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

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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 primordial 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, Sf1 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 Amh 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 failure of Sertoli cell differentiation in the mutant gonads. When Wt1(−KTS) knockout mice were crossed with the WT1(−KTS)−/− animals, 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 Velocity 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 testes 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.
Fig. 3C). 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(+KTS)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)2/2 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 /C2/C1/C1 P, 0.001). 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.

**Reduced Sox9 and Fgf9 expression in the Wt1(+KTS)2/2 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 Fgf92/2 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 Sox92/2 mice (28). Because Fgf9 expression has been shown to be SOX9-dependent (28), and Sox9 expression is SRY-dependent (29), the reduction in proliferation in the coelomic epithelium in Wt1(+KTS)2/2 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.
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 \( \text{Wt}1(+/\text{KTS})^{-/-} \) 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 \( \text{XY} \ Wt1(+/\text{KTS})^{-/-} \) gonads is due to reduced levels of \( \text{Sry}, \text{Sox9} \) and \( \text{Fgf9} \).

**DISCUSSION**

An important missing element in our knowledge of mammalian sex determination is an understanding of the molecular mechanisms involved in the regulation of \( \text{Sry} \). Genetic studies in mice have implicated several transcription factors in affecting overall expression levels of \( \text{Sry} \) in \( \text{XY} \) 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\(^{-/-}\)), up-regulate \( \text{Sry} \) 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 \( \text{XY} \) sex reversal due to a dramatic reduction in \( \text{Sry} \) mRNA levels (32). However, in those studies, the expression of \( \text{Sry} \) was not examined at the level of individual cells, and it is not possible to tell whether the putative regulators affect \( \text{Sry} \) expression levels cell-autonomously or whether their mutation results in fewer cells that express \( \text{Sry} \). By examining the cellular expression of \( \text{Sry} \) protein, we have for the first time demonstrated that one of these factors, \( \text{Wt1}(+/\text{KTS}) \), is an essential part of the molecular cascade of cell-autonomous \( \text{Sry} \) regulation in vivo.
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) regulate 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)(−/−) background. Even though endogenous Sox9 and Fgf9 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 /C24 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 littermates (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 \(\mu\)m 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 \(\mu\)m 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 [\(\alpha\)SRY (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-Ichirou 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 ingestion of cells (5) that in turn start to express SRY (6) and differentiate into Sertoli cells.

Image collection and cell counting
Images of the labelled tissue were collected using a Zeiss LSM-510 confocal microscope. To estimate the total volume of the gonads, overlapping Z-stack images were taken of whole gonad and mesonephroi, and each optical section was traced and reconstructed using 3dmod software (Qt/Qmake). To determine a representative number of SRY, SF1 and Ki67 cells per gonad Z-stack images were taken of whole gonad and mesonephroi, and each optical section was counted. Confocal sections were taken at ~5 μm thickness and to reduce the risk of counting the same cell twice, sections were spaced at ~10 μm intervals. Cell numbers were determined for three gonad pairs for each genotype. The following developmental stages were used: XY wild-type, 20, 21 and 22 ts; XY WT1(+KTS)−/−, 18, 19 and 21 ts; XX wild-type and WT1(+KTS)−/−, 17, 17 and 18 ts. The pixel intensity of SRY expression was determined by immunofluorescence of sectioned embryos with experiments performed in parallel and identical confocal settings used for imaging. The average image intensity of each cell above a defined threshold level was measured from five sections of each gonad, using the Volocity software (Improvision, version 3.7).

Statistical analysis was performed using Excel (Microsoft) and the statistical program Prism (GraphPad, version 4.0c). Pairwise comparisons (Figs 3, 4 and 6) were analysed using one-tailed Student t-tests, and multivariate comparisons (Fig. 5) using one-way ANOVA.

Quantitative real-time RT–PCR
Total RNA was extracted from dissected gonads plus mesonephroi of 11.5 dpc (18–22 ts) wild-type and WT1(+KTS)−/− embryos using TRIZol reagent (Invitrogen), following the manufacturer’s instructions, and reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Invitrogen) in presence of random primers. Quantitative RT–PCR was performed on a LightCycler2.0 system (Roche) using the Roche universal probe library. The amount of Sox9 and Fgf9 transcripts was calculated using LightCycler software 3.5. Data were normalized against the respective values of Hprt. Primers: CAGCAAGACTCTGGGCAAG and TCCAC GAAAGGTCTCTTCTC for Sox9, TGCAGACTGGATTT CATTAG and CCAAGGCCACTGCTTAATCG for Fgf9 and TCTCCTCAGACCGGTITT and CCTGGTTTAC TCGCTAATC for Hprt.

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