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Change of MAX Interactor 1 Expression in an Anti-Thy1 Nephritis Model and its Effect on Mesangial Cell Proliferation

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Key Words

Mxi1 • Mesangial cell • Cell proliferation • Cell cycle • Cell cycle regulatory proteins

Abstract

Background/Aims: During the disease process of mesangial proliferative glomerulonephritis, the expression of various factors that influence mesangial proliferation is altered. MAX interactor 1 (Mxi1) antagonizes the transcription factor Myc and is believed to be a tumor suppressor. However, no studies have investigated its effect on mesangial cell proliferation. Methods: To investigate the effect of Mxi1 on renal mesangial cell proliferation, we established a classic rat anti-Thy1 mesangial proliferative glomerulonephritis model. Mesangial proliferation was estimated by immunohistochemical analysis of Ki67. Mxi1 expression at each time point was assessed by real-time RT-PCR and Western blot analyses. Furthermore, we altered the expression level of Mxi1 by a plasmid and siRNA to detect its effect on rat mesangial cell proliferation *in vitro*.Results: Mxi1 expression decreased significantly during the proliferative period of anti-Thy1 nephritis model and then gradually increased as proliferation declined, indicating that Mxi1 may be linked to mesangial cell proliferation. Upregulation of Mxi1 expression via plasmid transfection *in vitro* reduced the expression of the positive-acting cell cycle regulatory proteins cyclin B1, cyclin D1, cyclin E, CDC2 and CDK2; significantly reduced mesangial cell proliferation; reduced the percentage of S phase cells; and increased the percentage of G2/M phase cells. Inhibition of Mxi1 expression by siRNA *in vitro* produced the opposite effects: increased expression of cyclin B1, cyclin D1, cyclin E, CDC2 and CDK2; markedly increased cell proliferation; higher percentage of S phase cells; and dramatically lower percentage of G2/M phase cells. Transcription factor c-myc protein expression showed no obvious difference after Mxi1 plasmid and siRNA transfection. The expressions of cell cycle regulatory proteins mentioned above were negative correlated with Mxi1 expression in anti-Thy1 nephritis model. Conclusion: These results suggest that Mxi1 expression levels were inversely correlated with proliferation in anti-Thy1 nephritis rats and it may influence cell cycle progression and thus the rate of mesangial cell proliferation by regulating the expression of c-myc target cell cycle regulatory proteins.

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Introduction

Mesangial proliferative glomerulonephritis (MsPGN), which includes IgA nephropathy, is the most common cause of glomerulonephritis worldwide. MsPGN is characterized by increased mesangial cell proliferation and mesangial matrix expansion. It is associated with proteinuria and hypertension and can ultimately lead to end-stage renal disease. Thus, it is important to investigate the pathogenesis of MsPGN and to explore appropriate treatment measures [1, 2].

Previous studies have shown that the expression of many proliferation-related factors is altered in MsPGN [3, 4]. Mesangial cell proliferation is increased as a consequence of disequilibrium between promoters and inhibitors of proliferation. Most studies have focused on proliferation promoters [5-7], and few have investigated factors that may inhibit mesangial cell proliferation. Identifying inhibitors of mesangial cell proliferation and enhancing their expression may help to inhibit proliferation and provide a new therapeutic strategy for combating mesangial proliferation and glomerular sclerosis.

MAX interactor 1 (Mxi1), a member of the Mad family of transcription factors, competitively inhibits the interaction between Myc and MAX, thereby impairing the ability of Myc to stimulate transcription [8]. Previous studies of Mxi1 have described its role in tumor biology, especially as a tumor suppressor. However, it is unknown whether Mxi1 can inhibit renal mesangial cell proliferation. The anti-Thy1 nephritis rat is a classic mesangial proliferative glomerulonephritis model and is useful for investigating mechanisms of mesangial cell proliferation [9]. In the present study, we investigated the change in Mxi1 expression at various time points during disease development and progression in anti-Thy1 nephritis model rats. We also studied the effect of Mxi1 on rat mesangial cell proliferation *in vitro* through positive and negative regulation of its expression and explored its possible mechanisms.

Materials and Methods

Anti-Thy1 nephritis rat model

Specific pathogen-free male Wistar rats (8 weeks old) were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed in the Experimental Animal Center of PLA General Hospital. Rats were randomly assigned to one of six groups (five experimental groups and one control group; 3 rats per group). Experimental mesangial proliferative glomerulonephritis was induced through a single intravenous injection of anti-Thy1 antibody (2.5 mg/kg). Control rats were injected with an identical volume of normal saline. Rats were killed on days 3, 5, 7, 10, and 14 post-injection. The renal tissues were examined by routine Periodic Acid-Schiff Stainings. RNA and protein were extracted from the renal cortex to perform the expression analyses on individual animals of each group at a given time point.

Immunohistochemical Analysis and Evaluation

Routine histologic paraffin sections were cut to 3-4-μm thickness and mounted on poly-L-lysine-coated slides. The sections were deparaffinized in xylene and rehydrated in alcohol. Endogenous peroxidase was blocked with 3% hydrogen peroxide. Then, the sections were heated in a microwave oven for 10 min in sodium citrate buffer (pH 6.0). The sections were incubated with 1.5% normal goat serum for 20 min, followed by incubation overnight with a rabbit polyclonal antibody against Ki67 antigen (Abcam, UK) as primary antibody. After removal of unbound primary antibody and rinsing with PBS, the sections were incubated with a biotinylated secondary antibody for 60 min at room temperature. The sections were rinsed and then incubated with avidin-biotinylated horseradish peroxidase (Vectastain Elite ABC kit, Vector Laboratories, USA) for 60 min. Incubation with 3,3-diaminobenzidine tetrahydrochloride was performed for 10 min as a substrate chromogen solution to produce a brown color. Finally, the sections were counterstained with hematoxylin. For negative controls, the anti-Ki67 antibody was replaced with PBS. In order to quantify the amount of proliferating cells in the glomeruli, 10 glomeruli per section obtained from 3 rats at each time point were evaluated under high power (x 400) light microscopy in a blinded fashion. Ki67 labeling index was calculated by Ki67 positive cells to total glomerular cells.

Cell culture

A rat mesangial cell line was purchased from the American Type Culture Collection (ATCC). Cells were cultured in RPMI 1640 medium (Gibco, USA) containing 10% fetal calf serum (FCS; Hyclone, Canada), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) at 37°C in a 5% CO_2 atmosphere.

Mxi1 plasmid construction

To construct a full-length Mxi1 expression plasmid, PCR products were cloned into pcR3.1. The resulting plasmid was named pcR3.1-Mxi1. After confirmation of its identity, it was transformed into *Escherichia coli* and extracted using a Maxipreps DNA purification system (Promega, USA).

siRNA design

A Mxi1-specific siRNA (siMxi1) and a control siRNA (siCon) were synthesized by Shanghai Jima Company. The Mxi1 RNAi target sequence and sequences of siMxi1 and siCon are shown in Table 1. Synthesized siRNA was suspended in DEPC-treated water and stored at -20°C until use.

Cell transfection

Mesangial cells in six-well plates were transfected with plasmid or siRNA using jetPRIME™ DNA and siRNA

Name	Sequences
siMxi1 target	5'-CAAAGCCAAAGCACACATC-3'
siMxi1	sense: 5'-CAAAGCCAAAGCACACAUCtt-3' antisense: 3'-ttGUUUCGGUUUCGUGUGUAG-5'
siCon	sense: 5'-UUCUCC GAACGUGUCACGUtt-3' antisense: 3'-ttTTAAGAGGCUUGCACAGUGCA-5'

Table 1. siRNA sequences

Target gene	Primers and TaqMan probes sequences
Mxi1	Forward: 5'-CGTCTGTGGCGCCTCCTGTCG-3'
	Reverse: 5'-GCTCCCTGCGCTCCAAAAACTCC-3'
	Probe: 5'-FAM- AGCCCATGGAGCGGGT -TAMRA -3'
GAPDH	Forward: 5'-GGCATGGACTGTGGTCATGAG -3'
	Reverse: 5'-TGCACCACCAACTGCTTA GC-3'
	Probe: 5'-FAM-CCTGGCCAAGGTCATCCATGACAACTT -TAMRA-3'

Table 2. Primers and TaqMan probes of real-time RT-PCR

transfection reagent (Polyplus-transfection, France) according to the following protocol. Mxi1 plasmid DNA (2 μg) or siRNA (5 μl of 10 μM) was added to 200 μl of jetPRIME™ buffer and mixed by vortexing. Next, 4 μl of jetPRIME™ were added to the sample, and the mixture was vortexed for 10 s, briefly centrifuged, and then incubated for 10-15 min at room temperature. The transfection mix (200 μl) was added drop-wise to each well of mesangial cells, for even distribution over the growing cells. After 4 h (for DNA) or 24 h (for siRNA), the transfection medium was replaced with 4 ml of growth medium. Co-transfection with eGFP-C1 plasmid was performed similarly. Mxi1 plasmid DNA (2 μg) and eGFP-C1 (2 μg) or Mxi1 siRNA (5 μl of 10 μM) and eGFP-C1 (400 ng) were added to 200 μl of jetPRIME™ buffer and mixed by vortexing. Then, 8 μl of jetPRIME™ were added to the sample, and the mixture was vortexed for 10 s, briefly centrifuged, and incubated for 15 min at room temperature. The transfection mix (200 μl) was added drop-wise to each well of mesangial cells. After 4 h, the transfection medium was replaced with 4 ml of growth medium. Fluorescence microscopy performed 24 h later confirmed a transfection efficiency >70% in all groups.

Real-time RT-PCR analysis

Relative Mxi1 expression was quantified by real-time RT-PCR, which was performed using a Rotor-Gene 3000 Real-Time PCR machine (Corbett Research, NSW, Australia). Total RNA was extracted from renal tissues or cells by using TRIzol reagent (Invitrogen, USA), and 5 μg of the total RNA was reverse-transcribed to cDNA. The primers and TaqMan probes (SBS Corp., Beijing, China), listed in Table 2, were designed using the full-length mRNA sequences of Mxi1 (GenBank Accession no. NM_013160) and GAPDH (internal reference) (GenBank Accession no. NM_017008). Each 25-μl reaction contained 12.5 μl of TaqMan PCR Master Mix (Toyobo, Japan), 0.2 μM primers, 0.2 μM TaqMan probes, and 1 μl of cDNA. PCR amplification was performed using the following conditions: initial denaturation at 95°C for 2 min, followed by 50 cycles of denaturation at 95°C for 30s, annealing at 58°C (Mxi1) or 60°C (GAPDH) for 30s (with fluorescence measured simultaneously), and extension at 72°C for 30s. Data analysis based on measurements of the threshold cycle was performed used the $2^{-\Delta\Delta Ct}$ method, as described by Livak [10]. We repeated the experiments three times.

Western blot analysis

RIPA buffer containing a protease inhibitor cocktail (1 μg/ml leupeptin, 1 μg/ml aprotinin, and 100 μM PMSF) was used to extract proteins from renal tissues and cells. Sample protein concentrations were measured using a BCA assay kit (Thermo Fisher Scientific, USA). Total cell or tissue lysates were separated by 12% SDS-PAGE and electroblotted to nitrocellulose membranes. The membranes were blocked by incubation in blocking buffer at room temperature for 1 h and were then incubated overnight at 4°C with primary antibodies against Mxi1 (Abcam, USA); c-myc, cyclin B1, cyclin D1, cyclin E, CDK2 and CDC2 (Santa Cruz Biotechnology, USA); and β-actin (Sigma, USA). After three washes with TBST buffer, the membranes were incubated with secondary antibody for 1 h. Immunoreactive bands were visualized using enhanced chemiluminescence followed by exposure to X-ray film. Protein band intensities were quantified using Quantity One software (Bio-Rad). We repeated the experiments three times.

Cell proliferation assay

As a measure of cell proliferation, the number of living cells was detected using a Cell Counting Kit-8 (Beyotime, China). Mesangial cells were seeded in 96-well plates at a density of 8,000 cells/well, synchronized by incubation in FCS(-) medium for 12 h, and then transfected with plasmid or siRNA. At 24, 36, 48, and 72 h post-transfection, the number of viable cells was measured by recording the optical density at 450 nm and generating cell growth curves. We repeated the experiments three times, six multiple holes were set up.

FACS analysis

Mesangial cells were seeded in six-well plates at a density of 2.5 x 10^5 cells/well, synchronized by incubation in FCS(-) medium for 12 h, and then transfected with plasmid or siRNA.

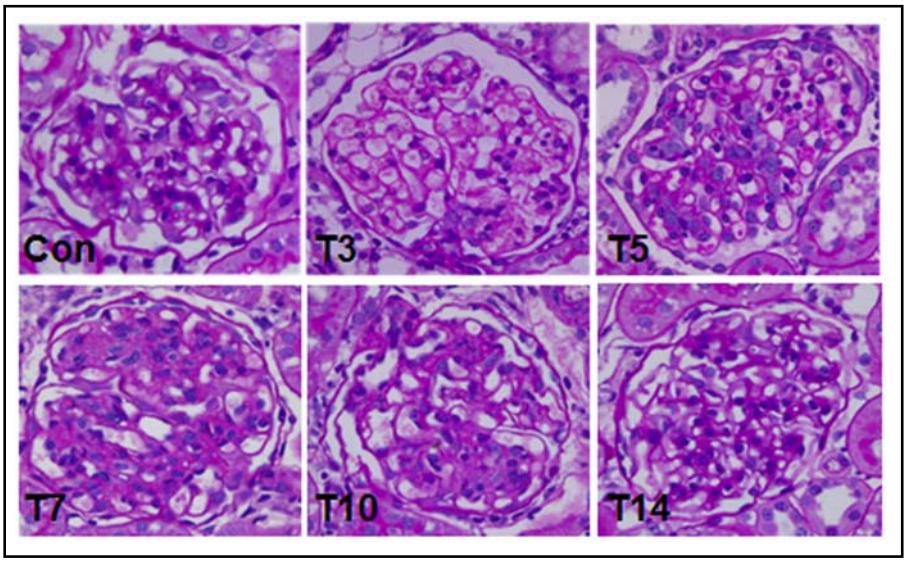


Fig. 1. Pathological changes in anti-Thy1 nephritis model rats. Periodic Acid -Schiff Stain (original magnification x 400). Con: control rats; T3, T5, T7, T10, T14: anti-Thy1 nephritis rats at day 3, 5, 7, 10, 14 separately.

The cells were washed twice with PBS, suspended in 75% ethanol, and fixed by incubation in the ethanol at 4°C overnight. The fixed cells were collected by centrifugation, washed with PBS, treated with RNase (50 μg/ml; Sigma), and stained with propidium iodide (50 μg/ml; Sigma). A FACS flow cytometer (BD Co., USA) was used to measure the cellular DNA content. The percentage of cells in G0/G1, S, and G2/M phases were counted using CellFIT Cell Cycle Analysis software (version 2.01.2; BD). When we performed double propidium iodide + BrdU stainings, BrdU (10μmol/L) was added before 2 hours of collection resulting in a 2-hour incorporation time. Cells were harvested and fixed same as above, resuspended in 2 mol/L HCl for 30 minutes at room temperature, and permeabilized in 0.5% Tween 20. Anti-BrdU antibody (Roche, USA) was added at a 1:10 dilution and incubated for 30 minutes at 37°C in the dark. The cells were washed in PBS, stained with FITC-conjugated anti-mouse-Ig-fluorescence at 1 in 10 for 30 minutes at 37°C in the dark. After 2 washes in PBS, propidium iodide and RNase were added and the cells were analyzed through the use of the FACS flow cytometer. We repeated the experiments three times.

Statistical analysis

Data are expressed as means ± SD. Statistical analyses were performed using SPSS version 16.0. Statistically significant differences between experimental groups were detected by one-factor analysis of variance. Values of $P < 0.05$ were considered to indicate statistical significance.

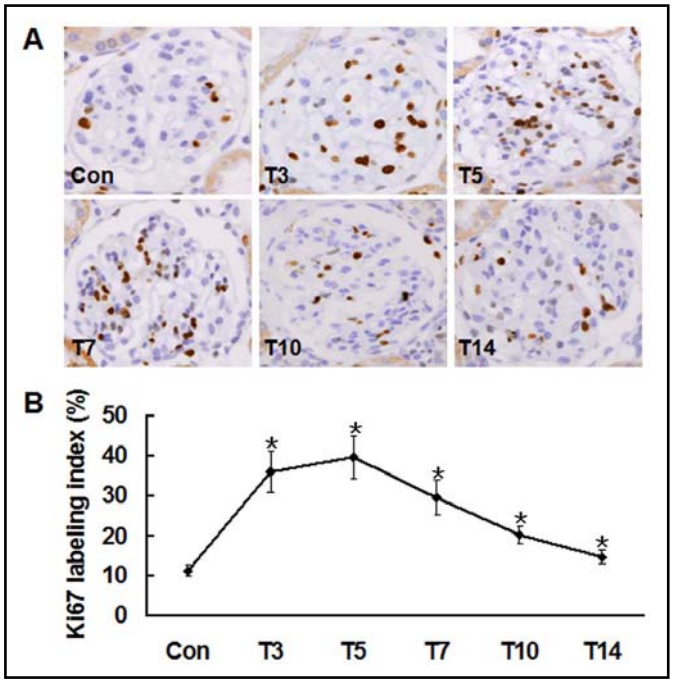


Fig. 2. Expression of Ki67 in anti-Thy1 nephritis model rats. A. Expression of Ki67 was detected with immunohistochemistry analysis. B. Ki67 labeling index was calculated by Ki67 positive cells to total glomerular cells. Con: control rats; T3, T5, T7, T10, T14: anti-Thy1 nephritis rats at day 3, 5, 7, 10, 14 separately. *$p < 0.05$ compared with control rats.

Results

Pathological changes in anti-Thy1 nephritis model rats

Mesangial dissolution was detected at day 3 after anti-Thy1 antibody injection. On days 5 and 7 (proliferative period), there were significant increases in glomerular cell number and marked expansion of the mesangial area.Then the mesangial cell proliferation gradually returned to nearly normal levels by days 10 and 14 (proliferation decline period) (Fig. 1). Ki67, a proliferation marker, was located in the nucleus. Ki67 expression was low in the control rats, then it was highly expressed after anti-Thy1 antibody injection. From day 7, its expression gradually declined to nearly normal levels (Fig. 2). These pathological changes were in agreement with previous results.

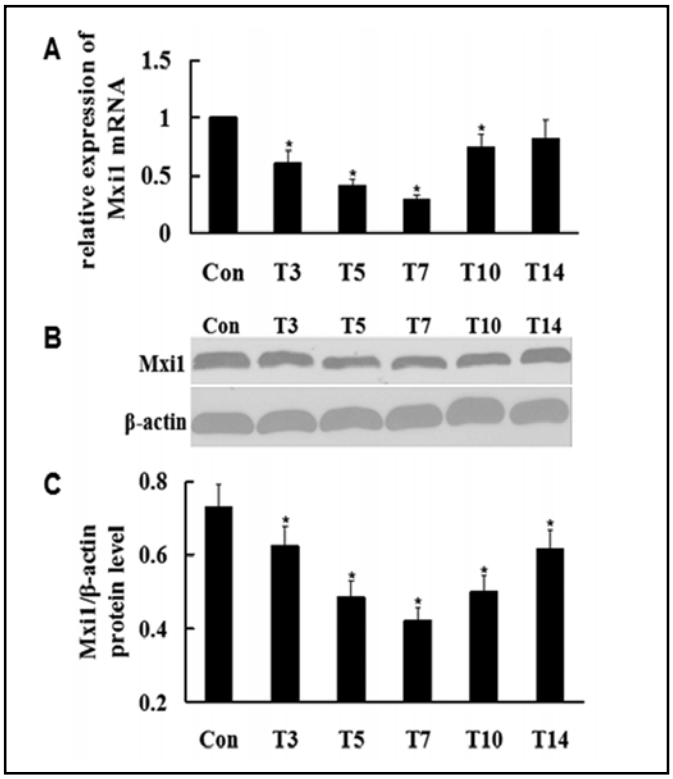


Fig. 3. Changes in Mxi1 expression in anti-Thy1 nephritis model rats. A. Mxi1 mRNA expression at each time point was assessed by real-time RT-PCR. The relative quantitation of Mxi1 mRNA was expressed as the ratio of Mxi1/GAPDH, which in control rats was set to 1. B. Mxi1 protein expression at each time point was assessed by Western blot analysis, specific bands were at 34kD. C.The relative quantitation of Mxi1 protein were expressed as the ratio of Mxi1/β-actin. Con: control rats; T3, T5, T7, T10, T14: anti-Thy1 nephritis rats at day 3, 5, 7, 10, 14 separately. *$p < 0.05$ compared with control rats.

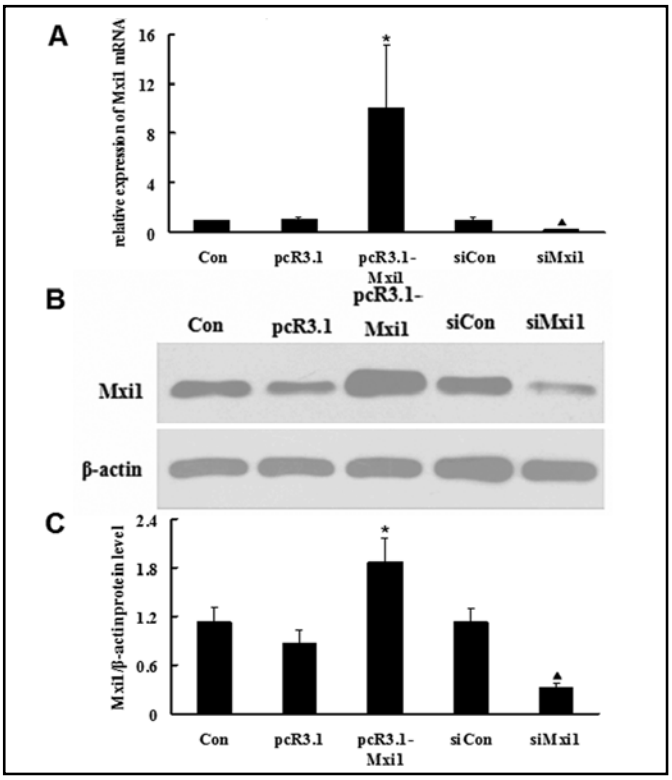


Fig. 4. Effects of pcR3.1-Mxi1 and siMxi1 transfection on Mxi1 expression in mesangial cells. A. To test whether a Mxi1 expression plasmid (pcR3.1-Mxi1) and siRNA (siMxi1) could influence Mxi1 expression, we used real-time RT-PCR to measure Mxi1 mRNA levels in mesangial cells after transfection. The relative quantitation of Mxi1 mRNA was expressed as the ratio of Mxi1/GAPDH, which in control cells was set to 1. B. Mxi1 protein levels in mesangial cells after transfection were assessed by Western blot analysis. C.The relative quantitation of Mxi1 protein were expressed as the ratio of Mxi1/β-actin. *$p < 0.05$ compared with pcR3.1-transfected cells, ▲$p < 0.05$ compared with siCon-transfected cells.

Changes in Mxi1 expression in anti-Thy1 nephritis model rats

Based on real-time RT-PCR and Western blot analyses, the expression of Mxi1 was significantly downregulated during the proliferative period, reaching its lowest level on day 7. Subsequently, Mxi1 expression gradually increased as proliferation declined. On day 14, Mxi1 mRNA expression had returned to normal, although the Mxi1 protein level was lower than that in control rats, perhaps owing to a time lag between changes in mRNA expression and changes in protein expression (Fig. 3).

Effects of pcR3.1-Mxi1 and siMxi1 transfection on Mxi1 expression in mesangial cells

To test whether a Mxi1 expression plasmid (pcR3.1-Mxi1) and siRNA (siMxi1) could influence Mxi1 expression, we used real-time RT-PCR and Western blot

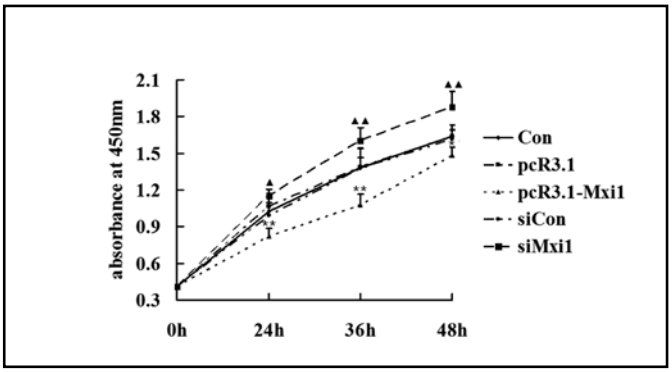


Fig. 5. Effects of pcR3.1-Mxi1 and siMxi1 transfection on mesangial cell proliferation. Cell proliferation was measured using a Cell Counting Kit-8 at 24, 36, and 48 h post-transfection. *$p < 0.05$ compared with pcR3.1-transfected cells, **$p < 0.01$ compared with pcR3.1-transfected cells, ▲$p < 0.05$ compared with siCon-transfected cells, ▲▲$p < 0.01$ compared with siCon-transfected cells.

Fig. 6. Effects of pcR3.1-Mxi1 and siMxi1 transfection on the cell cycle in mesangial cells. A. Cell cycle status were examined by flow cytometry. B. The percentage of mesangial cells in G0/G1, S, and G2/M phases were counted.*$p < 0.05$ compared with pcR3.1-transfected cells,▲$p < 0.05$ compared with siCon-transfected cells.

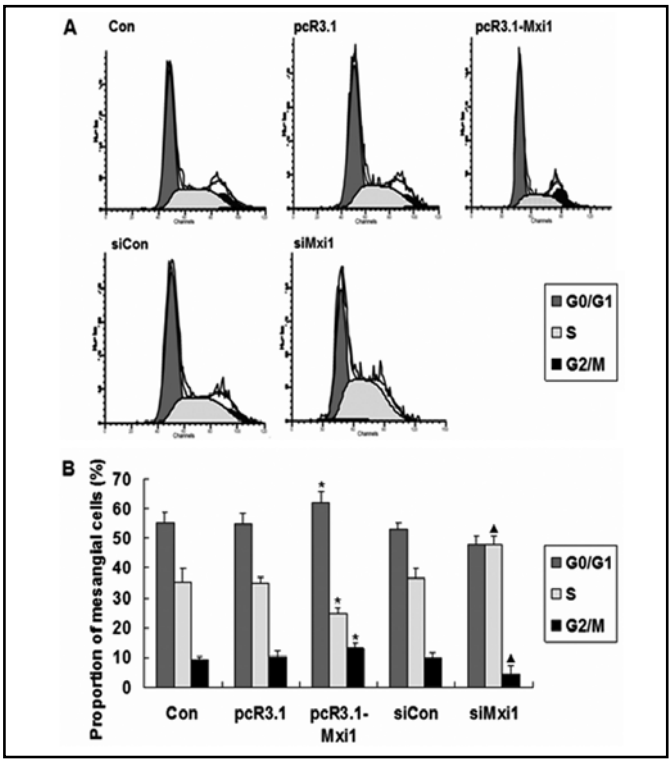
analyses to measure Mxi1 mRNA and protein levels, respectively, in mesangial cells after transfection. Compared with levels in control and pcR3.1-transfected cells, Mxi1 mRNA and protein levels were increased in pcR3.1-Mxi1-transfected mesangial cells ($p < 0.05$; Fig. 4). Conversely, mesangial cells transfected with siMxi1 displayed reduced Mxi1 mRNA and protein levels compared with the levels in control and siCon-transfected cells ($p < 0.05$; Fig. 4).

Effects of pcR3.1-Mxi1 and siMxi1 transfection on mesangial cell proliferation

After transfection of mesangial cells with pcR3.1-Mxi1 or siMxi1, cell proliferation was measured using a Cell Counting Kit-8. At 24, 36, and 48 h post-transfection, pcR3.1-Mxi1-transfected mesangial cells displayed a significantly lower proliferation rate compared with the rates of control and pcR3.1-transfected cells ($p < 0.01$ at 24 and 36 h; $p < 0.05$ at 48 h). Conversely, the cell proliferation rate was markedly increased in siMxi1-transfected cells compared with control and siCon-transfected cells ($p < 0.05$ at 24 h; $p < 0.01$ at 36 and 48 h). The proliferation rate at 72 h post-transfection did not differ significantly among the treatment groups (Fig. 5).

Effects of pcR3.1-Mxi1 and siMxi1 transfection on the cell cycle in mesangial cells

The percentage of mesangial cells in G0/G1, S, and G2/M phases were counted by flow cytometry. Compared with the cell cycle distribution of control and pcR3.1-transfected cells, a lower percentage of pcR3.1-Mxi1-transfected cells were in S phase and a higher percentage were in G2/M phase ($p < 0.05$ for each; Fig.6). Conversely, the percentage of siMxi1-transfected cells in S phase was higher and the percentage in G2/M phase was dramatically lower than the respective percentages of control and siCon-transfected cells ($p < 0.05$ for each; Fig. 6). BrdU is incorporated into the newly synthesized DNA of S-phase cells and may provide an estimate for cell proliferation. Double staining with propidium iodide and BrdU showed a lower percentage of cells had incorporated BrdU after pcR3.1-Mxi1 transfection as compared with control cells, on the contrary, more siMxi1-



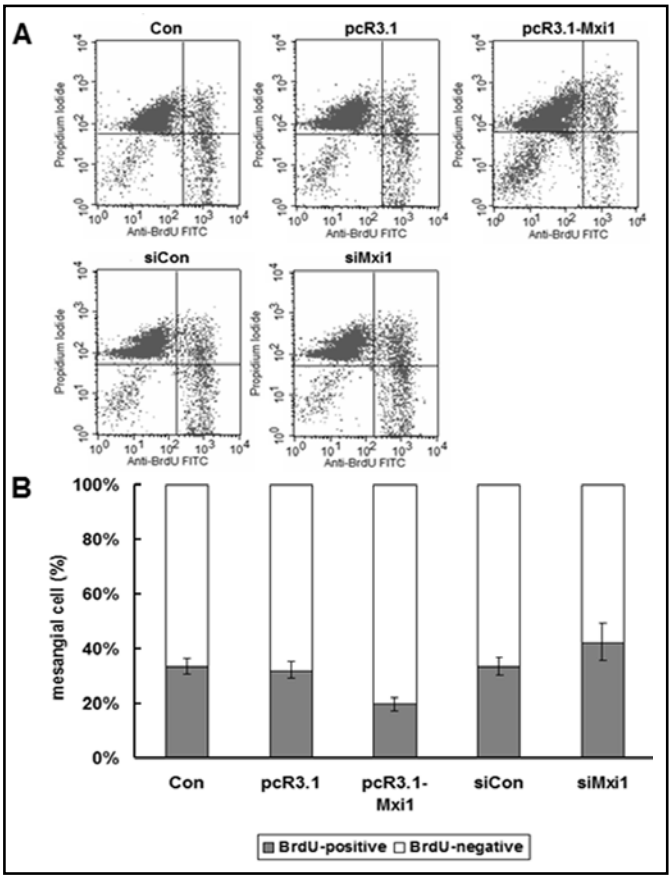


Fig. 7. Effects of pcR3.1-Mxi1 and siMxi1 transfection on the percentage of BrdU-positive cells. A. Double propidium iodide + BrdU stainings were performed by flow cytometry, the data were represented on dot plots. B.The percentage of BrdU-positive cells in each group.

Fig. 8. Effects of pcR3.1-Mxi1 and siMxi1 transfection on cell cycle regulatory protein expression. A. Cell cycle regulatory protein cyclin B1 (62kD), cyclin D1 (34kD), cyclin E (55kD), CDC2 (34kD) and CDK2 (34kD) expression was measured by Western blot analysis. B. The relative quantitation of cell cycle regulatory protein. β-actin was taken as an internal reference. *$p < 0.05$ compared with pcR3.1-transfected cells, ▲$p < 0.05$ compared with siCon-transfected cells.

transfected cells were BrdU-positive as compared with siCon-transfected cells ($p < 0.05$ for each; Fig. 7).

Effects of pcR3.1-Mxi1 and siMxi1 transfection on cell cycle regulatory protein expression

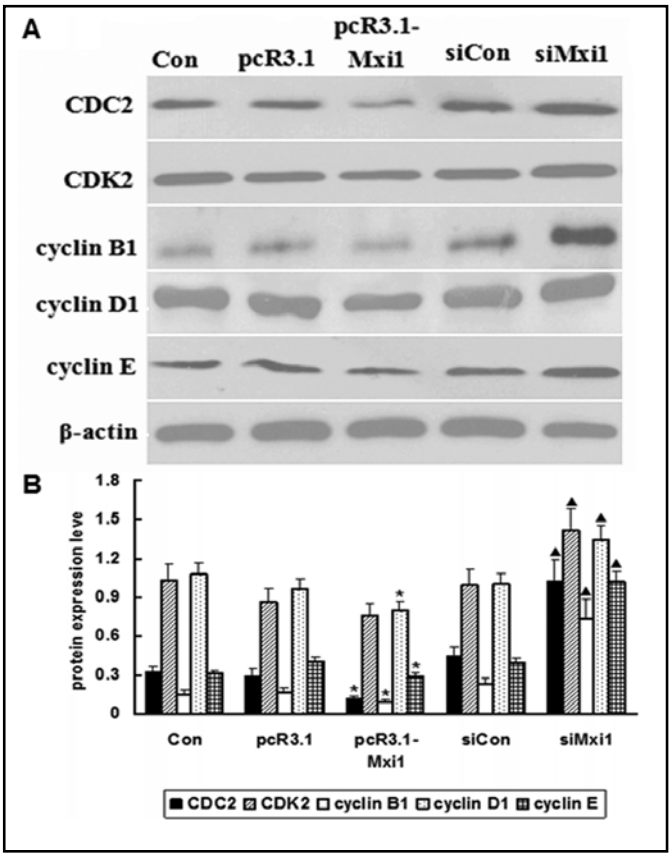
Cell cycle regulatory protein expression was measured by Western blot analysis. Compared with control and pcR3.1-transfected cells, pcR3.1-Mxi1-transfected cells displayed significantly reduced expression of cyclin B1,cyclin D1, cyclin E and CDC2 ($p < 0.05$ for each; Fig. 8). CDK2 expression tended to decline, but not to a significant extent. Compared with control and siCon-transfected cells, siMxi1-transfected cells showed significantly increased expression of cyclin B1, cyclin D1, cyclin E, CDC2 and CDK2 ($p < 0.05$ for each; Fig. 8).

Effects of pcR3.1-Mxi1 and siMxi1 transfection on c-myc expression

Mxi1 exhibited inhibition effect on proliferation mainly via the transcription factor Myc, so we tested whether pcR3.1-Mxi1 and siMxi1 transfection affected expression levels of c-myc. C-myc protein expression showed no obvious difference among all the groups (Fig. 9).

Changes in cell cycle regulatory protein expression in anti-Thy1 nephritis model rats

In order to further confirm our results *in vitro*, cell cycle regulatory protein expression at each time point of anti-Thy1 nephritis model was measured by Western blot analysis. The expressions of cyclin B1, cyclin D1, cyclin E, CDC2 and CDK2 were all upregulated in different degrees after anti-Thy1 antibody injection. By comparison with the proliferative period, their expression levels gradually reduced as proliferation declined (Fig. 10).



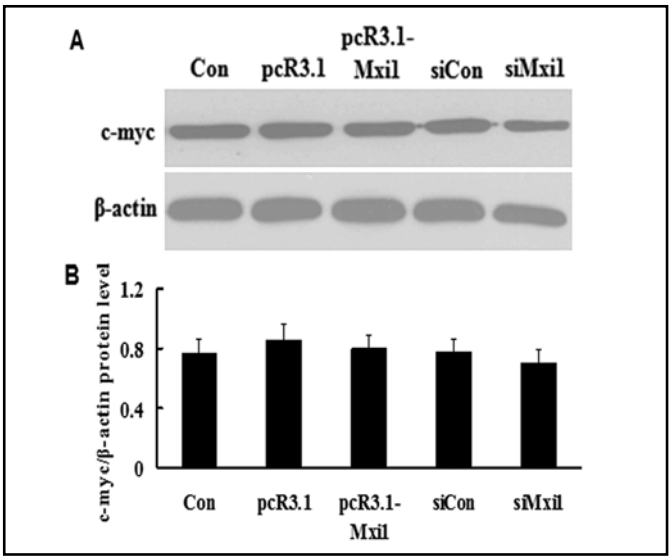


Fig. 9. Effects of pcR3.1-Mxi1 and siMxi1 transfection on c-myc expression. A.C-myc expression was measured by Western blot analysis, specific bands were at 67kD. B.The relative quantitation of c-myc protein were expressed as the ratio of c-myc/β-actin.

Discussion

In the present study, we first reported the expression of the transcriptional repressor Mxi1 was significantly downregulated during the proliferative period of MsPGN and subsequently increased as proliferation declined. Using a plasmid and siRNA to alter the expression level of Mxi1

in mesangial cells *in vitro*, we showed that Mxi1 inhibited mesangial cell proliferation by modulating the expression of cell cycle regulatory proteins. Therefore, we conclude that Mxi1 is an inhibitor of mesangial cell proliferation.

Mxi1 is a member of the Mad family of transcription factors. The human *MXI1* gene is localized to chromosome 10q24-q25, a region that contains mutations in kinds of tumors [11]. Mxi1 antagonizes the transcription factor Myc, which is overexpressed in a number of tumors and is considered to be a positive regulator of cell proliferation. Like Myc, Mxi1 contains a basic helix–loop–helix leucine zipper (bHLHZip) structural domain and can heterodimerize with MAX. Mxi1-MAX and Myc-MAX complexes recognize the same E-box sequences. Mxi1 competitively inhibits the Myc-MAX interaction, thereby antagonizing the proliferative effects of Myc [12, 13]. Mxi1 also contains an N-terminal Sin3-interacting domain, which recruits the general co-repressor protein Sin3 and an associated transcriptional repression complex containing histone deacetylases to the transcription regulatory regions of target genes. As a result, the core histones are deacetylated and the transcription of target genes is suppressed [14]. Some investigators have shown that Mxi1 inhibits the proliferation of DU145 human prostate cancer cells and U87 glioma cells [11, 15]. Mutations and deletions in the Mxi1 coding sequence have been described in patients with prostate cancer, neurofibrosarcoma, and neuroglioma [16, 17]. In addition, Mxi1-knockout mice exhibit a tumorigenic phenotype. On the basis of these findings, Mxi1 is considered to be a tumor suppressor. However, no studies have investigated its effect on mesangial cell proliferation.

The anti-Thy1 nephritis rat is a classic mesangial proliferative glomerulonephritis model. In our research, its typical pathological manifestations included significant mesangial proliferation at days 5 to 7 post-anti-Thy1 antibody injection which were confirmed by immunohistochemical assessment of Ki67. Thereafter, the lesions regressed spontaneously. Mxi1 expression was significantly reduced during the proliferative period in anti-Thy1 nephritis model rats, and it subsequently rose gradually as proliferation declined. Therefore Mxi1 expression levels were inversely correlated with proliferation.This expression pattern was similar to those of known inhibitors of mesangial proliferation such as secreted protein acidic and rich in cysteine (SPARC) [18], suggesting that Mxi1 may inhibit mesangial proliferation. Under normal conditions, mesangial cells express large amounts of Mxi1 and do not proliferate, as proliferation is inhibited. However, decreased Mxi1 expression in anti-Thy1 nephritis model rats at days 5 and 7 post-anti-Thy1 antibody injection allowed mesangial cells to proliferate. The subsequent return of Mxi1 expression to its normally high level reversed the increase in mesangial cell proliferation.

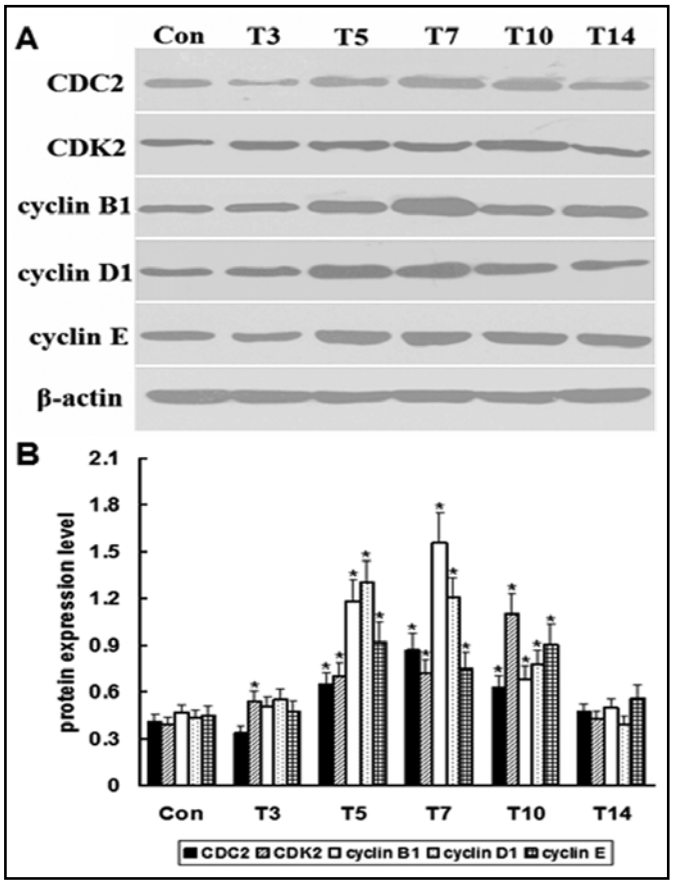


Fig. 10. Changes in cell cycle regulatory protein expression in anti-Thy1 nephritis model rats. A. Cell cycle regulatory protein expression at each time point was assessed by Western blot analysis. B. The relative quantitation of cell cycle regulatory protein. β-actin was taken as an internal reference. Con: control rats; T3, T5, T7, T10, T14: anti-Thy1 nephritis rats at day 3, 5, 7, 10, 14 separately. *$p < 0.05$ compared with control rats.

To further clarify the inhibitory effects of Mxi1 on mesangial proliferation, we generated the plasmid pcR3.1-Mxi1 and the small interfering RNA siMxi1, and tested their effects on rat mesangial cell proliferation *in vitro*. A cell proliferation assay and FACS analysis showed that overexpression of Mxi1 markedly reduced cell proliferation, the proportion of BrdU-positive cells and the percentage of cells in S phase, and increased the percentage of cells in G2/M phase. Thus, Mxi1 inhibits mesangial cell proliferation, in accordance with the findings of Manni et al., who reported that an artificial

increase in Mxi1 expression reduced glioblastoma cell proliferation [15]. In contrast, no previous studies have used siRNA to examine the effects of a reduction in Mxi1 on cell proliferation or cell cycle progression. In the present study, cells transfected with siMxi1 displayed markedly increased cell proliferation, an increased percentage of BrdU-positive cells and S phase cells, and a decreased percentage of G2/M phase cells, further confirming Mxi1 as an inhibitor of mesangial proliferation.

Completion of the cell cycle under the precise control of cell cycle regulatory proteins is the basis of cell proliferation [19]. The expression of cell cycle regulatory proteins is altered in anti-Thy1 nephritis model animals [20], and proliferation-related factors such as PDGF and IGF-1 regulate mesangial cell cycle progression by interfering with the expression of cell cycle regulatory proteins [4, 21]. We therefore assessed the effects of pcR3.1-Mxi1 and siMxi1 transfection on cell cycle regulatory protein expression. Cyclin B1, the master regulator of G2/M progression, can combine with CDC2 to form cyclin B1-CDC2 complex which is called mitosis promoting factor.This complex can promote the cells to pass through G2/M phase. Overexpression of Mxi1 has been shown to inhibit the expression of cyclin B1 and to thereby induce G2/M block and suppress glioblastoma cell growth [15]. CDC2-L1 has been identified to be a c-myc target gene in primary human umbilical vein endothelial cell [22]. In the present study, Mxi1 overexpression dramatically reduced the expression level of cyclin B1 and CDC2, and the inhibition of Mxi1 expression increased the expression level of them. These results explained the effects of pcR3.1-Mxi1 and siMxi1 transfection on the percentage of G2/M phase cells in cell cycle analysis. Cyclin E, a key regulator of G1 phase and a promoter of cell proliferation, is a c-myc target gene as well. It combines with CDK2 to form a complex which can accelerate G1 to S phase transition. Cyclin D1 is another key regulator of the G1/S transition. Researchers employing the MycER (Myc and estrogen receptor fusion gene)-inducible system in a non-transformed Rat-1 cell line showed that MycER was sufficient to induce cyclin D1 mRNA and protein expression [23]. In the present study, pcR3.1-Mxi1 transfection and Mxi1 overexpression reduced cyclin E, CDK2 and cyclin D1 expression, siMxi1 transfection significantly increased the above three protein levels. Mxi1 negatively regulated cyclin E, CDK2 and cyclin D1 expression perhaps by inhibiting Myc-stimulated transcription, reduced the percentage of cells entering S phase and inhibited cell proliferation.

The biological effects of Mxi are mediated through regulation of the transcription factor Myc. Since change in c-myc expression level would affect expression of c-myc target genes, we tested c-myc protein expression by Western blot analysis and found c-myc expression showed no obvious difference after pcR3.1-Mxi1 and siMxi1 transfection. So in this research, Mxi1 didn't change c-myc expression level in mesangial cells, perhaps it just competitively inhibited the Myc-MAX complex formation and thereby antagonized the transcriptioal activation function of c-myc, reduced the expression of c-myc target genes. All the results suggest that Mxi1 inhibits mesangial cell proliferation by regulating the expression of c-myc target positive-acting cell cycle regulatory proteins.

Finally, in order to support our *in vitro* data, cell cycle regulatory protein expression in anti-Thy1 nephritis model rats was measured by Western blot analysis. The expressions of cyclin B1, cyclin D1, cyclin E, CDC2 and CDK2 were all upregulated at different levels during the proliferative period, and then their expression levels reduced as proliferation declined. There was a negative correlation between their expression levels and Mxi1 expression that was similar with the results we found *in vitro*.

In conclusion, our study demonstrated that Mxi1 expression decreases significantly during the proliferative period in anti-Thy1 nephritis model rats and then gradually rises as proliferation declines, thereby suggesting a possible role for Mxi1 in mesangial proliferation. Furthermore, Mxi1 downregulates the expression of c-myc target positive-acting cell cycle regulatory proteins cyclin B1, cyclin D1, cyclin E, CDC2 and CDK2, through either direct transcriptional repression or antagonistic effects on Myc. This causes a G2/M block and a reduced percentage of cells entering S phase, with the ultimate result of reduced mesangial cell proliferation.

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