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Expression of Ets Transcription Factor Etv5 Subfamily in Mouse Testis

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Abstract: Spermatogenesis is a complicated biological process which involves the comprehensive regulation of the hormones, growth factors and transcription factors. It's demonstrated that the E26 transformation-specific (Ets) family transcription factor Etv5 (also named as Ets Related Molecule, ERM) plays a critical role in the maintenance of male germline stem cell niche. However, the basic expression levels of Etv5 and its peer member Etv1 (also known as ER81) and Etv4 (also known as Pea3) during the postnatal development and growth of the testis are still unclear. To know this, testicular samples were collected from 5-days-, 7, 16-weeks-and 10-months-old mice. Etv1, Etv4 and Etv5 were cloned using reverse transcription PCR (RT-PCR). Real-time PCR analysis showed that Etv1 remained higher levels at 7-weeks and 10-months stages compared with those of 5-days and 16-weeks stages ($p<0.05$, respectively). Etv4 expression was very weak at all four stages but also showing an increase tendency at 7-weeks. Etv5 expression was lower at 5-days, extremely increased at 7-weeks ($p<0.01$) and then dramatically decreased at 16-week and 10-months stages ($p<0.01$). Collectively, the mRNA expressions of Etv1 and Etv5 in mouse testis reach peak along with the sexual maturity, then decrease and maintain at a relatively high level which putatively suggests Etv1 may has similar function like Etv5 in spermatogenesis.

INTRODUCTION

The E26 transformation-specific (Ets) family includes approximately 30 members in mammals which are shown to encode nuclear transcription factors to regulate gene expression. The Ets Variant Gene 5 (Etv5 also named as Ets related molecule, ERM) is a member of the Ets family. Etv5 plays an important role during the development of mouse spermatogonial Stem Cells (SSCs)[1,2]. In the Ets family, the Etv5 subfamily has three members, Etv1 (also known as ER81), Etv4 (also known as Pea3) and Etv5. They share a conserved DNA domain which can bind to similar sites containing GGAA/T center sequence and participate in variety of cell development, differentiation, growth and transformation processes[3]. Previously, we briefly reported that the expressions of Etv4 in the busulfan-treated mouse testis and epididymis[4] as well as in the cryptorchidism mouse testis and epididymis[5]. However, the basic expression level and change patterns of Etv5 subfamily during the postnatal development and growth of the testis is still unclear. Here, we amplified Etv1, Etv4 and Etv5 from mouse testis and

initially analyzed their mRNA levels in mouse testis at different developmental stages using real-time PCR.

MATERIALS AND METHODS

Animals and reagents: The 16 male Kunming white mice (5-days-, 7, 16-weeks-and 10-months-old, four animals of each age) were provided by the Experimental Animal Center of Jilin University (SY201903003). All experiments were performed in accordance with the principles and procedures of Animal Ethics Committee of Jilin University. All reagents used for molecular biology were from TaKaRa (Dalian, China), unless stated otherwise.

RNA extraction: The testes were collected and put into liquid nitrogen immediately. The testicular samples were lysed with Trizol reagents. The total RNAs were extracted using RNeasy Micro Kit (Qiagen Hilden, Germany) and then treated with Rnase-free Dnase I to eliminate genomic DNA. The RNAs were then diluted into equal concentration with Rnase-free distilled water. Oligo (dT) 18 was used in reverse transcription reactions. The resultant cDNA was frozen at -20°C until use.

Reverse Transcription PCR (RT-PCR): The first strand cDNAs were synthesized from 2 ng of the total RNAs. Reverse transcription was conducted with the 1st Strand cDNA synthesis kit (Qiagen Hilden, Germany). The resultant cDNAs were frozen at -20°C until use. The Primers (Table 1) used for RT-PCR were synthesized by Shanghai Sangon Biological Engineering Technology and Services CO., Lt. (Shanghai, China). The PCR conditions were as follows: initial denaturation at 94°C for 5 min, 94°C denaturation for 30 sec, annealing 30 sec, extension at 72°C for 1 min and amplification for 35 cycles. Subsequently, the PCR products were preceded for electrophoresis with 1.5% agarose gel. The target fragments were recovered and connected with the pGEM-T vector for transformation, screening and sequencing.

Real-time PCR: Using the total RNAs extracted above and the specific primers listed in Table 2, the rst-strand cDNAs were synthesized. Real-time PCR reactions were performed in a 25 μL volume containing 2 μL of cDNA, 12.5 μL of SYBR green master mix, 9 μL of RNase-free water, 0.5 μL each of primers (the forward-F, reverse-R and probe-P, all 10 pmol) for each gene. The 5' ends of the probes were labeled with 6-carboxyl fluorescein (FAM) and their 3' ends labeled with Eclipse. A reaction mix was formulated for the sample and for the non-reverse transcriptase control reactions. The program used for the amplification of each gene consisted of a denaturing cycle of 5 min at 95°C and 40 cycles of PCR (94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min) and cooling at 4°C. Relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method[6]. Triplicate PCR amplifications were performed for each sample and the values were normalized to the internal control of mouse β-actin.

Statistical analysis: The PCR amplification products of Etv1, Etv4, Etv5, β-actin and DNA marker (DL-2000) were preceded for electrophoresis with 1.5% agarose gel. The data were the means±SD and analyzed with SPSS 16.0 using one-way ANOVA. Comparisons of mean values among animals at different ages were performed by Tukey's multiple comparison tests. $p<0.05$ was considered to be signi cant ($p<0.05$).

Table 1: The primer sequences and the product size of RT-PCR

Primer (Etv)	Primer pair sequences (5'-3')	Annealing temp (°C)×cycle No.	Product size (bp)
1	F-GGCGATGAACTATGACAAAC	50×35	173
1	R-CACATCAACGAAGAGGACAC		
4	F-TCTGGGGTATCCAGAAGAAC	60×35	195
4	R-CCTTCCCAGATAATCAACGT		
5	F-TGTACAGATTGGGTTTGGAG	60×35	137
5	R-GACAGGACACAGAGCTGACT		

Table 2: The primer sequences and product sizes for real-time PCR

Primer	Primer sequence (5'-3')	Produce size (bp)
Etv1	F- GCCTAGCTGCCACTCCATTT	129
Etv1	R-TGTGTGGTCCCGGAGAAGTT	
Etv1	P-(FAM) CAGAACAGAAGGCTGCATGTTTGAGAAGG (Eclipse)	
Etv4	F- GCCAGCCATGAATTATGACAAG	139
Etv4	R-TGTTCTCTCTGGCCTTCCCA	
Etv4	P-(FAM) ATCATGCAGAAGGTGGCTGGCGA (Eclipse)	
Etv5	F- GCTTGGTTAGCTGAAGCACAAGT	
Etv5	R- AGTTGTCGTCCTGTAGCCACG	148
Etv5	P-(FAM) CTTCATGCTCCACCTCCCACCAAGATC (Eclipse)	
β-actin	F- CCTGAGGCTCTTTTCCAGCC	
β-actin	R-ACGTTGACATCCGTA AAGACCTCTA	
β-actin	P-(FAM) TCCTTCTTGGGTATGGAATCCTGTGGC (Eclipse)	110

RESULTS AND DISCUSSION

The total RNAs were extracted and Etv1, Etv4 and Etv5 were amplified by using RT-PCR (Fig. 1).

Quantitative real-time PCR analysis indicated that Etv1 mRNA level increased dramatically at the 7-weeks and 10-months stages compared with those of 5-days and 16-weeks stages ($p<0.05$, Fig. 2a). The mRNA expression of Etv4 remained at very low levels for all 4 stages but relatively high in 7-weeks-old testes (Fig. 2b). While the mRNA expression of Etv5 extremely increased at the 7-weeks stage ($p<0.01$), then decreased remarkably and remain a relatively stable level in 16-weeks-and 10-months-old testes (Fig. 2c).

The family of Ets transcription factors has >30 members. They are widely involved in the cellular development, differentiation, growth and transformation processes and play essential roles in the regulation of various physiological and pathological processes[3, 7, 8]. Among them, the essential function of Ets factor Etv5 in the transcriptional regulation of SSCs niches has been demonstrated[1, 9, 10]. In Ets family, Etv5 subfamily comprises of three members Etv1, Etv4 and Etv5. In view of the homology in the structure, similarity in the function and the wide distribution of Etv5 and its peer member Etv1 and Etv4, we examined their mRNA levels in mouse testis at four postnatal development stages in this study.

Using high-quality total RNAs, RT-PCR analysis indicated that the cloned cDNAs of Etv1, Etv4 and Etv5 were consistent with the theoretical expectation. Subsequently, real-time PCR was employed to evaluate their mRNA levels at 5-days-, 7, 16-weeks-and 10-months-old stages. Previously, it's reported that Etv1 mainly functions in the nervous system and cancer cells[11, 12]. We demonstrated that Etv1 was also maintained at a relatively higher level in mouse testis, especially, in the adults (7-weeks-old) and aged animals (10-months-old) which might imply a possible role of Etv1 in the adult spermato genesis. In contrast, Etv4 expression was very low in the testis, no matter in young or aged animals. Yang *et al*.[13] showed that Etv4 was expressed in rat epididymis, however, no information is available for its expression in the testis. Based on its extreme low level, we supposed that Etv4 may not be closely related with the testicular functions. For Etv5 as reported by others[1, 9, 10], it's remarkably expressed in the mature testis (7-weeks-old) and remained a stable level afterwards which is fully consistent with its proven roles in the development of SSCs and the process of spermatogenesis.

Although, each of the three Etv5 subfamily members is similar in structure, they have unique characteristics that may be important for the differential regulation of transcriptional activity. Etv1, Etv4 and Etv5 all have a N-terminal transactivation domain characterized by a stretch of conserved acidic residues[14] but Etv5 and Etv1 have an additional transactivation domain in their C-terminus[15, 16]. This structural discrepancy might explain their expression differences in the testis.

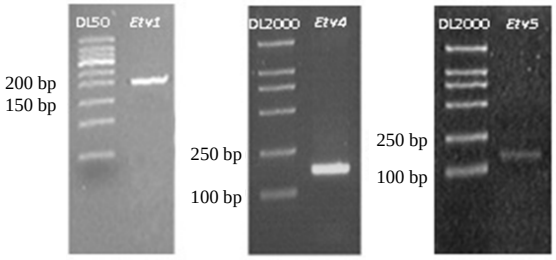

Fig. 1: RT-PCR analysis of Etv1, Etv4 and Etv5

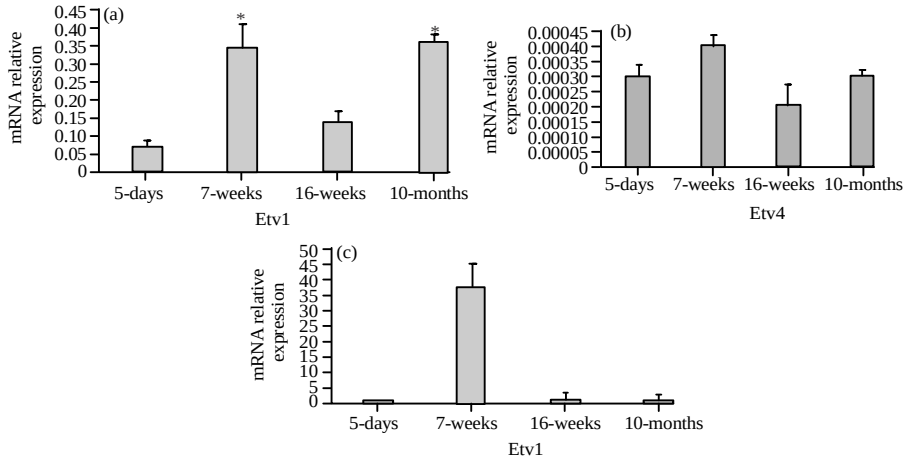

Fig. 2(a-c): Real-time analyses of Etv1 (a), Etv4 (b) and Etv5 (c). Different letters above the columns represent significant differences ($p<0.05$ in a; $p<0.01$ between (a) and (b), <0.05 between (a) and (c)

CONCLUSION

Together, we amplified Ets transcription factor Etv1, Etv4 and Etv5 and quantitatively investigated their mRNA levels in postnatal testis at different developmental stages. The relatively higher levels of Etv1 in mature and aged testis might suggest its possible role in the adult spermatogenesis which needs to be further addressed in the next step.

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