**Abstract**

Genome-wide recombination is essential for genome stability, evolution, and speciation. Mouse *Tex11*, an X-linked meiosis-specific gene, promotes meiotic recombination and chromosomal synapsis. Here, we report that *TEX11* is mutated in infertile men with non-obstructive azoospermia and that an analogous mutation in the mouse impairs meiosis. Genetic screening of a large cohort of idiopathic infertile men reveals that *TEX11* mutations, including frameshift and splicing acceptor site mutations, cause infertility in 1% of azoospermic men. Functional evaluation of three analogous human *TEX11* missense mutations in transgenic mouse models identified one mutation (V748A) as a potential infertility allele and found two mutations non-causative. In the mouse model, an intronless autosomal *Tex11* transgene functionally substitutes for the X-linked *Tex11* gene, providing genetic evidence for the X-to-autosomal retrotransposition evolution phenomenon. Furthermore, we find that *TEX11* protein levels modulate genome-wide recombination rates in both sexes. These studies indicate that *TEX11* alleles affecting expression level or substituting single amino acids may contribute to variations in recombination rates between sexes and among individuals in humans.

**Keywords** chromosomal synapsis; infertility; meiosis; recombination; X chromosome

**Subject Categories** Genetics, Gene Therapy & Genetic Disease; Urogenital System

**Introduction**

Infertility, defined as the inability to conceive after a prolonged period, is a worldwide reproductive health problem, affecting men and women about equally (Hull et al., 1985; Matzuk & Lamb, 2002). The underlying causes are multifaceted, including physiological, environmental, social, and genetic factors; and studies in various model organisms have identified multiple molecular and genetic pathways that regulate fertility (de Rooij & de Boer, 2003; Matzuk & Lamb, 2008). In particular, mouse models have identified more than 400 genes that are specifically or preferentially involved in the regulation of fertility, facilitating genetic studies of infertility in humans (Matzuk & Lamb, 2008; Handel & Schimenti, 2010; Jamsai & O’Bryan, 2011). In humans, male infertility is a more clearly defined entity in cases of azoospermia or severe oligospermia (Hull et al., 1985; Silber, 2000). Known genetic causes of azoospermia in humans include Y chromosome deletion and chromosomal abnormalities such as Klinefelter syndrome (47, XXX); these account for ~25% of spermatogenic failure in otherwise healthy men (Reijo et al., 1995, 1996; Van Assche et al., 1996). Therefore, the majority (~75%) of cases of spermatogenic failure in humans are idiopathic, and the underlying causes are postulated to be genetic. However, to date, efforts to uncover point mutations in single genes that contribute to human spermatogenic failure have been largely unsuccessful (Matzuk & Lamb, 2008; Nuti & Krausz, 2008; Jamsai & O’Bryan, 2011).

Two major hurdles complicate the molecular genetic analysis of human male infertility. Traditionally, many disease-causing monogenic mutations have been identified through pedigree-based linkage analyses, demonstrating that the mutation co-segregates with a disease in multi-generation families and can therefore be deemed causative. This conventional approach is not applicable to the study of human male infertility, because infertile men lack biological offspring; it is therefore difficult to determine whether a mutation is causative. The second hurdle is that clinical and ethical
considerations limit the availability of sufficient testis biopsy material from patients for further validations. Genome-wide association studies (GWAS) have identified risk loci for non-obstructive azoospermia in humans (Hu et al., 2011, 2014). While variants in a number of genes have been identified in infertile men, causality of these variants has not been definitively proven (Sun et al., 1999; Miyamoto et al., 2003; Stouffs et al., 2005a,b, 2006; Krausz et al., 2006; Rohozinski et al., 2006; Martinez et al., 2007; Luddi et al., 2009).

Genomic studies have revealed that germ cell-specific genes are not randomly distributed in the genome, and that, in particular, the unique hemizygous and transcriptional status of the X chromosome has shaped its germ cell-specific gene content (Wang et al., 2001; Khil et al., 2004; Namekawa et al., 2006; Turner et al., 2006; Mueller et al., 2008; Song et al., 2009). The mammalian X chromosome is enriched for germ cell-specific genes expressed during early spermatogenesis. A systematic genomic screen of mouse spermatogonia, which are diploid mitotic germ cells of the testis, identified dozens of genes that are expressed specifically in male germ cells, and nearly one-third of these genes map to the X chromosome, suggesting that genes encoded on the X chromosome play a preeminent role in spermatogenesis (Wang et al., 2001). Genetic studies in mouse models have shown that three of these X-linked genes (Tex11, Taf7 l, and Ns2) are important regulators of male fertility (Cheng et al., 2007; Yang et al., 2008; Pan et al., 2009; Zheng et al., 2010). As males are hemizygous for the X chromosome, mutations in single-copy X-linked genes cannot be compensated by a corresponding wild-type allele such as in heterozygous carriers of autosomal recessive mutations. Therefore, mutations in X-linked genes essential for fertility may represent a significant proportion of infertility-causing mutations in men.

Tex11 is essential for male fertility in mice. Disruption of Tex11 gene function causes meiotic arrest in males, resulting in azoospermia (Yang et al., 2008). Here, we report that the frequency of rare Tex11 mutations is significantly elevated in azoospermic men, suggesting that Tex11 is required for spermatogenesis in humans. In combination with analyses of genetically modified mice harboring Tex11 mutations analogous to those in human, our results demonstrate that in ~1% of azoospermic men, infertility is caused by mutations in a single X-linked gene—the Tex11 gene. Furthermore, our studies show that meiotic progression requires a critical threshold level of Tex11 protein and, significantly, that genome-wide meiotic recombination rates in both sexes are sensitive to Tex11 levels.

Results

Frequent singleton Tex11 mutations in men with spermatogenic failure

To evaluate the role of Tex11 in human fertility, we screened genomic samples from 246 azoospermic men with spermatogenic failure (no sperm in semen) and from 175 controls that included men who had fathered children (n = 93) and men of unknown fertility selected to represent worldwide genetic diversity based on their Y-chromosomal haplotypes (n = 82). All the infertile patients selected were pre-screened for the lack of Y chromosome microdeletions. Sequencing of amplicons covering the Tex11 exons and flanking intronic regions revealed 40 different sequence variants in Tex11 in our cohorts (Table 1 and Supplementary Table S1). Of these variants, 21 were singletons (observed in only one man; Table 1), whereas 19 were observed in two or more men and thus are not likely to be associated with spermatogenic failure (Supplementary Table S1).

We detected a significantly higher percentage of singleton variants in men with spermatogenic failure than in controls (7.3% versus 1.7%, P = 0.007, Fisher’s exact test, Table 1). Of the 21 singletons, 18 were found in azoospermic men and three in controls (Table 1). The significantly higher prevalence of singleton variants in azoospermic men strongly suggests that Tex11 is required for spermatogenesis in human.

With the exception of a frameshift mutation, 1258Ins(TT) in exon 16, and a splice site mutation, in intron 21, the mechanisms by which the singleton mutations may cause or predispose to azoospermia are not clear. Five of the singleton exonic mutations among azoospermic men were missense (W117R, V142I, Q172R, T244I, and V748A), and two were silent (Table 1). The remaining 10 were intronic mutations with undetermined functional consequences except for the splice site mutation (−1G→A) at the consensus 3’ splice acceptor site in intron 21, which would be expected to abolish splicing (Table 1).

Meiotic arrest in an azoospermic man with a frameshift mutation in Tex11

Among the singleton Tex11 mutations identified in azoospermic men, a frameshift mutation in exon 16 would predictably impair Tex11 protein function, yielding a severely truncated protein comprising only the N-terminal half. The patient carrying this mutation (WHT3759) and his brother were azoospermic, and two maternal uncles were childless (Fig 1A). Analysis of DNA samples from both parents of WHT3759 revealed that the mother was heterozygous for the mutation, whereas the father had the wild-type allele (Fig 1B), demonstrating inheritance of the mutation from the mother. We could not obtain DNA samples from the patient’s brother or maternal uncles and thus were unable to determine whether these relatives carried the same mutation as the proband. Nevertheless, the data in this family are consistent with X-linked spermatogenic failure.

Histological analysis of a testis biopsy obtained from the azoospermic patient WHT3759 revealed meiotic arrest at the pachytene stage (Fig 1C). No post-meiotic germ cells such as round spermatids and mature spermatozoa were observed in the seminiferous tubules, consistent with the diagnosis of azoosperma. Based on the histological data, we conclude that the primary defect caused by the Tex11 frameshift mutation of this patient is meiotic arrest, corresponding to the phenotype observed in Tex11-null (Tex11−/−) male mice (Yang et al., 2008).

Experimental transfer of Tex11 from the X chromosome to an autosome

To circumvent the inherent problems in the genetic dissection of human infertility as described earlier, we chose to analyze the consequences of mutant human Tex11 alleles in genetically
modified mice harboring analogous mutations in murine Tex11. The generation of mice with gene-specific mutations usually relies on gene targeting by homologous recombination in male (XY) embryonic stem (ES) cells and subsequent transmission of the modified allele through the germ line of male ES cell chimeras. However, this approach would predictably fail when modeling X-linked mutations causing male infertility, as these would not be transmitted through the male germ line. To overcome this impediment, we generated an experimental copy of the Tex11 gene at an autosomal locus, placing a Tex11 knockin allele under the transcriptional and translational control of Tex11 (Chr. 11; Fig 2A). We chose the Tex11 locus for several reasons. First, Tex19.1 and Tex11 exhibit similar temporal expression patterns during spermatogenesis. Previous studies have shown that both genes are expressed in spermatogonia and early spermatocytes (Wang et al., 2005; Yang et al., 2008, 2010). Furthermore, the Tex19.1 ORF is entirely encoded in one exon (exon 3) (Kuntz et al., 2008; Ollinger et al., 2008; Yang et al., 2010), permitting replacement of the Tex19.1 ORF with the Tex11 ORF (Fig 2A). As Tex19.1−/− mice display normal fertility (Ollinger et al., 2008; Yang et al., 2010), the deletion of one Tex19.1 copy to generate the Tex11 knockin allele would not be associated with phenotypic consequences.

Table 1. Singleton sequence variants in TEX11 found in infertile patients and controls.

<table>
<thead>
<tr>
<th>Position</th>
<th>Nucleotide change</th>
<th>Resultant change</th>
<th>Patient ID</th>
<th>Infertile males</th>
<th>Control males</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 6</td>
<td>349T→A</td>
<td>Missense mutation, W117R</td>
<td>WHT3150</td>
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<td>WHT3171</td>
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<td>424G→A</td>
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<td>WHT3417</td>
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<tr>
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<td>Missense mutation, Q172R</td>
<td>WHT3500</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Exon 10</td>
<td>731C→T</td>
<td>Missense mutation, T244I</td>
<td>WHT2546</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Exon 16</td>
<td>1258ins (TT)</td>
<td>Frameshift mutation; 1258CATG→TTGGTA</td>
<td>WHT3759</td>
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<tr>
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<td></td>
</tr>
<tr>
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<td>Silent mutation</td>
<td>WHT2546</td>
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<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>Intron 15</td>
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<td>Intronic alteration</td>
<td>WHT3158</td>
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<tr>
<td>Intron 21</td>
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<td>Alteration of splicing acceptor site</td>
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<td>−44C→T</td>
<td>Intronic alteration</td>
<td>1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AZ, azoospermic males.

*Number of individuals screened.

1 refers to the first base of a given intron, and −1, the last base.

An autosomal Tex11 knockin minigene rescues male infertility in adult Tex11−/−Y mice

We next determined whether the wild-type autosomal Tex11 knockin minigene (Tex19Tex11K) could functionally replace its X-linked progenitor and rescue infertility of azoospermic Tex11−/−Y males. In male meiotic germ cells, TEX11 localizes to recombination nodules and regulates both chromosomal synapsis and the formation of crossovers between homologous chromosomes. Tex11-deficient spermatocytes are eliminated at the pachytene and anaphase I stages of meiosis due to extensive asynapsis and a failure in chromosome segregation (Yang et al., 2008). We crossed Tex19Tex11K+/− males (Fig 2A) with Tex11+/− females to generate Tex19Tex11K+/− Tex11−/−Y males (hereafter referred to as Tex11 KI/KO males). Western blot analysis confirmed that TEX11 protein was expressed in the testes of adult (3-month) KI/KO male mice albeit at lower levels compared to wild-type testes (Fig 2B). Tex11 knockin males (Tex11 KI plus the wild-type Tex11 allele) exhibited higher testicular TEX11 protein levels compared to wild-type males. Therefore, TEX11 protein levels in these mouse models correlate with gene dosage (Fig 2B). Adult males of the four genotypes (Tex11−/−Y, Tex11 KI/Tex11−/−Y, Tex11+/−, Tex11−/−Y).
Tex11 KI/Tex11+/Y had comparable body weight (data not shown). Testes from adult KI/KO males weighed significantly more than those from Tex11-null males but less than those from wild-type males (Fig 2C). Strikingly, the sperm count of 3-month-old KI/KO males was comparable to that of wild-type males (Fig 2D). As expected, the KI/KO males were fertile.

Histological analysis revealed that KI/KO and wild-type testes contained the full spectrum of spermatogenic cells including mature spermatooza, whereas Tex11-null testes exhibited meiotic arrest of germ cells as previously reported (Fig 2E) (Yang et al., 2008). Chromosomal synopsis defects caused by Tex11 deficiency were rescued by the Tex11 KI minigene (Fig 2F).

Taken together, we conclude that the autosomal Tex11 KI minigene rescues meiotic arrest and male infertility in adult Tex11+/Y mice.

**Defective meiosis in the first wave of spermatogenesis in Tex11 KI/KO mice**

Testes from 8-week-old Tex11 KI/KO males were comparable in size and weight to wild-type testes (Fig 3A). However, testes from juvenile Tex11 KI/KO males (postnatal day 25, 36, or 49) were much smaller than wild-type testes (Fig 3A), suggesting a developmental delay or a failure in the first wave of spermatogenesis in Tex11 KI/KO males. Spermatogenesis proceeds through distinct stages that include mitotic proliferation of spermatogonial stem cells, meiotic division of spermatocytes, and spermiogenesis of haploid spermatids; this occurs in a locally synchronized and coordinated manner described as spermatogenic waves (McCarrey, 1993). In juvenile mice, the first wave of spermatogenesis is initiated within 1 week after birth, producing mature spermatooza by postnatal day 35. This wave bypasses a self-renewing spermatogonia stage and displays a synchronized appearance of differentiating germ cells in seminiferous tubules (de Rooij, 1998). In adult mice, waves of spermatogenesis are not synchronized among different seminiferous tubules. In Tex11-null male mice, lack of TEX11 affects juvenile and adult spermatogenic waves equally (Yang et al., 2008). In mature Tex11 KI/KO male mice, the chromosomal asynapsis defects caused by TEX11 deficiency were rescued by the Tex11 KI allele (Fig 2F). However, analysis of spermatocytes from juvenile Tex11 KI/KO males revealed that chromosomal synopsis remained severely impaired at day 25, that is, during the first spermatogenic wave (Fig 3B and Supplementary Fig S2). Evaluation of the number of MLH1 foci, which mark the site of future meiotic crossovers (Anderson et al., 1999), indicated that the meiotic recombination defect in juvenile testis was largely but not fully rescued by the Tex11 KI minigene.

These results suggest that defective meiosis rather than developmental delay causes the severe impairment of the first spermatogenic wave in juvenile Tex11 KI/KO males. Western blot analysis revealed substantially lower TEX11 protein levels in juvenile (day 25) KI/KO testes compared to wild-type testes (Fig 3B). These results suggest that the amount of TEX11 protein expressed from the Tex11 KI allele was too low to fully rescue meiotic defects in Tex11-null juveniles (Fig 3B), but was expressed at a higher level in adult mice, which is sufficient for meiotic progression in adult Tex11 KI/KO males (Fig 2B). Alternatively, different threshold levels of TEX11 may be needed for...
Figure 2. A wild-type Tex11 knockin allele at the autosomal Tex19.1 locus rescues male sterility in Tex11-null adult mice.
A Schematic diagram of Tex11 gene structure, Tex19.1 gene structure, targeting vector and the final Tex11 knockin allele containing the wild-type Tex11 ORF.
B Western blot analysis of TEX11 in the testes of 3-month-old mice with different Tex11 gene dosages. ACTB serves as a loading control.
C Testis weight of 3-month-old males.
D Sperm count of 3-month-old males.
E Histological analysis of testes from adult wild-type, Tex11 KI/KO, and Tex11 -/Y males. RS, round spermatids; ES, elongating spermatids; Pa, pachytene spermatocytes. Scale bar, 50 μm.
F The Tex11 knockin allele rescues chromosomal synapsis defects in Tex11 -/Y spermatocytes in 3-month-old males. Chromosomal synapsis defects were assessed by SYCP1 and SYCP3 immunostaining of spread nuclei from 100 pachytene spermatocytes per male; for each genotype, three males were analyzed. Abbreviations for Tex11 genotypes: -/Y, Tex11 knockout (Yang et al, 2008); KI:+/Y, Tex11 knockin and knockout; +/Y, wild type; KI:+/Y, Tex11 knockin plus wild-type Tex11.
Data information: All statistical analyses were performed using Student’s t-test. n.s.: not statistically significant.
progression of meiosis during the first wave of spermatogenesis compared to adult waves of spermatogenesis.

**Meiotic recombination levels are sensitive to the gene dosage of Tex11**

The presence of fewer MLH1 foci in spermatocytes from Tex11 KI/KO juvenile mice compared to wild type suggested that Tex11 dosage might influence the rate of meiotic recombination (Fig 3C). To further test this hypothesis, we analyzed the recombination rate in spermatocytes from 3-month-old males with increasing Tex11 gene dosages (genotypes: Tex11 −/−Y, Tex11 KI/Tex11 −/−Y, Tex11 +/+Y; Fig 4A and Supplementary Fig S3). The recombination rate was significantly different among these males, with the lowest rate in Tex11 −/−Y males and the highest rate in Tex11 KI/Tex11 +/+Y males, revealing a positive correlation between meiotic recombination rate and Tex11 dosage in males.

In female germ cells, both X chromosomes are active such that both copies of the Tex11 gene can be transcribed. Tex11 deficiency reduces the number of MLH1 foci in fetal oocytes (Yang et al., 2008). To ascertain whether the meiotic recombination rate in females depends on Tex11 gene dosage, we evaluated recombination frequencies in Tex11 −/−, Tex11 +/+ females and wild-type oocytes (Fig 4B). The average number of MLH1 foci in Tex11 −/− oocytes was significantly higher than in Tex11 +/+ oocytes but significantly lower than in wild-type oocytes, demonstrating that meiotic recombination rate in females is strongly affected by the Tex11 gene dosage. In conclusion, meiotic recombination rates in both sexes are sensitive to the Tex11 gene dosage.

**A TEX11 missense mutation found in an infertile man causes defects in chromosomal synapsis**

We identified five TEX11 missense mutations (W117R, V142I, Q172R, T244I, V748A) in azoospermic men (Table 1 and Fig 5A) that were not detected in any of the 175 control men, implying that they are likely to be genetic causes of infertility in humans. By sequence alignment analysis of TEX11 homologues from diverse species (human, mouse, rat, horse, cat, dog, marsupial, chicken, and fish), we found that three of these mutations affect residues that are highly evolutionary conserved and may therefore be important for functional and/or structural integrity (W117, Q172, and V748; Supplementary Fig S1). We therefore evaluated the consequences of missense mutations at these residues (W117R, Q172R, and V748A) in vivo by generating knockin mice harboring mutant Tex11 “retro-genes” at the Tex19.1 locus.

Using the same experimental approach as for the wild-type Tex11 KI allele (Fig 2A), we generated three different KI mouse lines, each harboring one TEX11 point mutation (W118R, Q173R, or V749A) (Fig 5B) analogous to the human mutations. All three KI
alleles were targeted to the Tex19 locus through homologous recombination in ES cells and thus were under the transcriptional and translational control of the Tex19 locus (Fig 5B). These three alleles are referred to as KI(W118R), KI(Q173R), and KI(V749A), respectively. Mutant KI males (Tex19\(^{KI-W118R}\)/+ Tex11\(^{+/+}\)) were fertile, suggesting that none of these point mutations are dominant negative. We then crossed KI males with Tex11\(^{+/+}\) females to generate KI/KO males.

Western blot analysis of testes from mature (3-month-old) males confirmed comparable expression levels of TEX11 from all KI alleles (Fig 6A). Mature KI/KO males with the three different Tex11 KI alleles (three point mutations) were comparable to KI/KO males with a wild-type Tex11 KI allele (hereafter referred to as wild-type KI/KO) in respect to body weight, testis weight, and fecundity (Fig 6B, C, and E). Sperm counts of KI(W118R)/KO and KI(Q173R)/KO males were also similar to wild-type KI/KO males (Fig 6D), correlating with rescue of chromosomal synapsis defects in males of these genotypes (Fig 6F and Supplementary Fig S4). Meiotic recombination rates, reflected by the number of MLH foci in pachytene stage spermatocytes, were comparable between KI(W118R)/KO and wild-type KI/KO males. Intriguingly, pachytene cells from KI(Q173R)/KO males contained more MLH foci than wild-type KI/KO germ cells, indicating a higher rate of recombination (Fig 6G). These results suggest that the TEX11 mutations (W117R and Q172R) may not cause male infertility in humans.

In striking contrast, the sperm count of KI(V749A)/KO males was significantly reduced compared to controls (Fig 6D). Surface spread analysis of pachytene spermatocytes revealed a similar proportion of asynapsis in germ cells from KI(V749A)/KO (33%; Fig 6F and Supplementary Fig S4) and KO males (34%; Fig 2F), revealing that the KI(V749A)/KO mutant phenocopies the KO mutant in terms of chromosomal synapsis in mice (Yang et al, 2008). Consistent with these observations, histological analysis of a testis biopsy obtained from patient WHT2499 carrying the TEX11 V748A missense mutation (Table 1) revealed meiotic arrest at the pachytene stage (Supplementary Fig S5). However, the meiotic recombination rate in KI(V749A)/KO males was comparable to the control (Fig 6G). We previously showed that TEX11 regulates two distinct processes during meiosis: chromosomal synapsis and meiotic recombination (Yang et al, 2008). Our current data therefore identify the single amino acid change (V749A) in TEX11 as a separation-of-function mutation that disrupts chromosomal synapsis but not meiotic recombination. Significantly, our results strongly support that the human TEX11 V748A mutation is likely a genetic cause of infertility in azoospermic men.

Discussion

Our studies show that TEX11, an X-linked meiosis-specific gene, is mutated in azoospermic men. A conservative calculation that considers only three TEX11 mutations (frameshift mutation in exon 16, splice site mutation in intron 21, and V748A missense mutation) indicates an infertility-causing mutation frequency in human TEX11 of \(~1\%\) (three mutations/246 azoospermic men screened, Table 1). Given that hundreds, if not thousands, of genes specifically regulate fertility, finding a causative mutation frequency of 1% in TEX11 is highly significant. This frequency is comparable with the mutation frequency of BRCA1 (\(~2\%\)) in breast cancers (Kurian, 2010). Finding a high mutation frequency of TEX11 among infertile men is not entirely unexpected: TEX11 is X-linked, such that any inherited or de novo mutations that
impair the function of this essential fertility factor would manifest as infertility.

Histological analysis of testis biopsies from two azoospermic men with TEX11 mutations revealed meiotic arrest at the pachytene stage, indicating that TEX11 plays a critical role in human meiosis (Fig 1C and Supplementary Fig S5). We have previously identified homologous recombination and meiotic arrest of TEX11, lead to defects in meiosis (Tsubouchi et al, 2006; Chelysheva et al, 2007). TEX11 therefore plays an evolutionarily conserved role in meiosis from budding yeast to humans. It is noteworthy that the azoospermic man with the V749A mutation exhibits complete meiotic arrest (Supplementary Fig S5), whereas Tex11 KO males with wild-type or mutant knockin allele (E) displays severe meiotic defects but no complete meiotic arrest. The differential effect of this missense mutation on the fertility of mouse and human may be attributed to evolving gene with only 56% protein sequence identity between mouse and human.

Crossovers are formed through at least two pathways: MLH1-dependent and Mus81-dependent (de los Santos et al, 2003; Holloway et al, 2008). The majority of crossovers in mice is processed through the MLH1-dependent pathway and is subject to crossover interference, a phenomenon that ensures wide spacing of crossovers on the same chromosome (Holliday, 1977; Bishop & Zickler, 2004). ZIP4/TEX11 belongs to the ZMM protein group and thus promotes crossover via the MLH1-dependent pathway (Perry et al, 2005; Tsubouchi et al, 2006; Chelysheva et al, 2007). In fact, disruption of ZIP4/TEX11 in yeast, Arabidopsis, and mouse causes reduction in MLH1-dependent crossovers (Tsubouchi et al, 2006; Chelysheva et al, 2007; Yang et al, 2008). Notably, the effect of Tex11 gene dosage on the number of MLH1 foci in mouse is nonlinear. This nonlinear effect may be largely attributed to crossover homeostasis (Martini et al, 2006; Zhang et al, 2014). Although Mus81-dependent crossovers are not affected in Arabidopsis Zip4 mutant (Chelysheva et al, 2007), further studies are needed.

Figure 5. Modeling human male infertility in mice using the autosomal knockin approach.
A Schematic representation of missense and nonsense mutations in human TEX11 found exclusively in men with azoospermia. The full-length TEX11 protein (GenBank accession number: NP_112566) contains 925 residues. The tetratricopeptide-like (TPR-like) helical domain, found in proteins that form large complexes, extends from residues 161 through 499 (Blatch & Lassle, 1999). Four of the residues mutated in azoospermic men (W117, V142, Q172, and V748) are conserved between human and mouse TEX11 proteins (Supplementary Fig S1).
B Generation of three lines of Tex11 knockin mice. Each harbors a single amino acid substitution in TEX11 (indicated by asterisks) analogous to a missense mutation identified in a human patient. Nucleotide and respective amino acid changes are indicated. Exons 1, 2, and 3’UTR are from the mouse Tex19.1 gene.

Figure 6. A single amino acid change (V749A) in TEX11 causes severe defects in chromosomal synopsis in mice.
Data shown are from 3-month-old Tex11 KO males with wild-type or mutant knockin allele (p, number of males analyzed per genotype) except for mating test (E).
A Western blot analysis reveals comparable levels of TEX11 protein in the testes of wild-type and point mutant KI/KO males. ACTB serves as a control.
B–D Body weight (B), testis weight (C), and significantly reduced sperm count (per pair of epididymides) in Tex11 KO (V749A)/KO males (D).
E Mating test of 3- to 5-month-old males.
F Dramatically increased chromosomal asynapsis in spermatocytes from Tex11 KI(V749A)/KO males. 100 pachytene spermatocytes per mouse were examined by surface spread analysis.
G Number of MLH1 foci in pachytene spermatocytes. Note a significantly higher number of MLH1 foci in spermatocytes from KI(Q173R)/KO males.

Data information: Values are shown as average ± standard deviation. n.s.: not statistically significant.
Figure 6.
to examine the formation of Mus81-dependent crossovers in Tex11-deficient and knockin mutant mice.

Recent linkage-based studies in humans and mice suggest that multiple loci regulate the levels of genome-wide meiotic recombination (Kong et al, 2008; Chowdhury et al, 2009; Murdoch et al, 2010). Variants in RNF212, a SUMO E3 ligase, is associated with recombination rate in human populations (Kong et al, 2008). Mouse RNF212 localizes to a subset of recombination sites (Lake & Hawley, 2013; Reynolds et al, 2013). HEI10, a ubiquitin E3 ligase, regulates meiotic recombination in mouse, rice, and Arabidopsis (Ward et al, 2007; Chelysheva et al, 2012; Wang et al, 2012). Interestingly, the levels of crossovers are sensitive to the gene dosage of Rnf212 and Hei10 (Lake & Hawley, 2013; Reynolds et al, 2013; Qiao et al, 2014). Seven recombination-associated loci have been mapped in the mouse genome, and the genetic locus with the highest LOD score in mouse maps close to Tex11 on the X chromosome (Murdoch et al, 2010). Non-synonymous Tex11 SNPs are associated with testicular size in cattle (Lyons et al, 2014). Our current results demonstrate that recombination rates in both sexes are sensitive to TEX11 levels. In addition, a missense mutation (Q173R) in Tex11 increases the recombination rate in mice (Fig 6G). Therefore, different expression levels of TEX11 or single amino acid substitutions may contribute to the variable genome-wide meiotic recombination rates between sexes and among individuals, and as such, low-expressing TEX11 alleles could be genetic causes of male infertility in humans.

We find that the engineered Tex11 minigene on the autosome rescues the infertility caused by the X-linked Tex11 deletion. The significance of this finding is two-fold. First, genetic modification of this allele can be used as a strategy to determine the consequences of human TEX11 mutations in vivo by introducing analogous mutations into the autosomal Tex11 knockin allele. Secondly, this allele resembles and therefore models endogenous testis-specific X-to-autosomal retrogenes. These genes originate from the retrotransposition of X-linked genes to autosomes during evolution and exhibit testis-specific expression patterns. A common hypothesis for the evolution of X-to-autosomal retrogenes is that they provide a backup source for gene expression during mammalian meiosis, which involves silencing of the X chromosome in males (McCarrey & Thomas, 1987; Emerson et al, 2004; Wang, 2004; Turner, 2007). The autosomal Tex11 knockin allele generated in our study carries the hallmarks of an X-to-autosomal retrogene, that is, lack of its introns, and therefore provides the first direct genetic evidence that such a gene can substitute for the function of its X-linked “ancestral” gene.

Materials and Methods

Ethical considerations and patient consents

The human mutation screening protocol was approved by the Institutional Review Board of the Massachusetts Institute of Technology. Informed consent was obtained from all participants. Experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. Standard barrier mouse housing conditions and all experiments involving mice were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Animals were used based on genotypes. No randomization and no blinding were used.

Population samples

We studied 246 patients with non-obstructive azoospermia. We excluded patients known to have had any of the following conditions or treatments that cause or predispose to spermatogenic failure: Y-chromosomal deletions (Reijo et al, 1995, 1996; Vogt et al, 1996; Kuroda-Kawaguchi et al, 2001; Repping et al, 2002); a 47, XXY karyotype; orchitis; cryptorchidism; radiation therapy; or chemotherapy. The 175 control subjects included men known to have fathered children (n = 93) and men of unknown fertility selected to represent worldwide genetic diversity based on their Y-chromosomal haplotypes (n = 82, samples from the NIH polymorphism discovery panel, Coriell Cell Repositories, and from our collection; Collins et al, 1998). We prepared DNA from peripheral blood leukocytes or EBV-transformed lymphoblastoid cell lines.

Mutation screening

The TEX11 exon/intron structure was determined by alignment of the TEX11 cDNA sequence (NM_031276) with its genomic sequence (Wang et al, 2001). We amplified the TEX11 coding exons (exons 2 through 30) by PCR using 28 primer pairs (GenBank dbSTS accession numbers: BV703476-BV703503). PCR was performed in a 25 µl reaction with 12.5 ng genomic DNA (94°C, 30 s; 56°C, 30 s; 72°C, 90 s; 35 cycles). PCR products were purified by Sephadex S-300 gel filtration. 12.5 µl of purified PCR product was sequenced in one of the PCR primers and ABI BigDye according to the manufacturer’s instructions. Reaction products were separated and read on an ABI 3700 sequencer. Sequence analysis was performed using the Sequencher software (Gene Codes Corporation), and sequence variants were identified by manual inspection of aligned sequences.

Generation of Tex11 knockin mice

The two homologous arms (2.1 kb each) of the Tex11 knockin targeting construct were amplified from a Tex11.1-positive ES clone (RP23-400P17) by high-fidelity PCR and were subcloned into the NeoA plasmid to flank a floxed PGK-Neo selection cassette, resulting in the parent vector pUP104/Call-EcoRV upstream of the PGK-Neo cassette, resulting in pUP115 (Fig 2). The construct was verified by sequencing. Hybrid V6.5 XY ES cells (C57BL/6 × 129/sv) were electroporated with the linearized Tex11 knockin targeting construct (pUP115/NotI), followed by culture in the presence of G418 (350 µg/ml). Seven days after electroporation, 96 G418-resistant ES cell clones were picked and screened by PCR for homologous recombination on both sides, identifying seven homologously targeted ES cell clones. Two targeted ES cell clones (C4 and E8) were injected into B6C3F1 (Taconic) blastocysts that were subsequently transferred to the uteri of pseudopregnant ICR females. Male chimeras were bred with Actb-Cre females to delete the PGK-Neo cassette, and germ-line transmission of the wild-type Tex11 knockin allele was obtained from chimeras derived from both ES cell clones.
To generate Tex11 alleles with missense mutations, nucleotide changes were introduced into the Tex11 ORF by overlapping PCR, followed by subcloning of the mutant Tex11 ORF into pUP104-12. The final Tex11 knockin targeting constructs for mutations T352A, A518G, and T2246C were pUP106-1, pUP107-4, and pUP108-5. Sequencing of the final constructs confirmed the desired mutation and revealed no other mutations. ES cells were targeted and screened as described above. One ES cell clone for each point mutation was injected into blastocysts. ES cell clones 1C2, 2D4, and 3G2 contained the Tex11 knockin allele bearing mutations T352A, A518G, and T2246C, respectively. These three nucleotide mutations correspond to amino acid changes W118R, Q173R, and V749A, respectively (Fig 5B). All knockin alleles were transmitted through the germ line from male chimeras, and point mutations were further confirmed by DNA sequencing of amplicons from tail genomic DNA. All knockin mice used in the study were from colonies that had been backcrossed to C57BL/6J for three generations. Offspring were genotyped by PCR of tail genomic DNA with the following primers: Tex11 knockin allele with PGK-Neo (510 bp), GCACC CTCAAAACAAGCTATG and CCTACCCTGTGAATGTGAATGTGTG; Tex11 knockin allele without PGK-Neo (252 bp), GCACCTCAA ACAAGCTATG and CTGAGCTTTAGTGTCTCAGG; Tex11 knockout allele (530 bp), ACTGTGTTACACTAGGTTGGA and TGAGGTCTCAGTGTCCAGG.

Histological and nuclear surface spread analysis

For histology, testes were fixed in Bouin’s solution, embedded in paraffin, sectioned, processed, and stained with hematoxylin and eosin. For meiotic nuclear surface spread analysis, spermatocytes or prophase I oocytes were prepared using the dry-down method as previously described (Peters et al., 1997; Kolas et al., 2005). Spread nuclei were immunostained with the following primary antibodies: Anti-SYCP1 (1:50; catalog no. ab15090, Abcam), anti-SYCP2 (1:100, sera 1918 and GP21; Yang et al., 2006), anti-MLH1 (1:50; catalog no. 550838, clone G168-15; BD Biosciences), followed by detection with FITC- and Texas red-conjugated secondary antibodies. Images were captured on a digital camera coupled to a Zeiss Axioskop 40 fluorescence microscope. The number of MLH1 foci per nucleus was counted.

Mating tests and sperm count

Each adult male (three males per genotype; Fig 6E) at the age of 3–5 months was housed with two 8-week-old wild-type C57BL/6J females for 4 months. Mice were checked daily, and litter size was recorded. Sperm count was performed as previously described (Cheng et al., 2007).

Western blot analysis

Testicular protein extracts were prepared by homogenization of testes from 3-month- or 25-day-old mice in SDS-PAGE buffer. Testicular protein (30 μg) was separated on a SDS–PAGE gel and blotted onto a nitrocellulose membrane. The blot was probed with the following primary antibodies; anti-TEX11 (Yang et al., 2008) and anti-ACTB (1 : 7,500; catalog no. A5441, clone AC-15; Sigma).

The paper explained

Problem

Infertility is a worldwide reproductive health issue. Azoospermia, characterized by the absence of sperm in semen, is a severe form of male infertility. The cause for the majority of azoospermia is unknown and likely to be genetic. Genetic studies of azoospermia in humans is complicated by the presence of hundreds, if not thousands, of candidate genes.

Results

We sequenced azoospermic patients (with no known causes) for mutations in TEX11, an X chromosome-linked germ cell-specific gene. We found one frameshift mutation, one point mutation at a splicing acceptor site, and a number of missense mutations in infertile patients. The frameshift mutation in the reported patient was passed from the mother, consistent with X-linked inheritance. Further analysis using mouse as an in vivo model demonstrated that one missense mutation (V748A) impairs meiosis. Our results show that mutations in the TEX11 gene account for 1% of infertility in non-obstructive azoospermic men. Furthermore, genome-wide recombination rates in both sexes depend on levels of TEX11 protein.

Impact

Our results suggest that mutations in TEX11 underlie non-obstructive azoospermia in a significant fraction of men. Identification of genetic causes of male infertility would improve genetic counseling for patients seeking infertility treatment.

Statistics

All data were analyzed using GraphPad Prism (GraphPad Software Inc) and KaleidaGraph (Synergy Software). Statistical analysis of singleton variants in infertile and control men was conducted using Fisher’s exact test (Table 1). Student’s t-test was performed for body weight, testis weight, sperm count, mating test, chromosomal asynapsis, and the number of MLH1 foci. Normal distribution of the number of MLH1 foci was tested using Shapiro–Wilks test and D’Agostino’s K-squared test in GraphPad Prism. A P-value ≤ 0.05 was considered significant.

Supplementary information for this article is available online:
http://embomolmed.embopress.org

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Author contributions

FY, DP and PW conceived and designed the experiments. FY, PW, NL, JM and LB performed the experiments. PW, FY, SR, DP and PW analyzed the data. SS, RO, and HS contributed reagents, materials, and/or analysis tools. PW and SR wrote the manuscript with comments and edits from all authors.

Conflict of interest

The authors declare that they have no conflict of interest.
For more information
Online Mendelian Inheritance in Man (OMIM)/TEX1, http://omim.org
Coriell Cell Repositories, https://catalog.coriell.org/

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X-linked regulation of male fertility

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