FERTILITY AND STERILITY®
VOL. 82, SUPPL. 3, OCTOBER 2004

Published by Elsevier Inc.
Printed on acid-free paper in U.S.A.

Normal expression of isoforms activating cyclic adenosine monophosphate responsive element modulator in patients with spermatid maturation arrest

Irina Palermo, Ph.D.,[a] *Giuseppe Arcidiacono, M.D.,*[b] *Nunziata Barone, Ph.D.,*[a] *Fabrizio Italia, M.D.,*[c] *Mario Arizzi, M.D.,*[d] *Giovanni Spera, M.D.,*[d] *Giovanni Bartoloni, M.D.,*[c] *Silvana Di Mercurio, M.D.,*[c] *Enzo Vicari, M.D.,*[a] *and Aldo E. Calogero, M.D.*[a]

Department of Biomedical Sciences, and Unit of Pathology and Cytodiagnosis, University of Catania, and Unit of Cardiology, Garibaldi Hospital, Catania, Italy; Department of Medical Pathophysiology, University "La Sapienza," Rome, Italy

Objective: To evaluate whether defective cyclic adenosine monophosphate responsive element modulator (CREM) expression is the causative factor of spermatid maturation arrest (SMA).

Design: Comparative evaluation of the testicular histology in patients with SMA or normal spermatogenesis.

Setting: University clinic of andrology.

Patient(s): Azoospermic patients undergoing testicular biopsy.

Intervention(s): None.

Main Outcome Measure(s): Expression of CREMτ in quantitative immunohistochemistry analysis of testicular biopsy samples.

Result(s): Regular CREM expression was observed in the tubules with round, but not elongated, spermatids of patients with SMA (n = 9). Quantitative analysis showed that round spermatids of patients with SMA had a staining intensity similar to that observed in controls (n = 7).

Conclusion(s): Lack of spermatid elongation was not due to defective CREM expression. Therefore, CREM did not play a pathogenetic role in the onset of SMA in humans. (Fertil Steril® 2004;82(Suppl 3):1072–1076. ©2004 by American Society for Reproductive Medicine.)

Key Words: CREM, spermatid maturation arrest, normal spermatogenesis, immunohistochemistry, spermatid, testicular biopsy, Johnson's score

Received January 12, 2004; revised and accepted May 26, 2004.

Reprint requests: Aldo E. Calogero, M.D., Sezione di Endocrinologia, Andrologia e Medicina Interna, Dipartimento di Scienze Biomediche, Università di Catania, Ospedale Garibaldi, Piazza S. Maria di Gesù, 95123 Catania, Italy (FAX +39-95-310899; E-mail: acaloger@unict.it).

[a] Section of Endocrinology, Andrology and Internal Medicine, Department of Biomedical Sciences, and Master in Andrological and Human Reproduction Sciences, University of Catania, Catania, Italy.

[b] Unit of Cardiology, Garibaldi Hospital, Catania, Italy.

[c] Unit of Pathology and Cytodiagnosis, Ascoli-Tomaselli Hospital, University of Catania, Catania, Italy.

[d] Chair of Internal Medicine III, Department of Medical Pathophysiology, University "La Sapienza," Rome, Italy.

0015-0282/04/$30.00
doi:10.1016/j.fertnstert.2004.05.077

Several studies have identified a family of transcription factors as a key component of spermatogenesis: the cyclic adenosine monophosphate (cAMP) responsive element–binding protein (CREB). The most important member of this family is the cAMP-responsive element modulator (CREM). Following follicule stimulating hormone (FSH)-induced phosphorilation at serine-117, CREM is activated and recognizes a 8-base pair (bp) palindromic sequence with the consensus motif 5′-TGACGTCA-3′ (CRE element). This results in the modulation of the expression of several genes coding the structural proteins required for mature spermatozoa development, such as protamine-1 and protamine-2, transition protein-1 and transition protein-2, and calspermin and RT7 (1–5).

The CREM gene has remarkable properties within its family. By mechanisms of alternative exon splicing, alternative promoter usage, and autoregulation of promoters, it generates both repressor and activator isoforms (6–9). One group of CREM activators consists of the CREMτ isoforms (τ, τ1, and τ2) that originate from the same promoter (P1) as the suppressor isoforms CREMα, CREMβ, and CREMγ. A second group of CREM activators, which originates from two alternative promoters (P3 and P4) and translation initiation codons, has recently been identified and named CREMθ1 and CREMθ2, respectively (10). In addition, by alternative us-

TABLE 1

Tubular and spermatid parameters of testicular biopsy specimens obtained from patients with normal spermatogenesis (controls) and patients with spermatid maturation arrest (SMA).

	Controls (n = 7)	SMA (n = 9)
Johnsen's score	9.9 ± 0.1	6.6 ± 0.2[a]
Number of tubules examined	14.8 ± 2.5	13.0 ± 1.6
Tubular area (μm^2)	46,598 ± 1,360	43,946 ± 1,403
Tubular perimeter (μm)	808.4 ± 13.6	802.2 ± 14.4
Number of spermatids/tubule	37.7 ± 3.0	16.1 ± 2.0[a]
Percentage of CREM + spermatids	85.8 ± 2.2%	72.0 ± 2.5%
Spermatid nuclear area (μm^2)	20.7 ± 0.5	24.3 ± 0.6

Note: Data are expressed as mean ± SEM.
[a] $P<.05$, unpaired Student's *t*-test.

Palermo. CREM immunohistochemistry and SMA. Fertil Steril 2004.

age of two translation initiation codons, the CREMτ transcript can generate two proteins with opposite functions: the activator CREMτ and the repressor S-CREM (11).

An important CREM repressor isoform is the inducible cAMP early repressor (ICER), which originates from an alternative intronic promoter (P2). Inducible cAMP early repressor, whose transcription can be activated by cAMP, binds to the CRE element and represses cAMP-induced transcription. The consequent fall in ICER protein levels eventually leads to repression release and permits a new cycle of transcriptional activation (12).

The role played by CREM in the male reproductive function has been demonstrated in the mouse model. A comparison of CREM expression in prepubertal and adult testes revealed a functional switch: while low levels of the repressor isoforms (CREMα, CREMβ, and CREMγ) are expressed in the immature testis, only the activator form CREMτ is detected in the adult testis at high levels (7). Antagonist forms of CREM are present in premeiotic germinal cells and early prophase spermatocytes. A prominent switch to the CREMτ and CREMθ activating isoforms starts in pachytene spermatocytes, leading to the activation of haploid genes important in the late round of spermatid development and elongation initiation.

The importance of CREM in male fertility was evident by the analysis of CREM knockout mice (13, 14). The homozygous males are sterile, having absolutely no mature spermatozoa. Seminiferous epithelium analysis of mutant male mice revealed postmeiotic arrest at the first step of spermiogenesis and a dramatic decline in postmeiotic gene expression. Testosterone and FSH levels were not affected by the mutation (15).

This experimental evidence suggests that CREM could also be involved in human spermatogenesis as a causal factor of round spermatid maturation arrest (SMA) in infertile men. In this regard, it has been reported that round spermatid maturation arrest is always associated with a lack of CREM expression (16, 17). However, Lin et al. (18) were unable to confirm these results. These contrasting results and the clinical implication for this key regulator of spermatogenesis led us to evaluate the expression of CREMτ by quantitative immunohistochemical analysis in testicular biopsy samples of selected azoospermic patients with round SMA and with normal spermatogenesis (control group).

MATERIALS AND METHODS

Patients

Sixteen men were selected for this study, and testicular biopsy samples were obtained from them as previously reported elsewhere (19). The testicular tissue was fixed in Bouin's solution. Based on quantitative evaluation of sections stained with hematoxylin and eosin (20), seven biopsy specimens from patients with obstructive azoospermia were classified as complete spermatogenesis (controls), and nine samples from patients with secretory azoospermia were classified as round SMA. Controls had a Johnson's score ranging from 9.30 to 10, while the patients with SMA had a Johnsen's score ranging from 5.30 to 7.25. The protocol was approved by the institutional review board, and informed written consent was obtained from each patient.

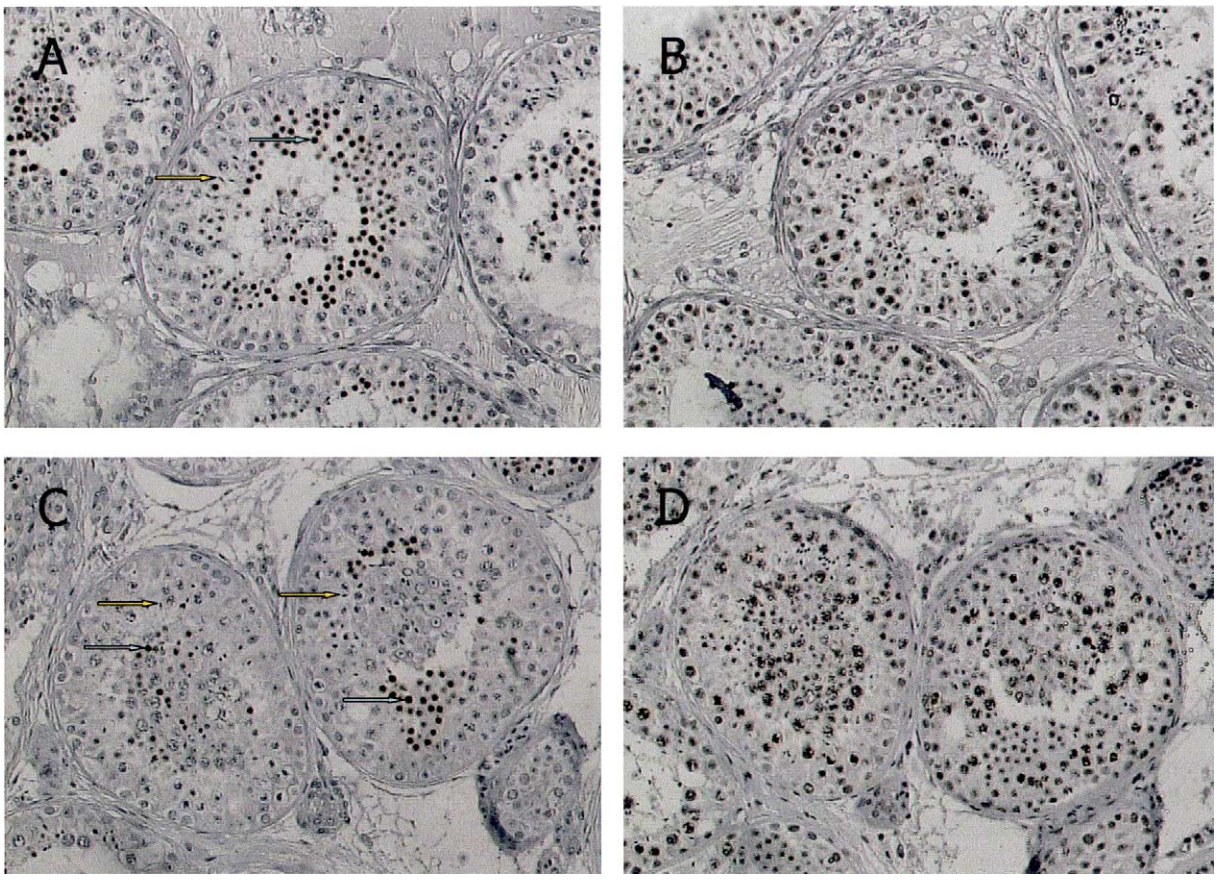
Immunohistochemistry

Tissue fixed with Bouin's (Sigma-Aldrich Srl, Milan, Italy) was dehydrated, embedded in paraffin, and cut into 6-μm sections for CREM localization. The paraffin-embedded tissue was deparaffinized by placing the slides in xylene for 30 minutes, then rehydrated by incubating it twice in 100% and 95% ethanol for 5 minutes each, followed by phosphate-buffered saline (PBS) 1× wash. After a wash in PBS/brij for 5 minutes, the slides were immersed into citrate buffer and placed in a microwave oven for 6 minutes and than cooled at room temperature for 25 minutes. The endogenous peroxidase activity was blocked by 30 minutes of incubation at 37°C in 4% freshly prepared hydrogen peroxide in PBS/brij. After being rinsed in PBS/brij, the sections were preincubated for 1 hour at 37°C in a humidified chamber with PBS containing 5% bovine serum albumin (BSA), then incubated overnight at 4°C with the monoclonal antibody against the τ isoform of CREM. The antibody, generously donated by Dr. P. Sassone-Corsi (Strasbourg, France), was used at 1:5000 dilution in PBS containing 5% BSA.

Following rinsing in PBS/brij for 15 minutes, the sections were incubated for 1 hour at room temperature with peroxidase-labeled goat anti-rabbit secondary antibody at 1:200 dilution in PBS containing 5% BSA. The sections were then rinsed in PBS/brij for 15 minutes and incubated for 20 minutes at room temperature in a humidified chamber with 3-3′diaminobenzidine tetrahydrochloride (DAB) (Sigma Chemical Company, St. Louis, MO) in PBS 1× containing

FIGURE 1

Testicular biopsy of an azoospermic patient with normal spermatogenesis (panels **A** and **B**) and of an azoospermic patient with spermatid maturation arrest (panels **C** and **D**) stained with anti-CREM antibody (panels **A** and **C**) or with a monoclonal antibody against the splicing factor SC35 (panels **B** and **D**). Light blue arrows indicate CREM-positive round spermatids, whereas yellow arrows indicate elongated spermatids. SC35 is expressed in all cell types.



Palermo. CREM immunohistochemistry and SMA. Fertil Steril 2004.

0.03% H_2O_2. The reaction was stopped by washing the sections in PBS/brij and then in distilled water two to three times until a uniform brown color became visible. The sections were counterstained in Mayer's hemalum solution (VWR International Srl, Milan, Italy) for 5 minutes, washed in distilled water two to three times, then dehydrated by incubating them twice in 95% and 100% ethanol for 5 minutes each. After a step in xylene for 30 minutes, the slides were mounted using Di-n-butyl-phthalate-polystyrene-xylene (DPX), (VWR International Srl, Milan, Italy) mountant (BDH Laboratory Supplies, Poole, United Kingdom).

Specificity control was obtained by replacing the primary antibody with PBS containing 5% BSA. To control for reactivity of the specimen, we used the monoclonal antisplicing factor SC35, a nuclear protein present in all cellular types. A section contiguous to that colored for CREM was stained using this antibody. All cells present in the sections of all biopsy specimens stained regularly with SC35.

Image Analysis

Quantitative analysis of immunostaining was performed using Leica's Windows-based image analysis system Qwin linked to a Leica DM RXA2 microscope (Wetzlar, Germany). Round tubules were evaluated from each biopsy specimen always using the same magnification (eyepiece 10×; objective 20×) and the same microscope light intensity. The software was set to recognize spermatid nuclear dimensions (to eliminate false-positive results) on the chromatic threshold level for DAB and on the algorithm to follow for the interpretation of the positively stained cell nuclei.

We first measured the circumference and the area of a tubule, then the image underwent analysis to reveal spermatid nuclei showing CREM-staining after having been cleaned from all interfering factors. The CREM-positive nuclei were identified and counted, and the staining intensity was decodified from the fundamental brown color to the single component of the RGB system to obtain objective information on the color intensity.

Data Analysis

Data are shown as mean ± SEM throughout the study. The mean CREM staining color intensity of controls was arbitrarily made equal to 1,000, and the staining intensity of each single control and SMA patient was then recalculated by applying a proportion. The data were analyzed by unpaired Student's *t*-test. The software SPSS 9.0 for Windows (SPSS, Inc., Chicago, IL) was used for statistical evaluation. $P<.05$ was considered statistically significant.

RESULTS

The number, area, and perimeter of the tubuli examined were similar in the testicular biopsy specimens of controls and SMA patients (Table 1). As expected, the patients with SMA had a statistically significantly lower number of round spermatids per tubule ($P<.05$). The CREM expression could always be detected in the nuclei of round spermatids of testicular biopsies from patients with SMA (Fig. 1). No CREM expression was detected after the replacement of the primary antibody with BSA.

The SMA patients had a lower percentage of CREM-positive round spermatids compared with controls, but the difference did not reach statistical significance. One patient had a number of CREM-positive spermatids below the lower range observed in controls. The round spermatid nuclear area was similar in controls and in patients with SMA (see Table 1).

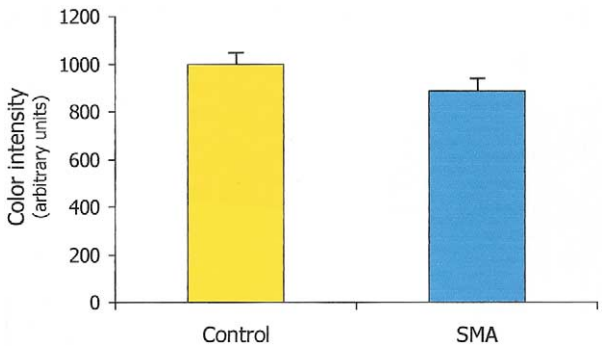
Testicular biopsy specimens from controls and SMA patients showed a strong expression of CREM in the nuclei of round spermatids. No statistically significant difference was detected in spermatid-staining intensity between controls and patients with SMA (Fig. 2).

DISCUSSION

Recent studies that have analyzed CREM expression in the human testis have reported contrasting results. Weinbauer et al. (16) showed an association between spermatid maturation arrest and CREM expression in the human testis. In their study, the testicular expression of CREM protein was analyzed by quantitative immunohistochemistry using a polyclonal antibody raised against recombinant mouse CREMτ that recognizes the CREM isoforms α, β, γ, τ1, and τ2 with similar affinity, ICER with lower affinity, and a rabbit polyclonal antiserum raised against full-length polyhistidine-tagged human CREM protein. Comparable results were obtained with both antibodies. In the 10 patients with SMA studied, the CREM signal was markedly reduced or undetectable. The reduction was significantly different when compared with the four patients with obstructive azoospermia and, hence, normal spermatogenesis. These observations led the investigators to conclude that CREM protein plays a relevant role in human spermatid development and that this transcription factor could be associated with spermatid maturation arrest in cases of idiopathic male infertility (16).

FIGURE 2

Color intensity of round spermatids stained with CREM antibody in testicular biopsies of patients with normal spermatogenesis (control) (n = 7) and in patients with spermatid maturation arrest (SMA) (n = 9).



Palermo. CREM immunohistochemistry and SMA. Fertil Steril 2004.

The essential role of CREM during human spermatogenesis was also underlined by Peri et al. (17). Using a different methodologic approach, these investigators showed that the lack of switch in the expression of CREM gene isoforms may be related to defective spermatid maturation in idiopathic male infertility. They performed reverse transcriptionbpolymerase chain reaction (RT-PCR) examinations of RNA extracted from germ cells at different maturation stages from two groups of men, the first with normal semen parameters and the second with severe oligozoospermia or azoospermia. They showed that a switch from CREM repressor to activator isoforms in postmeiotic germ cells occurs in the first group, but not in the second.

Those results were substantiated by in situ hybridization experiments performed on five testicular biopsies: two with normal spermatogenesis and three with SMA, using a probe that hybridated mRNA sequences common to CREMτ and τ1. Positive staining for CREM activators was present in all the sections of the patients with obstructive spermatogenesis, but was completely absent in the patients with SMA (17). Thus, the investigators suggested that a lack of switch in the expression of CREM gene isoforms is related to SMA.

Both of these studies suggested that a lack of CREM expression or of a switch from inhibitor to activator isoforms was the pathogenic mechanism responsible of SMA onset in patients with this type of spermatogenic failure. If this is true, CREM-activating isoform expression is the biochemical pathway where the different andrologic diseases convey their negative effects, thus blocking sperm maturation at the stage of round spermatids. However, a study of Lin et al. (18) was unable to confirm these results. They analyzed 48 testicular biopsy samples by immunohistochemical analysis, using a polyclonal rabbit antibody against the τ isoforms of CREM, which showed whenever present round spermatids exhibited a

regular staining for CREMτ. In fact, CREMτ immunoreactivity was observed in the round spermatids of testicular biopsy samples from patients with normal spermatogenesis, hypospermatogenesis, and SMA, but not in testicular specimens with Sertoli cell only syndrome or with primary spermatocyte maturation arrest. The lack of CREMτ expression in this latter group may not be regarded as a cause of the spermatogenic failure, but may simply reflect the absence of spermatids (18).

This discrepancy and our skepticism in considering all cases of SMA as due to a lack of CREM gene expression prompted us to evaluate the expression of activating isoforms of CREM in testicular biopsy specimens from patients with SMA. For this purpose, we carefully selected nine patients with round spermatids in their testicular biopsies. A strong expression of CREMτ was detected in the nuclei of round spermatids, as previously reported (18). Quantitative analysis showed that patients with SMA had a similar percentage of CREM-positive round spermatids, which also showed a similar staining intensity. Thus, these data suggest that patients with SMA had a CREM expression similar to that observed in the round spermatids of patients with normal spermatogenesis. In the tubules with round, but not elongated, spermatids, regular CREM staining was observed, confirming that the lack of spermatid elongation was due to a defective mechanism other than CREM expression.

Although we do not have a definitive explanation for this discrepancy, it may relate to a difference in the testicular biopsy sample selection among studies. Weinbauer et al. (16) based their histologic diagnosis of round SMA on Clermont (21) and Holstein and Roosen-Runge (22) criteria, so we cannot precisely compare their patients with ours. It should be highlighted that the European Association of Urology recently suggested that the most appropriate criteria to use in evaluating testicular biopsy specimens with nonobstructive azoospermia are those based on scoring systems (23). For this reason, we used Johnson's scoring criteria (20) to classify the patients enrolled in our study. Because our patients could be classified as having round spermatid maturation arrest in stages I to III, they had a spermatogenetic defect similar to the patients studies by Weinbauer et al. (16). Nevertheless, as suggested by the Weinbauer study, a SMA patient with presumably altered CREM expression could be identified among our patients when we considered the percentage of CREM-positive spermatids in relation to the range observed in controls. Indeed, this patient had a number of CREM-positive round spermatids below the lower range of controls. Therefore, one out of nine patients (11%) may have a CREM expression deficiency as a pathogenetic mechanism of their SMA. However, the staining intensity of his spermatids was similar to that observed in the patients with normal spermatogenesis.

Our data provide further evidence that CREM does not play a pathogenetic role in all cases of SMA in humans. However, we cannot exclude the suggestion that lack of a switch in the expression of CREM-activating isoforms could be responsible of sporadic cases of SMA.

References

1. Delmas V, van der Hoorn F, Mellstrom B, Jegou B, Sassone-Corsi P. Induction of CREM activator proteins in spermatids: down-stream targets and implication for haploid germ cell differentiation. Mol Endocrinol 1993;7:1502–14.
2. Kistler MK, Sassone-Corsi P, Kistler SW. Identification of a functional cAMP response element in the 5′-flanking region of the gene for transition protein 1 (TP1), a basic chromosomal protein of mammalian spermatids. Biol Reprod 1994;51:1322–9.
3. Sun Z, Sassone-Corsi P, Means A. Calspermin gene transcription is regulated by two cyclic AMP response elements contained in an alternative promoter in the calmodulin kinase IV gene. Mol Cell Biol 1995;15:561–71.
4. Lalli E, Lee JS, Lamas M, Tamai K, Zazopoulus E, Nantel F, et al. The nuclear response to cAMP: role of transcription factor CREM. Phil Trans R Soc Lond 1996;351:201–9.
5. Monaco L, Nantel F, Foulkes SN, Sassone-Corsi P. CREM: a transcriptional master switch governing the cAMP response in the testis. In: Hansson V, Levy FO, Tsken, K, eds. Signal transduction in testicular cells. Berlin: Springer-Verlag; 1996;69–94.
6. Foulkes NS, Borrelli E, Sassone-Corsi P. CREM gene: use of alternative DNA-binding domains generates multiple antagonists of cAMP-induced transcription. Cell 1991;64:739–49.
7. Foulkes NS, Mellstrom B, Benusiglio E, Sassone-Corsi P. Developmental switch of CREM function during spermatogenesis: from antagonist to transcriptional activator. Nature 1992;355:80–4.
8. Foulkes NS, Sassone-Corsi P. More is better: activators and repressors from the same gene. Cell 1992;68:411–4.
9. Laoide BM, Foulkes NS, Schlotter F, Sassone-Corsi P. The functional versatility of CREM is determined by its modular structure. EMBO J 1993;12:1179–91.
10. Daniel PB, Rohrbach L, Habener JF. Novel cyclic adenosine 3′, 5′-monophosphate (cAMP) response element modulator theta isoforms expressed by the two newly identified cAMP-responsive promoters active in the testis. Endocrinology 2000;141:3923–30.
11. Delmas V, Laoide B, Masquilier D, de Groot RP, Foulkes NS, Sassone-Corsi P. Alternative usage of initiation codons in CREM generates regulators with opposite functions. Proc Natl Acad Sci USA 1992;89:4226–30.
12. Molina CA, Foulkes NS, Lalli E, Sassone-Corsi P. Inducibility and negative autoregulation of CREM: an alternative promoter directs the expression of ICER, an early response repressor. Cell 1993;75:875–86.
13. Blendy JA, Kaestner KH, Schmid W, Gass P, Schutz G. Targeting of the CREB gene leads to up-regulation of a novel CREM mRNA isoform. EMBO J 1996;15:1098–106.
14. Nantel F, Monaco L, Foulkes NS, Masquilier D, LeMeur M, Henriksen K, et al. Spermiogenesis deficiency and germ cell apoptosis in CREM-mutant mice. Nature 1996;380:159–62.
15. Blendy JA, Kaestner KH, Weibauer GF, Nieschlag E, Schutz G. Severe impairment of spermatogenesis in mice lacking the CREM gene. Nature 1996;380:162–5.
16. Weinbauer GF, Behr R, Bergmann M, Nieschlag E. Testicular cAMP responsive element modulator (CREM) protein is expressed in round spermatids but is absent or reduced in men with round spermatid maturation arrest. Mol Hum Reprod 1998;4:9–15.
17. Peri A, Krausz C, Cioppi F, Granchi S, Forti G, Francavilla S, et al. cAMP-responsive element modulator gene expression in germ cells of normo- and oligoazoospermic men. J Clin Endocrinol Metab 1998;83:3722–6.
18. Lin WW, Lamb DJ, Lipshultz LI, Kim ED. Absence of cAMP responsive element modulator expression at the spermatocyte arrest stage. Fertil Steril 1998;69:533–8.
19. Calogero AE, Garofalo MR, Barone N, De Palma A, Vicari E, Romeo R, et al. Spontaneous regression over time of the germinal epithelium in a Y chromosome-microdeleted patient. Hum Reprod 2001;16:1845–8.
20. Johnsen SG. Testicular biopsy score count—a method for registration of spermatogenesis in human testes: normal values and results in 335 hypogonadal males. Hormones 1970;1:2–25.
21. Clermont Y. The cycle of the seminiferous epithelium in man. Am J Anat 1963;112:35–46.
22. Holstein AF, Roosen-Runge RC. Atlas of human spermatogenesis. Berlin: Grosse-Verlag; 1981:224.
23. Colpi GM, Hargreave TB, Papp GK, Pomerol JM, Weidner W. European Association of Urology: guidelines on infertility. European Association of Urology: Guidelines. Healthcare Office, eds. Arnhem, The Netherlands: Drukkerij Gelderland bv; 2001;1–57.