Asian Journal of Andrology (2011) 13, 446–452

www.nature.com/aja

ORIGINAL ARTICLE

LM23 is a novel member of the Speedy/Ringo family at the crossroads of life and death of spermatogenic cell

Yi-Ming Cheng[1,2,3], Mei-Ling Liu[2] and Meng-Chun Jia[2]

LM23 **is a gene specifically expressed in the testis of *Rattus norvegicus*, as previously reported by our laboratory. The aim of the study is to further investigate the biological function of LM23. Several bioinformatic tools were utilized, including PROSITE and BLAST. To determine the subcellular localization of LM23, a polyclonal antibody specific for LM23 was generated *via* the immunization of rabbits. The *LM23* gene was cloned from rat testis tissue, and LM23 protein was expressed in *Escherichia coli*. The biological function of LM23 was analyzed with microarray analysis and immunohistochemistry, using a rat model of *LM23* gene knockdown. The results suggested that LM23 belongs to the Speedy/Ringo family. *LM23* regulated the G_1/S and G_2/M transitions of the cell cycle during spermatogenesis. Downregulation of the *LM23* gene during spermatogenesis could lead to the activation of both the Fas–FasL pathway and the mitochondrial pathway. These novel findings indicate that LM23 has a diverse array of functions that are important in both the life and death of the spermatogenic cell.**
Asian Journal of Andrology (2011) **13,** 446–452; doi:10.1038/aja.2011.21

Keywords: cell cycle; LM23; spermatogenesis; spermatogenic cell

INTRODUCTION

Spermatogenesis is the process of differentiation of male germ cells into mature spermatozoa. It is divided into three distinct stages: the mitotic proliferation of spermatogonial stem cells, the meiotic division of spermatocytes and the maturation spermiogenesis of haploid spermatids.[1] These processes are subject to transcriptional, translational and post-translational regulation. The genes expressed during these processes encode proteins that are specific to the different phases of germ cell development. With the development and application of technologies such as gene cloning, gene expression and functional characterization, many spermatogenesis-related genes have been identified in the past few years. Some of these genes have been proven to play important roles in spermatogenesis.

LM23, a gene expressed specifically in the testis in a stage-specific manner, was previously identified by our laboratory.[2] The sequence of *LM23* was submitted to GenBank and the accession number is AF492385. Northern blot and RT-PCR analysis showed that *LM23* was specifically expressed in testis, and its expression was not detected in other tissues. Real-time PCR analysis showed that the expression level of *LM23* was the highest in spermatocytes and the lowest in spermatogonia. *In situ* hybridization revealed a strong positive signal in spermatocytes. These results indicate that *LM23* possesses testis-specific and stage-specific expression characteristics and is involved in rat spermatogenesis. To reveal the function of *LM23* in the testis, lentivirus-mediated RNA interference was used to knock down *LM23* expression in a tissue-specific manner *in vivo*. A lentiviral vector expressing a short hairpin RNA (shRNA)-targeting *LM23* was microinjected into the efferent ducts of *Rattus norvegicus* testis. The expression of *LM23* in the treated testis was markedly knocked down compared to controls. The *LM23*-shRNA testis contained germ cells that were arrested at the spermatocyte stage, and showed increased apoptosis and dysregulation of some meiotic genes.[3]

In this study, we presented novel functional and structural features of LM23, as predicted by bioinformatic analytical tools. The integrated bioinformatics database PROSITE and BLAST tools were used to analyse LM23. A BLAST homology search using against the NCBI non-redundant database and an *Ambystoma* EST database reveal that *LM23* is an *R. norvegicus* homolog of *Speedy A* (*Spdya*, *Spy*, also called *Ringo*). *Speedy* was initially identified in *Xenopus* as a protein, which induced the G_2/M transition during oocyte maturation by activating Cdk1 and Cdk2. No data regarding the role of Speedy in spermatogenesis have been published until now. Cheng *et al.*[4] have reported that all Speedy/Ringo proteins share a highly conserved approximately 133-amino acid domain, which has been termed the Speedy/Ringo box and is essential for Cdk binding. Its 51%–67% of region is identical to XRINGO/Speedy, spans 79 residues.[5] We investigated the function of LM23 with microarray, Western blot and immunohistochemistry using *LM23* gene knockdown rats. As there was increased apoptosis of spermatocytes in the *LM23* gene knockdown testis, the activation of caspase 3 was studied using an anti-activated caspase 3 antibody. Elucidation of the function of LM23 may promote insight into mammalian spermatogenesis.

MATERIALS AND METHODS

Animals

SPF female New Zealand white rabbits (8 weeks old) were purchased from the Animal Center of The National Institute for

[1]Graduate School of the Peking Union Medical College, Beijing 100005, China; [2]Department of Reproductive Endocrinology, National Research Institute for Family Planning, Beijing 100081, China and [3]Air Force Institute of Aviation Medicine, Beijing 100142, China
Correspondence: Professor MC Jia (jmchun48@yahoo.com.cn)
Received: 11 December 2010; Revised: 17 January 2011; Accepted: 28 February 2011

Control of Pharmaceutical and Biological Products (Beijing, China). *LM23* gene knockdown male *R. norvegicus* Sprague–Dawley rats (5 weeks old) were obtained from our animal facility.[2] Animals were maintained under controlled temperature (25 °C) and lighting (14 h light and 10 h dark) and were allowed free access to food and water. All experiments were conducted according to the guidelines of the Chinese Animal Care for Laboratory Animals, and the protocols were approved by the Animal Care and Use Committee in the National Research Institute for Family Planning (Beijing, China).

BLAST

Alignments of the conserved Speedy/Ringo boxes of mouse Speedy/Ringo A (79 residues) and LM23 were performed using the BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi? CMD=Web&PAGE_TYPE = BlastHome).

Structural domains and functional motifs of LM23

We used ScanProsite (Swiss Institute of Bioinformatics, Lausanne, Switzerland) at the EXPASY to identify PROSITE motifs in the LM23 protein. ScanProsite consists of documentation entries describing protein domains, families and functional sites, as well as associated patterns and profiles to identify these features.[6] The amino-acid sequence of LM23 in single-letter format was submitted to the ScanProsite server (http://www.expasy.org/prosite/).

Production of a polyclonal rabbit anti-LM23 antibody

A rabbit polyclonal antibody against the NH_2-terminal and COOH-terminal peptides of the LM23 protein was generated. According to the DNAStar soft,[7] analysis and prediction of the possible structure, hydrophilicity and antigenicity of LM23, two peptides (amino acids 1–20 and 274–291) of LM23 were synthesized using standard Fmoc-based Synthesis of Peptide Protocol. The synthesized peptides were purified by reversed phase high-performance liquid chromatography and crosslinked with keyhole limpet hemocyanin by 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide.[8] Rabbits were immunized three times (100 μg/rabbit) with the conjugated peptides. The sensitivity and specificity of the LM23–keyhole limpet hemocyanin-generated rabbit polyclonal antibody were evaluated by ELISA and Western blot.

Cloning of the *LM23* gene from rat testis and expression of the LM23 protein in *Escherichia coli*

LM23 RNA was extracted from rat testis tissue and amplified by RT-PCR. After purification and recovery, the cDNA fragment of *LM23* was cloned into a TA vector. The fragment was then sequenced, subcloned into the plasmid *pET28a(+)* (Novagen, Darmstadt, Germany), expressed in *E. coli* BL21, and identified by SDS-PAGE and Western blot analysis (anti-LM23, rabbit polyclonal peptide antibody, generated by our lab).

Microarray analysis to identify *LM23*-regulated genes in testis

To identify potential target genes that may be regulated by *LM23*, microarray analysis (service provided by Kangchen Biotech, Shanghai, China) was performed to survey the gene expression of *LM23*-shRNA testis and controls using Agilent rat whole genome arrays (design ID 14879).

Immunohistochemical analysis

Testis were fixed in Bouins solution, dehydrated and embedded in paraffin using standard methods. Immunostaining was performed using a commercial immunohistochemistry kit (Zhongshan Bio Co., Beijing, China) according to the suggested protocol of the manufacturer. Paraffin tissue sections (5 μm thick) were placed on the poly-L-lysine-coated slides and deparaffinized. To suppress endogenous peroxidase activity, the tissue sections were then treated with 3% H_2O_2 in methanol for 10 min. Antigen retrieval was performed by heating the sections in 10 $mmol^{-1}$ citric acid solution (pH 6.0) using an autoclave. The immunoreaction was detected by the streptavidin-horseradish peroxidase method. The primary antibodies (Ab) were as follows: anti-LM23 (rabbit, polyclonal peptide Ab; our lab), anti-Cdk2 (mouse, monoclonal Ab, MAB-390; Lab vision Co., CA, USA), anti-Cyclin A1 (rabbit, polyclonal peptide Ab, RB-1548-P1ABX; Thermo Fisher Scientific Inc., New York, USA) and anti-caspase 3 (mouse, monoclonal Ab, BA0588; Boshide Co., Wuhan, China). The optimal working dilution of each antibody was determined by incubating the sections with various concentrations of antibody. A final color reaction was performed using liquid 3,3′-diaminobenzidine tetrahydrochloride. The slides were then mounted with Canada balsam. The negative control was processed with non-immune serum instead of the polyclonal antibody. Each experiment was repeated at least three times, and similar results were obtained.

RESULTS

BLAST

LM23 was 97% identical to the Speedy/Ringo box of mouse Speedy/Ringo A, which is essential for Cdk binding (**Figure 1**).

Predicted functional motifs of LM23 protein by ScanProsite server

We used the ScanProsite server at the EXPASY (http://ca.expasy.org/tools/scanprosite/) to identify PROSITE motifs in the LM23 protein. PROSITE predicts that LM23 contains two *N*-glycosylation sites, seven protein kinase C phosphorylation sites, five casein

```
 Score =157 bits (398), Expect = 5e-44, Method: Composition-based stats.
 Identities = 77/79 (97%), Positives = 78/79 (98%), Gaps = 0/79 (0%)
Query  1   TAFFKLFDDDLIQDFLWMDCCCKIADKYLLAMTFVYFKRAKFTINEHTRINFFIALYLAN  60
           TAFFKLFDDDLIQDFLWMDCCCKIADKYLLAMTFVYFKRAKFTI+EHTRINFFIALYLAN
Sbjct  71  TAFFKLFDDDLIQDFLWMDCCCKIADKYLLAMTFVYFKRAKFTISEHTRINFFIALYLAN  130
Query  61  TVEEDEEEADYEIFPWALG  79
           TVEEDEEEA YEIFPWALG
Sbjct  131 TVEEDEEEAKYEIFPWALG  149
```

Figure 1 The results of the BLAST search of LM23.

kinase II phosphorylation sites, three *N*-myristylation sites and two amidation sites. These motifs suggest that LM23 may be subjected to modifications in the cell, which may be critical for its potential functions (**Table 1**).

LM23 polyclonal antibody production

Two synthesized peptides with a purity of 90% were prepared. The titers of the purified polyclonal antibodies were 1 : 64 000 and 1 : 128 000 detected by ELISA. The Western blot analysis showed that the antibody (amino acids 274–291) detected an immunoreactive protein with a molecular weight of 36 kDa in the total nucleoprotein of rat testis (**Figure 2**). The predicted molecular weight of the LM23 protein is 36 kDa.

Table 1 Analysis of the domain structure of LM23 using the ScanProsite server

Site	*Position*	*Domain*
N-glycosylation site	23	NRSH
	293	NETN
Protein kinase C phosphorylation site	22	SNR
	28	TRK
	33	SLK
	46	SEK
	177	SRR
	255	TGK
	289	SKK
Casein kinase II phosphorylation site	113	TISE
	131	TVEE
	223	TPVD
	247	SSSD
	289	SKKE
N-myristoylation site	21	GSNRSH
	204	GAARNY
	240	GLSSSS
Amidation site	230	CGKK
	255	TGKR

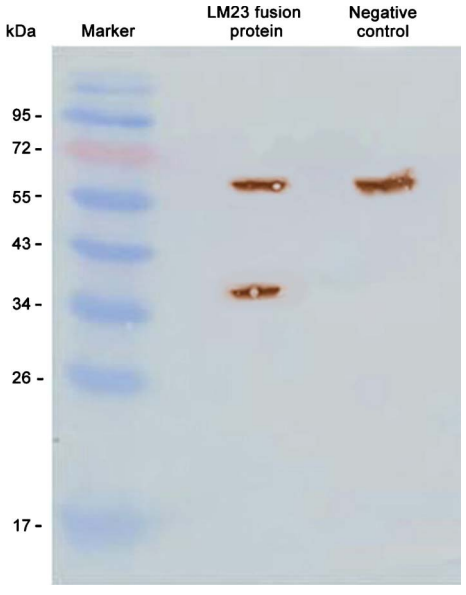

Figure 2 Western blot analysis of LM23 protein expression in testis of SD rat (DAB). Negative control, normal rabbit serum. DAB, 3,3′-diaminobenzidine tetrahydrochloride.

Cloning of the *LM23* gene from rat testis and expression of the LM23 protein in *E. coli*

A DNA fragment of approximately 900 bp was amplified. The sequencing result showed that this fragment contained only rat *LM23* cDNA (939 bp, Open Reading Frame). This *LM23* cDNA was subcloned into *pET28a*(+), and the recombinant 36-kDa LM23 protein was induced by IPTG. The LM23 fusion protein expressed by *E. coli* was analyzed by SDS-PAGE and Western blotting. The results of SDS-PAGE showed that the molecular weight of the fusion protein was 36 kDa and that the fusion protein was expressed in an inclusion body (**Figure 3a**). The Western blot analysis showed that the LM23 antibodies detected an immunoreactive protein with a molecular weight of 36 kDa in the *E. coli* expressing the LM23 fusion protein (**Figure 3b**).

Subcellular localization of LM23 by immunohistochemistry

To examine cell-specific expression in rat testis, a polyclonal rabbit anti-LM23 antibody was used for immunohistochemical detection.

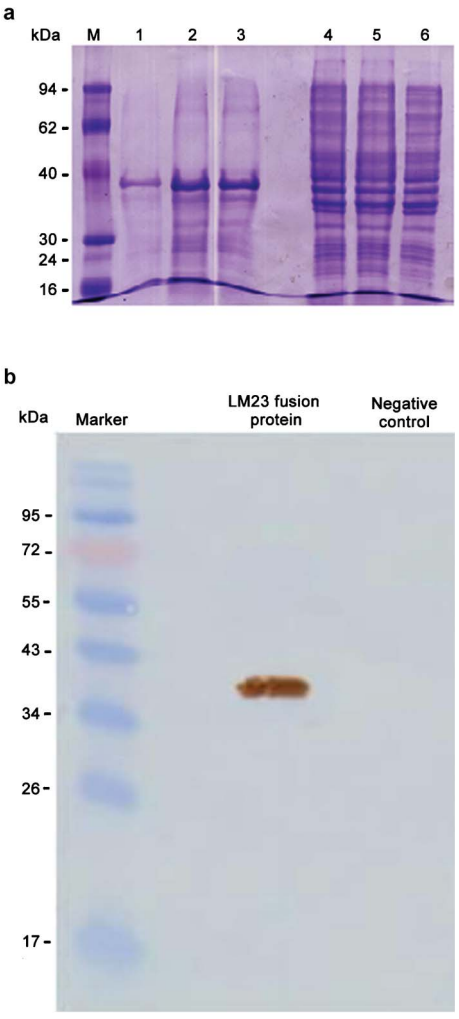

Figure 3 Analysis of the *E. coli* expressing the LM23 fusion protein by SDS-PAGE (**a**). Analysis of the *E. coli* expressing the LM23 fusion protein by Western blotting with the LM23 polyclonal antibody (**b**). Lane 1, inclusion body after induction by IPTG for 2 h; Lane 2, inclusion body after induction by IPTG for 4 h; Lane 3, inclusion body after induction by IPTG for 6 h; Lane 4, soluble protein after induction by IPTG for 2 h; Lane 5, soluble protein after induction by IPTG for 4 h; Lane 6, soluble protein after induction by IPTG for 6 h. Negative control, *E. coli*. M, marker.

Positive immunoreactivity was mainly located in spermatocytes. The subcellular localization of LM23 was primarily in the nucleus (**Figure 4**).

Microarray analysis and immunohistochemistry of a rat model of *LM23* gene knockdown

LM23 knockdown testis tissue and control tissue from *R. norvegicus* were used for histological analyses at 4 weeks post-transfection. The seminiferous tubule of the control testis contained a full spectrum of germ cells: spermatogonia, pachytene spermatocytes, round spermatids and elongated spermatids (**Figure 5b**). In contrast, the seminiferous tubules of *LM23* knockdown testis appeared disorganized, disrupted and were shedding germ cells into the lumina (**Figure 5a**). The germ cells exhibited complete meiotic arrest in spermatogenesis. Spermatocytes were accumulated, round spermatids were few, and elongating spermatids and spermatozoa were absent in certain *LM23* knockdown seminiferous tubules (**Figure 5a**). Three major types of seminiferous tubules were observed in *LM23* knockdown testis. Type A tubules contained many layers of spermatocytes and many heavily eosin-stained cells. Type B tubules contained 3–4 layers of spermatocytes. Type C tubules were characterized by a few layers of spermatogenic cells/Sertoli cells and large empty lumina.

The expression of some genes related to the cell cycle and apoptosis was markedly changed after *LM23* knockdown, as shown by the microarray analysis (**Tables 2 and 3**). The downregulation of *LM23* might block the G_1/S and G_2/M transitions of the spermatogenic cell (**Figure 6**). The downregulated *LM23* may active both the Fas–FasL pathway and the mitochondrial pathway during spermatogenesis.

Some cell cycle genes (*Cdk2*, *CyclinA1* and *Caspase 3*) were chosen for further analysis. Immunohistochemical analysis confirmed that expression of all of these proteins was markedly altered in *LM23* knockdown testis compared to the controls (**Figure 7**).

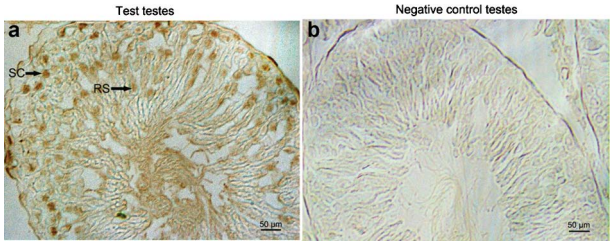

Figure 4 Immunohistochemical analysis of LM23 protein expression in testis of Sprague–Dawley rat. The positive immunological activity was mainly located in the nucleus of spermatocytes (SC) and round spermatids (RS), but few in elongating spermatids and mature sperm.

DISCUSSION

To visualize the subcellular localization of the LM23 protein, a rabbit polyclonal antibody against LM23 was generated. The LM23 polyclonal antibody can detect an immunoreactive protein with a molecular weight of 36 kDa in the total nucleoprotein of rat testis using Western blot analysis (**Figure 2**). The 60-kDa band may be a nonspecific band. However, the LM23 antibody also detected an immunoreactive protein with molecular weight of 36 kDa in the *E. coli* expressing the

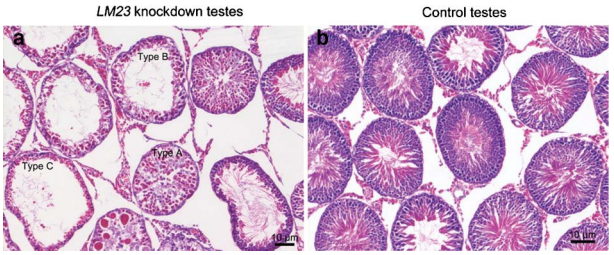

Figure 5 The seminiferous tubules, stained with hematoxylin and eosin, of *LM23* knockdown and the control testes in *R. norvegicus* at 4 weeks post-transfection.

Table 2 List of genes associated with cell cycle expression after *LM23* knockdown

Gene symbol	*Test/control*	*Array ID*	*ID number*	*Description*
Cdk4	3.369	A_44_P388782	NM_053593	Cyclin-dependent kinase 4
Cdk6	0.231	A_44_P405071	XM_342638	Cyclin-dependent kinase 6
Ccnd1	13.05	A_44_P189299	NM_171992	Cyclin D1
Ccnd2	6.244	A_44_P477768	NM_022267	Cyclin D2
Ccnd3	10	A_43_P15524	NM_012766	Cyclin D3
Cdkn2b (p15)	0.068	A_44_P313507	NM_130812	Cyclin-dependent kinase inhibitor 2B (p15, inhibits Cdk4)
Cdkn2a (p16)	24.3	A_44_P395885	NM_031550	Cyclin-dependent kinase inhibitor 2A
Cdkn2d (p19)	3.545	A_44_P444257	NM_001009719	Similar to cyclin-dependent kinase inhibitor 2D
Cdkn1a (p21)	2.394	A_44_P515275	NM_080782	Cyclin-dependent kinase inhibitor 1A
Cdkn1b (p27)	7.089	A_44_P508264	NM_031762	Cyclin-dependent kinase inhibitor 1B
Rb1	3.268	A_44_P229079	D25233	Retinoblastoma 1
Rbl2	4.108	A_43_P15508	NM_031094	Retinoblastoma-like 2
E2F1	3.042	A_44_P349002	XM_230765	E2F transcription factor 1
Cdk2	0.441	A_42_P785419	NM_199501	Cyclin-dependent kinase 2
Ccna1	0.0234	A_44_P995716	NM_001011949	Cyclin A1
Ccne2	0.341	A_44_P272210	XM_342804	Cyclin E2
Ccnb1	0.177	A_44_P534089	NM_171991	Cyclin B1
Ccnb2	0.309	A_44_P461544	NM_001009470	Cyclin B2
Ccnb3	0.0945	A_44_P422082	XM_228779	Cyclin B3
Apc	3.349	A_44_P276106	NM_012499	Adenomatosis polyposis coli, negative regulation of cyclin-dependent protein kinase activity
Cdc25c	0.465	A_44_P140314	XM_226071	Cell division cycle 25 homolog C
Mdm2	0.14	A_43_P16885	XM_235169	Transformed mouse 3T3 cell double minute 2 homolog

Table 3 List of genes associated with apoptosis with altered expression after *LM23* knockdown

Gene symbol	*Test/control*	*Array ID*	*ID number*	*Description*
Casp1	14.25	A_44_P468258	NM_012762	Caspase 1, induction of apoptosis
Casp2	2.411	A_44_P1019923	NM_022522	Caspase 2, induction of apoptosis
Casp3	63.61	A_44_P530813	NM_012922	Caspase 3, apoptosis-related cysteine protease
Casp4	14.13	A_44_P1028549	NM_053736	Caspase 4, apoptosis-related cysteine peptidase, induction of apoptosis
Casp6	17.11	A_44_P540910	NM_031775	Caspase 6, induction of apoptosis
Casp7	7.611	A_44_P183488	NM_022260	Caspase 7, induction of apoptosis
Casp8	34.47	A_44_P298210	NM_022277	Caspase 8, caspase activity
Myc	23.36	A_42_P493380	NM_012603	Myelocytomatosis viral oncogene homolog
p53	5.626	A_44_P118929	NM_030989	Tumor protein p53
Bid	52.64	A_44_P550497	NM_022684	BH3 interacting domain death agonist, positive regulation of apoptosis
Bax	10.66	A_43_P11800	NM_017059	Bcl2-associated X protein, induction of apoptosis
Apaf1	14.56	A_43_P12379	NM_023979	Apoptotic peptidase activating factor 1
Cycs	4.021	A_44_P851810	NM_012839	Cytochrome c
Faslg	4.408	A_44_P409820	NM_012908	Fas ligand (TNF superfamily, member 6)
Fadd	14.39	A_44_P258773	NM_1 52937	Fas (TNFRSF6)-associated *via* death domain

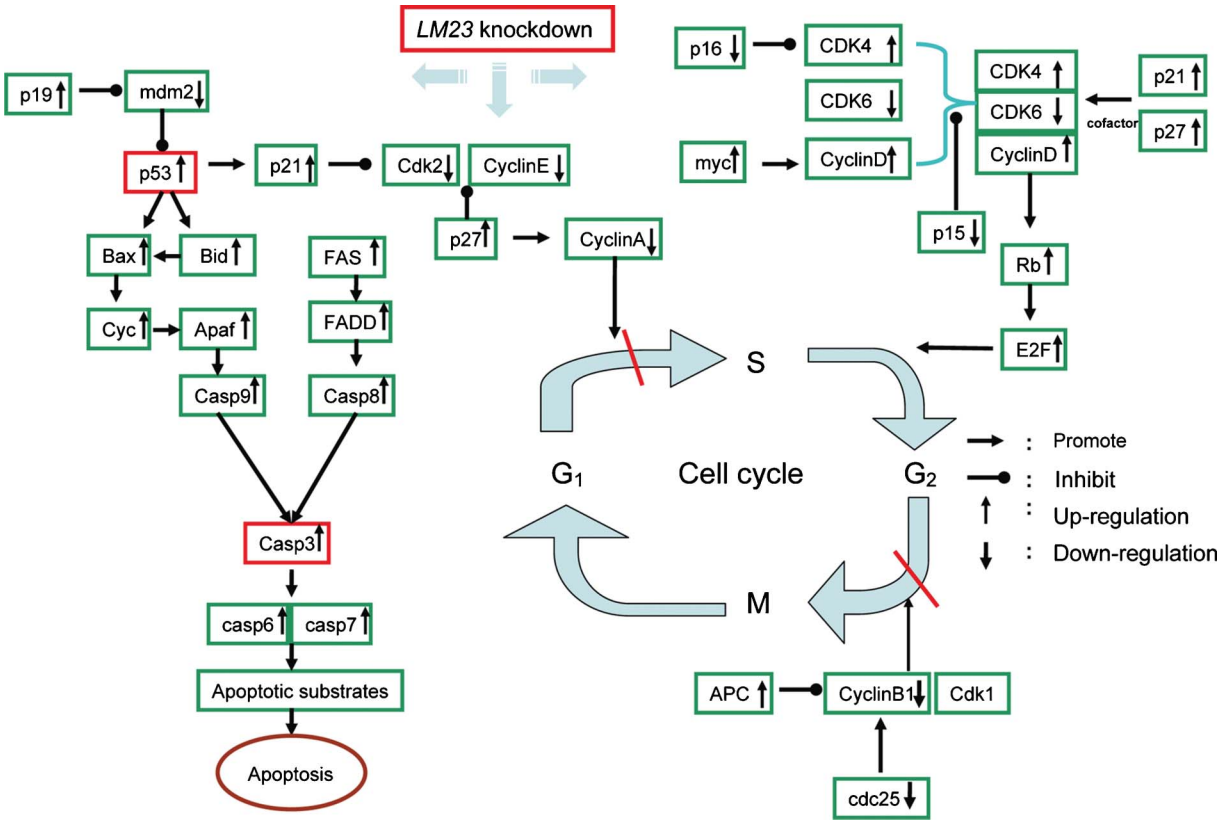

Figure 6 The expression of some genes related to the cell cycle and apoptosis were significantly changed after *LM23* knockdown, as shown by microarray analysis. The downregulation of *LM23* may block the G_1/S and G_2/M phase transitions of the spermatogenic cell. Both the Fas–FasL pathway and the mitochondrial pathway may be activated upon the downregulation of *LM23* during spermatogenesis.

LM23 fusion protein (**Figure 3**). These results further confirmed the reliability of the LM23 polyclonal antibodies and the LM23 fusion protein. Immunohistochemical analysis demonstrated that the positive immunoreactivity was mainly located in the nucleus of spermatocytes and round spermatids (**Figure 4**). These results indicated that LM23 is localized to the nucleus. The testis-specific and stage-specific expression characteristics of LM23 suggested that LM23 might be involved in *R. norvegicus* spermatogenesis. Therefore, the activation of LM23 is likely to promote meiosis in the spermatogenic cell.

LM23 is 97% identical to the Speedy/Ringo box of mouse Speedy/Ringo A. The EST database reveals that *LM23* is a *R. norvegicus* homolog of *RINGO/Speedy A*. Speedy A can activate Cdks that can regulate cell cycle progression.[4] The results from our immunohistochemical analysis showed that LM23 and Cdk2 were both located in the nucleus of spermatocytes and round spermatids (**Figures 4 and 7**). LM23 may also play a role the cell cycle regulation.

A rat *LM23*-deficient model reveals a significant role for LM23 in rat spermatogenesis. The germ cells of the *LM23* knockdown testis

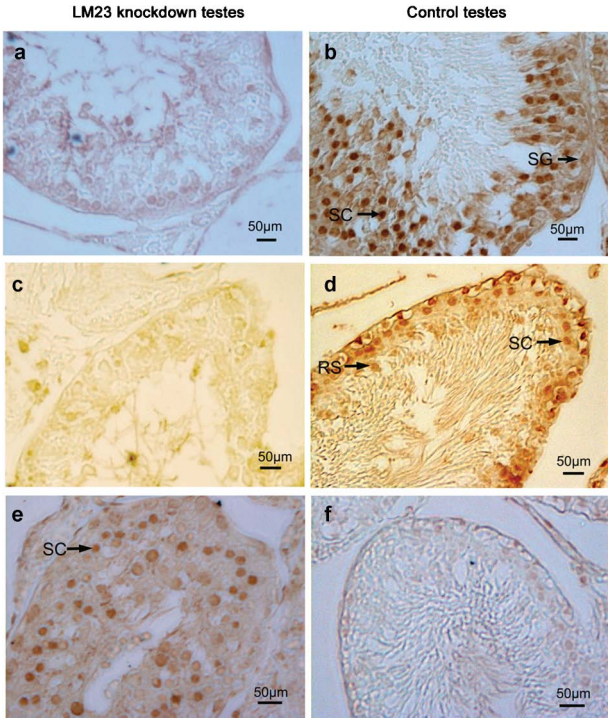

Figure 7 Immunohistochemical analysis with a cyclin A1 antibody. The positive immunoreactivity was mainly located in the nucleus of spermatogonia (SG) and spermatocytes (SC) in wild-type rat (**b**), but few in the *LM23* knockdown rat (**a**). Immunohistochemical analysis with a Cdk2 antibody. The positive immunoreactivity was mainly located in the nucleus of SC and round spermatids (RS) in wild-type rat (**d**), but few in the *LM23* knockdown rat (**c**). Immunohistochemical localization of activated caspase 3 in adult testis sections of wild-type rat (**f**) and the *LM23* gene knockdown rat (**e**). Activated caspase 3 subunit is detected only in the SC of the *LM23* gene knockdown testis sections, in the nucleus, as well as the cytoplasm.

exhibited a complete meiotic arrest in spermatogenesis. These results reveal that *LM23* expression in the testis is crucial for meiosis during spermatogenesis in *R. norvegicus*. Although a number of genes are known to be involved in spermatogenesis, only a few possess clean-cut arrest phenotypes, indicative of their role in the global regulation of key spermatogenic steps. *LM23* gene knockdown rats have a meiotic phenotype that is similar to Cdk2$^{-/-}$ and CyclinA1$^{-/-}$ mice, including germ cell arrest at the spermatocyte stage. Cdk2$^{-/-}$ seminiferous tubules are smaller than the wild type and show defective germ cell development. Post-meiotic cells (late spermatocytes, spermatids and spermatozoa) are absent.[9] Cyclin A1 is a partner of Cdk2. In Cyclin A1$^{-/-}$ mice, male germ cells of the testis cannot enter the first meiotic division.[10] The similarity in phenotypes of these models suggests that all of these proteins seem to be involved in a mechanism at the meiosis I–meiosis II transition in spermatogenesis.

Microarray analyses of the transcriptomes of the *LM23*-shRNA and control testes were performed to screen for the genes regulated by *LM23*. The results revealed that the expression of many genes associated with the cell cycle and apoptosis was markedly altered after *LM23* knockdown (**Tables 2 and 3**). Immunohistochemical analysis further confirmed that *CyclinA1* and *Cdk2*, which are very important in the cell cycle, displayed lower expression after *LM23* knockdown (**Figure 7**). The *LM23* gene knockdown testis showed significantly increased apoptotic cells compared to the control group.[3] Because most apoptotic signalling pathways culminate in the activation of caspase 3, enhanced caspase 3 activation was also found in the *LM23* gene knockdown testis by immunohistochemical analysis (**Figure 7e and f**). In our experiment, the expression of *Myc*, *Cyclin D*, *Cdk4*, *Rb*, *p21*, *p27* and *E2F* increased after *LM23* knockdown (**Table 2 and Figure 6**). Moreover, the expression of *p15* and *p16* decreased after *LM23* knockdown (**Table 2 and Figure 6**). Therefore, the downregulation of *LM23* cannot block the progression of the spermatogenic cell into S phase.[11–14]. Our study showed that the levels of *mdm2*, *Cdk2*, *CyclinE* and *CyclinA* significantly decreased, but the levels of *p19*, *p53*, *p21* and *p27* increased after *LM23* knockdown (**Table 2 and Figure 6**). Therefore, the downregulation of *LM23* may prevent the spermatogenic cell from entering S phase.[15–21] In our study, the expression of APC was increased after *LM23* knockdown (**Table 2 and Figure 6**). In addition, the expression of *CyclinB1* and *cdc25* decreased after *LM23* knockdown. Therefore, the downregulation of *LM23* may prevent the spermatogenic cell from entering M phase.[22] Our study showed that the levels of *p53*, *Bax*, *BID*, *Cytochrome C*, *Apaf-1*, *caspase 9*, *caspase 3*, *caspase 6* and *caspase 7* significantly increased after *LM23* knockdown (**Table 3 and Figure 6**). Therefore, the activation of caspase 3 was likely to be mediated by the mitochondrial pathway after *LM23* knockdown.[23–30] In our study, increased expression of *Fas*, *FADD*, *caspase 8*, *cytochrome c*, *caspase 3*, *caspase 6* and *caspase 7* was observed after *LM23* knockdown (**Table 3 and Figure 6**). Therefore, the activation of caspase 3 was also likely to be mediated by the Fas–FasL pathway after *LM23* knockdown.[31–35]

In conclusion, this study has shown that the knockdown of *LM23* could result in the downregulation of *Cyclin A1*, *Cdk2* and *CyclinB1* and a delay in the progression to S phase and G_2 phase and eventually leads to cell apoptosis. These results suggest that *LM23* belongs to the Speedy/Ringo family and that *LM23* regulates the G_1/S and G_2/M transitions of the cell cycle during spermatogenesis. Both the Fas–FasL pathway and the mitochondrial pathway may be activated by the downregulation of *LM23* gene during spermatogenesis. Future studies will further elucidate the function of *LM23* and provide insight into its physiological role.

AUTHOR CONTRIBUTIONS

MCJ conceived the original idea, designed the study, provided professional advice and edited the manuscript. YMC carried out all the experiments and prepared the original manuscript. MLL designed and carried out the experiments in **Figure 6**, **Table 2** and **Table 3**.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

ACKNOWLEDGMENTS

The authors thank Xin-Quan Shi, De-Yu Liu, Da-Guang Sun, Hong-Fei Xia, Qian-Xin Wang, Chong Wang, Dan Li, Xiang-Bo Xu, Yong-Jie Chang and Nan Yao for technical assistance. This work was supported by the National Natural Science Foundation of China (No. 30670784).

1 Zirkin BR. Regulation of spermatogenesis in the adult mammal: gonadotrophin and androgens. In: Desjardins CD, Ewing LL, editors. Cell and Molecular Biology of the Testis. New York: Oxford University Press; 1993. pp166–88.
2 Liu ML, Pei KY, Shi XQ, Liu DY, Jia MC. Identification and characterization of a novel spermatogenesis related gene *LM23* in rat testis. *Biochem Biophys Res Commun* 2007; **356**: 576–81.
3 Liu ML, Cheng YM, Jia MC. LM23 is essential for spermatogenesis in *Rattus norvegicus*. *Front Biosci* 2010; **2**: 187–94.

4 Cheng A, Xiong W, Ferrell JE Jr, Solomon MJ. Identification and comparative analysis of multiple mammalian Speedy/Ringo proteins. *Cell Cycle* 2005; **4**: 155–65.
5 Ana D, Laurent HP, Amparo D, Markus S, Tim H *et al*. Characterization of a new family of cyclin-dependent kinase activators. *Biochem J* 2005; **386**: 349–55.
6 Danishuddin, Khan AU. Analysis of PB2 protein from H9N2 and H5N1 avian flu virus. *Bioinformation* 2008; **3**: 41–6.
7 Kong L, Li Y, Yang Y, Li K. Expression of fusion IL2-B7.1(IgV+C) and effects on T lymphocytes. *Biochem Cell Biol* 2007; **85**: 685–95.
8 Naz RK. Immunocontraceptive effect of Izumo and enhancement by combination vaccination. *Mol Reprod Dev* 2008; **75**: 336–44.
9 Berthet C, Aleem E, Coppola V, Tessarollo L, Kaldis P. Cdk2 knockout mice are viable. *Curr Biol* 2003; **13**: 1775–85.
10 Liu D, Matzuk MM, Sung WK, Guo Q, Wang P *et al*. Cyclin A1 is required for meiosis in the male mouse. *Nat Genet* 1998; **20**: 377–80.
11 Blagosklonny MV, Pardee AB. The restriction point of the cell cycle. *Cell Cycle* 2002; **1**: 103–10.
12 Weintraub SJ, Chow KN, Luo RX, Zhang SH, He S *et al*. Mechanism of active transcriptional repression by the retinoblastoma protein. *Nature* 1995; **375**: 812–5.
13 Ohtani K. Implication of transcription factor E2F in regulation of DNA replication. *Front Biosci* 1999; **4**: D793–804.
14 Blain SW. Switching cyclin D–Cdk4 kinase activity on and off. *Cell Cycle* 2008; **7**: 892–8.
15 Pagano M, Pepperkok R, Verde F, Ansorge W, Draetta G. Cyclin A is required at two points in the human cell cycle. *EMBO J* 1992; **11**: 961–71.
16 van den Heuvel S, Harlow E. Distinct roles for cyclin-dependent kinases in cell cycle control. *Science* 1993; **262**: 2050–4.
17 Pagano M, Pepperkok R, Lukas J, Baldin V, Ansorge W *et al*. Regulation of the cell cycle by the cdk2 protein kinase in cultured human fibroblasts. *J Cell Biol* 1993; **121**: 101–11.
18 Ohtsubo M, Theodoras AM, Schumacher J, Roberts JM, Pagano M. Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol Cell Biol* 1995; **15**: 2612–24.
19 Kamijo T, Weber JD, Zambetti G, Zindy F, Roussel MF *et al*. Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. *Proc Natl Acad Sci USA* 1998; **95**: 8292–7.
20 Wulf GM, Liou YC, Ryo A, Lee SW, Lu KP. Role of Pin1 in the regulation of p53 stability and p21 transactivation, and cell cycle checkpoints in response to DNA damage. *J Biol Chem* 2002; **277**: 47976–9.
21 Gartel AL, Radhakrishnan SK. Lost in transcription: p21 repression, mechanisms, and consequences. *Cancer Res* 2005; **65**: 3980–5.
22 Morgan, David. The Cell Cycle: Principles of Control. London: New Science Press; 2007. pp96–8.
23 Matsui Y, Nagano R, Obinata M. Apoptosis of fetal testicular cells is regulated by both p53-dependent and independent mechanisms. *Mol Reprod Dev* 2000; **55**: 399–405.
24 Tim LB, Hermien LR, Iris SG, Paul PW, Gabriel GG *et al*. The role of the tumor suppressor p53 in spermatogenesis. *Cell Death Differ* 1998; **5**: 669–77.
25 Yin Y, Stahl BC, deWolf WC, Morgentaler A. p53-mediated germ cell quality control in spermatogenesis. *Dev Biol* 1998; **204**: 165–71.
26 Desagher S, Osen-Sand A, Nichols A, Eskes R, Montessuit S *et al*. Bid-induced conformational change of Bax is responsible for mitochondrial cytochrome c release during apoptosis. *J Cell Biol* 1999; **144**: 891–901.
27 Thornborrow EC, Manfredi JJ. The tumor suppressor protein p53 requires a cofactor to activate transcriptionally the human BAX promoter. *J Biol Chem* 2001; **276**: 15598–608.
28 Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S *et al*. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. *Cancer Cell* 2002; **2**: 183–92.
29 Kuwana T, Bouchier-Hayes L, Chipuk JE, Bonzon C, Sullivan BA *et al*. BH3 domains of BH3-only proteins differentially regulate Bax-mediated mitochondrial membrane permeabilization both directly and indirectly. *Mol Cell* 2005; **17**: 525–35.
30 Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M *et al*. Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* 2002; **111**: 331–42.
31 Pentikäinen V, Erkkilä K, Dunkel L. Fas regulates germ cell apoptosis in the human testis *in vitro*. *Am J Physiol* 1999; **276**: E310–6.
32 Medema JP, Scaffidi C, Kischkel FC, Shevchenko A, Mann M *et al*. FLICE is activated by association with the CD95 death-inducing signaling complex (DISC). *EMBO J* 1997; **16**: 2794–804.
33 Kabra NH, Kang C, Hsing LC, Zhang J, Winoto A. T cell-specific FADD-deficient mice: FADD is required for early T cell development. *Proc Natl Acad Sci USA* 2001; **98**: 6307–12.
34 Muzio M, Chinnaiyan AM, Kischkel FC, O'Rourke K, Shevchenko A *et al*. FLICE, a novel FADD-homologous ICE/CED-3-like protease, is recruited to the CD95 (Fas/APO-1) death-inducing signaling complex. *Cell* 1996; **85**: 817–27.
35 Boldin MP, Goncharov TM, Goltsev YV, Wallach D. Involvement of MACH, a novel MORT1/FADD-interacting protease, in Fas/APO-1- and TNF receptor-induced cell death. *Cell* 1996; **85**: 803–15.