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·*Original Article*·

Age-dependent expression of the cystatin-related epididymal spermatogenic (*Cres*) gene in mouse testis and epididymis

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Abstract

Aim: To investigate the spatial and temporal expression of the cystatin-related epididymal spermatogenic (*Cres*) gene in mouse testis and epididymis during postnatal development. **Methods:** The QuantiGene assay and indirect immunofluorescence technique were used to examine the *Cres* mRNA and Cres protein level in mouse testis and epididymis on postnatal days 14, 20, 22, 28, 35, 49, 70 and 420. **Results:** (1) In both the testis and epididymis, *Cres* mRNA was first detected on day 20, then it increased gradually from day 20 to day 70, and the high expression level maintained till day 420. (2) In the testis, the Cres protein was exclusively localized to the elongating spermatids and was first detected on day 22. The number of Cres-positive spermatids increased progressively till day 49. From day 49 to day 420, the number of Cres-positive cells was almost stable. (3) The Cres protein was first detected on day 20 in the proximal caput epididymal epithelium. By day 35, the expression level of the Cres protein increased dramatically and the high level was maintained till day 420. Moreover, the luminal fluid of the midcaput epididymis was also stained Cres-positive from day 35 on. No Cres-positive staining was observed in distal caput, corpus and cauda epididymis throughout. **Conclusion:** The *Cres* gene displays a specific age-dependent expression pattern in mouse testis and epididymis on both the mRNA and protein level. *(Asian J Androl 2007 May; 9: 305–311)*

Keywords: cystatin-related epididymal spermatogenic gene; spermatogenesis; sperm maturation; development

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1 Introduction

Mammalian spermatozoa are produced in the testis, and they progressively acquire their functional capacities of forward motility as they migrate through the epididymis. Many changes, both morphological and biochemical, take place during this development and maturation process. These changes are the result of protein interactions within germ cells and between germ cells

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and the microenvironment of the testis and epididymis. In other words, spermatogenesis and sperm maturation are regulated not only by gene expression in the germ cells but also by the microenvironment which is created and maintained through the precise expression of a series of genes in the testis and epididymis. To elucidate the molecular basis underlying sperm development and maturation, many genes which are specifically or highly expressed in the testis and/or the epididymis have been identified in the past few years [1–4] and the cystatin-related epididymal and spermatogenic (*Cres*) gene is among them [5].

The *Cres* gene encodes a protein which exhibits substantial homology with members of the family 2 cystatins in the cystatin superfamily of cysteine proteinase inhibitors [5]. This homology includes four highly conserved cysteine residues in exact alignment as that in the cystatins as well as other regions of sequence characteristic of the cystatins [6,7]. Despite of all the similarities, the Cres protein lacks two of the three motifs thought to be necessary for the inhibition of cysteine proteinases [7, 8]. Therefore, Cres protein may not function as a typical cystatin. Furthermore, unlike the ubiquitous expression of the members of the cystatin family, *Cres* gene shows a highly tissue-specific expression pattern. Cres mRNA is mainly expressed in postmeiotic germ cells and the proximal caput epididymis with less expression in anterior pituitary gonadotrophs and corpus luteum in the ovary [5, 9–11]. These observations suggest that Cres protein defines a new subgroup in the family 2 cystatins and may perform a unique function distinct from that of the classic cystatin proteins.

The purpose of this study is to investigate the spatial and temporal expression pattern of *Cres* gene in mouse testis and epididymis during postnatal development. By using a more sensitive method, this study further characterizes the age-dependent change of Cres mRNA expression.

2 Materials and methods

2.1 Animals

Intact male BALB/c mice were purchased from the Animal Center of the Chinese Academy of Sciences (Shanghai, China), and were divided into 8 groups (n = 3) according to their postnatal age (14, 20, 22, 28, 35, 49, 70 and 420 days). The testes and epididymides were removed immediately after the animals were killed by cervical dislocation. The tissues were either placed in RNAlater (QIAGEN, Hilden, Germany) until used for QuantiGene assay, or fixed in Bouin's solution for indirect immunofluorescence.

2.2 QuantiGene assay

Three sets of oligonucleotide probes were denoted capture extenders (CEs), label extenders (LEs), and blocking probes (BLs), respectively. The probe sets specific for the Cres and β-actin (constitutive control) mRNAs were designed and synthesized by Genospectra (Fremont, CA, USA). The common sequence of the two Cres transcripts was utilized for the probe design. The mRNAs were quantified using the QuantiGene Explore kit (Genospectra, Fremont, CA, USA) as per the manufacturer's protocol. Briefly, the testes and epdidymides were homogenized in lysis buffer (600 μL/10 mg tissue), and 10 μL of the tissue homogenate was added to each well of a 96-well Capture Plate with 80 μL lysis working reagent and 10 μL combined probe sets. The probes were allowed to hybridize to specific mRNAs at 53ºC for 18 h. Excess probes were removed by rinsing with wash buffer. The captured mRNAs were then hybridized with branched DNA (bDNA) amplifier and alkaline phosphatase-labeled probe subsequently at 53ºC for 60 min. After incubation with the substrate solution at 53ºC for 30 min, luminescence was measured with a Perkin-Elmer EnVision luminometer (Perkin-Elmer, Wellesley, MA, USA). All values are the ratio of relative light units (RLU) of the Cres to that of the β-actin, and data are expressed as the mean ± SE. Data are representative of three independent experiments.

2.3 Indirect immunofluorescence

Following fixation and embedding, tissue sections of 6 μm were cut. The sections were microwaved for 5 min in 0.01 mol/L citric acid buffer, and then incubated in 5% bovine serum albumin (BSA) for 60 min at room temperature to block nonspecific binding. The 1:400 diluted polyclonal rabbit anti-mouse CRES antiserum [9] was applied and incubated overnight at 4ºC. Negative control sections were incubated with preimmune rabbit serum (1:400) instead of the antiserum. The sections were rinsed in PBS and then incubated with a 1:100 diluted FITC-conjugated goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA, USA) for 1 h at room temperature. Following PBS washes, the slides were inverted onto coverslips containing glycerol/PBS. After taking photographs with an LSM-510 laser scanning

confocal microscope (Carl Zeiss, Oberkochen, Germany), the immunofluorescence-stained sections were re-stained with hematoxylin-eosin for cell discrimination.

3 Results

3.1 Cres mRNA expression

QuantiGene assay uses a signal-amplification probe to quantify mRNA without RNA purification and reverse transcription, and generate data which are consistent, reproducible and less prone to possible artifacts [12]. Since the two transcripts of the *Cres* gene showed similar expression patterns [13], common sequence between them was used for the probe design. Therefore, the quantification is a sum of the two transcripts.

QuantiGene assay showed that in the testis, a very low level of Cres mRNA was detected on day 20. The expression of Cres increased gradually thereafter, with significant increase from day 22 to day 28 (15 fold), and reached a peak by day 70 (Figure 1A). In the epididymis, a similar expression pattern was obtained except that the most dramatic increase occurred from day 28 to day 35 (4.5 fold) and remained elevated from day 70 to day 420 (Figue 1B). QuantiGene assay still detected a steady increase of Cres mRNA after day 35 in both the testis and epididymis, which was not observed by the less sensitive RT-PCR method[13].

3.2 Cres protein expression

In mouse testis, no Cres-positive cells could be detected from day 14 to day 20 (data not shown). On day 22, elongating spermatids could first be found in a few seminiferous tubules and these cells were identified as Cres-positive (Figure 2A, yellow arrow). However, non-specific positive cells could also be observed on the negative control sections at this stage (Figure 2B), and these cells proved to be preleptotene spermatocytes when the sections were restained with hematoxylin and eosin. The non-specific positive staining of preleptotene spermatocytes also appeared on day 28 (Figure 2D), but disappeared from day 35 on (Figure 2F, H). Moreover, non-specific staining was also found in the testicular interstitium (Figure 2B, D, F, H). Cres-positive cells increased dramatically from day 28 to day 35, and continued to increase till day 49 due to the increasing number of the elongating spermatids (Figure 2C, E, G). From day 49 to day 420, the number of Cres-positive cells was almost stable. Although the number of positively-stained cells was closely related to the age of the mouse, the stain intensity of Cres protein was mainly correlated with the stages of the cycle of the seminiferous epeithelium rather than the developmental stages of the mouse. That is, Cres protein was weakly positive in early elongating spermatids of stages IX–XI, and showed strong positive staining in mid-elongating spermatids of stages XII–V, then the stain intensity became weak again in late elongating spermatids of stages VI–VIII (Figure 3).

In mouse epididymis, no Cres-positive cells could be observed on day 14 when the epididymis consisted of low columnar undifferentiated cells (data not shown). From day 20 to day 28, weakly stained Cres-positive cells were identified in the proximal caput epididymal

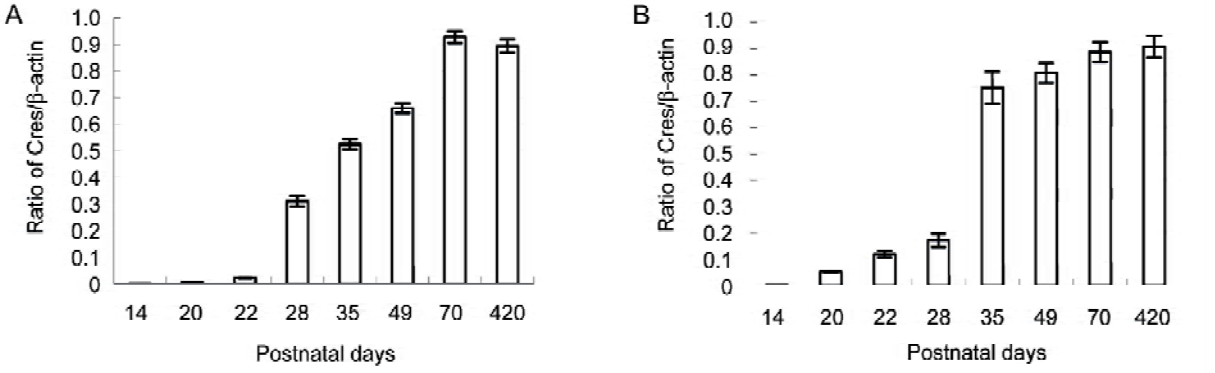

Figure 1. QuantiGene assay of Cres mRNA expression from mouse testis (A) and epididymis (B) at postnatal day 14, 20, 22, 28, 35, 49, 70 and 420. All values are the ratio of relative light units (RLU) of the Cres to that of the β-actin, and data are expressed as mean ± SE. Data are representative of three independent experiments.

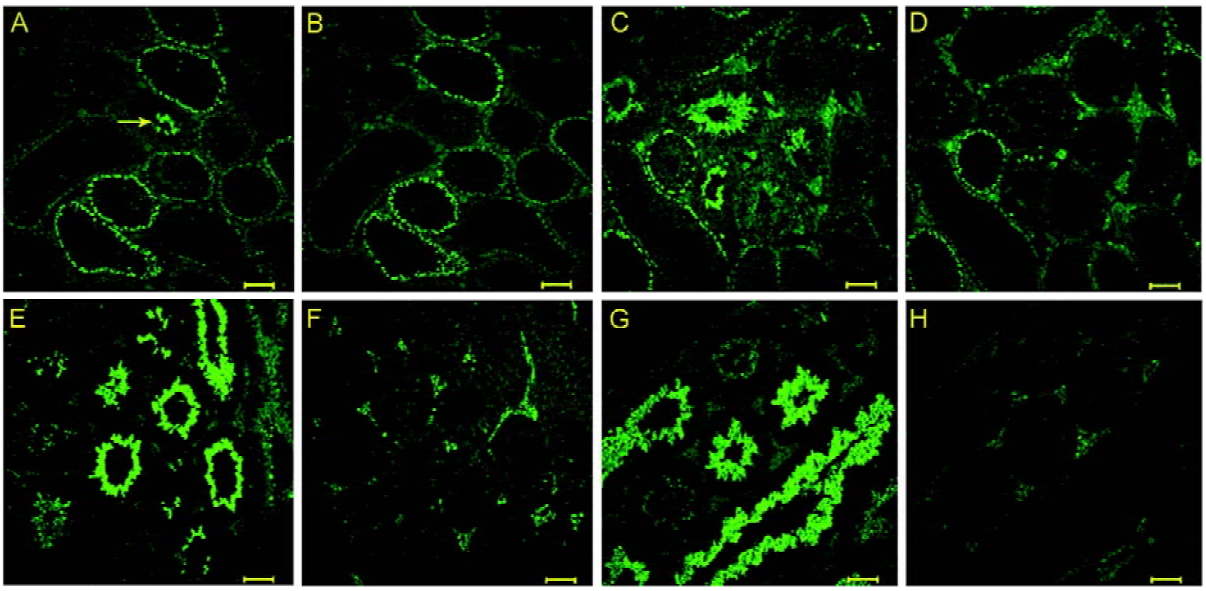

Figure 2. Cres protein expression in mouse testis at different postnatal stages. (A): Testicular section of postnatal day 22 stained with anti-Cres antiserum, the arrow indicates Cres-positive cells. (B): Negative control section of day 22 stained with preimmune serum, showing non-specific staining. (C, E, G): Testicular sections at postnatal day 28, 35, 49, respectively, stained with anti-Cres antiserum. (D, F, H): Negative control sections of postnatal day 28, 35, 49, respectively. Bar = 50 μm.

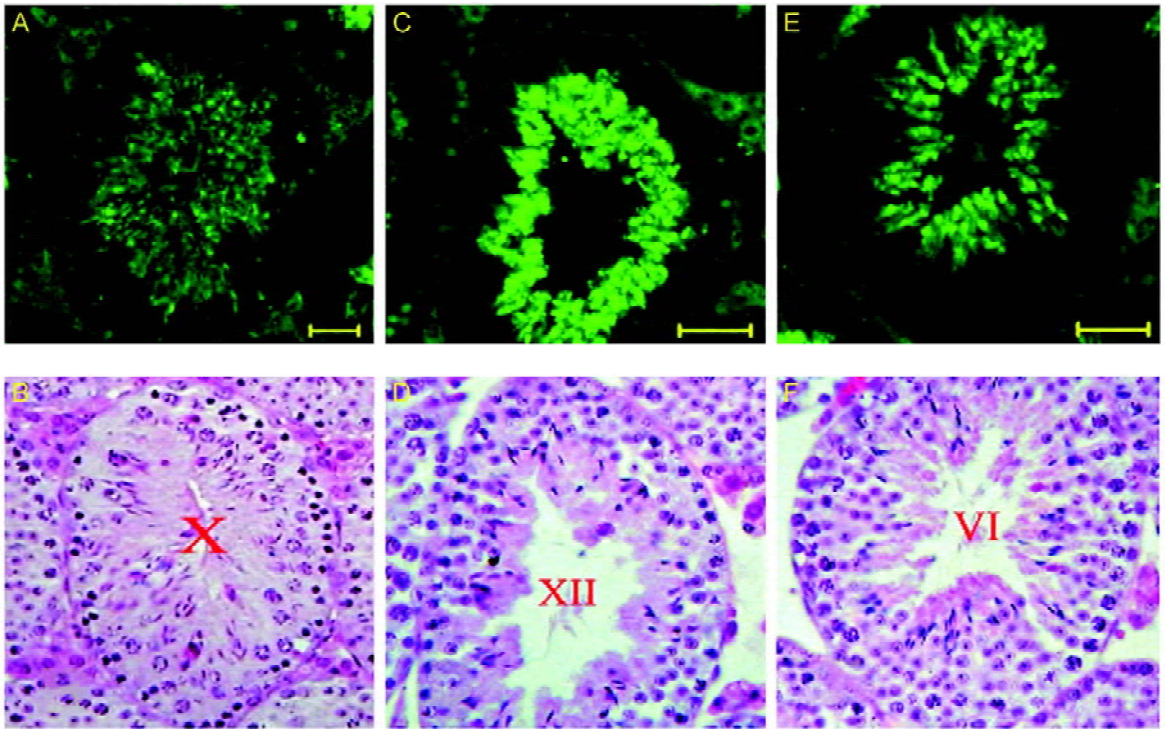

Figure 3. Immunostaining of Cres protein in elongating spermatids of different stages. The Cres-positive cells in sections A, C, E are elongating spermatids of stages X, XII, VI, respectively. B, D, F: The same sections to A, C, E, restained by hematoxylin and eosin after immunofluorescence staining. Bar = 20 μm.

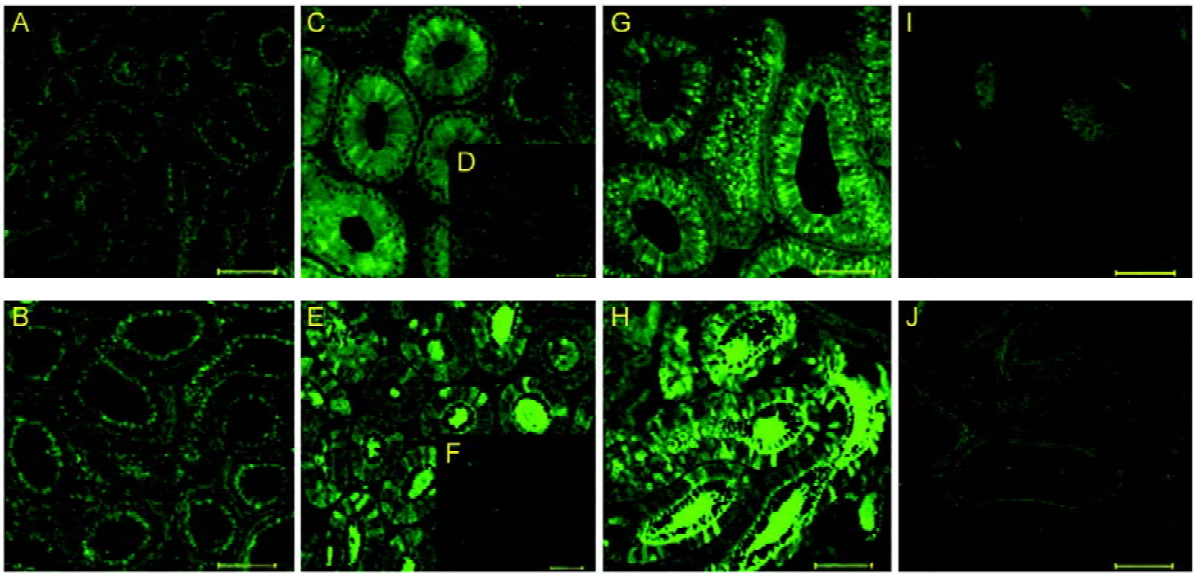

Figure 4. Cres protein expression in mouse epididymis at different postnatal stages. (A, B, C, G): Proximal caput epididimal sections on postnatal day 20, 28, 35, 70, respectively, stained with anti-Cres antiserum. (E, H): Midcaput epididymal sections on day 35, 70, respectively, stained with anti-Cres antiserum. (I, J): Distal caput and corpus epididymal sections on day 70, stained with anti-Cres antiserum. (D, F): Negative control sections of the proximal caput (D) and midcaput (E) on day 35, stained with preimmune antiserum. Bar = 50 μm.

epithelium, and the Cres protein was localized to the supranuclear region of the principal cells (Figure 4A, B). No positive staining was detected throghout the remainder of the epididymis. Cres protein expression increased dramatically from day 28 to day 35, then the high expression level maintained till day 420 (Figure 4C, E, G, H). Moreover, a region-specific expression pattern of Cres protein was observed from day 35 on, that is, Cres protein was restricted to the principal cells of the proximal caput epididymis (Figure 4C, G) as well as part of the cells and luminal fluid of the midcaput epididymis (Figure 4E, H). By the distal caput epididymis, the Cres protein had disappeared from both the tissue and luminal fluid and was not detected throughout the remainder of the epididymis (Figure 4I, J). No positive staining was observed on any of the negative control sections (Figure 4D, F).

4 Discussion

In this study, we demonstrated that Cres mRNA was first detected in mouse testis on day 20 of postnatal age when round spermatids first appeared in the seminiferous tubules [14]. Cres protein was first detected on day 22 when early elongating spermatids began to emerge. These results suggest that a delay exists between *Cres* gene transcription and translation. This is also the case in the adult mouse testis where Cres mRNA is mainly transcribed in round spermatids, while Cres protein is synthesized in elongating spermatids [9]. This is not surprising as all mRNA required for the later stages of spermatogenesis must be produced earlier and is stored until needed–known as translational delay. Hsia *et al* [15] found Cres mRNA was first detected on day 22 in mouse testis when round spermatids first appeared. It seemed that *Cres* gene transcription always corresponds with the appearance of round spermatids rather than the different birth date among various strains of mice. The Cres mRNA increased dramatically from day 22 to day 28, whereas the predominant increase of Cres protein occurred from day 28 to day 35. These results reconfirmed the translational delay. Non-specific immunofluorescence-staining in preleptotene spermatocytes was observed from day 14 to day 28. Cornwall *et al.* [9] found the Cres antibody cross-reacted with a 24 kDa protein when conducting Western blot analysis. However, Western blot using preimmune serum did not detect the Cres protein and the 24 kDa protein. So we don't think

the non-specific staining was caused by the 24 kDa protein. Moreover, the non-specific staining totally disappeared after day 35. This is probably because the gene expression and protein synthesis in early meiotic cells or spermatogonia are different at various postnatal stages. Our results also showed that with the proliferation of germ cells from puberty (day 35-49) to adult (day 70), a further increase of Cres mRNA and its protein was detected. No drastic decrease of *Cres* gene expression was observed in 420-day-old mouse testis. However, we can not rule out the possibility that a further decline of Cres mRNA and its protein might be observed if older mice are examined.

Indirect immunofluorescence also found that although the number of Cres-positive cells varied a lot at different ages, the staining intensity was mainly dependent on the stages of the cycle of the seminiferous epithelium rather than the developmental stages of the mouse. This stage-specific expression was consistent with previous studies in adult mouse testis [9]. According to Syntin *et al* [16], the Cres protein is packaged into the sperm acrosomes of late stage elongating spermatids and then is released during the acrosome reaction at the time of fertilization. The age-dependent and stage-specific expression pattern of the *Cres* gene strongly suggested its involvement in spermatogenesis, especially in the process of spermiogenesis since Cres protein expression corresponds approximately with the onset of spermatid elongation.

In mouse epididymis, the *Cres* gene also showed an obvious age-dependent expression manner. Different from the transcription-translation delay in the testis, the expression of the Cres protein in the epididymis closely parallels the expression of the Cres mRNA. Both of the mRNA and the protein were first detected on day 20, and the most dramatic increase happened from day 28 to day 35. It should be noted that from day 35 on, the spermatozoa produced in the testis continuously pass through the epididymis to acquire their forward motility and fertilizing capacity. And it is on day 35 that the Cres protein began to show a more distinct region-specific localizaion. Cres-positive staining was detected not only in the proximal caput epididymal epithelium, but also in the luminal fluid of the midcaput. However, the Cres protein totally disappeared from the distal caput epididymis. The staining was not likely caused by break down of dead spermatozoa or phagocytosis of these cells by the epididymal epithelium, because dead spermatozoa existed throughout all parts of the epididymis whereas the staining was region-specific. Taken together, the age-dependent and region-specific expression pattern of the *Cres* gene implied its importance in sperm maturation because the microenvironment of the caput region has been shown to be essential for sperm to acquire their forward motility [17].

Our results, together with previous reports, suggested the Cres protein may possess two different functions: one within the elongating spermatids and another within the epididymis. The Cres protein could thus be considered a "moonlighting" protein. Moonlighting proteins have been recently described as proteins that possess multiple functions and subcellular localizations. Many of these proteins have been reported. For example, the selenoprotein phospholipid hydroperoxide glutathione peroxidase (PHGPx) exists as a soluble peroxidase in spermatids and also as a structural protein on the tail midpiece of the fully differentiated spermatozoa [18]. Thus, depending upon its localization within the elongating spermatids or in the caput epididymis at various postnatal stages, the Cres protein may play multiple roles as do other moonlighting proteins.

In conclusion, the *Cres* gene shows a specific age-dependent expression pattern in mouse testis and epididymis on both the mRNA and protein level, which may indicate its dual functions of the *Cres* gene during spermatogenesis and its involvement in sperm maturation.

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