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A Novel Target of Mizoribine Inhibiting Mesangial Cell Proliferation: S Phase Kinase-Associated Protein 2

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Key Words
Mizoribine · Mesangial cell proliferation · Cell cycle proteins · S phase kinase-associated protein 2

Abstract
Background/Aims: Mizoribine (MZR) can inhibit mesangial cell (MC) proliferation, but the mechanism remains unknown. In this study, we investigated the inhibitory effect of MZR on MC proliferation via a cell cycle regulatory protein-dependent mechanism. ***Methods:*** We investigated the effect of MZR on MC proliferation and expression of cell cycle regulatory proteins such as cyclin D1, cyclin-dependent kinase 2 and $p27^{kip1}$ in primary cultured rat MCs. We further focused on analyzing the effects of MZR on S phase kinase-associated protein 2 (Skp2), which played a crucial role in $p27^{kip1}$ degradation. ***Results:*** MZR effectively inhibited MC proliferation in primary cultured rat MCs, reduced the expression of cyclin D1 and cyclin-dependent kinase 2, while it dramatically increased the protein level of $p27^{kip1}$, maintained the nucleus location of $p27^{kip1}$ and induced G1/S arrest. In contrast to the protein level, MZR produced no changes in $p27^{kip1}$ mRNA abundance. MZR impaired $p27^{kip1}$ degradation through downregulating the expression of Skp2 and this effect was not dependent on its inhibition on DNA synthesis. Skp2 overexpression abolished MZR-induced $p27^{kip1}$ accumulation. ***Conclusion:*** These results suggested that MZR-induced $p27^{kip1}$ accumulation was at least partly mediated by Skp2, and that Skp2 might be a novel target of MZR inhibiting MC proliferation.

Introduction

Mesangial cell (MC) proliferation is an important feature of many forms of glomerular diseases. Proliferative responses of MCs to a variety of stimuli are associated with extracellular matrix accumulation and the development of glomerulosclerosis, the final common pathway of progressive renal disease [1]. Thus, inhibiting MC proliferation is an important treatment strategy for proliferative glomerulonephritis, and searching for drugs that act via this mechanism is of great clinical importance.

Cell growth and proliferation is tightly regulated through a precise balance of positive and negative cell cycle regulatory proteins composed of cyclin-dependent kinases (CDKs), corresponding regulatory cyclins and CDK inhibitors (CKIs). Accumulating evidence has suggested

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that there are changes in the cell cycle regulatory proteins during MC proliferation [2]. The negative regulatory protein $p27^{kip1}$ is endogenously expressed at high levels in the normal rat glomerulus and quiescent MC, while it is dramatically decreased in anti-Thy-1 nephritis and mitogen-stimulated MC. Positive regulatory proteins such as cyclin D and CDK2 are increased while MCs proliferate. Furthermore, changes in the expression of specific cell cycle regulatory proteins such as $p27^{kip1}$ directly determine the onset and magnitude of renal cell proliferation [3]. Cell cycle proteins are also the therapeutic targets of many drugs that inhibit MC proliferation [4, 5].

Mizoribine (MZR) is an antibiotic agent produced by the soil fungus *Eupenicilium brefeldianum* [6]. MZR competitively inhibits inosine 5′-monophosphate dehydrogenase, the rate-limiting enzyme in the de novo pathway for synthesis of guanine nucleotide and guanosine monophosphate synthase, and depletes intracellular guanine nucleotide pools, resulting in inhibition of DNA synthesis [7]. It was first proven efficient in renal transplantation [8], and clinical studies further revealed its efficacy in the treatment of some patients with diffuse IgA nephropathy and proliferative lupus nephritis [9, 10]. Whether MZR can directly inhibit MC proliferation and its mechanism of action have still not been clarified. In view of the importance of cell cycle regulatory proteins in cell growth, we speculated that MZR might inhibit MC proliferation via a cell cycle regulatory protein-dependent mechanism. Thus, we investigated the effect of MZR on MC proliferation in primary cultured rat MCs. We further focused on analyzing the effects of MZR on $p27^{kip1}$ and S phase kinase-associated protein 2 (Skp2), a member of the SCF^{Skp2} ubiquitin ligase complex responsible for $p27^{kip1}$ degradation, to clarify the mechanism and therapeutic targets of MZR.

Methods

Materials

MZR was a kind gift from Asahi Kasei Pharma Corp. (Tokyo, Japan). FCS was supplied by Hycolon (USA), PPMI-1640 was from Gibco (Gaithersburg, Md., USA). A771726, hydroxycarbamide (HU), Hoechst-33342, and MTT were from Sigma (St. Louis, Mo., USA). MG-132 was from Calbiochem (San Diego, Calif., USA). BrdU kits were from Roche (Mannheim, Germany). Antibodies against cyclin D1, CDK2, $p27^{kip1}$, Skp2 and Skp1 were all purchased from Santa Cruz Biotechnology (Santa Cruz, Calif., USA). Penicillin, streptomycin, TRIzol reagent and Lipofectamine 2000 were from Invitrogen (Carlsbad, Calif., USA). Polybrene, leupeptin, aprotinin, antipain and phenylmethylsulfonyl fluoride were from Sigma. Plasmids of pIRES-GFP and pIRES-GFP-Skp2 were kind gifts from Mr. Z.X. Sun.

Rat MC Culture and Transfection

Primary rat MCs were obtained from male Wistar rats (3 months of age) as described previously [11]. The cells were grown in RPMI-1640 that was supplemented with 20% FCS, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37°C. MCs of passages 3–6 were used for experiments. Confluent cells were made growth-arrested by serum deprivation for 24 h. For transient transfection, MCs at 50% confluence were incubated for 6 h with 8 μg plasmid DNA and 20 μl Lipofectamine 2000. Cells were then replaced with fresh medium and cultured for an additional 18 h. After serum deprivation for 24 h, the cells were treated and harvested for protein extraction.

Assessment of Cell Proliferation, DNA Synthesis and Cell Cycle Progression

MTT assay was used to measure cell proliferation. Growth-arrested cells were stimulated with 20% FCS in the presence or absence of MZR. MZR concentrations were 50, 5, and 0.5 μg/ml. A final amount of 10% MTT was added into each well, and the absorbance at 490 nm was measured with an enzyme-linked immunosorbent assay reader. To assess DNA synthesis, 10 μM BrdU was added into the medium in the last hour of incubation.

After fixation in 4% paraformaldehyde, cells were incubated with anti-BrdU monoclonal antibody with nuclease for 30 min at 37°C. The cells were then covered with fluorescein-conjugated anti-mouse antibody for 30 min at room temperature, counterstained with Hoechst 33342 (1 μg/ml; Sigma), and examined by fluorescence microscopy. At least 500 cells were counted per experiment, and the results were expressed as the percentage of BrdU-labeled nuclei. To determine cell cycle progression, cells (1×10^6; each sample) were treated with 0.25% trypsin, washed twice with cold PBS, and fixed with 70% alcohol in PBS for 12 h at 4°C. The cells were then washed twice with PBS and stained for 30–60 min at 4°C in 100 μg/ml propidium iodide solution (with 100 μg/ml RNase). Stained cells were analyzed by flow cytometry (Becton Dickinson, Franklin Lakes, N.J., USA).

Real-Time PCR Assay

$P27^{kip1}$ and Skp2 mRNA expression was measured by real-time PCR with the following primers: $p27^{kip1}$ sense 5′-GGT GGA CCA AAT GCC TGA CT-3′ and antisense 5′-GCC CTT TTG TTT TGC GAA GA-3′; and Skp2 sense 5′-ACC AGC TTC ACG TGG GGA TGG G-3′ and antisense 5′-TTC GAC AGG TCC ATG TGC TGT AC-3′. Control glyceraldehyde-3-phosphate dehydrogenase primers were as follows: sense 5′-TGC ACC ACC AAC TGC TTA GC-3′ and antisense 5′-GGC ATG GAC TGT GGT CAT GAG-3′. Real-time PCR was performed with SYBR green I (1:20,000; Qiagen) with 1 cycle at 94°C for 5 min followed by 40 cycles at 94°C for 30 s, 56°C ($p27^{kip1}$)/68°C (Skp2) for 30 s, 72°C for 30 s, and 86°C for 15 s.

Western Blotting

Cells were lysed in RIPA buffer that was composed of 50 mM Tris-Cl (pH 7.6), 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, and 0.5% Triton X-100 and contained 1 μg/ml leupeptin, aprotinin, and antipain, 1 mM sodium orthovanadate, and 0.5 mM phenylmethylsulfonyl fluoride. Protein concentration was measured by the Bradford assay. A total of 80 μg of total protein was separated by 12% SDS-PAGE and then transferred to a membrane, which was blocked with 5% skim milk, probed with a primary antibody

overnight at 4°C, and incubated with a horseradish peroxidase-conjugated secondary antibody. Immunoreactive bands were visualized using enhanced chemiluminescence.

Immunofluorescence Detection

MCs were seeded on sterile glass coverslips and grown to confluence, fixed in 4% paraformaldehyde for 20 min at 4°C, washed in PBS, and then permeabilized with 1% Triton X-100. After blocking non-specific antibody binding with 1% BSA for 20 min, the cells were incubated overnight at 4°C in a moist chamber with a monoclonal mouse anti-rat $p27^{kip1}$ antibody diluted at 1:50 in PBS that contained 1% BSA. After washing with PBS three times, the cells were incubated for 1 h with FITC-conjugated anti-mouse IgG (Sigma) diluted at 1:50 in PBS that contained 1% BSA. After three PBS washes, the cells were counterstained with Hoechst-33342 (5 μg/ml) and photographed using a confocal microscope (Radiance 2000; Bio-Rad Laboratories) equipped with the LSM510 software.

Statistical Analyses

All data analyses were performed with SPSS 10.0 (SPSS Inc., Chicago, Ill., USA), and data are expressed as mean ± SD. The comparison among the groups was conducted with ANOVA. p values <0.05 were considered statistically significant.

Results

MZR Inhibits MC Proliferation

To study the effect of MZR on rat MC proliferation, growth-arrested cells were stimulated with 20% FCS with or without different concentrations of MZR. MZR caused a dose-dependent inhibition of cell proliferation, as determined by MTT assay (fig. 1a). To further characterize this antiproliferative effect of MZR, S phase entry was determined by BrdU labeling. As shown in figure 1b, in quiescent cells there were few BrdU-positive cells (8.25 ± 0.93%). After FCS stimulation for 24 h, 21.04 ± 5.38% cells entered S phase, and MZR decreased the positive ratio to 10.28 ± 1.26%. Cell cycle analysis by flow cytometry showed that MZR-treated MCs were arrested at the G1 phase of the cell cycle, indicating that 83.5% of the cells accumulated in the G1 phase after 24 h of the treatment in contrast to 71.2% in FCS-stimulated cells (fig. 1c). This antiproliferative effect could not be attributed to cell apoptosis, because there were no apoptosis signs by either Hoechst stain or cell cycle analysis, such as pre-G1 peak. Overall, these data indicate that MZR suppressed MC proliferation by inhibiting the FCS-stimulated G1 to S progression.

MZR Decreased Expression of Positive Cell Cycle Regulatory Proteins

We further examined the protein expression of cyclin D1 and CDK2 in FCS-stimulated rat MCs. As shown in figure 2, there were low levels of cyclin D1 and CDK2 in quiescent MCs. After stimulation by 20% FCS, the expression of cyclin D1 and CDK2 exhibited an increase at 12 h that was sustained up to 24 h, followed by withdrawal to baseline at 48 h. These stimulatory effects of positive cell cycle regulatory proteins were inhibited by MZR (5 μg/ml) at 12 and 24 h.

MZR Dramatically Increased the Negative Cell Cycle Regulatory Protein $p27^{kip1}$ Protein Level

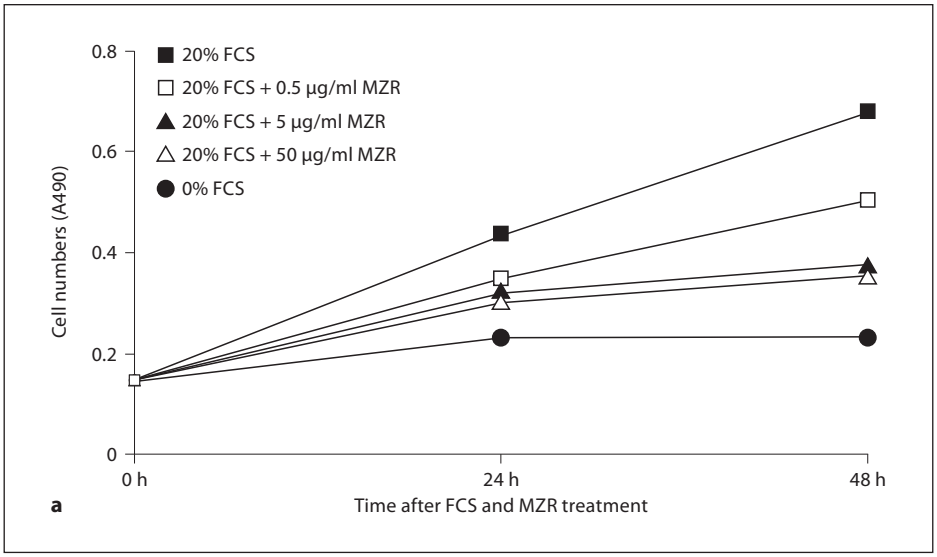
Western blot analysis showed that the $p27^{kip1}$ protein level was high in quiescent MCs, notably reduced at 12 and 24 h after FCS stimulation, and back to baseline at 48 h. MZR treatment (5 μg/ml) led to a dramatic increase in the $p27^{kip1}$ protein level beginning at 12 h and peaking at 24 h, as shown in figure 3a. Indirect immunofluorescence staining suggested that $p27^{kip1}$ was located in the nucleus in quiescent cells, whereas in FCS-stimulated cells, it was predominantly in the cytoplasm with significantly reduced fluorescence intensity, and MZR maintained the nucleus location and fluorescence intensity of $p27^{kip1}$ (fig. 3b).

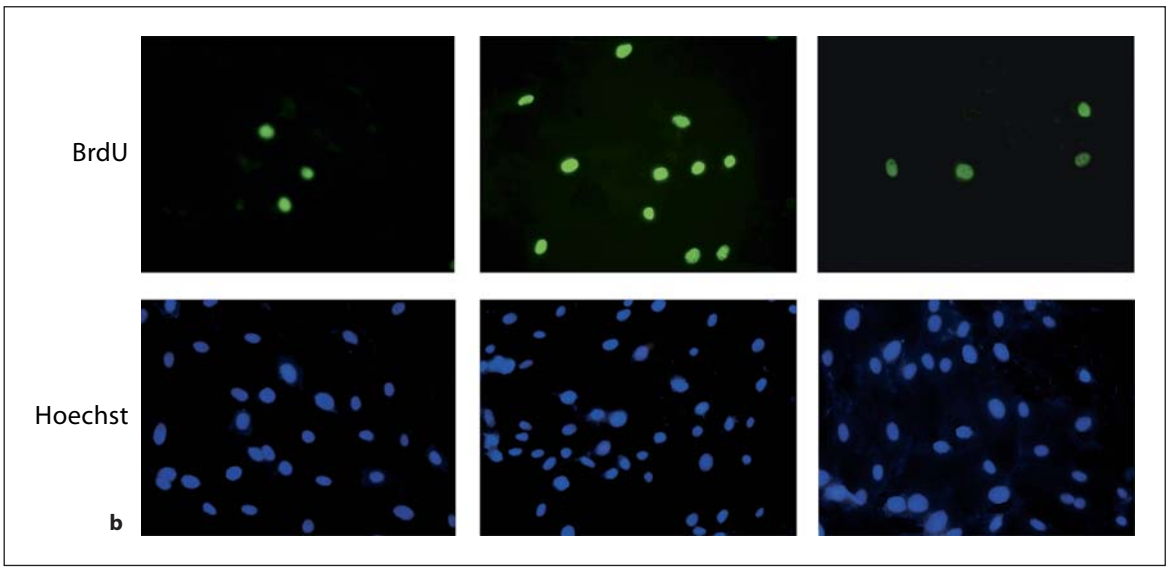
MZR Produced No Changes in $p27^{kip1}$ mRNA Levels

We detected $p27^{kip1}$ mRNA expression by real time-PCR in MCs. There were no significant changes either between quiescent and proliferative cells or between proliferative and MZR-treated cells at 12, 24 and 48 h (data not shown). Thus, although MZR significantly increased the $p27^{kip1}$ protein level, it produced no changes in the abundance of $p27^{kip1}$ mRNA. This discrepancy suggests that MZR regulated $p27^{kip1}$ by the post-transcription pathway or impaired degradation.

MZR Increased $p27^{kip1}$ Protein Level by Inhibiting Its Degradation, and This Effect Was Not Dependent on DNA Synthesis Inhibition

It has been reported that $p27^{kip1}$ expression is post-transcriptionally regulated by degradation through the ubiquitin-proteasome pathway and that the intracellular concentration of $p27^{kip1}$ is regulated predominantly by this proteolytic pathway. Since MZR increased $p27^{kip1}$ protein expression without changing its mRNA level, we postulated that MZR might impair $p27^{kip1}$ degradation. As shown in figure 4, the results for MZR (lane 3) were the same as those for proteasome inhibitor MG-132 (lane 6), as both of these agents significantly alleviated the decrease in $p27^{kip1}$ protein level induced by FCS. These results suggest that MZR might increase the $p27^{kip1}$ protein level by inhibiting its degradation through the ubiquitin-proteasome proteolytic pathway.





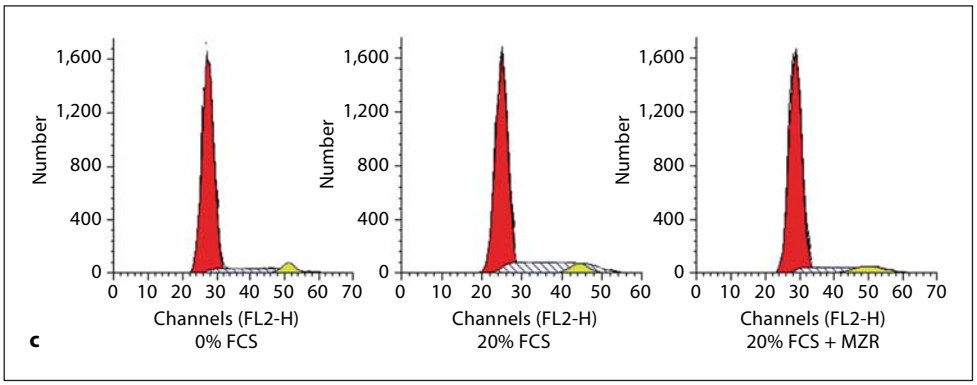


Fig. 1. MZR inhibits MC proliferation. **a** MZR caused a dose-dependent inhibition of MC proliferation, as determined by MTT assay. **b** In quiescent cells there were few BrdU-positive cells. After stimulation by 20% FCS for 24 h, BrdU-positive cells were increased, MZR decreased the ratio of BrdU-positive cells (×400). **c** Cell cycle analysis by flow cytometry showed that MZR-treated MCs were arrested at the G1 phase of the cell cycle after 24 h of the MZR treatment.

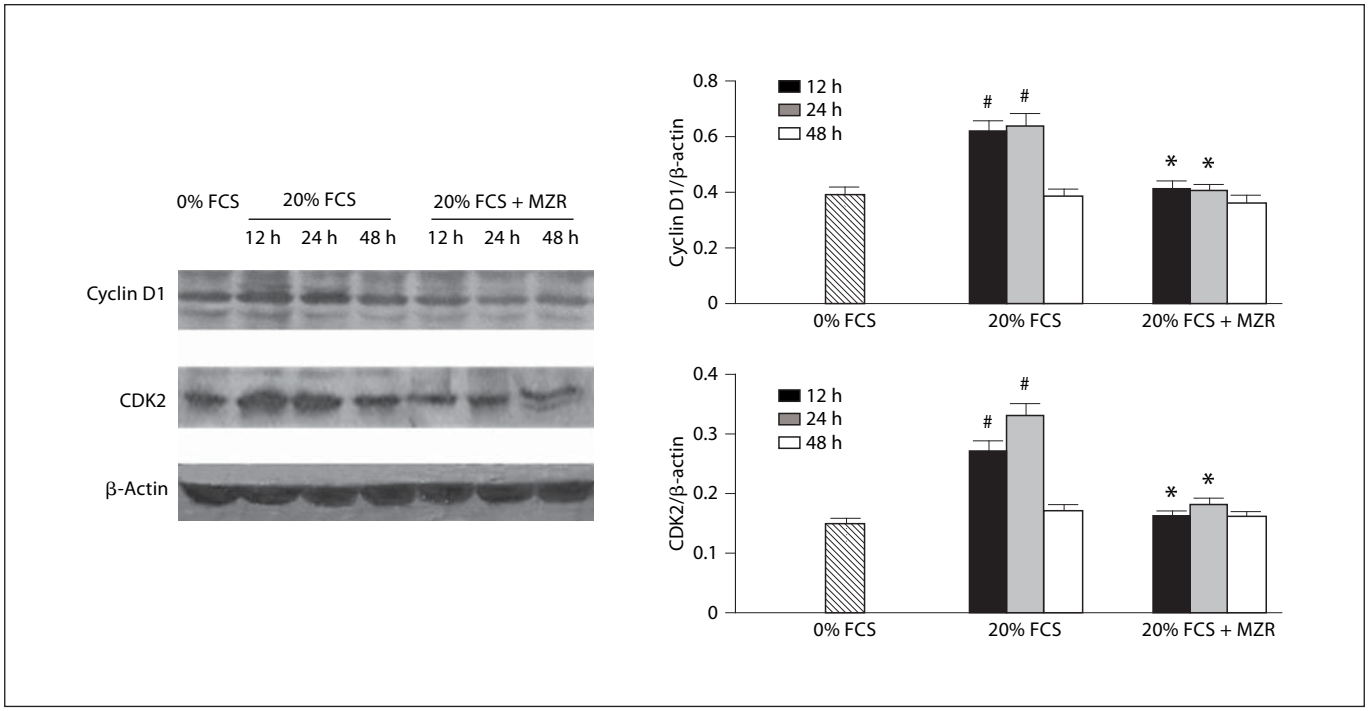


Fig. 2. MZR decreased expression of positive cell cycle regulatory proteins during MC proliferation. There were low levels of cyclin D1 and CDK2 in quiescent MCs. After stimulation by 20% FCS, the expression of cyclin D1 and CDK2 exhibited an increase at 12 and 24 h. These stimulatory effects were inhibited by MZR (5 μg/ml). Normalized signals from three independent experiments are shown in the bar charts. # $p < 0.05$ vs. growth-arrested cells; * $p < 0.05$ vs. 20% FCS-stimulated cells.

As a purine de novo synthesis inhibitor, MZR could increase the $p27^{kip1}$ protein level. Thus the question arises if this is a universal phenomenon for DNA synthesis inhibitors. That is, is the effect of MZR on $p27^{kip1}$ protein levels secondary to DNA synthesis inhibition? To answer this question we selected two other DNA synthesis inhibitors, A771726 and HU, to observe their effect on $p27^{kip1}$. A771726 is a pyrimidine synthesis inhibitor and HU is a DNA polymerase inhibitor. As demonstrated in figure 4, neither A771726 (lane 4) nor HU (lane 5) increased the $p27^{kip1}$ protein level similarly to MZR. The results suggest that the effect of MZR on the $p27^{kip1}$ protein level was unique and was not dependent on DNA synthesis inhibition.

Decreased Skp2 Expression in MZR-Treated MCs

Skp2 specifically interacts with the extreme COOH terminus of $p27^{kip1}$, and this association results in the recruitment of $p27^{kip1}$ to the SCF core complex, thereby promoting its ubiquitination and degradation. MZR inhibited $p27^{kip1}$ degradation through the ubiquitin-proteasome pathway, and one possible reason for this decreased degradation might be that MZR downregulated Skp2 expression. Just as seen in figure 5a, Skp2 protein expression was almost undetectable in quiescent MCs and was dramatically increased at 12 and 24 h after FCS stimulation, returning to baseline at 48 h. MZR significantly downregulated Skp2 expression at 12 and 24 h in concert with a reciprocal increase in the $p27^{kip1}$ protein level.

Skp2 Overexpression Abolished the MZR-Induced Increase in $p27^{kip1}$ Protein

We overexpressed Skp2 in MCs by transfection of the pIRES-GFP-Skp2 plasmid and investigated the effect of MZR on the $p27^{kip1}$ protein in cells overexpressing Skp2. As shown in figure 5b, an elevated expression of Skp2 was noted following Skp2 transfection (upper panel, lanes 4–6) compared with pIRES-GFP null plasmid-transfected cells (upper panel, lanes 1–3). Immunoblot analysis showed that $p27^{kip1}$ accumulation was abolished in Skp2-overexpressed cells, while the levels remained constant in null plasmid-transfected cells. In contrast, Skp1, a stable

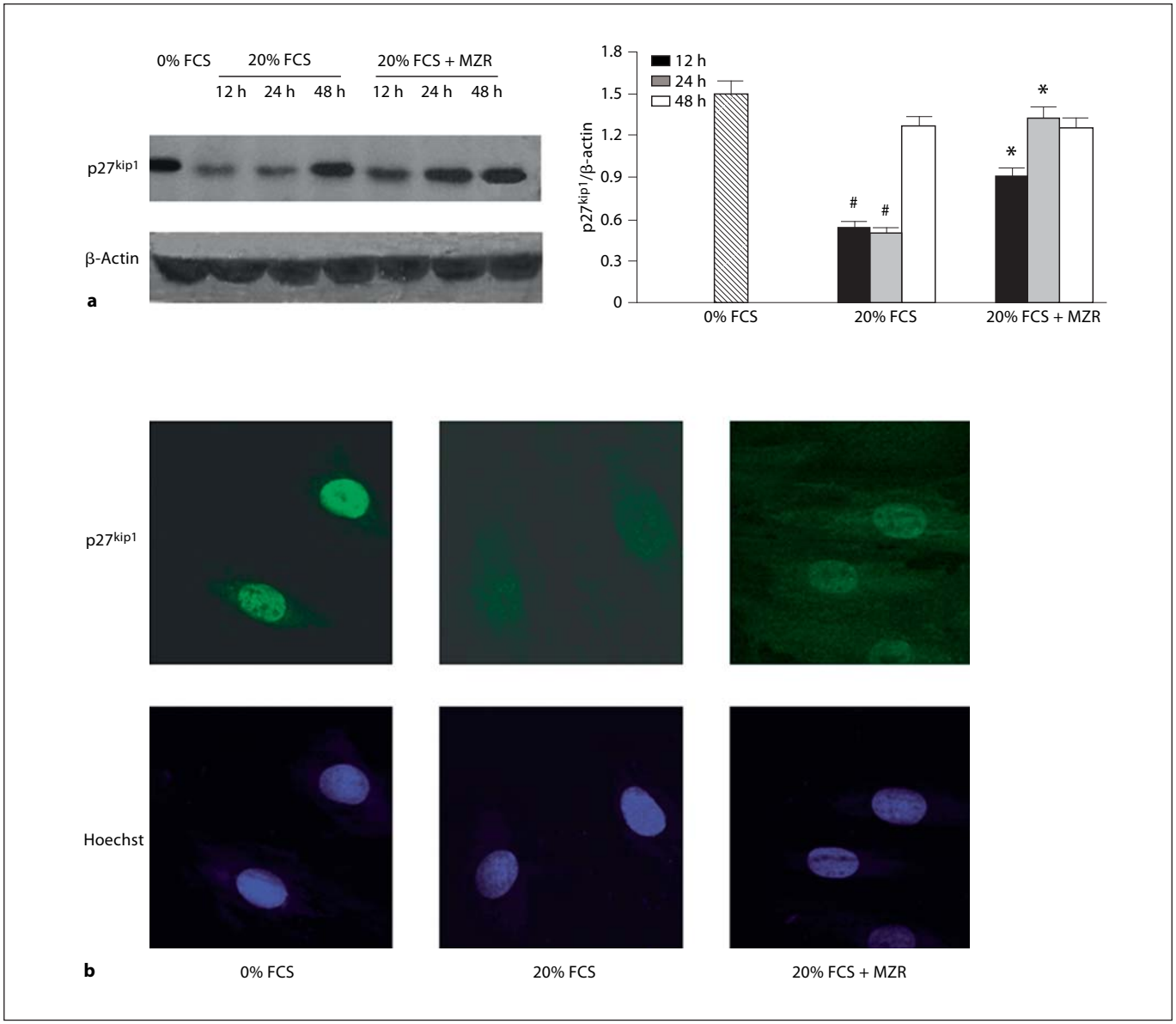


Fig. 3. MZR dramatically increased the negative cell cycle regulatory protein $p27^{kip1}$ protein level. **a** Western blot analysis showed that the $p27^{kip1}$ protein level was high in quiescent MCs, notably reduced at 12 and 24 h after FCS stimulation, and back to baseline at 48 h. MZR treatment (5 μg/ml) led to a dramatic increase in the $p27^{kip1}$ protein level. Normalized signals from three independent experiments are shown in the bar charts. # $p < 0.05$ vs. growth-arrested cells; * $p < 0.05$ vs. 20% FCS-stimulated cells. **b** Indirect immunofluorescence staining suggested that $p27^{kip1}$ was located in the nucleus in quiescent cells, whereas in FCS-stimulated cells, it was predominantly in the cytoplasm with significantly reduced fluorescence intensity, and MZR maintained the nucleus location and fluorescence intensity of $p27^{kip1}$. $p27^{kip1}$ immunofluorescence staining was observed under a laser scanning confocal microscope (×600).

component protein of the SCF complex during the cell cycle, did not show any change in expression in plasmid-transfected cells. These results suggest that the MZR-induced $p27^{kip1}$ accumulation was at least partly mediated by Skp2.

Discussion

In the present study, we demonstrated: (1) that MZR inhibits MC proliferation in culture conditions; (2) that MZR decreases expression of cyclin D and CDK2-positive

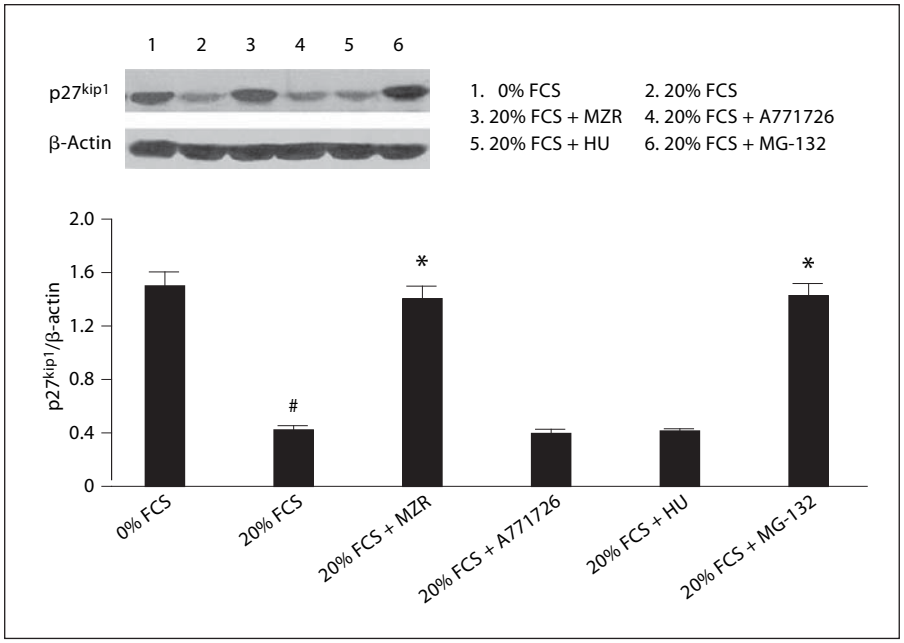


Fig. 4. Effect that MZR increased $p27^{kip1}$ protein was not dependent on DNA synthesis inhibition. MZR could increase the $p27^{kip1}$ protein level (lane 3), and the results were the same as those for proteasome inhibitor MG-132 (lane 6). DNA synthesis inhibitors A771726 (lane 4) and HU (lane 5) did not increase the $p27^{kip1}$ protein level similarly to MZR. Normalized signals from three independent experiments are shown in the bar charts. # $p < 0.05$ vs. growth-arrested cells; * $p < 0.05$ vs. 20% FCS-stimulated cells.

cell cycle regulatory proteins, and (3) that MZR increases expression of $p27^{kip1}$-negative cell cycle regulatory proteins. This increase in $p27^{kip1}$ happens without the increase in $p27^{kip1}$ mRNA levels and does not depend on DNA synthesis inhibition due to decreased degradation of $p27^{kip1}$. Skp2 is a protein-promoting degradation of $p27^{kip1}$ by enhancing the ubiquitin-proteasome pathway. We demonstrated that: (a) MZR downregulated levels of Skp2 with reciprocal increase in $p27^{kip1}$ levels, and (b) the MZR-induced increase in $p27^{kip1}$ was inhibited in cells that overexpressed Skp2. By inhibition of Skp2 expression, $p27^{kip1}$ is increased leading to the end result of decreased MC proliferation in vitro. Skp2 might be a novel target of the inhibitory effect of MZR on MC proliferation.

In vitro, MZR dose dependently inhibited the proliferation of MCs and prevented FCS-stimulated cells from entering S phase. Cell cycle analysis showed that MZR induced G1/S arrest. A previous study by Metz et al. [12] demonstrated that MZR reduced the incorporation of BrdU into pancreatic β cells in response to glucose, serum, or ketoisocaproate. MZR also inhibited T-lymphocyte proliferation and activation by guanine nucleotide depletion [13].

Transition from G1 into S is a coordinated, sequential process that is tightly regulated by the corresponding cyclin-CDK-CKI system. The main cell cycle regulatory proteins dominating G1/S progression are cyclin D, CDK2 and $p27^{kip1}$. Our results demonstrated that MZR decreased the expression of cyclin D and CDK2, while dramatically increasing the negative cell cycle regulatory protein $p27^{kip1}$ level during MC proliferation, suggesting that MZR inhibited MC proliferation via a cell cycle regulatory protein-dependent mechanism. Accumulating evidence has demonstrated that $p27^{kip1}$ is a crucial determinant of the renal response to immune or non-immune forms of injury. In experimental glomerulonephritic $p27^{kip1}$ –/– mice, the onset of glomerular cell proliferation occurred earlier, and the magnitude of proliferation was greater compared with $p27^{kip1}$ +/+ mice [3]. Thus, we postulated that the $p27^{kip1}$ accumulation largely contributed to the inhibitory effect of MZR on MC proliferation. MZR increased the $p27^{kip1}$ protein level and nucleus location during MC proliferation, thus maintaining the inhibitory effect of $p27^{kip1}$ on cyclins and CDKs, and therefore cells could not pass the 'restriction point' and go into the S phase. Other DNA synthesis inhibitors such as A771726 and HU could not increase the $p27^{kip1}$ protein level like MZR. This result demonstrated that $p27^{kip1}$ accumulation was not a universal phenomenon in DNA synthesis inhibitors.

In contrast to the increase in $p27^{kip1}$ protein level, MZR produced no changes in the abundance of $p27^{kip1}$ mRNA. This discrepancy suggests that stabilization of the $p27^{kip1}$ protein was responsible for the accumulation of $p27^{kip1}$. We further confirmed that MZR impaired $p27^{kip1}$ degradation through the ubiquitin-proteasome pathway. Much evidence has strongly suggested that the

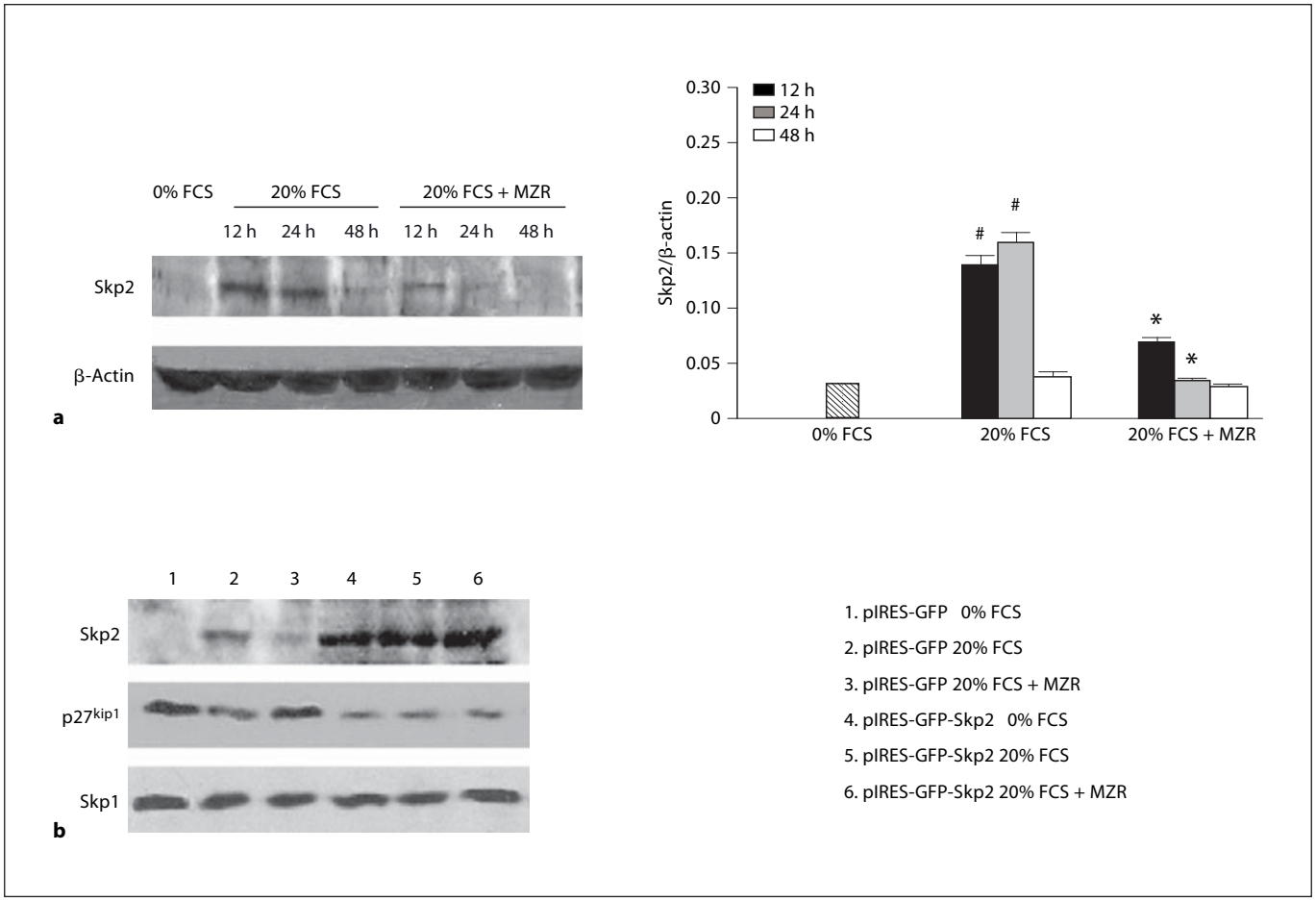


Fig. 5. Skp2 expression decreased in MZR-treated MCs, and Skp2 overexpression abolished the MZR-induced increase in $p27^{kip1}$ protein. **a** Skp2 protein expression was almost undetectable in quiescent MCs and was dramatically increased at 12 and 24 h after FCS stimulation. MZR significantly downregulated Skp2 expression at 12 and 24 h. Normalized signals from three independent experiments are shown in the bar charts. # $p < 0.05$ vs. growth-arrested cells; * $p < 0.05$ vs. 20% FCS-stimulated cells. **b** The transfection of the pIRES-GFP-Skp2 plasmid in MCs induced an elevated expression of Skp2 (upper panel, lanes 4–6) compared with pIRES-GFP null plasmid-transfected cells (upper panel, lanes 1–3). Immunoblot analysis showed that $p27^{kip1}$ accumulation was abolished in Skp2-overexpressed cells, while the levels remained constant in null plasmid-transfected cells. In contrast, Skp1, a table component protein of the SCF complex during the cell cycle, did not show any change in expression in plasmid-transfected cells.

intracellular concentration of $p27^{kip1}$ is regulated predominantly by Skp2, an F-box protein, through the ubiquitin-proteasome pathway [14, 15]. Mice lacking Skp2 have shown intracellular accumulation of $p27^{kip1}$, suggesting a pivotal role of Skp2 in the $p27^{kip1}$ degradation steps [16]. Thus, it was hypothesized that the MZR-induced increase in $p27^{kip1}$ protein should result from the decrease in Skp2 expression. Just as we postulated, MZR significantly downregulated Skp2 expression in concert with a reciprocal increase in the $p27^{kip1}$ protein level. Further study demonstrated that Skp2 overexpression abolished the MZR-induced $p27^{kip1}$ accumulation. These results suggest that the MZR-induced $p27^{kip1}$ accumulation was at least partly mediated by Skp2. Overexpression of Skp2 mRNA and protein levels was observed in many aggressive cancers and was commonly associated with downregulation of $p27^{kip1}$ levels and loss of tumor differentiation [17]. In addition, it has been reported that altered Skp2 levels are a major mediator of vascular smooth muscle cell proliferation in vitro [18] and in vivo [19]. Skp2-mediated $p27^{kip1}$ degradation also plays an essential role in growth and adaptive expansion of pancreatic β

cells [20]. In kidney, Suzuki et al. [21] reported that proliferation of tubular cells, which is a characteristic feature in obstructed kidneys, was significantly decreased in Skp2 –/– mice with obstructive nephropathy, with much greater $p27^{kip1}$ accumulation than in Skp2 +/+ mice. However, the above mechanism was studied only in the MC culture model. The mechanism of MZR action could be different between in vivo and in vitro, MCs and other cells (e.g. lymphocytes). In addition, whether Skp2 could directly regulate MC proliferation and the mechanism of downregulated Skp2 expression in MZR-treated MCs was not fully elucidated in our study.

In summary, our study demonstrated that MZR inhibits MC proliferation by downregulating Skp2 expression, consequently decreasing $p27^{kip1}$ degradation and resulting in $p27^{kip1}$ accumulation in vitro. Skp2 might be a novel target of the inhibitory effect of MZR on MC proliferation.

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Conflict of Interest Statement

None declared.

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