Implication of CD38 Gene in Podocyte Epithelial-to-Mesenchymal Transition and Glomerular Sclerosis

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ABSTRACT

CD38 is a multifunctional protein involving in a number of signaling pathways. Given that the lack of CD38 is considered as a dedifferentiation marker of lymphocytes and other cells, we hypothesized that CD38 and its signaling pathway may participate in the epithelial-to-mesenchymal transition (EMT) process of podocytes and thereby regulates the integrity of glomerular structure and function. Western blot analysis and RT-PCR demonstrated that renal tissue CD38 expression was lacking in CD38$^{-/-}$ mice or substantially reduced in renal CD38 shRNA-transfected WT (CD38-shRNA) mice compared to CD38$^{+/+}$ littermates. Confocal fluorescent microscopy demonstrated the reduced expression of epithelial markers (P-Cadherin, ZO-1 and podocin) and increased expression of mesenchymal markers (FSP-1, α-SMA and desmin) in the glomeruli of CD38$^{-/-}$ and CD38-shRNA mice compared to CD38$^{+/+}$ mice. Morphological examinations showed profound injury in the glomeruli of CD38$^{-/-}$ or CD38-shRNA mice compared to CD38$^{+/+}$ mice. This enhanced glomerular injury in CD38$^{-/-}$ or CD38-shRNA mice was accompanied by increased albuminuria and proteinuria. DOCA/high salt treatment further decreased the expression of epithelial markers and increased the abundance of mesenchymal markers, which were accompanied by more increased glomerular damage index and mean arterial pressure in CD38$^{-/-}$ and CD38-shRNA mice than CD38$^{+/+}$ mice. *In vitro* studies showed that inhibition of CD38 enhances the EMT in podocytes. In conclusion, our observations reveal that the normal expression of CD38 importantly contributes to the differentiation and function of podocytes and the defect of this gene expression may be a critical mechanism inducing EMT and consequently resulting in glomerular injury and sclerosis.

INTRODUCTION

Epithelial-mesenchymal transition (EMT) defines a phenotypic conversion in epithelial cells, leading to the loss of epithelial cell-cell-basement membrane contacts and structural/functional polarity and the acquisition of a fibroblastic phenotype [1, 2]. Emerging evidence has established EMT as a major mechanism of tubulointerstitial fibrosis and glomerulosclerosis [3-5]. EMT typically occurs in response to a number of environmental stresses and associated cytokine/growth factor stimuli such as mechanical stretch [6], cyclosporine treatment [2], exposure to advanced glycation end products (AGE consequence of hyperglycemia) [7], hypoxia [8], hyperhomocysteinemia, oxidative stress [9], aldosterone [10], activated monocyte supernatant, interleukin-1 [11], and oncostatin M treatment as well as the culturing of cells on collagen I [12]. However, the normal regulation of EMT in the kidney, in particular, in glomerular podocytes is still poorly understood.

CD38 first identified as a leukocyte differentiation antigen, subsequently has been found in many tissues including the liver, brain, heart and kidney [13, 14]. CD38 is a type II transmembrane glycoprotein and a member of a family of enzymes with multiple functions, including ADP–ribosylcyclase activity that cyclizes NAD^+ into cyclic ADP-ribose (cADPR), which is an important intracellular second messenger for the mobilization of Ca^{2+}. Recent studies have demonstrated that cADPR-mediated Ca^{2+} signaling plays an important role in the regulation of renal function through its action on different renal components such as renal arteries, the early distal tubule, and renal mesangium [15-17]. In addition, CD38-generated cADPR is importantly implicated in Ca^{2+}-induced insulin secretion [18], osteoclast-mediated bone resorption [19], and immune function [20, 21]. Furthermore, the lack of CD38 has been considered as a dedifferentiation marker of lymphocytes and other cells [22]. It is imperative

to know whether alterations of CD38 gene could also induce podocyte EMT and thereby alter the integrity of glomerular structure and function.

In this regard, podocytes are specialized, terminally differentiated visceral epithelial cells that reside on the glomerular basement membrane (GBM) outside the glomerular capillaries [23, 24]. In response to injurious stimuli, they often undergo a range of adaptive changes, including hypertrophy, dedifferentiation, detachment, and apoptosis, depending on the severity and duration of the injury [25, 26]. Because of their limited proliferative capacity, podocyte detachment from GBM and apoptosis will inevitably lead to cell depletion or drop out, which could reduce podocyte density, resulting in an impaired glomerular filtration and proteinuria [27-29]. This podocyte injury has been considered as the most important early event in initiating glomerulosclerosis and thereby resulting in end-stage renal disease in various animal models and humans [30, 31].

The present study hypothesized that CD38 and its signaling pathway may participate in the epithelial-to-mesenchymal transition (EMT) process of podocytes and thereby leads to glomerular injury or sclerosis. To test this hypothesis, we first performed a series of experiments using CD38$^{-/-}$ and their wild type littermates on the normal chow with or without treatment of DOCA + 1% NaCl to determine whether lack of CD38 gene induces podocyte EMT, podocyte injury and glomerular dysfunction and sclerosis. Then, we locally silenced renal CD38 gene using shRNA and observed the effects of renal CD38 deficiency on podocyte EMT and corresponding glomerular injury. We further used cultured murine podocytes to examine the direct effects of CD38 inhibition on podocyte EMT and injury. Our results demonstrate that the normal expression of CD38 importantly contributes to the

regulation of EMT in podocytes and that the defect of this gene expression may be a critical mechanism inducing EMT and thereby resulting in glomerular sclerosis.

MATERIALS AND METHODS

Animals: Eight weeks old male C57BL/6J WT, CD38$^{-/-}$ mice and their wild type littermates (The Jackson Laboratory, Bar Harbour, ME, USA) were used in the present study. The mice were fed with control diet (Dyets Inc, Bethlehem, PA, USA) and treated with or with out DOCA + 1%NaCl in drinking water for 4 weeks. DOCA + 1% NaCl dose was chosen based on earlier reports indicating the EMT transition in C57BL/6J WT mice and renal epithelial cells [10]. In another series of C57BL/6J mice, CD38 shRNA or a scrambled shRNA (Origene, Rockville, MD, USA) plasmid with a luciferase expression vector was co-transfected into the kidneys via renal artery injection using the ultrasound microbubble system as we described previously [32]. All protocols were approved by the Institutional Animal Care and Use Committee of the Virginia Commonwealth University.

Morphological examinations: The fixed kidneys were paraffin-embedded, and sections were prepared and stained with Periodic acid–Schiff stain. Glomerular damage index (GDI) was calculated from 0 to 4 on the basis of the degree of glomerulosclerosis and mesangial matrix expansion as described previously [33]. In general, we counted 50 glomeruli in total in each kidney slice under microscope, when each glomerulus was graded level 0-4 damages. 0 represents no lesion, 1+ represents sclerosis of <25% of the glomerulus, while 2+, 3+, and 4+ represent sclerosis of 25% to 50%, >50% to 75%, and >75% of the glomerulus. A whole kidney average sclerosis index was obtained by averaging scores from counted glomeruli

[34]. This observation was examined by two independent investigators in a blind way to the treatment of different experimental groups.

Urinary total protein and albumin excretion measurements: The 24-hour urine samples were collected using metabolic cages and subjected to total protein and albumin excretion measurements, respectively [34]. Total protein content in the urine was detected by Bradford method using a UV spectrophotometer. Urine albumin was detected using a commercially available albumin ELISA kit (Bethyl Laboratories, Montgomery, TX).

Western blot analysis: Homogenates were prepared from the kidney with a modified method as we described previously [35]. CD38 protein expression in the kidney from CD38$^{+/+}$ and CD38$^{-/-}$ mice was detected by Western blot analysis with a monoclonal antibody against CD38 (1:1000 dilution for overnight at 4°C, BD Biosciences, San Diego, CA, USA). For normalization, the blots were reprobed with alternative primary antibody against the housekeeping protein β-actin (1:4000 dilution for 1 hour, Sigma).

Monitoring of arterial blood pressure in conscious mice: Mean arterial pressure (MAP) was measured in CD38$^{+/+}$ and CD38$^{-/-}$ mice with or without DOCA + 1% NaCl treatment for 4 weeks as we described previously [36]. In brief, mice were anesthetized by inhalation of isoflurane, and then a catheter connected to a telemetry transmitter was implanted into the carotid artery and the transmitter was placed subcutaneously. The arterial blood pressure signal from the transmitter was received by a remote receiver and then recorded by a computer program (Data Sciences International, St. Paul, MN). Arterial blood pressure was continuously measured for one week after an equilibration period.

Delivery of CD38 shRNA into the kidneys by ultrasound-microbubble technique: CD38 shRNA or a scrambled shRNA plasmid with a luciferase expression vector was co-transfected into the kidneys via renal artery injection using the ultrasound-microbubble system. A full description of the procedures for the ultrasound-microbubble gene transfer technique can be found in our previous studies [32]. To monitor the efficiency of gene expression through somatic plasmid transfection daily, mice were anesthetized with isoflurane, and an aqueous solution of luciferin (150 mg/kg) was injected intraperitoneally 5 minutes before imaging. The anesthetized mice were imaged using the IVIS200 *in vivo* molecular imaging system (Xenogen, Hopkinton, MA, USA). Photons emitted from luciferase-expressing cells within the animal body and transmitted through tissue layers were quantified over a defined period of time ranging up to 5 minutes using the software program Living Image as program (Xenogen). The inhibitory efficiency of gene expression by CD38 shRNA was further confirmed by detection of CD38 mRNA level in mouse renal cortex using real-time RT-PCR.

Real-time reverse transcription polymerase chain reaction (RT-PCR): Total RNA from isolated mouse renal tissue was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the protocol as described by the manufacturer. RNA samples were quantified by measurement of optic absorbance at 260 nm and 280 nm in a spectrophotometer. The concentrations of RNA were calculated according to A260. Aliquots of total RNA (1 μg) from each sample were reverse-transcribed into cDNA according to the instructions of the first strand cDNA synthesis kit manufacturer (Bio-Rad, Hercules, CA). Equal amounts of the reverse transcriptional products were subjected to PCR amplification using SYBR Green as the fluorescence indicator on a Bio-Rad iCycler system (Bio-Rad, Hercules, CA) [34]. The

primers used in this study were synthesized by Operon (Huntsville, AL, USA) and the sequences were: CD38 sense GACGCTGCCTCATCTACACT, antisense TCTTGGAAACAAATGCTCCT; ZO-1 sense GAGCTACGCTTGCCACACTGT, antisense TCGGATCTCCAGGAAGACACTT; β-actin sense TCGCTGCGCTGGTCGTC, antisense GGCCTCGTCACCCACATAGGA.

Cell culture: Conditionally immortalized mouse podocyte cell line, kindly provided by Dr. Klotman PE (Division of Nephrology, Department of Medicine, Mount Sinai School of Medicine, New York, NY, USA), was cultured on collagen I-coated flasks or plates in RPMI 1640 medium supplemented with recombinant mouse interferon–γ at 33°C. After differentiated at 37°C for 10-14 days without interferon–γ, podocytes were used for the proposed experiments.

Indirect immuno-fluorescent staining: The cells were fixed in 4% PFA for 15 minutes. After rinsed with phosphate-buffer saline (PBS), cells were incubated with goat anti-FSP-1 (1:50), goat anti-ZO-1 (1: 50, Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA), rabbit anti-P-cadherin (1: 50), and mouse anti-α-SMA (1: 100, R&D system, Minneapolis, MN, USA) antibodies. After washing, the slides were incubated with Alexa 488-labeled secondary antibodies for 1 h at room temperature. After being mounted with DAPI-containing mounting solution, the slides were observed under a fluorescence microscope and photos were taken and analyzed. The fluorescent intensities were quantified by the Image Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA) and the data was normalized to control cells.

Double-immunofluorescent staining: Double-immunofluorescent staining was performed using frozen slides from mouse kidneys. After fixation, the slides were incubated with rabbit

anti-podocin antibody at 1: 200 (Sigma, St. Louis, MO, USA), which was followed by incubation with Alexa 555-labeled goat anti-rabbit secondary antibody. Then, goat anti-FSP-1, goat anti-ZO-1 1:50 (Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA), rabbit anti-P-cadherin 1:200, or mouse anti-α-SMA 1:200 (R&D system, Minneapolis, MN, USA) was used to incubate with the slides for overnight at 4°C. After washing, the slides were incubated with corresponding Alexa 488-labeled secondary antibodies. Finally, the slides were mounted and subjected to examinations using a confocal laser scanning microscope (Fluoview FV1000, Olympus, Japan). All exposure settings were kept constant for each group of kidneys. Colocalization was analyzed by Image Pro Plus software, and the colocalization coefficient was represented by Pearson's correlation coefficient represent the relationship between podocin and P-Cadherin or ZO-1 or α-SMA or FSP-1 [37, 38].

Statistical Analysis: Data are provided as arithmetic means ± SEM; *n* represents the number of independent experiments. All data were tested for significance using ANOVA or paired and unpaired Student's t-test as applicable. The glomerular damage index was analysed using a nonparametric Mann-Whitney rank sum test. Only results with $p < 0.05$ were considered statistically significant.

RESULTS

CD38 expression in the kidneys from CD38$^{+/+}$ and CD38$^{-/-}$ mice. By Western blot analysis using a monoclonal antibody against mouse CD38, CD38 protein was hardly detected in the kidneys from CD38$^{-/-}$ mice, but it was enriched in the kidney tissue from CD38$^{+/+}$ mice (data was not shown).

Epithelial to Mesenchymal transition in glomeruli of mice lacking CD38 gene. Earlier studies reported that lack of CD38 is considered as a dedifferentiation marker of lymphocytes and other cells. We determined whether mice lacking CD38 gene may induce the podocyte EMT in glomeruli of mice. Double immunofluorescent staining analysis was performed using podocin as a podocyte marker. As shown in Fig. 1A and 1B, the colocalization of podocin and epithelial markers such as p-cadherin or ZO-1 was decreased in glomeruli of CD38$^{-/-}$ mice compared to CD38$^{+/+}$ mice during a control diet. The DOCA/1%NaCl treatment decreased the colocalization of podocin and P-cadherin or ZO-1. The effect was more pronounced in CD38$^{-/-}$ mice. The Pearson correlation coefficient of podocin with P-Cadherin or ZO-1 was summarized in Fig. 1C and 1D, showing the quantitative colocalization of podocin with epithelial markers P-Cadherin (Fig. 1C) or ZO-1 (Fig. 1D) in glomeruli of mice. In contrast, the abundance of two mesenchymal marker, FSP-1 and α-SMA were weak in the glomeruli of CD38$^{+/+}$ mice, but it was dramatically increased in CD38$^{-/-}$ mice during a control diet (Fig. 2A and 2B). DOCA/1%NaCl treatment increased the colocalization of both CD38$^{+/+}$ and CD38$^{-/-}$ mice. The summarized data was shown in Fig. 2C and Fig. 2D

respectively. Taken together, these results suggest that enhanced EMT process mainly occurs in podocytes in mice lacking CD38 gene.

Pronounced DOCA/high salt-induced glomerular injury in mice lacking CD38 gene. As depicted in Fig. 3A and 3B, urinary protein and albumin excretion was significantly higher in $CD38^{-/-}$ than $CD38^{+/+}$ mice on the control diet. DOCA/high salt treatment significantly increased the urinary total protein and albumin excretion in $CD38^{+/+}$ mice, but it had no further effect in $CD38^{-/-}$ mice (Fig. 3A and 3B). By PAS staining, we observed a typical pathological change in glomerular sclerotic damage in $CD38^{-/-}$ but not in $CD38^{+/+}$ mice. This pathological change in glomeruli was more pronounced in $CD38^{-/-}$ mice with DOCA/high salt treatment. The average glomerular damage index (GDI) was significantly higher in $CD38^{-/-}$ than $CD38^{+/+}$ mice fed a control diet (Fig. 3D). However, the DOCA/high salt treatment significantly increased the GDI in both $CD38^{-/-}$ and $CD38^{+/+}$ mice compared to control diet fed mice (Fig. 3C and 3D).

Desmin is an intermediate filament protein and has been suggested as an injured podocyte marker. The expression of desmin which is often up-regulated in various glomerular diseases, in which podocyte damage is involved [34]. Immunofluorescent histological analysis showed that desmin staining was more pronounced in glomeruli of $CD38^{-/-}$ mice than $CD38^{+/+}$ mice fed a control diet, as shown by more remarkable red fluorescence detected with glomeruli, which indicates podocytes injury. The DOCA/high salt treatment increased glomerular desmin expression in both $CD38^{+/+}$ and $CD38^{-/-}$ mice (Fig. 4A). Conversely, another podocyte marker, podocin was found markedly reduced in glomeruli of $CD38^{-/-}$ mice compared to $CD38^{+/+}$ mice fed a control diet. However,   this reduced podocin expression or production was increased in both $CD38^{-/-}$ and $CD38^{+/+}$ mice following DOCA/high salt treatment (Fig. 4B). As illustrated in Fig. 5, the mean arterial pressure was similar in both

CD38$^{-/-}$ and CD38$^{+/+}$ mice fed a control diet. As expected, DOCA/high salt treatment significantly increased the mean arterial pressure in both CD38$^{-/-}$ and CD38$^{+/+}$ mice.

CD38 inhibition enhanced the EMT in podocytes. The above results demonstrated that mice lacking CD38 induces EMT in glomerular podocytes and podocyte injury. We further determined whether inhibition of CD38 may directly stimulate EMT in cultured podocytes. We examined the immunofluorescent expression of epithelial, mesenchymal and podocyte injury markers in well-established mouse murine podocyte cell line with or without CD38 shRNA transfection. As shown in Fig. 6, the CD38 shRNA transfection significantly decreased the CD38 expression in podocytes compared to scrambled shRNA treated cells. Real time PCR also confirmed such reduction of CD38 gene expression when podocytes were treated with its shRNA (not shown in the figure). These results confirm the efficiency of CD38 shRNA transfection in podocytes. In these podocytes with silenced CD38 gene, the epithelial markers P-cadherin and ZO-1 significantly decreased compared to control cells. In contrast, the mesenchymal markers FSP-1 and α-SMA significantly increased upon CD38 shRNA transfection. The podocytes injury markers desmin expression also significantly increased, while podocin expression significantly decreased in podocytes with silenced CD38 gene (Fig. 6).

Enhanced EMT and consequent glomerular injury in the kidney with local CD38 gene silencing. To further determine the implication of CD38 in podocyte EMT and consequent glomerular injury, shRNA strategy was used to locally silence this gene in the kidney and then observed the changes in glomerular function. As illustrated by images obtained by an *in vivo* molecular imaging system, luciferase gene expression co-transfected with CD38 shRNA could be detected even on the third day after the kidney was transfected by ultrasound-microbubble plasmid

introduction. In the hemi-dissected kidney, all of the cortical regions were observed to exhibit efficient gene transfection as shown in green and red fluorescence (Fig. 7A and 7B). This is consistent with previous studies showing that ultrasound-microbubble gene introduction is an efficient technique for delivery of the gene into glomerular cells, vascular endothelial cells and fibroblasts [32, 39]. The efficiency of local CD38 gene silencing was also examined by measurement of its expression when animals were sacrificed after completion of functional studies. Real time RT-PCR analysis demonstrated that CD38 mRNA expression was significantly decreased in C57BL/6J WT mice transfected with CD38 shRNA compared to control mice (Fig. 7C). Correspondingly, the ZO-1 mRNA expression significantly decreased in renal cortical tissue of CD38 shRNA transfected mice compared to control mice (Fig. 7D). Further, we determined whether CD38 gene silencing locally in the kidney may induce glomerular injury. It was found that the CD38 shRNA transfection significantly increased the urinary protein and albumin excretion compared to the control diet-fed mice (Fig. 7E and 7F). The colocalization of podocin and epithelial markers such as P-Cadherin or ZO-1 was decreased in glomeruli of CD38 shRNA transfected mice compared to control mice. In contrast, the mesenchymal markers (FSP-1 and α-SMA) were significantly increased in CD38 shRNA transfected mice (Fig. 8).

DISCUSSION

The goal of the present study is to determine whether CD38 is implicated in the podocyte EMT and glomerular injury. We found that lack of CD38 decreased the expression of epithelial markers (P-Cadherin and ZO-1), but increased the abundance of mesenchymal markers (FSP-1 and α-SMA) in *in vivo* and *in vitro* studies. Such enhanced EMT due to the lack of CD38 gene was accompanied by increased proteinuria and glomerular injury under normal condition and during DOCA + 1%NaCl treatment. Our results demonstrate for the first time that the defect of CD38 gene expression induces the podocyte EMT and glomerular injury in mice.

In the present study, we first characterized the CD38 expression in renal tissue of CD38$^{-/-}$ and CD38$^{+/+}$ mice. We found that CD38 expression was abolished in the kidneys of CD38$^{-/-}$ mice. These results suggest that CD38 gene is expressed in renal tissue and its expression disappeared in CD38$^{-/-}$ mice. EMT is a process of cell transition from epithelial to mesenchymal phenotype, which usually happens during normal developmental process, cancer progression and metastasis [40]. EMT in epithelial cells is characterized by the disruption of epithelial junctional complexes and the subsequent loss of cell polarity [41]. These actions are accompanied by morphological changes to a fibroblastoid morphology, downregulation of epithelial marker proteins such as E-Cadherin, ZO-1 and cytokeratin, and finally, upregulation of mesenchymal markers including vimentin, α-smooth muscle actin and fibroblast-specific protein-1 [42]. Podocytes and tubular epithelial cells are developmentally derived from the same origin (metanephric mesenchyme) [43], it is possible that podocytes, similar to tubular epithelial cells, may undergo a phenotypic conversion, namely, EMT under

specific injurious conditions. Several *in vivo* studies have demonstrated the existence of podocyte EMT in kidney diseases [24, 44-46]. In human biopsy samples of diabetic nephropathy and focal-segmental glomerulosclerosis, loss of nephrin and ZO-1 expression in glomerular podocytes is a common feature, where those cells express mesenchymal markers such as desmin, FSP-1 and α-SMA [24, 45]. Indeed, our results showed that epithelial markers, P-cadherin and ZO-1 expression were decreased in glomeruli of CD38$^{-/-}$ mice, indicating the loss of epithelial characteristics. In contrast to the loss of epithelial features, the expression of mesenchymal markers (FSP-1 and α-SMA) was increased. Using podocin as a podocyte marker, our confocal co-localization data demonstrated that decreased expression of podocin was associated with the loss of epithelial markers (P-Cadherin and ZO-1) and the increases in mesenchymal markers (FSP-1 and α-SMA) in glomeruli of CD38$^{-/-}$ mice. The pathological challenging with DOCA/high salt also further confirmed enhanced EMT in glomeruli of both CD38$^{-/-}$ mice. Taken together, these results suggest the occurrence of podocyte EMT in mice lacking CD38 gene. To our knowledge, these results represent the first experimental evidence demonstrating that renal residential cells could undergo EMT in mice lacking CD38 gene.

EMT is a potential pathway leading to podocyte dysfunction and represents an early cellular event causing a defective glomerular filtration and proteinuria. In response to various injurious stimuli, podocytes undergo a range of adaptive changes, including hypertrophy, dedifferentiation, detachment, and apoptosis, depending on the severity and duration of the injury [24, 26]. If the injury is progressive, podocytes will undergo EMT to escape from apoptosis, which results in the loss of highly specialized podocyte features and acquisition of new mesenchymal markers. This leads to an impaired glomerular filtration barrier, thereby ensuring the onset of proteinuria. More severe and/or longer injury induces podocyte

detachment from glomerular basement membrane and/or apoptosis, resulting in podocyte loss, which certainly exacerbates proteinuria and leads to glomerulosclerosis. In the present study, we tested whether such EMT is critically involved in the initiation or development of glomerular sclerosis. In accordance with the enhanced EMT in CD38$^{-/-}$ mice, urinary albumin, protein excretion and glomerular injury and sclerosis also significantly increased in CD38$^{-/-}$ mice compared to CD38$^{+/+}$ mice, suggesting that EMT-associated renal injury was pronounced when CD38 gene is lacking. In consistent with earlier observations [47], the mean arterial pressure was similar in both CD38$^{+/+}$ and CD38$^{-/-}$ mice under the control diet. However the DOCA/high salt treatment significantly increased the mean arterial pressure in both CD38$^{+/+}$ and CD38$^{-/-}$ mice. The increases in renal injury without a change in mean arterial pressure suggest that a CD38 gene deficiency impairs the renal function via pressure-independent mechanisms. Therefore, this CD38 signaling pathway could be a target of therapeutic strategy for EMT-associated glomerular injury or sclerosis.

To further explore the mechanism of glomerular injury, we observed changes in podocyte function in CD38$^{+/+}$ and CD38$^{-/-}$ mice. It has been well documented that podocyte loss and dysfunction occurs with the onset and magnitude of glomerulosclerosis. Since podocytes serve as the final barrier against urinary protein loss in the normal glomeruli, any change in podocyte structure and function may be intimately associated with proteinuria and consequent glomerular sclerosis [48]. The present study showed that podocin protein was markedly decreased in CD38$^{-/-}$ mice compared to CD38$^{+/+}$ mice. DOCA/high salt treatment further decreased the podocin protein in both CD38$^{-/-}$ and CD38$^{+/+}$ mice. In addition, we found that desmin an intermediate filament protein and a specific and sensitive podocyte injury marker increased in the glomeruli of CD38$^{-/-}$ mice. This increased desmin expression was further enhanced by DOCA/high salt treatment in both CD38$^{-/-}$ and CD38$^{+/+}$ mice. These

results support the view that CD38 deficiency-induced glomerular injury is associated with EMT in podocytes.

In addition to the whole animal experiments, we also used cultured murine podocytes to examine the direct effect of CD38 inhibition on podocyte EMT and podocyte injury. It was found that CD38 shRNA transfection in podocytes significantly decreased the epithelial markers (P-Cadherin and ZO-1) expression and increased the mesenchymal markers (FSP-1 and α-SMA) expression compared to scramble shRNA group. Additionally, the CD38 shRNA transfection also increased the desmin expression and decreased the podocin expression in podocytes. These results from cultured murine podocytes further confirm the findings from our *in vivo* studies, indicating that reduced CD38 expression enhances the podocyte EMT, consequently resulting in podocyte injury.

To further confirm the role of CD38 gene in podocyte EMT and glomerular injury, a local gene silencing strategy was used in the present study, where an ultrasound microbubble-mediated plasmid delivery was employed to introduce CD38 shRNA into the kidney. It was demonstrated that this method was highly efficient in delivering plasmids into renal cells *in vivo*, which led to gene transfection and expression in most renal cells (90%) as confirmed the earlier reports [32, 49-52]. Using *in vivo* molecular imaging system to daily monitor the efficiency of CD38 gene transfection in the kidney in living animals, we showed that the transgene or shRNA expression vector (with luciferase gene as an indicator) could be detected even 3 days after gene transfection and lasted for 4 weeks observed. This *in vivo* transgene monitoring importantly guided our functional studies to define the role of the CD38 gene in mediating podocyte EMT and glomerular injury. After completion of functional protocols, CD38 and ZO-1 mRNA expression, urinary protein and albumin excretion was

analyzed to confirm the efficient silencing of CD38 gene in shRNA transfected kidneys. In such local CD38 gene silenced kidney, we found that renal cortical tissue CD38 and ZO-1 mRNA expression level was significantly decreased. Moreover, silencing CD38 gene in the kidney decreased the expression of epithelial markers and enhanced the abundance of mesenchymal markers and proteinuria/albuminuria. These results from mice with local renal CD38 gene silencing further strengthen our above conclusion drawn from studies using CD38 knockout mice that normal expression of CD38 gene is essential in the maintenance of podocyte phenotype and function. The disturbance of CD38 gene expression may produce podocyte EMT and consequent glomerular injury.

In conclusion, the present study demonstrates that CD38 signaling pathway regulates the integrity of podocyte structure and function and that defect of CD38 gene expression results in EMT and thereby causes glomerular injury. Therefore, the defect of CD38 gene expression or function may be one of important mechanisms that may be implicated in the development of glomerular sclerosis and end-stage renal disease under different pathological conditions.

Conflict of Interest Statement

The authors confirm that there are no conflicts of interest.

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Figure 1: Expression of epithelial markers in the glomeruli of CD38$^{-/-}$ and CD38$^{+/+}$ mice with or without DOCA + 1% NaCl treatment. A: Typical confocal microscopic images of podocin (Alex555, red color) *vs*. P-Cadherin (Alex488, green color) staining in glomeruli from CD38$^{-/-}$ and CD38$^{+/+}$ mice with or without DOCA + 1%NaCl treatment (n=6/group). B: Typical confocal microscopic images of podocin (Alex555, red color) *vs*. ZO-1 (Alex488, green color) staining in glomeruli from CD38$^{-/-}$ and CD38$^{+/+}$ mice with or without DOCA + 1%NaCl treatment (n=6/group). C: Summarized data showing the colocalization coefficient represented by Pearson's correlation coefficient illustrating the relationship between podocin *vs*. P-Cadherin. D: Summarized data showing the colocalization coefficient represented by Pearson's correlation coefficient illustrating the relationship between podocin *vs*. ZO-1. * Significant difference (*P*<0.05) compared to the control CD38$^{+/+}$ mice (n=6).
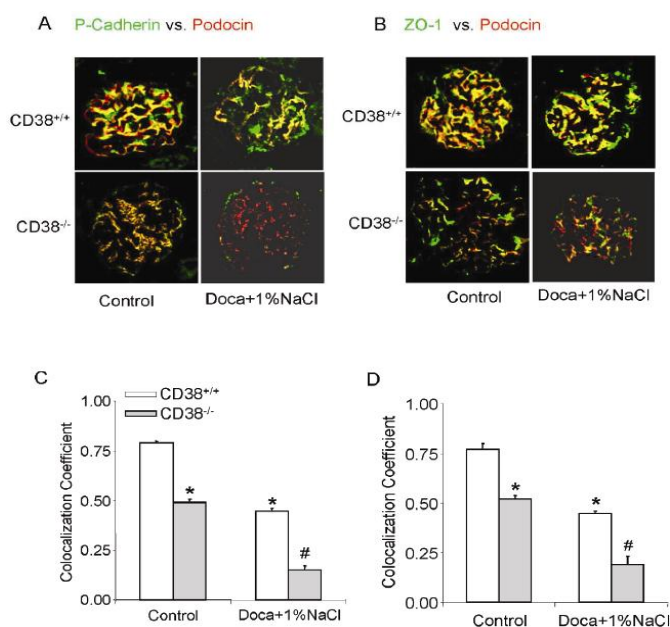
Figure 2: Expression of mesenchymal markers in the glomeruli of CD38$^{-/-}$ and CD38$^{+/+}$ mice with or without Doca + 1% NaCl treatment. A: Typical confocal microscopic images of podocin (Alex555, red color) vs. FSP-1 (Alex488, green color) staining in glomeruli from CD38$^{-/-}$ and CD38$^{+/+}$ mice with or without DOCA + 1% NaCl treatment (n=6/group). B: Typical confocal microscopic images of podocin (Alex555, red color) vs. α-SMA (Alex488, green color) staining in glomeruli from CD38$^{-/-}$ and CD38$^{+/+}$ mice with or without DOCA + 1% NaCl treatment (n=6/group). C: Summarized data showing the colocalization coefficient represented by Pearson's correlation coefficient illustrating the relationship between podocin *vs.* FSP-1. D: Summarized data showing the colocalization coefficient represented by Pearson's correlation coefficient illustrating the relationship between podocin *vs.* α-SMA.* Significant difference (*P*<0.05) compared to the control CD38$^{+/+}$ mice (n=6).
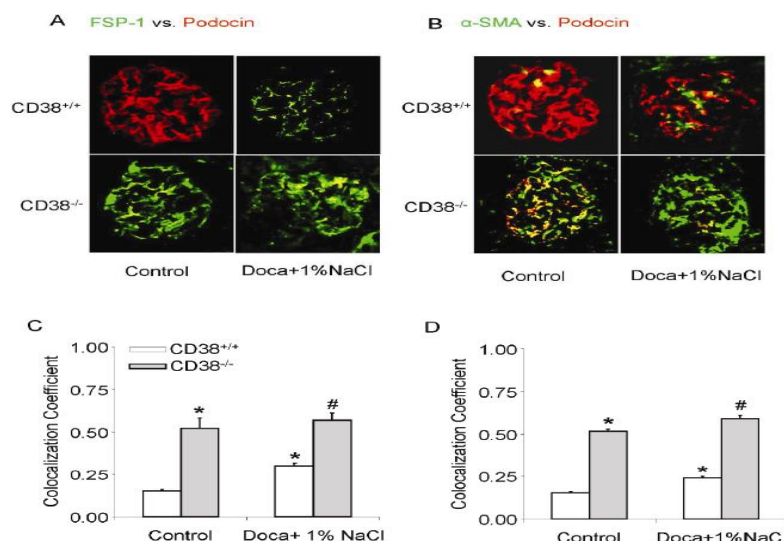
Figure 3: Effects of DOCA + 1% NaCl treatment on glomerular injury in CD38$^{-/-}$ and CD38$^{+/+}$ mice. A: Values are arithmetic means ± SEM (n=8 each group) of urinary total protein excretion, B: Urinary albumin excretion in CD38$^{-/-}$ and CD38$^{+/+}$ mice with or without DOCA + 1% NaCl treatment. C: Photomicrographs show typical glomerular structure (original magnification, x400) in CD38$^{-/-}$ and CD38$^{+/+}$ mice with or without DOCA + 1% NaCl treatment. D: Summarized data of glomerular damage index (GDI) by semi-quantitation of scores in 4 different groups of mice (n=6-9 each group). For each kidney section, 50 glomeruli were randomly chosen for the calculation of GDI. * Significant difference ($P<0.05$) compared to the values from CD38$^{+/+}$ mice, # Significant difference ($P<0.05$) compared to the values from mice on the normal diet.
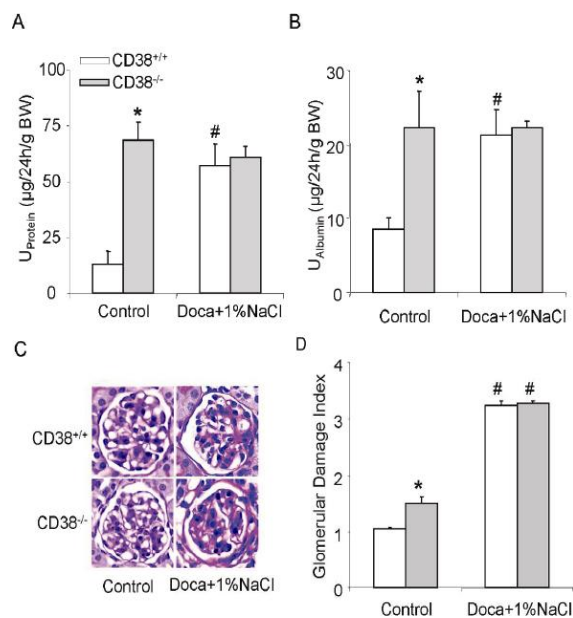
Figure 4: Immunofluorescence staining of desmin and podocin in glomeruli from CD38^{-/-} and CD38^{+/+} mice with or without DOCA + 1% NaCl treatment. A: Typical images of desmin staining in glomeruli from CD38$^{-/-}$ and CD38$^{+/+}$ mice with or without DOCA + 1% NaCl treatment (n= 6 each group). B: Typical images of podocin staining in glomeruli from CD38$^{-/-}$ and CD38$^{+/+}$ mice with or without DOCA + 1% NaCl treatment (n= 6 each group).
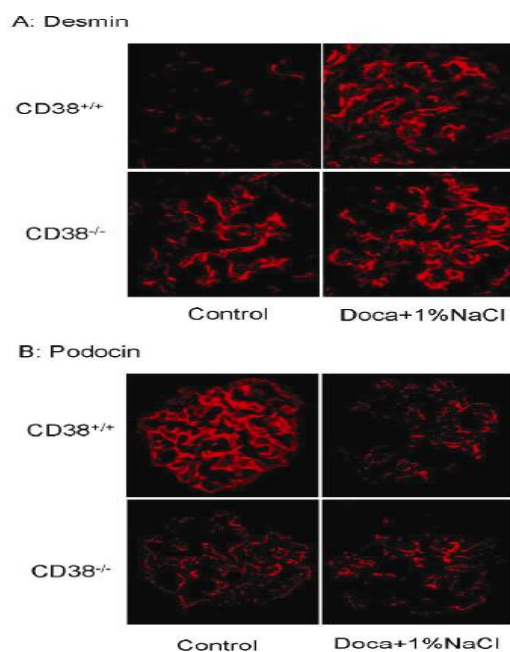
Figure 5: Mean arterial pressure in CD38$^{-/-}$ and CD38$^{+/+}$ mice with or without DOCA + 1% NaCl treatment. Values are arithmetic means ± SEM (n=4 each group) of mean arterial pressure in CD38$^{-/-}$ and CD38$^{+/+}$ mice with or without DOCA + 1% NaCl treatment. [#] Significant difference ($P<0.05$) compared to the values from mice on the normal diet.
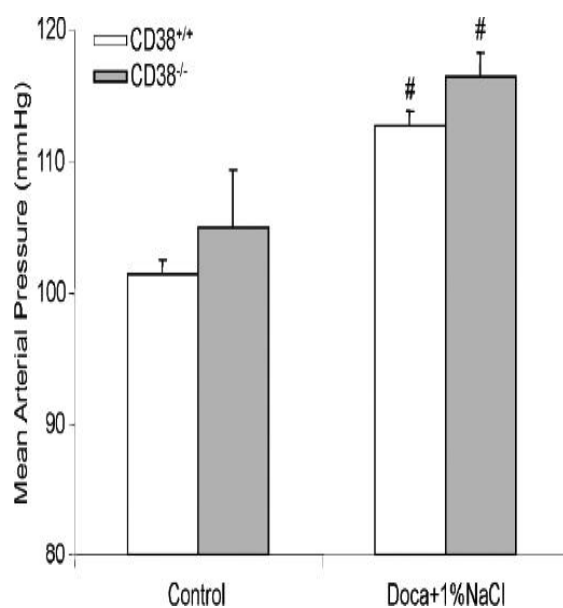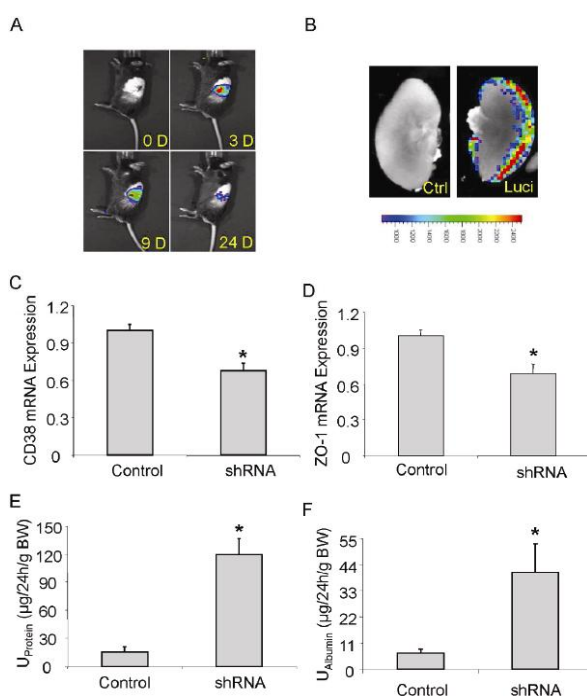
Figure 6: Expression of epithelial-to-mesenchymal transition of podocytes with or without CD38 shRNA transfection. Cultured podocytes were treated with or without CD38 shRNA transfection for 16 h. A: Typical fluorescent microscopic images of P-Cadherin, ZO-1, FSP-1, α-SMA in CD38 shRNA and scrambled shRNA treated podocytes (n=5/group). B: Values are arithmetic means ± SE (n=5 each group) of summarized data showing the percentage of positive staining cells of CD38, P-cadherin, ZO-1, FSP-1, α-SMA, desmin and podocin vs. scrambled shRNA group. * Significant difference ($P<0.05$) compared to the scrambled shRNA group.
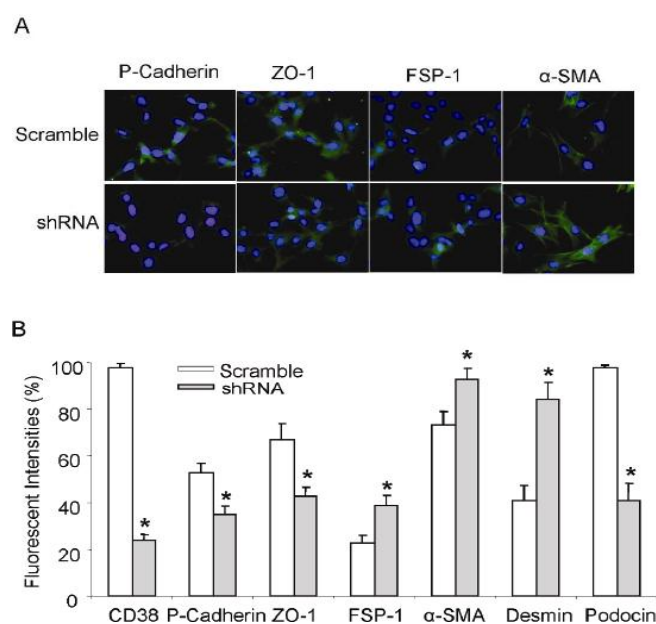
Figure 7: Effects of renal CD38 gene silencing on glomerular injury in C57BL/6J mice.

A: Daily imaging confirmation of gene transfection in the kidney by an *in vivo* molecular imaging system. B: Localization of transfected gene expression in the hemidissected kidney on day 14 after gene delivery. C: Values are arithmetic means ± SEM (n=5-8 each group) of CD38 mRNA expression. D: ZO-1 mRNA expression. E: Urinary total protein excretion. F: Urinary albumin excretion in C57BL/6J mice with or without CD38 shRNA transfection. * Significant difference (*P*<0.05) compared to the values from CD38$^{+/+}$ mice on the control diet.

Figure 8: Expression of epithelial and mesenchymal markers in the glomeruli of C57BL/6J WT mice with or without CD38 shRNA transfection. A: Typical confocal microscopic images of podocin (Alex555, red color) vs. P-Cadherin or ZO-1 or FSP-1 or α-SMA (Alex488, green color) staining in glomeruli of C57BL/6J WT mice with or without CD38 shRNA transfection (n=6/group). B: Summarized data showing the colocalization coefficient represented by Pearson's correlation coefficient illustrating the relationship between podocin *vs.* P-cadherin or ZO-1, C: Summarized data showing the colocalization coefficient represented by Pearson's correlation coefficient illustrating the relationship between podocin *vs.* FSP-1 or α-SMA. * Significant difference ($P<0.05$) compared to the values from CD38$^{+/+}$ mice on the control diet (n=6).