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Cloning, Characterization and Primary Function Study of a Novel Gene, *Cymg1*, Related to Family 2 Cystatins

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Abstract Cystatins are cysteine proteinase inhibitors. We found two expression sequence tags (ESTs), CA463109 and AV042522, from a mouse testis library using Digital differential display (DDD). By electrical hybridization, a novel gene, *Cymg1* (GenBank accession No. AY600990), which has a full length of 0.78 kb, and contains four exons and three introns, was cloned from a mouse testis cDNA library. The gene is located in the 2G3 area of chromosome 2. The full cDNA encompasses the entire open reading frame, encoding 141 amino acid residues. The protein has a cysteine protease inhibitor domain that is related to the family 2 cystatins but lacks critical consensus sites important for cysteine protease inhibition. These characteristics are seen in the CRES subfamily, which are related to the family 2 cystatins and are expressed specifically in the male reproductive tract. CYMG1 has a 44% (48/108) identity with mouse CRES and 30% (42/140) identity with mouse cystatin C. Northern blot analysis showed that the *Cymg1* is specifically expressed in adult mouse testes. Cell location studies showed that the GFP-tagged CYMG1 protein was localized in the cytoplasm of HeLa cells. Immunohistochemistry revealed that the CYMG1 protein was expressed in mouse testes spermatogonium, spermatocytes, round spermatids, elongating spermatids and spermatozoa. RT-PCR results also showed that *Cymg1* was expressed in mouse testes and spermatogonium. The *Cymg1* expression level varied in different developmental stages: it was low 1 week postpartum, steadily increased 2 to 5 weeks postpartum, and was highest 7 weeks postpartum. The expression level at 5 weeks postpartum was maintained during 13 to 57 weeks postpartum. The *Cymg1* expression level in the testes over different developmental stages correlates with the mouse spermatogenesis and sexual maturation process. All these indicate that *Cymg1* might play an important role in mouse spermatogenesis and sexual maturation.

Key words cystatins; gene cloning; testis; spermatogenesis; sexual maturation

Abnormalities in the structure and function of the testes can result in diseases that are seriously harmful to human reproduction and health. Although several external factors have been claimed to affect male reproduction, evidence that genetic factors play an important role is accumulating [1,2]. The cloning and study of testes-specific genes has important implications for theory and practice. Spermatogenesis is a complex process. Many genes have been confirmed to be essential for the process, but there are still other genes taking part in this process have not been identified. Identification of these new genes and their roles is of great importance in understanding the biology of spermatogenesis [3].

The cystatins are a superfamily of reversible competitive inhibitors of C1 cysteine proteases such as plant papain and the mammalian cathepsins B, H, and L [4]. The superfamily consists of three families: the stefins (family 1), cystatins (family 2), and kininogens (family 3). The family 2 cystatins, represented by cysteins C, D, E/M, F, S, SN, and SA, are secretory proteins, 13 kD in size, which contain two characteristic disulfide bonds in the C-terminus; some family members are glycosylated. Several genes, for example *Cres* (cystatin-related epididymal spermatogenic), have been identified to be related to the

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family 2 cystatins. They have a cysteine protease inhibitor domain, but lack critical consensus sites that are important for cysteine protease inhibition. In addition, these genes are primarily expressed in the reproductive tract, suggesting that they may have evolved to perform tissue-specific functions that are distinct from those of the typical cystatins [5].

In this study, we used Digital differential display (DDD) to search against ESTs in mouse testis library, and a novel gene, *Cymg1*, was cloned through electric elongation. *Cymg1* encodes a protein (CYMG1) of 141 amino acid residues that may be a new member of the CRES subgroup of the family 2 cystatins. PCR, RT-PCR, Northern blotting, cellar location in HeLa cells, protein expression in *E. coli* M15 and immunohistochemistry techniques were used to confirm its tissue-specific expression in mouse testes and the molecular mass of its expression product.

Materials and Methods

Materials

The 50×Advantage 2 DNA polymerase, mouse testis Marathon-Ready cDNA library, Northern blot membrane and Express hybridization solution were purchased from Clontech (San Jose, USA). Multiple tissue Northern (MTN) blot (8 tissues) was purchased from Jun-Xuan Bioengineering Co. (Shenzhen, China). The RNA isolation kit was product from Gentra (Minneaplis, USA). The RevertAid™ first strand synthesis kit was purchased from Fermentas (MBI, Lithuanian). The primers were synthesized by BioAisa (Shanghai, China). The reagents for electrophoresis, culture medium, and dNTPs were purchased from the Fermentas (MBI, Lithuanian). DNA sequencing was performed by BioAisa (Shanghai, China). Spermatogonial cell line was GC-1, and was purchased from the ATCC Company (Virginia, USA).

EST searching and gene cloning [6,7]

The DDD, BLAST [6] and ExPASy software packages were used to search for ESTs [7] in the mouse testis library, and these ESTs were electrically elongated by using the CAP Sequence Assembly Machine software. *Cymg1* was cloned, which was supported by 61 ESTs, two of which came from mouse ovary and brain, the others came from mouse testis.

Bioinformatic analysis of the cDNA sequence of *Cymg1* and protein characterization of CYMG1 [8,9]

Translation from the cDNA into the amino acid sequence was carried out using the ORF Finder program in the NCBI and ExPASy servers [8]. The protein sequence was analyzed by using the BLAST NR program, and SMART-Simple Modular Architecture Research in the NCBI server [9].

RNA isolation [10]

The fresh mouse testis, epididymis, ovary, kidney, heart, liver, muscle, spleen, lung, cerebellum and cerebra were from adult C57BL/6 mice [10]; spermatogonial cells were cultured by our stem cell centre; testes from BALB/c mice at different developmental stages were frozen in liquid nitrogen upon dissection and stored at –70 °C. Total RNA was isolated using the Gentra system of RNA isolation according to the manufacturer's instructions.

PCR amplification of the full length *Cymg1*

The primer pair 5′-GGGAGGAGAGGGAAGTCAGA-3′and 5′-GGTGGTCCACTATCTTACAA-3′were designed according to the full length *Cymg1* and were used in the PCR assay with Advantage 2 DNA polymerase and Marathon-Ready mouse testis cDNA as a template. PCR amplification cycles involved initial denaturation at 95 °C for 1.5 min; 35 cycles at 94 °C for 40 s, 60 °C for 40 s and 68 °C for 40 s; 68 °C for 7 min; and then holding at 4 °C. The PCR products were separated on 1.5% agarose gel and analyzed. These PCR fragments were cloned into PMD18T vectors and sequenced.

RT-PCR analysis

Synthesis of first strand cDNA A reaction mixture containing 9 μl ribonuclease-free water, 2 μl total RNA (0.23–0.26 μg/μl) and 1 μl oligo (dT) primer (0.5 μg/μl) was incubated at 70 °C for 5 min and chilled on ice. Then 4 μl 5×reaction buffer, 1 ml ribonuclease inhibitor (20 U/μl) and 2 μl 10 mM dNTP were added. The mixture was incubated at 37 °C for 5 min, and then incubated at 42 °C for 60 min after 1 μl RevertAid (MBI, Lithuanian) M-MuLV reverse transcriptase (200 U/μl) was added. The reaction was terminated by heating at 70 °C for 10 min and then the mixture was kept at 4 °C.

PCR amplification of Cymg1 Using the primer pair 5′-TCTGGAAAGAAAATAGGAACTTGG-3′ and 5′-AAGAAAAGTAAGAGTGGCAAGGTG-3′, PCR was performed to investigate the *Cymg1* expression pattern in C57BL/6 mouse tissues. The amplification conditions were the same as described above.

Analysis of Cymg1 by RT-PCR [11] The RNA isolated from different developmental stages of BALB/c mice was

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reverse transcribed into cDNA. The amplification reaction was then performed in a 20 μl reaction mixture containing 13.55 μl deionized water, 2 μl 10×PCR reaction buffer, 2 μl 2.5 mM dNTP, 0.4 μl *Taq* DNA polymerase, 0.45 μl primer pair mixture (0.5 μg/μl) and 1.6 μl cDNA [11]. In a programmable thermal cycler (PE 9600, Inland Southern California, USA), the following reaction was carried out: initial denaturation at 95 °C for 90 s, and then 35 cycles of 94 °C for 40 s, 60 °C for 40 s and 72 °C for 40 s. The extension step in the last cycle was at 72 °C for 5 min, and the mixture was finally kept at 4 °C. The PCR products were then separated on a 1.5% agarose gel and analyzed using *G3PDH* as contrast.

Northern blotting [12]

The hybridization probe was obtained by PCR; it was amplified from the total testis cDNA library by using the primer pair 5′-GGGAGGAGAGGGAAGTCAGA-3′ and 5’- GGTGGTCCACTATCTTACAA-3′. The PCR product was 0.78 kb in size. The probe was purified by using the PCR production purification kit (TaKaRa Co., Japan), according to the manufacturer’s instructions. Finally, the probe was labeled with digoxigenin, following the guidance of the manufacturer (Roche, Basel, Switzerland). After pre-hybridization at 65 °C for 1 h in 5 ml Express Hybrization solution, the membrance was hybridized with denatured cDNA probe at 65 °C overnight followed by washing with (i) 2×SSC and 0.1% SDS at 65 °C for 10 min three times; (ii) with 0.1×SSC and 0.5% SDS at 62 °C for 15 min twice; (iii) with detection buffer for 5 min; and then (iv) with NBT/BCIP (nitro blue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) overnight [12].

Protein expression

The *Cymg1* cDNA was amplified with RT-PCR from fresh mouse testis, then cloned into the pMD18-T vector and the recombinant plasmid was sequenced. Then *Cymg1* cDNA was cloned into the *Sac*I and *Hin*dIII sites of pQE30. *E. coli* M15 was used as host for prokaryotic expression of the pQE30/*Cymg1* and subject to IPTG (isopropyl-β-*D*-thiogalactopyranoside) induction.

Construction pEGFP-C3-*Cymg1* recombinant plasmid

To allow a directional insertion of the whole ORF of *Cymg1* into the pEGFP-C3 vector, primers 5′-CGGAGCTCATGGCCAGATTCTTACAG-3′ (*Sac*I) and 5′-CCGTCGACAAGTAAGAGTGGCAAGGTG-3′ (*Sal*I) were used to amplify the target gene. After PCR amplification, the *Cymg1* encoding sequences were subcloned in the pMD18-T vector. Subsequently, the recombinant pMD18-T-*Cymg1* and pEGFP-C3 vectors were digested with the restriction endonucleases *Sac*I and *Sal*I (MBI, Lithuanian). Both digestion reactions were checked by using 1.5% agarose gel electrophoresis, and then the pEGFP-C3 vector and the gene fragments digested using *Sac*I and *Sal*I were purified. Then, a ligation reaction was done according to the manufacturer’s instructions (TaKaRa Co., Japan). DH5α cells were transformed with 10 μl of the ligation reaction products and spread on an LB agar plate containing 100 μg/ml ampicillin. Finally, individual clones were checked using DNA-sequencing to ensure that the fusion plasmids were correctly constructed.

Cell culture and transfection

HeLa cells were grown in minimal essential medium supplemented with 10% fetal bovine serum and 50 μg/ml penicillin and streptomycin, respectively. Cells were seeded to a density of about 2×10^6 cells on 35 mm culture dishes and then transiently transfected with plasmid DNA using Lipofectamine 2000 (Invitrogen, America). After transfection, cells were replaced after transfection onto 35 mm glass bottom dishes (MakTek, Turkey) and grown in medium containing 20 mM HEPES buffer (pH 7.4). The correct colonies emerged out strong green fluorescence excited by blue light under the fluorescent microscope. Images were acquired using excitation (488 nm) and emission (515 nm) wavelengths for GFP (Green fluorescence protein). Images were captured digitally and imported into Adobe Photoshop 6.0 for formatting.

Immunohistochemistry

Adult C57BL/6 mice were killed by CO_2 inhalation, then their testes were dissected and fixed overnight in Bouin’s fluid. The fixed testes were washed with 70% ethanol to remove picric acid. The testes tissues were dehydrated in ethanol, embedded in paraffin, then cut into 5 μm thick sections. Then, the sections were gradually dewaxed using dimethylbenzene, 95% ethanol, 80% ethanol and water, 5 min for each. Immunohistochemistry was performed according to the procedure described by the manufacturer (SABC kit, Boster Co. Wuhan, China). Briefly, to neutralize endogenous peroxidase, the sections were immersed in methanol supplemented with 3% hydrogen peroxide. Then they were blocked with 10% normal goat serum in phosphate-buffered saline (PBS) for 10 min. After blocking, these sections were rinsed three times in PBS, each for 3 min and incubated with polyclonal rabbit anti-CTMG1 antibody diluted 1:50 in PBS for 30

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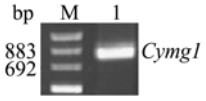

Fig. 1 PCR amplification of *Cymg1*
M, DNA marker (pUC mix 8); 1, *Cymg1*.

min at 37 °C. Being kept overnight at 4 °C, the sections were rinsed three times with PBS and then, the sections were incubated for 30 min at 37 °C with biotin peroxidase-conjugated goat anti-rabbit immunoglobulins diluted 1:100 in PBS. Finally, sections were rinsed three times with PBS and processed for staining with DAB substrate (0.05% DAB, 0.03% hydrogen peroxidase in PBS) for 1 min. Tissue sections were counterstained with methyl green (Sigma, America), then were observed and images were taken under the fluorescent microscope. The images were imported into Adobe Photoshop 6.0 for formatting.

Results

Cloning and sequencing of the full length cDNA

The full length cDNA was successfully cloned from adult mouse testes (**Fig. 1**). It was identical to the full-length cDNA cloned through assembling ESTs. The PCR product (780 bp) was sequenced and the result was identical to this gene. The novel gene was submitted to GenBank and was designated as *Cymg1* by the Mouse Genomic Nomenclature Committee.

Analysis of the sequence of *Cymg1* and encoding region

Analysis of conserved splice donor and acceptor dinucleotide sequences of Cymg1 The full-length cDNA was 0.78 kb in size, and contained 45.1% GC. BLAST searching against mouse chromosome mapping indicated that *Cymg1* is located in the mouse 2G3 area of chromosome 2 and contains four exons and three introns. The boundaries between exons and introns follow the gt-ag rule (**Table 1**).

Analysis of cDNA and protein sequences of Cymg1 *Cymg1* encodes a 141 amino acid residue protein. There is a start codon (ATG) in nucleotide position 280–282, a stop codon (TAA) at 703–705 and an additional stop codon (TGA) at 268–270 before the start codon. The ACCATGGCC sequence, in accordance with the Kozak rule, is found in the start region of the ORF and a potential polyadenylation signal (AATAAA), positioned at 750–754, is located at the 3′ end (**Fig. 2**).

Table 1 Exon-intron junctions in *Cymg1*

Exon	Exon size (bp)	5′ splice donor	Intron size (bp)	3′ splice acceptor	Intron
1	152	ATAAAG**gt**a			
2	357	GAGCAG**gt**g	2725	c**ag**CCGTGT	1
3	117	AAAAAG**gt**a	4822	c**ag**ATCACA	2
4	161		1993	t**ag**ATGGTG	3

Uppercase and lowercase letters indicate exon and intron sequences, respectively. Conserved splice donor and acceptor dinucleotide sequences are indicated in bold.

```
Gggaggagagggaagtcagaggccagaagcacctggggctcagatccccacagatgcctatctggaagcaggagcatgaacttggtatgctg
Gtgatcaagagcaacctgaggctagaggccatttaatcttagtcttcactacagataaagccgtgtcaggtgttgaagagaaaggatccatt
gacctgcagagaaagcaggggcctcactggctacattgacaccagctctggaaagaaaataggaacttggtatgttccttgaatgaagaagc
                                                                                          *
accatggccagattcttacagaccctgctgttcctggtgatcatggtggagtttgtatctagaagagtcgaagcctggggctccccacag
    M  A  R  F  L  Q  T  L  L  F  L  V  I  M  V  E  F  V  S  R  R  V  E  A  W  G  S  P  Q
attgtgaggccattcgaagacatccccaaatcctatgtctatgtgcagcatgcactctggtatgccatgaaagaatataacaaggccagc
 I  V  R  P  F  E  D  I  P  K  S  Y  V  Y  V  Q  H  A  L  W  Y  A  M  K  E  Y  N  K  A  S
aatgacctctacaacttcagggtggtggatatcctaaaatctcaggagcagatcacaggcagtctggagtattatcttgaagtaaacatt
 N  D  L  Y  N  F  R  V  V  D  I  L  K  S  Q  E  Q  I  T  D  S  L  E  Y  Y  L  E  V  N  I
gcccgaacaatgtgcaagaagattgcaggagataatgaaaactgcttgtttcaacaggatcctaaaatgaaaaagatggtgttttgcatt
 A  R  T  M  C  K  K  I  A  G  D  N  E  N  C  L  F  Q  Q  D  P  K  M  K  K  M  V  F  C  I
tttattgttagctccaaaccatggaagtttgaacttaaaatgctgaagaagcaatgcaaagatatctaatcagcattcgggacaccttgc
 F  I  V  S  S  K  P  W  K  F  E  L  K   M  L  K  K  Q  C  K  D  I  *
cactcttacttttcttttaactgaataaagatgcttgtaagatagtggaccacc
```

Fig. 2 cDNA and predicted protein sequences of *Cymg1*
The start codon sequence is underlined and bold; the polyadenylation signal is underlined and shadowed; stop codons are indicated by an asterisk and underline.

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Bioinformatics analysis of CYMG1

Analysis of the domain of CYMG1 The complete ORF of *Cymg1* encodes a predicted protein containing 141 aa with a signal peptide and without a trans-membrane region. CYMG1 has a theoretical molecular mass of 16.8 kD and a calculated isoelectric point of 9.0. PSORT WWW Server analysis showed that there is a 99.9% possibility of locating the protein in the cytoplasm. SMART results indicate that there is a cystatin domain from amino acid residue 43 to 139 (**Fig. 3**).

Blast homology of CYMG1 No gene completely identical to *Cymg1* was found through BLAST against NR. The results show that the largest ORF (1–141) of *Cymg1* has 100% identity with that encoded by mouse RIKEN cDNA 1700006C19, and 44% identity and 64% similarity with that encoded by mouse CRES (**Fig. 4**).

Analysis of the conserved regions of CYMG1 CYMG1 has significant sequence similarity to the family 2 cystatins, but lacks some of the motifs believed to be important for inhibition of cysteine proteinases. Analysis (**Fig. 5**) of the sequences of CYMG1, CRES and Cystatin C showed that, compared with cystatin C, CYMG1 and CRES lacked N-terminal glycine and a glutamine_/valine_/glycine (QX/VX/G) loop segment, which is important for the inhibition of cysteine proteinases, but retained the C-terminal PW site. In addition, the N-terminal region of cystatin C, which is responsible for its tight binding to cysteine proteases [16], is poorly conserved in CYMG1 and CRES, suggesting that like CRES, the function of CYMG1 may be also quite different from cystatin C.

RT-PCR in mouse multi-tissues and different developmental stages

The results (**Fig. 6**) show that *Cymg1* (512 bp) was expressed strongly in adult testes but not in other tissues. *G3PDH* was expressed in all kinds of tissues. **Fig. 7** shows that *Cymg1* was expressed in spermatogonial cells. **Fig. 8** shows that *Cymg1* was expressed in the testes of mice at different developmental stages, and that *G3PDH* was expressed in the testes of mice at all different development stages.

Northern blotting

To further investigate the transcription level of *Cymg1* in mouse adult multi-tissues and the transcript length, Northern blot analysis was carried out. Northern blotting (**Fig. 9**) showed that there was a 0.78 kb transcript only from adult testes. These results further confirmed the results above.

Expression of CYMG1 in *E. coli* M15

In order to get enough protein to carry out further research on function and to prepare antibodies against

Fig. 3 Cystatin domain of the CYMG1 protein

```
CYMG1   FEDIPKSYVYVQHALWYAMKEYNKASNDLYNFRVVDILKSQEQITDSLEYYLEVNIARTM  93
        F  I  S   V+  +W+AMKEYNK S D Y F V  IL ++ QITD +EY ++V I+R+
CRES    FGSINISNANVKQCVWFAMKEYNKESEDKYVFLVDKILHAKLQITDRMEYQIDVQISRSN  91

CYMG1   CKKIAGDNENCLFQQDPKMKKMVFCIFIVSSKPWKFELKMLKKQCKDI              141
        CKK   + ENC+ Q+ P+++K + C F+V + PW  E  +L K+CKD+
CRES    CKKPLNNTENCIPQKKPELEKKMSCSFLVGALPWNGEFNLLSKECKDV              139
```

Fig. 4 Comparison of CYMG1 with mouse CRES

```
CYMG1       MARFLQTLLFLVIMVEFVSRRVEAWGSPQIVRPFEDIPKSYVYVQHALWYAMKEYNKASNDLYNFRVVDILKSQ
CRES        MAKPLWLSLILFIIPVALAVGVDQSKNEVKAQNYFGSINISNANVKQCVWFAMKEYNKESEDKYVFLVDKILHAK
Cystatin C  MASPLRSLLFLLAVLGVAWAATPKQGPRMLGAPEEADANEEGVRRALDFAVSEYNKGSNDAYHSRAIQVVRAK

CYMG1       EQITDSLEYYLEVNIARTMCKKIAGDNENCLFQQDPKMKKMVFCIFIVSSKPWKFELKMLKKQCKDI
CRES        LQITDRMEYQIDVQISRSNCKKPLNNTENCIPQKKPELEKKMSC SFVGALPWNGEFNLLSKECKDV
Cystatin C  KQKVAGVNYFFDVEMGRTTCTKSQTNLTDCPFHDQPHMRKALCS FQIYSVPWKGTHSLTKFSCKNA
```

Fig. 5 Alignment of the CYMG1 amino acid sequence with mouse Cres and cystatin C

The three highly conserved regions important for inhibition of cysteine proteinases are shadowed and underlined.

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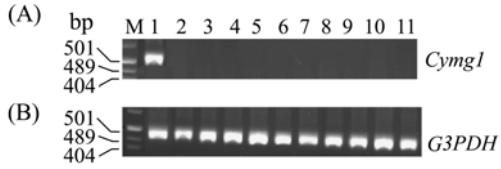

Fig. 6 RT-PCR results of *Cymg1* in multiple tissues
(A) 1, testis; 2, epididymis; 3, ovary; 4, kidney; 5, heart; 6, liver; 7, skeletal muscle; 8, spleen; 9, lung; 10, cerebellum; 11, cerebra; M, marker (pUC Mix8). (B) *G3PDH* in multiple-tissues.

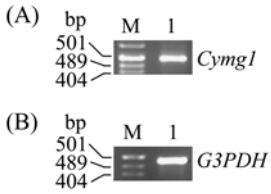

Fig. 7 RT-PCR results of *Cymg1* in spermatogonial cells
(A) M, marker (pUC Mix8); 1, spermatogonial cells. (B) M, marker pUC Mix8; 1, *G3PDH* in spermatogonial cells.

CYMG1, a pQE30-*Cymg1* recombinant plasmid was constructed. After IPTG induction, a sole fusion protein, which has a predicted molecular weight of 18.1 kD (1.3 kD+16.8 kD) was expressed in *E. coli* M15 and the band can be seen in the 12% gel (**Fig. 10**), although CYMG1 was expressed weakly in *E. coli* M15.

Cellular location of GFP-tagged CYMG1 and GFP protein

In HeLa cells transfected with pEGFP-C3-*Cymg1*, GFP-tagged CYMG1 protein was localized in the cytoplasm, but when the cells were transfected with pEGFP-C3, the GFP protein was dispersed throughout the cells (**Fig. 11**).

Immunohistochemistry

The results (**Fig. 12**) show that CYMG1 was expressed in mouse testis spermatogonium cells, spermatocytes, round spermatids, elongating spermatids and spermatozoa.

Discussion

In recent years, spermatogenesis has been a hot topic for discussion. The discovery of spermatogenesis-related genes is a major challenge in human reproduction genetics. It is becoming increasingly clear that multiple genes are responsible for testis development. In our study, a novel

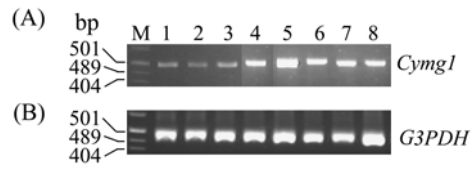

Fig. 8 RT-PCR results of *Cymg1* in testis tissues from mice at different developmental stages
(A) M, marker pUC Mix8. 1–8, RNAs from the testes of mice of 1, 2, 3, 5, 7, 13, 26 and 57 weeks.(B) *G3PDH* in testis tissues.

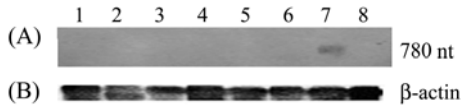

Fig. 9 Northern blot analysis of *Cymg1*
(A) 1, heart; 2, kidney; 3, skeletal muscle; 4, liver; 5 lung; 6, epididymis; 7, testis; 8, ovary. (B) β-actin.

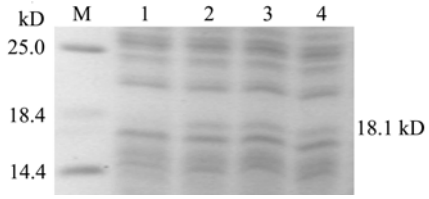

Fig. 10 Expression of the CYMG1 in E. coli M15
M, protein marker; 1, uninduced M15 control; 2–4, IPTG-induced M15 at 1, 2 and 3 h, respectively.

testis-specific gene, *Cymg1*, was cloned from a mouse testis library. The gene encoded an amino acid sequence that contains a cystatin domain. Northern blotting showed that *Cymg1* is 0.78 kb in length and expressed in the testes, but not in other tissues, such as heart, kidney, ovary, epididymis, skeletal muscle, liver, or lung. This suggests that *Cymg1* may have a tissue-specific function in the testes.

In mouse testis development, at 16 days postpartum, there are germ cells in all stages of the first meiotic prophase, and at 20 days postpartum, the early type of haploid cells first appear in the seminiferous epithelium [10]. Spermatogenesis is a complex process. During spermatogenesis, the production of sperm occurs within the testicular seminiferous tubules in three separate phases. First, diploid germ cells, primitive spermatogonium, will self renew to amplify and produce type A and B spermatogonium. Type B spermatogonium will differentiate into primary spermatocytes. The meiotic division of

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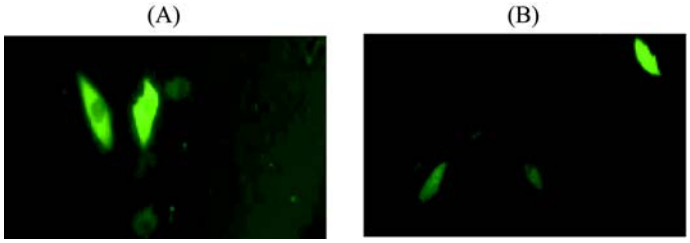

Fig. 11 Cellular location of GFP-tagged CYMG1 and GFP protein in HeLa cells

(A) GFP-tagged CYMG1 protein localized in the cytoplasm; (B) GFP protein dispersed throughout the cells. Magnification 10×20.

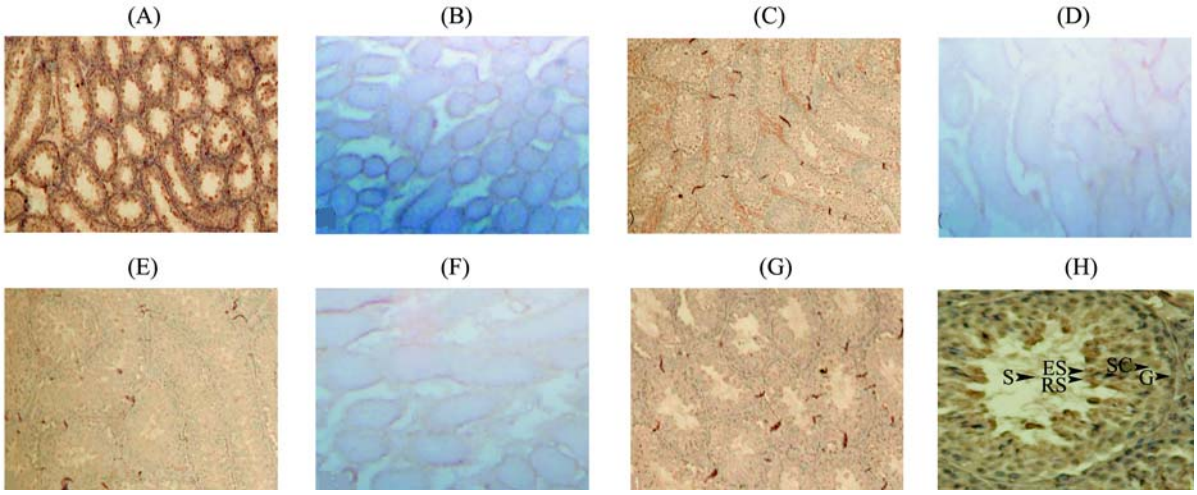

Fig. 12 Immunohistochemistry of CYMG1 in testicular germ cells

CYMG1 was detected in the testes of mice at different developmental stages with polyclonal rabbit anti-CTMG1 antibody and biotin peroxidase-conjugated goat anti-rabbit through immunohistochemistry. The presence of CYMG1 was revealed by brown staining (A, C, E, G and H). G, spermatogonium; SC, spermatocytes; RS, round spermatid; ES, elongated spermatid; S, spermatozoa. No significant signals were detected in the seminiferous tubules of controls (B, D and F). Testis were used at the following developmental stages: (A–B), 20 days postpartum; (C–D), 40 days postpartum; (E–F), 60 days postpartum; (G–H), 80 days postpartum. (A–G) 100×100 magnification. (H) 400×100 magnification.

spermatocytes will produce round spermatids. Finally, after a series of biochemical and morphological changes, spermatids elongate and spermatozoa are generated [11]. Mouse sex maturation appears early; at the earliest, sperm appear at 5 weeks postpartum and mice mature sexually at 6–8 weeks postpartum. In the present study, Northern blot analysis showed that *Cymg1* is specifically expressed in adult mouse testes. Cell location studies showed that the GFP-tagged CYMG1 protein is localized in the cytoplasm of HeLa cells, so CYMG1 is a cytoplasm protein. Immunohistochemistry revealed that the CYMG1 protein is expressed in mouse testis spermatogonium, spermatocytes, round spermatids, elongating spermatids and spermatozoa. RT-PCR results also showed that *Cymg1* is expressed in mouse testis and spermatogonium. Studies of multi-tissues and testes at different developmental stages showed that *Cymg1* is expressed specifically in adult testis. These results are in accordance with the results of Northern blot analysis, which also showed that *Cymg1* is specifically expressed in adult mouse testes. In addition, the RT-PCR results also showed that the *Cymg1* expression level varied in testis tissue in different developmental stages: it was low 1 week postpartum, steadily increased from 2 to 5 weeks postpartum, then was highest in 7 weeks postpartum. The expression level at 5 weeks postpartum was maintained during 13 to 57 weeks postpartum. These results correlate with the mouse spermatogenesis and sexual maturation process. All these indicate that *Cymg1* might play an important role in mouse spermatogenesis and sexual maturation.

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Cres is primarily expressed in the reproductive tract, is cell-specific, and is present in the stage-specific germ cells in the testes [13,14]. Studies have revealed that CRES is present in the tissues and luminal fluid of the proximal epididymis as well as in the sperm acrosome, suggesting a putative role in sperm maturation and fertilization [14,15]. *Cres* mRNA and protein in the anterior pituitary gland gonadotroph cells, and in particular, a colocalization of *Cres* with LHb in the secretory pathway have been studied [16]. *Cres* mRNA is present in the corpora lutea of the ovary as well. These data suggest that *Cres* may perform highly cell-specific and regulative functions that are unlike that of typical cystatins [5]. Like *Cres*, the full length *Cymg1* cDNA encompasses the entire open reading frame, which encodes 141 amino acid residues, has a cysteine protease inhibitor domain, but does not have critical consensus sites that are important for cysteine protease inhibition, which may be related to the CRES subfamily. In addition, all the above indicate that CYMG1 may have an important role in mouse spermatogenesis and the sexual maturation process.

In summary, we have cloned a novel gene, *Cymg1*, which is specifically expressed in mouse testis tissue over the different developmental stages. This new gene may be a new member of the *Cres* subgroup of family 2 cystatins and it may play an important role in spermatogenesis and sexual maturation. Further studies are being carried out to examine the role of *Cymg1*.

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