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ORIGINAL ARTICLE

Role of LM23 in cell proliferation and apoptosis and its expression during the testis development

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***LM23*, a gene expressed specifically in the testis in a stage-specific manner, has a diverse range of functions that are important in both the life and death of spermatogenic cells. The aim of this study was to further investigate the expression of LM23 in the developing rat testis and the biological function of LM23 in proliferation and antiapoptosis *in vitro*. Semiquantitative reverse transcription (RT)-PCR and real-time PCR were used to examine the expression of LM23 in testis at different developmental stages. The results suggested that LM23 mRNA levels in the testis increased progressively after birth. The role of LM23 in proliferation was analyzed with cell counting kit-8 (CCK8), colony-forming efficiency (CFE) and flow cytometry assays. The results indicated that ectopic expression of LM23 in 293T cells significantly promoted cell proliferation by increasing cell numbers in S phase. Several methods were used, including CCK8, annexin V and propidium iodide staining and western blotting, to determine the role of LM23 in apoptosis. The results showed that LM23 played a protective role in H_2O_2-induced apoptosis of 293T cells, mediated at least in part through the Akt/PI3K signal pathway. Taken together, these results provide new insights into the role of LM23 in the development of the testes and spermatogenesis.**

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INTRODUCTION

The mammalian testis is a complex organ that serves two important functions: synthesis of steroids and production of spermatozoa. Spermatogenesis, or the differentiation of male germ cells to produce mature spermatozoa, is controlled by gonadotrophins and numerous locally synthesized factors.[1] Spermatogenesis is divided into three distinct stages: the mitotic proliferation of spermatogonia, meiotic division of spermatocytes and spermiogenesis of haploid spermatids. This process of spermatogenesis involves transcriptional, translational and posttranslational regulation. Many spermatogenesis-related genes, which are specific to different phases of germ cell development, have been found in the past few years, and some, such as *LM23*, have been proven to play important roles in spermatogenesis.[2] *LM23* (GenBank accession no. AF492385) cDNA consists of 1896 base pairs (bp) with a complete open reading frame of 936 bp, and encodes a putative protein including 312 amino acids. It has been shown that the *LM23* gene is expressed specifically in the testis in a stage-specific manner, and may be involved in rat spermatogenesis, as previous reports from our laboratory have suggested.[3]

Cell proliferation, differentiation, senescence and apoptosis are cell cycle-dependent, and the basic regulatory mechanisms in cell cycle progression rely on a multicomponent system. At different phases, progression through the cell cycle is regulated by sequential activation and subsequent inactivation of a series of cyclin-dependent kinases, whose activity depends on interactions with cyclins and cyclin-dependent kinase inhibitors.[4–6] Apoptosis is a type of programmed cell death characterized by the morphological changes of nuclear condensation and cell shrinkage.[7,8] The process of apoptosis is regulated by several proteins, including members of the Bax, p53, Bcl-2 and caspase 3 protein families.[9] A recent report has provided persuasive evidence that the knockdown of LM23, by lentivirus-mediated RNA interference, may result in downregulation of Cyclin A1, Cdk2, and CyclinB1, S and G2 phase delay, and eventually lead to apoptosis.[10] This report also revealed that LM23 expression in the testis is crucial for meiosis during spermatogenesis in *Rattus norvegicus*.[10] Further research in our laboratory has suggested that LM23 belongs to the Speedy/Ringo family and can regulate the G1/S and G2/M transitions of the cell cycle during spermatogenesis.[2] However, the expression of LM23 in the developing rat testis has not been fully investigated, and a direct role for LM23 in cell proliferation and apoptosis or other LM23-associated functions is yet to be established.

Additional, the testes of 35-day-old rat after LM23 knockdown by lentivirus-mediated RNA interference showed that the germ cells arrested at the spermatocyte stage, and apoptosis of pathytene spermatocytes was increased.[10] The expression of some genes related to the cell cycle and apoptosis was markedly changed after LM23 knockdown, as

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shown by the microarray analysis. The downregulation of LM23 might block the G1/S and G2/M transitions of the spermatogenic cell.[2] Since no suitable male germ cell lines are available now, we have to use somatic cell line to investigate the function of LM23 in apoptosis, cell proliferation and cell cycle.

The objective of the present study was to address both these issues. The *in vivo* expression of LM23 in the developing rat testis was examined using semiquantitative reverse transcription (RT)-PCR and real-time PCR. To investigate the role of LM23 in apoptosis, cell proliferation and cell cycle progression *in vitro*, 293T cells transfected with LM23 expression plasmids were used in cell counting kit-8 (CCK8) and flow cytometry assays. Western blotting, annexin V and propidium iodide staining and proliferation assays were used to investigate the effect of LM23 overexpression in H_2O_2-induced apoptosis and its possible mechanisms of action in 293T cells. The results provide new insights into the role of LM23 in the development of the testes.

MATERIALS AND METHODS

Animals and tissue preparation

Male Sprague–Dawley rats were purchased from Peking University Laboratory Animal Center. Rats were obtained at 0, 5, 14, 21, 30 and 65 days after birth. The testes were obtained from three rats at each age.

RT-PCR and real-time PCR

Total RNA was extracted from the testes according to the manufacturer's protocol for Trizol Reagent (Invitrogen, Carlsbad, CA, USA). For each sample, cDNA synthesis was carried out using 0.25 μg of total RNA and PrimeScript RT Master Mix Perfect Real-Time (TaKaRa, Tokyo, Japan). Specific transcripts of LM23 were amplified by quantitative PCR using the following primers: LM23, 5′-AGATACGTGAGACTGGGACTGT-3′(forward) and 5′-CGTGCTTTAGTTGAGCCTTG-3′(reverse); GAPDH, 5′-AAGAAGGTGGTGAAGCAGGC-3′(forward) and 5′-TCCACCACCCTGTTGCTGTA-3′(reverse). GAPDH was used as an internal control. The real-time PCR was conducted using SYBR Green PCR Master Mix Reagent (SYBR Premix Ex Taq kit; TaKaRa) and an ABI Stepone Sequence Detection System (PE Applied-Biosystems, Streetsville, Canada). The level of mRNA was expressed as the threshold cycle values of the target gene and reference gene, GAPDH, which is constitutively expressed. Comparison of threshold cycle values was used to determine the relative mRNA levels, expressed as the fold change of the target gene relative to the reference gene.

Plasmid construction

The generation of vector (pGEM-T-LM23) has been previously described.[2] Full-length LM23 fragment was obtained by double enzyme digestion from pGEM-T-LM23 and cloned into the pcDNA3.1/myc-His (−) B vector (pcDB; Invitrogen), using the restriction enzymes *Eco*RI and *Bam*HI.

Cell culture and transient transfection

HEK 293T (human embryonic kidney cell line, a kind gift from T. Matsuda, Japan) cells were maintained in Dulbecco's modified Eagle's medium (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and were incubated in a 5% CO_2 incubator at 37 °C. Plasmids were transfected into 293T cells using VigoFect (Vigorous Biotechnology, Beijing, China), according to the manufacturer's instructions.

CCK8 assay

293T cells transfected with pcDB vector control or pcDB-LM23 were plated in a 96-well plate at a concentration of 2000 cells per well. At 0, 1, 2, 3, 4 and 5 days after transfection, the cell proliferation assay was performed by the addition of 10 μl CCK8 solution (Dojindo, Tokyo, Japan) to each well, followed by incubation at 37 °C for 2 h. Absorbance was measured at a wavelength of 450 nm using a microplate reader (Synergy 2 Multi-Mode Microplate Reader; BioTek, Winooski, VT, USA).

Flow cytometry with propidium iodide staining

293T cells were synchronized with 12 h of serum starvation, and then transfected with 2.5 μg of pcDB vector control or pcDB-LM23. Cells were trypsinized at 24 h after transfection, washed twice with phosphate-buffered saline (PBS), and fixed with ice-cold 70% ethanol. The fixed cells were pelleted, washed and resuspended in PBS. Samples were treated with 1 μl of a 10 mg ml^{-1} stock of DNase-free RNase A (Promega, Madison, WI, USA) and incubated at 37 °C for 30 min. They were then treated with 50 μl of 300 mg ml^{-1} propidium iodide (Boehringer, Indianapolis, IN, USA) containing Triton X-100 and incubated in the dark within 30 min. Data were collected on a flow cytometer (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Colony-forming efficiency (CFE)

293T cells transfected with pcDB or pcDB-LM23 were plated in a 6-well plate at a concentration of 500 cells per well and incubated at 37 °C for 14 days. Colonies were then stained with crystal violet solution, and the number of colonies was counted. The CFE of each group was expressed as a percentage of the total number of colonies in pcDB-transfected controls.

Western blotting

293T cells were transfected with pcDB or pcDB-LM23. After 24 h, cells were washed twice with ice-cold PBS and lysed in cell lysis buffer (1× PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodycl sulfate, with freshly added proteinase inhibitor cocktail) for 30 min at 4 °C. Cell lysates were clarified by centrifugation at 4 °C at 16 000 *g* for 15 min. The total protein concentration was determined using the BCA protein assay kit (Vigorous Biotechnology). Equal amounts of protein were separated by 12.5% sodium dodycl sulfate–polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membrane. Membranes were blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat milk for 2 h, and incubated overnight at 4 °C with the appropriate primary antibody. After washing in Tris-buffered saline containing 0.1% Tween-20 buffer, membranes were incubated for 1 h in the dark with the appropriate horseradish peroxidase-conjugated secondary antibodies. Antibody reactivity was visualized with an enhanced chemiluminescent substrate (Invitrogen). Antibodies specific for the following proteins were used: Bcl-2-associated protein (Bax), p53, caspase 3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), Bcl-2 (Cell Signaling Technology, Beverly, MA, USA) and actin (Sigma, St Louis, MO, USA). Dilution (1 : 1000) was used to detect actin, and all the other antibodies were used at the 1 : 500 dilution.

Transient expression dual-luciferase reporter assay

293T cells were seeded into a 96-well plate at 1×10^4 cells per plate. After 24 h, the cells in each well were cotransfected with 80 ng of the pcDB-LM23 or pcDB vector control plasmids, 40 ng of the pNF-κB-Luc plasmids containing the firefly luciferase reporter gene (PathDetect; Stratagene, La Jolla, CA, USA) and 4 ng of the pRL-TK

plasmid as the internal control containing the *Renilla* luciferase gene (Promega). Each transfection experiment was performed in triplicate wells. At 24 h after transfection, the cells were lysed in standard lysis buffer (Promega). The cell lysates were assayed for both firefly and *Renilla* luciferase activities with the dual-luciferase reporter assay system (Promega), according to the manufacturer's instructions, using a GENios Pro reader (Tecan, Mannedorf, Switzerland). Luciferase activity was normalized to the *Renilla* luciferase activity. All experiments were performed in duplicate.

Flow cytometry analysis with annexin V and propidium iodide staining

293T cells were transfected with 80 ng of the pcDB-LM23 or pcDB plasmids. At 24 h after transfection, 293T cells were stained according to the manufacturer's instructions of Annexin V-FITC/PI kit (PharMingen). Flow cytometry was conducted on an FACS calibur system and the results were analyzed by CellQuest software (Becton Dickinson, Mountain View, CA, USA).

RESULTS

Expression of LM23 mRNA in testis development

LM23 is known to be specifically expressed in testis,[3] but its expression in the developing rat testis has not been established. In order to investigate the expression of LM23 in testis development and spermatogenesis, the semiquantitative RT-PCR and real-time PCR were performed to examine the expression of LM23 at different periods of testicular development. The results showed that the LM23 mRNA level in the testis was low at day 0 after birth, and then increased progressively during postnatal testis development (**Figure 1a and 1b**). The expression of LM23 mRNA reached a peak level at day 30 after birth, and then decreased at day 65 after birth (**Figure 1b**). These data indicate that LM23 expression is stage-specific during testis development.

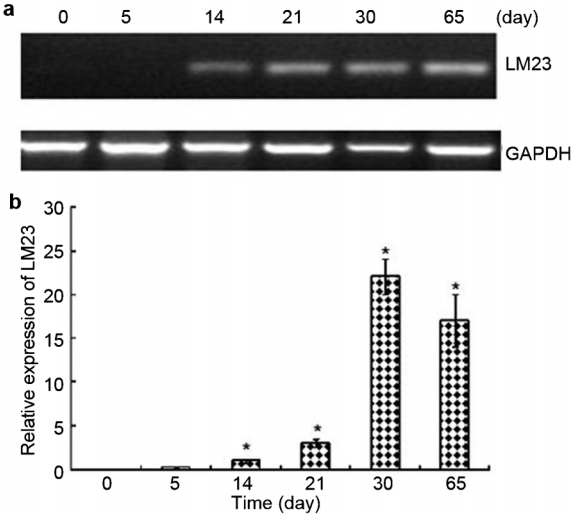

Figure 1 Quantitative analysis of LM23 expression during postnatal development in the rat testis by semiquantitative RT-PCR (**a**) and real-time PCR analysis (**b**). The RNA prepared from testes illustrates the changing levels of LM23 mRNA in the testis at days 0, 5, 14, 21, 30 and 65 postpartum (dpp). The data are representative images from groups of rats (n=3 per group). All values are expressed as mean±s.d.*P<0.05 in an unpaired t test when compared with vector alone. RT, reverse transcription; s.d., standard deviation.

Overexpression of LM23 enhanced cell proliferation

We have previously reported that LM23 may regulate cell cycle progression during spermatogenesis.[2] In the present study, the effect of LM23 on cell cycle progression of 293T cells was investigated using a range of cell proliferation assays. As shown in **Figure 2a**, cell numbers in LM23-overexpressing cells increased significantly after 2 days, compared to cells transfected with the empty vector. **Figure 2b** shows that, in LM23-overexpressing cells, CFE was also increased compared to the control cells. The CFE ratio was 3.08-fold higher than in controls. These results indicated a promoting effect of LM23 on the proliferation of 293T cells. Furthermore, flow cytometric measurements of LM23-overexpressing 293T cells after propidium iodide staining confirmed an increase in the number of cells in S phase. Twenty-four hours after transfection of 293T cells with LM23 expression plasmid or empty vector control, the percentage of cells in S phase was 53.66%±0.7% in the LM23-overexpressing sample, and 42.37%±0.8% in controls (**Figure 2c**). The results indicated that LM23 reduced the proportion of cells in G0/G1 phase, but increased the number of S phase cells. Taken together, these data suggest that LM23 significantly promotes cell cycle progression into S phase in 293T cells.

Overexpression of LM23 activated nuclear factor (NF)-κB

In order to investigate the function of LM23, a dual-luciferase reporter assay system was used to examine LM23 expression in mammalian cells. It was found that overexpression of LM23 activated the NF-κB reporter gene in 293T cells when compared to control 293T cells transfected with empty vector controls (**Figure 2d**).

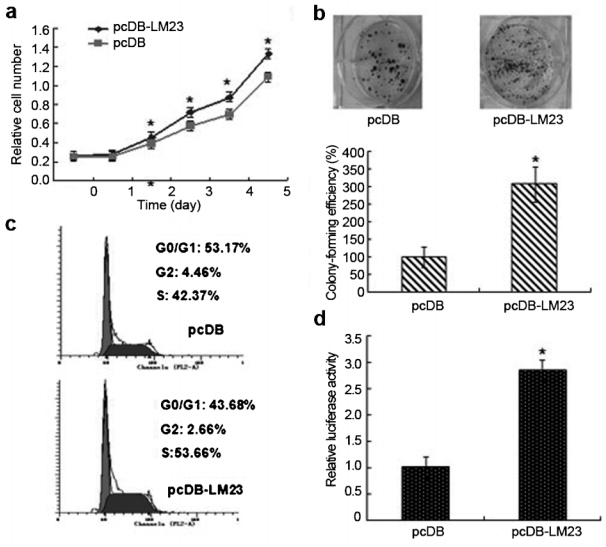

Figure 2 Overexpression of LM23 promoting cell proliferation and inducing NF-κB activation. (**a**) 293T cells transfected with pcDB or pcDB-LM23 were assayed with a CCK8. Cells were collected every 24 h, and the absorbance was measured at 450 nm. (**b**) 293T cells were transfected with pcDB or pcDB-LM23, and colony-forming efficiency was analyzed for 14 days after transfection. The colony-forming efficiency was expressed as a percentage of the total number of colonies in the pcDB controls. (**c**) 293T cells transfected with pcDB or pcDB-LM23 were harvested, fixed and stained with PI, and then analyzed by flow cytometry. *P<0.05 in unpaired t test when compared with vector alone. (**d**) 293T cells were transfected with either the empty pcDB vector control or pcDB-LM23 along with pNF-κB-luc and pRL-TK. A dual-luciferase reporter gene assay was performed using the lysates from transfected cells. *P<0.05 in unpaired t test when compared with empty vector control. CCK8, cell counting kit-8; NF-κB, nuclear factor-κB; PI, propidium iodide.

LM23 protects against H_2O_2-induced apoptosis

Exogenous H_2O_2 was used to induce free radical-mediated apoptosis in 293T cells. Cell apoptosis was quantified by CCK8 assays. As shown in **Figure 3a**, H_2O_2 at concentrations between 100 and 500 μmol l^{-1} induced apoptosis of 293T cells in a dose-dependent manner. To evaluate the effects of LM23 on H_2O_2-induced apoptosis, 293T cells were transfected with pcDB-LM23 or pcDB vector control plasmids for 24 h, and then treated with various concentrations of H_2O_2 for 12 h. The effects of H_2O_2-induced apoptosis were reduced in the LM23-overexpressing cells (**Figure 3b**). A concentration of 200 μmol l^{-1} H_2O_2 was chosen for subsequent studies, because this dose induced moderate apoptosis. Annexin V and propidium iodide staining of 293T cells confirmed the antiapoptotic effects of LM23. The treatment with H_2O_2 caused a significant increase in late apoptotic and early apoptotic 293T cells in comparison to the untreated controls, whereas LM23 overexpression in 293T cells reduced the H_2O_2-induced apoptosis (**Figure 3c**). These observations suggest that LM23 plays a protective role against H_2O_2-induced apoptosis in 293T cells.

To understand the molecular changes involved, western blots was performed to examine the effects of LM23 and H_2O_2 on the expression of the apoptotic proteins Bax, p53, Bcl-2 and caspase 3. 293T cells were transfected with pcDB-LM23 or pcDB vector control plasmids for 24 h, followed by the addition of 200 μmol l^{-1} H_2O_2 for 12 h. Overexpression of LM23 in 293T cells decreased the expression of Bax, p53 and caspase 3, in the absence of H_2O_2. Similarly, the levels of H_2O_2-induced Bax, p53 and caspase 3 expression were significantly reduced by LM23 overexpression. No differences in Bcl-2 expression were detected (**Figure 3d**). These data suggest that LM23 prevents H_2O_2-induced apoptosis in 293T cells *via* a decrease in the expression of Bax, p53 and caspase 3.

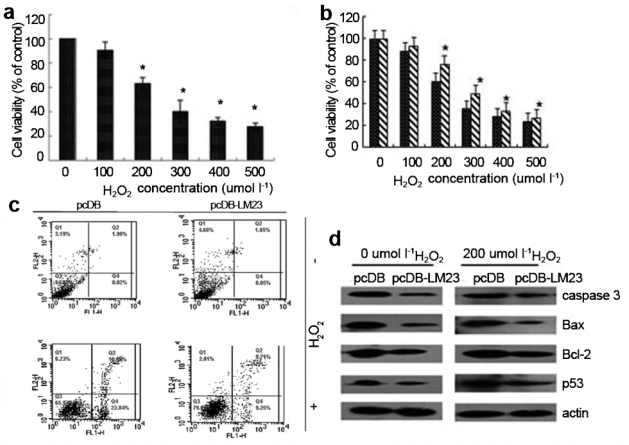

Figure 3 LM23 decreasing H_2O_2-induced cell apoptosis. (**a**) 293T cells were treated with 0, 100, 200, 300, 400 or 500 μmol l^{-1} H_2O_2 and harvested after 12 h. Cell viability was determined by CCK8 assay. The mean and s.d. values were calculated from six replicates. (**b**) 293T cells were transfected with pcDB or pcDB-LM23. After 24 h, the cells were treated with 0–500 μmol l^{-1} H_2O_2 as described above, and cell viability was measured by CCK8 assay. (**c**) 293T cells transfected with pcDB or pcDB-LM23 for 24 h, and untransfected control 293T cells, were cultured with or without 200 μmol l^{-1} H_2O_2 for a further 12 h. 293T cells were fixed and stained with annexin V/PI, and then analyzed by flow cytometry. FL1-H and FL2-H correspond to annexin V and PI staining respectively. Quadrants Q1, Q2, Q3 and Q4 correspond to necrotic cells, late apoptotic cells, viable cells and early apoptotic cells, respectively. *P<0.05 in unpaired t test when compared with vector alone. (**d**) LM23 downregulates Bax, p53 and caspase 3 expression while protecting against H_2O_2-induced apoptosis. 293T cells were transfected with pcDB (control) or pcDB-LM23 vector. After 24 h, the cells were treated with 200 μmol l^{-1} H_2O_2 for 12 h. The levels of Bax, p53, Bcl-2 and caspase 3 proteins were measured by western blotting. Actin was used as control. CCK8, cell counting kit-8; PI, propidium iodide; s.d., standard deviation.

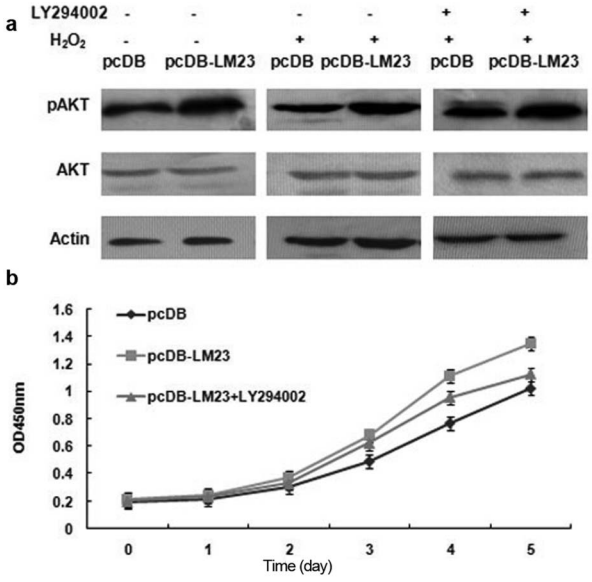

Figure 4 Effect of LM23 on cell survival is mediated by activation of Akt/PI3K signaling. (**a**) 293T cells transfected with pcDB or pcDB-LM23 vectors for 24 h were cultured with or without, 200 μmol l^{-1} H_2O_2 and 5 μmol l^{-1} LY294002. The levels of AktT, pAKT and actin were measured by western blotting. Actin was used to normalize the AktT and pAKT levels. (**b**) CCK8 assays were used to verify that LY294002 at least partially blocked the LM23-induced increase in cell viability of 293T cells. CCK8, cell counting kit-8.

Effects of LM23 on Akt/PI3K signaling

Akt/PI3K signaling plays a crucial role in a variety of cellular events including apoptosis and proliferation.[11–13] In the present study, western blotting was used to investigate whether the Akt/PI3K signaling pathways contributed to the antiapoptotic function of LM23. **Figure 4a** shows that overexpression of LM23 in 293T cells significantly activated phosphorylation of Akt, in the presence or absence of H_2O_2, in comparison with control cells transfected with empty vector. Given that LM23 overexpression resulted in a strong activation of the Akt signal pathway (**Figure 4a**), 293T cells were transfected with LM23 expression plasmid or empty vector control for 24 h followed by treatment with 5 μmol l^{-1} PI3K inhibitor LY294002 for 3 h, and then treated with 200 μmol l^{-1} H_2O_2 for 12 h. Western blotting showed that blockade of the Akt/PI3K signaling pathway by LY294002 dramatically weakened LM23-induced expression of pAkt (**Figure 4a**). Cell viability was also determined by CCK8 assay. The results showed that LY294002 at least partially blocked the LM23-induced increase in cell viability (**Figure 4b**). These data therefore imply that the protective effect of LM23 in H_2O_2-induced apoptosis is mediated at least in part through the Akt/PI3K signaling pathway.

DISCUSSION

In higher eukaryotes, spermatogenesis is a very complex process of differentiation. During this process, primordial germ cells firstly form gonocytes, or prespermatogonia. After birth, gonocytes reenter the cell cycle and then become spermatogonia. Some of these spermatogonia form the self-renewing stem cell pool, while others differentiate immediately to spermatocytes. Spermatocytes undergo meiosis to further

differentiate into haploid spermatids, and are finally transformed into mature sperm. Spermatogenesis is influenced by many genes and cellular factors in a time-dependent and cycle-specific manner, resulting in coordinated and orderly development. We previously demonstrated that LM23 was one of the genes involved in spermatogenesis.[3]

Previously we detected expression of LM23 mRNA by SYBR Green-based quantitative PCR in type A spermatogonia, pathytene spermatocytes and round spermatids, respectively. The results suggested that the expression level of LM23 was highest in spermatocytes, moderate in round spermatids and very low in spermatogonia.[3] We also examined cell-specific expression in rat testis using a polyclonal rabbit anti-LM23 antibody by immunohistochemical analysis. The positive immunological activity was mainly located in the nucleus of spermatocytes and round spermatids, but few in elongating spermatids and mature sperm.[2]

In this study, the RNA prepared from testes illustrated changing levels of LM23 mRNA in the testis at 0, 5, 14, 21, 30 and 65 days postpartum (dpp) (**Figure 1a**). None or very low expression of LM23 was detected at 0 and 5 dpp, since there are only primitive type A spermatogonia and Sertoli cells in the testis. In 14 dpp (prepuberty), the expression of LM23 was started, while the meiotic prophase is initiated and the germ cells reach the early and late pachytene stages in testis. The expression of LM23 reached the peak level at 30 dpp (prepuberty), then decreased at day 65 dpp (postpuberty).[14,15] In the testis of 65 dpp rat, ther are a large amount of elongating spermatids and mature sperms which have no or very low expression of LM23, so expression of LM23 is decreased. The changes of LM23 are coincided with the development of germ cells in the rat testis.

NF-κB is a generic term for a dimeric transcription factor formed by the heterodimerization or homodimerization of members of the Rel/NFκB family.[16,17] NF-κB-regulated genes are important for cell differentiation, embryonic development, the immune response and inflammation,[18–20] as well as the development, progression and drug resistance of cancer cells.[17,21] Our study demonstrated that LM23 activated the NF-κB pathway, suggesting that it may be an important regulator of a variety of cell functions.

In mammals, there are at least five different Speedy/Ringo homologs and all of them can bind to and activate cyclin-dependent kinases, which suggests that Speedy/Ringo proteins may play versatile roles in cell cycle control.[22–24] Speedy A (Spdya), a human Speedy homolog, is a novel cell cycle protein capable of promoting cell proliferation.[25] LM23 is a *R. norvegicus* homolog of Speedy A.[2] Our results showed that LM23 promoted 293T cell cycle progression by increasing cell numbers in S phase of the mitotic cell cycle, suggesting that LM23 may be an important regulator of cell cycle progression. Liu *et al.*[10] have shown that the knockdown of LM23 may result in downregulation of Cyclin A1, Cdk2 and CyclinB1. However, they noted that the mechanism through which LM23 promoted HEK 293 cell proliferation required further elucidation.

Speedy A, also known as SPY1, not only promotes cell proliferation under normal circumstances, but also promotes cell survival under conditions of genotoxic stress.[25,26] Barnes *et al.*[26] showed that Spy1 could override the DNA damage response, by inhibiting DNA damage-induced apoptosis. As LM23 is homologous to Spy1, it may also play a key role in regulating both cell growth and apoptosis. Spy1 expression in mammalian cells enhances survival under conditions of genotoxic damage, including treatment with CPT, cisplatin and HU.[26] These results are consistent with our findings that LM23 markedly promotes the survival of 293T cell after H_2O_2 treatment. Apoptosis is a process of programmed cell death, and involves a series of morphological changes, including cell detachment, cell shrinkage, mitochondria leakage, chromatin condensation and DNA fragmentation.[27,28] Our results found that LM23 decreased the apoptosis of 293T cells induced by H_2O_2. These data suggest that LM23 has a protective role in H_2O_2-induced apoptosis in 293T cells.

The process of apoptosis is regulated by several proteins, including members of the Bax, p53, Bcl-2 and caspase 3 protein families.[9] Caspases, which are cysteine aspartic proteases, play essential roles at various stages of the apoptotic process.[29] Caspase 3 is a key mediator of apoptosis and a common downstream effector of multiple apoptotic signaling pathways.[30] These results are consistent with our findings that the protective effect of LM23 in H_2O_2-induced apoptosis was associated with an obvious decrease in the expression of Bax, p53 and caspase 3 in 293T cells. Akt, a serine/threonine kinase, plays a crucial role in a variety of cellular events including apoptosis and proliferation.[11–13,27] Akt activation is a critical component of the PI3K downstream cascade during growth factor stimulation.[31] The results presented in this study also showed that prevention of H_2O_2-induced apoptosis by LM23 was mediated at least in part through the Akt/PI3K signal pathway.

In conclusion, LM23 promoted 293T cell cycle progression and played a protective role during H_2O_2-induced apoptosis of 293T cells. We therefore propose that LM23 might act as a potential regulatory factor at the crossroads between proliferation and apoptosis in cell. However, the *in vivo* mechanisms of action of LM23 are likely to be far more complex. Further investigation *in vivo* is required to elucidate the exact role of LM23 in postnatal testis development and spermatogenesis.

AUTHOR CONTRIBUTIONS

QL, YJS and LJM conceived and designed the study. FH, LXG, CHJ, HMT and WJW collected the data. QL, YJS and ML performed the statistical analyses. QL, XJZ and MCJ wrote the manuscript with input from all of the coauthors. All of the authors revised the manuscript and approved the final version.

COMPETING FINANCIAL INTERESTS

All authors declare that there are no competing financial interests.

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