Expression analysis of genes encoding TEX11, TEX12, TEX14 and TEX15 in testis tissues of men with non-obstructive azoospermia

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ABSTRACT

Objective: Spermatogenesis is a complex process controlled by a plethora of genes. Changes in expression and function of these genes may thus lead to spermatogenic deficiency and male infertility. TEX11, TEX12, TEX14 and TEX15 are germ cell-specific genes expressed in the testis. TEX11, involved in the initiation and maintenance of chromosome synapsis in meiotic chromosomes, has been shown to be essential for meiosis and fertility in males. TEX14, a component of intercellular bridges in germ cells, is required for spermatogenesis and fertility. TEX12 and TEX15 are essential for correct assembly of the synaptonemal complex and thus meiosis progression.

Methods: In order to examine whether changes in expression of these genes is associated with impaired spermatogenesis, expression levels of these genes were quantified by RT-qPCR on samples retrieved from infertile patients submitted to diagnostic testicular biopsy at Royan institute. Samples were divided into two groups of 18 patients with non-obstructive azoospermia considered as case; nine patients with obstructive azoospermia were included in the control group.

Results: A significant down-regulation of these genes was observed in the SCOS group when compared to the control group.

Conclusion: This result suggests that regular expression of TEX11, TEX12, TEX14 and TEX15 is essential for the early stages of spermatogenesis.

Keywords: Male infertility, Gene expression, Non-Obstructive Azoospermia, Intercellular bridges, Synaptonemal complex

INTRODUCTION

Male-factor infertility apparently accounts for 40% to 50% of infertile complications and may be defined by environmental reasons, infections, immunological or hormonal insufficiencies, while many are regulated by genetic factors (Hirsch, 2003; Brugh & Lishultz, 2004; Boyle et al., 1992; Ma et al., 2016). Spermatogenesis is a complex process controlled by thousands of genes, where any change in the expression or function of these genes may lead to spermatogenic failure and male infertility (Zhou et al., 2009; Westerveld, 2008). Identification of stage-specific genes controlling spermatogenesis is thus important.

Previous studies have revealed that TEX11, TEX12, TEX14 and TEX15 expression is restricted to germ cells and is not detectable in somatic tissues of humans and mice (Wang et al., 2001; Adelman & Petrini, 2008). Multiple studies have shown that X-linked germ cell-specific genes such as testis-expressed gene 11 (Tex11) have significant roles in regulating male fertility (Zheng et al., 2010; Matzuk & Lamb, 2008). Furthermore, TEX11 expression has been associated with the onset of spermatogenesis, although restricted to spermatocytes and round spermatids (Wang et al., 2005; Tang et al., 2011).

The synaptonemal complex (SC) is a large protein structure needed for synopsis and the successful completion of meiotic cell division. Incorrect assembly of this complex in mice results in maturation arrest and infertility (Bolcun-Filas et al., 2009; Hamer et al., 2008; Kouznetsova et al., 2011), and may similarly lead to infertility, recurrent miscarriage and aneuploidies such as Down syndrome in humans (de Vries et al., 2005; Gerton & Hawley, 2005; Page & Hawley, 2003). Tex11, Tex12 and Tex15 are needed in chromosomal synopsis and meiotic recombination. More specifically, Tex11 forms distinct foci on homologous chromosomes that synapse with each other and seem to be a new component of meiotic nodules needed for recombination. In the absence of this protein, chromosomal synopsis stops and crossover formation is reduced (Stouffs & Lissens, 2012; Yang et al., 2008b). Tex12 is a meiosis-specific protein essential for the progression of synopsis between homologous chromosomes in male and female germ cells (Hamer et al., 2008). Loss of Tex15 leads to meiotic recombination failure, since this protein is responsible for the transfer of DNA repair proteins onto double strand break (DSB) locations (Yang et al., 2008a). Tex12 is small with no known domains, but has orthologs in other mammals such as mice, cows, and dogs (Hamer et al., 2006; Wang et al., 2001). Tex15 has orthologs in mammals and zebra fish (Yang et al., 2008a). It is abundantly expressed in post-meiotic germ cells, spermatogonia, and early spermatocytes, showing that this gene plays a role in different stages of spermatogenesis (Yang et al., 2008a; Wang et al., 2005). Interestingly, it is expressed in both the testis and ovaries, as is its mouse ortholog (Wang et al., 2001).

Germ cell intercellular bridges are required for fertility in invertebrates (Brill et al., 2000; Robinson et al., 1994; Robinson & Cooley, 1996) and have been preserved from invertebrates to humans (Fawcett et al., 1959), indicating their importance in fertility (Greenbaum et al., 2006;
Mammalian intercellular bridges connect hundreds of germ cells in syncytia originated from a single spermatogonial stem cell (Huckins, 1971; Greenbaum et al., 2006; 2009). In the absence of cell bridges in mammalians, syncytium is thus not formed and as a result spermatogenesis is impaired (Greenbaum et al., 2006; 2009). TEX14 is needed to generate the germ cell intercellular bridges from the midbody (Greenbaum et al., 2006). Human TEX14 is preferentially expressed in the testis with its highest levels observed in spermatocytes and early round spermatids (Wu et al., 2003). Greenbaum et al. (2006) showed that Tex14/-/- mutant male mice do not form intercellular bridges, thus confirming its essentiality in mammalian spermatogenesis. It was later revealed that Tex14 is also essential for complete spermatogenesis in Finnish Yorkshire boars as in mice (Sironen et al., 2011). Chung et al. (2013) recognized new loci showing association with development of testicular germ cell cancer (TGCT), one of which (rs9905704) located in TEX14. It has also been shown that common genetic variations in TEX14, which is over-expressed in breast tumors, is associated with risk of breast cancer in Caucasians (Kelemen et al., 2009). Recent studies showed that although Tex11-/-, Tex14-/- and Tex15-/- mutant male mice were sterile because of meiotic arrest and disturbance of spermatogenesis, mutant females were fertile. They suggested that the infertility observed in mutant male mice was caused by loss of function of these genes and that Tex11, Tex14 and Tex15 were needed for meiosis progression and fertility only in male mice (Yang et al., 2008a; Greenbaum et al., 2006; Sironen et al., 2011). However, TEX12 has identical localization patterns in oocytes and spermatocytes, revealing that this protein is a common part of the central element of the synaptonemal complex in mammals and is not sex-specific (Hamer et al., 2006). Tex12-/- mutant male mice were azoospermic and therefore sterile because of failure in chromosomal synapsis progression, while Tex12-/- female mice were sterile because of loss of ovarian follicles (Hamer et al., 2008).

The homology of amino acid sequences and expression patterns of the human and murine TEX11, TEX12, TEX14 and TEX15 genes (Wang et al., 2001; Zheng et al., 2010; Tang et al., 2011; Stouffs et al., 2009; Greenbaum et al., 2006; Wu et al., 2003; Hamer et al., 2006; Yang et al., 2008b) suggests that these genes may play some a few in human spermatogenesis. Given that deficiency of these genes results in azoospermic mice (Zheng et al., 2010; Greenbaum et al., 2006; Wu et al., 2003; Hamer et al., 2006; Yang et al., 2008a), severe spermatogenic failure may thus be caused by their disruption. Therefore, this study aimed to replicate this finding in humans by examining the expression levels of TEX11, TEX12, TEX14, and TEX15 in the testis tissue of patients with non-obstructiveazoospermia and comparing them against controls.

**MATERIAL AND METHODS**

**Patients**

Twenty-seven tissue samples were obtained from 18 patients with non-obstructiveazoospermia (NOA) and nine subjects with obstructiveazoospermia. All participating infertile men had undergone testicular sperm extraction (TESE) procedures at the Royan Institute. All patients gave written informed consent and the Ethical Review Board of the Royan Institute approved the study (reference number EC/90/1050).

Nine of the 18 patients with NOA were diagnosed with Sertoli cell-only syndromes (SCOS), while the other nine were diagnosed with maturation arrest at the spermatocyte stage. The control group consisted of nine patients with obstructiveazoospermia. Patients with abnormal karyotypes and Y chromosome microdeletions were excluded from the study.

The age range of the patients at the time of diagnosis was 30 to 50 years and their mean age was 36.9±4.39 years. Hormonal levels were measured using a competitive ELISA kit (Monobind, CA, USA) according to the procedure described by Zangeneh et al. (2015). Cytogenetic analysis was performed based on standard methods (Asia et al., 2014).

**Histological evaluation**

After TESE, a portion of the testicular samples was submerged in Bouin’s solution and sent for standard histopathological analysis (McLachlan et al., 2007). Residual tissues were collected by the Royan Tissue Bank. Testicular histopathology was categorized according to the most recognized pattern of spermatogenesis process: samples with complete spermatogenesis (obstructive-azoosperma), samples with spermatogenic maturation arrest at the spermatocyte stage or SCOS.

**RNA extraction**

Total RNA from frozen testis tissue was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Approximately 50-100 mg of testis tissue was pipetted into one mL of TRIzol. RNA was detached with chloroform, precipitated with isopropanol, washed with 75% ethanol, and finally dissolved in DEPC treated water (Li et al., 2006).

The concentration of RNA samples was determined spectrophotometrically by measuring absorption at 260 nm (Li et al., 2006), while DNA and protein contamination were checked by optical density (OD) measurements at 260/280 nm.

The integrity of total RNA was evaluated by measuring 260:280 nm absorption ratios and by gel electrophoresis on 1.2% agarose gel (Yu et al., 2007; Tang et al., 2006).

**DNase treatment and cDNA synthesis**

Before total RNA reverse transcription (RT), the samples were treated with DNase to eliminate DNA contamination using the DNase I (RNase free) kit (Fermentas, Life Sciences, UK). Total RNA (2 µg) was reverse-transcribed into cDNA in a reaction primed with random hexamer primers using the RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas, Life Sciences, UK) according to the manufacturer's recommendations. Polymerase chain reaction (PCR) primers for TEX11, TEX12, TEX14, and TEX15 were designed using PerlPrimer v1.1.20 (Table 1).

After RNA extraction from testis tissue samples and cDNA synthesis, RT-PCR was performed with the related primers to confirm their expression in the samples of the three groups.

**Quantitative RT-PCR (RT-qPCR)**

RT-qPCR was carried out to confirm and analyze the expression levels of target genes in the SCOS, MA, and control groups. The reactions were processed on a 7500 Real Time PCR machine (Applied Biosystems, Carlsbad, CA, USA) using the Power SYBR Green PCR master mix (Applied Biosystem, EU). The amplification solution contained 10 µl of Power SYBR Green PCR master mix, 50 ng of cDNA and 5 pmolmes of each primer, yielding a final volume of 20 µl. Cycling conditions included an initial step of enzyme activation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 62°C for 30 seconds, and extension at 72°C for 30 seconds. Transcript levels normalized to human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) showed minimum variation among individual samples. GAPDH was used as an internal
positive control. Each sample was run in duplicate and the mean value was calculated. No primer-dimer formation was observed during PCR amplification.

**Immunohistochemistry**
The expression of **TEX14** was analyzed in the SO/H (n=3), SCOS (n=3), and MA (n=3) groups by immunohistochemistry. First, testis tissue sections were deparaffinized in xylene washes and then rehydrated in descending concentrations of ethanol washes. Endogenous peroxidase activity was blocked for 1 h at 37°C with goat serum (Sigma-Aldrich, St Louis, MO, USA), and triton X 100 was used to permeabilize cell membranes. Primary (**Tex14** Antibody, NB1-85424 Novus, Biologicals USA, 1:100) and secondary antibodies (goat anti-rabbit HRP, ab97051, 1:100) were added and protein localization was visualized using a Dako Liquid DAB+ Substrate Chromogen System (Dako, Glostrup, Denmark). Finally, cells were imaged using an Olympus IX 71 microscope (Julaton & Pera, 2011). Negative controls were generated by the same method as the positive controls; however, the primary antibody was replaced with PBS solution.

**Statistical analysis**
Data on clinical characteristics were shown as mean ± SEM. The normality of variables was analyzed with the Kolmogorov test. Differences in the mean values among the three groups were analyzed by one-way analysis of variance (ANOVA). The tests cited above were performed using SPSS statistical software package (SPSS Inc, Chicago, IL, USA) version 22.0. Real-time data were processed and analyzed using a two-tailed t-test. Differences with p-values <0.05 were considered significant.

**RESULTS**

**Patient clinical characteristics**
The characteristics of patients including age and LH, FSH, and testosterone levels are listed in Table 2. There was no significant difference in age, LH or testosterone serum levels between the three groups; however, the FSH serum levels between these groups were significantly different (p=0.01).

**Gene expression analyses**
The RT-qPCR results demonstrated that transcripts of all four **TEX** genes existed in all samples of the SCOS, MA, and control groups. Differential expression analysis showed that expression of **TEX** genes is significantly lower in SCOS testes samples when compared with controls (p<0.01) (Figures 1-4). The expression of **TEX11** and **TEX14** was also significantly lower in MA samples compared with controls (p≤0.038) (Figures 1,3); however, expression of **TEX12** and **TEX15** was not significantly different (**TEX12,15 p>0.05**) (Figures 2,4). Expression levels of **TEX12, TEX14** and **TEX15** in SCOS and MA samples were not significantly different (**TEX12, 14,15 p>0.05**) (Figures 2,3, and 4); however, **TEX11** expression was significantly lower in SCOS samples (p=0.003) (Figure 1).

**DISCUSSION**
The same expression pattern of **TEX11, TEX12, TEX14** and **TEX15** genes in mice and humans indicates that these conserved genes may have an important role in the early stages of mammalian spermatogenesis (**Zheng et al., 2010; Tang et al., 2011; Stouffs et al., 2009; Greenbaum et al., 2006; Wu et al., 2003; Hamer et al., 2006; Yang et al., 2008a**). Although the function of these genes in humans is not well-known, their roles in the progression of mouse spermatogenesis (**Zheng et al., 2010; Greenbaum et al., 2006; Wu et al., 2003; Hamer et al., 2006; Yang et al., 2008b**) led us to hypothesize that they were likely to have the same roles in humans. To test this hypothesis, we compared the expression levels of these genes in testicular tissue samples of infertile men with two distinct histological patterns of spermatogenic failure (MA and SCOS) - our case group - to the levels observed in a control group including men with complete spermatogenesis (obstructive azoospermia).

Given that there was no germ cell in the testicular tissue samples of SCOS patients and all four **TEX** genes are germ cell-specific, down-regulation of these genes was expected. This observation is in line with the findings in mice, in which the lack of these genes disrupts the development of meiosis, resulting in male infertility (**Zheng et al., 2010; Greenbaum et al., 2006; Wu et al., 2003; Hamer et al., 2006; Yang et al., 2008b**). The similar expression pattern and sequence conservation of **TEX11** seen in mice, humans, and pigs indicate that this gene is highly conserved and may have an important role in mammalian testicular function, including spermatogenesis (**Zheng et al., 2010; Tang et al., 2011; Stouffs et al., 2009**). **TEX11** expression in testis is correlated with the onset of spermatogenesis and is restricted to spermatocytes and round spermatids (**Tang et al., 2011**). Stage-specific expression of **TEX11**
Porcine meiotic germ cells is in agreement with findings in mice, where the lack of Tex11 disturbs the progression of meiosis and thus results in infertility. The loss of function of Tex11 in Tex11-null mice resulted in meiotic arrest and deletion of spermatocytes (Yang et al., 2008b). In summary, the expression pattern of Tex11 is highly preserved in rodents and higher mammals such as pigs and humans (Tang et al., 2011).

Yang et al. (2015) demonstrated that the frequency of rare TEX11 mutations is significantly higher in azoospermic men, suggesting that TEX11 is essential for human spermatogenesis and mutations in this single X-linked gene is the cause of infertility in ~1% of azoospermic men. The authors also reported that the level of TEX11 protein must be above a critical threshold for meiosis to progress, and that low-expressing TEX11 alleles may thus result in human male infertility. Yatsenko et al. (2015) hypothesized that mutations in human TEX11 disrupt the formation and function of the synaptonemal complex, resulting in disturbance of pachytene synapsis, meiotic arrest, and azoospermia. The authors reported that hemizygous TEX11 mutations were a common cause of meiotic arrest and azoospermia in infertile men.

Our results showed that TEX11 expression was significantly decreased in MA patients when compared to controls. As dysfunction of TEX11 results in meiotic arrest and sterility in mice (Adelman & Petrini, 2008; Yang et al., 2008b; Stouffs et al., 2009), impairment of spermatogenesis and maturation arrest in this group may be linked to TEX11 down-regulation. This down-regulation was significantly more pronounced in SCOS patients than in patients with MA. Our results confirmed the involvement of TEX11 in human spermatogenesis. Furthermore, our findings indicated that expression of this gene is required for the completion of spermatogenesis.

### Table 2. Clinical characteristics of patient groups. Values are expressed as mean±SEM

| Patients groups                          | Age (years) | FSH (mIU/mL) | LH (mIU/mL) | Testosterone (ng/mL) |
|-----------------------------------------|-------------|--------------|-------------|----------------------|
| Maturation arrest (MA)                  | 37.28±3.28 (30-50) | 5.62±1.51    | 4.32±1.26   | 5.28±0.84            |
| Sertoli-cell-only syndrome (SCOS)       | 37.33±1.20 (33-42) | 18.57±3.90   | 3.73±1.82   | 3.27±0.59            |
| Control                                 | 36.33±1.24 (30-42) | 8.37±1.73    | 3.70±0.87   | 5.61±3.57            |

*p-value 0.924 0.010* 0.941 0.237

Values are expressed as mean±SEM. *Significant difference based on ANOVA.

Normal range of FSH: 1.5-12.4 mIU/mL
Normal range of LH: 1.0-10.0 mIU/mL
Normal range of Testosterone: 2.0-8.0 ng/mL
Tex12 expression is limited to meiotic cell division and its promoter, like many other germ cell-specific genes, is quenched in somatic cells by transcription factor E2F6 (Pohlers et al., 2005). Previous studies showed that TEX12 localizes to the central element of the synaptonemal complex. Their results also revealed that without TEX12, the synaptonemal complex lacks a correct central element structure, synopsis cannot complete, and meiotic recombination and crossing-over do not occur (Hamer et al., 2006). Since loss of TEX12 results in the inaccurate assembly of the synaptonemal complex and non-chromosomal synopsis (Hamer et al., 2006), reduced expression of this gene in the MA group in relation to the control group was expected; however, our results did not reveal a significant difference in expression between these two groups.

Previous studies of Tex15 in mice showed this gene is necessary for chromosomal synopsis, DSB repair, and meiotic recombination during meiosis; therefore, TEX15 is thought of as a possible factor in spermatogenic failure risk (Yang et al., 2008a). Okutman et al. (2015) showed that a nonsense mutation in TEX15 is the cause of spermatogenic defect in familial cases of teratozoospermia and...
infertility. Immunohistochemistry confirmed these findings showing high levels of expression in germ cells and low levels of expression in Sertoli cells. The tests also revealed that Tex15 knock-out produced spermatogenesis meiotic arrest and severe reduction in testicular size. Therefore, we expected to observe down-regulation of Tex15 in the MA group when compared to controls. However, our results did not show any significant difference. This may possibly be due to high levels of expression in spermatogonia and early spermatocytes in the testicular tissue of MA patients. Previous studies have shown spermatogenic disruption in Tex14 knockout mice before the first meiotic division due to loss of intercellular bridges (Bolcun-Filas et al., 2009; Greenbaum et al., 2006; 2009). Moreover, a study on Tex14 mutant pigs showed that Tex14 is also essential for the completion of spermatogenesis in pigs (Sironen et al., 2011). Although Tex14 localizes to intercellular bridges of both female and male mice germ cells, disruption of this gene leads to sterility only in males (Brill et al., 2000; Greenbaum et al., 2006). The comparison of Tex14 expression between control and MA groups showed that Tex14 expression had a significant decrease in MA samples. As dysfunction of Tex14 results in meiotic arrest and sterility in mice (Greenbaum et al., 2006; 2009; Wu et al., 2003), impairment of spermatogenesis and maturation arrest may be linked to the down-regulation of Tex14. These results were confirmed by immunohistochemistry, with Tex14 expressed in type A spermatogonia, primary spermatocytes, and early round spermatids in control samples. Conversely, this protein was absent or hardly visible in type A spermatogonia, primary spermatocytes, and early round spermatids of MA samples. These results support the possibility that Tex14 under-expression in MA patients is etiologic, suggesting that the low expression of this protein in these patients is unlikely to be caused by the lack of germ cells.

CONCLUSION

This study demonstrated that Tex11, Tex12, Tex14 and Tex15 are essential for the completion of spermatogenesis. Down-regulation of these genes, especially Tex14 and Tex11, may lead to impaired spermatogenesis in infertile men. Studies on other independent sample sets are required to confirm this association.

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CONFLICTS OF INTEREST

None to declare

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