Rapamycin ameliorates IgA nephropathy via cell cycle-dependent mechanisms

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Abstract

IgA nephropathy is the most frequent type of glomerulonephritis worldwide. The role of cell cycle regulation in the pathogenesis of IgA nephropathy has been studied. The present study was designed to explore whether rapamycin ameliorates IgA nephropathy via cell cycle-dependent mechanisms. After establishing an IgA nephropathy model, rats were randomly divided into four groups. Coomassie Brilliant Blue was used to measure the 24-h urinary protein levels. Renal function was determined using an auto-analyzer. Proliferation was assayed via Proliferating Cell Nuclear Antigen (PCNA) immunohistochemistry. Rat mesangial cells were cultured and divided into the six groups. Methylthiazolyldiphenyl-tetrazolium bromide (MTT) and flow cytometry were used to detect cell proliferation and the cell cycle phase. Western blotting was performed to determine cyclin E, cyclin-dependent kinase 2, p27^{Kip1}, p70S6K/p-p70S6K, and extracellular signal-regulated kinase 1/2/p- extracellular signal-regulated kinase 1/2 protein expression. A low dose of the mammalian target of rapamycin (mTOR) inhibitor rapamycin prevented an additional increase in proteinuria, protected kidney function, and reduced IgA deposition in a model of IgA nephropathy. Rapamycin inhibited mesangial cell proliferation and arrested the cell cycle in the G1 phase. Rapamycin did not affect the expression of cyclin E and cyclin-dependent kinase 2. However, rapamycin upregulated p27^{Kip1} at least in part via AKT (also known as protein kinase B)/mTOR. In conclusion, rapamycin can affect cell cycle regulation to inhibit mesangial cell proliferation, thereby reduce IgA deposition, and slow the progression of IgAN.

Keywords: Rapamycin, IgA nephropathy, mesangial cell, cell cycle proteins

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Introduction

IgA nephropathy (IgAN) is the most frequent type of glomerulonephritis worldwide.¹ The primary form of the disease used to be considered quite harmless. Now, IgAN is recognized as a major health problem because 20–40% of patients progress to end-stage renal disease within 20 years of disease onset, and an additional 20% exhibit progressive kidney damage.^{1,2}

Currently, IgAN is considered an immune complex-mediated glomerulonephritis characterized by IgA deposition, mesangial cell (MC) proliferation, and mesangial matrix expansion.³ The importance of MC proliferation has been shown in experimental and human glomerular disease.⁴ However, we have focused on the role of cell cycle proteins (CCPs) in MC proliferation because of the compelling view that cell proliferation is ultimately controlled in the nucleus by these proteins.⁵

The role of cell cycle regulation in the pathogenesis of IgAN has been largely characterized by *in vitro* and

human IgAN studies. In cultured MCs, the expression of cyclins D1, E, A, and cyclin-dependent kinase 2 (CDK2) was increased upon stimulation with different mitogens,^{6–8} while the proliferation of rat MCs induced by platelet-derived growth factor was significantly inhibited by p27^{Kip1} and moderately inhibited by p21^{Waf1}.^{7–9} Qiu et al.¹⁰ reported that the onset and magnitude of MC proliferation and glomerulosclerosis are associated with the upregulation of E2F1 by MCs and the downregulation of p27^{Kip1} and p57^{Kip2} by glomerular epithelial cells in human IgAN. Their results suggest that cell cycle regulation in glomerular intrinsic cells may underlie glomerular lesions from MC proliferation to glomerulosclerosis, leading to the progressive deterioration of renal function. Therefore, we can intervene in these processes to slow the progression of renal disease.

Rapamycin, also known as sirolimus, belongs to a novel class of immunosuppressive drugs that inhibit the mammalian target of rapamycin (mTOR).^{11,12} Moreover, mTOR plays a central role in regulating cell proliferation, growth,

differentiation, migration, and survival.¹³ The activation of mTOR leads to an interaction with downstream effectors, such as p70 ribosomal S6 kinase (p70S6K) and eukaryotic initiation factor-4E-binding protein-1.¹⁴ Through these pathways, mTOR controls cellular proliferation, thereby promoting processes such as DNA translation, RNA transcription, ribosomal biogenesis, and cell cycle progression.¹⁵

Rapamycin binds to an intracellular cytoplasmic receptor, FK506-binding protein-12, and this complex disrupts mTOR function. In T cells stimulated by IL-2, rapamycin prevents the activation of the cdk2-cyclin E complex, blocking the G1-to-S transition in the cell cycle.¹⁶ In addition to lymphocytes, rapamycin acts antiproliferatively for several other cell types, such as vascular smooth muscle cells, MCs, and endothelial cells.¹³ In particular, low-dose rapamycin (1 ng/mL) can inhibit MC proliferation^{17,18} and slow the progression to chronic renal fibrosis and insufficiency in a model of mesangioproliferative anti-Thy1.1 nephritis.¹⁹ However, whether rapamycin can modulate cell cycle regulation proteins to affect the progression of IgAN and the underlying mechanisms of this modulation have not been widely investigated.

The present study was designed to explore the renoprotective potential of low-dose rapamycin in an IgAN rat model. Furthermore, we investigated the effect of rapamycin on cell cycle regulatory proteins in MCs and explored the protective mechanism of rapamycin.

Materials and methods

Animals

Young (6–7 weeks of age) male Sprague–Dawley rats (specific pathogen free) weighing 150 g (± 10) were purchased from the Experimental Animal Center of Shanxi Medical University (Taiyuan, China) and housed under controlled environmental conditions (temperature 22°C, 12-h dark period). The animals were given free access to water and fed a standard laboratory diet. All of the rats were active and had glossy hair before the start of the experiments. The protocol was approved by the Institutional Animal Care and Use Committee at Shanxi Medical University.

Experimental design

The rats ($n = 40$) were randomly divided into the following four groups, with 10 rats in each group: control with vehicle treatment (control), control with rapamycin treatment (control + Rapa), IgAN model with vehicle treatment (IgAN), and IgAN model with rapamycin treatment (IgAN + Rapa). Four rats (one rat from each group) were sacrificed respectively at the end of weeks 8 and 12 to detect IgA deposition. No rat died for any other reason.

After the IgAN model was established at the end of week 12, rapamycin (1.0 mg/kg per day, Adamas-beta, Switzerland) was dosed intragastrically from weeks 12–16. This dose was based on a previous experiment²⁰ and did not adversely affect renal function. Four weeks after the rapamycin treatment (at week 16), the rats ($n = 8$ per group) were deeply anesthetized, and tissue samples were

processed and stored. Blood from the rats was allowed to clot at room temperature and was centrifuged at $13,000 \times g$ for 2 min; the serum was stored at -70°C .

The body weight of the rats was measured weekly. At the end of weeks 0, 4, 8, 12, and 16, 24-h urine collection was obtained using metabolic cages.

Establishment of the IgAN model

An IgAN model was induced by continuous oral immunization with 0.1% bovine gamma globulin (BGG) (Sigma, St Louis, MO, USA) with 6 mM HCL in tap water for eight weeks, followed by a tail intravenous injection of 1 mg BGG daily for three consecutive days.^{21–23} For the control group, saline replaced the BGG. The IgAN model was established at the end of the 12th week.

At the end of weeks 8, 12, and 16, direct immunofluorescence was used to visualize IgA deposition in the glomeruli.

Renal function

Coomassie Brilliant Blue was used to measure the 24-h urinary protein levels. Renal function was analyzed by measuring the serum creatinine, urea nitrogen, total protein, and albumin levels with an autoanalyzer (Beckman Instruments, Palo Alto, CA, USA).

Immunofluorescence

Four-micrometer frozen sections were prepared and fixed with cold acetone for 3 min. To evaluate IgA deposition in the glomeruli, 1:100 diluted Fluorescein Isothiocyanate (FITC)-conjugated goat anti-rat IgA antibody (Abcam, Cambridge, UK) was used for direct immunofluorescence. Digital images were obtained using a confocal laser fluorescence microscope (Olympus, Tokyo, Japan).

Immunohistochemistry

Consecutive sections were deparaffinized in xylene, and antigen retrieval was performed by high-pressure repair for 2 min. Nonspecific staining was blocked with 1.5% blocking serum for 30 min. The sections were incubated with PCNA mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA., USA) at a 1:200 dilution overnight at 4°C . Signals were detected using the Avidin-biotin-peroxidase complex (ABC) staining system (Santa Cruz Biotechnology) according to the manufacturer's instructions. An isotype-matched control antibody was used as a negative control.

All of the images were acquired using an Olympus BX51 clinical microscope and a DP70 digital camera and software (Olympus, Tokyo, Japan). The images were analyzed using Image-Pro Plus (Media Cybernetics).

Cell culture and treatments

All reagents used for cell culture were purchased from Sigma (USA), unless noted otherwise. The rat mesangial cell line HBZY-1 was obtained from the China Center for Typical Collection (Wuhan, China). Cells were grown in

Dulbecco's Modified Eagle's Medium Nutrient (DMEM) with 15 mM 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer, 2 mM L-glutamine, penicillin (100 U/mL)/streptomycin (100 µg/mL), and 10% fetal bovine serum in humidified air at 37°C with 5% CO₂. After preincubation in DMEM supplemented with 0.5% fetal bovine serum to synchronize cells for 24 h, the cells were divided into the following groups ($n = 6$ per group): normal; normal with Platelet-derived growth factor-B (PDGF-B) (20 ng/mL); PDGF-B + rapamycin 1 nmol/L; PDGF-B + rapamycin 10 nmol/L; PDGF-B + rapamycin 100 nmol/L; PDGF-B + rapamycin 1000 nmol/L.

Cell proliferation assay

The MTT assay was used as a qualitative index of cell proliferation. We cultured MC cells in 96-well plates (5×10^3 cells/well). After 24 or 48 h of incubation with the above compounds, 20 µL MTT (5 mg/mL, Invitrogen, CA, USA) was added to each well. The plate was incubated for 4 h at 37°C in a CO₂ incubator. Then, the cell medium was removed, and 150 µL dimethyl sulfoxide (Sigma) was added to each sample. When the formazan crystals were completely dissolved, the optical density (OD) was measured at 492 nm using a Microplate Reader (Bio-Rad Model 550, Bio-Rad, Hercules, CA, USA). Inhibition ratio = $(1 - \text{OD drug treated} / \text{OD control}) \times 100\%$.

Flow cytometry

Cell cycle analysis was performed by flow cytometry. The cells were harvested 24 h after treatment and fixed with 70% ethanol at 4°C for 24 h. After the washes, the cells were treated with ribonuclease (RNase) (0.5 mg/mL) at 37°C for 30 min, followed by staining with propidium iodide (PI, 50 µg/mL) at 4°C for 30 min in the dark. The cells were analyzed by flow cytometry using an FC500 flow cytometer (Coulter, Beckman, Palo Alto, CA, USA) to determine the proportion of cells within the G1, S, and G2/M phases.

Western blot analysis

The cells were divided into the following groups: normal; normal with PDGF-B (20 ng/mL), PDGF-B + rapamycin 10 nmol/L, and PDGF-B + rapamycin 100 nmol/L. The cells were harvested after drug intervention at 2 and 24 h to detect signaling pathway and CCPs.

The cells were lysed in lysis buffer (KeyGEN Biotech, Nanjing, China). The lysates were centrifuged at $12,000 \times g$ for 30 min at 4°C. The supernatant was collected, and the protein concentrations were determined using a Bicinchoninic acid (BCA) assay kit (KeyGEN Biotech, Nanjing, China). Samples containing 50 µg of protein were separated on 10% Sodium Dodecyl Sulfonate (SDS)-polyacrylamide gels. After blocking, the following primary antibodies were used overnight at 4°C: (1) cyclin E antibody (M20), dilution 1:200 (Santa Cruz Biotechnology); (2) CDK2 antibody (D12), dilution 1:200 (Santa Cruz Biotechnology); (3) p27 kip1 antibody, dilution 1:1000 (Cell Signaling Technology, Beverly, MA, USA); (4) p70 S6 kinase antibody and phosphor-p70 S6 kinase (Thr389) antibody, dilution 1:1000 (Cell Signaling

Technology); (5) p44/42 Mitogen-activated protein kinase (MAPK) (extracellular signal-regulated kinase (Erk)1/2) (137F5) rabbit mAb and phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (197G2) rabbit mAb, dilution 1:1000 (Cell Signaling Technology); and (6) β-actin antibody (C4), dilution 1:500 (Santa Cruz Biotechnology).

On the second day, the membranes were hybridized for 2 h at 37°C with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) at a 1:2000 dilution. After washing three times, specific bands were visualized by fluorography using an enhanced chemiluminescence kit (Pierce, Rockford, IL, USA). The relative densities were quantified using the Quantity One analysis system (Bio-Rad, Hercules, CA, USA).

Statistical analysis

The results are presented as the mean $\pm$ SD. The data were analyzed by one-way analysis of variance with the Bonferroni correction to compare the mean values in multiple treatment groups. $P < 0.05$ was considered significant.

Results

Effect of rapamycin on body weight, proteinuria, and renal function in IgAN rats

Proteinuria was observed as early as week 4 in the IgAN rats and increased to 32.61 ± 6.46 mg/24 h by the end of the experiment. The rats that received low-dose rapamycin experienced no further increase in proteinuria after week 12, and the final amount of proteinuria decreased to 15.52 ± 2.76 mg/24 h and was significantly lower compared with the IgAN rats (Figure 1(a)). Proteinuria in normal control group was constantly low (week 16: 3.20 ± 0.64 mg/24 h).

Although the IgAN rats had a significant amount of urinary protein, no evidence of renal damage was observed during the study period. Only creatinine slightly increased in the IgAN rats compared with the control rats at week 16 (Figure 1(c-f)).

The animals' mean body weight during the experiment did not change compared with that of the nonnephritic controls (Figure 1(b)), indicating that neither IgAN nor low-dose rapamycin affected the rats' body weight.

Rapamycin inhibited IgA deposition and cellular proliferation in glomeruli

Immunofluorescence staining for IgA indicated that the control rats had very little IgA deposition in the glomeruli. By contrast, the IgAN rats had increased IgA expression at week 8, which continued to increase until week 16. However, after low-dose rapamycin treatment at week 12, IgA deposition did not increase (Figure 2(a)).

PCNA is widely used as a marker of replicated DNA and cell proliferation. Representative immunohistochemistry images are presented in Figure 2(b). The IgAN rats showed marked glomerular proliferation at week 16, and the PCNA-positive renal cell staining in the animals treated with low-dose rapamycin was reduced markedly in the glomerular compartment (IgAN 12 ± 3 vs. IgAN + Rap 5 ± 2 , $P < 0.05$).

Figure 1 Functional parameters in IgA nephropathy after rapamycin treatment. (a) 24-h Urinary protein levels. Four weeks after BGG was administered intragastrically, proteinuria developed in the IgAN rats. Rapamycin treatment (initiated in week 12) significantly reduced protein excretion, but the proteinuria remained more severe compared with the control rats at week 16 ($n = 10/\text{group}$ at week 4; $n = 9/\text{group}$ at week 8; $n = 8/\text{group}$ at week 12–16). (b) The mean body weight of the rats. IgA nephropathy and low-dose rapamycin did not affect the body weight of the rats ($n = 10/\text{group}$ at week 3, 6; $n = 9/\text{group}$ at week 9; $n = 8/\text{group}$ at week 12–16). (c) Serum creatinine slightly increased in the IgAN rats at week 16 ($n = 8/\text{group}$). For urea nitrogen (D), total protein (E), and albumin (F), there was no significant difference among the groups ($n = 8/\text{group}$). The data are presented as the means $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ vs. control; # $P < 0.05$, ## $P < 0.01$ versus IgAN

Rapamycin reduced MC proliferation and arrested the cell cycle in the G1 phase

The MTT assay was used to assess the impact of rapamycin on MC proliferation. Rapamycin significantly suppressed

PDGF-B-induced MC proliferation in a dose- and time-dependent fashion, but with the dose increased (1–100 nmol/L), the time dependence gradually weakened. A minimum inhibition of rapamycin was 1 nmol/L at 48 h,

Figure 2 Rapamycin inhibited IgA deposition and cellular proliferation in glomeruli. (A) IgA deposition in the glomeruli was visualized by direct immunofluorescence. (a) control (16 weeks); (b) control with rapamycin treatment (16 weeks); (c) IgA nephropathy model (8 weeks); (d) IgA nephropathy model (12 weeks); (e) IgA nephropathy model (16 weeks); and (f) IgA nephropathy model with rapamycin treatment for 4 weeks (16 weeks). All of the sections were imaged with a confocal immunofluorescence microscope (200 × magnification). (B) Cell proliferation was detected via PCNA immunohistochemistry at week 16 ($n = 8/\text{group}$, 400 × original magnification). The data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ versus control; *** $P < 0.01$ versus IgAN. (A color version of this figure is available in the online journal.)

but not 24 h (inhibition ratio: 28.2% vs. 3.6%, $P < 0.01$). In the 1000 nmol/L rapamycin-treated cells, at 24 or 48 h, the inhibition ratios were not higher than the 100 nmol/L group (24 h: 39.1% vs. 46.2%; 48 h: 52.9% vs. 53.8%) (Figure 3(a)).

To further examine the role of rapamycin in MC cell cycle progression, we performed flow cytometry analysis. PDGF-B induced a decrease in the number of cells in G1 phase but increased the number of cells in S phase, indicating that PDGF-B promotes cell cycle progression (Figure 3(b)). By contrast, rapamycin dose-dependently arrested cells in the G1 phase ($P < 0.05$); therefore, the number of cells in S phase was significantly lower ($P < 0.05$). These results suggest that rapamycin can block PDGF-B-induced cell cycle progression by inhibiting the G1-S phase transition and arresting cells in the G1 phase.

Effect of rapamycin on cyclin E and CDK2

Cyclins and CDKs are essential for cell cycle progression in MCs. To determine the possible mechanism of action of rapamycin on MC proliferation, we evaluated the expression of cyclin E and CDK2 using Western blotting (Figure 4(a, b)). PDGF-B at 20 ng/mL significantly increased the expression of cyclin E and CDK2 by 2- and 2.36-fold, respectively, compared with the control. However, no inhibitory effect was observed at the concentrations of 10 and 100 nmol/L of rapamycin ($P > 0.05$).

PDGF decreases CKI p27^{Kip1} expression: Modulation by rapamycin

The expression of p27^{Kip1} in quiescent and proliferating MCs was determined by Western blot analysis. Detectable

Figure 3 Rapamycin reduced mesangial cell proliferation and arrested the cell cycle in the G1 phase. (a) MTT assay for cell proliferation. Rapamycin inhibited cell proliferation induced by PDGF-B at 24 and 48 h. Rapamycin treatment at 1 nmol/L for 48 h but not 24 h significantly suppressed MC proliferation. There was no significant difference among the groups using 100 nmol/L or 1000 nmol/L ($n = 6/\text{group}$). (b) Flow cytometry was used to analyze the cell cycle. Rapamycin dose-dependently arrested cells in the G1 phase; therefore, the number of cells in the S phase was significantly lower ($n = 6/\text{group}$). The data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$ versus control, # $P < 0.05$, ## $P < 0.01$ versus PDGF. (A color version of this figure is available in the online journal.)

Figure 4 The effect of rapamycin on cell cycle proteins. (a and b) The expression of cyclin E (a) and CDK2 (b) was increased by PDGF-B, but no inhibitory effect was observed using 10 or 100 nmol/L of rapamycin. (c) The level of p27^{Kip1} was analyzed by Western blotting. Rapamycin upregulated p27^{Kip1} expression at 10 and 100 nmol/L (two samples per group). Data are representative of three independent experiments. * $P < 0.05$ versus control; # $P < 0.05$ versus PDGF; mean $\pm$ SD

levels of p27^{Kip1} were expressed in quiescent MCs. However, incubation with PDGF caused a decrease in p27^{Kip1} expression at 24-h poststimulation. These decreases were significantly abrogated by rapamycin (Figure 4(c)).

Rapamycin suppressed p-p70S6K but did not affect p-ERK1/2

PDGF-B activates several protein kinase pathways, including the ERK1/2 and AKT pathways, which downregulate CDK inhibitors. Thus, we further assessed the effect of rapamycin on p27^{Kip1} protein expression via the ERK1/2 and AKT/mTOR pathways. PDGF induced the phosphorylation of p44/42 MAPK and increased the phosphorylation of p70S6K, a downstream target of AKT/mTOR that is frequently used as an indicator of mTOR activity. Treatment

with rapamycin inhibited p70S6K phosphorylation (Figure 5(b)) but did not affect the phosphorylated p44/42 MAPK expression (Figure 5(a)).

Discussion

In IgAN rats, low-dose rapamycin prevented an additional increase in proteinuria and protected kidney function. In our IgAN model, despite heavy proteinuria, the animals had normal renal function that remained stable for several months. Only at week 16 were the serum creatinine levels in the IgAN group slightly higher than those of the control group. These data prove that substantial proteinuria for a long period of time can affect kidney function. Proteinuria is a recognized risk factor for chronic kidney disease progression.²⁴ Rapamycin attenuated any additional increase

Figure 5 Rapamycin inhibited p70S6K phosphorylation, but did not affect p44/42 MAPK phosphorylation. (a) PDGF induced p44/42 MAPK phosphorylation, and rapamycin did not reduce p44/42 MAPK phosphorylation. (b) The levels of p-p70S6K and p70S6K were analyzed by Western blotting. Phosphorylated p70S6K was elevated in the PDGF group, and effectively inhibited by rapamycin in a dose-dependent manner. The data are presented as the fold increase in the ratio of phosphoprotein to total protein. The bar graph illustrates the mean $\pm$ SD from three independent experiments. * $P < 0.05$ versus control, # $P < 0.05$, ## $P < 0.01$ versus PDGF

in proteinuria, as evidenced by periodic 24-h urinary protein excretion measurements, to protect kidney function. Although the proteinuria did not return to normal, with a longer treatment time, the proteinuria might decrease further.

IgAN is characterized by IgA deposition, MC proliferation, and mesangial matrix expansion. IgA deposition in the mesangium is a critical step in the development of IgAN.²⁵ Low-dose rapamycin reduced IgA deposition in the mesangium; after initiating rapamycin treatment in week 12, the fluorescence intensity of IgA did not increase. Changes in the physiology of MCs appear to be an early event in progressive glomerular injury, resulting in a modified composition of the extracellular matrix and glomerular sclerotic changes.²⁶ The key action of rapamycin is the inhibition of cell proliferation. Our study proved that this pathway was involved. Using PCNA staining, low-dose rapamycin treatment corresponded with significantly less cell proliferation in glomeruli.

Because MC proliferation is a key pathological feature in IgAN, MCs were cultured for further *in vitro* studies. Here, our cell experiment data show that low-dose rapamycin treatment inhibited MC proliferation and arrested the cell cycle in the G1-phase, blocking the G1-to-S transition. High-dose rapamycin (1000 nmol/L) is not more effective in inhibiting cell proliferation than low-dose rapamycin (100 nmol/L), so we selected 10 and 100 nmol/L rapamycin to continue our experiment.

Each phase of the cell cycle is controlled by specific positive (cyclins and CDKs) and negative (cyclin kinase inhibitors) cell cycle regulatory proteins.²⁷ Cyclin D1, along with its associated CDK4 and 6, is a key regulatory protein for

controlling the reentry of quiescent cells in G0 into the cell cycle at G1. Cyclin E expression increases in late G1, when it associates with and activates CDK2, thereby playing a pivotal role in the G1/S transition. The transition from late G1 to the S phase determines the cell's growth characteristics. Cyclin A expression peaks in late G1, is maximal during the S phase and persists through G2. Cyclin A activates CDK2, which is essential for DNA synthesis.^{28–30} Low-dose rapamycin mainly blocked the G1-to-S transition, so we tested the protein expression of cyclin E and CDK2. PDGF-B induced increases in cyclin E and CDK2. To our surprise, rapamycin did not decrease the expression of cyclin E and CDK2.

The cyclin-CDK complex is inhibited by CDK inhibitor. The interaction of p27^{Kip1} with cyclin-CDK complexes is more complicated than previously thought because p27^{Kip1} can be both an inhibitor and a substrate for cyclin E-CDK2. Cyclin E-CDK2 may be inhibited in G0 by p27^{Kip1}.²⁹ In normal rat glomeruli, CKI p27^{Kip1} expression is high, in accordance with the quiescent nature of MCs in healthy kidneys,³¹ but p27^{Kip1} expression decreases dramatically in IgAN.¹⁰ Our MC culture experiments revealed that PDGF-B induced decreased expression and that rapamycin inhibited cell cycle-dependent proliferation in MCs by increasing p27^{Kip1} protein expression.

The serine-threonine kinase Akt and the ERK 1/2 are the two major signaling molecules in regulating extracellular matrix (ECM) protein synthesis and cell proliferation.³² The expression of p27^{Kip1} is regulated by multiple signaling pathways, including the Ras/MAPK/ERK1/2^{33–35} and PI3K/Akt/mTOR pathways.^{36,37} These observations prompted us to examine the impact of rapamycin on

p27^{Kip1} via ERK1/2 or AKT/mTOR in MCs. We found that PDGF-B enhanced the levels of phosphorylated p70S6K and ERK1/2 in MCs. Rapamycin treatment significantly decreased the level of p-p70S6K, but did not affect p-ERK1/2. Thus, we may infer that rapamycin upregulated p27^{Kip1} at least in part via AKT/mTOR, but not ERK1/2.

In conclusion, this study demonstrated that rapamycin upregulated p27^{Kip1} expression at least in part by blocking the mTOR pathway, thus arresting MCs in the G1 phase and reducing MC proliferation. Therefore, rapamycin can modulate cell cycle regulation to inhibit MC proliferation, reduce IgA deposition, so slow the progression of IgAN. Rapamycin ameliorates IgAN via cell cycle-dependent mechanisms.

Author contributions: All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript; J.T., Y.W., and Z.X. conducted the experiments and analyzed the data; J.T. wrote the manuscript; and R.L. and X.L. contributed constructive suggestions to the writing of the manuscript.

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