Kruppel-like Factor-15 Inhibits the Proliferation of Mesangial Cells

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Key Words

Kruppel-like factor-15 • Mesangial cell proliferation • Cell cycle regulatory proteins

Abstract

Background/Aims: The Kruppel-like factor-15 (KLF15), a DNA-binding transcription factor, is highly expressed in endothelial and mesangial cells of the kidney. However, its effects on mesangial cell proliferation have not previously been investigated. In this study, we investigated the effect of KLF15 on mesangial cell proliferation. **Methods:** We established a classic rat anti-Thy1 mesangial proliferative nephritis model. Affymetrix rat U230 2.0 chip was used to detect the gene expression profiles at different time point in the model. The different expression of KLF15 was shown during mesangial cell proliferation period and proliferation declined period of anti-Thy1 nephritis model by microarray analysis, Real-time PCR and Western blotting. Then we determined the effects of KLF15 and its downstream target, cell cycle regulation factor E2F1 on the proliferation of mesangial cells and the expression of the positive-acting cell cycle regulatory proteins, cyclinD1 and CDK2, by means of positive and negative interference experiments in cultured rat mesangial cells. We detected also protein

expression of E2F1, cyclinD1 and CDK2 *in vivo*. **Results:** By real-time PCR, Western blotting, and microarray analysis, KLF15 expression was shown to be lower during mesangial cell proliferation period and higher during proliferation declined period and under normal conditions. The mesangial cell proliferation was reduced and the expression of E2F1, cyclin D1 and CDK2 was downregulated in mesangial cells overexpressing KLF15. When KLF15 expression was inhibited by siRNA, the expression of E2F1, cyclin D1 and CDK2 and mesangial cell proliferation were increased. When E2F1 was inhibited by siRNA, protein level of CDK2 and cyclin D1 were lower than control. When siE2F1 was co-transfected with siKLF15 into mesangial cells, the increase of cell proliferation induced by siKLF15 was eliminated partly by siE2F1. Moreover, E2F1, cyclin D1 and CDK2 were higher expression during mesangial cell proliferation period, and were downregulated during proliferation declined period *in vivo*. **Conclusions:** These results suggest that KLF15 inhibits mesangial cell proliferation, possibly by regulating the expression of cell cycle regulation proteins through E2F1. Thus, KLF15 may be a useful target for therapeutic intervention in mesangial proliferative glomerulonephritis.

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Introduction

Mesangial proliferative glomerulonephritis (MesPGN) is a common kidney disease whose main pathological changes are mesangial cell proliferation and extracellular matrix accumulation, which ultimately lead to glomerular sclerosis and loss of renal function [1]. The rat anti-Thy-1 model, a classic animal model with mesangial cell proliferated at day 5/7 and gradually recovered after day 10/14, was used to study the pathogenesis and treatment of MesPGN [2-4].

Previous studies have suggested that changes in the expression levels of many proliferation-related factors such as transcription factors and cell cycle regulation factors [5] promote or inhibit mitosis and proliferation in mesangial cells [6, 7]. A shift in the balance between proliferation-promoting and proliferation-inhibiting factors influences the rate of cell proliferation. The ability to restore the balance among proliferation-related factors may suggest a new therapeutic approach for MesPGN. Earlier studies addressing this issue have focused mainly on blocking the high expression of proliferation-promoting factors [8], rather than promoting the expression of proliferation-inhibiting factors.

In this study, we took a different approach and used microarray analysis to identify part of genes as being downregulated during mesangial cell proliferation period, and more highly expressed during proliferation declined period and under normal circumstances. We considered these genes might be related with the proliferation of mesangial cells as there was a negative correlation between the expression trends of these genes and the pathological features of the model. Among of which, reports have demonstrated that the DNA-binding transcription factor Kruppel-like factor 15 (KLF15) is highly expressed in endothelial and mesangial cells of the kidney and can bind to the GC-rich region of the promoter for cell cycle regulation factor E2F1 to regulate cell proliferation and differentiation [9-11]. Based on the above, we hypothesis KLF15 might regulated the proliferation of mesangial cell, and performed positive and negative interference experiments *in vitro* to demonstrated that KLF15 may inhibit mesangial cell proliferation by downregulating the expression of cell cycle regulation proteins.

Materials and Methods

Anti-Thy1 nephritis animal model

An anti-Thy1 nephritis model was set up as described previously [2]. Male Wistar rats (Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing, China), weighing between 200 and 220 g, were randomly allocated to control and anti-Thy1 groups. Anti-Thy1 nephritis was induced by a single intravenous injection of a monoclonal anti-Thy1 antibody (2.5 mg/kg) produced by OX-7 cells. Controls were injected with the same volume of normal saline. Anti-Thy1-treated animals were sacrificed at days 5, 7, 10, and 14 post-injection (three rats per time point), and the control animals were sacrificed at day 0 (n = 3). The kidney tissues were used to PAS staining, immunohistochemical staining, microarray analysis, qRT-PCR and western blotting detection. All rats were provided a diet of standard laboratory chow and free access to water.

Immunohistochemical Analysis and Evaluation

The renal tissues were fixed in formalin and embedded with paraffin. The histological paraffin sections were cut to 3 μ m thickness and mounted on poly-L-lysine-coated slides and stained with periodic acid Schiff (PAS). Endogenous peroxidase was blocked with 3% hydrogen peroxide. The sections were heated in a microwave oven for 10 min in sodium citrate buffer (pH 6.0), incubated with 1.5% normal goat serum for 20 min, followed by incubation overnight with 1:100 diluted primary antibody (Ki67). For negative control, the sections were incubated with PBS. After removal of unbound primary antibody, the sections, including negative control, were incubated with a biotinylated secondary antibody for 60 min at room temperature. The sections were rinsed and incubated with avidin-biotinylated horseradish peroxidase (Vectastain Elite ABC kit, Vector Laboratories, USA) for 60 min. Incubation with 3, 3'-diaminobenzidine tetra hydrochloride was performed for 10 min as a substrate chromogen solution to produce a brown color. Finally, the sections were counterstained with hematoxylin. Ten glomeruli per section at each time point were evaluated under high power light microscopy (x400) in a blinded fashion. Ki67 labeling index was calculated by Ki67 positive cells to total glomerular cells.

Microarray analysis

Microarray analysis of the normal rat kidney tissue and the tissue of anti-thy1 model at day 5,7,10 and 14 was performed by CapitalBio Corp. (Beijing, China) using Affymetrix rat U230 2.0 chips. Data normalization and analysis were performed as previously [12]. GoMiner software was used to analyze gene function.

Cell culture

Rat mesangial cells (RMCs) were obtained from the American Type Culture Collection (ATCC) of the United States. Cells were grown in RMPI 1640 medium containing 10% fetal calf serum at 37°C in a humidified incubator with an atmosphere containing 5% CO₂. Trypsin (0.25%) was used for cell passage.

Name	Sequence
siRNA1(siKLF5)	Sense: 5'-GCCUUCUGUUCCUGCUACAAtt-3' Antisense: 5'-CGGAAGACAAGGACGACGAUGUtt--3'
siRNA2	Sense: 5'-GAGAACAUGGAGGCUGAGGtt -3' Antisense: 5'-CCUCAGCCUCCAUGUUCUCtt-3'
siRNA3	Sense: 5'-UUGCGCCUGUGCCCAUUGCtt -3' Antisense: 5'-GCAAUGGGGCACAGGCGCAAtt-3'
Control(siCon)	Sense: 5'-UUCUCCGAACGUGUCACGUtt-3' Antisense: 5'-ACGUGACACGUUCGGAGAATTtt-3'

Table 1. siRNA sequences

Quantitative real-time PCR (qRT-PCR)

RNA was extracted from the tissues and cells using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA) and was reverse transcribed to cDNA using M-MLV reverse transcriptase (2 µg) (Invitrogen). The cDNA was used as a template in quantitative real-time PCR reactions performed using TaqMan PCR Master Mix and an ICycler system (Bio-Rad, Hercules, CA, USA). The following primers and probe were designed from the full-length KLF15 mRNA sequence (GenBank Accession no. NM_053536) and synthesized by SBS Biotechnology Corp. (Beijing, China): sense, 5'-CAT GAG TTG TCA CGG CAC C-3'; antisense, 5'-CAC TGC GCT CAG TTG ATG G-3'; and TaqMan probe, 5'-FAM-CCA GTG TCC CGT ATG CGA GAA GAA-TAMRA-3' (product size, 152 bp). As an internal control, rat GAPDH was amplified using the following: sense, 5'-GGC ATG GAC TGT GGT CAT GAG-3'; antisense, 5'-TGC ACC ACC AAC TGC TTA GC-3'; and TaqMan probe, FAM-5'-CCT GGC CAA GGT CAT CCA TGA CAA CTT-TAMRA-3' (product size, 87 bp). Relative expression (fold-change vs. control) was quantified by the $2^{-\Delta\Delta Ct}$ method.

Western blot analysis

Proteins were extracted from the cells and tissues using RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% deoxycholate, 1% Nonidet P-40, 0.1% SDS, 1 mM PMSF, and protease cocktail at 1 µg/ml). Protein concentrations were determined using a BCA kit. Protein samples (80 µg per lane) were separated by 10% SDS-PAGE electrophoresis and transferred to PVDF membranes. Then the membranes were incubated overnight in 5% non-fat milk at 4°C, followed by incubation with primary antibody against KLF15, CDK2, cyclin D1, E2F1 or β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were visualized using ECL reagent (Santa Cruz Biotechnology), according to the manufacturer's instructions, and exposure to X-ray film. Protein band intensities were quantified using Quantity One software (Bio-Rad).

siRNA transfection

Specific siRNAs were designed to target to rat KLF15 (Genbank No.NM_053536.1) by the online siRNA finder tools (http://www.ambion.com/techlib/misc/siRNA_finder.html),

target sequences and control were shown in Table 1. These were synthesized by Shanghai GenePharma Co.,Ltd (Shanghai, China). Synthesized siRNA was suspended in DEPC treated water (100 µM) and stored at -20°C until use, the final concentration was 20 nM each. E2F-1 siRNA was purchased from Santa Cruz Biotechnology (sc-61861). A TransPass R2 kit (New England Biolabs, Inc., Ipswich, MA, USA) was used to transfect RMCs with the siRNAs.

KLF15 plasmid construction and transfection

For the expression plasmid carrying full-length KLF15, a PCR product (1,463 bp) was generated using a pair of specific primers (sense, 5'-AGA GAC GTT GTG CTG CTT TCC TG-3' and antisense, 5'-CAC GGC GGC TCT GGG TCA TA-3') and was cloned into pcDNA3.1(+) by *KpnI* / *Xho I*, producing pcDNA-KLF15. After confirmatory DNA sequencing, it was used to transfect RMCs by electroporation (Gene Pulser Xcell electroporation system; Bio-Rad).

MTT cell proliferation assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, first described by Mosmann in 1983 [13, 14] was used to detected the cell proliferation activity. Rat mesangial cells were seeded in 96-well plates (10,000 cells/well) and transfected with either siRNA or plasmid. After incubation at 37°C for 24 or 48 h, the culture medium in each well was discarded and replaced with 20 µl of MTT (final concentration, 5 mg/ml), followed by incubation at 37°C for 4 h. The supernatants were discarded, 200 µl of dimethyl sulfoxide were added to each well, and the plate was allowed to sit at room temperature for 15 min. The optical density of each sample was measured at a wavelength of 490 nm using a spectrophotometer (Bio-Rad).

FACS analysis of the cell cycle distribution

Cells were harvested using 0.25% trypsin, washed with PBS, and incubated overnight at 4°C in 75% ethanol (pre-cooled to 4°C). After being washed with PBS and treated with RNase (50 µg/ml), the cells were incubated with propidium iodide (PI, 50 µg/ml) at room temperature in the dark for 30 min. Fluorescence-activated cell sorting (FACS) was used to measure the DNA content of the cells, and the data were

Fig.1. Pathological changes of anti-Thy1 model were analyzed by using of PAS or Ki67 staining. At day 5 and 7, mesangial cell number was significantly increased when compared with day 0, however it was declined gradually at day 10 and day 14. At day 14, it was nearly to day 0. * $p < 0.05$, v.s. day 0; # $p < 0.05$, v.s. day 5 and day 7. Magnification, x400.

analyzed using CellFIT Cell Cycle Analysis software Version 2.01.2 (BD Biosciences, Franklin Lakes, NJ, USA). The percentage of cells in each cell cycle phase was calculated.

Statistical analysis

Data are expressed as means $\pm$ SD. Statistical analyses were performed using SPSS version 16.0. Statistically significant differences were identified using one-way analysis of variance. Values of $P < 0.05$ were taken to indicate statistical significance.

Results

Pathological changes in anti-Thy1 nephritis model rats

In order to detect the pathological change of anti-Thy1 nephritis model, we performed PAS stain and Ki67 immunochemical stain. The results indicated day 5 and day 7 after anti-Thy1 antibody injection were the mesangial cell proliferation period, the glomerular cell number was outstanding increased. From day 10, mesangial proliferation gradually reduced. Day 10 and day 14 were considered as the proliferation declined period (Fig. 1). The expression of Ki67, a proliferation marker, was low in normal rats. Then it was highly expressed at day 5 and day 7 after anti-Thy1 antibody injection. From day 14, its expression declined to normal (Fig. 1).

Identification of KLF15 in a microarray screen

Kidney cortex RNA samples taken from normal and MesPGN model animals at different time points were subjected to microarray analysis. As we know, the genes which expression was changed during the proliferative period or proliferation declined period of anti-Thy1 nephritis model might be related with mesangial proliferation. So we selected the genes which expression was low during mesangial cell proliferation period and increased during proliferation declined period for further research, especially the genes associated with cell proliferation. In this way, we identified 389 genes (<http://www.sendspace.com/file/ujqyx6>), including KLF15, which were highly expressed under normal conditions, downregulated at days 5 and 7 (the mesangial cell proliferation period) after anti-Thy1 antibody injection, and subsequently upregulated at days 10 and 14 (the proliferation declined period). GO analysis indicated that there about 30 genes were related with cell proliferation including KLF15, TIMP-1, USP, S100A4 and so on. It has been reported that KLF family proteins regulate proliferation in various cell types [15-17], and KLF15 is highly expressed in the kidney [18]. Therefore, we validated expression of KLF15 mRNA and protein *in vivo* by qRT-PCR and Western blotting and evaluated the function of KLF15 *in vitro*.

Fig. 2. Verification of KLF15 mRNA and protein expression in anti-Thy1 nephritis model rats. A. On days 5, 7 and 10 post-injection of anti-Thy1, kidney expression of KLF15 mRNA was lower in anti-Thy1-injected rats than in controls ($*p < 0.05$, vs. Normal group, 0d, $n = 3$), and KLF15 mRNA expression was significantly higher on day 14 than on day 5, 7 and 10 in anti-Thy1-injected rats ($\dagger p < 0.05$, v.s. 5d, 7d, 10d, $n = 3$). The tendency of mRNA expression was in accordance with the results of gene chip, pearson correlation coefficient was above 0.90. B. On days 5, 7 and 10 post-injection of anti-Thy1, kidney expression of KLF15 protein was lower in anti-Thy1-injected rats than in controls ($*p < 0.05$, vs. Normal group, 0d, $n = 3$), and KLF15 protein expression was significantly higher on day 14 than on day 5, 7 and 10 in anti-Thy1-injected rats ($\dagger p < 0.05$, v.s. 5d, 7d, 10d, $n = 3$). M; DNA molecular marker; N: Normal group.

KLF15 mRNA and protein expression in anti-Thy1 model rats

The expression of KLF15 mRNA and protein in anti-Thy1 model rats was analyzed by qRT-PCR and Western blotting, in order to verify the results of microarray. KLF15 mRNA expression levels were comparable to those determined in the microarray analyses (Fig. 2A). KLF15 mRNA expression was downregulated at days 5 and 7, and was significantly higher at day 14, although still lower than at day 0. KLF15 protein expression was similarly downregulated at days 5 and 7, and upregulated at day 14 (Fig. 2B).

Inhibition of KLF15 mRNA and protein expression by siRNA

To test the ability of the siRNA to inhibit KLF15 expression, we analyzed expression levels of KLF15 mRNA and protein in RMCs following their transfection with siRNA. KLF15 siRNA (siKLF15) reduced KLF15 mRNA expression by approximately 50%, to a level significantly lower than that in cells transfected with a

non-specific siRNA (siCon) and in non-transfected cells (Fig. 3A), and we select siRNA1 and siRNA2 as followed experiment. Based on Western blot analysis, siKLF15 reduced KLF15 protein expression, seen as a specific band at 44 kDa, by approximately 50%, to a level significantly lower than those in siCon-transfected and non-transfected cells (Fig. 3B).

Inhibition of KLF15 expression increases mesangial cell proliferation and cell proliferation index

The MTT assay was used to assess mesangial cell proliferation after transfection with siKLF15. There were no significant differences in cell proliferation among the groups at 24 h after transfection. However, at 48 h after transfection, cell proliferation was significantly greater in siKLF15-transfected cells than in siCon-transfected and non-transfected cells (Fig. 3C). These results suggest that cell proliferation can be promoted by short-term inhibition of KLF15 expression. In subsequent experiments, we used 48 h as the time point for assessment of the cells.

Fig. 3. Effect of KLF15 down-expression on mesangial cell proliferation and cell cycle regulatory proteins. A. Cells were transfected with siRNAs, 48h later, total RNA was extracted with Trizol. RT-PCR products were analyzed by electrophoresis, and a 152-bp product of KLF15 amplification was detected. KLF15 mRNA expression was lower in KLF15 siRNAs (siRNA1,2,3)-transfected cells than in siCon-transfected and non-transfected control cells ($*p < 0.05$ vs. Con; $\dagger p < 0.05$, vs. siCon, $n = 3$). B. Cells were transfected with siRNAs, 48h later, protein was extracted with RIPA lysis buffer. KLF15 protein expression was assessed by Western blotting, and a specific 44-kDa band was detected. KLF15 protein expression was lower in siRNAs (siRNA1,2,3)-transfected cells than in siCon-transfected and non-transfected control cells ($*p < 0.05$ vs. Con; $\dagger p < 0.05$, vs. siCon, $n = 3$). siRNA1 was shown to take much more effected on inhibiting KLF15 protein expression level as well. C. Cells transfected with siRNA1, siRNA2 or siCon were analyzed by MTT assay at 24 h and 48 h. At 48 h, the optical density at 490 nm was higher, indicating higher proliferative activity, in siRNA1 or siRNA2-transfected cells than in siCon-transfected and non-transfected control cells ($*p < 0.05$ vs. Con; $\dagger p < 0.05$, vs. siCon, $n = 3$). D. Flow cytometric analysis of the cell cycle distribution showed fewer siRNA1 or siRNA2-transfected cells were in G0/G1 phase, compared with siCon-transfected and non-transfected control cells ($p < 0.05$, $n = 3$). The proliferation index (S + G₂M) of siKLF15-transfected cells was higher than those of siCon-transfected and non-transfected control cells at 48 h after transfection ($p < 0.05$, $n = 3$). E. Protein expression of the cell cycle regulatory proteins CDK2 and cyclin D1 were assessed by western blotting, they were upregulated in mesangial cells at 48 h after transfection with KLF15 siRNAs (siRNA1 and siRNA2) ($*p < 0.05$ vs. Con, $\dagger p < 0.05$ vs. siCon, $n = 3$). M; DNA molecular marker.

Fig. 4. Effect of KLF15 over-expression on mesangial cell proliferation and cell cycle regulatory proteins. A. KLF15 mRNA expression was increased in mesangial cells at 48 h after transfection with pcDNA-KLF15 ($*p < 0.05$ vs. Con; $†p < 0.05$ vs. pcDNA, $n = 3$). There was no significant difference in KLF15 mRNA expression between pcDNA-transfected and non-transfected control cells. B. Expression of KLF15 protein, detected as a specific 44-kDa band on Western blotting, was increased in mesangial cells at 48 h after transfection with pcDNA-KLF15 ($*p < 0.05$ vs. Con; $†p < 0.05$ vs. pcDNA, $n = 3$). C. At 48h post-transfection, the proliferative activity of pcDNA-KLF15-transfected cells was assessed by MTT assay, it was lower than that of pcDNA-transfected and non-transfected control cells ($*p < 0.05$ vs. Con; $†p < 0.05$ vs. pcDNA, $n = 3$). D. Flow cytometric analysis of the cell cycle distribution showed a greater proportion of pcDNA-KLF15-transfected cells were in G0/G1 phase, compared with pcDNA-transfected and non-transfected control cells ($p < 0.05$, $n = 3$). The proliferation index (S + G₂M) of pcDNA-KLF15-transfected cells was lower than those of pcDNA-transfected and non-transfected control cells ($p < 0.05$, $n = 3$). E. Protein expression of the cell cycle regulatory proteins CDK2 and cyclin D1 was assessed by Western blotting, they were downregulated in mesangial cells at 48 h after transfection with pcDNA-KLF15 ($*p < 0.05$ vs. Con, $†p < 0.05$ vs. pcDNA, $n = 3$). M; DNA molecular marker.

Flow cytometry was used to analyze the cell cycle distribution of mesangial cells. Compared with siCon-transfected and non-transfected cells, the

siKLF15-transfected cells had a lower proportion of cells in G0/G1 phase. The cell proliferation index of siKLF15-transfected cells was increased by 55% and

siRNA	G0-G1 phase (%)	S phase (%)	G2-M phase (%)	S + G2-M phase (%)
Control	51.18±1.26	36.46±1.47	12.36±0.92	48.82±3.18
siCon	52.36±3.15	36.07±2.82	11.57±1.65	47.64±2.32
siRNA1(siKLF5)	44.40±2.39*†	42.31±3.20*†	13.29±1.19	55.60±3.19*†
siRNA2(siKLF5)	45.39±2.65	41.83±3.73*†	12.78±1.25	54.61±3.48*†

Table 2. Proportion of siRNA-transfected mesangial cells in each cell cycle phase. * $p < 0.05$ vs. Control, † $p < 0.05$ vs. siControl, $n = 3$.

Plasmid	G0-G1 phase (%)	S phase (%)	G2-M phase (%)	S + G2-M phase (%)
Control	50.41±1.45	37.94±1.46	11.64±1.11	49.58±1.45
pcDNA control	50.94±1.75	31.65±1.03	17.41±1.11	49.06±1.75
pcDNA-KLF15	60.23±5.63*†	21.67±6.50*†	18.11±3.53	39.78±5.63*†

Table 3. Proportion of plasmid-transfected mesangial cells in each cell cycle phase. * $p < 0.05$ vs. Control, † $p < 0.05$ vs. pcDNA control, $n = 3$.

was significantly higher than those in the other two groups (Fig. 3D, Table 2).

Inhibition of KLF15 expression increases expression of cell cycle regulatory proteins

The expression levels of the cell cycle regulatory proteins CDK2 and cyclin D1 in mesangial cells at 48 h post-transfection were significantly higher in siKLF15-transfected cells than in siCon-transfected and non-transfected cells (Fig. 3E). Thus, effect of KLF15 on mesangial cell proliferation may be mediated by a reduction in CDK2 and cyclin D1 expression.

Overexpression of KLF15 decreases mesangial cell proliferation and cell proliferation index

The expression levels of KLF15 mRNA and protein were significantly higher in pcDNA-KLF15-transfected cells than in pcDNA-transfected and non-transfected cells at 48 h post-transfection (Fig. 4A, B). KLF15 expression did not differ significantly between non-transfected and pcDNA-transfected cells.

According to the MTT assay results, the rate of proliferation 48 h post-transfection was lower in pcDNA-KLF15-transfected cells than in non-transfected and pcDNA-transfected cells ($p < 0.05$) (Fig. 4C). This suggests that KLF15 overexpression may contribute to a reduction in mesangial cell proliferation. Both the

proportion of cells in S phase and the proliferation index (S + G2-M) were significantly lower in pcDNA-KLF15-transfected cells than in pcDNA-transfected cells ($p < 0.05$) (Fig. 4D, Table 3).

Overexpression of KLF15 decreases expression of cell cycle regulatory proteins

The protein levels of CDK2, cyclin D1, two cell cycle regulatory proteins, were significantly lower in pcDNA-KLF15-transfected mesangial cells compared with pcDNA-transfected cells ($p < 0.05$) (Fig. 4E). The decreased expression of CDK2 and cyclin D1 may be a factor in the inhibition of proliferation in KLF15-overexpressing mesangial cells.

Expression changes of cell cycle regulatory proteins when RMCs transfected with E2F1 siRNA

Wade et al. report that KLF15 can bind to the promoter of E2F1 [11]. In order to verify whether KLF15 could affect cell cycle regulatory proteins via E2F1, siRNAs used to inhibit the expression of E2F1 and KLF15. The results indicated when E2F1 was inhibited by siRNA, expression level of CDK2 and cyclin D1 protein were lower than control. Expression level of two cell cycle regulatory proteins in the cells transfected with E2F1 and KLF15 siRNAs together were lower than KLF15 siRNA transfected group, however higher than E2F1 siRNA transfected group ($p < 0.05$) (Fig. 5A). When

Fig. 5. Expression changes of cell cycle regulatory proteins in anti-Thy1 nephritis model by Western blot. A. Cells were transfected with siRNAs (siKLF15 and/or siE2F1), western blot detected the protein expression level. CDK2 and CyclinD1 expression were significantly decreased in siE2F1 transfected group, and increased in siKLF15 transfected group. However, two protein expression level in the cells transfected with siKLF15 and siE2F1 was significantly downregulated when compared with siKLF15 transfected group. * $p < 0.05$, v.s. control; # $p < 0.05$, v.s. siKLF15 group, $n = 3$. B. Cells were transfected with siRNAs (siKLF15 or siE2F1), western blot detected the protein expression level. The expression level of E2F1 in siE2F1 transfected group was downregulated, and KLF15 did not change. However, when cell was transfected with siKLF15 or pcDNA-KLF15, the expression level of E2F1 was upregulated and downregulated respectively, *, #, † $p < 0.05$, v.s. control, $n = 3$; C. Western blot detected the protein expression level. At day 5 and 7, E2F1, CDK2 and CyclinD1 expression were significantly increased when compared with day 0, however their expressions were declined gradually at day 10 and day 14. At day 14, they were nearly to day 0. * $p < 0.05$, v.s. day 0; # $p < 0.05$, v.s. day 5 and day 7, $n = 3$.

siE2F1 was co-transfected with siKLF15 into mesangial cells, the increase of cell proliferation induced by siKLF15

was eliminated partly by siE2F1. At the same time, after cell was transfected with siE2F1, the expression level of

KLF15 did not change. However, when cell was transfected with siKLF15 or pcDNA-KLF15, the expression level of E2F1 was upregulated about 3 folds and downregulated respectively ($p < 0.05$, Fig.5B).

Expression changes of cell cycle regulatory proteins in anti-Thy1 nephritis model

In order to further confirm our results *in vitro*, positive cell cycle regulatory protein expression at each time point of anti-Thy1 nephritis model was measured by Western blot analysis. The expressions of E2F1, CDK2 and cyclinD1 were all upregulated in different degrees in mesangial cell proliferation period. By comparison with the proliferative period, their expression levels gradually reduced as proliferation declined period (days 10 and 14) (Fig. 5C).

Discussion

KLF15 is a member of the Kruppel-like factor (KLF) family of DNA-binding transcription regulatory proteins. The three conserved zinc finger motifs located in the C-terminal region of these proteins bind to GC-rich sequences in target genes to regulate target gene transcription and cell proliferation and differentiation [9, 10]. KLF15 plays various regulatory roles. The current study examined the role of KLF15 in the regulation of mesangial cell proliferation.

It has previously been shown that KLF15 promotes the differentiation of 3T3-L1 pre-adipocytes into fat cells [19, 20]. Moreover, it activates the expression of BMPER, which is essential for angiogenesis, in endothelial cells [21]. KLF15 negatively regulates cardiac hypertrophy factor, thereby inhibiting cardiac hypertrophy and fibrosis [22, 23]. Other research results have shown that KLF15 can inhibit Gata-4 and myocyte factor 2 (Mef2) functions respectively in animal models of cardiac hypertrophy, and KLF15 may help to prevent cardiac hypertrophy in mice [24, 25].

KLF15 is sometimes referred to as kidney-enriched Kruppel-like factor (KKLF), because it is highly expressed in endothelial and mesangial cells of the kidney, where it regulates the expression of the kidney-specific chloride channels CCL-K1 and CLC-K2 [18]. KLF15 is also known to regulate the expression of collagen type IV [26], which promotes renal fibrosis. In contrast, its effects on mesangial cell proliferation have not previously been investigated. The present study reveals for the first time that overexpression of KLF15 can inhibit

mesangial cell proliferation.

Through a combination of microarray, PCR, and Western blot analyses, this study showed that the transcription factor KLF15 was only weakly expressed during mesangial cell proliferation period, but was more strongly expressed under normal conditions and during proliferation declined period in an anti-Thy-1 mesangial proliferative glomerulonephritis model. Overexpression of KLF15 caused the downregulation of cyclin D1 and CDK2 expression and the inhibition of mesangial cell proliferation, whereas inhibition of KLF15 expression caused increased cyclin D1 and CDK2 expression and increased cell proliferation. These results suggest that KLF15 may regulate the expression of cell cycle regulatory proteins and thereby inhibit mesangial cell proliferation. Thus, KLF15 may represent a new target for therapeutic intervention in mesangial proliferative glomerulonephritis.

Cell proliferation is determined by the levels of cell cycle regulatory proteins, and numerous transcription factors regulate the expression of these proteins. In mesangial cells, changes in proliferation are accompanied by the altered expression of cyclins, CDKs, and CKIs [27-30]. KLF15 binds to the GC-rich region of the promoter for transcription factor E2F1 [11], which regulate the expression of cell cycle proteins [31]. The initiation and amplification of mesangial cell proliferation have been linked to glomerular sclerosis and the downregulation of mesangial cell E2F1 expression in human IgA nephropathy [32]. In this study, siRNA target to E2F1 was co-transfected with siKLF15 into RMCs; the results indicated that increase of cell proliferation induced by siKLF15 can be eliminated partly by siE2F1. At the same time, if E2F1 was inhibited by RNAi, the expression of KLF15 did not change. But when to knockdown or overexpress the KLF15, the expression of E2F1 have an upregulation or downregulation respectively, which demonstrated E2F1 may play an important role in this process as downstream target of KLF15. It was also indicated that KLF15 could inhibit E2F1 expression of RMCs, which was contrary to previous report [11]. The different result might be Wade et al use the breast cancer cell lines, however we used RMCs. Similarly, other studies reported KLF5 could take a reverse effect on the proliferation with different type of cell [33, 34].

Then we analyzed E2F1, cyclinD1 and CDK2 expression *in vivo*. At days 5 and 7 of anti-Thy1 nephritis model, three proteins expression level were significantly higher than day 0, moreover their expression level downregulated at day 14, it further supports that KLF15

may inhibit cell cycle regulatory protein expression. How the expression of KLF15 downregulated at day 5 and 7. Liu et al. [35] suggested that KLF5 is a downstream signal of the ERK 1/2 and p38 MAPK pathways, and activates the transcription of cyclin D1 gene via functional interaction with c-Jun in Ang II-induced VSMC proliferation. Our study focus on how KLF15 could regulate the proliferation of RMCs, therefore, in our investigation we did not determinate the mechanism that reduce KLF15 in glomerulonephritis. Of course, it should be verified by furthermore test.

The inhibition of KLF15 expression may promote cell proliferation by upregulating positive-acting cell cycle regulatory protein expression. Conversely, the overexpression of KLF15 may inhibit cell

proliferation by downregulating positive-acting cell cycle regulatory protein expression. The results suggest that KLF15 may be a new target for the treatment of mesangial proliferative glomerulonephritis, although the anti-proliferative effects of KLF15 need to be confirmed *in vivo*.

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