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Original Article

NGAL regulates extracellular matrix related factors and cell cycle distribution in mesangial cells: possible roles in the progression of renal fibrosis

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Abstract: This study aimed to examine the effects of neutrophil gelatinase-associated lipocalin (NGAL) on extracellular matrix (ECM) secretion and cell cycle of human mesangial cells (HMC), and to investigate the possible roles of NGAL in the progression of renal fibrosis. The effects of NGAL on secretion and cell cycle of HMC and the potentially involved mechanisms (fibronectin, collagen IV, CTGF, p44/42MAPK, phospho-smad2/3, smad2/3, smad7, CDK2, CDK4, and phospho-AKT/AKT) were investigated by immunoblotting, real-time PCR, siRNA, immunofluorescence, CCK8 assay, cell cycle analysis, and apoptosis. NGAL (50 ng/ml) induced the mRNA and protein expression of fibronectin, collagen IV and CTGF. NGAL activated the Smad2/3 pathway but had no effect on MAPK pathway. When NGALR was down-regulated by siRNA in HMC, the increased protein expression of collagen IV and CTGF by NGAL was attenuated. Incubation of cells with NGAL caused a G0/G1 stagnation in cell cycle progression, but no increased apoptosis. Cyclin D1, p21, and p27 were increased and CDK2 was decreased in HMCs treated with 50 ng/ml NGAL. There was no change in CDK4. The PI3K/Akt kinase pathway was inactivated by NGAL. NGAL could induce human mesangial cells to secrete ECM (fibronectin, collagen IV) and CTGF, this process involves activation of the Smad2/3 protein. And NGAL caused G0/G1 stagnation through increased cyclin D1, p21, and p27 and decreased CDK2.

Keywords: Neutrophil gelatinase-associated lipocalin, mesangial cells, extracellular matrix, glomerulonephritis, fibrosis

Introduction

Chronic kidney disease (CKD) is characterized by abnormalities of kidney structure or function that are present for >3 months and have implication for health [1]. In the United States alone, 341 people per million start dialysis each year [1]. The prevalence of CKD is estimated to be 10.8% in China [2]. Aberrant cell cycle and secretion of extracellular matrix (ECM) by mesangial cells are key processes during the progression of nephropathy [3, 4].

Neutrophil gelatinase-associated lipocalin (NGAL) is a 25-kDa protein that belongs to the lipocalin superfamily [5]. NGAL was initially detected in activated neutrophils, but it was later found that many other types of cells may produce NGAL in response to various injuries [5]. NGAL levels are significantly increased in patients with acute kidney injury (AKI) 24 to 48 h before a detectable increase of serum creatinine [6, 7]. Patients with systemic lupus erythematosus, IgA nephropathy, membranous (MGN), membranoproliferative glomerulonephritis (MPGN), or cardiorenal syndrome have higher urinary and serum NGAL levels compared to normal controls [8-10]. Urinary NGAL is associated with severity of renal disease in proteinuric patients [5]. Plasma NGAL demonstrated a higher diagnostic value to detect kidney impairment in the early stages of CKD as compared to serum cystatin C and serum creatinine in hypertensive patients [11]. For all these reasons,

NGAL may become one of the most promising next-generation biomarkers in clinical nephrology [12, 13]. Recent evidence suggests that NGAL somehow may be involved in the pathophysiological process of CKD [12, 14-17]. NGAL can promote renal tubular cell proliferation and increase renal tubular damage degree [18].

Mesangial cells are smooth muscle-like pericytes that abut and surround the filtration capillaries within the glomerulus. Mesangial areas are a source of kidney cytokines and play very important roles in the pathophysiological process of glomerulonephritis [19]. However, there is no study that has investigated the relationship between NGAL and mesangial cells. According to our previous study, the basal mRNA levels of NGAL could be detected in human mesangial cells (HMC) and the protein level of NGAL is aberrant, even with IL-1β treatment [20].

Therefore, the present study aimed to examine the effects of NGAL on ECM secretion and cell cycle of HMC, and to investigate the possible roles of NGAL in the progression of renal fibrosis.

Materials and methods

Cell culture

HMC were purchased from ScienCell Research Laboratories (Carlsbad, CA, USA). HMC were incubated in RPMI-1640 with 10% fetal bovine serum (Biowest, Teco Medical, Sissach, Switzerland) at 37°C in 95% air and 5% CO_2 at a concentration of 2×10^5 cells/2 ml in a 6-well tissue culture plate. After 24 h, the HMC were treated with 50 ng/ml NGAL (R&D Systems, Minneapolis, MN, USA) for various durations. The DLD1 and EC18 cell lines were purchased from the Cell Resource Center of the Shanghai Academy of Life Sciences (Shanghai, China).

Total RNA isolation and RT-PCR

Total RNA was extracted from cells with TRIzol (Invitrogen Inc., Carlsbad, CA, USA). First-strand cDNA was reverse transcribed from 2 μg of total RNA in a total volume of 20 μl using the Reverse Transcription System (Takara Bio, Otsu, Japan), according to the manufacturer's instructions.

Semiquantitative PCR was performed using Taq DNA polymerase (Takara Bio, Otsu, Japan) in a Bio-Rad S1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA. After initial denaturation for 5 min at 94°C, cDNA was subjected to 30 cycles of PCR.

The oligonucleotide primers designed for fibronectin, collagen IV, CTGF, and β-actin were: fibronectin, 5'-CCA GGA GAC TGT GAG CAC TGG-3' and 5'-CTC CAA GTA CCC CCT GAG GAA-3'); collagen IV, 5'-TGG CGC ACT TCT AAA CTC CT-3' and 5'-CTC TAC GTG CAA GGC AAT GA-3'; CTGF, 5'-CGG ATG CAC TTT TTG CCC TT-3' and 5'-GCT TAC CGA CTG GAA GAC ACG-3'; and β-actin, 5'-CAA CTG GGA CGA CAT GGA GAA A-3' and 5'-GAT AGC AAC GTA CAT GGC TGG G-3'. Each primer spanned a different exon to avoid amplification of contaminating genomic DNA.

Real-time quantitative PCR was performed using Rotor-Gene 3000 (Corbett Research, Australia). Briefly, each PCR mixture contained cDNA, Premix ExTaq (Takara), and a primer pair in a final volume of 20 μl. After activation of Taq polymerase at 95°C for 10 s, PCR was performed for 40-50 cycles, with each cycle consisting of a denaturation step at 95°C for 5 sec, annealing, and extending at 60°C for 30 sec. The amount of target gene expression was calculated from the standard curve, and quantitative normalization of fibronectin, collagen IV, and CTGF cDNA in each sample was performed using β-actin as an internal control.

Western blot

Cells were lysed quickly using 1x SDS buffer on ice, boiled for 5 min and then stored at -80°C until use. Samples were subjected to 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes (Millipore corp., Billerica, MA, USA). The membranes were probed with antibodies against NGAL receptor (NGALR) (polyclonal rabbit anti-human) (Prosci Inc, Poway, CA, USA), NGAL (polyclonal rabbit anti-human), fibronectin (monoclonal rabbit anti-human), collagen IV (monoclonal rabbit anti-human), CTGF (monoclonal rabbit anti-human) (Abcam, Cambridge, MA, USA), phospho-p44/42MAPK (monoclonal rabbit anti-human) (Cell Signaling, Danvers, MA, USA), p44/42MAPK (monoclonal rabbit anti-human) (Cell Signaling, Danvers, MA, USA), phospho-smad2/3 (monoclonal rabbit antihuman) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), smad2/3, smad7, (Cell Signaling, Danvers, MA, USA), CDK2 (monoclonal mouse

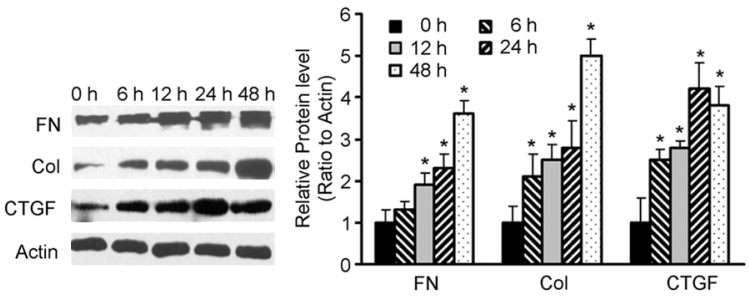

Figure 1. Increased expression of fibronectin, collagen IV, and CTGF proteins in HMC stimulated by NGAL. HMCs were stimulated using NGAL (50 ng/ml) for the indicated durations. Data are shown as the mean ± SD of three independent experiments. **P<0.05*, versus 0 hour.

anti-human), CDK4 (monoclonal rabbit antihuman) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-AKT/AKT (Abcam, Cambridge, MA, USA), Cy3-IgG (monoclonal goat antihuman), rhodamine-IgG (monoclonal goat antihuman) (Proteintech Group inc., Chicago, IL, USA) and β-actin (monoclonal mouse antihuman) (Sigma, St Louis, MO, USA) as a loading control for 1 h at 37°C, then incubated overnight at 4°C. Following incubation with HRP-conjugated secondary antibodies (Cell Signaling, Danvers, MA, USA), the immunoreaction was visualized by enhanced chemiluminescence and film exposure. Each experiment was repeated at least three times.

siRNA transfection

The NGALR siRNA1 and 2 (Invitrogen Inc., Carlsbad, CA, USA) transfection for HMC cells was performed according to the manufacturer's instruction using siRNA Transfection Reagent (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Briefly, 1×10^5 cells in 1 ml of complete medium were seeded into each well of an uncoated six-well plate 24 h before transfection. After 24 h of incubation, the medium was changed to 800 μl of siRNA Transfection Medium and 6 μl of NGALR siRNA1/2 or negative control siRNA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) in 200 μl of siRNA Transfection Medium containing 6 μl of siRNA transfection reagent. After 5-7 h of incubation, 1 ml of complete medium (containing 20% fetal serum) was added into each well and the cells were incubated. After 24 or 48 h post-transfection, the cells were incubated in RPMI-1640 with 10% fetal serum with 50 ng/ml NGAL (R&D Systems, Minneapolis, MN, USA) for 48 or 72 h.

Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometry. Cells were harvested after 24 h, and treated 24 h or 48 h with 50 ng/ml NGAL. Then, the cells were washed in cold PBS two times. The cells were resuspended cells in 500 μl of PBS and fixed in 700 μl of 70% cold ethanol at 4°C overnight. Flow cytometry was performed with an Epic flow cytometry system (Beckman Coulter, Brea, CA, USA). The experiment was repeated three times.

Apoptosis analysis

Apoptosis was detected using a fluorescein isothiocyanate (FITC) Annexin V staining kit (Life Technologies, Grand Island, NY, USA) followed by fluorescence-activated cell sorting (FACS) analysis, according to the manufacturer's protocol.

Cell proliferation analysis

HMC cells were seeded at 3000 cells/well in three 96-well plates. After 24 h, 50 ng/ml NGAL was added and cell proliferation was assayed with 10 μl of CCK8 (Dojindo Molecular Technologies, Kimamoto, Japan) at 12, 24, and 48 h).

Statistical analysis

Data are expressed as the mean ± standard deviation. Statistics were calculated using SPSS software. The Student's t test was applied to compare data between the 2 groups, and analysis of variance (ANOVA) was applied to compare data among multiple groups. Differences in means were evaluated by a two-tailed t-test assuming unequal variances. A *P*-value of < 0.05 was defined as statistically significant.

Results

Elevated protein levels of fibronectin, collagen IV, and CTGF in HMC stimulated by NGAL

According to previous studies, we chosed 50 ng/ml NGAL for stimulating HMC for 6, 12, 24,

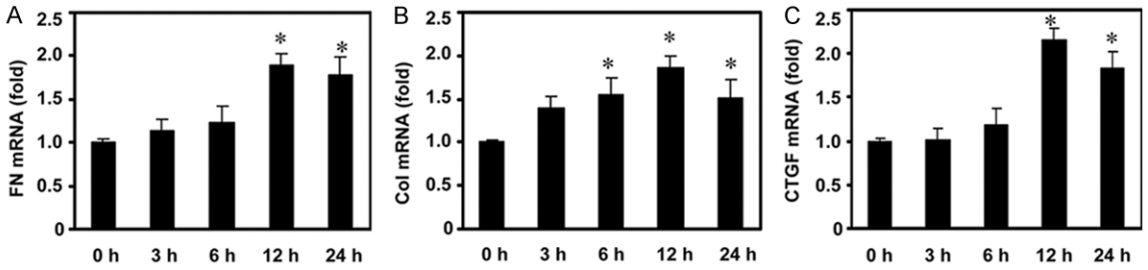

Figure 2. NGAL induced the mRNA expression of fibronectin (A), collagen IV, (B) and CTGF (C). HMC were exposed to NGAL (50 ng/ml) for the indicated durations. The results show the time-dependent up-regulation of these proteins mRNA expression by NGAL. Relative levels of individual mRNAs were normalized to those of β-actin mRNA. Data are shown as the mean ± SD of three independent experiments. *$P<0.05$, versus 0 hour.

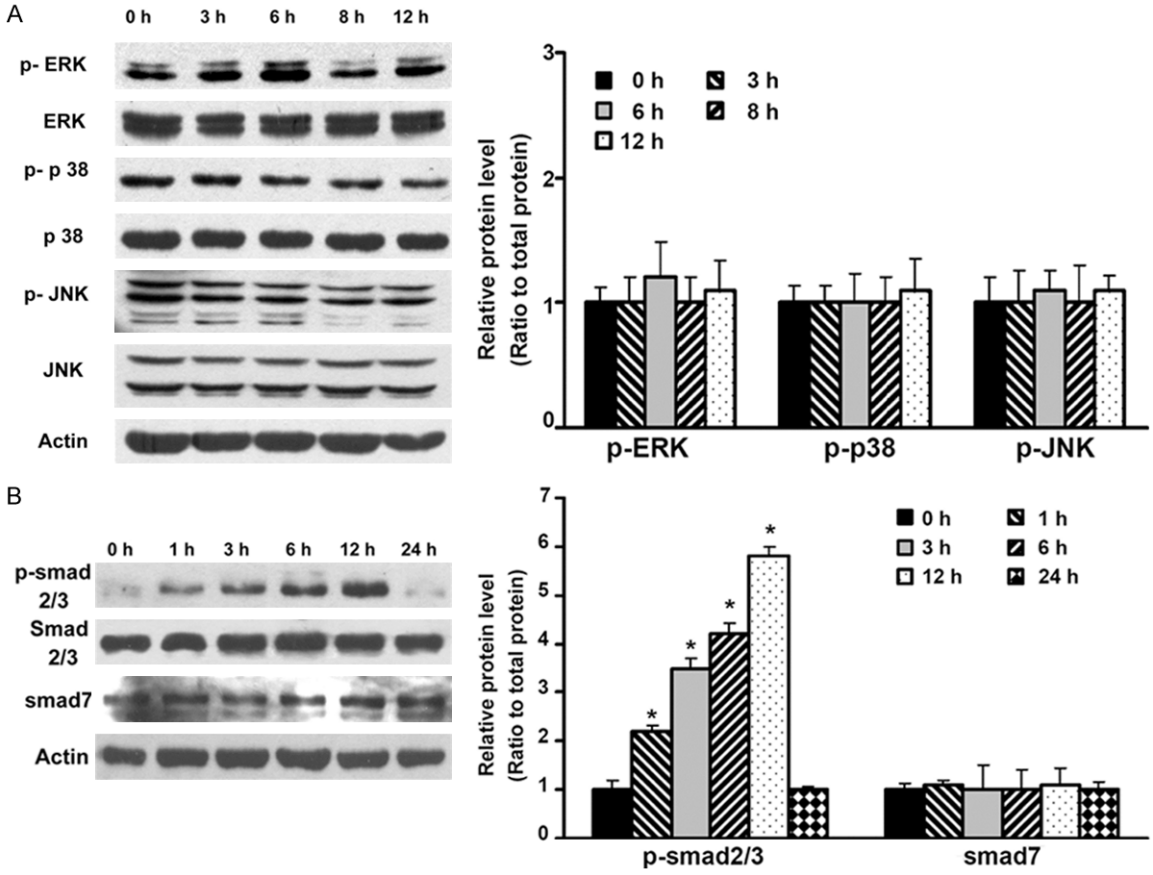

Figure 3. NGAL induced the activation of Smad2/3. Cells were stimulated with NGAL (50 ng/ml) for the indicated durations. A. The relative ERK, p-ERK, JNK, p-JNK, p38, and p-p38 protein levels were determined by western blotting. B. The relative levels of Smad2/3, p-Smad2/3, and Smad7 were determined by western blotting. β-actin was used as a loading control. Data are shown as the mean ± SD of three independent experiments. *$P<0.05$, versus 0 hour.

and 48 h [21]. The protein levels of fibronectin, collagen IV, and CTGF were up-regulated in a time-dependent manner in HMC stimulated by NGAL. The expression of fibronectin, collagen IV, and CTGF reached a peak at 48 h, 48 h, and 24 h, respectively ($P<0.05$) (**Figure 1**).

NGAL induced the mRNA expression of fibronectin, collagen IV, and CTGF

HMC exposed to NGAL (50 ng/ml) for 6, 12, 24, and 48 h. As determined by real-time PCR, the mRNA expression of fibronectin, collagen IV,

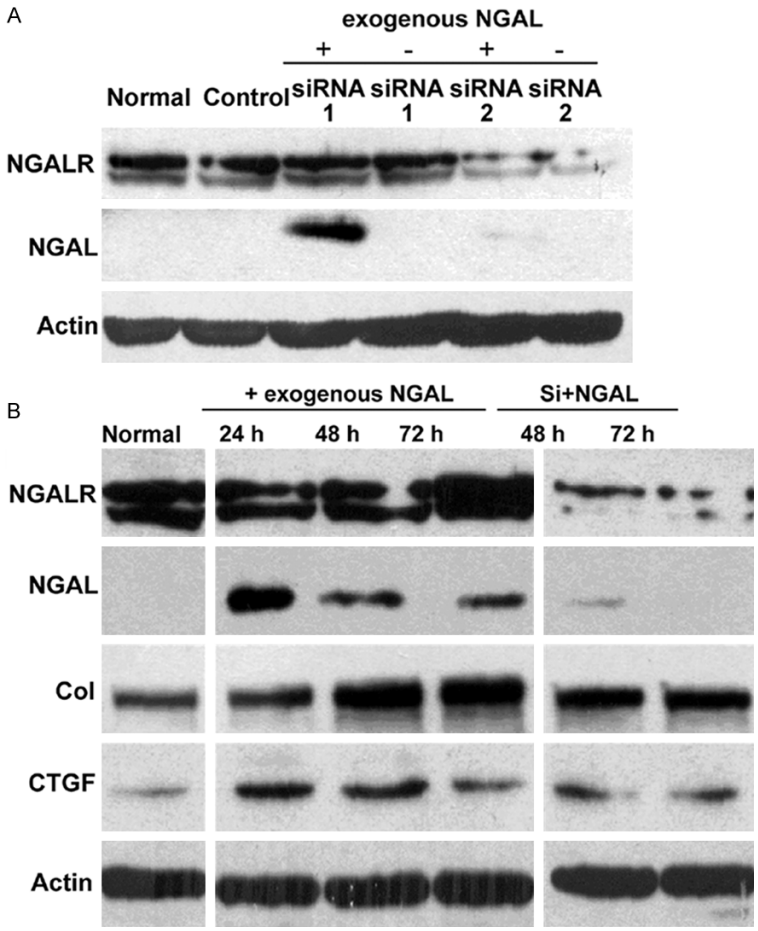

Figure 4. Effects of down-regulation of NGALR in HMC. A. Based on the expression of NGALR and NGAL in HMC, siRNA2 was the best to down-regulate NGALR expression. Control, transfected with control siRNA; +, cultured with exogenous NGAL (50 ng/ml). B. HMC transfected with NGALR siRNA were exposed to 50 ng/ml NGAL for 48 and 72 h, using the administration of exogenous recombinant NGAL (50 ng/ml) in cells as positive control and normal cells as negative control. si+NGAL, transfected with NGALR siRNA and cultured with exogenous NGAL (50 ng/ml). The increased protein expression of collagen IV and CTGF by cultured HMCs with NGAL is attenuated by siRNA NGALR.

and CTGF were increased in a time-dependent manner in HMC stimulated by NGAL. The expression of fibronectin, collagen IV, and CTGF reached a peak at 12 h ($P<0.05$) (**Figure 2**).

NGAL induced the activation of Smad2/3

Studies showed that MAPK and Smad signaling pathways are involved in the metabolic process of ECM [22-25]. To further understand the signaling pathways involved in NGAL playing its biological role in HMC, We carried out the following experiments: Cells were stimulated with NGAL (50 ng/ml) for 1, 3, 6, 8, and 12 h. The MAPK and Smad signaling pathways were examined by determining the changes of the related protein expression levels. The expression of ERK, p-ERK, JNK, p-JNK, p38, and p-p38 were determined by western blotting. These proteins in HMC stimulated by NGAL do not change obviously compared with control HMC ($P>0.05$) (**Figure 3A**). The levels of Smad2/3, p-Smad2/3, and Smad7 were also determined by western blotting. The activation of Smad2/3 was induced by NGAL, while Smad7 was not. pSmad2/3 was up-regulated at 1 h after NGAL stimulation, peaked at 12 h, and fell back to normal at 24 h ($P<0.05$) (**Figure 3B**). We conclude that this may be related to feedback inhibition and that when the amount of Smad2/3 in the cell is sufficient, it is regulated by the negative feedback of the organism.

Down-regulation of NGALR related to the induction decreased of NGAL in HMC

As a specific receptor, NGALR plays a key role in the biological effects of NGAL on HMC. When the two are combined, the NGAL can move into HMC and work further. Therefore, when the expression of NGALR is inhibited, the binding between the two is bound to decrease, which leads to the fact that NGAL cannot effectively enter HMC, and its biological effects will be affected. In order to prove our inference, we carried out the following experiments: the two types of siRNA were used to down regulate NGALR in HMC for 48 h, then these cells were exposed to NGAL (50 ng/ml) for 24 h. These results of western blot showed that compared with siRNA1, the interference effects of siRNA2 on NGALR protein expression of HMC had a higher level. The amount of NGAL entering HMC decreased significantly was associated with the down regulation of expres-

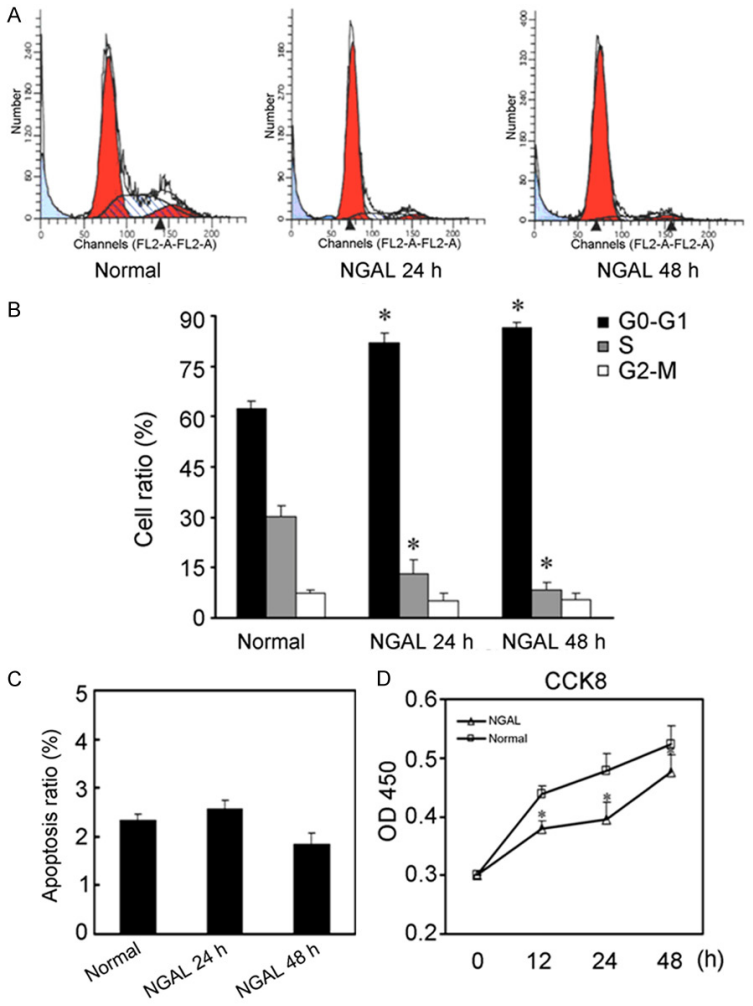

Figure 5. Changes of cell cycle distribution in HMC treated with NGAL. A. Cells were harvested after 24 h, and treated for 24 or 48 h with 50 ng/ml NGAL. Cell cycle distribution was analyzed by flow cytometry. B. The percentage of cells present in G0/G1, S, and G2/M phases. C. Fluorescence-activated cell sorting shows no increased apoptosis in HMC cultured with 50 ng/ml NGAL for different durations. D. NGAL decreases HMC proliferation as determined by the CCK8 assay. The cells were cultured with 50 ng/ml NGAL for 12, 24, or 48 h. Data are shown as the mean ± SD of three independent experiments. *$P<0.05$, versus 0 hour.

sion of NGALR induced by siRNA2 (**Figure 4A**). Following, after NGALR was down-regulated by siRNA2 for 24 h, HMCs were exposed to NGAL (50 ng/ml) for 48 and 72 h. The increased protein expression of collagen IV and CTGF by NGAL was attenuated by siRNA against NGALR, according to western blot (**Figure 4B**).

Cell cycle distribution of HMC treated with NGAL

In chronic kidney disease, there is usually a change in the MC cell cycle, so we also carried out a preliminary study on this. HMC were harvested after 24 h, treated for 24 or 48 h with 50 ng/ml NGAL, and analyzed by flow cytometry. Incubation of HMC with NGAL caused a G0/G1 stagnation in cell cycle progression and no increased apoptosis. The ratio (%) of G0/G1 phase cells increased from 62.1±3.1% to 81.6±4.3% at 24 h and 86.7±3.6% at 48 h ($P<0.05$). The ratio (%) of S phase cells decreased from 30.1±3.0% to 13.1±6.3% at 24 h and 8.5±2.3% at 48 h ($P<0.05$), while the proportion of G2/M cells remained unchanged (**Figure 5A**, **5B**). There was no increased apoptosis in HMC cultured with 50 ng/ml NGAL for different times (**Figure 5C**). NGAL decreased HMC proliferation as determined by CCK8 assay. The cells were cultured with 50 ng/ml NGAL for 12, 24, or 48 h. The OD450 values were down to 0.38±0.01, 0.40±0.03, and 0.47±0.02, respectively, from 0.43±0.01, 0.50±0.02, and 0.52±0.01 at 12, 24, and 48 h, respectively (**Figure 5D**).

Impact of NGAL on cyclin proteins in HMC

NGAL regulated the protein expression of G1 phase cyclins and cyclin-dependent kinase inhibitors (CDKI) in HMC. Using western blotting, cyclin D1, p21, and p27 were increased, whereas CDK2 was decreased in HMCs treated with 50 ng/ml NGAL. CDK4 did not change. Expression of β-actin protein was examined as a quantity loading control (**Figure 6A**). Cells were stimulated with 50 ng/ml NGAL for 1, 3, and 6 h, respectively. The PI3K/Akt kinase pathway was inactivated in the progression and peaked at 6 h ($P<0.05$) (**Figure 6B**).

Discussion

The mechanisms of progression of CKD are poorly understood. Viau et al. [18] used two mouse strains that react differently to nephron

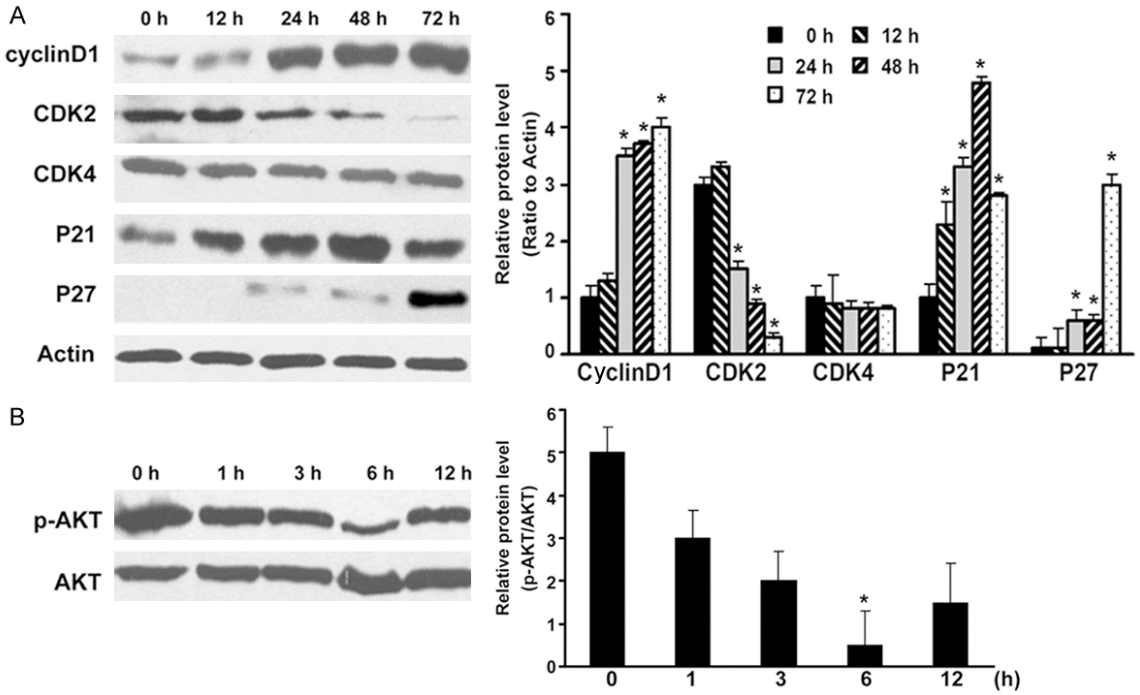

Figure 6. Impact of NGAL on cyclin proteins in HMC. A. NGAL regulates protein expression of the G1 phase cyclins and cyclin-dependent kinase inhibitors (CDKI) in HMC. Using western blotting, cyclin D1, p21 and p27 were shown to be increased, whereas CDK2 was decreased in HMCs treated with 50 ng/ml NGAL. There was no change in CDK4 levels. β-actin was used as a quantity loading control. B. Inactivation of the AKT kinase pathway by NGAL. Cells were stimulated with 50 ng/ml NGAL for 1, 3, and 6 h. Data are shown as the mean ± SD of three independent experiments. *$P<0.05$, versus 0 hour.

reduction (the FVB/N mice, which develop severe renal lesions, and the B6D2F1 mice, which are resistant to early deterioration) coupled with genome-wide expression to explore the molecular nature of CKD progression. The results showed that NGAL, the most highly upregulated gene in the FVB/N strain, was not simply a marker of renal lesions, but an active player in disease progression. In addition, NGAL is involved in the pathophysiolog ical process of polycystic kidney disease and glomerulonephritis [26]. Therefore, this study aimed to examine the effects of NGAL on ECM secretion and cell cycle of HMC, and to investigate the possible roles of NGAL in the progression of renal fibrosis. Results showed that NGAL induced the mRNA and protein expression of fibronectin, collagen IV, and CTGF by activating Smad2/3. NGAL caused G0/G1 stagnation through increased cyclin D1, p21, and p27 and decreased CDK2.

Glomerular mesangial cells are the source of a variety of cytokines, are a target for a variety of cytokines, and play many important physiological functions such as ECM secretion. Through contraction and expansion, they are involved in the regulation of glomerular hemodynamics. In pathological conditions, mesangial cells release a variety of ECM components, which themselves promote the proliferation of mesangial cells, thus increasing renal damage. ECM components are closely associated with renal fibrosis and mainly included fibronectin and collagen IV. Excess ECM is detected in various glomerular diseases [27, 28]. CTGF is also a very important cytokine in the process of fibrosis. In the present study, HMC were stimulated with NGAL and the protein levels of fibronectin, collagen IV, and CTGF were up-regulated in a time-dependent manner. Silencing of NGALR attenuated the upregulation of these proteins when HMC are submitted to NGAL.

MAPK and Smad signaling pathways are involved in ECM metabolism [22-25]. In the present study, Smad2/3 was activated, while the MAPK pathway showed no reaction to NGAL

challenge. The cell cycle is coordinated by positive (cyclins and cyclin-dependent kinases) and negative (cyclin-dependent kinase inhibitors) regulators [29, 30]. Under normal conditions, mature glomerular cells have a low turnover rate but under pathological conditions, a variety of pathophysiological stimuli can lead to disturbances in glomerular cell biology (including toxins, immune-mediated stresses, metabolic derangements, drugs, infections, hemodynamic changes, growth factors, and cytokines) and the responses are coordinated by the same regulators [29, 31]. A study showed that kidney and glomerular hypertrophy caused by hyperglycemia was associated with specific G1 phase cell-cycle events such as early and sustained increase in expression of cyclin D1 and activation of cyclin D1CDK4 complexes [32]. High glucose levels also induce the NGAL pathway in mesangial cells, leading to kidney fibrosis [33]. Fan et al. [34] examined methods to attenuate p21 levels in wild-type kidney cells; attenuation of p21 in mesenchymal cells leads to a dose-dependent reduction of hypertrophy. In the present study, cyclin D1, p21Cip1, and p27Kip1 were increased by NGAL, while the expression of CDK2 was decreased, as supported by previous studies [32, 34, 35].

The PI3K/Akt signal transduction cascade has been investigated extensively for its roles in oncogenic transformation. PI3K/Akt signaling plays an important part in cell proliferation [36]. The PI3K/Akt pathway play a key role in cell cycle transition [37, 38] via target of rapamycin (TOR) [39]. The present study suggested that the activity of PI3K/Akt pathway was inhibited by NGAL, which may be related to cell arrest in G1/S phase. Also it was be observed that the expression of p-AKT was suppressed at 6 h and then increased again at 12 h. This was maybe because HMCs stimulated by exogenous NGAL were induced a stress response at first, later produced a feedback regulation. But the relationship between cell cycle regulatory proteins and PI3K/Akt signaling pathway, and which is more important, we still need further experiments to verify.

The present study had some limitations. It was solely performed in cells and these results will have to be confirmed in animal models. In addition, only a small panel of proteins was observed and more comprehensive analyses are necessary to grasp the mechanisms responsible for the effects of NGAL. Nevertheless, the present study provided important knowledge for our understanding of the progression of kidney diseases and could pave the way for new treatments.

In conclusion, our experiments revealed that NGAL can induce mesangial cells to secrete ECM and CTGF, this process involves activation of the Smad2/3 protein. And NGAL caused G0/G1 stagnation through increased cyclin D1, p21, and p27 and decreased CDK2.

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Disclosure of conflict of interest

None.

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