SPANDIDOS PUBLICATIONS

Involvement of ALF in human spermatogenesis and male infertility

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Abstract. We conducted this study to explore functions of TF II Aα/ß-like factor (ALF) during human spermatogenesis, and the relationship of its expression levels with male infertility. The RT-PCR and Western blot analyses illustrated that ALF was highly expressed in adult testis. Immunohistochemistry and immunoflurescence showed that ALF is located in the spermatid nuclei and in the annulus of spermatozoa. Further, to reveal whether ALF is related to male infertility, we performed the same experiments in infertility patients. The changes in the expression levels of ALF in the male infertility samples lead us to believe that ALF may function in spermatogenesis, especially in spermiogenesis. We also detected the ALF DNA methylation level by real-time methylation-specific PCR (MSP) both in testes of adult, fetal and infertile patient. The differential expression level of ALF gene in different types of testes was regulated by DNA methylation. Our research identified ALF as a human spermatogenesis related gene, the abnormal expression of ALF might be the partial cause for human infertility.

Introduction

Spermatogenesis can be subdivided into proliferative, meiotic and spermiogenesis phases (1). This highly ordered process requires a precise and well-coordinated program that regulates the constantly changing patterns of gene expression. As in all other cells, the ultimate regulation of gene expression in male germ cells occurs first at the level of transcription (2). Han and coworkers (3) determined unique gene expression patterns in testis for the general transcription factors (GTFs; TF II A, -B, -D, -E, -F, and -H) and for several GTF-related factors. For example, the TF II Aα/ß-like factor (ALF), TF II Aα/ß, TF II Aγ, TF II B, TATA-binding protein (TBP) and TBP-related factor (TRF) are expressed at higher levels in the testis than in any other tissue (4-8). These high levels of GTFs in the testis are required to express sufficient spermatogenesis-related genes for proper spermatogenesis regulation.

Among these transcription factors, ALF has recently received notable attention. Upadhyaya and coworkers (5) isolated human ALF and determined its exclusive expression in testis. Subsequently, ALF was found to be highly expressed in the elongated spermatids of mouse (3) and rat testes. Other studies verified expression of ALF in mouse and *Xenopus* ovaries (9,10), underscoring the importance of ALF in testis biology and spermatogenesis. However, none of these studies have extended the detection of ALF expression to its functionality in human testis (5,11).

In this report, we strove to determine whether ALF contributes to human spermatogenesis and if there is a relationship between ALF and human male infertility. For these studies we detected the expression and the distribution of the ALF gene in the testes and spermatozoa from fertile and infertile humans, respectively. The results showed that ALF is a spermatogenesis-related gene and moreover that its abnormal expression may contribute to our molecular understanding of male infertility.

Materials and methods

ALF gene cloning using cDNA microarrays. We cloned the full length of ALF from a human testicular cDNA library. The detailed protocol for the construction of a human testis cDNA microarray and sequence cloning was described previously (12).

Sample preparation. All participants signed the consent forms, and the approval to conduct this research was granted by the Ethics Committee of Nanjing Medical University. Three normal human testis specimens were obtained from healthy volunteers. One embryonic testis was obtained from an accidentally aborted 6-month fetus. The testes specimens of 16 infertile patients (7 spermatogenesis arrest, 7 spermatogenesis disturbance and 2 Sertoli cell-only syndrome) were obtained from clinical biopsies for pathologic diagnosis after informed consent. Human semen samples were collected for the experiments according to WHO criteria (13). In total, 12 normal semen samples and 20 teratospermia samples were processed. Detailed parameters of semen samples and the subjects are listed in Table I.

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Table I. Characteristics of the semen samples.

	Sperm parameters						
	Sperm concentration (10^6/ml)	Viability (%)	Motility (a, b, c %)	Morphology (% abnormal)	Total of sperm counted	No. of sperm with ALF	Missing rate of ALF (%)
N	73.25±24.73	64.08±2.06	35.4±2.5, 22.8±2.7, 5.7±1.6	<20	100.58±10.08	91.17±9.99	9.27±4.48
T	73.01±42.68	42.85±16.72	18.3±9.7, 15.5±7.0, 9.05±3.3	68.25±13.70	106.85±20.41	47.55±26.07	55.92±21.52

N, normal (n=12); T, teratospermia (n=20).

RNA extraction and RT-PCR. Total RNA samples from normal (adult and fetal) and 16 infertile human testes were isolated using TRIzol reagent (Invitrogen, Paris, France). Reverse transcription reactions were performed using random hexamers and SuperSuperscript II reverse transcriptase following the vendor protocols (Invitrogen). The PCR was performed using specific ALF primers (5'-GTGCCAGACCTGTTTGAC-3' and 5'-GCTCTGTGCTGGACTATATTC-3' as the sense and the anti-sense primers, respectively). Expression of ß-actin in each sample was performed as the positive control (forward primer was 5'-CGGTTGGCCTTGGGGTTCAGGGG-3'; reverse primer was 5'-ATCGTGGGGGCGCCCCAGGCAC CA-3'). Each PCR was performed as follows: 5 min at 95˚C followed by a total of 30 cycles of 30 sec at 95˚C, 30 sec at 55˚C and 1 min at 72˚C. Amplified products (257 bp for ALF, 265 bp for actin) were analyzed on 1.5% agarose gels.

Production of recombinant ALF protein and antibodies. Recombinant ALF was expressed as a His tag fusion protein (His-ALF) in *E. coli*. The open reading frame encoding human ALF was sub-cloned into the *Bam*HI-*Eco*RI sites of the PET28a expression vector (Amersham Biosciences, GE, USA) and used to transform *Escherichia coli* BL21 (DE3). After isopropyl-1-thio-ß-D-galactopyranoside (IPTG) was added to a final concentration of 1 mM, cells were collected and were resuspended in 200 ml of 20 mM Tris-HCl, 500 mM NaCl, 8 M urea buffer. The cells were sonicated and the clear supernatant was filtered through a 0.22 μm membrane and purified by AKTA Basic (Amersham Biosciences) using Hitrap Chelating 1 ml HP according to the manufacturer's instructions. The fractions containing pure recombinant protein were pooled and dialyzed against a linear decrease gradient of 6, 5, 4, 3, 2 M urea buffer, respectively. The polyclonal anti-ALF antibody was generated by immunizing Babl/c mouse with the purified recombinant ALF protein. Reactivity of preimmune and immune sera was determined using ELISA.

Western blot analysis. Protein extracts were prepared by treatment of normal testis tissues and semen samples with lysis buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (w/v) DTT] in the presence of a protease inhibitor cocktail (Pierce Biotechnology, Rockford, IL, USA). The concentration of the extracted protein was determined by Bradford micro-protein assay (14) using BSA as a protein standard. An aliquot of each sample and an internal control (50 μg protein/lane) were resolved by 12% SDS-PAGE and electro-transferred to nitrocellulose membranes. The membranes were incubated with mouse ALF antiserum (1:1000) diluted in blocking solution at 4˚C overnight and then with 1/1000 peroxidase conjugated goat anti-mouse IgG (Beijing Zhong Shan Biotechnology Co., China) for 1 h at 37˚C. Specific proteins were detected using an ECL kit and AlphaImager™ (Amersham Life Science). Molecular weights of detected sperm proteins were deduced by comparison with pre-stained recombinant molecular weight standards (New England BioLabs). Negative controls were performed by the replacement of the first antibody with non-immune mouse serum.

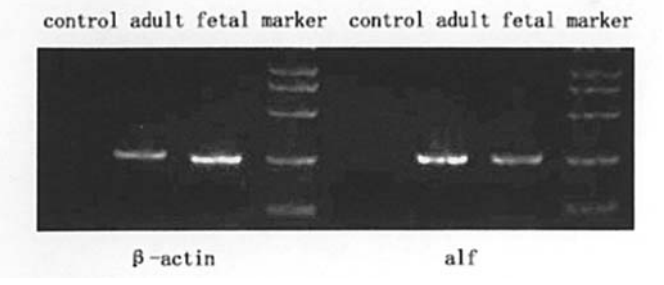

Figure 1. ALF mRNA expression in human adult and fetal testes shown with ß-actin mRNA expression as a positive control.

Immunohistochemistry. Formalin-fixed adult normal testis and the testes of 6 patients (5 spermatogenetic disturbance patients and 1 SCOS patient) were processed and mounted on silane-coated slides. Testis sections were blocked with 10% goat serum, 0.1% saponin in PBS for 1.5 h at room temperature and exposed to the primary polyclonal anti-ALF antibody (1:1000 diluted) overnight at 4˚C. After washing with 0.1% PBS, sections were incubated for 1.2 h with HRP conjugated

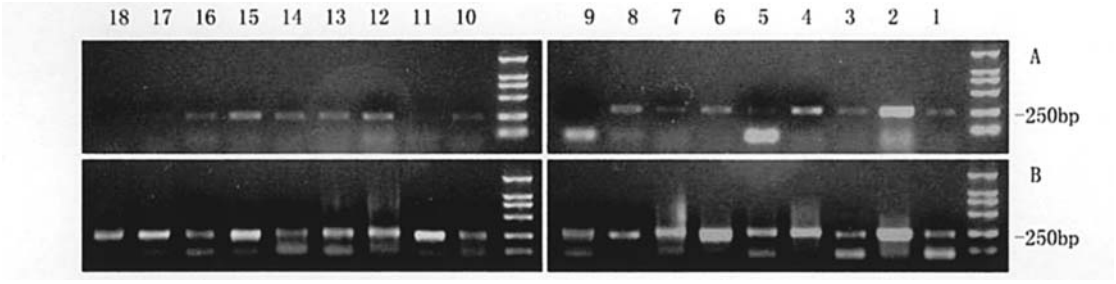

Figure 2. ALF mRNA expression in infertile patients (A) shown with ß-actin mRNA expression as positive control (B). Lanes 1 and 2, ALF expression in normal spermatozoa and adult human testis; lanes 3-9, spermatogenesis arrest samples; lanes 10-16, spermatogenesis disturbance. Note that ALF expression was reduced relative to that in normal testis; lanes 17 and 18, Sertoli cell-only syndrome. No ALF signal was detectable.

goat anti-mouse secondary antibody (Beijing Zhong Shan Biotechnology Co.). For staining, the sections were exposed to di-aminobenzamidine (DAB). Prior to inspection the sections were counterstained with hematoxylin. Control staining was performed appropriately using a diluted preimmune serum in the place of the primary antibody.

Real-time quantitative methylation-specific PCR. Genomic DNA was extracted and purified from human adult, fetal and infertile patient testes with Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Approximately 1-3 μg DNA was denatured in 3 M NaOH for 15 min at 37˚C. And then incubated in 3 M $NaHSO_3$ (pH 5.0) and 0.5 mM hydroquinone at 50˚C for 10-16 h. The sample was desalted with the Wizard DNA Clean-Up System (Promega), desulfonated in 0.3 M NaOH at room temperature for 5 min, then ethanol precipitated of the sodium-bisulfite-treated DNA. Real-time quantitative methylation-specific PCR (MSP) was performed with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The primers for detection of methylated sequence of ALF were 5'-TTTATATAGGTCGATCGTTTTTTCG-3' (forward) and 5'-ACCTAACCCTTTACGCCTACG-3' (reverse), and the unmethylated sequences were 5'-TTATATAGGTTGATT GTTTTTTTGT-3' (forward) and 5'-AACACCTAACCTTTA CACCTACAC-3' (reverse) according to Xie *et al* (15). The reaction mixture contained 1X SYBR Green PCR Master Mix (Applied Biosystems), 5 pmol each of methylated or unmethylated primers of ALF gene as described above, and 2 μl of DNA in a total volume of 20 μl. Each reaction (each sample was done three times to eliminate the difference) was performed as follows: 10 min at 95˚C followed by a total of 40 cycles of 30 sec at 95˚C, 30 sec at 56˚C, and 1 min at 72˚C. Following the PCRs, the Ct values for each sample of the three groups (adult, fetal and patient) were analyzed and compared with a two-sample t-test using SPSS software 12.5. Comparisons with P-values <0.05 were considered significant.

Immunofluorescence. Human ejaculate specimens were obtained from 12 normal and 20 teratospermia subjects, as described above. Freshly prepared, washed spermatozoa (washed with 0.01 M PBS, pH 7.2) were smeared on a microscope slide at a density of 30000 cells/cm^2, air-dried, fixed with 4% paraformaldehyde/PBS for 30 min, permeabilized with 0.2% Triton X-100/PBS for 20 min at 37˚C, and then blocked with goat serum (Beijing Zhong Shan Biotechnology Co.) for 2 h at room temperature. Then incubated with a 1:1000 dilution of mouse anti-ALF-serum overnight at 4˚C and with the secondary anti-mouse IgG labeled with FITC (Beijing Zhong Shan Biotechnology Co.) at 1:100 dilution for 1 h at room temperature. The slides were observed under a Zeiss fluorescent microscopy and recorded with a digital camera. Negative controls were performed by the replacement of the first antibody with non-immune mouse serum.

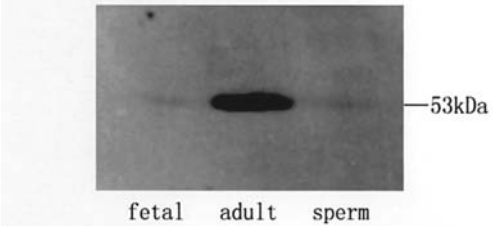

Figure 3. Western blot shows enrichment of ALF in adult testis tissue relative to fetal testis and spermatozoa.

Results

Differential expression patterns of ALF mRNA in normal and abnormal human testes. RT-PCR studies showed that ALF mRNA was present at a higher level in adult than in fetal testis (Fig. 1). Furthermore, ALF was not expressed or was expressed at low levels in the testes of 16 infertile patients (Fig. 2). In the testes of 2 SCOS patients, ALF was not detected. ALF was not expressed in the testis of 2 subjects among the 7 spermatogenesis disturbance and 7 spermatogenesis arrest patients, respectively. ALF showed lower expression in the testis samples from the other infertile patients compared with its expression in human adult testis.

Expression of ALF protein in human testis and spermatozoa. The ALF protein and mRNA expression levels were similar between the human adult and fetal testis. The target band was 53 kDa. We also detected lower level of ALF protein expression in human spermatozoa (Fig. 3).

ALF protein is expressed during the final stages of spermatogenesis. Immunohistochemistry studies showed that ALF immunoreactive protein was mostly expressed in the round and elongating spermatids in human adult testis. Moreover,

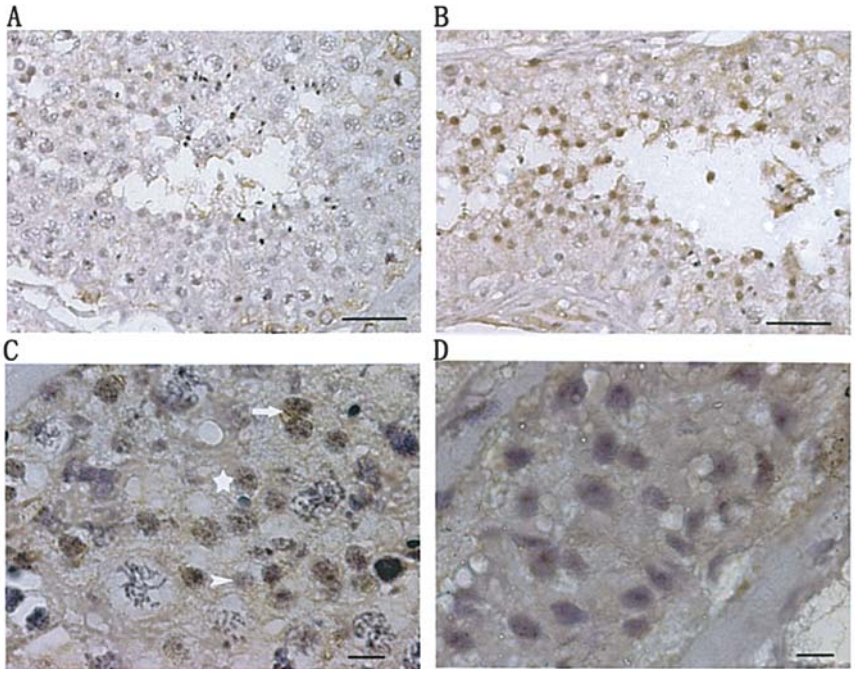

Figure 4. Immunohistochemical localization of ALF in normal (A and B, bar 100 μm) and abnormal (C and D, bar 20 μm) human testis sections. (A) Negative control. (B) Normal adult testis. (C) Spermatogenic disturbance sample. Cells with ALF labeling that was similar to (arrow) and lower than (star) that in the normal sample were observed; spermatids with no ALF labeling were also present (arrowhead). (D) SCOS sample.

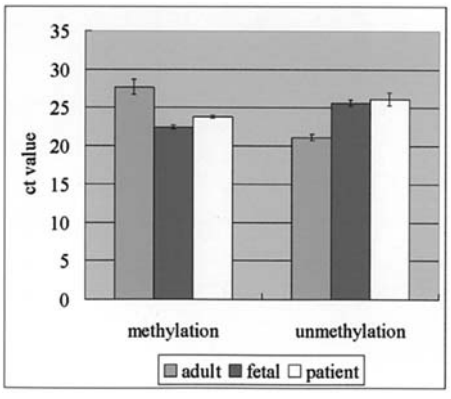

Figure 5. Levels of methylated and unmethylated ALF DNA in the human testes of adult, fetal and infertile subjects.

the immunostaining investigation of the human spermatids revealed the strongest signal in the nucleus and a relatively weak staining occurred in the cytoplasm (Fig. 4A and B). As with the mRNA, the ALF protein was expressed at lower levels in infertile patients with spermatogenetic disturbances. The number of cells that showed positive signals was dramatically decreased (Fig. 4C). ALF was not expressed in patients with SCOS (Fig. 4D).

Methylation level of ALF in human testis. Quantitative detection and statistical analysis of the methylation level of ALF in normal adult, fetal and infertile testis revealed significant differences among the groups of adult and fetal, and adult and infertile patients (Fig. 5; P<0.05). Since the Ct value is related with the input copies of transcript logarithmically and conversely, these results revealed that the DNA methylation level of ALF was lower in the adult testis than in the fetal testis (P<0.05). The methylation level is comparatively increased in infertile patients relative to that in the normal adult testis (P<0.05). Similarly, the level of unmethylated ALF DNA in the adult testis was higher than that in the fetal testis (P<0.05) and in the patient testis (P<0.05), respectively.

Localization of ALF protein in sperm of normal and teratospermia subjects. The signal of ALF in sperm was predominantly localized in the annulus and partly in acrosomal cap area as detected by immunostaining. Furthermore, the fluorescence signals of ALF in the spermatozoa of teratospermia subjects were dramatically decreased or undetected (Fig. 6). The absence of ALF in the teratospermia subject group was significantly higher than that in the normal samples (Fig. 7; P<0.05).

Discussion

The ALF gene encodes a germ cell-specific form of the large (α/ß) subunit of the GTF TF II A (3,5). In humans, the TFs II Aα/ß and II Aγ are the large and small subunits of TF II A, respectively (16). They co-work with the TBP to stabilize the binding to the core promoter DNA to enable the formation of a transcriptionally active preinitiation complex (PIC) (11). A previous study suggested the possibility of ALF as a substitute for TF II A in male germ cells. Evidently, ALF can form a heterodimeric complex with the small (γ) subunit of TF II A and stabilize the binding of TBP to the core promoter DNA (17) in various genes, as determined by *in vitro* experiments. Barring any functional data, it remains unclear, however, what specific role ALF plays during human germ cell development.

To explore this issue, we first characterized ALF expression in normal human testes and spermatozoa. At the transcription

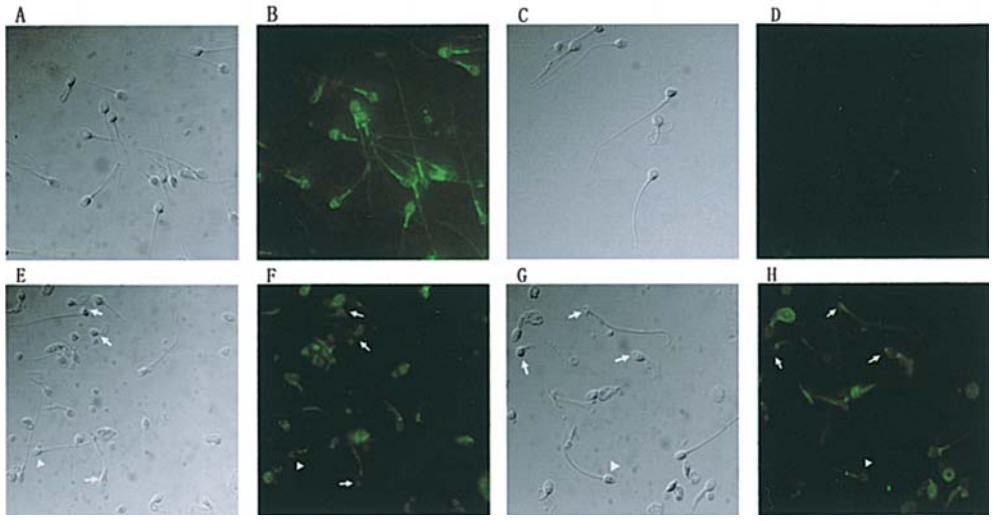

Figure 6. Immunofluorescence localization of ALF in human spermatozoa from normal (A-D) and teratospermia subjects (E-H). (A and B) Localization of ALF in normal spermatozoa. (C and D) Negative controls. (E-H) Localization of ALF in spermatozoa of teratospermia patients. White arrow, sperm that is not showing ALF protein signal; white angle, sperm that can be detected with ALF protein.

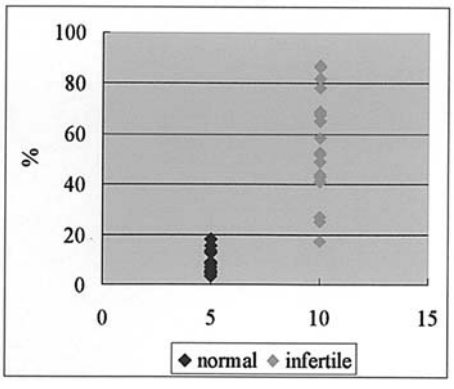

Figure 7. Percent of spermatozoa not expressing ALF protein in 12 normal and 20 teratospermia subjects.

and translation levels, ALF showed a significant increase in the adult testis over that in the fetal testis (Figs. 1 and 3). Immunohistochemistry showed that ALF resides in the spermatid nuclei (Fig. 4B). These results revealed that ALF is a testis development-related gene, and its localization is restricted to haploid spermatids in human seminiferous tubules. Thus, ALF is considered a spermatogenesis-related gene in human testis.

A genetic understanding of male-factor infertility requires knowledge of gene expression patterns associated with normal germ cell differentiation (19). Many testis-specific genes or genes that are expressed differentially during testis development were considered as infertility factor candidates (20). For example, NYD-SP16, a human adult testis highly expressed gene, was reported being expressed abnormal in the testis of infertile patients (21). And human inhibitor of apoptosis survivin showed a decreased expression in patients with spermatogenesis disorders (22). To reveal whether the expression level of ALF is related to human infertility and to confirm the function of ALF during spermatogenesis, we explored ALF expression in the testis of infertile patients. The RT-PCR experiments showed that ALF mRNA was absent or low in 16 infertile patients lacking spermatogenesis function and possessing spermatogenesis disturbance and SCOS syndrome. ALF was not expressed in 2 of the patients with SCOS syndrome (Fig. 2). The pathological diagnosis showed that SCOS patients had only Sertoli cells with no spermatogenic cells in the testicular seminiferous and tubules. This result indicated that ALF is not expressed in somatic cells, such as Sertoli and Leydig cells, but only in spermatogenetic cells. Our immunohistochemical analysis of infertile patients with spermatogenetic disturbance and SCOS showed that the positive signal was relatively weak in spermatogenic disturbance patients, an absent in patients with SCOS (Fig. 4C and D). Combined with the testis developmental expression of ALF both in mRNA and protein levels, the spermatid-specific distribution of ALF protein and its abnormal expression in infertile patients suggested that ALF is strongly linked to the spermatogenic cell function.

Previous studies with mice revealed that transcriptional control of ALF in male germ cells involves DNA methylation (15). In the present study, we detected DNA methylation in ALF in normal adult, fetal and infertile testis. We demonstrated that the higher expression of ALF in the adult testis was associated with reduced methylation, whereas silencing in the fetal and infertile patient testis was associated with increased methylation (Fig. 5). Therefore, as in mice, transcription of ALF in human testis might be regulated by DNA methylation.

Since most of the ALF signal occurs in spermatids, we suspected a role for ALF in spermiogenesis. To explore this potential, we examined the distribution of ALF in spermatozoa samples using immunofluorescence analysis. The results revealed that ALF is localized in the annulus on the mature sperm, and weakly in the acrosome (Fig. 6A-D). Although immunohistochemistry showed ALF to be a nuclear protein, we also detected relatively weak staining in the cytoplasm. Some of the transcription factors or transcription regulators in the testis were reported to being transferred from nucleus

to cytoplasm. For example, the RNA-binding protein Translin (TSN) was found in the cytoplasm in post-meiotic male germ cells, whereas in pachytene spermatocytes, TSN is predominant in the nucleus (23). Meanwhile, mouse ALF is detectable in spermatozoa cytoplasm, but almost absent in the nuclei (18). So ALF might be transported from the nucleus to the cytoplasm and predominantly localized in the annulus and partly in acrosomal cap area during spermiogenesis.

Surprisingly, when we investigated the distribution of ALF in the spermatozoa from 20 teratospermia subjects, we found that we could barely detect the ALF protein (Fig. 6E-H). The rate of missing ALF in the teratospermia group was significantly higher than that in the normal samples (Fig. 7, $P<0.05$). Based on previous morphological observations, the annulus has an active role to establish mitochondrion distribution (24); its absence may induce abnormal sperm, possessing several distinct structural defects, including defective mitochondria architecture, bent and non-motile tails, and defects in removing residual cytoplasm. Thus, the above results further elucidate the function of ALF in the process of shaping the spermatozoa. Moreover, a lack of ALF protein on the sperm annulus may induce abnormal sperm formation.

The results of this study reveal the primary functions of ALF in human testis biology: ALF may regulate spermatogenesis as a testis-specific transcription factor, and the abnormal expression of ALF is related to abnormal human spermatogenesis and the formation of normal spermatozoa that results in infertility.

Acknowledgements

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