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LM23 is essential for spermatogenesis in *Rattus norvegicus*

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1. ABSTRACT

LM23 is a gene with testis-specific expression in Rattus norvegicus. To reveal the function of *LM23* in the testis, we used lentivirus-mediated RNA interference (RNAi) 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 *R.* norvegicus testes. The expression of *LM23* in the treated testes was significantly knocked down compared with controls. These *LM23*-shRNA testes contained germ cells arrested at the spermatocyte stage, and showed increased apoptosis and disregulation of some meiotic genes. The results demonstrate the validity of the RNAi approach for targeting *LM23* and reveal that *LM23* expression in the testis is crucial for meiosis during spermatogenesis in *R. norvegicus*.

2. INTRODUCTION

LM23 (AF492385) is a gene specifically expressed in the testes of *Rattus norvegicus* previously reported by our laboratory. *LM23* mRNA was detected in the testis, but not in other tissues including heart, liver, spleen, lung, kidney, brain, muscle and ovary. Real-time PCR analysis showed that the expression level of *LM23* was highest in spermatocytes and very low in spermatogonia and spermatids. *In situ* hybridization revealed a strong positive signal in the cytoplasm of spermatocytes and a weak signal in spermatids and spermatogonia (1). This testis-specific and stage-specific expression pattern suggested that *LM23* might be involved in R. norvegicus spermatogenesis. A BLAST homology search against the NCBI non-redundant database and an Ambystoma EST database revealed that *LM23* is a R. norvegicus homologue of Speedy A (Spdya). Speedy (Spy,

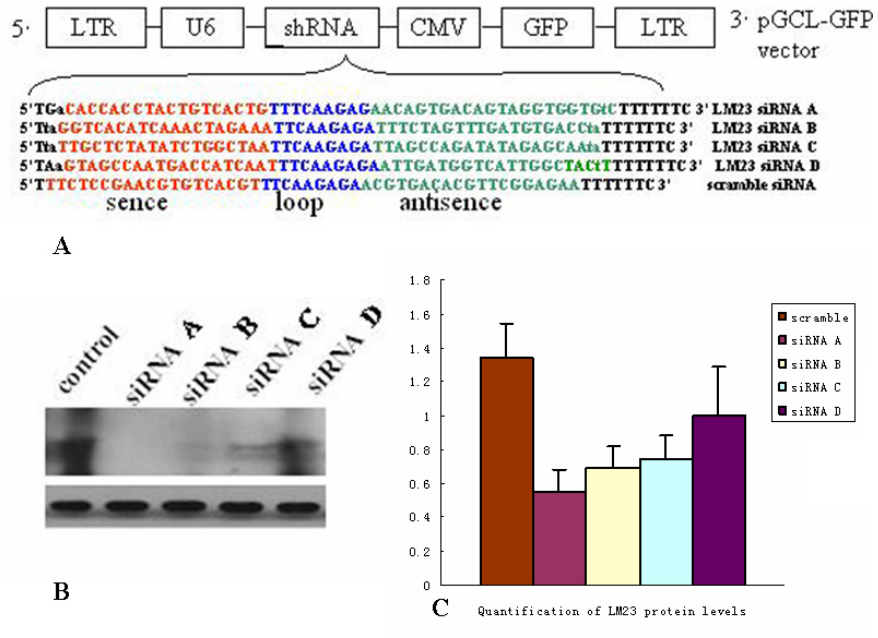

Figure 1. RNAi inhibition of LM23 expression *in vitro*. (A) Schematic of pGCL-GFP lentiviral vector showed long terminal repeats (LTR), U6 promoter-short hairpin RNA (shRNA) cassette, and cytomegalovirus (CMV)–GFP cassette. The sequences of the 4 *LM23*-specific and the scramble shRNAs were shown in the lower panel. (B) Representative immunoblot of LM23 in 293T cells cotransfected pEGFP-C1-*LM23* with siRNA expression vector. Actin loading control was shown in the lower panel. (C) Quantification of LM23 protein levels; LM23 protein abundance in 293T cells transfected with scramble-shRNA was assigned a value of 100%. All data represent the mean+/-SD.*P less than 0.05;**P less than 0.01 compared with control cells.

also called Ringo) was initially found in Xenopus as a protein able to induce the G2/M transition during oocyte maturation. The multiple members of the Speedy family discovered recently represent a novel class of CDK activators and play important roles in cell cycle progression. All Speedy proteins are highly expressed in the testis; some of them are also found in a variety of tissues and cell lines (2). This family of proteins is required for and enhances meiotic maturation in Xenopus oocytes, increases cell proliferation in mammalian cells, and promotes cell survival through prevention of apoptosis in cell lines challenged with DNA-damaging agents.

However, the functional significance of Speedy exclusively expressed in the testis is not known. To investigate its biological function, we used a lentivirus-mediated RNA interference (RNAi) approach to knock down testicular expression of *LM23 in vivo*.

3. MATERIALS AND METHODS

3.1. Experimental animals

SPF male R. norvegicus Sprague-Dawley were purchased from Peking University Laboratory Animal Center. Animals were maintained under controlled temperature (25^{o} C) and lighting (14 hours light: 10 hours dark), and 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 at National Research Institute for Family Planning, P.R. China.

3.2. Construction of fusion protein expression vector

A full-length fragment of LM23 was amplified and cloned into the multiple cloning site of pEGFP-C1 vector containing the enhanced green fluorescent protein gene in accordance with the manufacturer's guidelines to obtain the recombined plasmid pEGFP-C1-LM23. 293T cells were transfected with plasmid pEGFP-C1- LM23 by Lipofectamine 2000 and stably expressed the LM23 fusion protein. The primers pairs used for PCR were CAGATCTCGAGCTCAAGCTTGGATGCGGCATAATC AGATGTGTTG and TATCTAGATCCGGTGGATCCTCATTCTTCGCTCTCT GCAAAC.

3.3. Lentivirus construction

Four pairs of antisense oligonucleotides were designed to generate short hairpin RNA (shRNA) complementary to R. norvegicus *LM23* mRNA transcript (GenBank NCBI accession AF492385, with the ATG start codon taken as position 1; *LM23* RNAi A, 29-47; *LM23* RNAi B, 71-89; *LM23* RNAi C, 370-389; *LM23* RNAi D 848-865. The sequences are shown in Figure 1A). A control shRNA with a nonspecific (NS) nucleotide sequence was also designed (scrambled RNAi; Figure 1A). BLAST analysis (3) verified that these oligonucleotides were specific for *LM23* and that the scrambled RNAi sequence was not homologous to any region of the R. norvegicus genome. Lentiviral vector pGCL-GFP

(Shanghai GeneChem Co. Ltd.) with human U6 promoter (Figure 1A) was used to express the short interfering RNA (siRNA). Oligonucleotides encoding the *LM*23-siRNA or NS-siRNA sequence and a loop sequence separating the complementary domains were synthesized and inserted into pGCL-GFP. *LM23* RNAi A had the best interference efficiency in 293T cells cotransfected with pEGFP-C1-*LM23* and siRNA expression vector, as revealed by Western blot assays (Figure 1B), and consequently it was selected to knock down the endogenous *LM23 in vivo*. Scrambled RNAi was used as a control.

3.4. Generation of high-titer lentivirus

Recombinant lentiviral vectors were produced by co-transfecting 293T cells with the lentiviral expression plasmid pGCL-GFP-*LM23*, RNAi A, and packaging plasmids (pHelper 1.0 including gag/pol and pHelper 2.0 including VSVG) using the calcium phosphate method (4).

Viral supernatant was harvested at 48 hours after transfection, centrifuged to get rid of cell debris, and then filtered through 0.22 μm cellulose acetate filters (5). The infectious titer was determined by fluorescence-activated cell sorting analysis of GFP-positive 293T cells. The infectious lentivirus virus titers were in the range of 10^9 transducing units/ml medium.

3.5. RNAi *in vivo*

Rattus norvegicus males aged 5 weeks old (pubertal) were anesthetized by ether inhalation. One testis was pulled out from the abdominal cavity or scrotum, and approximately 30 μl of the lentivirus preparation described above was injected into the seminiferous tubules by efferent duct injection using glass capillaries under a binocular microscope as Ogawa described (6).The testis was then returned to the abdominal cavity. The scrambled RNAi was injected into the other testis as a control, using the same method. The abdominal wall and skin were closed with sutures.

3.6. Histological and TUNEL analysis.

Freshly harvested *LM23*-shRNA and control testes were dissected at four weeks post-transfection, fixed in Bouin's solution, and then embedded in paraffin. Five-μm testis sections were cut and used for histological and Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL) assay. Sections were stained routinely with hematoxylin and eosin for histological examination. TUNEL assays were performed with the *In situ* Cell Death Detection Kit according to the manufacturer's instructions (Roche). Samples were counterstained briefly in 0.5% (wt/vol) methyl green and examined under a microscope (NIKON H600L, Japan).

3.7. Real-time PCR

The total mRNA from testes was isolated and the cDNA templates were synthesized as described previously (1). A cDNA sample expressing the target gene was selected as template to amplify a target gene segment by conventional PCR. The PCR product was used in 10-fold serial dilutions from 10^1 to 10^6 to construct a standard curve. Quantitative PCR was conducted using SYBR Green PCR Master Mix Reagent (SYBR(R) *Premix Ex Taq*™ kit, TaKaRa) and an ABI 7700 Sequence Detection System (PE Applied-Biosystems). PCR reaction mixes for each standard and sample were prepared in separate tubes, using SybergreenI, universal PCR master mix, primers, and cDNA. All samples were assayed in triplicate and a 25 μl aliquot of each reaction mix was transferred to a well of a MicroAmp optical 96-well reaction plate (Applied Biosystems, USA) to perform reactions. The primers are shown in Table 1. The expression of the housekeeping gene GAPDH was detected in each sample using the same procedure. Target gene expression was normalized with GAPDH gene expression; the ratio between the target and GAPDH was calculated in each sample.

3.8. Statistical analysis

All values are expressed as mean +/- SD. Significant differences were determined by Student's t-test using a p-value of less than 0.05.

4. RESULTS

4.1. Lentivirus-mediated RNAi efficiently inhibited testicular *LM23* expression *in vivo*

A lentiviral vector pGCL-GFP, in which the *U6* promoter drives ubiquitous expression of an *LM23*-specific antisense shRNA and the CMV promoter drives expression of GFP, was used to determine whether LM23 expression could be inhibited. Lentiviruses containing nonspecific control shRNA (scrambled RNAi) and 4 independent shRNAs directed against the LM23 mRNA were designed (LM23 RNAi A-D; Figure 1A). Their ability to reduce LM23 expression through RNAi was first assessed in 293T cells cotransfected with pEGFP-C1-LM23 expressing LM23 fusion protein. Comparing 293T cells transfected with LM23 RNAi A-D with cells transfected with scrambled RNAi, LM23 RNAi A reduced LM23 protein levels by about 60% (Figure 1B,C); LM23 RNAi B, LM23 RNAi C, and LM23 RNAi D reduced LM23 protein levels by about 48%, 44%, and 25% respectively (Figure 1B,C). On the basis of these results, LM23 RNAi A (hereafter simply termed LM23 RNAi) was used in all subsequent experiments. Then the high-titer lentivirus was generated.

The infectious lentivirus was microinjected into testes of 5-week-old R. norvegicus just completing the first wave of spermatogenesis. The enhanced green fluorescent protein (EGFP) signal in about 75% of whole testes of R. norvegicus at four weeks post-transfection is shown in a stereomicroscope view (SteREO Lumar.V12,Carl Zeiss) in Figure 2A.

Next, to examine the efficiency of *LM23* RNAi, we analyzed the expression levels of *LM23* mRNA in testes by real-time PCR at two weeks and four weeks post-transfection. Compared with scrambled RNAi-transfected testes, *LM23* mRNA expression was significantly reduced (69% and 87%, respectively) (Figure 2B). There was no difference in *LM23* mRNA level between scrambled RNAi-transfected testes and wild type testes.

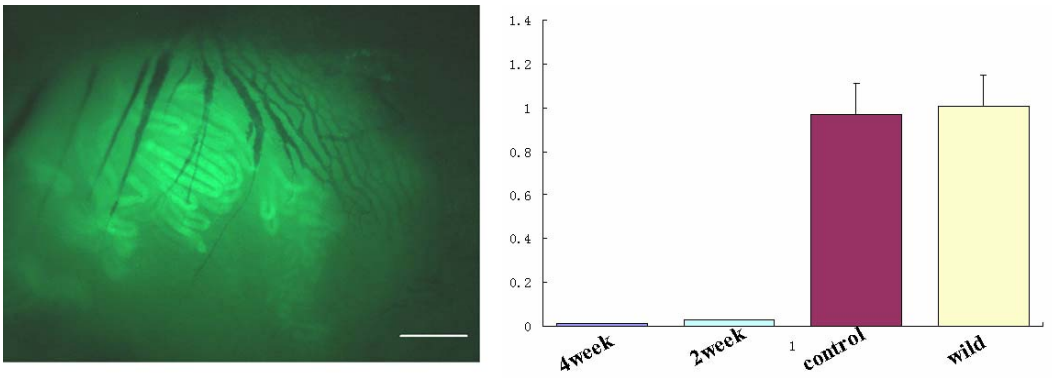

Figure 2. Lentivirus-mediated RNAi efficiently inhibited testicular *LM23* expression *in vivo* (A) EGFP expression (green) was observed in about 75% of whole testes at four weeks post-transfection. Scale bars: 0.3 cm; (B) *LM23*mRNA expressions relative to beta-actin mRNA at two weeks and four weeks post-transfection were measured by real-time RT-PCR. All data represent the mean+/-SD.*P less than 0.05;**P less than 0.01 compared with control testes.

Table1. primers of real-time PCR

Gene	primers	Product size(bp)
LM23	F:5'AGATACGTGAGACTGGGACTGT3' R:5'CGTGCTTTAGTTGAGCCTTG3'	257
Sycp1	F:5'TGGAATCACCTGAAGCCACT3' R:5'GCAGATGCCCGCAGACTAT3'	130
Sycp2	F:5'CTTTATATGGAACCCGAATCTC3' R:5'TCCCTTTCTTCTGCCTCTT3'	248
Sycp3	F:5'CTTCTTTCAAAGCCAGTAACCAG3' R:5'CTTCTCCACATCCTCCAAACTC3'	281
Msh5	F:5'AAGTTGTCCCATACCAAGGTCA3' R:5'CCTGGGTAATGTCCCGAAA3'	127
stage3	F:5'TCAGTATGAGGCAGAACGAAAC3' R:5'CCCTGTACCGATGAACAAAGAC3'	158
rec8L1	F:5'GAAGACATCCAGCATATCCTAGAG3' R:5'CCACAGACATCGTCCCAAAA3'	163
GAPDH	F:5'AAGAAGGTGGTGAAGCAGGC3' R:5'TCCACCACCCTGTTGCTGTA3'	203

These data showed that the specific *in vivo* knockdown of *LM23* in testes of *R. norvegicus* via lentivirus-mediated RNAi was effective and stable.

4.2. Impaired spermatogenesis in the *LM23* knockdown Rattus norvegicus

The size and weight of *LM23*-shRNA testes had no significant differences from the controls (data not shown). Seminiferous tubules of control testes were well organized and contained a full spectrum of spermatogenic cells, including spermatogonia, spermatocytes, spermatids and spermatozoa (Figure 3 A). In contrast, seminiferous tubules of *LM23*-shRNA testes appeared disorganized, disrupted, and shedding germ cells into the lumina; the germ cells exhibited complete meiotic arrest in spermatogenesis (Figure 3, B–D). spermatocytes were accumulated, round spermatids were few and elongating spermatids, spermatozoa were absent in certain *LM23*-shRNA seminiferous tubules. Three major types of seminiferous tubules were observed in *LM23*-shRNA testes. Type I tubules contained 3-4 layers of spermatocytes (Figure 3 B). In type II tubules, there were more layers of spermatocytes and many heavily eosin-stained cells, which might be apoptotic cells (Fig.3C). Type III tubules were characterized by a few layers of spermatogenic cells / Sertoli cells and big empty lumina (Figure 3 D). The epididymal tubules of control R. norvegicus were filled with spermatozoa, whereas those of *LM23*-shRNA testes R. norvegicus were empty (data not shown).

4.3. Increased apoptosis in *LM23*-knockdown Rattus norvegicus

A TUNEL assay showed the presence of many apoptotic cells in certain tubules, which were likely type II tubules (Figure 3G). In contrast, few apoptotic cells were present in type I (Figure 3F) or type III tubules (Figure 3H). Few apoptotic cells were detected in tubules of control testes (Figure 3E). One possible explanation for the presence of three types of tubules in *LM23*-knockdown testes might be coordinated differentiation of the germ cells in a given tubule. In *LM23*-knockdown testes, spermatogenesis proceeded from spermatogonia to spermatocytes, but further differentiation was blocked, resulting in the accumulation of spermatocytes in type I tubules. Subsequently, these spermatocytes failed to further differentiate and underwent apoptosis in type II tubules. Eventually, most apoptotic spermatocytes were eliminated in type III tubules.

4.4. *LM23*-regulated genes in testes

To identify *LM23*-regulated genes that may cause or contribute to these phenotypic effects, gene expression was surveyed by microarray analysis (service provided by Kangchen Biotech, Shanghai, China) on *LM23*-shRNA

Table2. List of genes altered expression after *LM23* knockdown

Array ID	ID munber	Genesymbol	Description
		Up regulation	
A_44_P473159	NM_012955	Fshprh1	FSH primary response 1
A_44_P294687	NM_080885	Cdk5	cyclin-dependent kinase 5
A_44_P388782	NM_053593	Cdk4	cyclin-dependent kinase 4
A_44_P276106	NM_012499	Apc	adenomatosis polyposis coli
A_44_P404198	NM_017347	Mapk3	mitogen activated protein kinase 3
A_44_P530813	NM_012922	Casp3	caspase 3, apoptosis related cysteine protease
A_44_P360756	AF149299	Bcl2	B-cell leukemia/lymphoma 2
A_44_P489512	AF235993	Bax	Bcl2-associated X protein
		Down regulation	
A_44_P149138	NM_013086	Crem	cAMP responsive element modulator
A_44_P304930	NM_001025742	Dazap1	DAZ associated protein 1
A_44_P982926	BQ190235	Tegt	Testis enhanced gene transcript
A_44_P1024125	NM_031694	Hsf2	heat shock factor 2
A_44_P555309	XM_344105	Piwil1_predicted	piwi like homolog 1 (Drosophila) (predicted)
A_44_P443479	NM_001007752	Mns1	meiosis-specific nuclear structural protein 1
A_44_P412236	NM_053730	**stage3**	stromal antigen 3
A_44_P253175	XM_345476	Spo11_predicted	sporulation protein, meiosis-specific, SPO11 homolog (S. cerevisiae) (predicted)
A_43_P11573	NM_012810	**Sycp1**	synaptonemal complex protein 1
A_44_P320812	NM_001011916	**Rec8L1**	REC8-like 1 (yeast)
A_44_P459191	NM_013041	**Sycp3**	synaptonemal complex protein 3
A_44_P995716	NM_001011949	Ccna1	cyclin A1
A_44_P185215	NM_130735	**Sycp2**	synaptonemal complex protein 2
A_43_P19334	NM_212536	**Msh5**	mutS homolog 5 (E. coli)
A_44_P107461	NM_130406	Faf1	Fas-associated factor 1
A_42_P785419	NM_199501	Cdk2	cyclin dependent kinase 2

Genes shown in bold were analyzed further

Table3. *LM23*-regulated genes in testes

Symbel	Test AVG	Control AVG	Test/Control	Fold change	P value
Sycp1	0.00411	0.02050	0.20049	-4.98783	0.00449
Sycp2	0.00064	0.30867	0.00230	-476.82801	0.00058
Sycp3	0.00479	0.29400	0.01632	-61.29256	0.00023
Stag3	0.00058	0.04157	0.01394	-71.70788	1.91251E-07
Msh5	0.00107	0.16467	0.00650	-153.75039	0.00023
rec8L1	0.00051	0.20333	0.00251	-398.17232	3.94614E-06

testes and controls using Agilent rat whole genome arrays (design ID 14879). Interestingly, the expression of some genes related to spermatogenesis, meiosis, the cell cycle, and apoptosis was significantly changed after *LM23* knockdown (Table 2). Some meiotic genes involved in synapsis, recombination (Sycp1, Sycp2, Sycp3, Msh5) and meiotic sister-chromatid cohesion (Stag3, rec8L1) were chosen for further analysis. Real-time PCR analysis confirmed that all these genes exhibited significantly altered expression in *LM23*-knockdown testes compared with controls. The expression level of the six genes was significantly lower in testes with *LM23* knockdown than in the control group (Table 3).

5. DISCUSSION

Studies of genes that regulate spermatogenesis have been carried out mostly via the production and analysis of mutant mice carrying transgenes or targeted gene disruptions (7). However, these methods are laborious, time-consuming, and expensive. In recent years RNA interference (RNAi) has come to the fore as an efficient alternative for studying gene function. In mammals, the introduction of chemically synthesized siRNA or a vector-based system expressing the short hairpin type of siRNA (shRNA) induces sequence-specific gene silencing in various cell types and tissues (8). Shoji (9) first carried out *in vivo* the DNA electroporation of the testis during the first wave of spermatogenesis to enable foreign gene expression at each stage of differentiation during spermatogenesis. His results showed that RNAi was effective throughout spermatogenesis, including during meiosis and in haploid cells. This RNAi system *in vivo* affords a rapid means of assessing the physiological roles of spermatogenic genes. However, the transfection efficiency of electroporation is limited and transient at present. A lentiviral system had high transduction efficiency and is effective for expressing siRNAs in early embryos (10,11). Lentivirus has emerged as a highly effective vector for introducing transgenes into such animals as mice (12), *R. norvegicus* (13,14), and chickens (15). Possibly the most important aspect of lentiviral transgenesis is the ease and efficiency with which it can be applied.

To reveal the function of LM23, we have introduced a lentivirus-mediated RNAi approach to knock down *LM23* expression *in vivo* in *R. norvegicus.* In this approach a lentiviral vector expressing a short hairpin RNA targeting *LM23*, which strongly inhibited *LM23* expression in transfected cells, was microinjected into the efferent duct of the testis. The expression of *LM23* in LM23-shRNA

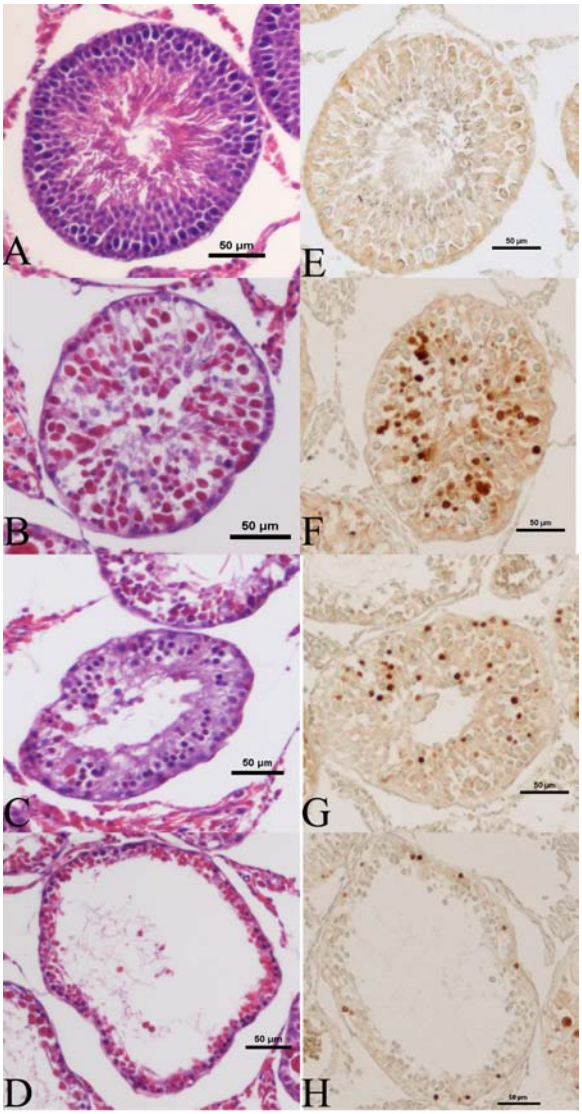

Figure 3. Impaired spermatogenesis and apoptosis of germ cellsin in *LM23*-shRNA testes. **C**ontrol and *LM23*-shRNA testes of Rattus norvegicus were used for histological and apoptosis analyses at four weeks post-transfection. (A) The seminiferous tubule of control testes contained a full spectrum of germ cells: spermatogonia, pachytene spermatocytes, round spermatids, and elongated spermatids. (B–D) The seminiferous tubules of *LM23*-shRNA testes appeared disorganized and disrupted. Three major types of seminiferous tubules were observed in *LM23*-shRNA testes. (B) Type I tubules contained 3-4 layers of spermatocytes. (C) In type II tubules, there were more layers of spermatocytes and many heavily eosin-stained cells (D). Type III tubules were characterized by a few layers of spermatogenic cells / Sertoli cells and big empty lumina. (E-H) A TUNEL assay showed the presence of many apoptotic cells in certain tubules, which were likely type II tubules (G). In contrast, few apoptotic cells were present in type I (F) or type III tubules (H). Few apoptotic cells were detected in tubules of control testes (E). Bars, 50 µm.

testes decreased significantly. In LM23-shRNA testes the seminiferous tubules appeared disorganized and disrupted. The spermatogenic cells developed into spermatocytes, but failed to progress to postmeiotic germ cells; some spermatocytes suffer apoptosis and shed into the lumina. We presume that *LM23* knockdown blocks the meiosis of spermatogenesis. The test results demonstrated the validity of our RNAi approach and unambiguously revealed that *LM23* expression in testes is crucial for meiosis during spermatogenesis.

Microarray analyses of the transcriptomes of the LM23-shRNA and control testes were performed to screen for genes regulated by *LM23*. The results revealed that the expression of some genes related to spermatogenesis, meiosis, the cell cycle, and apoptosis were significantly changed after *LM23* knockdown. Real-time PCR analysis confirmed that some meiotic genes involved in synapsis, recombination (*Sycp1, Sycp2, Sycp3, Msh5*) and meiotic sister-chromatid cohesion (*Stag3, rec8L1*) had lower expression.

Recent studies have demonstrated the essential roles of many genes involved in meiosis, including those related to synapsis and meiotic recombination. Sycp1, Sycp2 and Sycp3 are important synaptonemal complex (SC) proteins. SC proteins have provided novel mechanistic insights into the regulation of meiosis, in particular the assembly of the SC. In mice lacking Sycp1, axial elements appeared to be assembled normally; homologous chromosomes paired with each other but failed to undergo synapsis; meiotic recombination was initiated, but crossovers were not formed (16). Sycp2 and Sycp3 are integral components of the axial/lateral elements (AE/LE). Genetic studies of mutant mice demonstrated that both proteins were required for formation of axial elements and thus chromosomal synapsis (17,18). Msh5 is a member of the mammalian protein family of MutS homologues that repair mismatches during meiotic recombination. De Vries found that mice carrying a disruption in Msh5 showed a meiotic defect leading to male and female sterility; Msh5 promoted synapsis of homologous chromosomes in meiotic prophase I (19). Sister chromatid cohesion is a key event in chromosomal segregation during the cell cycle; it is maintained by a multi-subunit protein complex termed cohesin (20). Cohesins are also intertwined with the SC structure, and meiosis-specific cohesin complexes are present in mammalian germ cells. REC8 and STAG3 are subunits of the meiotic cohesin complex that are specific to germinal cells; they are colocalized along the chromosome arms in pachytene (21,22). Recent evidence has indicated that an intact cohesin complex is essential for progression through prophase I of meiosis (21).

Real-time PCR analysis confirmed that the expression levels of these six genes were significantly lower in testes with *LM23* knockdown than in controls. We speculate that these six genes are target genes regulated by *LM23* and may cause or contribute to its phenotypic effects. Interestingly, the phenotype of the disruption of *Sycp2* in mouse was similar to *LM23* knockdown in *R. norvegicus* (17). Both brought about meiotic arrest and subsequent

spermatocyte apoptosis in the testis. Thus, it is conceivable that *LM23* plays an important role in meiotic progression.

Collectively, these studies demonstrate that *LM23* is required for meiosis in spermatogenesis. To our knowledge, this is the first definitive assignment of the function of *LM23* and mammalian Speedy homologues in spermatogenesis. This experimental system provides a novel tool for the effective assessment of the physiological functions of spermatogenic genes *in vivo*.

6. ACKNOWLEDGEMENTS

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