miR-148a-3p overexpression contributes to glomerular cell proliferation by targeting PTEN in lupus nephritis

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Submitted 11 May 2015; accepted in final form 29 December 2015

SYSTEMIC LUPUS ERYTHEMATOSUS (SLE) is a complex, systemic autoimmune disease that is characterized by autoantibody production, complement activation, and immunocomplex deposition, which causes tissue and organ damage (22, 23). Renal injury is a significant cause of death and disability (4), with excessive mesangial cell proliferation being the main pathological change in lupus nephritis (LN) (26). The glomerulosclerotic changes can be alleviated by inhibiting the proliferation of the mesangial cells (12). Therefore, it is crucial to determine the precise mechanism of disease progression and identify ways to inhibit mesangial cell proliferation in LN.

High-mobility group box 1 (HMGB1) is a nuclear protein that controls chromatin architecture. Once released into the cytoplasm or other extracellular spaces, HMGB1 mediates the pathogenesis of chronic inflammatory and autoimmune diseases as an important inflammatory mediator. The expression of HMGB1 has been shown to increase in the renal tissue of patients and mice with SLE. HMGB1 may also participate in antibody-induced kidney damage in SLE. Thus HMGB1 has important biological effects in autoimmunity and may be a promising therapeutic target for SLE (22). Our previous studies have revealed that HMGB1 is an important cytokine in LN pathogenesis and that HMGB1 can induce mesangial cell proliferation in mice (6) and lipid deposition in mouse mesangial cells (MMCs) (9). However, the signaling pathways involved in these processes and the precise mechanisms are still unknown.

Phosphatase and tensin homology deleted on chromosome ten (PTEN), a tumor suppressor gene on chromosome 10, is frequently lost in advanced human cancers such as prostate cancer and breast carcinoma (18). Mutations, deletions, transcriptional silencing, and posttranscriptional modifications to PTEN also induced decreases in PTEN protein levels or PTEN inactivation in a variety of human cancers. PTEN is a lipid phosphatase that catalyzes the reaction that turns phosphatidylinositol 4,5-bisphosphate (PIP2) and inhibits Akt phosphorylation (16). PTEN also induced decreases in PTEN protein levels or PTEN transcriptional silencing, and posttranscriptional modifications to PTEN gene expression at the posttranscriptional level. They bind to gene expression or induce mRNA degradation and contribute to LN progression by targeting PTEN.

miR-148a-3p; lupus nephritis; PTEN; cell proliferation; diagnosis; therapy

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cancer (15, 21). The abnormal expressions of some miRNAs have been shown in the blood serum, urine, and renal tissue of patients with LN; however, these results were not explored extensively.

In our previous study, we used a microassay to analyze the differential expression of miRNAs in control and HMGB1-stimulated MMCs and identified a set of 13 miRNAs whose expression was abnormal in the presence of HMGB1. miR-148a-3p is one of the most upregulated miRNAs in the HMGB1-stimulated MMCs. The targetScan assay predicts that PTEN is a possible miR-148a-3p target gene.

In this study, we aimed to examine the expression of miR-148a-3p in the renal tissues and blood serum of mice and patients with LN and to evaluate the possible role of miR-148a-3p and its related target gene in LN pathogenesis.

**Materials and Methods**

**Patients.** Thirty patients (12 males and 18 females, aged between 18 and 55 yr) diagnosed with SLE and type II (n = 15) or type IV LN (n = 15) (BSN/RPS2003 classification criteria) were enrolled in this study between 2012 and 2014 at the Inpatient Department of Nephrology at the Second Hospital of Hebei Medical University and Hebei Provincial People’s Hospital, and mesangial cell proliferation was the main pathological change. Twenty control renal tissue samples were obtained from the healthy tissues away from the tumor tissues in renal tumor patients without history of primary glomerulonephritis, hypertension, or diabetic nephropathy. The study was approved by the Research Ethics Board of Hebei Medical University. Renal tissues were fixed with 4% formaldehyde for in situ hybridization and immunohistochemistry. The corresponding serum samples from the patients and the control groups were obtained for RNA extraction and real-time quantitative PCR (qPCR).

**Animals.** Six MRL/MJP mice (3 females and 3 males, weight 45–55 g) were designated as the control group. Six female and six male MRL/Faslp mice (28 wk old on average, weight 45–55 g) were randomly divided into LN group and treated group with three female and three male mice in each group. The animals were kindly provided by the Model Animal Research Center of Nanjing University. Experimental protocols were approved by the Institutional Animal Care and Use Committee of Hebei Medical University (approval ID: HebMU 20080026). Hereafter, the mice of the treated group received a single intraperitoneal injection of anti-miR-148a-3p-adenovirus (In vitro) at a dose of 1 × 10^9 plaque-forming units/mouse at 28 wk, while the mice of LN group and control group received an injection of empty-adenovirus at the same dose. All of the animals were killed after 2 wk. Serum, urine, and renal cortex samples were collected for further study.

**Cell culture and groups.** MMCs (ATCC No. CRL-1927) were obtained from the Chinese Academy of Sciences, Shanghai Institute for Biological Sciences Cell Resource Center and stored in our laboratory. The MMCs were cultured in DMEM/F12 (3:1) medium (GIBCO-BRL) supplemented with 10% fetal bovine serum (GIBCO-BRL). To observe the effect of miR-148a-3p on cell proliferation, MMCs were randomly divided into the following six groups: control group, HMGB1 group, miR-148a-3p mimic + HMGB1 group, pre-miR-148a-3p mimic negative control + HMGB1 group, miR-148a-3p inhibitor + HMGB1 group, and anti-miR-148a-3p inhibitor negative control + HMGB1 group (HMGB1 was obtained from Sigma and miR-148a-3p mimic and inhibitor were purchased from Invitrogen). All of the cells were collected at 10 or 30 min or 8 h after being exposed to HMGB1 and Western blotting was used to detect the expressions of PTEN, p-Akt-S473, Akt, PCNA, cyclin D1, CDK4, and p16 protein. Immunocytochemistry was used to detect the expression of PCNA. 5-Bromo-2-deoxyuridine (BrdU) incorporation and immunofluorescent staining were used to detect cell proliferation. To explore whether miR-148a-3p mediated MMCs proliferation by targeting PTEN, the cells were divided into the following six groups: control group, HMGB1 group, miR-148a-3p mimic + HMGB1 group, pcDNA3.1-PTEN + HMGB1 group, miR-148a-3p mimic + pcDNA3.1-PTEN + HMGB1 group, and pcDNA3.1 vector + pre-miR-148a-3p mimic negative control + HMGB1 group. Western blotting was used to detect the PCNA protein expression.

RNA extraction and real-time qPCR. Total RNA from mouse blood serum, cultured cells and glomeruli was extracted using the mirNA Isolation Kit (Qiagen) according to the manufacturer’s instructions. Normal glomeruli and proliferative glomeruli were sieved from the renal cortical tissue of normal and MRL/lpr mice using a microdissection technique described previously (31). qPCR assays were carried out to detect miRNA expression using Taqman miRNA assays. The 2^−ΔΔCT method was used to normalize the qPCR cDNAs [ΔΔCT = (C_Target − C_T_Actin) × timeX − (C_T_Target − C_T_Actin) × time0]. All of the experiments were repeated at least three times.

In situ hybridization. The miR148a-3p probe was purchased from Exiqon and the in situ hybridization kit was purchased from Roche. Paraffin sections were used for in situ hybridization to detect the expression of miR-148a-3p according to the manufacturer’s instructions. After equilibration, the sections were prehybridized for 2 h at 37°C. Hybridization was carried out for 18 h at 70°C. Then, the slides were washed with washing buffer for 15 min at 37°C. The sections were stained with BM purple and the nucleus were dyed with nuclear fast red. Pink blue granules in the cytoplasm were considered to be a positive region. The sections were imaged with an Olympus microscope and quantitated by digital image analysis using the Image Pro-Plus 5.0 software (6).

Transfection of miR-148a-3p mimic and inhibitor. MMCs were seeded in a six-well plate. To induce or decrease the expression of miR-148a-3p, MCC cells induced by HMGB1 were transfected with miR-148a-3p mimic (In vitro) or miR-148a-3p inhibitor using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. In addition, pre-miR-148a-3p mimic negative control or anti-miR-148a-3p inhibitor negative control was transfected into MMCs using the same protocol as control. All of the experiments were repeated at least three times.

Transfection of wild-type PTEN. The PTEN expression vector (pcDNA3.1-PTEN-EGFP) and the control vector (pcDNA3.1-IRES-EGFP) were designed and produced by the Beijing Fugenome Technology (Beijing, China). Transfections were performed using Lipofectamine 2000 reagent (Invitrogen) as described previously (7). At least three independent experiments were completed.

**Protein extraction and Western blotting.** MMCs and renal glomeruli were collected and protein extraction was performed as described previously (6). The protein extracts were separated by 10% SDS-PAGE and then transferred to PVDF membranes. The membranes were incubated overnight at 4°C with anti-Akt (1:1,000; Epitomics), p-Akt (P-S473; 1:1,000; Epitomics), PTEN (1:1,000; Epitomics), PCNA (1:1,000; Abcam), cyclin D1 (1:1,000; Epitomics), CDK4 (1:1,000; Epitomics), p16 (1:1,000; Epitomics), or β-actin (1:1,000; Epitomics) antibodies. Then, the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:5,000; ZSGB-BIO) and imaged using the LI-COR Odyssey Infrared Imaging System.

**Immunofluorescence and immunocytochemistry or immunohistochemistry.** After HMGB1 treatment, the cells were mixed with BrdU (10 μmol/l; Sigma) for 2 h before collection. Then, the cells were fixed in a 4% paraformaldehyde solution and permeabilized with 0.5% Triton X-100. The cells were then treated with 2 N HCl for 2 h at 37°C to denature the DNA. Next, the cells were washed with PBS and then incubated with an anti-BrdU antibody (1:100; Abcam) overnight at 4°C. After conjugation with a rhodamine-labeled affinity purified mouse IgG antibody and DAPI staining (10 μmol/l; KPL), the sections were visualized using an Olympus microscope (Olympus BX71). During the second day, the sections were incubated with
polymer helper and polyperoxidase-anti-rabbit IgG at 37°C and stained with diaminobenzidine.

PCNA expression was detected by immunocytochemistry or immunohistochemistry as described (6). The cells or slides were incubated with an anti-PCNA antibody (1:100; Abcam) overnight at 4°C. The remaining operations were performed as above.

Double staining of immunofluorescence was performed as described previously (6). Antigen recovery was done by the method of anticyclone and the negative control was performed by replacing the primary and secondary antibodies with PBS buffer. The slides were incubated with anti-PCNA and anti-Thy1 antibodies (PCNA, 1:100; Thy1, 1:50; Abcam) overnight at 4°C. On the following day, slides were incubated with FITC-conjugated goat anti-rat IgG and TRITC-goat anti-mouse IgG (1:100; Proteintech, Chicago, IL) simultaneously at 37°C for 1 h. Images were examined with an Olympus BX71 digital microscope. The positive signal of PCNA was red located in nuclei and Thy1 was green located in cytoplasm.

PTEN gene 3′-UTR luciferase reporter assay. To create the PTEN 3′-UTR luciferase reporter construct, sequences were synthesized and ligated into the pGL3-REPORTER vector. The following primers were used to amplify the 3′-UTR of PTEN: 5′-ATC GAC TAG TGA ATA ATG ACA TTA CAA TGG GCT GTT GCA CTG TTA ATA TTT TTC CTT TGG AAG CTT ATC G-3′ and 5′-ATC GAC TAG TGC AGC AGT GGC TCT GTG TGT AAA TGC TAT GCA CTG AGG ATA CAC AAA TAT GAA GCT TAT GCA CGT GAG AGG ATA CAC AAA TAT GAA GCT TAT CG-3′. The amplified fragment was cloned into the pGL3 luciferase reporter vector between the SpeI and HindIII sites. The sequence of the plasmid (pGL3-PTEN) was confirmed by DNA sequencing.

miR-148a-3p expression in the glomeruli of mice by qPCR (mean ± SD, n = 6). *P < 0.05 vs. control.
MMC were cultured in a 24-well plate the day before transfection and then cotransfected with the firefly luciferase-3'-UTR (pGL3-PTEN; 500 ng) and the pRL-TK vector (Promega), along with the miR-148a-3p inhibitor or the control sequences. After 2 days, the firefly and Renilla luciferases were measured using an HT microplate reader (Biotek, Beijing, China) with the Dual-Glo Luciferase assay system (Promega). Luciferase activities were normalized to Renilla luciferase activity. All of the experiments were repeated at least three times.

Statistical analysis. The quantitative data were presented as the mean ± SD. Statistical analyses were performed using one-way ANOVA with the Student-Newman-Keuls test. Statistical significance was defined as \( P < 0.05 \).

RESULTS

Upregulated expression of miR-148a-3p was inversely associated with glomeruli cell proliferation in LN. Microarray analysis results showed that miR-148a-3p was overexpressed in HMGB1-stimulated MMCs (Fig. 1). To determine the clinicopathological significance of the aberration in miR-148a-3p expression, we evaluated miR-148a-3p levels in 30 renal tissues with LN and control renal tissues using in situ hybridization and determined miR-148a-3p expression in blood serum by qPCR. MiR-148a-3p expression in the glomeruli and blood serum of patients with LN was higher than that in normal people (Fig. 2, A and B). A positive signal was located in cytoplasm of glomeruli. Additionally, the positive expression of miR-148a-3p in their glomeruli and the increased expression of PCNA protein in glomeruli were both detected in patients with LN, compared with the control group (Fig. 2C), and there was positive correlation between the PCNA expression and miR-148a-3p expression in glomeruli of LN group (\( r = 0.998, P = 0 \)). Thy1 is considered as a marker of the mesangial cell. In order to disclose that the proliferative cell in glomeruli was mesangial cell, we carried out immunofluorescence double staining. As shown in Fig. 2D, the PCNA protein expression in mesangial cell of mice with LN upregulated compared with that in control mice.

Meanwhile, the qPCR method was also used to detect miR-148a-3p expression in the renal glomeruli of MRL/MPJ mice (control group) and MRL/faslp mice (LN group). The result showed that miR-148a-3p expression in the glomeruli of MRL/faslp mice was remarkably higher than that in control mice (Fig. 2E). Furthermore, MRL/faslp mice, whose glomeruli displayed higher miR-148a-3p expression levels, simultaneously demonstrated increased amounts of proliferative mesangial cells and increased PCNA protein expression in mesangial cells of glomeruli (see Fig. 7D). In addition, the expression of miR-148a-3p in glomeruli was positively correlated with PCNA expression (\( r = 0.941, P = 0.005 \)).

![Fig. 3. miR-148a-3p accelerates MMC proliferation. A: forced expression of miR-148a-3p in HMGB1-induced MMCs accelerated cell proliferation as shown in the BrdU incorporation assay (mean ± SD, \( n = 3 \)). B: ectopic expression of miR-148a-3p in MMCs upregulated the expression of PCNA protein by immunocytochemistry. C, control group; H, HMGB1 group; M, miR-148a-3p mimic group; M + H, miR-148a-3p mimic + HMGB1 group; and MC + H, pre-miR-148a-3p mimic negative control + HMGB1 group (mean ± SD, \( n = 3 \)). *\( P < 0.05 \) vs. C; #\( P < 0.05 \) vs. H and M.](http://ajpcell.physiology.org/)
From above all, we speculated that high miR-148a-3p expression was closely related to glomeruli cell proliferation in LN.

Overexpression of miR-148a-3p accelerated cell proliferation and reduced expression of miR-148a-3p restrained cell proliferation in MMCs induced by HMGB1. Because high levels of miR-148a-3p expression in the LN group correlated with glomeruli cell proliferation, we hypothesized that ectopic reduction or overexpression of miR-148a-3p in MMCs would be able to exert inhibitory or acceleratory effects on cell growth, respectively. To validate this hypothesis, we transfected an miR-148a-3p mimic, inhibitor, or scrambled sequence into MMCs that had been exposed to HMGB1 or not (these cells had low basal levels of miR-148a-3p). Successful overexpression or downregulation of miR-148a-3p expression in cells was confirmed by qPCR. Immunofluorescence and immunocytochemistry staining demonstrated that BrdU and PCNA protein levels in the HMGB1 group were both higher than those in the control group and that PCNA protein levels and the number of BrdU-positive cells were significantly higher in the miR-148a-3p mimic + HMGB1 group compared with those in the HMGB1 group and the miR-148a-3p mimic group (Fig. 3, A and B).

The results from Western blotting showed that the expression of PCNA protein in the miR-148a-3p mimic + HMGB1 group was 1.04 ± 0.045 and significantly higher than that in pre-miR-148a-3p mimic negative control + HMGB1 group, suggesting that ectopic expression of miR-148a-3p could accelerate the proliferation of MMCs induced by HMGB1 (Fig. 4B). However, as shown in Fig. 4A, miR-148a-3p mimic or inhibitor alone could not affect the expression of PCNA protein.

In contrast, PCNA expression was reduced in the miR-148a-3p inhibitor + HMGB1 group compared with that in the HMGB1 group (Fig. 4C). There were no significant differences between the HMGB1 group and anti-miR-148a-3p inhibitor negative control + HMGB1 group (Fig. 4C).

To explore the possible mechanism of miR-148a-3p overexpression on cell proliferation, we detected the expression of cyclin D1/CDK4/p16 (the check point of G0/G1 to S phase). The analysis of the Western blots showed that the miR-148a-3p mimic upregulated the expression of CDK4, while the miR-148a-3p inhibitor reduced the expression of CDK4 and cyclin D1 in MMCs induced by HMGB1 (Fig. 4, B and C). Meanwhile, the expression of p16 in the miR-148a-3p mimic + HMGB1 group was significantly lower (0.32 ± 0.019) than that in the HMGB1 group (Fig. 4B). The expression of p16 was significantly higher in the miR-148a-3p inhibitor + HMGB1 group (0.84 ± 0.02) compared with that in the HMGB1 group (Fig. 4C).

As shown in Fig. 4D, the expression of p-Akt-S473 in the miR-148a-3p mimic + HMGB1 group was 1.085 ± 0.067 and significantly higher than that in the HMGB1 group, following a 20-min exposure to HMGB1. However, there were no significant differences between the HMGB1 and pre-miR-148a-3p negative control + HMGB1 groups, and the expressions of p-Akt-S473 in both groups were markedly higher than that in the control group (Fig. 4D). The expression of p-Akt-S473 in the miR-148a-3p inhibitor + HMGB1 group was significantly lower (0.467 ± 0.013) than that in the HMGB1 group. How-

**Fig. 4.** miR-148a-3p upregulates proliferation-related protein expression and Akt phosphorylation. *A:* effects of miR-148a-3p mimic and inhibitor alone on the PCNA and phosphatase and tensin homology deleted on chromosome ten (PTEN) expression were detected by Western blot (mean ± SD, n = 3). *B:* ectopic expression of miR-148a-3p increased PCNA, cyclin D1, and CDK4 expression and increased p16 expression in HMGB1-induced MMCs by Western blot (mean ± SD, n = 3). *C:* MiR-148a-3p inhibited PCNA, cyclin D1, and CDK4 expression and decreased p16 expression in HMGB1-induced MMCs by Western blot (mean ± SD, n = 3). *D:* transfection of miR-148a-3p mimic upregulated p-Akt expression in HMGB1-induced MMCs (mean ± SD, n = 3). *E:* transfection of miR-148a inhibitor downregulated p-Akt expression in HMGB1-induced MMCs (mean ± SD, n = 3). *F:* transfection of anti-miR-148a-3p inhibitor in HMGB1-induced MMCs (mean ± SD, n = 3). *G:* anti-miR-148a-3p inhibitor negative control + HMGB1 group; IC + H, anti-miR-148a-3p inhibitor + HMGB1 group; *P < 0.05 vs. C; #P < 0.05 vs. H.
ever, there was no significant difference in the total Akt level among the six groups (Fig. 4D).

**Anti-oncogene PTEN was a direct target of miR-148a-3p.** Our previous studies have identified that PTEN mediates HMGB1-induced MMC cell proliferation (7). To elucidate the mechanisms responsible for the ability of miR-148a-3p to stimulate cell proliferations, we used bioinformatics analysis to identify its target genes. PTEN was identified as one of the miR-148a-3p candidate targets. miR-148a-3p can potentially bind to the PTEN 3'-UTR. Our studies have revealed that HMGB1 can inhibit PTEN protein expression and upregulate the expression of miR-148a-3p; they have also shown a negative correlation between PTEN expression and miR-148a-3p expression. To determine whether miR-148a-3p regulated PTEN, we performed a luciferase reporter assay. The luciferase activity of the PTEN 3'-UTR was increased by 94.42% in cells transfected with miR-148a-3p mimic (mean ± SD, n = 3). PTEN protein expression level was increased in HMGB1-induced MMCs transfected with miR-148a-3p mimic (mean ± SD, n = 3). *P < 0.05 vs. C; #P < 0.05 vs. H.

**PTEN was involved in miR-148a-3p-induced MMC proliferation.** To elucidate whether the downregulation of PTEN by miR-148a-3p was involved in cell proliferation induced by HMGB1, MMCs were transfected with the miR-148a-3p mimic and the pcDNA3.1-PTEN vector. Compared with the pcDNA3.1-PTEN vector group (P + H), the cells transfected with miR-148a-3p mimic displayed lower PTEN expression, while cells cotransfected with both the miR-148a-3p mimic and the pcDNA3.1-PTEN vector exhibited lower PTEN expression (Fig. 6A). Interestingly, the cells transfected with pcDNA3.1-PTEN displayed lower cell proliferation potential compared with the cells transfected with both the miR-148a-3p mimic and the pcDNA3.1-PTEN vector (Fig. 6B). As shown in Fig. 6C, the PCNA protein level in the pcDNA3.1-PTEN group was significantly lower (0.22 ± 0.02) compared with that in the HMGB1 group. Upon transfection with the miR-148a-3p mimic and the pcDNA3.1-PTEN at the same time, PCNA protein expression was 0.64 ± 0.02 and statistically higher than that in the pcDNA3.1-PTEN group. These observations suggested that the downregulation of cell proliferation was seen when the cells were transfected with the pcDNA3.1-PTEN vector, which could be diminished by miR-148a-3p. Thus PTEN might have an important role in the cell proliferation of HMGB1-induced MMCs mediated by miR-148a-3p.

**miR-148a-3p inhibition improved renal function and cell proliferation levels in the glomeruli in lupus mice in vivo.** To further explore the regulatory effects of miR-148a-3p on PTEN and renal function, MRL/lpr/lpr mice were treated with either

Fig. 5. PTEN is a miR-148a-3p target gene. A: luciferase reporter assay in MMCs showed that luciferase activity controlled by the PTEN 3'-UTR is dramatically increased by an miR-148a-3p inhibitor transfection (mean ± SD, n = 3); B: PTEN protein expression levels were reduced in HMGB1-induced MMCs transfected with miR-148a-3p mimic (mean ± SD, n = 3). C: PTEN protein expression level was increased in HMGB1-induced MMCs transfected with miR-148a-3p inhibitor (mean ± SD, n = 3). *P < 0.05 vs. H, #P < 0.05 vs. H.

Fig. 6. PTEN is involved in the miR-148a-3p-induced acceleration of cell proliferation. A: PTEN protein expression levels increased in MMCs treated with the miR-148a-3p mimic and pcDNA3.1-PTEN vector by Western blotting (mean ± SD, n = 3). B: cell proliferation levels were detected by immunocytochemistry with the BrdU antibody (mean ± SD, n = 3). The amount of proliferation was reduced in MMCs treated with the miR-148a-3p mimic and pcDNA3.1-PTEN vector. C: MMCs treated with the miR-148a-3p mimic and pcDNA3.1-PTEN vector exhibited reduced PCNA expression by Western blotting (mean ± SD, n = 3). P + H, pcDNA3.1-PTEN + HMGB1 group; M + P + H, miR-148a-3p mimic + pcDNA3.1-PTEN + HMGB1 group; and MC + PV + H, pre-miR-148a-3p mimic negative control + pcDNA3.1 vector + HMGB1 group. *P < 0.05 vs. C; #P < 0.05 vs. H; ΔP < 0.05 vs. P + H.
anti-miR-148a-3p adenovirus or empty-adenovirus. Anti-miR-148a-3p adenovirus, but not empty-adenovirus, decreased miR-148a-3p expression and increased PTEN expression in renal glomeruli as determined by qPCR and Western blotting (Figs. 7, A and B). Anti-miR-148a-3p adenovirus significantly decreased 24-h urine protein (upro) (Fig. 7C) and the expression of PCNA protein in glomeruli, as shown by immunohistochemistry staining and Western blotting (Fig. 7, B and D). In contrast, empty-adenovirus had no effect on renal glomeruli cell proliferation and renal function. Anti-miR-148a-3p adenovirus treatment also downregulated p-Akt expression and decreased Cyclin D1 expression (Fig. 7B), as shown by Western blotting. These results suggested that miR-148a-3p promoted renal glomeruli cell proliferation in vivo.

**DISCUSSION**

PTEN is a phosphatase with a central function in counteracting PI3K signaling (20). PTEN removes a phosphate from the D3 position of PIP3 to generate PIP2, thereby converting the “on” switch of the PI3K pathway into an “off” switch (19). By reducing the generation of the phosphorylated form of the serine and threonine kinase Akt (p-Akt) via PI3P downstream of PI3K, the phosphatase activity of PTEN directly regulates diverse cellular processes, including cell proliferation, growth, survival, apoptosis, and metabolism (10, 14). PTEN is inhibited and promotes the proliferation of HMGB1-induced mesangial cells in sequence by enhancing the phosphorylation level of Akt. Additionally, PTEN is also repressed in LN glomeruli and negatively correlates with cell proliferation (7). The reason for the abnormally low PTEN expression level in the renal tissues during LN remains unclear.

In our studies, we have demonstrated that decrease in PTEN protein level occurs in the glomeruli of patients and mice with LN, even though the mRNA level does not change. Here, we demonstrate a new regulatory mechanism whereby PTEN expression is regulated via miR-148a-3p-mediated posttranscriptional inhibition. In this study, we first find that miR-148a-3p is frequently upregulated in LN renal tissues and HMGB1-induced MMCs and that the increased miR-148a-3p expression is closely related to renal glomeruli cell proliferation. Furthermore, we demonstrate that anti-miR-148a-3p inhibition could suppress MMC proliferation, while miR-148a-3p overexpression could accelerate MMC proliferation in vitro and in vivo. We also identify PTEN as a target of miR-148a-3p. Therefore, upregulation of miR-148a-3p might contribute to LN by regulating PTEN.

Elucidating the molecular mechanisms involved in cell proliferation is a critical issue, as excessive mesangial cell proliferation is the main pathological change in LN (26). These glomerulosclerotic changes could be effectively alleviated by inhibiting the proliferation of mesangial cells (12). The cell cycle mechanism is regulated by cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinases inhibitors (CDKIs) (13). The cyclin D1/CDK4/p16-pRb pathway is one of the key signal transduction pathways at the G1/S checkpoint during cell cycle (25). Our previous study has shown that HMGB1 induces MMC proliferation and promotes cell cycle transition from G0/G1 to S phase. Recent studies have also shown that many signaling pathways are related to cell proliferation, including the MARK/ERK (28), PI3K/Akt (20), Wnt/β-catenin (17), and NF-κB pathways (1). The PI3K/Akt pathway is a classic signaling pathway that could induce cell prolifer-
showed that and the BrdU-positive expression ratio. Our functional analysis produced MMC proliferation. PTEN overexpression also inhibits growth, while PTEN overexpression prevents HMGB1-induced MMC proliferation. PTEN overexpression also inhibits Akt phosphorylation and decreases the cyclin D1 expression and the BrdU-positive expression ratio. Our functional analysis showed that miR-148a-3p overexpression inhibits PTEN expression and accelerates MMC growth, which is parallel to the small interfering RNA-mediated knockdown of PTEN. In vivo, anti-miR-148a treatment upregulates the PTEN expression and inhibits the phosphorylation of Akt and Cyclin D1 expression. These observations suggest new insights regarding the essential mechanisms regulating the Akt pathway during LN progression. These findings reveal that PTEN is a biologically significant target gene of miR-148a-3p. Additionally, the miR-148a-3p/PTEN pathway might be a previously unrecognized regulator involved in LN progression. Therefore, miR-148a-3p may serve as a potential target for therapeutic interventions against LN cell proliferation.

PTEN has been identified as a target of miR-148a-3p. However, the effects of miR-148a-3p upregulation might not be solely explained by its ability to regulate PTEN alone, as a single miRNA can regulate numerous genes. Using bioinformatics prediction analysis, we have identified at least eight other miR-148a-3p potential targets including some proliferation-related genes. For example, RUNX3 has recently been proposed to be a biologically relevant miR-148a-3p target. MiR-148a-3p may regulate RUNX3 expression by modulating DNMT1-dependent DNA methylation in gastric cancer (33). Porstner et al. (24) and other researchers have revealed that miR-148a is the most abundant miRNA in primary human and murine plasma cells and its expression is upregulated in activated murine B cells and targets Bach2 and Mitf and proapoptotic factors such as PTEN and Bim. Similarly, Yuan et al. (32) also report that miR-148a regulates the expression of PTEN in hepatic cell lines, apparently via the 3’-UTR. In contrast, the bioinformatics analysis also suggests that the PTEN may be targeted by more than 10 different miRNAs, implying that other miRNAs may also mediate the function of PTEN in LN. For example, miR-216a has been predicted to regulate PTEN (30, 31). Interestingly, Akt activation by TGF-β due to PTEN downregulation by miR-216a/217 is an important step in kidney dysfunction (15). Therefore, it is necessary to identify additional miR-148a-3p targets and other miRNAs that can also regulate PTEN in the future studies. These studies will allow us to have a deeper understanding of the mechanism underlying the development and progression of LN.

In conclusion, increased miR-148a-3p expression is common in the glomeruli of patients and mice with LN and HMGB1-induced MMCs and is closely associated with LN cell proliferation. MiR-148a-3p could induce proliferation during the development and progression of LN by targeting PTEN. With more understanding of its function, miR-148a-3p may be used as a potential prognostic marker and therapeutic target in treating LN.

GRANTS
This work was supported by National Natural Science Foundation Grant 81000301, 2012 Annual Program for New Century Excellent Talents Grant 0300690202, Department of Education of Hebei Grant ZH2012002, and Doctoral Program of the Education Ministry of China Grant 20131230001.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: L.Q., L.H., and Z.S. performed experiments; L.Q., F.X., and Y.M. analyzed data; F.X. and L.S.-X. drafted manuscript; Z.W., W.C., and K.P. prepared figures; Z.W. edited and revised manuscript; W.C. and H.J. interpreted results of experiments; L.S.-X. conception and design of research.

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