Gene xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Differentially expressed genes in the testicular tissues of adenylyl cyclase 3 knockout mice

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ARTICLE INFO

Article history:
Received 5 July 2016
Received in revised form 10 November 2016
Accepted 14 November 2016
Available online xxxx

Keywords:
Adenylyl cyclase 3
Differentially expressed genes
Testicular tissues
cAMP
Gene chip hybridization

ABSTRACT

Adenylate cyclase 3 (*AC3*) is an important component of the cyclic adenosine 3′,5′-monophosphate (cAMP) signaling pathway and converts adenosine triphosphate into cAMP. Male mice with *AC3* deletion ($AC3^{-/-}$) are sterile. However, the mechanical mechanism remains unclear. By TUNEL staining, we found that cell apoptosis in the testicular tissues of $AC3^{-/-}$ mice increased significantly compared with that in the wild-type ($AC3^{+/+}$) mice. Differentially expressed genes regulated by *AC3* in the testicular tissues were identified by gene chip hybridization. We observed that the expression of 693 genes was altered in the testicular tissues of $AC3^{-/-}$ mice, including 330 up-regulated and 363 down-regulated gene expression with fold changes higher than 2 (≥2) as the standards. Furthermore, part of these differentially expressed genes was verified by the real-time fluorescence quantification PCR and immunofluorescent staining. The expression levels of the genes related to olfactory receptors, cell apoptosis, transcriptional activity, defensive reaction, cell adhesion, cell death, and immunoreactions were significantly altered in the testicular tissues of $AC3^{-/-}$ mice compared with $AC3^{+/+}$ mice. In addition, the corresponding Ca^{2+}, cAMP, and cell adhesion signaling pathways, as well as the signaling pathways related to axon guidance and cell interaction, were altered significantly in the $AC3^{-/-}$ mice. These data would help elucidate the general understanding of the mechanisms underlying the sterility in $AC3^{-/-}$ male mice.

1. Introduction

Cyclic adenosine 3′,5′-monophosphate (cAMP) is a universal broad-spectrum messenger in cells and plays a wide role in various biological systems (Sadana and Dessauer, 2009; Dessauer, 2009). cAMP is important during spermatogenesis, particularly in cooperating with Ca^{2+} to maintain sperm activity. To maintain normal reproductive capacity, cAMP must adjust the functions of sperm from different aspects, including the capacitation and acrosomal reaction (Flesch et al., 2001; Gadella and Harrison, 2000; Demarco et al., 2003; Wang et al., 2003; Breitbart and Spungin, 1997; Wertheimer et al., 2013), membrane lipid reconstruction (Flesch et al., 2001; Gadella and Harrison, 2000), hyperpolarization of sperm membrane (Demarco et al., 2003), increase in pH inside cells (Demarco et al., 2003; Wang et al., 2003), increase in Ca^{2+} cell content, enhancement of the tyrosine phosphorylation of proteins (Visconti et al., 1995), and adenosine triphosphate (ATP) synthesis (Xie et al., 2006). Studies indicates that the Ca^{2+} control of sperm movement is realized by regulating Ca^{2+} concentration in the sperms (Felix, 2005), and cAMP levels in the testes can increase the sperm Ca^{2+} concentration. Most members of the cAMP signal pathway are olfactory receptors (ORs). The *OR* gene is expressed not only in olfactory tissues, but also in some male germ cells of mammals, including human beings (Zhang et al., 2004; Parmentier et al., 1992). Adenylate cyclase 3 (*AC3*) is an important component of the cAMP signal transduction pathway; *AC3*'s main biological function is converting ATP to cAMP (Johnson and Leroux, 2010; Defer et al., 2000). *AC3* in bodies inhibits protein kinase II, which is activated by calmodulin through phosphorylation (Defera et al., 1998). More importantly, male mice with *AC3* deletion present sperm motility defects (Livera et al., 2005) and weak ability of sperms penetrating oocyte complexes (Livera et al., 2005) as manifested by sterility.

Spermatogenesis in the testis is a highly complicated process that requires the coordinated and ordered interaction of spermatogenic, sustentacular, and interstitial cells of different periods. This process involves various biological processes, such as cell adhesion, maintenance of cell polarity, control of cell cycle, regulation of various hormones, and balance of cell apoptosis. Spermatogenesis is a highly strict process

Abbreviations: AC3, adenylate cyclase 3; cAMP, cyclic adenosine 3′,5′-monophosphate; ATP, adenosine triphosphate; GO, gene ontology; QRT-PCR, real-time fluorescence quantification PCR; OR, olfactory receptor.

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http://dx.doi.org/10.1016/j.gene.2016.11.026
0378-1119/

of generating mature sperms that involves mitosis, meiosis, and spermiogenesis (Skinner, 1991). The gene expression at specific phases regulated by transcription factor importantly controls the effect of this process. In particular, some important changes occur in early spermiogenesis, when the transcription mechanism is the most active (Sassone-Corsi, 1997). *AC3* is expressed in this phase, but the importance of *AC3* during spermatogenesis remains to be determined.

Gene chip technology is characterized by high speed, high throughput, intensification, and low cost. This technology is widely applied in the screening and identification of differentially expressed genes. Guo et al. identified 1176 differentially expressed genes in spermatogenic cells by gene chip technology and found 115 genes expressed in different developmental stages of specific spermatogenic cells (Guo et al., 2004). By gene chip hybridization with testicular tissues of *CREM* wild and deletion mice, Kosir et al. identified 4706 differentially expressed genes. Among these genes, 1822 are down-regulated and 2884 are up-regulated. The expression of genes related to spermatogenesis, the cAMP signaling pathway, cell-cycle adjustment, and cell junctions are altered (Kosir et al., 2012).

In this study, we analyzed cell apoptosis in the testicular tissues of $AC3^{+/+}$ and $AC3^{-/-}$ mice and found that cell apoptosis in the testicular tissues of the $AC3^{-/-}$ mice increased significantly. Differentially expressed genes regulated by *AC3* in the testicular tissues were identified by gene chip hybridization method. The bioinformatics analysis of these differentially expressed genes revealed that the expression levels of many genes related to cell apoptosis, cell junction, olfactory receptors and cAMP pathway were altered in the testicular tissues of $AC3^{-/-}$ male mice.

2. Materials and methods

2.1. Animals and sample treatment

$AC3^{+/-}$ mice were obtained from the Storm Laboratory of the University of Washington at Seattle. $AC3^{+/+}$ and $AC3^{-/-}$ mice were bred from heterozygotes in the SPF room of the Laboratory Animal Center of the Hebei University and genotyped using PCR as previously described (Wang and Storm, 2006). Briefly, genomic DNA was isolated from mouse tails. 1 μL DNA, about 100 ng, was used as templates in a 25-μL PCR volume. The primers used were listed in Table S1. The PCR amplifications were performed in a 2720 thermal cycler (Applied Biosystems, NY, USA) as follows: 5 min at 94 °C, 32 cycles of 45 s at 94 °C, 1 min at 58 °C, and 1 min 30 s at 72 °C, followed by a 7-min hold at 72 °C. 10 μL of PCR product were electrophoresed to differentiate the genotypes of mice. Three-month-old male mice with identified genotypes were sacrificed by the dislocation method, and testes were collected and placed in liquid nitrogen. The testes were subsequently stored under −80 °C for later RNA extraction and PCR testing. In a histological section, three-month-old male mice with identified genotype were injected with pentobarbital sodium (100 mg/kg body weight) for anesthesia. Animals were transcardially perfused with 4% paraformaldehyde (PFA, pH 7.4) and testis were collected and placed in 4% PFA for fixing for 3 days. Then, the organs were dehydrated with 30% sucrose for 3 days and then treated with OCT embedding. Afterward, the testes were cut into 10 μm-thick slices by the freezing microtome, which were stored under −80 °C for the following tissue dyeing test. All the treatment methods and operations of testing animals conformed to the *Guidance Suggestions of Caring Laboratory Animals* issued by the Ministry of Science and Technology of the People's Republic of China and were approved by Animal Ethics and Caring Committee of the Hebei University.

2.2. TUNEL staining

To test the DNA integrity of the testis, a TUNEL in situ apoptosis detection kit (Cat#G3250; Promega, Madison, WI, USA) was used for staining of the frozen testicular sections. First, testicular sections were cleaned with PBS for three times and then digested by proteinase K (20 g/mL) under room temperature for 15 min. Subsequently, equilibrium liquid was added for incubation (10 min) under room temperature.

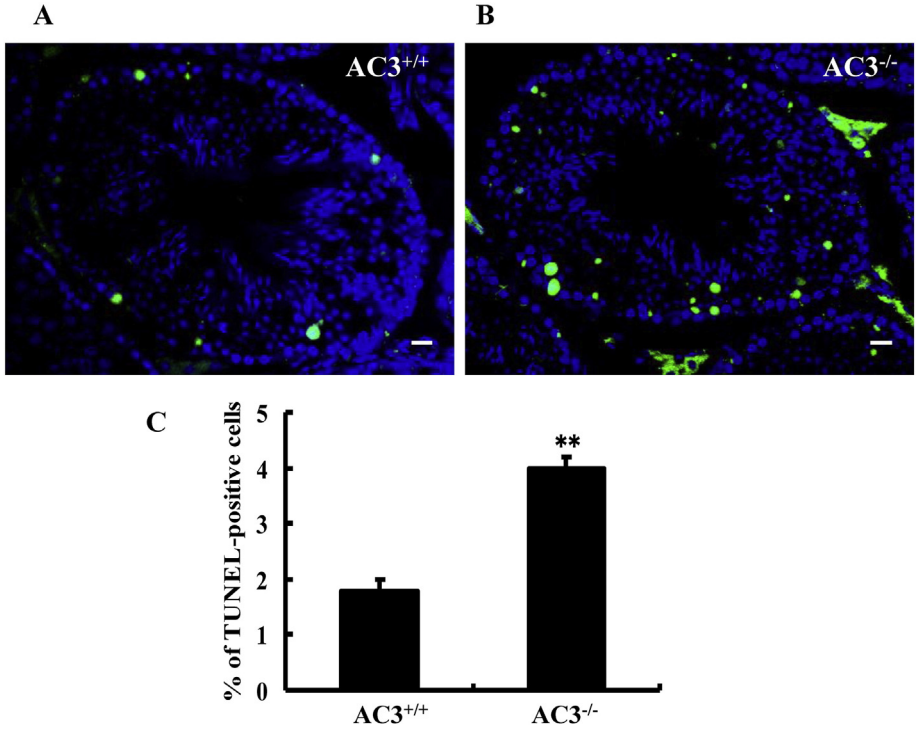

Fig. 1. Apoptotic cells in the testicular tissues of the $AC3^{-/-}$ mice increased significantly. TUNEL immunofluorescent staining was performed on the testicular tissue sections of the $AC3^{+/+}$ (A) and $AC3^{-/-}$ mice (B). Green fluorescence indicates the TUNEL-positive cells. (C) is the proportion of TUNEL-positive cells in the seminiferous tubule (n = 3; **P < 0.01, scale plate = 20 μm).

Please cite this article as: Zhang, J., et al., Differentially expressed genes in the testicular tissues of adenylyl cyclase 3 knockout mice, Gene (2016), http://dx.doi.org/10.1016/j.gene.2016.11.026