lower dotted line the border of mesenchyme and mesonephros. CE, coelomic epithelium; Mes, mesenchyme; M, mesonephros. Scale bar, 50 μm. (B) Quantification of the number of SRY-positive cells per optical section in wild-type and mutant gonads, three gonads were used per condition between 18 and 22 ts. Error bars represent standard error. **P < 0.01. (C) Representative images of the reconstructions of the volume as determined by the SRY-expressing regions in the genital ridges of XY wild-type and Wt1(+KTS)−/− embryos. Scale bar, 200 μm. (D) Quantification of the relative gonadal volume determined by the reconstruction of SRY-expressing regions of XY wild-type and mutant gonads, three gonads between 18 and 22 ts were used per genotype. Error bars represent standard error. ***P < 0.001.

Reduced Sox9 and Fgf9 expression in the Wt1(+KTS)−/− mice

The male-specific burst of proliferation at the coelomic epithelium during normal XY gonadal development is thought to be dependent on the action of FGF9. Genital ridges from XY Fgf9−/− mice show a reduced level of proliferation in the coelomic epithelium, approximately equivalent to that seen in XX genital ridges (25). A similar decrease in proliferation in the XY coelomic epithelium has been observed in Sox9−/− mice (28). Because Fgf9 expression has been shown to be SOX9-dependent (28), and Sox9 expression is SRY-dependent (29), the reduction of proliferation in the coelomic epithelium in Wt1(+KTS)−/− mice might be regulated via Sox9 and Fgf9.

To test this hypothesis, we measured the expression levels of Sox9 and Fgf9 using quantitative RT-PCR in urogenital ridges of wild-type and mutant animals at 11.5 dpc.
Expression of Sox9 and Fgf9 were not significantly reduced in XX \( Wt1(+\text{KTS})^{-/-} \) gonads compared with wild-type (Fig. 6A and B). In contrast, the levels of both Sox9 and Fgf9 transcripts in the XY \( Wt1(+\text{KTS})^{-/-} \) gonads were significantly reduced compared with wild-type XY and were at a level similar to that found in XX gonads (Fig. 6A and B). The level of Sox9 in XY mutants was less than 30% of XY wild-type \( (P < 0.01) \) (Fig. 6A). These data corroborate previous \textit{in situ} data describing a female-like expression pattern of Sox9 mRNA in the gonads of XY \( Wt1(+\text{KTS})^{-/-} \) mice (12). The level of Fgf9 expression was less than 50% of wild-type XY \( (P < 0.01) \) and was lower than that seen in mutant and wild-type XX gonads (Fig. 6B).

In addition, we examined SOX9 expression at the protein level using immunofluorescence. SOX9 was present in wild-type Sertoli cells, but was not detectable in the gonads of \( Wt1(+\text{KTS})^{-/-} \) mice (Fig. 6C) thus confirming, at a cellular level, that Sertoli cells fail to differentiate from those pre-Sertoli cells that are present. In summary, our results support the hypothesis that the reduction in proliferation observed in the coelomic epithelium of XY \( Wt1(+\text{KTS})^{-/-} \) gonads is due to reduced levels of \textit{Sry}, Sox9 and Fgf9.

Addition of FGF9 \textit{in vitro} rescues the male pathway in \( Wt1(+\text{KTS})^{-/-} \) gonads

\textit{Sry} normally initiates a positive feedback mechanism where SOX9 expression results in the upregulation of Fgf9, and FGF9 is required for the maintenance of Sox9. This regulatory loop is necessary to allow Sertoli cells to differentiate (28). We hypothesized that the reduced levels of Sox9 mRNA, and therefore Fgf9, in XY \( Wt1(+\text{KTS})^{-/-} \) gonads are insufficient to induce or maintain the required feedback mechanism and thus Sertoli cell differentiation is aborted, demonstrated by the absence of SOX9 positive cells as measured by immunofluorescence analysis (Fig. 6C). If this was the case then the treatment of XY \( Wt1(+\text{KTS})^{-/-} \) gonads with exogenous FGF9 should rescue Sertoli cell differentiation and the sex reversal phenotype \textit{in vitro}.

To test this hypothesis, genital ridges from 11.5 dpc embryos were incubated in the presence (left genital ridge) or absence (right genital ridge) of recombinant FGF9 and the effect on the male differentiation pathway was determined by the presence of AMH, a direct target of SOX9 (30) and marker of Sertoli cell differentiation (31). Immunofluorescence analysis of wild-type XY genital ridges showed AMH expression in both control and FGF9-treated gonads (Fig. 7A and D). Although no AMH was detectable in wild-type XX untreated controls (Fig. 7C), a few AMH-positive cells were detected in FGF9-treated XX genital ridges (data not shown). XY \( Wt1(+\text{KTS})^{-/-} \) genetic ridges, cultured in the absence of FGF9, had no AMH positive cells (Fig. 7B). In contrast, XY \( Wt1(+\text{KTS})^{-/-} \) genetic ridges cultured with exogenous FGF9 showed large numbers of AMH-positive cells with an immunofluorescent signal similar to that seen in XY wild-type cells (Fig. 7E), confirming that FGF9 is sufficient to induce Sertoli cell differentiation and rescue the male pathway in XY \( Wt1(+\text{KTS})^{-/-} \) mice.

DISCUSSION

An important missing element in our knowledge of mammalian sex determination is an understanding of the molecular mechanisms involved in the regulation of \textit{Sry}. Genetic studies in mice have implicated several transcription factors in affecting overall expression levels of \textit{Sry} in XY genital ridges. Mice homozygous for a loss of function mutation in the gene encoding the cofactor FOG2, and mice with a targeted GATA4 mutation that abrogates the interaction of GATA4 with FOG co-factors (Gata4\textsuperscript{−/−}), up-regulate \textit{Sry} to only 25% of normal mRNA expression levels and show delayed testis development (11). Likewise, mice lacking all three members of the insulin receptor tyrosine kinase family show XY sex reversal due to a dramatic reduction in \textit{Sry} mRNA levels (32). However, in those studies, the expression of \textit{Sry} was not examined at the level of individual cells, and it is not possible to tell whether the putative regulators affect \textit{Sry} expression levels cell-autonomously or whether their mutation results in fewer cells that express \textit{Sry}. By examining the cellular expression of \textit{Sry} protein, we have for the first time demonstrated that one of these factors, WT1(+KTS), is an essential part of the molecular cascade of cell-autonomous \textit{Sry} regulation \textit{in vivo}.

Figure 4. No reduction in somatic cell number or gonadal volume in XX \( Wt1(+\text{KTS})^{-/-} \) embryos. (A) Whole mount immunofluorescence of 11.5 dpc XX gonads. SF1 (green) marks somatic cells and Kit67 (red) marks proliferating cells; cells positive for both markers are yellow. The upper dotted line denoted the border of the coelomic epithelium and mesenchyme, the lower dotted line the border of mesenchyme and mesonephros. CE, coelomic epithelium; Mes, mesenchyme; M, mesonephros. Scale bar, 50 \( \mu \)m. (B) Quantification of the number of SF1-positive cells per optical section in wild-type and mutant gonads, three gonads used per genotype between 17 and 18 ts. (C) Quantification of the gonadal volume using 3D-reconstructions of the SF1 expressing region of female wild-type and mutant gonads. Three gonads were used per genotype between 17 and 18 ts. Neither parameter is significantly different between wild-type and mutant samples. Error bars represent standard error. NS, not significant \( (P > 0.05) \).
The role of WT1 in regulating Sry expression

How WT1 controls sex determination and gonad development has remained unclear, partly because WT1 has complex isoform- and context-dependent functions. In vitro evidence suggests that WT1(−KTS) acts as transcriptional regulator, binding to the human SRY regulatory region and activating its transcription (20,21). However, no in vivo data have emerged to support these findings. In mice carrying null mutations for the −KTS isoforms, the testis pathway is still induced as shown by the expression of the Sry-downstream genes Sox9 and Amh (12). In these mice, both XY and XX genital ridges showed a dramatic reduction in size and differentiated tissue (12), suggesting that rather than directly regulating Sry, WT1(−KTS) are instead essential for cell survival or proliferation in the primordial gonad in a manner that does not depend on Sry.

The molecular mode of action of WT1(−KTS) remains controversial. On the one hand, WT1(+KTS), in synergy with GATA4, has been shown to up-regulate transcription of the mouse and pig Sry promoter in vitro (22). However, in most other studies, WT1(+KTS) has been found to be a poor transcriptional regulator and instead has been implicated in RNA processing in vitro (15,16) and shown to enhance mRNA translation (33). Our data would support a model in which WT1(+KTS) regulates the expression of Sry by either mechanism. A further possibility arises from the fact that the absence of WT1(+KTS) results in an over-expression of −KTS isoforms, so that the overall WT1 expression levels are not altered in WT1(−KTS)−/− mice (12). An excess of the −KTS product might lead, directly or indirectly, to the transcriptional repression of Sry, since WT1(−KTS) can function as transcriptional activators and repressors, depending on the cellular and experimental context (reviewed in 34). This possibility could be tested in vivo by specifically over-expressing the −KTS isoforms in XY genital ridges.

Sertoli cell differentiation and FGF9

It has been demonstrated previously that FGF9 is required for the maintenance of Sox9 expression and that the addition of FGF9 to XX 11.5 dpc dissociated gonadal cells induces SOX9 expression (28). However, the addition of recombinant FGF9 to ex vivo cultures of whole XX gonads was insufficient to induce a general upregulation of SOX9, possibly due to the lack of a sufficiently high local concentration of FGF9 to override inhibiting signals present in the XX gonad (35). Here we demonstrate that exogenous FGF9 is capable of inducing Sertoli cell fate on a sex-reversed XY WT1(+)KTS−/− gonad, while not in XX gonads, suggesting that Sry expression levels in these gonads were the same or lower than those in wild-type XX gonads, the addition of FGF9 was sufficient to induce Sertoli cell differentiation in XY WT1(+)KTS−/−, but not in XX gonads, suggesting that Sry expression levels in XY WT1(+)KTS−/− gonads are sufficient to tip the balance towards Sertoli cell differentiation when FGF9 is added. These findings support a model in which FGF9 not only maintains Sox9 expression, but also is able to induce Sertoli cell differentiation, presumably through Sox9, as indicated by robust AMH expression.

Proliferation and sex determination

High levels of proliferation in the XY gonad are considered to be crucial for testis differentiation. An increase in size of the XY gonad over the XX gonad has been observed in all mammals examined so far, in addition to a number of non-mammalian vertebrates such as chicken, alligator and turtle (reviewed in 36). Male-specific proliferation occurs in cells of the coelomic epithelium in two waves, the first giving rise to Sertoli cells and the second contributing to other somatic cell lineages (27). Our analysis of the WT1(−KTS)−/− mice has provided the first in vivo evidence associating a decrease in cell
proliferation specifically within the coelomic epithelium with a significantly lower number of pre-Sertoli cells marked by SRY protein expression. Numerous studies have demonstrated that a threshold number of Sry-expressing pre-Sertoli cells are necessary to drive testis development (37,38), the reduced numbers in the Wt1(+KTS)−/− mutants may therefore contribute to sex reversal.

Taken together, our data lead us to propose a model for the action of WT1(+KTS) (Fig. 8), which allows us to explain the development of the sex-reversal phenotype associated with Frasier syndrome. First, WT1(+KTS) play a cell-autonomous role in regulating Sry expression in individual cells of the supporting cell lineage in vivo. Normal levels of SRY are required for Sox9 and subsequently Fgf9 expression and Sertoli cell differentiation. Secondly, FGF9 affects Sertoli cell differentiation via two processes: (i) by acting directly on pre-Sertoli cells to increase the expression of Sox9 and thus drive Sertoli cell differentiation, (ii) to induce male-specific proliferation in the coelomic epithelium. In the absence of appropriate levels of WT1(+KTS), as in the

Figure 6. Reduced Sox9 and Fgf9 expression in Wt1(+KTS)−/− gonads. Real-time RT–PCR analysis of wild-type (WT) and mutant (−/−). XY and XX gonads for Sox9 (A) and Fgf9 (B) transcript levels relative to Hprt (mean ± SEM of three independent experiments). **P < 0.01. Three gonads were used per genotype between 18 and 22 ts. (C) Section immunofluorescence of 11.5 dpc XY wild-type and Wt1(+KTS)−/− mice. SOX9 (green), the germ cell marker OCT4 (red); cells were counterstained with DAPI (blue). No SOX9-positive cells could be detected in the mutant gonads. Dotted line delineates the genital ridges. Scale bar, 50 μm.
Wt1(+/KTS)−/− mice and in patients with Frasier syndrome, this chain of events breaks down with the result that XY gonads are small and contain insufficient pre-Sertoli cells to ensure testis development.

MATERIALS AND METHODS

Mouse strains

Embryos were collected from timed matings of Wt1(+KTS) heterozygous mice (12) with noon of the day on which the mating plug was observed designated 0.5 dpc. For more accurate staging, the ts stage of the embryo was determined by counting the number of somites posterior to the hind limb (5). Using this method, 10.5 dpc corresponds to 8 ts, 11.5 dpc to 18 ts and 12.5 dpc to 30 ts. Mice were genotyped as described previously (12). The presence of the Y chromosome was determined by PCR using Zfy primers.

Gonad explant cultures

Genital ridges were microsurgically isolated from wild-type and mutant littersmates (four XY Wt1(+KTS)-/− embryos at 15, 17, 17 and 18 ts, and 16 XY and 16 XX wild-type embryos stages 15−21 and 13−19 ts, respectively, were used) and transferred on 0.1 μm pore size polycarbonate membranes (Whatman) supported by a metal grid. Genital ridges were cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% newborn calf serum (Gibco) and 0.5% penicillin−streptomycin (Gibco). Murine FGF9 (Peprotech) was added to a final concentration of 50 ng/ml. The media was replaced once at 36 h and cultures stopped after 72 h.

Tissue processing and immunofluorescence

Embryos and dissected gonads were fixed in 4% paraformaldehyde in phosphate buffered saline for several hours and embryos were embedded in paraffin. Cultured gonads were fixed in 100% methanol on ice for 10 min, before being blocked and processed as described below. Section immunofluorescence of 7 μm paraffin sections was performed as described previously (7).

Whole gonads were rehydrated through a gradient as above and incubated in blocking solution for 5 h. Following this, primary antibodies were added and left overnight at 4°C. Tissues were then washed five times for at least 1 h each in PBTX before being blocked for at least 1 h at 4°C. After incubation with secondary antibodies overnight at 4°C, gonads were washed five times for 1 h, stained with DAPI as above and mounted for confocal imaging.

Antibodies

Primary antibodies and dilutions used were: Anti-SRY [αSRY (7,24)] and anti-SOX9 (7) at 1:100, anti-Ki67 (DakoCytomation, Clone TEC-3), anti-MVH (Abcam DDX4), anti-OCT3/4 (Santa Cruz) and anti-AMH (Santa Cruz MIS (C-20)) at 1:200, anti-SF1 (a kind gift of Dr Ken-Ichiro Morohashi) and DAPI (Sigma Aldrich) at 1:2000. Secondary antibodies (all from Molecular Probes) used were: goat anti-rabbit Alexa Fluor 488, donkey anti-rat Alexa Fluor 647, goat...
Figure 8. Model of the molecular function of WT1(+KTS) during sex determination. WT1(+KTS) is involved in upregulating SRY in a cell-autonomous manner (1), which in turn results in the up-regulation of SOX9 (2). Recent studies have shown that SOX9 is essential for the expression of FGF9 (3), which is secreted to play a role in the male-specific increase in proliferation (4) observed in the coelomic epithelium of the testis at 11.5 dpc. The proliferation within the coelomic epithelium results in the delamination and ingress of cells (5) that in turn start to express SRY (6) and differentiate into Sertoli cells.

ACKNOWLEDGEMENTS

We thank Caroline Hopkins, Danielle Badro and Alexander Combes for technical assistance. Confocal microscopy was performed at the Institute for Molecular Biosciences (IMB) Dynamic Imaging Facility for Cancer Biology, established with the support of the Australian Cancer Research Foundation.

Conflict of Interest statement. None declared.

FUNDING

Australian Research Council (ARC); National Health and Medical Research Council (NHMRC) of Australia; University of Queensland Graduate Student Research Travel Award; ARC/NHMRC Network in Genes and the Environment in Development; ARC/NHMRC Network in Genes and the Environment in Development Laboratory Interchange Award; Institute for Molecular Bioscience Postgraduate Research Award (to S.T.B.); NHMRC Career Development Award (to D.W.); Fondation pour la Recherche Médicale (FRM) and ANR (ANR-05-MRAR-019-01) (to A.S.); ARC Federation Fellowship (to P.K.).

REFERENCES

1. Berta, P., Hawkins, J.R., Sinclair, A.H., Taylor, A., Griffiths, B.L., Goodfellow, P.N. and Fellous, M. (1990) Genetic evidence equating Sox9 and the male sex determining gene. Nature, 348, 448–450.
2. Jäger, R.J., Anvret, M., Hall, K. and Scherer, G. (1990) A human XY female with a frame shift mutation in the candidate testis-determining gene SRY. Nature, 348, 452–454.
3. Koopman, P., Gubbay, J., Vivian, N., Goodfellow, G. and Lovell-Badge, R. (1991) Male development of chromosomally female mice transgenic for Sry. Nature, 351, 117–121.
4. Albrecht, K. and Eicher, E. (2001) Evidence that Sry is expressed in pre-Sertoli cells and Sertoli and granulosa cells have a common precursor. Dev. Biol., 240, 92–107.
5. Hacker, A., Capel, B., Goodfellow, P. and Lovell-Badge, R. (1995) Expression of Sry, the mouse sex determining gene. Development, 121, 1603–1614.
6. Sekido, R., Bar, I., Narvaez, V., Penny, G. and Lovell-Badge, R. (2004) SOX9 is up-regulated by the transient expression of SRY specifically in Sertoli cell precursors. Dev. Biol., 274, 271–279.
7. Wilhelm, D., Martinson, F., Bradford, S., Wilson, M.J., Combes, A.N., Beverdam, A., Bowles, J., Mizusaki, H. and Koopman, P. (2005) Sertoli cell differentiation is induced both cell-autonomously and through prostaglandin signaling during mammalian sex determination. Dev. Biol., 287, 111–124.
8. Miyamoto, N., Yoshida, M., Kuratani, S., Matsuo, I. and Aizawa, S. (1997) Defects of urogenital development in mice lacking Emx2. Development, 124, 1653–1664.
9. Birk, O., Casiano, D., Wassif, C., Cogliati, T., Zhao, L., Zhao, Y., Grinberg, A., Huang, S., Kreidberg, J., Parker, K. et al. (2000) The LIM homeobox gene Lhx9 is essential for mouse gonad formation. Nature, 403, 909–913.

10. Luo, X., Ieda, Y. and Parker, K.L. (1994) A cell-specific nuclear receptor is essential for adrenal and gonadal development and sexual differentiation. Cell, 77, 481–491.

11. Tevssion, S.G., Albrecht, K.H., Crispino, J.D., Fujiwara, Y., Eicher, E.M. and Orkin, S.H. (2002) Gonadal differentiation, sex determination and normal Sry expression in mice require direct interaction between transcription partners GATA4 and FOG2. Development, 129, 4627–4634.

12. Hossain, A. and Saunders, G. (2001) The human sex-determining gene

13. Kreidberg, J.A., Sariola, H., Loring, J.M., Maeda, M., Pelletier, J., Housman, D. and Jaenisch, R. (1993) WT-1 is required for early kidney development. Cell, 74, 679–691.

14. Haber, D.A., Sohn, R.L., Buckler, A.J., Pelletier, J., Call, K.M. and Housman, D.E. (1991) Alternative splicing and genomic structure of the Wilms tumor gene WT1. Proc. Natl Acad. Sci. USA, 88, 9618–9622.

15. Caricasole, A., Duarte, A., Larsson, S.H., Hastie, N.D., Little, M., Holmes, G., Todorov, I. and Ward, A. (1996) mRNA binding by the Wilms tumor suppressor zinc finger proteins. Proc. Natl Acad. Sci. USA, 93, 7562–7566.

16. Kennedy, D., Ramsdale, T., Mattick, J. and Little, M. (1996) An RNA recognition motif in Wilms' tumor protein (WT1) revealed by structural modelling. Nat. Genet., 12, 329–331.

17. Gao, F., Matti, S., Alain, N., Zhang, Z., Deng, J.M., Behringer, R.R., Lecureuil, C., Guillon, F. and Huff, V. (2006) The Wilms tumor gene, Wt1, is required for Sox9 expression and maintenance of tubular architecture in the developing testis. Proc. Natl Acad. Sci. USA, 103, 11987–11992.

18. Klattig, J., Stierig, R., Kruspe, D., Besenbeck, B. and Englert, C. (2007) Wilms' tumor protein Wt1 is an activator of the anti-Mullerian hormone receptor gene Amh2. Mol. Cell. Biol., 27, 4355–4364.

19. Wilhelm, D. and Englert, C. (2002) The Wilms tumor suppressor WT1 regulates early gonad development by activation of Sf1. Genes Dev., 16, 1839–1851.

20. Shimamura, R., Fraizer, G.C., Trapman, J., Lau YF, C. and Saunders, G.F. (1997) The Wilms' tumor gene WT1 can regulate genes involved in sex determination and differentiation: SRY, Mullerian-inhibiting substance, and the androgen receptor. Clin. Cancer Res., 3, 2571–2580.

21. Mittwoch, U. (1986) Males, females and hermaphrodites. An inaugural lecture delivered by Professor Ursula Mittwoch at University College London on 24 October 1985. Ann. Hum. Genet., 50, 103–121.

22. Nagamine, C., Morohashi, K., Carlisle, C. and Chang, D. (1999) Sex reversal caused by Mis cephalus domesticus Y chromosomes linked to variant expression of the testis-determining gene. Sex. Dev. Biol., 216, 182–194.

23. Palmer, S.J. and Burgoyne, P.S. (1991) In situ analysis of fetal, prepuberal and adult XX×XY chimaeric mouse testes: Sertoli cells are predominantly, but not exclusively, XY. Development, 112, 265–268.