Characteristics of Testicular Dysgenesis Syndrome and Decreased Expression of *SRY* and *SOX9* in Frasier Syndrome

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ABSTRACT Frasier syndrome (FS) is characterized by chronic renal failure in early adulthood, varying degrees of gonadal dysgenesis, and a high risk for gonadal germ cell malignancies, particularly gonadoblastoma. Although it is known to arise from heterozygous splice mutations in intron 9 of the Wilms' tumor gene 1 (*WT1*), the mechanisms by which these mutations result in gonadal dysgenesis in humans remain obscure. Here we show that a decrease in *WT1* + KTS isoforms due to disruption of alternative splicing of the *WT1* gene in a FS patient is associated with diminished expression of the transcription factors *SRY* and *SOX9* in Sertoli cells. These findings provide the first confirmation in humans of the results obtained by others in mice. Consequently, Sertoli cells fail to form the specialized environment within the seminiferous tubules that normally houses developing germ cells. Thus, germ cells are unable to fully mature and are blocked at the spermatogonial–spermatocyte stage. Concomitantly, subpopulations of the malignant counterpart of primordial germ cells/gonocytes, the intratubular germ cell neoplasia unclassified type (ITGCN), are identified. Furthermore, dysregulated Leydig cells produce insufficient levels of testosterone, resulting in hypospadias. Collectively, the impaired spermatogenesis, hypospadias and ITGCN comprise part of the developmental disorder known as 'testicular dysgenesis syndrome' (TDS), which arises during early fetal life. The data presented here show that critical levels of *WT1* + *KTS*, *SRY* and *SOX9* are required for normal Sertoli cell maturation, and subsequent normal spermatogenesis. To further study the function of human Sertoli cells in the future, we have established a human cell line. *Mol. Reprod. Dev.* © 2008 Wiley-Liss, Inc.

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Key Words: WT1; gonadal dysgenesis; intratubular germ cell neoplasia unclassified (ITGCN); sertoli cells

INTRODUCTION

Sex determination and gonadal differentiation in humans is critically dependent on the transcriptional regulation of specific genes. The interaction of three transcription factors, SRY (sex-determining region on Y), SOX 9 (SRY box protein 9), and WT1 (Wilms' tumor-1 protein) plays a key role in the testis determination pathway (for review see Koopman et al., 2001; Park and Jameson, 2005). In mammals, differentiation of the testes from the indifferent gonad involves both the transcriptional activation of *SRY* by WT1 (Shimamura et al., 1997; Hossain and Saunders, 2001) and probably the stabilization of *SRY* mRNA transcripts by WT1 in pre-Sertoli cells (Hammes et al., 2001). These two distinct functions are performed by different WT1 protein isoforms, namely the +KTS or −KTS isoforms, which differ by the insertion or omission of lysine-threonine-serine (KTS) between zinc fingers 3 and 4. WT1 + KTS associates with spliceosomes

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and binds to RNA (Larsson et al., 1995; Caricasole et al., 1996), suggesting a role in RNA processing, whereas WT1 – KTS binds to DNA and behaves as a classical transcription factor (Madden et al., 1991).

Once SRY is turned on, it activates transcription of *SOX9* (Sekido et al., 2004) which is likely a critical factor in Sertoli cell maturation (Chaboissier et al., 2004). Appropriate differentiation of Sertoli cells is necessary for the maturation of other testicular cells, such as Leydig cells that produce the masculinizing hormone testosterone. In addition, Sertoli cells secrete anti-Mullerian hormone (AMH) to effect regression of the Mullerian ducts. Later in development, Sertoli cells function to support spermatogenesis. Without this support, germ cell differentiation, meiosis and transformation into spermatozoa are all arrested (reviewed in Sharpe et al., 2003). Thus, Sertoli cells play two functionally different roles during male gonadal development, namely, the promotion of testis differentiation and the support of spermatogenesis.

Failure of Sertoli and Leydig cells to mature are believed to result in 'testicular dysgenesis syndrome' (TDS), which presents with impaired spermatogenesis, undescended testes, hypospadias and testicular germ cell cancer (Skakkebaek et al., 2001). Histological signs of TDS are: testicular microcalcifications, immature seminiferous tubules with undifferentiated Sertoli cells and arrest of spermatogenesis, and seminiferous tubules containing intratubular germ cell neoplasia unclassified type (ITGCN), the noninvasive precursor of malignant testicular germ cell tumors (Skakkebaek et al., 1987; Oosterhuis and Looijenga, 2005).

Gonadal and kidney development depends on the appropriate expression of *WT1*. Mutations in the exon 9 splice donor site of *WT1* lead to an imbalance of the WT1 ± KTS isoforms, and cause Frasier syndrome (FS; Barbaux et al., 1997; Klamt et al., 1998). This syndrome is characterized by chronic kidney failure during early adulthood, complete gonadal dysgenesis and a high risk for malignant germ cells (either ITGCN or gonadoblastoma; Moorthy et al., 1987; Melo et al., 2002). To gain further insight into how gonadal defects arise in FS, Hammes et al. have generated mice lacking the WT1 + KTS isoforms (Hammes et al., 2001). These mice have reduced levels of *Sry* and *Sox9*, and as a consequence, genotypically male (XY) mice develop along the female pathway.

Although the genetic cause of FS is known and a mouse model exists, the mechanisms whereby a disturbed ratio of WT1 ± KTS isoforms results in testicular dysgenesis in humans remain largely obscure. We show here a marked decrease in *SRY* and *SOX9* expression in the Sertoli cells of a patient with FS displaying characteristics of TDS. Our results confirm for the first time in humans the findings obtained in mice (Hammes et al., 2001).

MATERIALS AND METHODS

Patient and Tissue Samples

Formalin-fixed, paraffin-embedded or snap-frozen testicular specimens from one FS patient, three adult controls, one child control (6 years old), and one seminoma taken at the time of surgery or autopsy were included in this study. Adult control testes were obtained from patients with testicular cancers in the course of a former study (Schmidt et al., 2001). Testicular tissue from a FS individual was obtained at the time of prophylactic gonadectomy. The patient was informed about the study and gave written consent.

WT1 Mutation Analysis

WT1 mutation analysis was performed by amplification and direct sequencing of exon 9 according to the manufacturer's recommendations (Big Dye Terminator Kit; Applied Biosystems, Foster City, CA).

Antibodies and Immunostaining

For immunohistochemistry, 4 μm thick formalin-fixed and paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in graded ethanols. For concentration and suppliers of the primary antibodies see Table 1. Standard horseradish peroxidase detection systems were used for WT1, Inhibin α, Cytokeratin 18, PLAP, c-KIT, OCT3/4, Vimentin, and SMA. SCF was detected with an alkaline phosphatase based system. All slides were counterstained with hematoxylin, mounted, and coverslipped. Negative controls consisted of replacement of the primary antiserum with the corresponding irrelevant IgGs. Cells were cultured on glass slides,

TABLE 1. Overview of the Origin and the Protocols Used for Different Antibodies on Formalin Fixed and Paraffin Embedded Tissue

	Supplier, clone	Dilution	ER	Chromogen
WT1	Dakocytomation, 6F-H2, Denmark	1:50	Protease Type XIV	DAB
Inhibin α	Dakocytomation, R1, Denmark	1:10	Citrate	DAB
Cytokeratin18	BioGenex, San Ramon, CA	1:5,000	Citrate	DAB
PLAP	Dakocytomation, Denmark	1:200	Citrate	DAB
c-KIT	Dakocytomation, Denmark	1:200	Citrate	DAB
OCT3/4	Santa Cruz, Biotechnology, Santa Cruz, C20, CA	1:200	Citrate	DAB
Vimentin	Dakocytomation, Denmark	1:20	Citrate	DAB
SMA	Dakocytomation, Denmark	1:100	Citrate	DAB
SCF	Santa Cruz, N19, CA	1:500	Citrate	NF

ER, epitope retrieval; Citrate, citrate buffer pH 6.0, DAB, 3,3′-diaminobenzidinetetrahydrochlorid dehydrate; NF, new fuchsin.

washed once with 1× PBS, fixed for 10 min in methanol/acetone (1:1) at −20°C, dried and rehydrated in 1× PBS. They were then blocked with 4% fetal calf serum, 0.1% Tween 20 in 1× PBS for 30 min and stained with the Labeled StreptAvidin Biotin kit according to the manufacturer's instructions (Dakocytomation, Hamburg, Germany). Negative controls consisted of omission of the primary antibody.

Semiquantitative RT-PCRs

cDNA was synthesized from 1 μg of DNA-free total RNA (RNAeasy Mini Kit, Qiagen, Hilden, Germany), using Superscript II reverse transcriptase and hexanucleotide random primers according to the manufacturer's instructions (Invitrogen, Carlsbad, CA). PCR was performed as described previously (Schumacher et al., 2003) using primers and cycle conditions listed in Table 2 and 5 μl cDNA (1:24 diluted for GAPDH and 1:3 for all other genes). To determine the ratio of *WT1* ± KTS aliquots were run on a 15% polyacrylamide gel and visualized by ethidium bromide staining. The band intensities of RT-PCR products were evaluated using a Phosphoimager FLA 3000 (Fuji Europe, Duesseldorf, Germany) and the AIDA Image Analysis 2.11 (Raytest Germany, Sprockhoevel, Germany) software. The ratio was determined by measuring the intensity of the band corresponding to the +KTS isoform and the −KTS isoform and by calculating the ratio of both.

Real-Time RT-PCR

Real-time RT-PCR reactions were performed as described earlier (Schumacher et al., 2007) in triplicate by using cDNA transcribed from 10 ng of total RNA. The ready to use 'Assay on Demand System' from Applied Biosystems was used for *WT1* (HS 00240913_m1) and for *18S* rRNA (Hs 99999901_s1) according to the manufacturer's recommendations. Cycle conditions for the TaqMan ABI 7700 Sequence Detection System (PE Biosystems, Weiterstadt, Germany) were as follows: 10 min at 95°C, 50 cycles at 95°C for 15 sec and at 60°C for 60 sec. The expression of *SRY* and *SOX9* was determined using the qPCR MasterMix for SYBR Green (Eurogentec, Seraing, Belgium) according to manufacturer's recommendations and the QuantiTect Primer Assay Hs_SRY_1_SG and Hs_SOX9_1_SG (Qiagen). Cycle conditions were: 2 min at 50°C, 10 min at 95°C, 40 cycles at 95°C for 15 sec, at 55°C for 20 sec and 72°C for 40 sec. Nonspecific amplification products were excluded by running an agarose gel and performing a melting curve analysis for the SYBR Green assays. Expression of *SRY* and *SOX9* was determined relative to the expression of *18S* rRNA or of *WT1* after normalization to *18S* rRNA using the formula $2^{-\Delta\Delta CT}$.

Primary Cell Culture

Fresh testis tissue from the FS patient was cut into 2–3 mm^3 small pieces, washed three times with cold 1× PBS and placed into DMEM supplemented with 20% FCS (Sigma–Aldrich), 1 mM Sodium Pyruvate (Invitrogen-Gibco BRL, Carlsbad, CA), 2 mM L-Glutamine (Gibco BRL), 1× nonessential amino acids (Gibco BRL), and 1× Penicillin/Streptomycin (Gibco BRL). Cells were cultured at 37°C and 5% CO_2.

Soft Agar Test

A base layer of 0.5% agar, overlaid with cells suspended in 0.25% agar was used. Cells were incubated at 37°C and 5% CO_2. Every 3–4 days fresh medium was added onto the cells and growth was checked using an inverted microscope.

Karyotyping

After cell cultivation cytogenetic analysis was performed by standard procedures. Twenty-two metaphases were examined after GTG banding and were karyotyped according to the ISCN (2005) guidelines.

RESULTS

Clinical and Mutational Findings

We report a 19-year-old phenotypic male who underwent correction of glandular hypospadias and herniotomy

TABLE 2. RT-PCRs: Primer Sequences and Cycle Conditions

Gene	Sequence 5′–3′	Annealing temperature (°C)	Denaturing, annealing, elongation (min)
AR	For ACAGGATGTTCCTGCATGGT Rev TGCCCTTTCTGGTATCAACTG	58	1,1,1
GAPDH	For GGCTCTCCAGAACATCATCCCTGC Rev GGGTGTCGCTGTTGAAGTCAGAGG	68	1,1,1
LHR	For CAGAGGCTAATTGCCACGTCATCC Rev CAGGAGCACATCGGGGTGTCTTG	58	1,1,1
MIC2	For GGTGGTTTCGATTTATCCGA Rev CTCCATCAACAACAGCATCT	53	1,1,1
SOX9	For CCCTTCAACCTCCCACACTACAGC Rev TGTGTAGACGGGTTGTTCCCAGTG	68	1,1,1
VASA	For ACAGGATGTTCCTGCATGGT Rev TGCCCTTTCTGGTATCAACTG	52	0.5,0.5,1
WT1	For CCGGTGCTTCTGGAAACTACCAGGTG Rev GGCTGACCTCGGGAATGTTAGACAAGAT	68	1,1,1
WT1 ± KTS	For CTTGTCAGCGAAAGTTCT Rev GACAACTTGGCCACCGA	52	1,1,1

at the age of 5 years. Gross proteinuria (1.7 g/m^2/day) was noted at 5 years. A renal biopsy revealed focal segmental glomerulosclerosis (FSGS), which subsequently progressed to end-stage renal failure by 15 years of age. Following 2 years of peritoneal dialysis, the patient received a deceased donor kidney transplant.

At 15 years of age, his Tanner pubertal stage was PH3G1, with a testicular volume of only 2 ml. Ultrasound examination revealed bilateral testicular microcalcifications, which is a risk factor for ITGCN (de Gouveia Brazao et al., 2004). The karyotype was 46 XY. Based on the findings of very low basal levels of testosterone (1.02 ng/ml) and high basal levels of LH (19 U/L) and FSH (66.5 U/L), which increased upon a 30 min GnRH stimulation (up to 70.6 U/L for LH and 125.8 U/L for FSH) hypergonadotropic hypogonadism was diagnosed. To induce puberty, the patient was treated with increasing doses of testosterone until the testosterone levels rose to 4.06 ng/ml, while LH and FSH levels diminished to LH <0.1 mU/ml and FSH 0.3 mU/ml. For a more detailed clinical description of this patient, please see Benz et al. (2006).

Molecular analysis revealed a heterozygous *WT1* mutation in the splice donor site in intron 9 (IVS9 $+5G>A$). Although genitalia were only mildly affected, the presence of this typical splice site mutation permitted the diagnosis of FS in this patient.

Characteristics of Testicular Dysgenesis Syndrome (TDS)

Since FS patients have a risk of about 44% to develop gonadal germ cell tumors (Melo et al., 2002), especially gonadoblastoma in dysgenetic gonads (represents the parallel pathology of ITGCN of the testis (Kersemaekers et al., 2005)), prophylactic bilateral orchiectomy was carried out at the age of 19 years and testicular protheses were implanted. Histological examination revealed testicular atrophy. Seminiferous tubules showed a thick basal membrane, diffuse Sertoli cell proliferation and arrest of spermatogenesis at the level of spermatogonia to spermatocytes (see Fig. 1). To confirm the presence of Sertoli cells and their increase in seminiferous tubules, we have stained sections using antibodies against WT1, Inhibin α and Vimentin. Indeed, the majority of cells in the seminiferous tubules stained positive for these Sertoli cell markers (Fig. 1). Further, the interstitium displayed hyperplastic Leydig cells with Leydig cell "nodules" (not shown). No manifest tumor was seen, but ITGCN (Fig. 1) and intratubular microcalcification were seen focally (not shown). The presence of ITGCN was confirmed using a set of well-established markers: octamer binding transcription factor 3/4 (OCT3/4), placental/germ alkaline phosphatase (PLAP), stem cell factor receptor (c-KIT), and stem cell factor (SCF; Looijenga et al., 2003; Cools et al., 2005; de Jong and Looijenga, 2006). OCT3/4, c-KIT and PLAP are normally expressed in embryonic germ cells, and their presence in ITGCN cells demonstrates the immature nature of these transformed cells (Looijenga et al., 2003). Sertoli cells secrete SCF, the ligand of c-KIT. As seen in Figure 1, tubules with signs of ITGCN expressed this marker set, whereas adjacent tubules were negative. The segmental involvement of tubules, together with the finding that OCT3/4, PLAP and c-KIT positive cells were attached to the basal lamina, are in accord with ITGCN, and exclude germ cell maturation delay (Cools et al., 2005).

It is known that the arrest of spermatogenesis and the presence of transformed germ cells, are at least in part related to impaired Sertoli cell function. In order to evaluate the differentiation status of Sertoli cells in ITGCN-containing tubules and adjacent tubules, we assessed cytokeratin 18 expression (Kliesch et al., 1998). All seminiferous tubules containing ITGCN cells showed positive cytokeratin 18 expression in associated Sertoli cells, whereas Sertoli cells in adjacent tubules were negative (Fig. 1). This indicates that the Sertoli cells in ITGCN-containing tubules were more immature than those in seminiferous tubules showing arrest of spermatogenesis.

In summary, the features present in our patient are concordant with TDS. The patient presents with an arrest in spermatogenesis, ITGCN, hypospadias and Leydig cell dysfunction, which is manifested by decreased testosterone levels and hypergonadotropic hypogonadism.

Reversion of the Ratio of *WT1* + KTS/−KTS Isoform Transcripts

The ratio of the *WT1* + KTS/−KTS isoform transcripts was determined in material from one testis of a child, three adult testes and the testis from the FS patient. As shown in Figure 2, the ratio is very similar in the testes of a child and the adults with a mean of 2.6 (2.4–2.7), suggesting that this ratio is almost constant during gonadal differentiation. Compared to the controls, the ratio of +KTS/−KTS mRNA isoforms is reversed in the FS patient, due to the presence of a splice donor site mutation. The ratio of 0.3 is in accordance with previously published data (Barbaux et al., 1997; Denamur et al., 2000; Melo et al., 2002).

Expression of *SRY* and *SOX9* is Markedly Diminished in Frasier Syndrome

We next asked whether the altered ratio of *WT1* ± KTS in Sertoli cells of FS patients would result in a reduction of *SRY* and *SOX9* in these cells, and might therefore account for a delay in their maturation.

The expression levels of *SRY* and *SOX9* were determined by real time RT-PCR in material from the whole testis of a child, three adults and one FS patient. In addition, total *WT1* mRNA was measured in the same samples. We first determined the relative expression normalized to 18S rRNA levels. The FS testis expressed almost comparable *WT1* levels as the testis from a child and twofold (1.4–3.0) more *WT1* than three adult controls (Fig. 3A). This may reflect the increase in Sertoli cell numbers in the Frasier patient as shown in Figure 1, but alternatively, may be related to the age of the patient. Despite the higher proportion of Sertoli cells

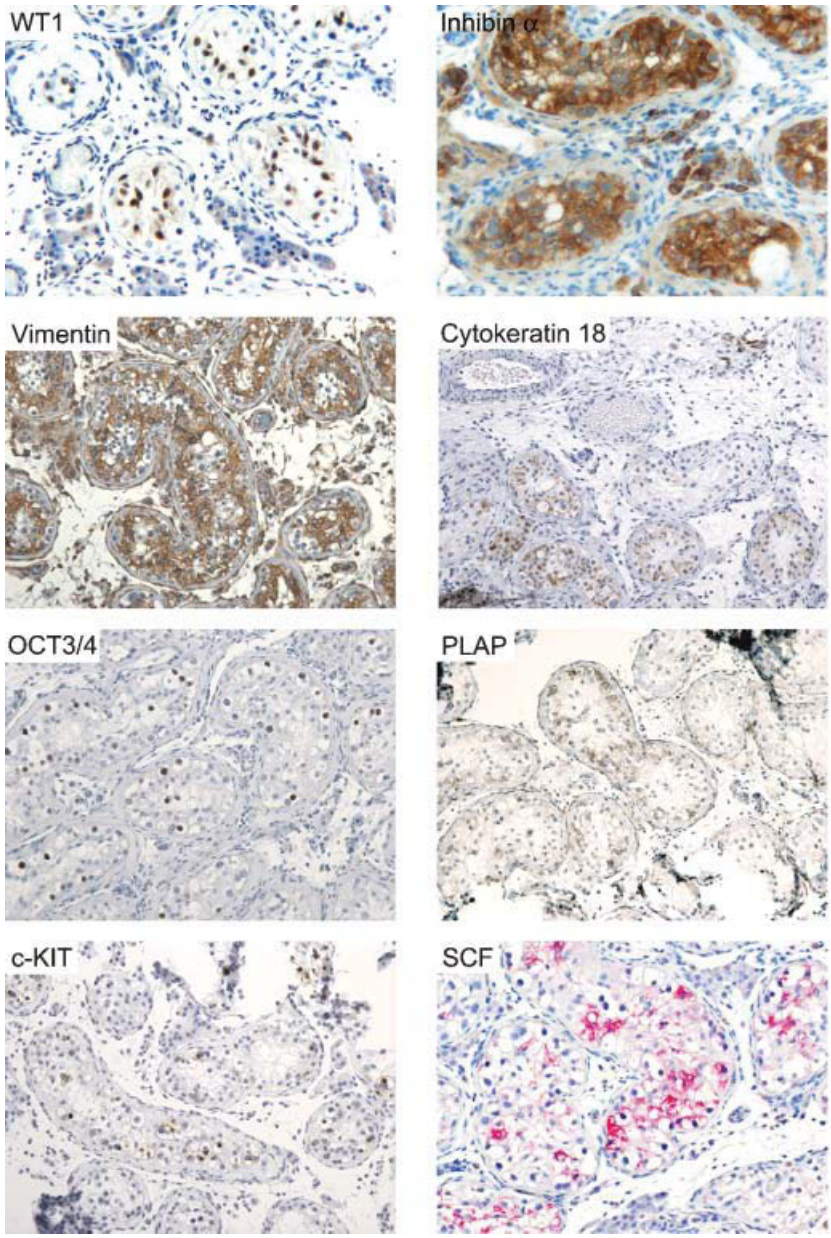

Fig. 1. Immunoperoxidase staining of WT1, Inhibin α, and Vimentin demonstrates diffuse Sertoli cell proliferation in the seminiferous tubules of a patient with Frasier syndrome (FS). Most of the cells in the seminiferous tubules stain positive for these Sertoli cell markers. Spermatocytes are absent due to an arrest at the spermatogonia level. In accord with intratubular germ cell neoplasia (ITGCN), OCT3/4, PLAP and c-KIT positive cells are found focally attached to the basal lamina. Sertoli cells in tubules with ITGCN express Cytokeratin 18 and SCF, demonstrating their immature state.

in the FS testis, the expression levels of *SRY* and *SOX9* were both diminished when compared to controls (Fig. 3B, C). The FS testis expressed twofold less *SRY* than the testis from a child and 4.8 (2.5–7.2)-fold less than three adult controls. For *SOX9*, the FS testis expressed ninefold less than the testis from a child and 3.3 (1.9–4.3)-fold less than adult controls. This decrease became even more evident when taking into account the Sertoli cell proliferation in the patient by normalization to *WT1* levels. In this case, the FS testis expressed threefold less *SRY* and sevenfold less *SOX9* as the testis from a child and 25 (13.5–33.0)-fold less *SRY*, and 7.3 (5.4–10.8) less *SOX9* than adult controls (Fig. 3D,E). Both genes, *SRY* and *SOX9* are known to be expressed in Sertoli cells during early testicular development, and the persistence of both transcripts in three adult controls (Fig. 3B,C) suggests that they may also be essential throughout adult life, although there are conflicting reports in the literature as to the exact location of expression in adult humans.

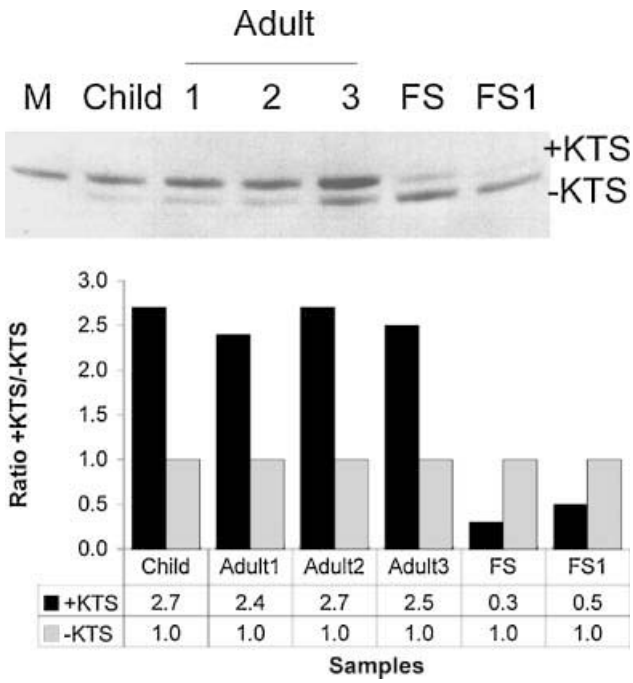

Fig. 2. RT-PCR analyses to determine the ratio of ± KTS isoforms of *WT1* in RNA from testicular tissue of one child, three adults and a FS patient as well as in RNA from the primary testicular cell culture of the patient (FS1). The *WT1* splice donor site mutation (IVS9 +5G > A) alters the ± KTS isoform ratio of *WT1*. The **upper panel** shows the agarose gel electrophoresis; the **lower panel** shows the calculated ratio after quantifying the intensity of the bands.

In summary, testes from a FS patient show a marked decrease in *SRY* and *SOX9* expression, both of which may play a crucial role in Sertoli cell maturation.

Establishing a Primary Sertoli Cell Culture From a Frasier Syndrome Patient

To enable further studies on the role of WT1 in Sertoli cell maturation and homeostasis in the future, we have established a primary cell culture (FS1) from the testis of the FS patient which has been immortalized with both the wildtype large T-antigen and the temperature sensitive large T-antigen. At the moment, we are in the process of expanding single clones. The primary cell culture exhibits homogenous cell morphology with prominent nucleoli and long cytoplasmic processes. The culture shows no signs of spontaneous transformation: upon frequent passaging, cells become more differentiated and lose their ability to proliferate (Fig. 4A), they do not grow in soft agar (Fig. 4B) and show a stable and normal 46 XY karyotype (Fig. 4C).

To characterize the FS1 cells, RT-PCR was performed for a set of markers that distinguish between different cell types (Table 3). As controls we used RNA from the testis of one adult and one seminoma, and compared these with the testis RNA from the FS patient and the corresponding primary cell culture FS1. FS1 cells expressed *WT1* and showed the expected reversion of the ±KTS isoform ratio (Fig. 2 and supplemental Fig. 1). They expressed the Sertoli cell markers *SOX9*, *MIC2*, androgen receptor (*AR*) and Inhibin α (supplemental Fig. 1). The low levels of *SOX9* are in accordance with the real time RT-PCR results described above. FS1 cells were negative for the germ cell marker *VASA1* and for the Leydig cell marker luteinizing hormone receptor (*LHR*; supplemental Fig. 1). Additional immunocytochemistry revealed that they are strongly positive for Vimentin (Fig. 5), which is expressed in Sertoli cells. Very few cells (approx. 1%) weakly expressed cytokeratin 18, which was shown to be only expressed in Sertoli cells adjacent to ITGCN cells (Figs. 1 and 5). Approximately 3% weakly expressed smooth muscle actin, a marker for peritubular myoepithelial cells. All data are included in Table 3.

In summary, the FS1 primary cell culture is chromosomally stable, shows no sign of spontaneous transformation and is almost entirely composed of cells expressing Sertoli cell markers. It represents the first human Sertoli cell line.

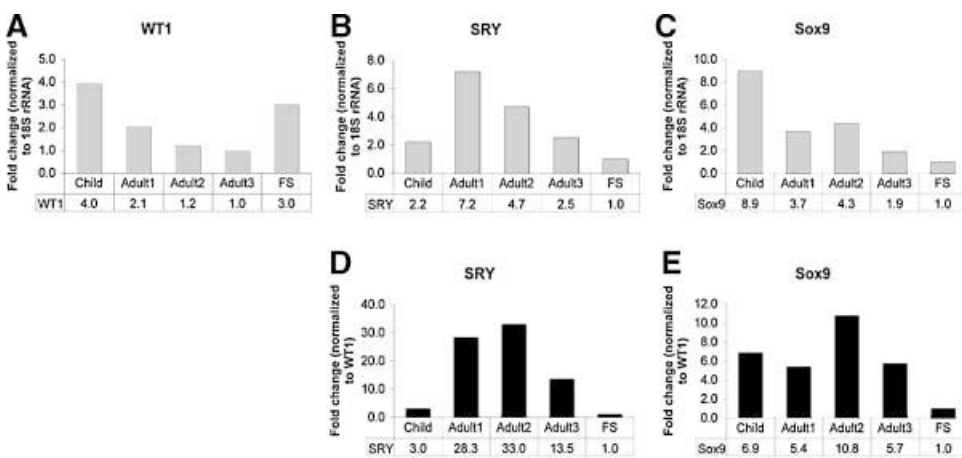

Fig. 3. Real-time RT-PCR for *WT1*, *SRY* and *SOX9* on RNA from the testicular tissue of one child, three adults and a FS patient. **A**,**B**,**C**: demonstrate the fold changes after normalization to 18S rRNA and (**D**), (**E**) after normalization to *WT1*. Both the *SRY* and *SOX9* expression are markedly decreased in the testicular tissue of the FS patient when compared to three adult control testes.

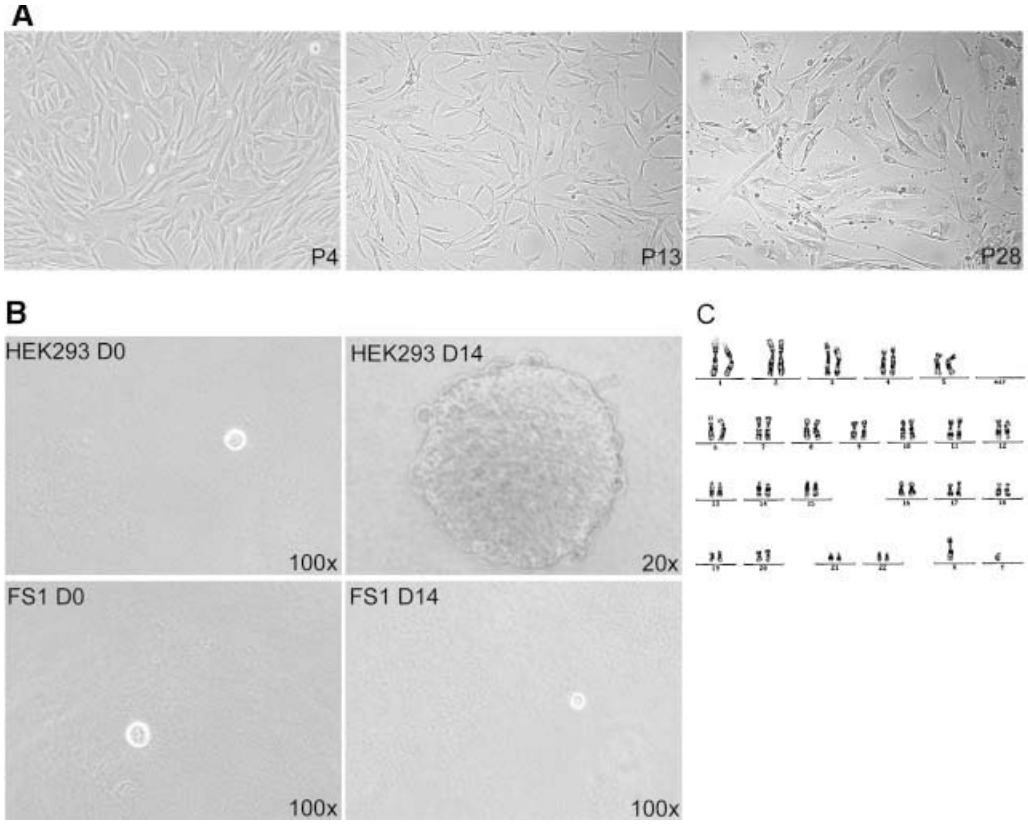

Fig. 4. Characteristics of a primary cell culture established from testis tissue of a FS patient. **A**: Cells stop proliferating and begin to differentiate upon frequent passaging. **B**: Compared to HEK293 cells, FS1 cells do not grow in soft agar. At Day 0 (D0) single HEK293 and FS1 cells are seen. At Day 14 (D14) only HEK293 cells have formed a colony, while FS1 cells are still single cells. **C**: The karyogram of FS1 cells shows no cytogenetic visible alterations.

DISCUSSION

The data presented here suggest that the WT1 + KTS isoforms function in terminal Sertoli cell differentiation and homeostasis through the maintenance of a critical level of SRY and SOX9 expression. Decreased expression *of WT1 + KTS, SRY* and *SOX9* affects Sertoli cell maturation, and therefore their ability to support germ cell differentiation. Ultimately, this may result in arrest of spermatogenesis and malignant transformation of germ cells, both of which are characteristics of TDS.

Variable Clinical Expression in Frasier Syndrome Patients

FS usually presents with complete gonadal dysgenesis, streak gonads and male to female sex reversal (summarized in (Melo et al., 2002)). Recently, three

TABLE 3. Summary of the RT-PCRs and Immunohistochemistry Done With the FS1 Cell Culture

Marker	Gene/protein	RT-PCR	Immunhistochemistry
Sertoli cell	*WT1*	+	n.d.
	SOX9	+	n.d.
	MIC2	+	n.d.
	AR	+	n.d.
	Vimentin	n.d.	+ (100%)
	Cytokeratin 18	n.d.	+ (1%)
Leydig cell	*LHR*	+	n.d.
Germ cell	*VASA*	+	n.d.
Peritubular myoepithelial cell	SMA	n.d.	+ (3%)

AR, androgen receptor; LHR, luteinizing hormone receptor; SMA, smooth muscle actin; n.d., not done.

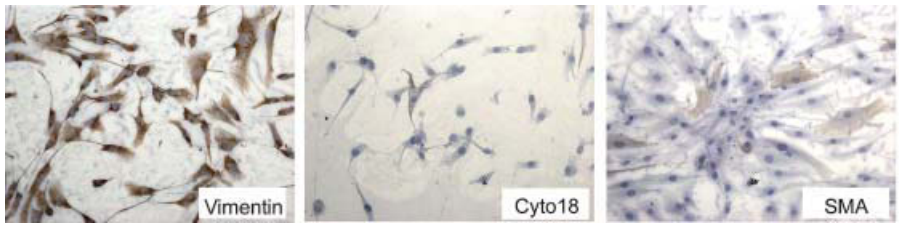

Fig. 5. Immunocytochemistry on a primary cell culture established from the testicular tissue of a patient with FS. Cells are strongly positive for Vimentin, which is expressed in Sertoli cells. Only a few cells are weakly positive for Cytokeratin 18, previously shown to be expressed by Sertoli cells adjacent to ITGCN cells. A small proportion of cells expresses the peritubular myoepithelial marker smooth muscle actin (SMA).

cases with predominantly male genitalia were reported (Denamur et al., 2000; Melo et al., 2002; Tajima et al., 2003). Histological examination was performed in one case and showed hypotrophic testes without signs of spermatogenesis, hyperplastic Leydig cells and bilateral gonadoblastoma. These three cases and the case presented here have either the IVS9 + 4C > T or the IVS9 + 5G > A *WT1* mutation, which are the most common mutations seen in FS. Notably, different patients with the same mutation and expression of similar ratios of the +KTS/−KTS isoforms may exhibit variable phenotypes. This is exemplified by our patient with an IVS + 5G > A mutation and a ratio of +KTS/−KTS of 0.3, and patient JA with the same mutation and a ratio of 0.57, but with complete gonadal dysgenesis and streak gonads (Klamt et al., 1998). These data suggest that modifier genes and/or environmental factors may be responsible for this phenotypic variability.

Reduced Levels of *WT1* + KTS Result in Testicular Dysgenesis Syndrome (TDS)

Abnormal spermatogenesis, testicular cancer, undescended testis and hypospadias collectively comprise TDS (Skakkebaek et al., 2001). TDS is thought to arise as a result of genetic and/or environmental factors (Clark et al., 2000; Colvin et al., 2001; Jeffs et al., 2001; Fisher et al., 2003) that cause a delay in Sertoli and Leydig cell maturation.

Here we present evidence that splice donor mutations in the *WT1* gene, and thus reduced levels of WT1 + KTS, may cause a delay in Sertoli and Leydig cell maturation, and consequently lead to arrest of spermatogenesis, ITGCN and hypospadias in FS. Figure 6 schematically represents the clinical and histopathological findings of the Frasier patient described here (modified from Skakkebaek et al., 2001). Two genes, *SRY* and *SOX9*,

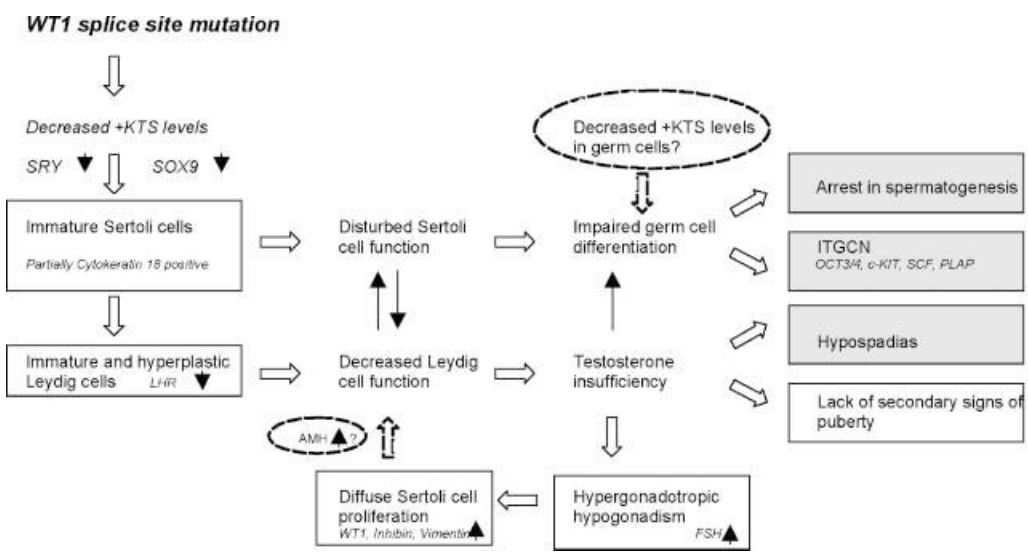

Fig. 6. Schematic diagram (modified from Skakkebaek et al., 2001) illustrating the clinical and histopathological findings (boxes) in the Frasier patient together with the data obtained by the analyses presented here. Dashed circles: not examined in this study. SRY: sex-determining region on the Y chromosome; SOX9: SRY-like HMG box; LHR: luteinizing hormone receptor; AMH: anti-Müllerian hormone; FSH: follicle stimulating hormone; OCT3/4: octamer binding transcription factor 3/4; c-KIT: stem cell factor receptor; SCF: stem cell factor; PLAP: placental/germ alkaline phosphatase.

both of which are known to be critical factors in sex determination and testis differentiation (Houston et al., 1983; Koopman et al., 1991, 2001; Kent et al., 1996; Morais da Silva et al., 1996; Vidal et al., 2001; Chaboissier et al., 2004; Bouma et al., 2005), were found to be down regulated. These findings are in accord with those in a Frasier mouse model described by Hammes et al. (2001). Complete absence of WT1 + KTS in male karyotype mice leads to a dramatic reduction of *Sry*, and results in development along the female pathway. Their findings illustrate the critical importance of WT1 + KTS isoforms in the activation of *Sry* transcription and/or stabilization of *Sry* transcripts, which then upregulates *Sox9* expression. Subsequently, a protein complex which includes SOX9 and WT1-KTS activates *AMH* transcription in Sertoli cells (Nachtigal et al., 1998; Park and Jameson, 2005), ultimately leading to regression of the uterine anlagen and Fallopian tubes in the male fetus.

Unlike in the Frasier mouse model, sex determination was barely affected in our FS patient. This difference between the mouse model and the human patient is most likely caused by the fact that the mouse phenotype results from a complete loss of the WT1 + KTS isoform, whereas in the human the heterozygous *WT1* mutation leads to a reduction but not complete loss of this specific isoform. As a consequence SOX9 levels might be sufficient to activate *AMH* and cause regression of the Müllerian ducts. Alternatively, high FSH levels may have led to Sertoli cell proliferation and increased production of AMH (Lukas-Croisier et al., 2003). Although sex determination was mostly unaffected by the presence of a *WT1* splice site mutation, germ cell differentiation was severely impaired. In vivo assessment of a possible role for WT1 in the XY gonad after testis determination has previously been difficult; however recent mouse models have begun to address this question. Mice with a transgene encoding *Wt1*-KTS on a *Wt1* heterozygote background (thus altering the WT1 isoform ratio) are infertile, and histological examination of the testes revealed thickening of the tubular basement membrane, many seminiferous tubules severely depleted of spermatogenic cells, and expansion of interstitial cells (Lahiri et al., 2007). Another group undertook an siRNA approach to knock down WT1 specifically in Sertoli cells at the beginning of spermatogenesis (Rao et al., 2006). These mice displayed increased germ cell apoptosis and impaired fertility. Gao et al. generated mice with ablated WT1 function in Sertoli cells after testis determination. A dramatic and progressive loss of seminiferous tubular architecture and Sertoli cells, and a subsequent loss of germ cells was observed. Interestingly, these authors found a loss of *Sox9* expression at a time point when *Sry* was already down regulated in mice, and concluded that WT1 may directly or indirectly be required for continued *Sox9* expression (Gao et al., 2006). Finally, Natoli et al. describe that highly *Wt1*−/− chimeric mice show hypoplastic and dysgenic testes, with seminiferous tubules lacking spermatogonia. Interestingly, *Wt1* was expressed in germ cells, suggesting that it may be important for germ cell proliferation, maturation and survival (Natoli et al., 2004).

In summary, these findings in the mouse models, and ours in a FS patient, support the role of WT1 + KTS in terminal Sertoli cell differentiation and maintenance. The diminished expression of SRY and SOX9 may result in TDS. Whether the reduction in SRY and SOX9 levels is directly involved in causing the TDS phenotype or is rather a consequence of TDS is not distinguishable at the moment.

An In Vitro Model to Further Study the Function of WT1 + KTS in Human Sertoli Cells

The primary cell culture generated from the testis of a FS patient expresses primarily Sertoli cell markers, and is currently in the process of being expanded from immortalized clones. This system will allow the study of the role of the WT1 + KTS isoforms in Sertoli cells. One interesting aspect would be to examine Sertoli–Sertoli cell and Sertoli–germ cell interactions, which are known to be critical for spermatogenesis (reviewed in Lui et al., 2003).

CONCLUSIONS

Our results further support the importance of the WT1 + KTS isoforms as a critical factor for germ cell maturation by contributing to the correct microenvironment, namely functional Sertoli cells. The maintenance of Sertoli cells in their fully differentiated state is dependent on the correct expression of *WT1 + KTS*, *SRY* and *SOX9*.

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