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.

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 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 CAGATCTCGAGCTCAAGCTTGGATGCGGCATAATCAGATGTGTTG and TATCTAGATCCGGTGATCCATTCTTCGCTCTCTGCAAAC.

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 anaesthetized 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® 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 Sybergreen, 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>primers</th>
<th>Product size(bp)</th>
</tr>
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<tbody>
<tr>
<td>LM23</td>
<td>F:5'AGATACGTGAGACTGGGACTGT3' R:5'CGTGCTTTATGTTGAGCCCTTG3'</td>
<td>257</td>
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<tr>
<td>Syct1</td>
<td>F:5'TGGAATCACCTGGAAGCCTACT3' R:5'GCAGATGCCGGCAGACATAT3'</td>
<td>130</td>
</tr>
<tr>
<td>Syct2</td>
<td>F:5'CTTTTATAGGAAACCCGAACTC3' R:5'TCCTTCCTCTCTCTCCTT3'</td>
<td>248</td>
</tr>
<tr>
<td>Syct3</td>
<td>F:5'CTTCTTCAGGAACCGATAAACAG3' R:5'CCTCTCCACATCTCCAAAATCT3'</td>
<td>281</td>
</tr>
<tr>
<td>Msh5</td>
<td>F:5'AAAGTTGTCATTCCATCAAGGCACT3' R:5'CTCGGTTAATGTGCGGGAA3'</td>
<td>127</td>
</tr>
<tr>
<td>stage3</td>
<td>F:5'TCAGATAGGAACGGAGAAACGAAC3' R:5'CCTGGTACGATGAAACAAAGAC3'</td>
<td>158</td>
</tr>
<tr>
<td>rec8L1</td>
<td>F:5'GAAGACATCAGCTATATTAGAGA3' R:5'CCTCCAGAGACGTCCAAAA3'</td>
<td>163</td>
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<tr>
<td>GAPDH</td>
<td>F:5'AAGAAGGTGGTGAAGCAGGC3' R:5'TCCACCACCCTGTGCTGTA3'</td>
<td>203</td>
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</table>

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
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Table 2. List of genes altered expression after LM23 knockdown

<table>
<thead>
<tr>
<th>Array ID</th>
<th>ID number</th>
<th>Genesymbol</th>
<th>Description</th>
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<tr>
<td>A_44_P473159</td>
<td>NM 012955</td>
<td>Fshprh1</td>
<td>FSH primary response 1</td>
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<tr>
<td>A_44_P294687</td>
<td>NM 080885</td>
<td>Cdk5</td>
<td>cyclin-dependent kinase 5</td>
</tr>
<tr>
<td>A_44_P388782</td>
<td>NM 053593</td>
<td>Cdk4</td>
<td>cyclin-dependent kinase 4</td>
</tr>
<tr>
<td>A_44_P267106</td>
<td>NM 012499</td>
<td>Apc</td>
<td>adenomatosis polyposis coli</td>
</tr>
<tr>
<td>A_44_P401998</td>
<td>NM 017347</td>
<td>Mapk3</td>
<td>mitogen-activated protein kinase 3</td>
</tr>
<tr>
<td>A_44_P358813</td>
<td>NM 012892</td>
<td>Casp3</td>
<td>caspase 3, apoptosis related cysteine protease</td>
</tr>
<tr>
<td>A_44_P360756</td>
<td>AF149299</td>
<td>Bcl2</td>
<td>B-cell leukemia/lymphoma 2</td>
</tr>
<tr>
<td>A_44_P489512</td>
<td>AF235993</td>
<td>Bax</td>
<td>Bcl2-associated X protein</td>
</tr>
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</table>

Genes shown in bold were analyzed further

Table 3. LM23-regulated genes in testes

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Test AVG</th>
<th>Control AVG</th>
<th>Test/Control</th>
<th>Fold change</th>
<th>P value</th>
</tr>
</thead>
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<tr>
<td>Sycp1</td>
<td>0.00411</td>
<td>0.02050</td>
<td>0.20049</td>
<td>-4.98783</td>
<td>0.00449</td>
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<tr>
<td>Sycp2</td>
<td>0.00064</td>
<td>0.30867</td>
<td>0.0230</td>
<td>-476.82601</td>
<td>0.00058</td>
</tr>
<tr>
<td>Sycp3</td>
<td>0.00479</td>
<td>0.29400</td>
<td>0.01362</td>
<td>-61.2956</td>
<td>0.00023</td>
</tr>
<tr>
<td>Stag3</td>
<td>0.00058</td>
<td>0.04457</td>
<td>0.01394</td>
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<tr>
<td>Msh5</td>
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<td>0.16467</td>
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<td>rec8L1</td>
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<td>0.20333</td>
<td>0.00251</td>
<td>-398.17232</td>
<td>3.94614E-06</td>
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</tbody>
</table>

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 electroportion 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. Lentivirus has emerged as a highly effective vector for introducing transgenes into such animals as mice (10,11), R. norvegicus (12,13), and chickens (15). Possibly the most important aspect of lentiviral transgenesis is the ease and efficiency with which it can be applied.
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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.

6. ACKNOWLEDGEMENTS

The project was supported by the National Natural Science Foundation of China (No. 30670784).

7. REFERENCES


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

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