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Original Article

The role of Stim1 in the progression of lupus nephritis in mice

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Abstract: Objective: To investigate the expression of Stim1 in the kidneys of mice with lupus, and the effect of Stim1 on the progression of renal interstitial fibrosis. Methods: Mice (MRL/lpr) with spontaneous lupus nephritis (LN) and normal control mice (C57/BL) were selected. Immunohistochemistry and Masson staining were used to determine the degree of renal interstitial fibrosis in kidney tissues. The expression of Stim1 and fibronectin in tissues was measured by qRT-PCR, western blotting, and immunohistochemistry. Urine protein, blood urea nitrogen, and serum creatinine levels in the mice were analyzed, and Spearman analysis was conducted to determine the correlation with Stim1 expression levels. Mouse renal tubular epithelial cells (mRTECs) were chosen as the experimental objects. After various treatments, the cells were divided into the blank control group, lipopolysaccharide (LPS) treatment group, LPS+siRNA-NC group and LPS+siRNA-Stim1 group. Western blotting and immunofluorescence were used to measure epithelial-mesenchymal transition (EMT)-related protein levels. Results: There was significant interstitial fibrosis in the kidneys of LN mice. Compared with that in normal mice, the expression of Stim1 in the kidney tissues of LN mice was significantly increased, and Stim1 expression was positively correlated with fibronectin, urine protein, blood urea nitrogen and serum creatinine levels. LPS induced the expression of Stim1, fibronectin, and α-SMA in mRTECs and decreased the protein level of E-CA, while silencing Stim1 effectively alleviated the effects of LPS. Conclusion: Stim1 is significantly increased in the kidneys of lupus mice, and it is possible to promote EMT in renal tubular epithelial cells and renal interstitial fibrosis by elevating fibronectin, which ultimately contributes to renal damage.

Keywords: Lupus nephritis, renal interstitial fibrosis, EMT, Stim1

Introduction

Systemic lupus erythematosus (SLE) is a severe chronic autoimmune disease involving multiple organs that is not uncommon in young women, with recurrent episodes and remission alternating [1]. SLE pathogenesis is not obvious, and it is universally acknowledged to be a complex disease involving multiple gene regulation and multiple mechanisms [2]. The kidney is the most commonly involved organ in the pathogenesis of SLE. According to clinical statistics, the incidence of nephritis in lupus patients is 40%-70%, and nephritis is also the main cause of death in patients [3]. The immune complex formed by autoantibodies has a tendency to deposit in the glomerulus, causing renal damage through complement-mediated local inflammatory responses and cytokine- or chemokine-mediated infiltration of hematopoietic cells, ultimately leading to lupus nephritis (LN) [4]. LN is mostly characterized by glomerulonephritis, but it is often accompanied by renal tubular, renal interstitial, and capillary damage [5]. It has been reported that the incidence of tubulointerstitial fibrosis in LN is as high as 50% [6]. Renal tubulointerstitial fibrosis is not only a concomitant manifestation of glomerulopathy but may also be an independent participant in LN and is closely linked to the

prognosis of LN [7]. Therefore, renal tubulointerstitial lesions in LN are gaining attention in research.

Calcium ions (Ca^{2+}), a type of second messenger, transmit information in cells and participate in the regulation of a series of life activities [8]. Intracellular Ca^{2+} homeostasis is essential for maintaining the normal morphology and function of cells, and imbalanced Ca^{2+} homeostasis is closely related to a variety of diseases [9]. A study showed that in LN renal tubular interstitial fibrosis during the development of kidney tissue in the body or outside of the cell, calcium channel expression or intracellular Ca^{2+} concentration increased to different degrees, and a certain range of intracellular Ca^{2+} concentrations was positively related to the process of interstitial fibrosis [10]. Abnormal intracellular Ca^{2+} control in LN and the occurrence of renal tubular interstitial fibrosis development are likely to be effective targets for the prevention and control of LN. Stromal interaction molecule 1 (Stim1) is one of the major proteins that constitutes the calcium channel in the calcium storage and can maintain the dynamic balance in Ca^{2+} concentrations inside and outside the cell. When Stim1 expression is abnormal or dysfunctional, it leads to an imbalance in Ca^{2+} concentrations inside and outside the cell, resulting in the loss of homeostasis [11, 12]. Studies have confirmed that the abnormal expression of Stim1 is closely related to a variety of diseases [13]. In view of the relationship between the occurrence and development of renal tubulointerstitial fibrosis in SLE and the imbalance in Ca^{2+} homeostasis in cells, we believe that Stim1 may be abnormally expressed in renal tissue in LN and participate in the process of renal tubulointerstitial fibrosis in LN. This study aimed to observe the expression of Stim1 in the kidney tissues of normal mice and spontaneous lupus mice by using a variety of experimental methods, and to explore the regulatory effect of Stim1 on EMT by silencing the expression of Stim1 in renal tubular cells from mice using small interfering fragments. This might provide new effective targets for the prevention and treatment of LN.

Materials and methods

Animal samples and cell culture

MRL/lpr mice (LN mice) and C57/BL mice (negative control animals) were purchased from Jackson Laboratory (USA). All experiments for animal studies were performed in accordance with animal protocols approved by the Animal Care and Use Committee of Guizhou Medical University. The animals were raised in an SPF environment with controlled a temperature controlled at of 21±2°C, a relative humidity of approximately 55%, a freely available diet, and a light cycle of 12 h day and night. The urine was collected by the metabolic cage, and the urine protein content was determined by the Coomassie bright blue method. Bovine serum albumin (BSA) was used to generate the standard curve. The measured value was multiplied by the dilution ratio of the sample to determine the urine protein concentration. Following standard practices, blood urea nitrogen (BUN) and serum creatinine (CRE) concentrations were measured by a Hitachi-7080 fully automatic biochemical analyzer. Renal tubular epithelial cells (mRTECs) from normal mice were purchased from ATCC (USA). F-12 culture medium was placed in a cell culture flask and cultured in an incubator (37°C, 5% CO_2).

Histologic analysis

After the animals were sacrificed by intraperitoneal injection of an overdose of 0.6% sodium pentobarbital, tissue sections were obtained and fixed in 4% paraformaldehyde at postoperative days 7 and 14. The harvested tissues were gradually dehydrated and embedded in paraffin. Then, the paraffin-embedded tissues were sliced into 5-μm-thick sections, which were used for Masson's trichrome staining to observe the degree of collagen deposition.

Immunohistochemical staining

For immunohistochemical (IHC) staining, the harvested sections were deparaffinized and rehydrated. Subsequently, the deparaffinized sections were processed with secondary antibody and ABC complex following incubation with the primary antibody (Stim1, α-SMA, and E-cadherin (abbreviated E-CA); Proteintech, USA). Finally, the samples were visualized by the chromogenic substrate diaminobenzidine (DAB). An optical microscope (Olympus, Japan) was used to obtain images of the stained sections.

Western blotting

Proteins were extracted from fresh kidneys using a protein extraction kit (KeyGen Biotech, China) and quantified using a Pierce Rapid Gold BCA protein assay kit (Thermo Fisher Scientific, USA). Equivalent amounts of lysates were separated in SDS-PAGE gels and transferred onto PVDF membranes (Roche Applied Sciences, Switzerland). Then, the membranes were blocked in PBS-T containing 5% skim milk and incubated with primary antibodies overnight at 4°C, followed by incubation with the appropriate secondary antibodies (Bioworld Technology, USA). Signals were detected using a Pierce Fast Western blot kit and ECL substrate (Thermo Fisher Scientific, USA).

RNA extraction and quantitative real-time PCR

TRIzol reagent (Takara, Japan) was used to extract RNA from the tissue samples according to previously established protocols. Reverse transcription was performed with a reverse transcription kit (Takara, Japan). The expression of Stim1, fibronectin (FN), and GAPDH was analyzed by quantitative real-time PCR (qRT-PCR) using SybrGreen qPCR master mix (Takara, Japan). The expression levels were normalized to the expression of GAPDH. The sequences of PCR primers were as follows: Stim1 forward, 5'-TGACAGGGACTGTACTGAAGATG-3' and reverse, 5'-TATGCCGA-GTCAAGAGAGGAG-3'; FN forward, 5'-ATGGCGACGGTATTC TGTAAAG-3' and reverse, 5'-TTGGCAGTTGGTCAATCACAT-3'; and GAPDH forward, 5'-AGCTTCGGCACATATTTCATCTG-3' and reverse, 5'-CGTTCACTCCCAT-GACAAACA-3'.

Cell transfection

The mRTEC lines selected for transfection were inoculated into 6-well plates at (0.5-1) × 10^6 cells/well before transfection, and the plates were incubated for 12-18 h. The cells were transfected until they reached approximately 50-70% confluence. The old medium was discarded, the cells were washed with PBS 3 times, and 2 ml of new medium was added to each well. Liquid A consisted of 500 livability +10 livability lsiRNA, and liquid A and liquid B were mixed well at room temperature for 25 min. Then, the mixture was added to the 6-well plate, and was shaken and mixed gently. After 24-36 h, the cells were collected, and RNA was extracted for fluorescence quantitative PCR and western blot analysis to verify the interference efficiency. The medium was washed away with PBS, LPS was added, and the cells were incubated at 37°C. si-Stim1 was purchased from GenePharma, and the sequences were as follows: sense, 5'-GGAAGACCUCAAUUACCAUTT-3', antisense, 5'-AUGGUAAUUGAG-GUCUUCCTT-3', for Stim1, and sense, 5'-UUCUCCGAACGUGUCACGUTT-3', and antisense, 5'-ACGUGACACGUUCGGAGAATT-3', for the negative control.

Confocal immunofluorescence analysis

First, 4% paraformaldehyde prefixed-cells were rinsed in phosphate-buffered saline (PBS) and then incubated in PBS containing 0.5% Triton X-100 for 20 min. Afterwards, the samples were blocked in PBS containing 1% bovine serum albumin (BSA) for 30 min. The sections were incubated with primary antibodies overnight at 4°C. Subsequently, the sections were incubated with secondary antibodies conjugated to FITC or TRITC for 1 h at 37°C. Finally, the samples were incubated in methanol containing 1% DAPI. Images were captured using a confocal microscope (Olympus, Japan).

Statistical analysis

SPSS 20.0 statistical software was used to analyze the experimental data. Analysis of variance was used for comparison between groups, and Spearman correlation analysis was used to analyze the correlation between two variables. A value of $P<0.05$ was significant, and the data are expressed as the mean ± standard deviation ($x \pm s$).

Results

Analysis of renal tissue pathology in mice

Masson's trichrome staining is a classic technique for collagen fiber staining and can selectively distinguish between collagen fibers and muscle fibers [14]. To explore the differences in kidney morphology between lupus mice and normal mice, Masson's trichrome staining was performed on kidney sections from 6 lupus mice and 6 normal control mice, and immunohistochemical staining was used to verify the related indexes of renal tubulointerstitial fibrosis. Compared with that of the normal control

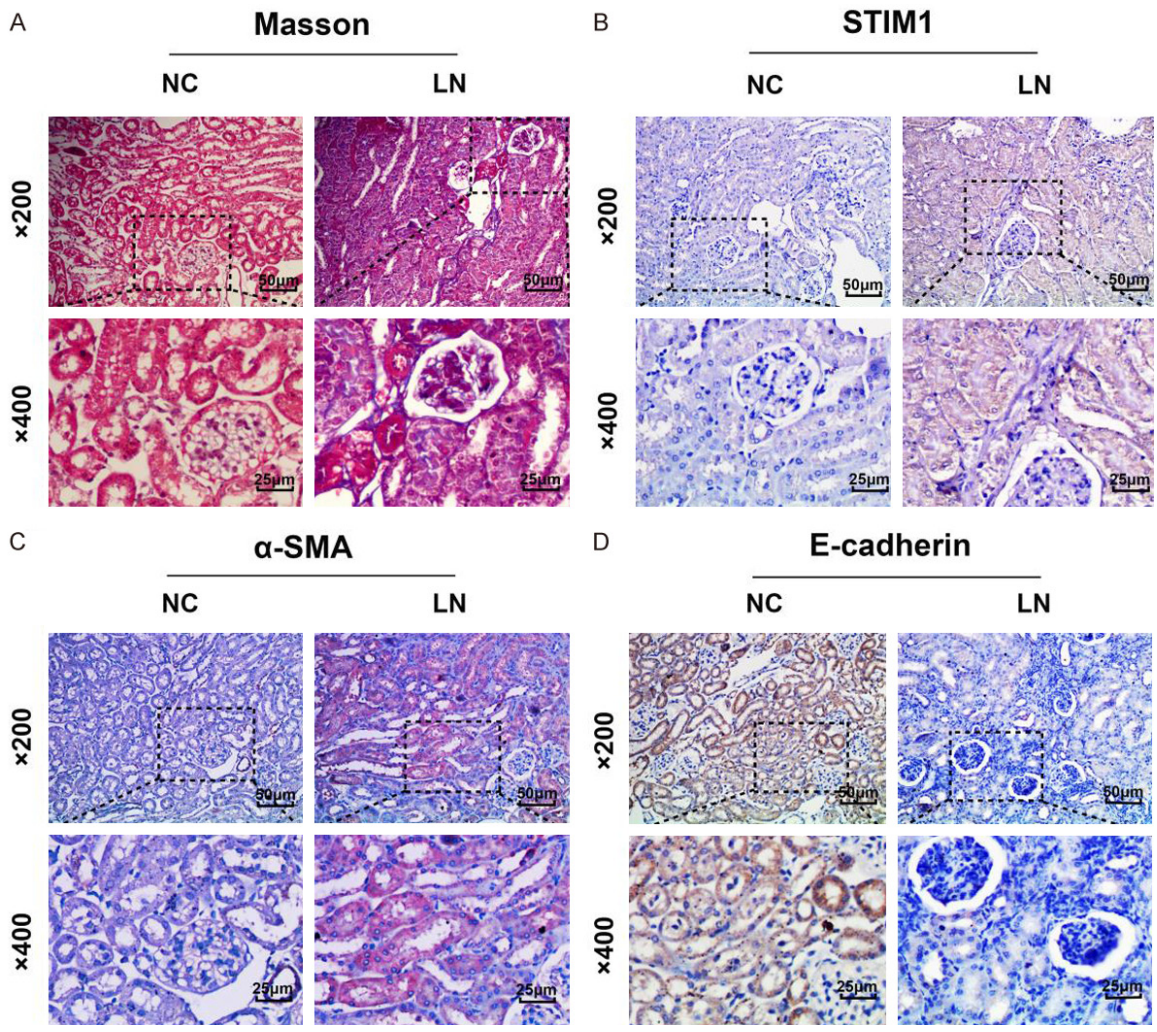

Figure 1. Pathologic analysis of renal tissue in mice. A. Tissue sections of kidneys from MRL/lpr mice and age-matched C57BL/6 mice were subjected to Masson staining. B-D. The kidneys of mice were subjected to immunohistochemical analysis of Stim1, α-SMA, and E-cadherin.

(NC) group, collagen-positive staining was significantly increased in the LN group (**Figure 1A**). The immunohistochemical results demonstrated that the protein expression of Stim1 and α-SMA was significantly increased and E-CA protein expression was decreased in the LN group compared with the NC group (**Figure 1B-D**). These results indicated that the kidneys of lupus mice produced large amounts of collagen, exhibited matrix deposition and had relatively evident fibrotic lesions.

Correlation between Stim1 and FN expression in mouse kidney tissue

FN, an adhesion glycoprotein in the extracellular matrix, is produced by fibroblasts and plays an important role in fibrosis in the lung, liver, and kidney [15]. Consequently, to verify the expression of Stim1 in the kidneys of lupus mice and explore the potential disease-promoting mechanism, we used western blotting and qRT-PCR to measure the protein and mRNA expression, respectively, of Stim1 and FN in the kidney tissues of the two groups of mice and analyzed the correlation between their expression. Western blot results showed that the protein levels of Stim1 and FN were significantly upregulated ($P<0.05$; **Figure 2**). The qRT-PCR results revealed that the mRNA levels of Stim1 and FN in the kidneys of LN mice were significantly higher than those of NC mice (**Figure 3A** and **3B**), and Stim1 was highly positively correlated with FN expression in the kidneys ($P<0.0001$, $r = 0.9855$; **Figure 3C** and **3D**.

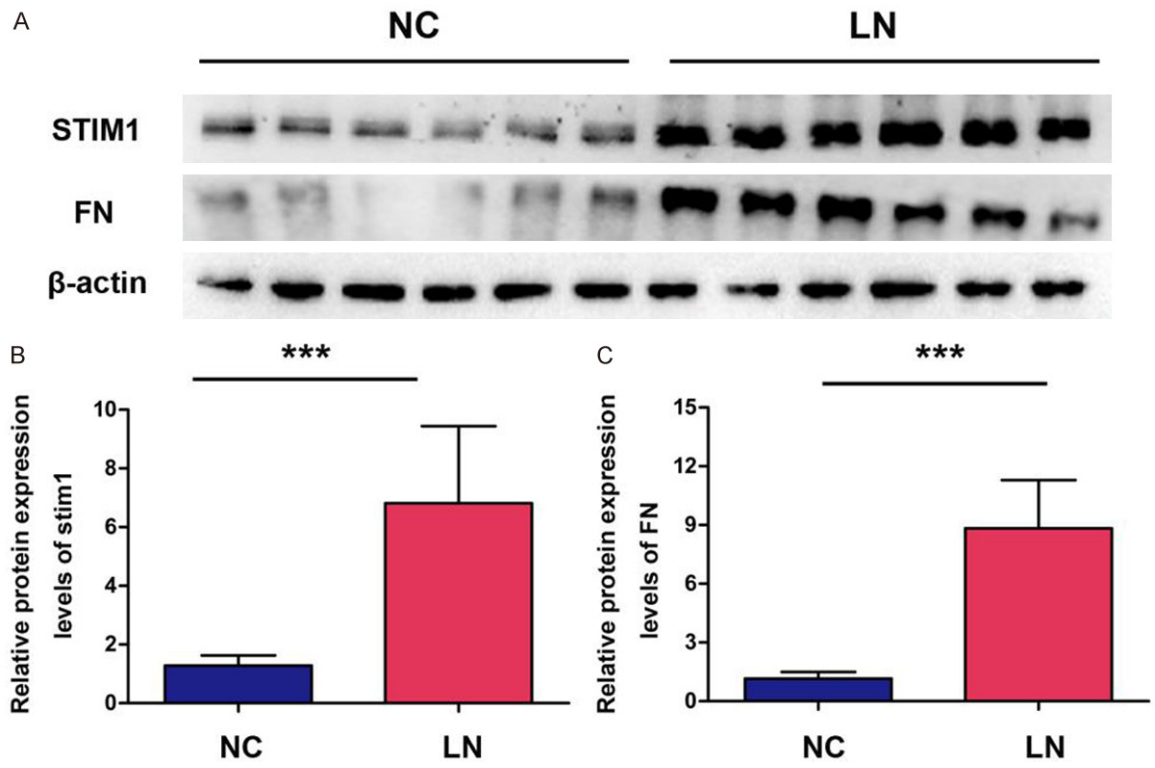

Figure 2. Protein expression of Stim1 in mouse kidneys. A. The protein expression of Stim1 and fibronectin (FN) in the kidneys of MRL/lpr mice and age-matched C57BL/6 normal controls was measured by western blotting (n = 6 mice per group). B, C. Protein expression was analyzed by densitometry.

This result indicates that Stim1 may induce a marked effect on the occurrence and development of LN, and this effect may be related to the induction of high FN expression.

Correlation of renal tissue Stim1 with urinary protein, BUN, and serum CRE levels in mice

In the course of LN pathogenesis, renal injury occurs, resulting in decreased glomerular filtration and renal tubular reabsorption capacity and increased protein concentration in urine. Along with the progression of LN in mice, urine protein levels increased gradually. Urea nitrogen and creatinine are metabolites of protein and muscle, respectively, and the kidney is its definitive excretory organ. After renal impairment, BUN and serum CRE concentrations increase rapidly due to retention. As a consequence, serum BUN and CRE levels and urinary protein levels are the main clinical indicators of renal function [16]. In this study, the urine protein concentration, BUN, and serum CRE levels of LN mice were significantly higher than those of NC mice (**Figure 4A**, **4C**, **4E**), indicating significantly impaired renal function in LN mice. To explore the relationship between Stim1 expression and renal impairment in mice, we verified the correlation between the mRNA level of Stim1 and urine protein, BUN, and serum CRE levels in mice. The results showed that the mRNA level of Stim1 was positively correlated with urine protein, BUN, and serum CRE (**Figure 4B**, **4D**, **4F**). This finding indicates that there is an apparent relationship between Stim1 expression and renal function impairment in mice.

Silencing Stim1 can effectively inhibit the EMT process in renal tubular epithelial cells

Studies have demonstrated that EMT of renal tubular epithelial cells plays a crucial role in the occurrence and development of LN-associated renal tubular interstitial fibrosis, with more than 1/3 of mesenchymal fibroblasts derived from local renal tubular epithelial cell EMT [17]. To investigate the effect of Stim1 on EMT in LN tubular epithelial cells, this study used lipopolysaccharide (LPS) to simulate LN cells [18], and siRNA knockdown was used to reduce Stim1 expression in LPS-stimulated

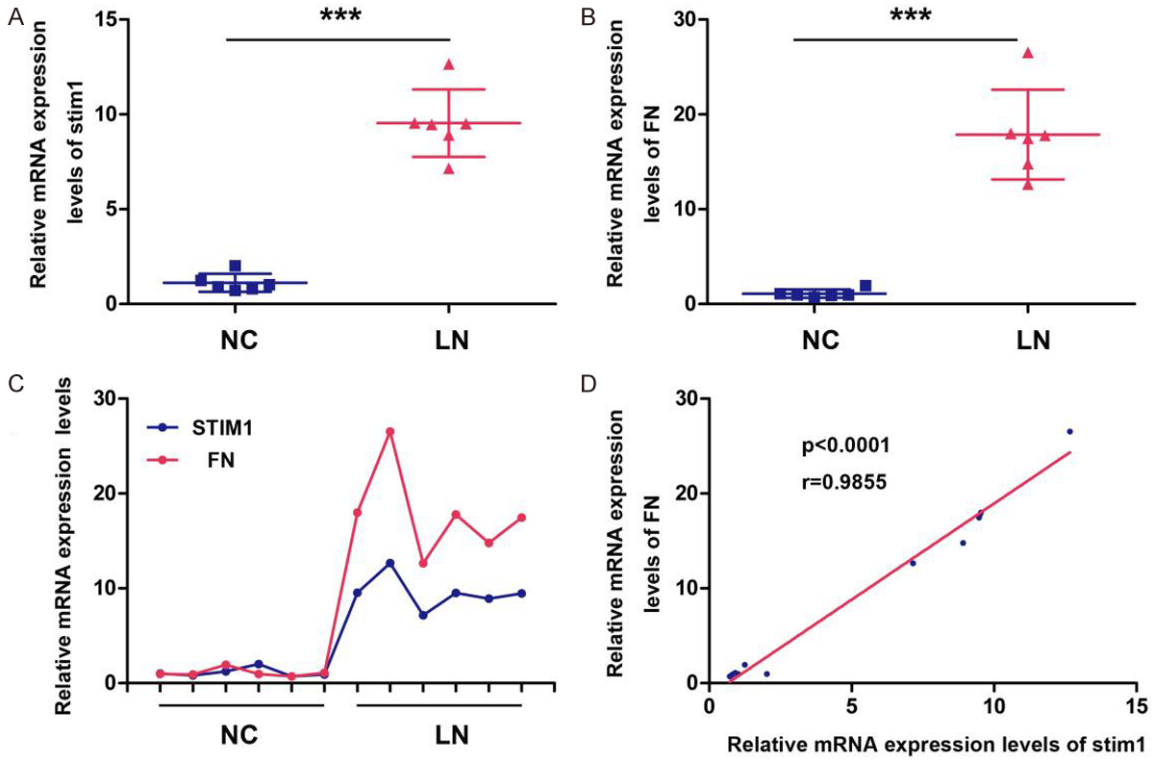

Figure 3. Correlation between the mRNA expression levels of Stim1 and fibronectin (FN) in renal tissue. A. mRNA expression of Stim1 in the kidneys of MRL/lpr mice and age-matched C57BL/6 normal controls (n = 6 mice per group). B. mRNA expression of FN in the kidneys of MRL/lpr mice and age-matched C57BL/6 normal controls (n = 6 mice per group). C, D. Relationship between the mRNA expression levels of Stim1 and FN by Spearman correlation analysis.

renal tubular epithelial cells to verify the effect of this intervention on EMT. After transfection, the cells were divided into four groups: the blank control group (group 1), the LPS treatment group (group 2), the LPS+siRNA-NC group (group 3), and the LPS+siRNA-Stim1 group (group 4). Stim1, α-SMA, E-CA, and FN protein levels were measured by western blotting and immunofluorescence. The results showed that the levels of Stim1, α-SMA and FN were significantly increased, while the levels of E-CA were significantly decreased. Compared with those of group 3, the levels of Stim1, α-SMA and FN in group 4 decreased dramatically, while the expression of E-CA increased. The confocal immunofluorescence results supported the western blot results (**Figure 5**). This finding indicates that silencing Stim1 can effectively inhibit EMT in renal tubular epithelial cells.

Discussion

Lupus nephritis (LN) is one of the most serious complications of SLE. Clinically, LN is often accompanied by simple proteinuria, hematuria, azotemia and other symptoms, which manifest as chronic glomerulonephritis or nephrotic syndrome. Prolongation of the disease is likely to lead to end-stage renal disease, which seriously threatens the life of patients [19, 20]. At present, LN is clinically treated mainly through immunotherapy and complication treatment. The main intention is to improve the renal function of patients, prevent the occurrence and development of the disease, promote the quality of life of patients, and improve the survival rate, but the therapeutic effect is not ideal, and there are many side effects [21, 22]. Therefore, nothing is more important than exploring LN pathogenesis. Renal damage in LN involves various renal structures, including the glomeruli, tubules, renal interstitium and blood vessels [23]. Studies have shown that renal tubular damage caused by LN promotes the EMT of renal tubular epithelial cells, transforming these cells into myofibroblasts, which produce a large amount of extracellular matrix (ECM), leading to the deposition of ECM and accelerating the occurrence of renal interstitial

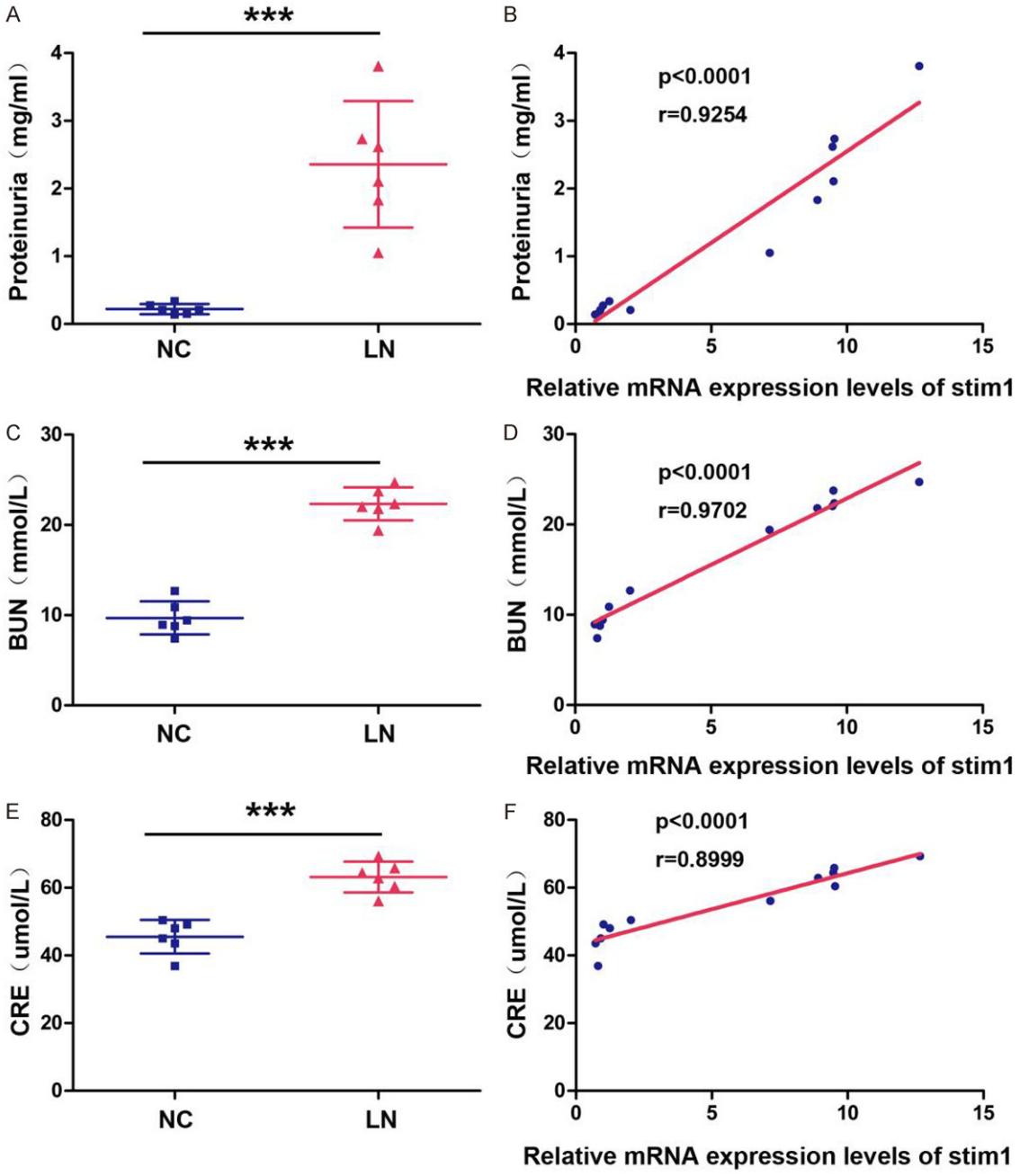

Figure 4. Correlation between Stim1 expression in renal tissue and renal function injury. A. Levels of proteinuria were immediately measured at the end of the study. B. The relationship between the Stim1 mRNA expression level and the proteinuria was determined by Spearman correlation analysis. C. The levels of urea nitrogen in serum were immediately measured at the end of study. D. The relationship between the Stim1 mRNA expression level and the urea nitrogen level was determined by Spearman correlation analysis. E. The levels of creatinine in serum were immediately measured at the end of study. F. The relationship between the Stim1 mRNA expression level and the creatinine level was determined by Spearman correlation analysis. The data shown are the mean ± SD of 3 independent experiments.

fibrosis and the development of end-stage renal disease [24]. EMT refers to the transformation of epithelial cells into mesenchymal cells, which have the capacity to migrate to distant anatomical sites through specific processes during the course of the disease, which usually manifests as decreased expression of E-CA and increased expression of α-SMA and FN [25]. Hence, in this study, the relevant protein indicators of EMT and ECM were repeated-

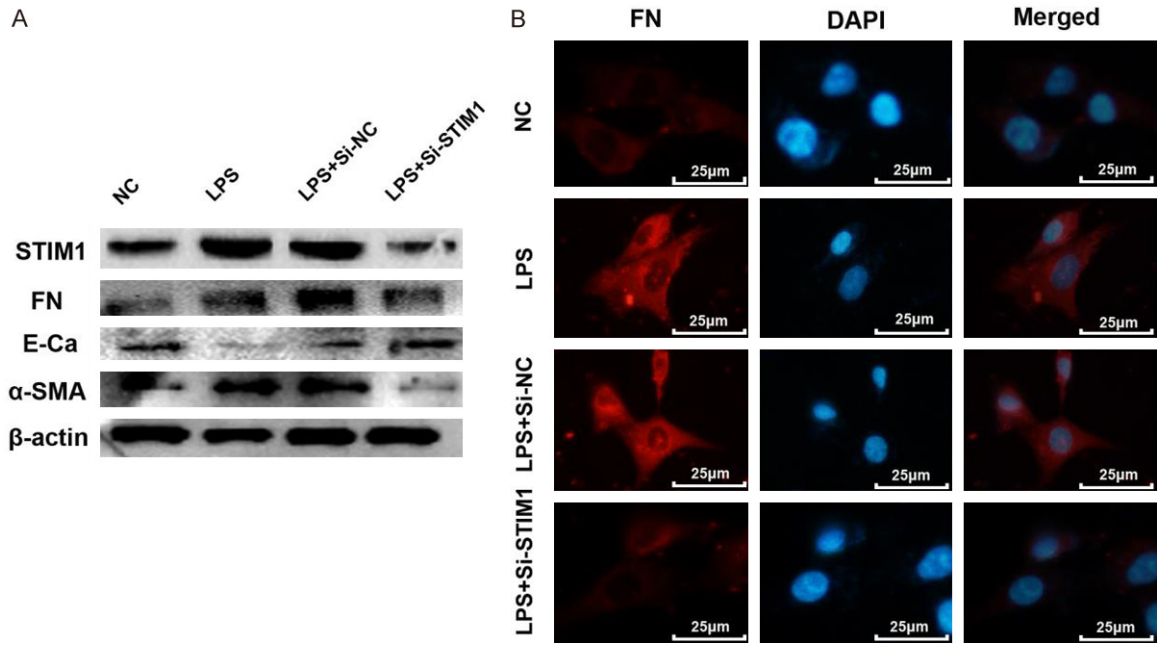

Figure 5. Effects of Stim1 silencing on EMT in renal tubular epithelial cells in LPS-stimulated mice. A. Stim1, FN, E-cadherin, and α-SMA levels were measured by western blotting in the 4 indicated groups. B. FN levels were measured by immunofluorescence in the 4 indicated groups. Nuclei were stained with DAPI.

ly verified to examine the fibrosis process of renal tissue cells.

Calcium channels can be divided into three categories according to the extracellular Ca^{2+} inflow pathway: voltage-dependent Ca^{2+} channels (VDCCs), receptor-energized calcium channels (ROCCs), and store-energized Ca^{2+} channels (SOCCs) [26]. SOCCs are widely distributed in nonexcitatory cells and some excitatory cells and are mainly composed of the Stim and Orai family proteins [27]. Stim proteins are type I transmembrane proteins and can be divided into two subtypes, Stim1 and Stim2, of which Stim1 is highly correlated with SOCC activation [28]. When the concentration of Ca^{2+} stored in the endoplasmic reticulum drops, the transmembrane region of Stim1 senses this signal, which is transmitted by the cytoplasmic region of Stim1 to Orai1 located in the membrane. This signal initiates the opening of Orai1 channels and leads to the influx of Ca^{2+} from the extracellular environment, thus supplementing Ca^{2+} in the cytoplasm and the calcium pool, maintaining a stable intracellular Ca^{2+} concentration [29]. In 2005, researchers found that after inhibiting Stim1 in Drosophila S2 cells, extracellular Ca^{2+} could not enter the cells through SOCC channels even if the endoplasmic reticulum Ca^{2+} store was empty. Stim1 was first identified as an important signaling molecule involved in SOCCs, and it was suggested that Stim1 was a Ca^{2+} receptor in the endoplasmic reticulum of mammalian cells [30]. Other studies have shown that silencing the Stim1 gene in HeLa cells can block SOCC currents [31]. Given that Ca^{2+} homeostasis imbalance is closely related to a variety of diseases and that SOCCs are regulated by Stim1 signaling, Stim1 is thought to play an important role in a variety of diseases. A large number of studies have shown that Stim1 is involved in the progression of various diseases, such as Alzheimer's disease, pathological cardiac hypertrophy, high blood pressure, diabetes, immunological diseases and tumors [32, 33]. In 2009, the New England Journal of Medicine reported that Stim1 may also play a role in the pathogenesis of nephrotic syndrome [34]. Recent studies have shown that Stim1 can induce Ca^{2+} influx to promote the EMT process in tumor cells by mediating abnormal activation of SOCCs [35]. Thus, we hypothesized that Stim1, an important regulator of native channels Ca^{2+}, may play an essential role in the progression of renal interstitial fibrosis in LN.

The present study used animal models of lupus referred to as MRL/lpr mice by Murphy and Roths, which were first established in 1978 and were called MRL/lpr mice due to lack of the Fas gene and ease of lymphadenopathy induction; thus, Fas-induced apoptosis was mediated by this interference, and the activation of lymphocytes, autologous T cells, and B cells significantly prolonged survival time, eventually stimulating an excessive immune response and inducing the pathological features of lupus [36]. Significant systemic lymph node enlargement was observed in MRL/lpr mice at 3 months of age and increased gradually with time. The level of immunoglobulin in blood was significantly increased and was 5 times as high as that of normal mice at 5 months of age, among which IgG was approximately 6-7 times as high as that of normal mice. The complement titer in blood decreased with increasing age, which was similar to the pathological process of human SLE [37]. Various antibodies, such as ssDNA antibody, DS-DNA antibody, Sm antibody and ANA, also increased daily with age at 2-3 months. Proteinuria and impaired renal function occurred in MRL/lpr mice older than 3-6 months [38]. As observed in the biochemical results of this study, the urine protein, blood urea nitrogen and blood creatinine levels of MRL/lpr mice were higher than those of normal mice. Moreover, the renal morphological results showed that the renal tubules in the normal control group exhibited clear outlines and were nearly arranged, and the morphology of each part was normal. However, the renal tubules of MRL/lpr mice were disordered and had a large amount of collagen deposition. These results indicated that the MRL/lpr mice exhibited obvious renal damage and renal interstitial fibrosis lesions. The immunohistochemical analysis of E-CA and α-SMA further confirmed the reliability of the model.

In this study, a variety of experimental methods were used to measure expression, and it was confirmed that Stim1 expression in the kidney tissues of LN mice was significantly higher than that of normal mice. Expression of Stim1 was not only highly consistent with FN expression but also significantly positively correlated with the levels of urine protein, blood urea nitrogen, and blood creatinine of mice. Additionally, Stim1 silencing effectively inhibited the promoting effect of LPS on FN expression and EMT. This finding suggests that Stim1 expression may be related to renal function injury and the development of renal interstitial fibrosis. Recently, it was reported that the expression of Stim1 is significantly increased in DKD injury and that the level of urine protein in patients and db/DB diabetic mice are positively correlated with the expression of Stim1. The researchers attributed this to the fact that Stim1 may induce epithelial-mesenchymal transition in podocytes by activating FcγRII [39]. Additionally, other studies have found that the mRNA levels of Stim1 were increased in adriamycin-induced nephropathy mice. Stim1 overexpression significantly decreased the expression of Podocin and CD2-associated protein (CD2AP), whereas it increased the expression of α-actinin-4. This suggested that Stim1 overexpression could affect the cell permeability and the expression of partial podocyte-associated proteins, which may ultimately result in podocyte injury [40]. In view of the above reports, we reckon that the demonstrated role of Stim1 in the pathogenesis of lupus nephritis, as well as the regulation of FN expression and EMT in renal tubular epithelial cells, which is likely to be related to these underlying mechanisms. Considering that Stim1 is a key protein in calcium channels, its increased expression and activation may lead to severe calcium overload in renal tubular epithelial cells. This may also cause increased FN expression and progression of lupus nephritis and fibrosis. It should be noted that we have only hypothesized about the underlying mechanism based on these phenomena, and the molecular regulatory mechanism needs to be verified in future experiments. We will fther explore the detailed molecular mechanism of the Stim1-induced EMT process in renal tubular epithelial cells. Moreover, the search for transcription factors that are upstream of Stim1 will be another important target to explain high Stim1 expression in lupus nephritis renal tissue. Ultimately, these findings are expected to provide a new and effective means for the prevention and treatment of LN.

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Disclosure of conflict of interest

None.

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