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Identification of a novel male germ cell-specific gene TESF-1 in mice

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Abstract

Mammalian spermatogenesis is precisely regulated by many germ cell-specific factors. In search for such a germ cell-specific factor, we have identified a novel mouse gene testis-specific factor 1 (TESF-1). Messenger RNA of TESF-1 was found only in the testis and its expression appeared to be regulated in a developmental manner. Further analysis demonstrated that the expression of TESF-1 was specifically in male germ cells, supported by the observation that we were not able to detect the TESF-1 mRNA from *at*/*at* homozygous mutant testes, which lack germ cells. The deduced amino acid sequence of TESF-1 contains a leucine-zipper motif, a potential nuclear localization signal, and two cAMP- and cGMP-dependent protein kinase phosphorylation sites. The green fluorescent protein (GFP)-tagged TESF-1 fusion protein was expressed in COS-7 cells and localized primarily in the nucleus. Taken together, these results indicate that TESF-1 is a novel male germ cell-specific gene, and its protein product may function as a nuclear factor involved in the regulation of spermatogenesis.

Keywords: Spermatogenesis; Germ cell-specific; Novel gene; TESF-1

Mammalian spermatogenesis is a highly ordered, precisely orchestrated developmental process in which germ cells sequentially undergo mitotic, meiotic, and post-meiotic phases to execute specialized proliferation and differentiation programs [1–5]. Throughout this process, an undifferentiated diploid spermatogonium is developed into four highly differentiated haploid sperm, with enormous morphogenetic transformation taking place in post-meiotic spermiogenesis. Type A spermatogonia are stem cells, and thus can renew themselves through proliferation to maintain spermatogenesis over a man's reproductive life. Sperm are functional male gametes, which possess unique cellular structures, acrosome, flagellum, compacted chromatin, among others, to qualify them as carriers of the paternal genome in sexual reproduction.

The process of spermatogenesis occurs in the seminiferous tubules of testes. It begins at puberty under the sophisticated control of endocrine hormones and paracrine factors [6,7]. Besides this extrinsic control, recent studies indicate that spermatogenic cells also manage a number of intrinsic, unique regulatory programs executed by many germ-cell-specific molecules, e.g., protamine-1, an indispensable molecule for spermatid chromatin compaction [8,9]; cAMP-responsive element modulator (CREM), an essential transcription factor for spermatid development [10,11]. Thus, to further understand how regulatory programs govern the course of spermatogenesis in detail, it is now important to establish the identity of the full complement of molecules active in this process. In this report, we describe the identification and characterization of a novel, germ-cell-specific gene testis-specific factor-1 (TESF-1).

Materials and methods

Chemicals. All chemicals were purchased from Sigma unless stated elsewhere.

Isolation of the full-length TESF-1 cDNA. In the search for a testis-specific cyclin, we coincidentally obtained a clone with a 1.4 kb,

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poly(A)-containing cDNA insert from the mouse testis marathon-ready cDNA (BD Clontech). The sequence of this cDNA was not found in the National Center for Biotechnology Information (NCBI) GenBank, based on the BLAST analysis. To obtain the full-length cDNA sequence, we performed 5′RACE (rapid amplification of cDNA ends) using the mouse testis marathon-ready cDNA (BD Clontech) as PCR template. The primers used were the forward primer, adaptor primer 1 (AP1) (5′-CCATCCT AATACGACTCACTATAGGGC-3′) and the reverse primer, gene-specific primer 1 (GSP1) (5′-CTGAGAGGTCCCAGTCTTCTGCC TTTGGCA-3′). PCR parameters were 94 °C for 2 min; 94 °C for 30 s, 72 °C for 4 min, 5 cycles; 94 °C for 30 s, 70 °C for 4 min, 5 cycles; 94 °C for 30 s, 68 °C for 4 min, 25 cycles; 72 °C for 6 min. The PCR product was cloned into the pT-Adv vector (BD Clontech) and sequenced from both strands. The sequence data from both the initial clone and the 5′ RACE product were used to compile the full-length cDNA.

Northern blot. To determine the tissue transcription profile of TESF-1, we performed the Northern blot assay on the Multiple Tissue Northern blot (MTN) membrane (BD Clontech). The digoxigenin-labeled DNA probe was prepared by using a Dig Labeling DNA Kit (Roche). The DNA template was the gel-purified PCR product generated using the forward primer GSP2 (5′-GCCAGCCTGGAGGTCCTCAA-3′) and the reverse primer GSP3 (5′-TGTCTTATCTTCAATTCTTGCT-3′). DNA hybridization and detection were performed according to the manufacturer's protocol (Roche).

Germ cell isolation and testis collection. Male ICR mice were obtained from Hilltop Lab Animals (Scottdale, PA). Enriched germ cell fractions were separated by velocity sedimentation at unit gravity [12]. Spermatogonia were obtained from 8-day-old mice. Leptotene/zygotene spermatocytes were isolated from 17-day-old mice. Adult mice at least 8 weeks old were used to obtain pachytene spermatocytes, round spermatids, and residual bodies. Testes were obtained from atrichosis (*at*) mutant mice and their normal littermates (The Jackson Laboratory, Bar Harbor, ME). The testes of the homozygous mutant mice are germ cell free [13]. Testes from 8- to 100-day-old ICR mice were also collected. The cells and testes were frozen in liquid nitrogen and stored at −80 °C until used for RNA isolation.

RT-PCR. RNA was extracted from germ cells and testes using the method of Chomczynski and Sacchi [14]. One microgram total RNA was used as template for reverse transcription by the random primer method. The primers used in the PCR were GSP2 and GSP3 (see above). For the control β-actin, the primers were the forward primer Actin-5′ (5′-GTG GGC CGC TCT AGG CAC CAA-3′) and the reverse primer Actin-3′ (5′-CTC TTT GAT GTC ACG CAC GAT TTC- 3′).

Expression of TESF-1-GFP (green fluorescent protein) in COS-7 cells. The whole open-reading frame of TESF-1 was amplified by PCR using the forward primer GFP-5′ (5′-ACTG ctgcag ATG GGT TCC CAA AGC CTG AA-3′, the *Pst*I site in lowercase letters) and the reverse primer GFP-3′ (5′- ACTG ggatcc GC TCC TCC TCT CTT CAA TAA-3′, the *Bam*HI site in lowercase letters), sequenced to rule out any mutations, and cloned into the *Pst*I/*Bam*HI sites of the vector pEGFP-N1 (BD Clontech). This construct pTESF-1-EGFP was transiently transfected into COS-7 cells using the Polyfect Transfection Reagent (Qiagene). Twenty-four hours

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                    5′    TATTGATTTCAGGGTAGGACTTAGGAGGGTAAAGCCTCCAAGGCAAGAGGAC
ATGGGTTCCCAAAGCCTGAATTCCACCTTTCTAAAAGTCAGGAGACTTTCATTAATTTCCAGTAATGAAAGATTA
 M  G  S  Q  S  L  N  S  T  F  L  K  V  R  R  L  S  L  I  S  S  N  E  R  L
CTTGGACAAACCTCTGGATTGGCCACTGGGTCGAGGTTGGTAACCCAGGAGGTTGATGACCTCAGGGTCATCCCA
 L  G  Q  T  S  G  L  A  T  G  S  R  L  V  T  Q  E  V  D  D  L  R  V  I  P
GGAAGTAGACCGGATCTGGATGATCATCAGCCTCAGTGTAGCCTGGCGGAGCTGCCTAGCACTGCTCATGGCAAG
 G  S  R  P  D  L  D  D  H  Q  P  Q  C  S  L  A  E  L  P  S  T  A  H  G  K
AGGAAGCCAGGCCATCTTCCACGCCTTAGAAGCAGTGCTGTCAAGGGCCATGCCCCTGACCCTAATCCAAGCTTG
 R  K  P  G  H  L  P  R  L  R  S  S  A  V  K  G  H  A  P  D  P  N  P  S  L
TCTATAGTCTCCAAAAGAATCTTTAAGGGCGAATCTGTGATCAAAGGGCCCGAGGACAGGCAGACATTTGTTGGG
 S  I  V  S  K  R  I  F  K  G  E  S  V  I  K  G  P  E  D  R  Q  T  F  V  G
CCCAGTGGCTTGCCAAAGATTAGCCCCAAAGCAACAGCAGGGGAGGCCCAAGGGAAGAAGAGGACAATGGAGTTG
 P  S  G  L  P  K  I  S  P  K  A  T  A  G  E  A  Q  G  K  K  R  T  M  E  L
CTTAACAAGGCTCGAAAGCAAGAAGAAAAAGTCTCAAACCTACTAGATATCAGACAGCTCCCGAAGCAAGAGGTG
 L  N  K  A  R  K  Q  E  E  K  V  S  N  L  L  D  I  R  Q  L  P  K  Q  E  V
TTTATTAACAACACACACCCATGCAAGAAACATCTGAAACAGCAACCAATGAGCCTTGAGGAATGGAGGAGGGGC
 F  I  N  N  T  H  P  C  K  K  H  L  K  Q  Q  P  M  S  L  E  E  W  R  R  G
CATCTAGGGGGAGACAACACAGGACTCATATCACAAGAACCTTTCAGGTGCTGCAAACGTCTTGGAAAGAAGGCA
 H  L  G  G  D  N  T  G  L  I  S  Q  E  P  F  R  C  C  K  R  L  G  K  K  A
CAATGTCAACTGTTGGAAGTAACTTCCCTAGAGGCAGAAGCCAGCCTGGAGGTCCTCAAGAGGAGGAGGAGGATG
 Q  C  Q  L  L  E  V  T  S  L  E  A  E  A  S  L  E  V  L  K  R  R  R  R  M
CAGGCTATGGAGATGTCCAAAAAACCTCAGGACAGAGGACTCGGTCAAGAAAAAGCAGTCTTCCTAAGCAGAGAG
 Q  A  M  E  M  S  K  K  P  Q  D  R  G  L  G  Q  E  K  A  V  F  L  S  R  E
AAAGTAAAGCCATCTTCTCATGACATGCATCTGAGCACTGCTGAAAGAAGCTTTAAACCTAAATCAATGCCAAAG
 K  V  K  P  S  S  H  D  M  H  L  S  T  A  E  R  S  F  K  P  K  S  M  P  K
GCAGAAGACTGGGACCTCTCAGTCCAGGGAACTCCTGTAGTGCTTACAGTTCGGGACCACAGCAATGTCTCACAG
 A  E  D  W  D  L  S  V  Q  G  T  P  V  V  L  T  V  R  D  H  S  N  V  S  Q
GCTCAGAAACATTTGGGCTGTGCTGAAATATTTCATTCCAGAGATGGGCGATGTACTTTATTGAAGAGAGGAGGA
 A  Q  K  H  L  G  C  A  E  I  F  H  S  R  D  G  R  C  T  L  L  K  R  G  G
GCCTAGAGAGAGTAGACACTTTCTCTGCATCCTGCTCGAGCACAATACAGGAACGAGGACCAGATTACCTTCACC
 A  .
AGCGACTGTGACTTCAATGGGCCAGGTGACTGAGGCACCCATTGTTCCACATGGGAATCAGACAGAATTCCAAGA
GTCCGTCACCATCCCGTGTATTCCATGTGAAAGCAAGAATTGAAGATAAGACAACTTTAGGAAACATCAATGAGG
AGAACAAAATAGAGTCTTGTCAAGTGTGACGAGATTATCAGTCAATTGGATGGAAATCGTACTGCACTGTAGGAT
TAGCAGCATCAGACAATGGGGGACTAGACTATTCAGTGGTTGGTGACATATGAGGCAGAATACCTGGGGCCCTGG
GCTTCTCTCTTGGGGGAGCTCCCCTATGGTGGTGCTTTAGGACCCACTAAGGAACAAATTGCTGAAGGAAATATA
AGTAGAGATATAGTATTTTTCCGGAGAAATATTTTTGTTGGACCTCAATCCACCAAGTTCCAGGCAGTTGAGCTG
CTACAGTAATTTCCTGGACACTCCAGATCTTTGGATATTGACCACCTTTACAGGGAAAAAAAAAGAATGATGTGT
TCTGTAAACACCGAGCAAGGCATAGAAAATGTAATGTCTTAAATAAAACTGTTTCAGATCTGCAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAA      3′
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Fig. 1. The cDNA and deduced amino acid sequence of TESF-1. The first methionine represents the potential starting codon of the longest open-reading frame. The conserved leucine-zipper motif is shown in bold. The underlined sequence indicates a potential nuclear localization signal. The strike through sequences represent two cAMP- and cGMP-dependent protein kinase phosphorylation sites.

after transfection, the cells were fixed with formaldehyde, stained with 4′,6-diamidino-2-phenylindole (DAPI), and observed for fluorescent signals under the Olympus BX51 microscope equipped with a digital camera.

Results

Cloning and sequencing of TESF-1

We initially obtained one partial-length, poly(A)-containing cDNA of 1408 bp from the mouse testis marathon-ready cDNA (BD Clontech). The length of the 5′RACE PCR product was 974 bp. The overlap region between the initial cDNA clone and the 5′RACE cDNA was 582 bp. These sequence data were used to compile the complete cDNA sequence (GenBank Accession No. AY552600). The gene encoding this cDNA was named as testis-specific factor 1 (TESF-1). The full-length 1800 bp cDNA contains a potential open-reading frame (ORF) of 1056 bp, a 5′ untranslated region (UTR) of 52 bp, and a 3′ untranslated region (UTR) of 657 bp (Fig. 1). TESF-1 is predicted to encode a ~39 kDa protein of 351 amino acid residues containing a conserved leucine-zipper motif (aa 11–32), two cAMP- and cGMP-dependent protein kinase phosphorylation sites (aa 14–17 and aa 144–147), and a potential nuclear localization signal (aa 245–262) (Fig. 1). The deduced protein has an isoelectric point of 9.95 and 24.72 positive charge at pH 7.0.

Tissue distribution of TESF-1

To analyze the transcript expression profile of TESF-1 in different tissues, the multiple tissue mRNA blot from mouse (BD Clontech) was probed with a TESF-1 cDNA fragment. Organs or tissues tested included: heart, brain, spleen, lung, liver, skeletal muscle, kidney, and testis. A positive signal corresponding to about 1.8 kb was detected exclusively in testis (Fig. 2). We could not detect the expression of TESF-1 either in the ovary or in oocytes by RT-PCR (data not shown). These results collectively suggest that TESF-1 is a testis-specific gene.

Time of expression in testis development

In order to determine when TESF-1 is expressed in testis development, we performed the RT-PCR experiment, using the total RNA samples from the testes harvested at different postnatal days. The result shows that TESF-1 has a highly specific developmental pattern of expression in testis, and an adult level expression is reached around the 25th postnatal day (Fig. 3).

Expression of TESF-1 in germ cells

The first round of spermatogenesis in the mouse starts at birth and is completed in about 34 days. The developmental expression of TESF-1 in testis after birth suggests that TESF-1 may be a germ cell-specific gene. To test this, we used the total RNA of purified germ cells and of *at*/*at* mutant testes in the RT-PCR. As shown in Fig. 4, TESF-1 is expressed in spermatocytes of leptotene, zygotene, and pachytene, round spermatids, but not in *at*/*at* mutant testes. It is known that testes of homozygous *at*/*at* mutant mice are germ cell free [13]. Taken together, these data suggest that TESF-1 is a germ-cell-specific gene.

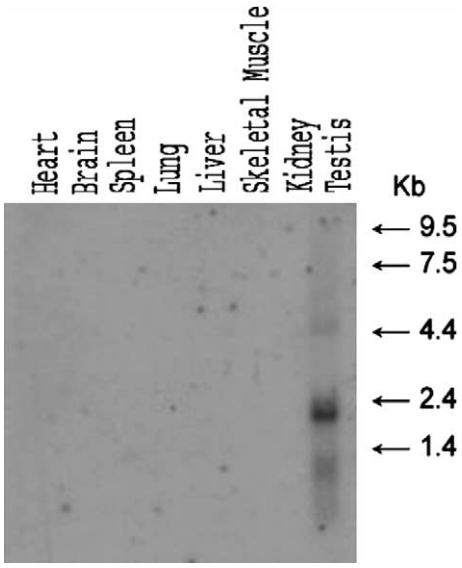

Fig. 2. Tissue distribution of TESF-1. The MTN membrane was probed with a TESF-1 cDNA fragment as described in the Materials and methods. The tested tissues are shown above the lanes.

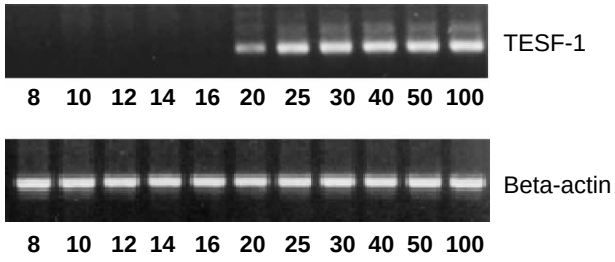

Fig. 3. Time of TESF-1 expression in testis development detected by RT-PCR. The total RNA samples were prepared from the mouse testes harvested on different postnatal days. RT-PCR then was performed to detect the expression of TESF-1. The number below each lane indicates the postnatal day. β-Actin was used as the RT-PCR control.

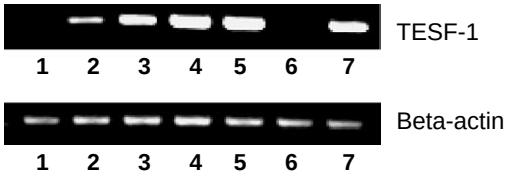

Fig. 4. Expression of TESF-1 in spermatogenic cells and *at*/*at* mutant testes. RT-PCR was performed on RNA isolated from spermatogonia (lane 1), leptotene/zygotene spermatocytes (lane 2), pachytene spermatocytes (lane 3), round spermatids (lane 4), testes of *at*/+ heterozygous littermate control mice (lane5), *at*/*at* homozygous mutant testes (lane 6), and testes of normal adult mice (lane 7).

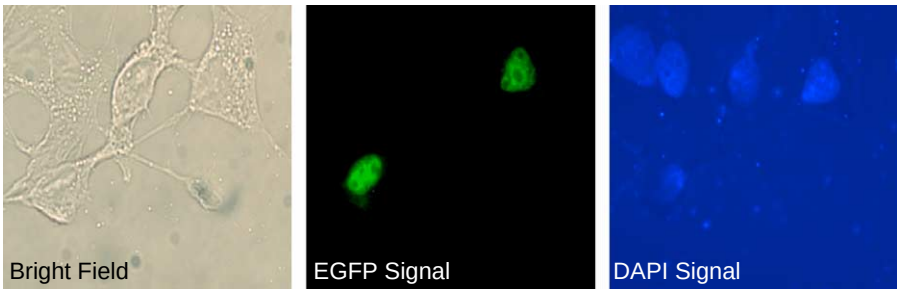

Fig. 5. Nuclear localization of TESF-1-EGFP in COS-7 cells. COS-7 cells were transiently transfected with the expression vector pTESF-1-EGFP. The fluorescent signals of EGFP and DAPI were obtained with a digital camera (original magnification, 1000×).

Expression and cellular location of TESF-1-EGFP in transiently transfected COS-7 cells

The deduced protein sequence of TESF-1 contains a nuclear localization signal (see above), suggesting that TESF-1 may function as a nuclear factor. To test this, we constructed the vector pTESF-1-EGFP and transfected this vector into COS-7 cells. The expression and cellular location of the fusion protein TESF-1-EGFP was determined by the EGFP fluorescent signal. We observed the EGFP signal in 45% of cells. As shown in Fig. 5, TESF-1-EGFP is primarily localized in the nucleus. The green fluorescence in the COS-7 cells transfected with the control vector pEGFP-N1 was distributed throughout the cytoplasm (data not shown).

Discussion

Mammalian spermatogenesis occurs in a developmentally regulated manner in the seminiferous tubules of testes [1–5]. It consists of several complex cellular processes, including the mitotic proliferation and self-renewal of spermatogonial stem cells, meiotic division of spermatocytes, and morphogenesis of haploid spermatids. It is known that these processes are highly regulated by many germ cell-specific factors [15]. Therefore, identifying the full complement of these so-called germ cell-specific factors will undoubtedly lay the solid base for our further understanding and better manipulation of spermatogenesis. Herein, we reported a novel mouse germ-cell-specific gene TESF-1.

On Northern blot (Fig. 2), TESF-1 was found only in the testis, displaying a distinct transcript size of about 1.8 kb. The other two vague signals, one at ~4.6 kb and the other at ~1.1 kb, may reflect different transcript isoforms or cross-hybridizations. The RT-PCR result shows that the TESF-1 transcript was first detected around the 20th postnatal day in the testis development (Fig. 3). This timing of expression suggests that TESF-1 could be a germ-cell-specific gene. This was confirmed by the results that the TESF-1 transcript was expressed in the purified germ cells as early as in the leptotene/zygotene stage, but not in the *at/at* mutant testis that is free of germ cells (Fig. 4).

TESF-1 may encode a unique protein in the mouse. Despite a comprehensive search of the available public databases no sequence homology to any other known protein could be detected. Interestingly, we found several structurally conserved motifs in the deduced amino acid sequence of TESF-1 by the PROSITE analysis (http://ca.expasy.org/) (Fig. 1). First, it contains a potential nuclear localization signal (NLS) in the middle of the molecule. The finding that the expressed fusion protein TESF-1-EGFP was predominantly localized in the nucleus clearly supports that the NLS of TESF-1 is functional (Fig. 5), indicating that TESF-1 may function as a nuclear factor. Second, TESF-1 contains a conserved leucine-zipper motif at its amino terminus. Leucine-zipper motif is a proposed DNA-binding domain that can be found in many transcription factors [16,17], including the cAMP response element binding proteins (CREB) and the cAMP responsive element modulators (CREM). It has been well-documented that CREB and CREM play pivotal roles in spermatogenesis [10,11]. Moreover, TESF-1 may also functionally interact with the cAMP/cGMP signaling pathway since it contains two potential cAMP- and cGMP-dependent protein kinase phosphorylation sites. Taken together, these structural features suggest that TESF-1 may function as a nuclear transcription factor in spermatogenesis, an intriguing speculation that will be addressed in the further investigation.

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References

[1] D.M. de Kretser, K.L. Loveland, A. Meinhardt, D. Simorangkir, N. Wreford, Spermatogenesis, Hum. Reprod. 13 (1998) 1–8.
[2] D.G. de Rooij, Proliferation and differentiation of spermatogonial stem cells, Reproduction 121 (2001) 347–354.
[3] E.M. Eddy, Male germ cell gene expression, Recent Prog. Horm. Res. 57 (2002) 103–128.
[4] S. Kimmins, N. Kotaja, I. Davidson, P. Sassone-Corsi, Testis-specific transcription mechanisms promoting male germ-cell differentiation, Reproduction 128 (2004) 5–12.

[5] S. Kimmins, P. Sassone-Corsi, Chromatin remodelling and epigenetic features of germ cells, Nature 434 (2005) 583–589.
[6] W.F. Crowley, R.W. Whitcomb, J.L. Jameson, J. Weiss, J.S. Finkelstein, L.S. O'Dea, Neuroendocrine control of human reproduction in the male, Recent Prog. Horm. Res. 47 (1991) 27–67.
[7] M.K. Skinner, J.N. Norton, B.P. Mullaney, M. Rosselli, P.D. Whaley, C.T. Anthony, Cell-cell interactions and the regulation of testis function, Ann. NY Acad. Sci. 637 (1991) 354–363.
[8] C. Cho, H. Jung-Ha, W.D. Willis, E.H. Goulding, P. Stein, Z. Xu, R.M. Schultz, N.B. Hecht, E.M. Eddy, Protamine 2 deficiency leads to sperm DNA damage and embryo death in mice, Biol. Reprod. 69 (2003) 211–217.
[9] C. Cho, W.D. Willis, E.H. Goulding, H. Jung-Ha, Y.C. Choi, N.B. Hecht, E.M. Eddy, Haploinsufficiency of protamine-1 or -2 causes infertility in mice, Nat. Genet. 28 (2001) 82–86.
[10] J.A. Blendy, K.H. Kaestner, G.F. Weinbauer, E. Nieschlag, G. Schutz, Severe impairment of spermatogenesis in mice lacking the CREM gene, Nature 380 (1996) 162–165.
[11] F. Nantel, L. Monaco, N.S. Foulkes, D. Masquilier, M. LeMeur, K. Henriksen, A. Dierich, M. Parvinen, P. Sassone-Corsi, CREM: a transcriptional master switch during the spermatogenesis differentiation program, Nature 380 (1996) 159–162.
[12] A.R. Bellvé, Purification, culture, and fractionation of spermatogenic cells, Methods Enzymol. 225 (1993) 84–113.
[13] M.A. Handel, J.J. Eppig, Sertoli cell differentiation in the testes of mice genetically deficient in germ cells, Biol. Reprod. 20 (1979) 1031–1038.
[14] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[15] P. Sassone-Corsi, Unique chromatin remodeling and transcriptional regulation in spermatogenesis, Science 296 (2002) 2176–2178.
[16] W.H. Landschulzm, P.F. Johnson, S.L. McKnight, The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins, Science 240 (1988) 1759–1764.
[17] S.J. Busch, P. Sassone-Corsi, Dimers, leucine zippers and DNA-binding domains, Trends Genet. 6 (1990) 36–40.