Original Paper

MALAT1 Promotes Cell Apoptosis and Suppresses Cell Proliferation in Testicular Ischemia-Reperfusion Injury by Sponging MiR-214 to Modulate TRPV4 Expression

Wei Li^a Jin-zhuo Ning^b Fan Cheng^b Wei-min Yu^b Ting Rao^b Yuan Ruan^b
Run Yuan^b Xiao-bin Zhang^b Yang Du^b Cheng-cheng Xiao^b

^aDepartment of Anesthesiology, Renmin Hospital of Wuhan University, Wuhan, Hubei Province,

^bDepartment of Urology, Renmin Hospital of Wuhan University, Wuhan, Hubei Province, P.R.China

Key Words

lncRNA MALAT1 • MiR-214 • TRPV4 • Testicular IRI • Apoptosis • Proliferation

Abstract

Background/Aims: Accumulating evidences has indicated that aberrant expression of long non-coding RNAs (lncRNAs) is tightly associated with the progression of ischemia-reperfusion injury (IRI). Previous studies have reported that lncRNA MALAT1 regulates cell apoptosis and proliferation in myocardial and cerebral IRI. However, the underlying mechanism of MALAT1 in testicular IRI has not been elucidated. **Methods:** The levels of MALAT1, some related proteins and apoptosis in the testicular tissues were determined by quantitative real-time PCR, HE staining, immunohistochemistry, western blot and TUNEL assays. Relative expression of MALAT1, miR-214 and related proteins in cells were measured by western blot and quantitative real-time PCR. Cell viability and apoptosis were examined using MTT assay and flow cytometry. **Results:** In the present study, we found that MALAT1 was up-regulated in animal samples and GC-1 cells. The expression level of MALAT1 was positively related to cell apoptosis and negatively correlated with cell proliferation as testicular IRI progressed. In gain and loss of function assays, we confirmed that MALAT1 promotes cell apoptosis and suppresses cell proliferation *in vitro* and *in vivo*. Furthermore, we found that MALAT1 negatively regulates expression of miR-214 and promotes TRPV4 expression at the post-transcriptional level. Consequently, we investigated the correlation between MALAT1 and miR-214 and identified miR-214 as a direct target of MALAT1. In addition, we found that TRPV4 acted as a target of miR-214. Over-expression of miR-214 efficiently abrogated the up-regulation of TRPV4 induced by MALAT1, suggesting that MALAT1 positively regulates the expression of TRPV4 by sponging miR-214. **Conclusion:** In sum, our study indicated that the lncRNA MALAT1 promotes cell apoptosis and suppresses cell proliferation in testicular IRI via miR-214 and TRPV4.

W. Li and J.-Z. Ning contributed equally to this work.

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Dr. Fang Cheng

Department of Urology, Renmin Hospital of Wuhan University
Wuhan, 430060 Hubei (China)
Tel. +86-138-5534-8212; Fax +86-027-8804-1911, E-Mail chenfan_93@126.com

Introduction

Testicular torsion is an acute urological emergency involving aberrant twisting of the spermatic cord and mostly affects young and adolescent males [1]. Since prolonged ischemia of the testis can lead to irreversible damage, prompt clinical treatment should be adopted to restore blood flow within an appropriate time frame [2, 3]. However, testicular detorsion can cause progressive reperfusion injury and its damage can be more serious than ischemic injury [4]. The pathological process exhibits many features of ischemia reperfusion injury (IRI). A growing body of evidence suggests that testicular IRI usually causes extensive spermatogenic cell apoptosis and testicular dysfunction [5, 6]. Undoubtedly, it is essential to identify the mechanism underlying testicular IRI development and determine novel effective molecular biomarkers.

According to the human genome project, only approximately 2% of DNA sequences in the human genome are protein-coding genes and the rest of the genome is not responsible for protein coding transcribed as non-coding RNAs (ncRNAs) [7]. Long non-coding RNA (lncRNA) represents a newly discovered class of ncRNAs of more than 200 nucleotides in length [8]. Recently, accumulating studies have demonstrated that lncRNAs are widely involved in various biological processes such as cell apoptosis, proliferation, differentiation and angiogenesis [9, 10]. Moreover, they can also modulate gene expression through a variety of mechanisms, including chromatin structure remodeling, regulating cutting and splicing machinery, adjusting transcription factor activity and controlling RNA decay and epigenetics [11, 12]. Therefore, lncRNAs may possess great potential as new therapeutic targets during the progression of IRI.

The abnormal expression of lncRNAs has been found in the progression of a variety of organ IRIs. Increasing evidence has suggested that lncRNAs may participate in diverse biological processes in different contexts [13]. For example, Zhao et al. reported that up-regulated lncRNA MALAT1 functions as a pro-apoptotic regulator in myocardial IRI through the inhibition of miR-145 [14]. Yu et al. found that lncRNA PRINS contributes to inflammatory reactions in kidney IRI by regulating RANTES expression [15]. Wu et al. showed that lncRNA N1LR reduces neuronal apoptosis and enhances cell proliferation in cerebral IR by targeting Nck1 gene expression [16]. Chen et al. demonstrated that silencing of AK139328 inhibits NF-κB and caspase-3 activity with activation of the Akt signaling pathway in hepatic IRI [17]. However, the biological effect of MALAT1 in testicular IRI has not been elucidated. Therefore, our study hypothesized that lncRNA MALAT1 could serve as a novel biomarker during the progression of testicular IRI.

In this study, we found that the up-regulation of MALAT1 is a characteristic molecular change in testicular IRI and that the expression of MALAT1 is positively associated with cell apoptosis and negatively correlated with cell proliferation at different reoxygenation/reperfusion time intervals. Furthermore, we predicted and identified miR-214 as a direct downstream target of MALAT1 and demonstrated that MALAT1 played a key role in regulating IR-induced apoptosis and proliferation *in vitro* and *in vivo*. In addition, TRPV4 was identified as a direct target of miR-214 and acted as a sensor for testicular IRI. Together, these results provide a new outlook on the role of MALAT1. Additionally, lncRNA MALAT1 may be used as an effective therapeutic target for predicting apoptosis and proliferation in the treatment of testicular IRI.

Materials and Methods

Experimental animals and IRI model

Male C57BL/6J mice (8–10 weeks of age, 20–25 g) were purchased from the Hubei Center for Disease Control. Twenty-five rats were randomly divided into 5 groups with 5 rats in each group. All experimental procedures were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Wuhan University. Prior to the experiments, the mice were housed in cages maintained in a standard temperature-controlled

room ($22 \pm 2^\circ\text{C}$) and were subjected to alternating 12-h light/dark cycles. They also had access to food and water without restriction. In brief, the mice were anesthetized via intraperitoneal administration of 2% sodium phenobarbital (50 mg/kg) and rectal temperature was maintained at $37.0 \pm 0.5^\circ\text{C}$. The left testis was twisted 720 degrees clockwise and fixed to the skin of the scrotum with a 5/0 silk suture. After 1 h of ischemia, the testis was restored to the natural position to allow reperfusion for 0, 4, 8 or 16 h. Sham-operated mice underwent a scrotal incision that was sutured with 5/0 silk without additional intervention. After reperfusion, the mice were deeply anesthetized and the left testis of each rat was removed quickly for later analysis. Additionally, an adenoviral vector containing si-MALAT1 and its negative control (si-NC) (n=5, each group) were injected into seminiferous tubules while the mice were under anesthesia.

Cell culture and the OGD/R model

GC-1 spermatogenic cells were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO, MA, USA) mixed with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2 . To mimic OGD/R (oxygen glucose deprivation/reoxygenation) injury *in vitro*, the cells were kept in an anaerobic chamber filled with 5% CO_2 and 95% N_2 at 37°C for 3 h. After OGD exposure, the medium was replaced with glucose-containing DMEM under normoxic conditions for 0, 6, 12, 24 or 48 h to reoxygenate.

Plasmid construction and cell transfection

Full-length MALAT1 from GC-1 cells was amplified by RT-PCR and then cloned into a pcDNA3.1 vector (Invitrogen, USA). Recombinant adenoviruses were packaged in HEK293T cells. Forty-eight h later, adenoviruses containing MALAT1, si-MALAT1 and their respective negative controls sequence were harvested and purified. The wild-type sequence of TRPV4 3'UTR containing miR-214 binding sites was also amplified from GC-1 cells. The mutant 3'UTR sequence of TRPV4 was generated using overlap extension PCR and then the full sequences were cloned into a psiCHECK-2 vector (Promega, Madison, WI, USA). Pri-miR-214, anti-miR-214 and their respective negative controls were purchased from Bioss Company (Wuhan, China). All transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

HE staining

After the testis tissues were fixed in 4% paraformaldehyde, they were paraffin-embedded and cut into 4- μm sections. The sections were then stained with hematoxylin and eosin (HE) after routine procedures, including deparaffinization and hydration.

TUNEL assays

Spermatogenic cell apoptosis was evaluated using a transferase-mediated dUTP nick-end labeling (TUNEL) method with a detection kit (Roche, Mannheim, Germany) following the manufacturer's instructions. The nuclei that stained brown were considered TUNEL-positive cells. Five visual fields were randomly selected from each slide and approximately 200 cells were counted per field. The apoptosis index (AI) was determined as follows: $\text{AI} = (\text{positive cells} / \text{total cells counted}) \times 100\%$.

Quantitative Real-time PCR

Total RNA was extracted from GC-1 cells and testis samples using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and the purity of RNA was examined with a DU800 UV/Vis Spectrophotometer (Beckman Coulter, CA, USA). First-strand cDNA was synthesized using a reverse transcription Reagents kit or the TaqManH MicroRNA Reverse Transcription Kit (Takara Biotechnology, Dalian China) following the manufacturer's protocol. Real-time quantitative PCR was performed using the Applied Biosystems SYBR Green mix kit and the ABI 7900 Real-Time PCR system (Applied Biosystems Life Technologies, Foster City, CA, USA). All results were normalized to the expression of GAPDH or snRNA U6. The quantitative analysis was conducted using the $2^{-\Delta\Delta\text{Ct}}$ method. The primer sequences used are shown in Table 1.

Cell viability

The viability of GC-1 cells was measured by a CCK-8 cell viability test kit (Beyotime Institute of Biotechnology, Jiangsu, China). Cells were seeded in 96-well plates at a concentration of 2×10^3 cells/well.

Twenty-four hours after transfection, the cells were stained with 20 μ L of CCK8 reagent for 4 hours. Cell viability was detected according to the absorbance at 450 nm using a spectrophotometer (Thermo, multiskan FC, USA).

Cell apoptosis

Cell apoptosis was measured using the Annexin V-FITC/Propidium Iodide (PI) staining kit (DOJINDO, Japan; AD10). GC-1 cells were seeded in 6-well plates at a density of 10^6 cells/mL. The cells were labeled with Annexin V-FITC for 5 min in the dark. Then, 5 mg of mlPI was added to each sample for 30 min. For the statistical analysis, more than 20000 total cells per sample were measured.

Western blot analysis

Proteins from testis tissues or cultured GC-1 cells were collected using RIPA Lysis Buffer (Beyotime, Beijing, China) and quantified with a bicinchoninic acid assay (BCA) kit (Beyotime, Beijing, China). Equivalent amounts of protein samples (40 μ g/lane) were separated by 10% sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel electrophoresis and then transferred to a polyvinylidene fluoride membrane (PVDF; Billerica, MA, USA). Membranes were blocked in Tris-buffered saline (TBS) containing 5% nonfat milk and then incubated with the following primary antibodies at 4 °C overnight: TRPV4 (ab94868; Abcam, Cambridge, UK), caspase-3 (sc7148; Santa Cruz, CA), Bax (sc493; Santa Cruz, CA) and Bcl-2 (sc7382; Santa Cruz, CA). After rinsing twice with TBST buffer, the membranes were incubated with secondary antibodies at room temperature for 1 h. All specific bands were detected by an ECL system kit (Pierce Biotechnology, Beijing, China) and estimated by ImageJ software (NIH, Bethesda, MD, USA).

Luciferase reporter assays

MALAT1 containing the predicted miR-214 binding site was amplified by PCR and then cloned into a pmirGLO Dual-luciferase miRNA Target Expression Vector (Promega, Madison, WI, USA). To mutate the putative binding site of miR-214 in MALAT1, the sequence of the putative binding site was replaced as indicated and named as MALAT1-mutated-type (MALAT1-Mut). MALAT1-Wt or MALAT1-Mut was co-transfected with miR-214 NC or miR-214 mimics using Lipofectamine 2000 (Invitrogen, USA). After 48 h of transfection, luciferase activity was analyzed using a Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The putative and mutated miR-214 target binding sequences in TRPV4 were synthesized and cloned into luciferase reporters to form the wild-type (TRPV4-Wt) or mutated-type (TRPV4-Mut) reporter plasmids. The transfection procedure and measurement was similar as described previously.

RNA immunoprecipitation assay

An RNA immunoprecipitation assay was conducted using the EZ-Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Billerica, MA, USA) following the manufacturer's instructions. GC-1 cells were lysed by RIP lysis buffer with RNase inhibitor and subsequently incubated with RIP immunoprecipitation buffer. An antibody against argonaute2 (Ago2) (Millipore) was used to form conjugated magnetic beads. The MALAT1 fold enrichment of RNA immunoprecipitation was normalized to the RIP fraction of negative control antibody IgG (Millipore), and was subjected to qRT-PCR analysis.

Immunohistochemistry

The expressions of Bax and Bcl-2 was evaluated by immunohistochemical staining. The testis tissues were fixed in 4% paraformaldehyde, embedded in paraffin and then sliced in 4- μ m sections. The primary antibodies against Bax (sc493; Santa Cruz, CA) and Bcl-2 (sc7382; Santa Cruz, CA) were used at a dilution of 1:100. All sections were analyzed by comparing staining intensities under microscopic examination.

Statistical analysis

All data are represented as the means $\pm$ standard deviations (SD). Differences were assessed by one-way analysis of variance (ANOVA), followed by all pairwise multiple-comparison procedures using the

Table 1. RT-PCR primer sequences

GENE	Primer sequences (5'-3')
lncRNA MALAT1	F: GGTAACGATGGTGTGAGGTC
	R: CCAGCATTACAGTTCTTGAACATG
TRPV4	F: CGCTCCTTCCCGTATTCTCT
	R: TTGATGATGCCCAAGTTCTGGTT
miR-214	F: ACAGCAGGCACAGACAGGCAGU
	R: CAGACGAGGCTCCGTGGT
U6 snRNA	F: CTCGCTTCGGCAGCACATATACT
	R: ACGCTTCACGAATTTGCGTGTC
GAPDH	F: ACAGCAACAGGGTGGTGGAC
	R: TTTGAGGGTGACGCAACTT

Bonferroni test. $P < 0.05$ was considered statistically significant. All experiments were performed at least 3 times. The data analysis was performed using SPSS 20.0 (SPSS, Inc., Chicago, IL, USA).

Results

lncRNA MALAT1 was involved in the response to testicular IRI and cellular OGD/R

To determine the role of lncRNA MALAT1 during the progression of testicular IRI, we first established a mouse IRI model. The animal samples were examined by HE staining and TUNEL assays at 0, 4, 8 or 16 h of reperfusion after 1 h of ischemia. As shown in Fig. 1A and B, testicular spermatogenic function was significantly injured and markedly elevated during prolonged reperfusion and the apoptosis index increased gradually and peaked at 8 h of reperfusion compared with the sham group. These pathophysiological changes indicated successful establishment of the testicular IRI model in our study. Next, we measured the expression of MALAT1 in mice testes samples by qRT-PCR. The results showed that the level of MALAT1 abruptly increased, reaching its maximum at 1 h of ischemia/8 h of reperfusion (Fig. 1C). We further investigated MALAT1 expression in a cellular OGD/R model using mice GC-1 cells. First, data from the CCK-8 assay showed that OGD/R reduced the viability of GC-1 cells, which continuously decreased to approximately 40% at 3 h of OGD/24 h of reoxygenation, with a significant difference from the normoxia group (Fig. 1D). Second, 3 h of OGD followed by 24 h of reoxygenation induced significantly higher MALAT1 expression compared with the normoxia group (Fig. 1E), which was consistent with the decrease in cell viability and was similar to the trend observed in the *in vivo* IRI model. Collectively, these data suggested that MALAT1 may be involved in the progression of testicular IRI.

Fig. 1. lncRNA MALAT1 was involved in the response to testicular IRI and cellular OGD/R. (A) HE staining in the testes exposed to 1 h ischemia followed by reperfusion of different durations. $n = 5$ per group; (B) QRT-PCR of MALAT1 level expression at different reperfusion durations after 1 h ischemia in animal samples. $*p < 0.05$ vs. sham, $n = 5$ per group; (C) TUNEL assays were performed to investigate the cell apoptosis at 1 h ischemia followed by reperfusion of different durations. $*p < 0.05$ vs. sham, $n = 5$ per group; (D) CCK-8 assays were performed to determine cellular viability in GC-1 cells at 3 h OGD exposure followed by different reoxygenation conditions. $*p < 0.05$ vs. normoxia, $n = 6$ per group; (E) Relative expression level of MALAT1 in GC-1 cells under different reoxygenation conditions after 3 h OGD exposure. $*p < 0.05$ vs. normoxia, $n = 6$ per group.

Influence of lncRNA MALAT1 on spermatogenic cell apoptosis and proliferation in vitro

To investigate the effect of lncRNA MALAT1 on OGD-induced cell injury, we increased the expression of MALAT1 via transfection of recombinant adenoviruses and suppressed that by small interfering RNA (siRNA) in GC-1 cells. The qRT-PCR analysis suggested that the expression of MALAT1 significantly increased in adenovirus-MALAT1-transfected cells and remarkably decreased in si-MALAT1-transfected cells at 3 h of OGD/24 h of reoxygenation compared with their negative controls, respectively (Fig. 2A). In addition, the results of

Fig. 2. Influence of lncRNA MALAT1 on spermatogenic cell apoptosis and proliferation in vitro. (A) Relative expression of MALAT1 in GC-1 cells after transfection with MALAT1 or si-MALAT1. * $p < 0.05$ vs. respective control group, $n = 6$ per group; (B) CCK-8 assays were performed to evaluate cellular viability in GC-1 cells transfected with MALAT1 or si-MALAT1 in normoxia and 3 h OGD/24 h reoxygenation treatments. * $p < 0.05$ vs. non-trans, $n = 6$ per group; (C,D) Cell apoptosis of GC-1 cells transfected with MALAT1 or si-MALAT1 was examined by flow cytometry in normoxia, without OGD and 3 h OGD/24 h reoxygenation treatments. * $p < 0.05$ vs. non-trans, $n = 6$ per group; (E-H) Cleaved caspase-3, Bax and Bcl-2 protein levels transfected with MALAT1 or si-MALAT1 were examined in normoxia and 3 h OGD/24 h reoxygenation treatments by western blot analysis. * $p < 0.05$ vs. non-trans, $n = 6$ per group.

the CCK8 assay showed that the over-expression of MALAT1 remarkably inhibited cellular viability induced by 3 h of OGD/24 h of reoxygenation. However, knockdown of MALAT1 caused an opposite result (Fig. 2B). Flow cytometry data also showed that GC-1 cell apoptosis was induced by 3 h of OGD/24 h of reoxygenation. Our data showed that MALAT1 over-expression markedly promoted cell apoptosis, but MALAT1 knockdown significantly suppressed cell apoptosis in GC-1 cells (Fig. 2C, D). Furthermore, the western blot analysis showed that MALAT1 over-expression increased the expressions of caspase-3 and Bax and decreased the expression of Bcl-2. Consistent with this result, the inhibition of MALAT1 in GC-1 cells resulted in decreased caspase-3 and Bax levels and increased Bcl-2 expression (Fig. 2E-H). Taken together, these findings indicated that the over-expression of MALAT1 promoted cell apoptosis and inhibited cell proliferation in testicular spermatogenic cells.

Fig. 3. lncRNA MALAT1 inhibits miR-214 expression by directly targeting it. (A) Relative expression level of miR-214 in GC-1 cells under different reoxygenation conditions after 3 h OGD exposure. * $p < 0.05$ vs. normoxia, $n = 6$ per group; (B) A two-tailed Pearson's correlation analysis was performed to determine the relationship between miR-214 and lncRNA MALAT1 ($p < 0.05$); (C) qRT-PCR assays were performed to analyze the expression level of miR-214 after transfection with pri-miR-214 or anti-miR-214 in 3 h OGD/24 h reoxygenation treatments. * $P < 0.05$ vs. respective control group, $n = 6$ per group; (D) Relative expression of MALAT1 transfected with pri-miR-214 or anti-miR-214 was determined by qRT-PCR in 3 h

OGD/24 h reoxygenation treatments. * $P < 0.05$ vs. respective control group, $n = 6$ per group; (E) Relative expression of miR-214 transfected with MALAT1 and si-MALAT1 was examined by qRT-PCR in 3 h OGD/24 h reoxygenation treatments. * $P < 0.05$ vs. respective control group, $n = 6$ per group; (F) Putative binding site of miR-214 on MALAT1. (G) Dual-luciferase reporter assay was performed to determine luciferase activity in GC-1 cells co-transfected with pri-miR-214 and MALAT1-Wt or MALAT1-Mut. * $P < 0.05$ vs. pri-miR-214 ctrl group, $n = 6$ per group; (H) RNA-IP assay was performed in GC-1 cells transfected with pri-miR-214 and pri-miR-214 ctrl. The expression of MALAT1 was determined by qRT-PCR. * $P < 0.05$ vs. pri-miR-214 ctrl group, $n = 6$ per group.

lncRNA MALAT1 inhibits miR-214 expression by directly targeting it

It has been elucidated that lncRNAs can competitively binding to miRNAs and function as a competing endogenous RNAs (ceRNAs), consequently modulating the depression of miRNA targets [18, 19]. Previous studies have reported that miR-214 regulates cell proliferation and apoptosis in ovarian and nasopharyngeal cancer [20, 21]. Moreover, it has been found that miR-214 is tightly associated with the progression of myocardial IRI [22]. Therefore, we intended to delineate the interaction between lncRNA MALAT1 and miR-214 in testicular IRI. First, the qRT-PCR analysis showed that the expression of miR-214 was low in OGD-induced cell injury compared with the normoxia group (Fig. 3A), which was inversely associated with the expression of MALAT1 (Fig. 3B). Next, we transfected the GC-1 cells with pri-miR-214 and anti-miR-214 to change the miR-214 levels in GC-1 cells and a qRT-PCR analysis was performed to verify the efficiency of transfection (Fig. 3C). To further investigate whether MALAT1 was regulated by miR-214, we detected the expression level of MALAT1 in GC-1 cells transfected with pri-miR-214, anti-miR-214 and their respective negative controls. The results suggested that the over-expression of miR-214 could not reduce the expression of MALAT1 in GC-1 cells (Fig. 3D). However, the over-expression of MALAT1 significantly decreased the expression of miR-214 compared to the negative control (Fig. 3E). To validate that lncRNA MALAT1 directly binds to miR-214, we performed an *in silico* prediction of target sites in the sequence of miR-214 using the LncBase Experimental v.2 database (Fig. 3F). We found that miR-214 overexpression could decrease ATB-WT luciferase activity but could not affect ATB-Mut luciferase activity compared with miR-214 NC (Fig. 3G). Previous studies have demonstrated that microRNAs degrade RNA or repress translation in an Ago2-dependent manner. To further support this conclusion, we employed an anti-Ago2 RIP assay in GC-1 cells transfected with pri-miR-214. Endogenous MALAT1 was pulled down specifically in miR-214-transfected cells, suggesting that miR-214 is a direct inhibitory target of lncRNA MALAT1 (Fig. 3H). Therefore, these results demonstrated that miR-214 was an inhibitory target of MALAT1 during the progression of testicular IRI.

TRPV4 is a target gene of miR-214 and is regulated by MALAT1

To explore the relationship between miR-214 and TRPV4, an *in silico* prediction was performed using open access software (TargetScan, PicTarget and miRanda). To verify the prediction, we constructed luciferase reporter plasmids containing the wild-type 3'UTR sequence of TRPV4 or the mutant 3'UTR sequence. A mutant reporter vector of 3'UTR of TRPV4 containing a luciferase reporter was used as a negative control. The data from the luciferase reporter assay showed that the over-expression of miR-214 significantly decreased the reporter vector activity of TRPV4 3'UTR in GC-1 cells, but there was no change in the TRPV4-Mut group (Fig. 4A, B). To confirm the regulation of TRPV4 by miR-214, we examined the expression of TRPV4 after miR-214 over-expression or knockdown. The western blot analysis showed that miR-214 over-expression decreased the expression of TRPV4 and that miR-214 knockdown caused a significant increase in TRPV4 expression (Fig. 4C, D). To further explore whether TRPV4 and cleaved caspase-3 were regulated by MALAT1, we determined the protein expression of TRPV4 and cleaved caspase-3 after cells were transfected with MALAT1 or si-MALAT1 and pri-miR-214 or anti-miR-214. We found that MALAT1 over-expression markedly promoted the expression of TRPV4 and cleaved caspase-3, but miR-214 abrogated this increase in TRPV4 and cleaved caspase-3 expression induced by MALAT1. On the other hand, MALAT1 knockdown led to a reduction in TRPV4 and cleaved caspase-3, which could be rescued by miR-214 inhibition (Fig. 4E-L). Therefore, these findings revealed that the regulation of TRPV4 and cleaved caspase-3 by MALAT1 required the activity of miR-214.

lncRNA MALAT1 promotes spermatogenic cell apoptosis caused by testicular IRI in vivo

To further determine the effect of MALAT1 on spermatogenic cell apoptosis *in vivo*, we injected si-MALAT1 or si-NC into mouse seminiferous tubules. The transfection effect was confirmed by a qRT-PCR analysis and the expression level of MALAT1 was remarkably down-

Fig. 4. TRPV4 is a target gene of miR-214 and is regulated by MALAT1. (A) Predicted miR-214 binding sites in the 3'UTR of TRPV4 (TRPV4-Wt) and its mutated sequence (TRPV4-Mut) was shown; (B) Luciferase reporter assay was performed to determine luciferase activity in GC-1 cells co-transfected with pri-miR-214 and luciferase reporters containing 3'UTR sequence of TRPV4-Wt or TRPV4-Mut. * $P < 0.05$ vs. pri-miR-214 ctrl group, $n = 6$ per group; (C,D) TRPV4 protein levels of GC-1 cells transfected with pri-miR-214 or anti-miR-214 in 3 h OGD/24 h reoxygenation treatments. * $P < 0.05$ vs. non-trans, $n = 6$ per group; (E,F) TRPV4 protein levels of GC-1 cells transfected with MALAT1 or si-MALAT1 in 3 h OGD/24 h reoxygenation treatments. * $P < 0.05$ vs. non-trans, $n = 6$ per group; (G-I) Relative TRPV4 and cleaved caspase-3 protein expressions co-transfected with MALAT1 and pri-miR-214 or anti-miR-214 in 3 h OGD/24 h reoxygenation treatments. * $P < 0.05$ vs. NC group, $n = 6$ per group; (J-L) Relative TRPV4 and cleaved caspase-3 protein expressions co-transfected with si-MALAT1 and pri-miR-214 or anti-miR-214 in 3 h OGD/24 h reoxygenation treatments. * $P < 0.05$ vs. si-NC group, $n = 6$ per group.

regulated in si-MALAT1-transfected GC-1 cells at 1 h of ischemia/8 h of reperfusion compared with the negative control group (Fig. 5A). A TUNEL assay was used to investigate whether lncRNA MALAT1 could affect spermatogenic cell apoptosis in response to 1 h of ischemia/8 h of reperfusion. The results of the TUNEL assay showed that IR-induced spermatogenic cell apoptosis was significantly reduced by the injection of si-MALAT1 (Fig. 5B, C). We detected the expressions of Bax and Bcl-2 in testicular samples via immunohistochemical staining. As the results showed, the number of Bax-positive cells in the si-MALAT1 group was obviously fewer than in the negative control group, but Bcl-2 expression was moderately increased in the si-MALAT1 group compared to the negative control group (Fig. 5D). Moreover, the western blot data showed that si-MALAT1 treatment decreased the protein levels of TRPV4 and caspase-3 compared to the negative control group (Fig. 5E-G). Together, these data

Fig. 5. lncRNA MALAT1 promotes spermatogenic cell apoptosis caused by testicular IRI in vivo. (A) QRT-PCR assay was performed to analyze the relative level of MALAT1 after transfection with si-MALAT1 or si-NC at 1 h of ischemia followed by 8 h of reperfusion. * $p < 0.05$ vs. none, $n = 5$ per group; (B,C) TUNEL assays was performed to investigate the cell apoptosis after transfection with si-MALAT1 or si-NC at 8 h of reperfusion after 1 h of ischemia. * $p < 0.05$ vs. none, $n = 5$ per group; (D) Immunohistochemistry staining of Bax and Bcl-2 after transfection with si-MALAT1 or si-NC at 1 h of ischemia/8 h of reperfusion. $n = 5$ per group; (E-G) TRPV4

and cleaved caspase-3 protein levels expression after transfection with si-MALAT1 or si-NC were detected at 1 h of ischemia followed by 8 h of reperfusion by western blot analysis. * $p < 0.05$ vs. none, $n = 5$ per group.

suggested that lncRNA MALAT1 could promote spermatogenic cell apoptosis in testicular IRI *in vivo*.

Discussion

The pathophysiological mechanisms of testicular T/D center around testicular IRI, in which torsion results in ischemic damage and detorsion leads to enhanced blood flow and subsequent reperfusion injury, resulting in spermatogenic cell apoptosis and the disruption of spermatogenesis, eventually leading to testicular dysfunction and infertility [23, 24]. The two major factors affecting testicular damage are the duration and degree of spermatic cord torsion, with testicular salvage rates from manipulative and operative reduction ranging from 42% to 88% [25, 26]. Due to the fast development of basic medical research, the molecular mechanisms underlying the pathogenesis of testicular IRI have emerged gradually over the past few years. Extensive research has indicated that the dysregulation of lncRNAs may contribute to IRI progression in many systems [27-29]. Therefore, elucidating the role and biological functions of lncRNAs in testicular IRI is critical to better identify diagnostic tools and treatments in clinical settings.

MALAT1 is an 8-Kb nuclear-residing lncRNA that is capable of interacting with serine/arginine (SR) proteins and influencing their cellular levels in a phosphorylation-dependent manner [30]. It was initially identified to control tumor metastasis and cancer cell survival and plays an important role in endothelial cell function and dysfunction [31]. Recent

studies have demonstrated that MALAT1 was aberrantly expressed in several pathological processes, including gastric cancer, colorectal cancer myocardial IRI and cerebral IRI [14, 32-34]. In the current study, we found that the expression of MALAT1 in testicular IR tissues was remarkably increased compared with normal tissues. High MALAT1 expression levels were positively associated with cell apoptosis and negatively correlated with cell proliferation *in vitro* and *in vivo*. Furthermore, in gain and loss of function assays, we verified that the overexpression of MALAT1 induced significant suppression of cell proliferation. On the other hand, knockdown of MALAT1 led to an inverse result. These data suggest that lncRNA MALAT1 may exert regulatory effects on apoptosis and proliferation in testicular IRI.

Emerging evidence has reported that lncRNAs may act as competing endogenous RNAs (ceRNAs) and regulate the derepression of miRNA target genes at the post-transcriptional level [18, 19]. On the basis of this theory, lncRNAs can antagonize the inhibitory ability of miRNA by competitively binding to it. For example, lncRNA XIST inhibited neuronal apoptosis by targeting miR-494 in a rat spinal cord injury model [35] and lncRNA H19 contributed to intestinal epithelial barrier function by epigenetically repressing miR-675 [36]. lncRNA MALAT1 promoted cardiomyocyte apoptosis in myocardial IRI by down-regulating miR-145 [14]. In the present study, we confirmed that miR-214 expression is significantly down-regulated in the progression of testicular IRI and inversely associated with the expression of MALAT1. Using bioinformatics predictions, we found that miR-214 may serve as a direct downstream target of MALAT1. To verify whether MALAT1 directly binds to miR-214, luciferase reporter assays and an RNA pull down assay were performed. Furthermore, we identified the direct binding site between MALAT1 and miR-214. Taken together, these data provide evidence that MALAT1 regulates miR-214 expression by directly targeting it.

Transient Receptor Potential Vanilloid 4 (TRPV4) is a non-selective cation channel that belongs to the transient receptor potential (TRP) family [37]. TRPV4 has been found to be involved in the regulation of various cellular activities and is widely expressed in the kidneys, brain, lungs, heart, liver and testes [38, 39]. It has also been reported that excessive activation of this channel may be correlated with myocardial, lung and cerebral IRIs. For example, Dong et al. showed that TRPV4 inhibition alleviates myocardial IRI by reducing infarct size and apoptosis [40]. Jie et al. found that TRPV4 activation promotes cell apoptosis by regulating the PI3K/Akt and p38 MAPK signaling pathways in cerebral IRI [41]. A recent study suggested that an increase in TRPV4 is related to neurological injury, which may contribute to the activation of Ca²⁺-dependent signaling pathways [42]. However, the relationship between lncRNA and TRPV4 in testicular IRI has not been elucidated. In our studies, we identified that MALAT1 could positively regulate the expression of TRPV4 post-transcriptionally by targeting miR-214 in testicular spermatogenic cells. In contrast, miR-214 overexpression did not change the expression level of MALAT1, but abolished the up-regulation of TRPV4 induced by MALAT1 overexpression. Collectively, these results suggested that lncRNA MALAT1 could function as a ceRNA and that this positive regulatory effect of MALAT1 on TRPV4 required the inhibition of miR-214.

Conclusion

In conclusion, our study demonstrated that lncRNA MALAT1 was remarkably increased during the progression of testicular IRI, which played a key role in regulating testicular spermatogenic cell apoptosis and proliferation *in vitro* and *in vivo*. In addition, we revealed that lncRNA MALAT1 positively regulated TRPV4 expression at the post-transcription level by directly targeting miR-214 in testicular IRI. Therefore, our study indicated that lncRNA MALAT1 could potentially serve as a novel biomarker in the treatment of testicular IRI.

Disclosure Statement

No conflict of interest exists.

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