Characterization of the porcine testis-expressed gene 11 (*Tex11*)

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Testis expressed gene 11 (*Tex11*) is essential for meiosis and male fertility in the mouse. Currently, little is known about the control of spermatogenesis in non-rodent animal models such as pigs. Here, we characterized the sequence and expression profile of the porcine *Tex11* gene. We showed that the porcine *Tex11* is an X-linked gene that is exclusively expressed in germ cells in the adult pig testis. The expression of porcine *Tex11* is correlated with the onset of meiosis and the expression pattern is highly conserved between the *Tex11* homologs in pig and mouse. The DNA sequence analysis and the estimated molecular weight also suggested a high level of homology across species. As the mouse *Tex11* proved to be essential for male fertility, the important biological function during meiosis is likely conserved in the porcine *Tex11*.

Introduction

Spermatogenesis is an elaborate, complex biological process during which spermatogonia undergo a series of highly organized events of proliferation, differentiation, meiosis and morphogenesis to form sperm. Genetic defects that disrupt a single or multiple stages of spermatogenesis generally result in infertility, or to a lesser extent, subfertility.^{1,2} Although the structural and morphological characteristics of spermatogenesis are well-defined, the underlying molecular mechanisms are much less well-understood.

The Testis-expressed gene 11 (*Tex11*) was originally identified as an X-linked, germ cell-specific gene in the mouse during an effort to search for spermatogonia-specific transcripts.³ More detailed analysis of the expression pattern revealed that the *Tex11* transcript is present in germ cells at different spermatogenic stages, including type A and type B spermatogonia, meiotic spermatocytes and round spermatids.⁴ During meiosis, a high level of *Tex11* is maintained from the preleptotene stage to the zygotene stage, however, the expression is downregulated in pachytene spermatocytes.^{4,5}

The TEX11 protein was found to form discrete foci along the synaptonemal complexes (SCs) on meiotic chromosomes and was shown to be essential for meiosis in males.⁶ The conditional deletion of 27 exons (out of a total of 30 exons) of *Tex11* in mice resulted in defects in synapsis formation and meiotic

crossovers, leading to infertility in males.⁶ Interestingly, another study reported that the deficiency of *Tex11* led to impaired crossover and achiasmate chromosomes at Meiosis I; however, males were fertile.⁵ This discrepancy in phenotype could be attributed to the differential efficiency of gene knockout (a null allele vs. a hypomorphic allele), given that only one exon (exon3) was conditionally deleted in the *Tex11* knockout animal in the second study. TEX11 was also detected in fetal oocytes; however, female mutants were fertile with a decreased litter size.⁶

While extensive efforts utilizing rodent models have contributed greatly to the understanding of spermatogenesis,¹ little information is available on this process in larger species such as pigs. Pigs are of increasing importance as a non-rodent research model, providing additional or novel knowledge essential for a better understanding of spermatogenesis in higher mammals and humans. The objective of this study was to characterize the porcine *Tex11* gene during spermatogenesis.

Results

Characterization of the sequence of the porcine *Tex11* gene. With a goal of cloning the porcine *Tex11* gene, primers were designed based on the homologous regions between the human and mouse *Tex11*. The designed *Tex11* primers yielded a fragment slightly over 1 Kb from adult pig testis tissue by reverse transcription PCR (RT-PCR) (Fig. 1). The PCR fragment was cloned into the pET28a vector for sequencing. The resulting

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Figure 1. Cloning of the porcine *Tex11* from adult pig testis. A partial sequence (1,028 bp) of the porcine *Tex11* (slightly over 1 Kb) was amplified from adult pig testis by RT-PCR and resolved on a 1.2% agarose gel. Lane a: DNA ladder. Lane b: *Tex11*. Lane c: *Gapdh* (983 bp).

1,028 bp sequence represents a partial sequence of the porcine *Tex11* transcript. This partial sequence shares 83% and 73% sequence identity to the corresponding homologous regions of human *Tex-11* (1,221 bp–2,277 bp of the isoform 1; NM_001003811.1) and mouse *Tex11* (1,178 bp–2,235 bp of the isoform 1; NM_031384.2), respectively (Clustal W2: www.ebi.ac.uk/Tools/clustalw2/index.html; Fig. 2).

BLAST search of the partial *Tex11* sequence against the pig genomic reference sequences available on the NCBI database indicated a significant alignment only to the *Sus scrofa* breed mixed chromosome X genomic scaffold (NW_003301797). This suggests that the porcine *Tex11* is an X-linked gene, which is consistent with previous findings for human and mouse *Tex11* homologs.

The expression profile of the porcine *Tex11* gene. To confirm that porcine *Tex11* is a testis-specific gene, we performed RT-PCR on various pig tissues. *Tex11* was only detected in the mature porcine testis, not in adult somatic tissues such as lung, brain, heart, liver, spleen, skeletal muscle and kidney (Fig. 3). This expression pattern of porcine *Tex11* confirmed that *Tex11* is indeed a testis-specific gene.

The *Tex11* expression is also age-dependent. While expressed in the mature testis, the transcript was not detectable in the 2-week and 10-week old pig testes (data not shown). The absence or low expression of *Tex11* in the pre-pubertal testis was also confirmed by western-blot. TEX11 protein was present in the cell lysate from the adult testis, but undetectable in the 10-week old testis lysate (Fig. 4). Interestingly, two distinct bands were picked up by the TEX11 antibodies we used. The lower band is slightly over 100 KD, similar to the mouse TEX11 protein with a predicted size of 109 KD. It is unknown whether the upper band represents a TEX11 with post-translational modifications or a distinct isoform. Given that both human and mouse *Tex11* have two isoforms of various sizes (NCBI database, www.ncbi.nlm.nih.gov/), we cannot exclude the possibility of the presence of two porcine *Tex11* isoforms.

The cellular localization of the porcine TEX11 protein. To identify the cell-type specific expression of TEX11, we performed immunofluorescent staining on mature pig testis sections (Fig. 5).

Similar to the mouse TEX11, porcine TEX11 was highly expressed in spermatocytes (Fig. 5a and b). Round spermatids were also immune-reactive, albeit with a decreased staining intensity, which is in line with previous findings indicating the presence of TEX11 in spermatids.⁴ No somatic cells in the testis reacted with TEX11 antibodies, suggesting that TEX11 is also germ cell specific in the pig. In the 10-week old testis, TEX11 was barely detectable (Fig. 4). The cellular localization of TEX11 is cytoplasmic in all three positive cell types.

Discussion

In this study, we cloned a segment of the porcine *Tex11* gene and showed that the porcine *Tex11* is an X-linked gene that is exclusively expressed in germ cells in the mature pig testis. The partial sequence of porcine *Tex11* shares a high degree of sequence identity with human and mouse homologs. The sequence conservation, combined with the nearly identical expression pattern of *Tex11* between pig and mouse, suggested that *Tex11* is likely highly conserved across species. The gene prediction algorithm used by the NCBI database predicted *Tex11* homologs in a few other species such as rat, cow, dog, chicken and horse. However, experimental data are required to validate the computer-based predictions.

Tex11 is a testis-specific transcript that is not detectable in somatic tissues investigated in this study. In the testis, expression of TEX11 is correlated with the onset of spermatogenesis and is restricted to spermatocytes and round spermatids. TEX11 was not detected at the ages of 2 weeks and 10 weeks. At 2 weeks of age, pig germ cells are at the gonocyte stage and are surrounded by immature Sertoli cells in the primitive seminiferous cords. By 10 weeks, gonocytes have differentiated into spermatogonia, but meiosis has not been initiated yet. The lack of TEX11 expression at these early stages suggested that the expression is likely correlated with the onset of meiosis. However, we cannot exclude the possibility that the expression level of porcine TEX11 in gonocytes and spermatogonia may be too low to be detected by the experimental conditions currently used. Stage-specific expression of porcine TEX 11 in meiotic germ cells is in line with observations in the mouse that the lack of *Tex11* disrupted the progression of meiosis and resulted in infertility.⁶ In *Tex11* null mice, meiotic arrest occurred through stage-specific germ cell loss at stage XII.^{5,6}

Cellular localization of mouse TEX11 in spermatocytes has been reported in references 5 and 6. In one study⁶ rabbit-anti-mouse and guinea pig-anti-mouse TEX11 antibodies were used to show TEX11 formed arrays of discrete foci along synaptonemal complexes at the zygotene and early pachytene stages. In the other study⁵ goat-anti-mouse and rat-anti-mouse TEX11 antibodies were used, yet discrete foci of TEX11 localization along synaptonemal complexes in spermatocyte were not detected. In both studies, different C-terminal sequences of mouse TEX11 were used as antigen. The reason for the discrepancy in the localization of mouse TEX11 in spermatocytes between these two studies is unknown. In the current study, a rabbit-anti-rat TEX11 antibody generated against a synthetic peptide was used

pig	-----AGAACGAATCCAGTGTTC-CACA-CATTCTGTGGAAAAAGCTGCCAGAAG	49
human	ACTGACAGCAGAATCAATGAAGTGGTTACACAACATTCTGTGGAGACAAGCTGCCAGTAG	60
mouse	ACTGTCAAGAGATTTGGTAAATTGGTTACACAACATTCTGTGGGGAAGGCTTCCAGAAG	60
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pig	TTTTGAGGTACAAAATATGCTGATGCTCTACACTGGTACTATTATTCTCTGAGACTTTA	109
human	TTTTGAGGTACAAAATTACACTGATGCCCTACAATGGTACTATTATTCTCTGAGGTTT	120
mouse	TGTTAAGGTCCAAAAATATGCTGATGCCCTACACTGGTACAGTTATCTCTGAAGTTGTA	120
	* * * * * *	
pig	TGCATCTGATCAAACGGATCTGGACTTGGCCAAGCTGCAGAGGAACATGGCTTCTTGTTA	169
human	TTCAACTGATGAAATGGATCTGGACTTCACCAAGCTGCAGAGGAACATGGCTTGTGTGTA	180
mouse	TGAGTATGATAAAGCAGATCTGGATTTGATCAAGCTGAAGAGGAACATGGTTTCTTGTTA	180
	* * * * * *	
pig	CCTGCATTTGGAACAATTTGATAAGGCTAAAGAAGCAGTGACAGAAGCAGAACGATGTGA	229
human	CCTGAATTTGCAACAACCTTGATAAGGCCAAAGAGGCAGTGGCAGAAGCTGAACGACATGA	240
mouse	CTTATCTTTGAAACAACCTTGATAAGGCTAAAGAGGCCATAGCAGAAGTTGAGCAAAAGGA	240
	* * * * * *	
pig	CCCTACAAATATTTTCACTCAATTTTATGTATTCAAGATTGCAGTCCTAGAAGGCAGCTC	289
human	CCCTAGGAACGTTTTCCTCAATTTTATATATTCAAGATTGCAGTCATAGAGGGCACTC	300
mouse	TCCTACACATGTTTTCCTCGGTATTATATATTCAAGATCGCAATCATGGAGGGTGATGC	300
	*** * * * * *	
pig	TGACAGAGCTTTGCAAGCAATTACTGCTTTAGAGAATATACTAACAACCTACAGAACAAGA	349
human	TGAAAGAGCTTTGCAGGCAATAATTACTTTAGAGAATATATTAACAGATGAAGAGTCAGA	360
mouse	TTTCAGAGCTTTACAGGTGGTCAGTGTCTTAAAGAAATCATTAAATGGATGGAGAATCAGA	360
	* * * * * *	
pig	AGAGAATGATGTACTTACAGATAGAGGTTCTGCTGTATGGTCTTACTTTAGCTGCCCA	409
human	AGATAATGATCTAGTTGCAGAGAGAGGTTACCTACCATGCTTCTAAGTTTAGCTGCCCA	420
mouse	AGATCGTGGACTAATTGAAGCTGGAGTTTCAACTCTCACAATCCTAAGTTTATCTATAGA	420
	*** * * * * *	
pig	GTTTGCTCTAGAGAATGAACACAAATTTGTGGCAGGAAAAGCTTTGGAATGTTTAGCTCA	469
human	GTTTGCTCTAGAGAATGGACAACAAATTTGTGGCAGAAAAGCTTTGGAATATTTAGCTCA	480
mouse	TTTTGCTCTAGAGAATGGACAGCAATTTGTGGCAGAAAGAGCTTTGGAATATTTATGTCA	480

pig	GTATTGAGAAGACCCACAACAAGTCCTTACAGCTTTAAAGTGTTTGTGTCGTCTTATTCT	529
human	ACATTGAGAAGACCAGGAACAAGTTCTTACAGCTGTAAAGTGTTTGTGCTTCTTCTCT	540
mouse	ACTTTCAAAGACCCAAAAGAAGTACTTGGAGGTTTAAAGTGTCCTCATGCGGATTATTCT	540
	*** * * * * *	
pig	TCCAGGAGTTTCTCAAATGACAGACTCTGAAAACAAAAAGAAGGAAATGGATAGACTTTT	589
human	TCCAAAAATTGCTGAAATGCCGGAATCTGAAGATAAGAAGAAAGAAATGGATCGACTTTT	600
mouse	TCCACAAGCTTTTCATATGCCAGAATCTGAATATAAAAAGAAAGAAATGGGTAGACTTTG	600
	*** * * * * *	
pig	GACTTGCTTAAATACAGCACTCCTGAAACTTGGTCAGTGTGTTTATAGAAGAGCTTTGCC	649
human	GACTTGCTTGAATAGAGCCTTTGTGAAACTTTCTCAGCCTTTTGGTGAAGAAGCCTTAAG	660
mouse	GAACTACTTGAATACAGCACTCCTGAAATTTTCTGAATATTTAATGAAGCTCCCTCAAC	660
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pig	TTCAGACTCAAGGACTAATGAAGCTCATTTGGTTTCGAAAAATAGCTTGGAACTTGGCTGT	709
human	TTTGGAGTCAAGAGCTAATGAAGCTCAGTGGTTTCGAAAAACAGCTTGGAACTTGGCTGT	720
mouse	TTTGGATTATATGGTTAATGATGCCAATTGGTTTCAGGAAAAATAGCTTGGAACTTAGCTGT	720
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Figure 2. Multiple alignments of the partial porcine *Tex11* sequence with the corresponding mouse and human *Tex11* sequences.

Figure 3. The expression profile of *Tex11* in pig tissues. The porcine *Tex11* is specifically expressed in the adult pig testis. It is not detected in somatic tissues examined in this study. The size of the PCR product for porcine *Tex11* and *Gapdh* is 1,028 bp and 983 bp, respectively.

and specific localization of TEX11 to synaptonemal complexes was not apparent at the level of detail analyzed. It is also possible that post-translational modifications are important for chromosome localization of TEX11 and that antibodies used in the current study were not able to recognize modified form(s) of TEX11. Commercial antibodies against human TEX11 have more recently become available, yet cross-reactivity with porcine TEX11 or localization in spermatocytes has not yet been reported for those antibodies.

In *Tex11* null mice, the loss-of-function of TEX11 resulted in arrest of meiosis and elimination of spermatocytes.⁶ This precludes the analysis of TEX11 functions in round spermatids. The only known domain in TEX11 is a tetratricopeptide repeat (TPR). TPR family members are involved in a variety of biological processes such as organelle fission, protein transport, protein folding, chaperone binding and establishment of protein localization (<http://supfam.org/SUPERFAMILY/cgi-bin/scop.cgi?sunid=48453b>). Given that TEX11 interacts with components of chromosome/DNA-binding protein complexes (such as SYCP2 in synaptonemal complex and NBS1 in MRN complex), it is possible that TEX11 is involved in chromatin packaging during spermiogenesis.

In summary, the expression pattern of *Tex11* is highly conserved between rodents and higher mammals such as the pig, adding to our understanding of the control of spermatogenesis in non-rodent animal models.

Materials and Methods

RNA isolation and RT-PCR. Total RNA was isolated from the testes of 2 week-old, 10 week-old and adult pigs (>12 months of age), as well as from adult somatic tissues (lung, brain, heart, liver, spleen, stomach, skeletal muscle and kidney). RNA isolation was performed using the RNeasy kit (Qiagen, catalog number: 74104) according to the manufacturer's instructions. For RT-PCR, genomic DNA was removed by DNase digestion using the RNase-free DNase kit (Qiagen, catalog number: 79254) and the first-strand cDNA synthesis was performed using the RT-for-PCR kit (Clontech Laboratories, catalog number: 639505). *Gapdh* was used as an internal control to ascertain the quality of cDNA synthesized. The primers used for *Gapdh* (NM_002046.3) amplification are: forward primer 5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3' and reverse primer

Figure 4. TEX11 protein is detected in the mature pig testis. TEX11 protein was only detected in the adult pig testis where complete spermatogenesis occurs. In the 10 week old testis, the only germ cells present were spermatogonia and TEX11 was undetectable. The estimated size of porcine TEX11 is slightly over 100 kD, which is similar to the size of homologs reported in mice and humans.

5'-CAT GTG GGC CAT GAG GTC CAC CAC-3'. To amplify the porcine homolog of *Tex11*, primers (forward 5'-GCT CAC CAA ACA GGA AGA CA-3', Reverse 5'-AAC TCC CAC ACT GAT TCC AG-3') for PCR were designed based on the homologous regions of the *Tex-11* coding sequences of between the mouse (NM_031384.2) and human (NM_001003811.1). PCR conditions were used as follow: 30 seconds at 94°C, 45 seconds at 60°C and 2 minutes at 72°C each cycle for 25 cycles.

Cloning of the *Tex11* sequence for sequencing. The RT-PCR product of *Tex11* was cloned into the pET28a vector (Novagen, EMD catalog number: 69864-3). BamHI and SalI restriction sites were incorporated into the PCR primers to clone the *Tex11* fragment into the multiple cloning site of pET28a.

Immunofluorescent staining on frozen sections. Testicular tissues were collected from 10 week-old and mature boars, and frozen sections were prepared as follow: Blocks of tissues of ~2 mm thickness were fixed overnight at 4°C in 2% paraformaldehyde. Tissue blocks were then dehydrated in 10% sucrose followed by 30% sucrose at 4°C for 4 hours each prior to snap freezing. Freezing was performed by coating tissues with the Tissue-Tek OCT embedding medium (Fisher, catalog number: 14-373-65) and submerging in 2-methylbutane chilled in liquid nitrogen to approximately -50°C. Sections were cut on a Leitz cryostat.

Immunofluorescent staining was performed using routine procedures. Briefly, sections were washed with three changes of Phosphate Buffered Saline (PBS) buffer, blocked in PBS containing 0.1 M glycine for 15 minutes, followed by an additional blocking step in 3% preimmune donkey serum for 15 minutes. Incubation with rabbit anti-rat TEX11 antiserum (generously provided by Dr. F. Kent Hamra, University of Texas Southwestern Medical Center) was carried out at a 1:500 dilution overnight at 4°C. The antibodies were raised in rabbits to a synthetic peptide with rat Tex11 sequences deduced from cDNAs amplified and cloned from rat spermatogonial RNA. The antibodies have been tested on frozen rat testis sections (26 days old) and selectively labelled spermatocytes (Hamra FK, personal communication). After three changes of PBS washes, sections were incubated for one hour at room temperature with Alexafluor 488 conjugated donkey anti-rabbit IgG antiserum (Invitrogen Molecular Probes, catalog number: A-21206) at a 1:500 dilution. Sections were washed again with three changes of PBS and mounted in Vectashield mounting medium with DAPI for examination and imaging (Vector Laboratories, catalog number: H-1200). Control sections were treated non-immunized serum in place of

Figure 5. The cell-type specific expression of TEX11 in the porcine testis. TEX11 is highly expressed in spermatocytes in mature (>12 months of age) porcine testis (a). The expression pattern is very similar to that of the mouse TEX11 in mature mouse testis (b). Spermatocytes are labelled with white arrows. Stages of spermatogenesis are indicated with Roman numerals. Scale bar: 10 μ m.

the primary antibody. Images were captured with a Leica microscope (Leica Microsystems Inc.).

Western blot for TEX11. Testicular tissues from 10-week-old and adult boars were homogenized in RIPA buffer with the presence of protease inhibitor cocktail (Sigma, catalog number: P2714). 30 μ g of cleared lysates from each sample was loaded onto a precast linear gradient polyacrylamide gel (4%–15%, Bio-Rad, catalog number: 456-1083EDU) for separation. Proteins were then transferred onto an Immun-Blot PVDF membrane (Bio-Rad, catalog number: 162-0177) and probed with rabbit anti-rat TEX11 polyclonal antibodies (kindly provided by Dr. Kent Hamra) at a

dilution of 1:500. HRP conjugated donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch, catalog number: 711-035-152) were used at a dilution of 1:5,000. Blots were developed using Amersham ECL western blotting detection reagents and hyperfilm (GE Healthcare, catalog number: RPN2109 and 28-9068-35).

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