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Molecular Cloning of *MSRG-11* Gene Related to Apoptosis of Mouse Spermatogenic Cells

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Abstract Beginning with a new contig of the expressed sequence tags (Mm.63892) obtained by comparing testis libraries with other tissue and cell line libraries using the digital differential display program, we cloned a new gene which is related to the apoptosis of mouse spermatogenic cells using the Genscan program and polymerase chain reaction (PCR) technology. The sequence data have been submitted to the GenBank database under accession number AY747687. The full cDNA length is 1074 bp, and the gene with 7 exons and 6 introns is located in mouse chromosome 1 H5. The protein is recognized as a new member of calmodulin (CaM) binding protein family because the sequence contains three short calmodulin-binding motifs containing conserved Ile and Gln residues (IQ motif) and is considered to play a critical role in interactions of IQ motif-containing proteins with CaM proteins. The putative protein encoded by this gene has 192 amino acid residues with a theoretical molecular mass of 23.7 kDa and a calculated isoelectric point of 9.71. The sequence shares no significant homology with any known protein in databases. RT-PCR and Northern blot analyses revealed that 1.3 kb *MSRG-11* transcript was strongly expressed in adult mouse testis but weakly expressed in the spleen and thymus. The *MSRG-11* gene was expressed at various levels, faintly at two weeks postpartum and strongly from three weeks postpartum in adult testes. The green fluorescence produced by pEGFP-C2/*MSRG-11* was detected in the cytoplasm of COS7 cells 24 h post-transfection. The pcDNA3.1(–)/*MSRG-11* plasmid was constructed and introduced into COS7 cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, USA). MSRG-11 can accelerate COS7 cell apoptosis, which suggests that this gene may play an important role in the development of mouse testes and is a candidate gene of testis-specific apoptosis. Based on these observations, it was considered that we cloned a new gene which probably accelerates spermatogenetic cell apoptosis in mouse.

Key words *MSRG-11* gene; testis-specific expression; spermatogenetic cell apoptosis; IQ motif

Loss of germ cells is very common during various stages of mammalian spermatogenesis. It has been observed that the amount of mature sperms in mouse testis is 20%–75% less than that expected though the testis is a tissue with high proliferation ability. The explanation is that the apoptosis in testis results in spontaneous degeneration of spermatogenic cells [1–4]. The apoptosis could be induced by many signals including the Fas and Fas ligand (FasL) system and/or the Bax and Bcl-2 system in normal testis in adult mouse. So the disturbance of spermatogenic cell apoptosis, which involves multi-gene, is an important problem to improve male infertility [5–8]. Although cell death, particularly apoptosis, has been implicated, our understanding of the mechanisms underlying germ cell death is still limited. Cloning of new spermatogenic cell-specific genes related to apoptosis is of physiological and pathological significance to illustrate both the apoptosis mechanism and the biological process of spermatogenic cells.

Calmodulin (CaM) is recognized as a major calcium sensor and orchestrator of regulatory events through its interaction with a diverse group of cellular proteins. Three

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classes of recognition motifs exist in many of the known CaM binding proteins; the short calmodulin-binding motif containing conserved Ile and Gln residues (IQ motif) as a consensus is for Ca^{2+}-independent binding and two related motifs are for Ca^{2+}-dependent binding. The IQ motif is widely distributed in both myosins and non-myosins and is quite common in the database that includes more than 900 Pfam entries. An examination of IQ motif-containing proteins that are known to bind CaM indicates a wide diversity of biological functions that parallel the Ca^{2+}-dependent targets. These proteins include a variety of neuronal growth proteins, myosins, voltage-operated channels, phosphatases, Ras exchange proteins, sperm surface proteins, a Ras Gap-like protein, spindle-associated proteins and several proteins in plants [9–14].

Beginning with a new contig of the expressed sequence tags (ESTs) obtained by comparing the testis libraries with other tissue and cell line libraries using the digital differential display (DDD) program [15], we cloned a novel mouse full-length cDNA sequence, *MSRG-11*, from a mouse testis cDNA library using Genscan software and PCR technology. The sequence may be a member of the IQ motif of proteins and may be related to spermatogenic cell apoptosis.

Materials and Methods

Materials

50×Advantage 2 DNA polymerase, mouse testis Marathon-ready cDNA library, Northern blot membrane (MTN) and Express Hyb hybridization solution were purchased from Clontech (San Jose, USA). Digoxigenin labeling and detection kit were purchased from Roche (Basel, Switzerland). Primers were synthesized by BioAsia (Shanghai, China). The mRNA isolation kit was purchased from Invitrogen (Carlsbad, USA) The RT-PCR kit was from Promega (Madison, USA). The reagents for electrophoresis, culture medium, pUCm-T vector and dNTP were purchased from Biological Engineering company (Shanghai, China). Sequencing was performed by BioAsia (Shanghai, China). Monkey kidney cell line, COS7, was purchased from ATCC company (Virginia, USA).

Isolation of *MSRG-11* cDNA from mouse ESTs

Using DDD, we screened multiple cDNA libraries to identify ESTs which were present in libraries derived from the testis but absent in libraries derived from other tissues. The contig of the ESTs predicted to be testis-specific was chosen for further study. Beginning with the contig of the ESTs Mm.63892, we used BLAST algorithms available at the National Center for Biotechnology Information (NCBI) and found a series of ESTs. The full-length cDNA sequence of a predicted novel mouse gene was obtained from these ESTs by splicing on an EST annotation machine at IFOM (http://bio.ifom-firc.it/EST_MACHINE/index.html) and using the Genscan program (http://genes.mit.edu/GENSCAN) [16,17].

Molecular cloning of *MSRG-11* full-length cDNA

The primers LM-1F (5′-AGTCGACTCAAGTCTGGTCTCA-3′) and LM-1R (5′-GCAGTTTAATAGGAAGGCGAGA-3′) were designed according to the above full-length cDNA sequence and used in polymerase chain reaction (PCR) assay with Advantage 2 DNA polymerase and Marathon-ready cDNA of mouse testis as a template to confirm the open reading frame (ORF). PCR amplification cycles were as follows: initial denaturation at 95 °C for 2 min; 35 cycles of reaction at 94 °C for 40 s, at 59 °C for 40 s and at 72 °C for 2 min; and at 72 °C for 5 min, holding at 4 °C. This PCR fragment was subcloned into pUCm-T vectors and the recombinant plasmid was sequenced. We checked the nucleotide sequence by sequencing the clone in two directions and submitted it to GenBank. The primers LM-3A (5′-GGAATTCCAGTTGCCATGGAGACTAAT-3′) and LM-3B (5′-GGTCGACAATCCTGTTGGTGAGCTT-3′) or LM-4B (5′-GGAAGCTTAATCCTGTTGGTGAGCTT-3′) were designed according to the *MSRG-11* full-length cDNA sequence. LM-3A/LM-3B sequence was inserted with *Eco*RI and *Sal*I sites to construct EGFP-C2/*MSRG-11* expression plasmid. LM-3A/LM-4B sequence was inserted with *Eco*RI and *Hin*dIII sites to construct pcDNA3.1(–)/*MSRG-11* expression plasmid. Then LM-3A/LM-3B or LM-3A/LM-4B was used in PCR assay with the PCR products mentioned above as template. The PCR amplification procedure was as follows: initial denaturation at 95 °C for 5 min; 35 cycles of reaction at 94 °C for 40 s, at 54 °C for 40 s, at 72 °C for 1 min; and extension at 72 °C for 5 min, holding at 4 °C. The PCR fragment was cloned into pUCm-T vector and the resulting plasmid was sequenced.

Bioinformatics analysis of *MSRG-11*

The translation program at ExPASy (http://us.expasy.org/tools/dna.html) was used to identify the ORF. Comparison of *MSRG-11* against mouse genome draft sequences in GenBank database was performed to locate the new gene in mouse chromosome. The ProtParam tool was used to identify the physico-chemical parameters of

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the new protein sequence. TMpred was used to predict the transmembrane regions and protein orientation. SignalP V1.1 was performed to predict signal peptide cleavage sites [18]. PSORT WWW Server was utilized to predict protein subcellular localization. SMART (Simple modular architecture research tool, http://smart.embl-heidelberg.de/) was used to analyze motifs. Both the BLAST program at NCBI and the CLUSTAL program were performed to analyze similarities for nucleotides and proteins.

Identification of *MSRG-11* mRNA in adult mouse tissues by RT-PCR

Total RNAs from multiple adult mouse tissues (brain, heart, liver, lung, spleen, kidney, thymus, skeletal muscle, ovary and testis) were extracted using an RNA isolation kit (Invitrogen, Carlsbad, USA). cDNA was synthesized according to the instructions (Promega, Madison, USA) and was used as a template in the following PCR amplification. The PCR was performed using the primer pair LM-1F/LM-1R with DNA polymerase (Fermentas, MBI, Lithuanian). For the conservative *G3PDH* gene in mouse, the forward primer was 5′-GACCCCTTCATTGACCTCAA-3′ and the reverse primer was 5′-GCATGGACTGTGGTCATGAGT-3′. PCR was performed as follows: initial denaturation at 95 °C for 5 min; 35 cycles of at 94 °C for 40 s, at 59 °C for 40 s, at 72 °C for 1 min; and at 72 °C for 5 min, holding at 4 °C. RT-PCR product was separated on 2.0% agarose gel. *G3PDH* was amplified as the control.

Northern blot analysis

To confirm the tissue distribution of the new gene mRNA, Northern blot analysis was performed using the PCR amplified 641 bp segment of the *MSRG-11* cDNA as a probe. The PCR products were recovered as described above using primer pair LM-3A/LM-3B and labelled in the presence of a digoxigenin labeling system according to the manufacturer's protocol (Roche, Basel, Switzerland). Hybridization of the probe to the MTN-membrane-bound RNA was performed following the manufacturer' s instructions (Clontech, San Jose, USA). Equal loading was conformed using the β-actin gene probe as a control.

Identification of *MSRG-11* mRNA of mouse testis at different developmental stages using semi-quantitative RT-PCR

Total RNAs isolated from the mouse testes at different developmental stages were used to perform RT-PCR. cDNA was synthesized according to the manufacturer's instructions (Promega, Madison, USA) and was used as a template. PCR was performed in a 10 μl reaction volume containing 5.8 μl nuclease-free water, 1 μl 10×reaction buffer, 1 μl dNTP mixture, 0.8 μl 2.5 mM $MgSO_4$, 0.2 μl *Taq* DNA polymerase, 0.2 μl 20 mM LM-1F/LM-1R and *G3PDH* primers and 0.8 μl cDNA. PCR was performed for 30 cycles to obtain transcripts to the maximal extent at different stages. The reaction parameters were as follows: initial denaturation at 95 °C for 5 min; 30 cycles of reaction at 94 °C for 40 s, at 59 °C for 40 s, at 72 °C for 60 s; and at 72 °C for 5 min, holding at 4 °C for *MSRG-11* and 20 cycles for *G3PDH* as control. The PCR fragments were separated on 2.0% agarose gel and cloned into pUCm-T vectors. the recombinant plasmids were sequenced. The *G3PDH* product was 499 bp.

pEGFP-C2/*MSRG-11* plasmid construction and transfection

An EGFP-C2/*MSRG-11* expression plasmid was constructed by inserting *MSRG-11* cDNA which had cloned into pUCm-T vector into pEGFP-C2 (Clontech, San Jose, USA) vector at the downstream of EGFP with *Eco*RI and *Sal*I sites. Correct insertion was confirmed by restriction digestion and DNA sequencing. COS7 cells were seeded on a culture dish at a density of 4×10^5 cells/dish and transfected with pEGFP-C2/*MSRG-11* plasmid DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions. When GFP-MSRG-11 fusion protein was detected, the cells were twice washed with 0.1 M PBS and fixed with 4% paraformaldehyde. Observation of fluorescence of the GFP-MSRG-11 fusion protein were performed with a Nikon fluorescence microscope. The correct colonies displayed strongly and evenly green fluorescence excited by blue light. The COS7 cells transfected with pEGFP-C2 vector were used as a control.

Expression of *MSRG-11* in COS7 cells

An eukaryotic expression vector pcDNA3.1(–)/*MSRG-11* for expression of MSRG-11 protein was constructed by inserting *MSRG-11* cDNA ligated to pUCm-T vector into pcDNA3.1(–) (Invitrogen, Carlsbad, USA) with *Eco*RI and *Hin*dIII sites. Restriction endonuclease digestion and sequencing were used to confirm the correct insertion. COS7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, UAS), supplemented with 10% fetal calf serum (Gibco Carlsbad, USA), 2 mM *L*-glutamine (Sigma, St. Louis, USA), and 1% non-essential amino acids (Sigma, St. Louis, USA). The cultures were incubated at 37 °C in a humanized 5% CO_2 atmosphere and subcultured every 4 days. Cells were cul-

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tured at 50% confluence in 100 mm dishes. About 15 μg plasmid DNA was used for each transfection at a final concentration of 1 mM in the presence of 10 mg/ml Lipofectamine 2000 (Invitrogen, Carlsbad, USA). Approximately 4 h after the mixture was added, the medium was changed to standard DMEM and the cells were cultured for 36 h. Control cells were transfected with pcDNA3.1(–) vector using Lipofectamine 2000. The cells were washed with PBS, fixed with ice-cold 70% ethanol, and stored at 4 °C for 2 h. Subsequently, the cells were incubated with 50 μl of l mg/ml RNase A for 60 min at 37 °C, 400 μl of 50 μg/ml PI was added to the cells, and the mixture was incubated for 60 min on ice in the dark. The effect of pcDNA3.1(–)/*MSRG-11* plasmids on cell cycle dynamics was examined using flow cytometry. At least 1×10^4 cells were analyzed.

Results

Cloning of a novel mouse gene cDNA from mouse ESTs

Beginning with the contig of the ESTs Mm.63892, a series of ESTs, CA494625, BU936559, BY715324, BY715324, CA465532, CA465033 and BQ839904, derived from mouse testes with high sequence similarity, were obtained. Homology comparison of those ESTs against mouse EST databases was carried out. A 1075 bp full-length cDNA sequence of a predicted novel mouse gene, which included the part-length sequence of hypothetical mouse testis gene NM_028848, was obtained from these ESTs by splicing on an EST annotation machine at IFOM (http://bio.ifom-firc.it/EST_MACHINE/index.html). An ORF sequence containing these ESTs was obtained using Genscan software. Results of a comparison against the nr database showed that the sequence represented a new gene, termed *MSRG-11* (GenBank accession number AY747687).

Molecular cloning of *MSRG-11* full-length cDNA

The PCR was performed using primers LM-1F/LM-1R and the PCR product (1015 bp) was sequenced. The result showed that it was identical to the predicted sequence. The full length of the cDNA is 1074 bp, encoding a protein of 192 amino acids (**Fig. 1**). There is a start codon ATG from nucleotide position 136 to 138 and a stop codon TGA from 713 to 715. GCCATGGAG sequence consistent with the Kozak rule was found in the start region of ORF and potential polyadenylation signal (ATTAAA) was found at the 3′ end. The boundaries between exons and

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1     aggagaaaggggcgagtcgactcaagtctggtctcacgtgtacaaatccgatttctctgtc   61
62    cagttgcttgaaactgaaacagagtgaaaggaaaggccgataagtcggaagtgaccttgg   121
                          *                       *
122   ttcagcccagttgccatggagactaatagtaacaattttggtgaactccaggagttgaag   181
1                   M  E  T  N  S  N  N  F  G  E  L  Q  E  L  K      15
182   gacatggctactttagccaagctgctggcgcgggcacccttctagaaagtcagtactac   241
16     D  M  A  T  L  A  K  L  L  A  R  A  P  F  L  E  S  Q  Y  Y     35
242   tttaggaaccgcgccgttgattcatttaggaaattcgagaatgatgcagctgtgatgatc   301
36     F  R  N  R  A  V  D  S  F  R  K  F  E  N  D  A  A  V  M  I     55
302   cagagctggttccgagggtgccaagtccgggcctatatgaggcacttgaacagagtagtg   361
56     Q  S  W  F  R  G  C  Q  V  R  A  Y  M  R  H  L  N  R  V  V     75
362   acaattattcaaaagtggtggagaagctacttaggcagaaaatttttaccaacttgttgtt   421
76     T  I  I  Q  K  W  W  R  S  Y  L  G  R  K  F  Y  Q  L  V  V     95
422   gaggcagcatattatactatgaagatgaatctctacaatgaaatggctgtcaggattcag   481
96     E  A  A  Y  Y  T  M  K  M  N  L  Y  N  E  M  A  V  R  I  Q    115
482   agacgatggcgtggctttaggatccggaaatattgctttaattattattatttgaaggaa   541
116    R  R  W  R  G  F  R  I  R  K  Y  C  F  N  Y  Y  Y  L  K  E    135
542   tatttaagagctgtttcagaaaccaatgatgcaattcgggaggctttggaggagttcgca   601
136    Y  L  R  A  V  S  E  T  N  D  A  I  R  E  A  L  E  E  F  A    155
602   gaaatgaaagagagagaagagagaaaggttctcctggaacgagaggagaagcaaaaagat   661
156    E  M  K  E  R  E  E  R  K  V  L  L  E  R  E  E  K  Q  K  D    175
662   taccaagcccgcaagatgcattacctgctcagcacaaagcagcataaacattgacaaccc   721
176    Y  Q  A  R  K  M  H  Y  L  L  S  T  K  Q  H  K  H  *          192
722   tccgctattgcttccgccctgcttctcagtctttccttccttgctcttcagtgtggtca   781
782   tcccaccgcagcctgacattatgctcaagaaaagctcaccaacaggattttctaacatat   841
842   ggatgaagctgattccttcttacagctagttacagtctggacgttcagagtcctgctctc   901
902   atgccaaagggcagttccaacaacttcccacaattccaacaacttcccacaattcctaca   961
962   acttttcaccacactgacctttccctttccctttcctttaaagatgttctcgccttcctat  1021
1022  taaactgcgttgtcaatgaaaatctgaaaaaaaaaaaaaaaaaaaaaaaaaa          1073
```

Fig.1 ***MSRG-11*** **cDNA and the predicted protein sequence**

Primers are marked in bold and italic. Polyadenylation signal is underlined. Stop codon is indicated by an asterisk (*).

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Table 1 Exon-intron junctions of *MSRG-11* gene

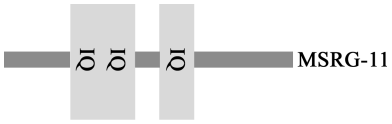
Exon	Exon size (bp)	5′ splice donor	Intron size (bp)	3′ splice acceptor	Intron
1	250	GAACCG**gt**g	20,175		1
2	90	TATGAG**gt**a	905	t**ag**CGCCGT	2
3	82	GTTGAG**gt**a	9785	t**ag**GCACTT	3
4	53	GTCAGG**gt**a	8741	c**ag**GCAGCA	4
5	104	AATTCG**gt**g	33,525	t**ag**ATTCAG	5
6	124	AAGCAG**gt**t	4738	c**ag**GGAGGC	6
7	339			c**ag**CATAAA	

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

introns are coincident with the gt-ag rule (**Table 1**).

Bioinformatics analysis of *MSRG-11*

The gene was located in mouse chromosome 1 H5, containing 7 exons and 6 introns. There existed no transmembrane region and signal peptide in the predicted protein with a theoretical molecular mass of 2.37 kDa and a calculated isoelectric point of 9.71. PSORT WWW Server analysis showed that there was a 56.5% possibility to locate the protein in cytoplasm. SMART analysis results indicated there existed three IQ motifs from amino acid residue 47 to 69, 70 to 92, 106 to 128 (**Fig. 2**). BLAST results illustrated that there was no significant homology with any known protein in databases.



IQ motif	Begin (aa)	End (aa)	*E*-value
1st	47	69	5.27e+00
2nd	70	92	1.77e–02
3rd	106	128	1.40e+01

Fig.2 The predicted IQ motifs of MSRG-11

aa, amino acid residues.

Expression of *MSRG-11* gene in adult mouse tissues

In order to understand the expression profiles of the *MSRG-11* gene in various tissues, the expression of *MSRG-11* in multiple mouse tissues was examined by RT-PCR. The results showed that the *MSRG-11* gene was expressed strongly in adult testis tissue and faintly in spleen and thymus tissue (**Fig. 3**), whereas the control, the *G3PDH* gene, was expressed equally in all kinds of tissues.

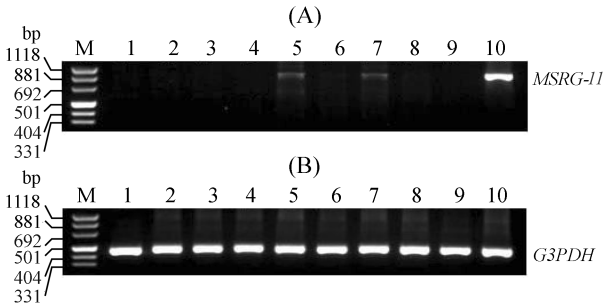


Fig. 3 RT-PCR analysis of *MSRG-11* gene expression in various adult mouse tissues

(A) Amplification of *MSRG-11* in multiple tissues of adult mouse. (B) Amplification of *G3PDH* in multiple tissues of adult mouse. M, pUC Mix8 marker; 1, brain; 2, heart; 3, liver; 4, lung; 5, spleen; 6, kidney; 7, thymus; 8, skeletal muscle; 9, ovary; 10, testis.

Northern blot analysis

The expression of the *MSRG-11* gene was detected in eight different mouse tissues using Northern blot analysis. A single strongly expressed mRNA band of 1.3 kb was observed exclusively in adult testis and weakly in ovary; no hybridization signal was detected in other tissues (**Fig. 4**).

MSRG-11 expression in mouse testes at different developmental stages

The full mRNAs of 1015 bp from the testes at representative stages of development were analyzed by semi-quantitative RT-PCR. The results showed that *MSRG-11* was expressed at various levels, faintly at two weeks post-

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partum and strongly from three weeks postpartum in adult testis (**Fig. 5**), whereas the internal control, *G3PDH* was expressed equally in each developmental stage in normal testis tissues.

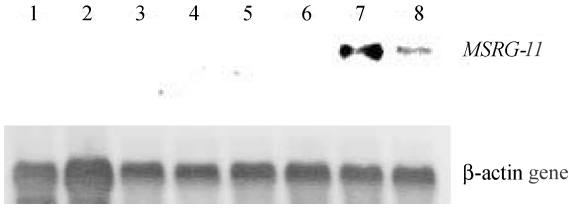

Fig. 4 Northern blot analysis of *MSRG-11* in eight mouse tissues

A single strongly expressed mRNA band of 1.3 kb was observed exclusively in adult testis; no hybridization signal was detected in other tissues. 1, brain; 2, heart; 3, liver; 4, lung; 5, spleen; 6, kidney; 7, testis; 8, ovary.

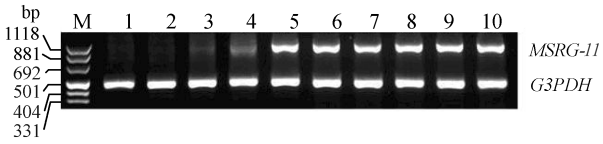

Fig. 5 Semi-quantitative RT-PCR analysis of *MSRG-11* in mouse testes at different developmental stages

1, mouse testis at one day postpartum; 2, three days postpartum; 3, one week postpartum; 4, two weeks postpartum; 5, three weeks postpartum; 6, four weeks postpartum; 7, five weeks postpartum; 8, six weeks postpartum; 9, seven weeks postpartum; 10, eight weeks postpartum; M, pUC mix8 marker.

Localization of EGFP-MSRG-11 fusion protein

To determine EGFP-MSRG-11 fusion protein expression in mammalian cells, a pEGFP-C2/*MSRG-11* fusion plasmid was constructed and transiently introduced into COS7 cells by liposome transfection. Under fluorescence microscope, the green fluorescence produced by pEGFP-C2/*MSRG-11* was detected in the cytoplasm of COS7 cells 24 h post-transfection, while the fluorescence produced by pEGFP-C2 was detected throughout the cells. Consistent with the prediction by bioinformatics, this result suggested that *MSRG-11* encoded protein is located in cytoplasm (**Fig. 6**).

The effects of MSRG-11 on cell growth and apoptosis

To determine whether the *MSRG-11* gene resulted in changes in culture cell proliferation or apoptosis, we examined transient expression of the MSRG-11 protein in COS7 cells. The effects of such treatment on tumor cell growth were examined *in vitro* using flow cytometry. The

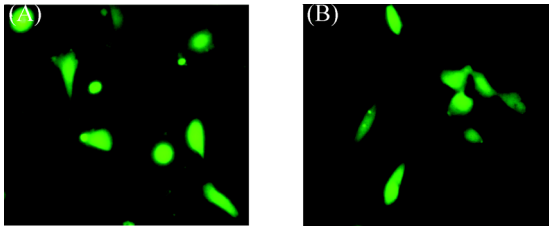

Fig. 6 Subcellular localization of MSRG-11 protein

(A) Expression of the EGFP protein in COS7 cell line (400×). (B) Expression of the GFP-MSRG-11 fusion protein in COS7 cell line (400×).The results indicated that GFP-MSRG-11 fusion protein was localized in the cell cytoplasm *in vitro*.

percentage of cells resident in each cell-cycle phase was indicated (**Fig. 7**). In the control COS7 cells transfected with pcDNA3.1(–), the cell cycle distribution is 51.0% of cells in G_1, 41.3% in S-phase, and 7.7% in G_2. The apoptosis rate is 9.3%. In COS7 cells transfected with pcDNA3.1(–)/*MSRG-11*, the cell-cycle distribution is 40.5% in G_1, 47.1% in S-phase, and 12.5% in G_2. The percentage of the apoptosis cells is 24.2%. The results indicated that MSRG-11 could accelerate COS7 cell apoptosis.

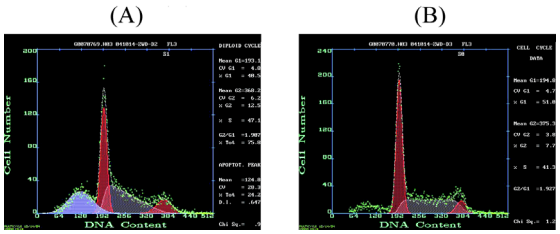

Fig. 7 The cell cycle distribution of COS7 cells transfected with *MSRG-11*

(A) COS7 cells transfected with pcDNA3.1(–)/*MSRG-11* plasmid. The cell-cycle distribution is 40.5% in G_1, 47.1% in S-phase, and 12.5% in G_2. The percentage of the apoptosis cells is 24.2%. (B) COS7 cells transfected with pcDNA3.1(–) plasmid. The cell-cycle distribution is 51.0% of cells in G_1, 41.3% in S-phase, and 7.7% in G_2. The apoptosis rate is 9.3%. MSRG-11 can accelerate COS7 cells to apoptosis or traverse the S-phase and enter the G_2-phase (A) compared with the control without *MSRG-11* transfection (B).

Discussion

Previous research data have shown that spermatocyte apoptosis is related to many factors, such as: (1) the *p53* gene, which is highly expressed in spermatocytes from the leptotene to pachytene stage and is related to apoptosis of spermatogenic cells induced by heat pressure [19–21]; (2) the FAS pathway, which is the key factor to activate

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the apoptosis of spermatogenic cells at initiation stage of apoptosis [22–26]; (3) apoptosis inhibitor Bcl-2 and apoptosis inducer Bax at apoptosis effector stage [27,28]; (4) protease caspase at apoptosis execution stage. No datum has been reported that IQ motif protein can accelerate apoptosis in the testis.

Analyzing the resource of ESTs, we found that most of them were derived from testis libraries and a few of them from testis spermatocytes. For example, BQ839904, a 564 bp mRNA linear EST, was derived from McCarrey Eddy 18-day preleptotene spermatocytes, suggesting that *MSRG-11* may play some roles in mouse spermatogenic cells. The gene, whose full cDNA length is 1074 bp containing 7 exons and 6 introns, is located in mouse chromosome 1 H5. The putative protein of this gene has 192 amino acid residues with a theoretical molecular weight of 23.7 kDa and a calculated isoelectric point of 9.71. The sequence shares no significant homology with any known protein in databases.

RT-PCR and Northern blot analysis revealed that *MSRG-11* transcript of 1.3 kb was detected strongly in mouse testis and weakly in spleen and thymus. Gene expression patterns revealed that *MSRG-11* is a testis-specific high expression gene that may play an essential role in testis function [29–31]. MSRG-11 was located in the cytoplasm of COS7 cells 24 h post-transfection. The protein was mostly found in the cell cytoplasm and could accelerate cell apoptosis.

MSRG-11 was expressed at various levels in testis at different developmental stages, strongly in adult testis after three weeks postpartum and faintly before two weeks postpartum. This revealed that the gene performs its function by different amounts of expression in spermatogenesis cells at different developmental stages, which coincides with the time of apoptosis peak, observed approximately 10–13 days after birth, when the first wave of spermatogenesis had started and active spermatogonial proliferation was present [32]. MSRG-11 protein can accelerate COS7 cells to apoptosis compared with the control without transfection of the *MSRG-11* gene, which suggested that this gene plays an important role in the development of testis and may be related to maintaining equilibrium between normal spermatogenesis and high rate apoptosis. The gene probably plays a role in the cell apoptosis of spleen and thymus too. The *MSRG-11* gene is a testis apoptosis candidate gene.

The action of the IQ motif may result in complex signaling as observed for myosins and the *L*-type Ca^{2+} channels and is highly localized as required for sites of neuronal polarized growth and plasticity, fertilization, mitosis and cytoskeletal organization. The IQ motif associated with the unconventional myosins also promotes Ca^{2+} regulation of the vectorial movement of cellular constituents to these sites. Additional regulatory roles for this versatile motif seem likely. Another class of IQ motif-containing proteins that are induced by the plant hormone ethylene have been identified. These proteins bind CaM and promote plant senescence and death. The association between CaM signaling and cell death is not unique, but to our knowledge it has not been found in mammalian cells. The present study indicated that MSRG-11 protein containing 192 amino acid residues is recognized as a new member of CaM binding protein family because the sequence contains the highly conservative IQ motif and is considered to play a critical role in interactions of IQ motif-containing proteins with CaM protein in different periods of testis development.

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