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 deregulation 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,
LM23 is essential for spermatogenesis

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°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 recombinant 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 CAGATCTCGAGCTCAAGCTTGGATGCGGCATAATCAGATGTGTTG and TATCTAGATCCGGTGGATCCTCATTCTTCGCTCTCTGCAAAC.

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
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(Shanghai GeneChem Co. Ltd.) with human U6 promoter (Figure 1A) was used to express the short interfering RNA (siRNA). Oligonucleotides encoding the LM23-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® Green 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 Sybgreen, 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.
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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

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<tr>
<th>Gene</th>
<th>primers</th>
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<tr>
<td>LM23</td>
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<tr>
<td>Syca1</td>
<td>F:5’TGGAAATCCCATGGAACGACTG3’ R:5’GCGAGATGCCCGAGACTATA3’</td>
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<td>Syca2</td>
<td>F:5’CTTTATGGAACCGGAACATGCTC3’ R:5’TCCCTCTTTCTCTGCTCTC3’</td>
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<td>Syca3</td>
<td>F:5’CTTCTTCAAACGAGATACCCAG3’ R:5’TCTCTACACATCTCCAAAAC3’</td>
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</tr>
<tr>
<td>Msh5</td>
<td>F:5’AAATGTTATCCCATACCAAGAC3’ R:5’CTTGGGGATGAATGCAAAAC3’</td>
<td>127</td>
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<tr>
<td>Stage3</td>
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<tr>
<td>Rec8L1</td>
<td>F:5’GAAGACATTCACATCAGTACAG3’ R:5’CCACAGACATGCTGCCAAAAC3’</td>
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<td>GAPDH</td>
<td>F:5’GAGAAGGTTGGTGAAGACGCG3’ R:5’TCCACCACCTGTTGACTA3’</td>
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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
Table 2. List of genes altered expression after LM23 knockdown

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<th>Genesymbol</th>
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<td>NM_012955</td>
<td>Fshprh1</td>
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<td>cyclin-dependent kinase 5</td>
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<td>A_44 P388782</td>
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<td>A_44 P276106</td>
<td>NM_012499</td>
<td>Apc</td>
<td>adenomatosis polyposis coli</td>
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<td>A_44 P401998</td>
<td>NM_01347</td>
<td>Mapk3</td>
<td>mitogen-activated protein kinase 3</td>
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<td>A_44 P350813</td>
<td>NM_013922</td>
<td>Casp3</td>
<td>caspase 3, apoptosis-related cysteine protease</td>
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<td>AF149299</td>
<td>Bcl2</td>
<td>B-cell leukemia/lymphoma 2</td>
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<td>A_44 P489512</td>
<td>AF235993</td>
<td>Bax</td>
<td>Bcl2-associated X protein</td>
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Up regulation

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<td>cAMP responsive element modulator</td>
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<tr>
<td>A_44 P304930</td>
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<td>Dazap1</td>
<td>DAZ associated protein 1</td>
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<td>A_44 P982926</td>
<td>BQ190235</td>
<td>Teg1</td>
<td>Testis enhanced gene transcript</td>
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<tr>
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<td>AF149299</td>
<td>Bcl2</td>
<td>B-cell leukemia/lymphoma 2</td>
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<td>A_44 P489512</td>
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<td>Bax</td>
<td>Bcl2-associated X protein</td>
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Down regulation

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<td>AF149299</td>
<td>Bax</td>
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Genes shown in bold were analyzed further

Table 3. LM23-regulated genes in testes

<table>
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<tr>
<th>Symbol</th>
<th>Test AVG</th>
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<th>Test/Control</th>
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<td>0.29400</td>
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<td>Sycp4</td>
<td>0.00157</td>
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<td>Sycp6</td>
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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 electroperoration 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), 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 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 synopsis, 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).
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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 synopsis, 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 synopsis 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 synopsis; 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 synopsis (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 synopsis 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

Figure 3. Impaired spermatogenesis and apoptosis of germ cells in LM23-shRNA testes. Control 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.
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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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7. REFERENCES


LM23 is essential for spermatogenesis


**Key Words:** LM23; RNAi; lentiviral vector; spermatogenesis; Rattus norvegicus

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