Biochemistry and Biophysics Reports 28 (2021) 101145

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

GAP-43 ameliorates Podocyte injury by decreasing nuclear NFATc1 expression

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ARTICLE INFO

Keywords:
Growth associated protein-43
Podocyte injure
Apoptosis
Nuclear factor of activated T cells c1

ABSTRACT

Podocyte injury is sufficient to cause glomerulosclerosis and proteinuria, eventually leading to kidney failure. Previous studies found that podocytes and neurons had similar biological characteristics. Growth-associated protein-43 (GAP-43) is a growth cone protein in neurons, and a marker of axonal and synaptic growth. However, it is not known whether GAP-43 is expressed in podocytes. Compared with normal glomerular podocytes, GAP-43 was significantly reduced in patients with glomerular diseases. GAP-43 also significantly reduced in lipopolysaccharide (LPS)-treated podocytes. We found that the decreased expression of nephrin, the cell marker of the podocyte, was significantly recovered with GAP-43 overexpression. In contrast, the migration ability in LPS-treated podocyte was reduction after GAP-43 overexpressing. Moreover, overexpression of GAP-43 attenuated podocyte apoptosis by up-regulating the ratio of Bcl-2/Bax with LPS treatment. Finally, Plaue and Rcan1 which are downstream target gene of NFATc1 decreased with overexpression of GAP-43 podocytes. We concluded that GAP-43 attenuated podocyte injury by inhibiting calcineurin/NFATc1 signaling. The findings may provide a promising treatment for podocyte injury-related diseases.

1. Introduction

Podocytes are located at around the glomerular basement membrane. They form part of the structures of the glomerular filtration barrier that regulates the passage of plasma protein into the urine [1,2]. Podocytes are terminally differentiated and have a similar structure and morphology to that of neurocytes [3]. Injury and loss of podocytes have been considered to cause proteinuria and the development of glomerulosclerosis [4–6]. Podocytes play a vital part in maintaining glomerular structure and function, and the progression of chronic kidney diseases always has podocytes involvement [7]. Although accumulating evidence has indicated that signaling pathways play a pivotal role in the pathogenesis of podocyte injury [8,9], the underlying mechanisms of podocyte-associated damage remain to be elucidated.

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https://doi.org/10.1016/j.bbrep.2021.101145
Received 27 May 2021; Received in revised form 27 September 2021; Accepted 28 September 2021

Activated T nuclear factor (NFAT) is the most widely studied calcineurin substrate (can), which belongs to the $Ca2^{+}$ dependent transcription factor family. Many experimental studies have already shown that the activation of NFATc1 is a significant cause of podocyte injury, apoptosis, and glomerulosclerosis [10–14]. Currently, the calcineurin-NFAT axis is an essential therapeutic targets in most immunosuppressive treatments, through which NFAT exerts the detrimental action on podocytes. Meanwhile, mounting evidence suggest that the activation of calcium/calcineurin/NFATc1 signaling is the most crucial event linked to the development of podocyte injury [10–13]. However, the mechanism mediating NFATc1 is still not fully understood. Previous studies have shown that growth-associated protein-43 (GAP-43) constrained the calcium (Ca^{2+}) signal transduction system in the neuron [15], and that GAP-43 is upregulated with stroke, traumatic brain injury, and epilepsy [16–19]. However, the expression and the mechanism underlying the effect of GAP-43 on podocyte injury remains unclear.

This paper aims to investigate the expression and role of GAP-43 in podocyte injury. We discussed the underlying molecular mechanism in an *in vitro* model of podocyte injury using lipopolysaccharide (LPS). We found that GAP-43 ameliorated podocyte injury by suppressing calcineurin/NFATc1 signaling.

2. Materials and methods

2.1. Patients

Renal biopsy samples were used for immunofluorescence staining. The Human study project was approved by the Ethics Committee at Guangdong Provincial People's Hospital. Kidney biopsies were obtained from patients diagnosed with focal segmental glomerulosclerosis (FSGS, n = 3), membranous nephropathy (MN, n = 3), and minimal change disease (MCD, n = 3), as were normal kidney tissues from patients who suffered from surgical nephrectomies.

2.1.1. Cell culture and treatment

The conditionally immortalized mouse podocyte cell line was provided by Dr. Jochen Reiser (Rush University Medical Center, Chicago, IL, USA). Podocytes were cultivated at 33 °C in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, USA) and 50 U/mL˙1 IFN-γ (growth permissive conditions; CYT-358, ProSpec, Tany Technogene Ltd., Ness Ziona, Israel). Podocytes were differentiated at 37 °C in the absence of IFN-γ (non-permissive condition) for 10–14 days. Podocytes were incubated in serum-free RPMI-1640 medium for 24 h to synchronize into dormancy, and then incubated with LPS (100 μg/ml) for 24 h, 48 h, and 72 h, respectively. To overexpress GAP-43 in podocytes, the adenovirus containing GFP-GAP-43 (Hanbio, China) was employed. For intervention experiments, adenovirus GFP-GAP-43 (5 μL) was transfected cells with LPS (100 μg/ml) for 72 h. Each reaction was repeated in triplicate at least.

2.2. Immunofluorescent staining

Kidney cryosections or the cells (2*10ˆ4) cultured on the cover glass were fixed with pre-cold methanol for 20 min at −20 °C, followed by infiltration with 0.1% Triton X-100. After 10 min, 5% BSA was used to block nonspecific binding for 20 min at 37 °C. For immunofluorescent staining, the cells were incubated with rabbit anti-GAP-43 (Abcam, 1:500) and goat anti-synaptopodin (Santa Cruz, 1:100) overnight at 4 °C. After washing with PBS three times, the secondary antibodies, Alexa Fluor 488 donkey anti-rabbit and Alexa Fluor 546 donkey anti-mouse (Life technologies, 1:250) were incubated for 1 h at room temperature. 4′-6- diamidino-2-phenylindole (DAPI) was used to stain slides for 15 min to visualize the nuclei. Finally, all images were analyzed by laser confocal microscopy (LCSM, Germany).

2.3. Real-time quantitative-PCR

Total RNA isolation and quantitation were carried out according to the manufacturer's instructions. Complementary DNA synthesis was carried out using a PrimerScript real-time reagent kit (Takara Biotechnology, China). Real-time quantitative-PCR was performed with Power SYBR Green PCR Master Mix (Takara Biotechnology, China). The primers used for qPCR are listed as follows: GAP-43, forward 5′-ACCACCATGCTGTGCTGTATGAG-3′,reverse 5′-TCCGGCTTGACACCATCTTG-3′; Plaur, forward 5′-GACTACCGTGCTTCGGGAATG-3′, reverse 5′-ATGGTCCTGTTGGTCTTTTCG-3′; β-actin,forward 5′-GCTTCTAGGCGGACTGTTAC-3′, reverse 5′-CCATGCCAATGTTGTCTCTT-3′; Rcan1, forward 5′-CTCCTCCCGTTGGCTGGAAA-3′, reverse 5′-CTGGGAGTGGTGTCTGTCGC-3′. For each reaction we used *GAPDH* mRNA as an internal control and quantified the relative expression levels of mRNA using the $2^{-\Delta\Delta Ct}$ method.

2.4. Western blotting

After subjecting to different experimental conditions, podocytes were washed twice by cold PBS, and then RIPA buffer was added for lysing. The nuclear protein was extracted from podocytes using the Nuclear and Cytoplasmic Protein Extraction Kit (Nanjing Key GEN Biotech, China). The protein assay reagent kit (Invitrogen, Waltham, MA) was used to evaluate the protein concentration. An equal amount of protein was separated on 7.5% SDS-PAGE gels electrophoresis, followed by transfer to PVDF membranes (Millipore, USA). The membranes were incubated antibodies overnight at 4 °C after blocking with 5% non-fat dry milk for at least 1 h. The primary antibodies used were as follows: rabbit anti-GAP-43 (Abcam, 1:500), rabbit anti-NFATc1 (Abcam, 1:1000), rabbit anti-histone (Cell Signaling Technology, 1:1000), rabbit anti-nephrin (Abcam, 1:2000), rabbit anti-β-actin (Affinity, 1:10000), rabbit anti-calcineurin (Abcam, 1:2000), rabbit anti-Bax (Abcam, 1:1000), and rabbit anti-Bcl-2 (Abcam, 1:1000). The next day, the anti-rabbit IgG (Cell Signaling Technology, 1:5000) was incubated for 1 h at 37 °C. Membranes were visualized using ECL Western Blotting Detection Reagents (Advansta, USA). β-actin or histone as the internal control.

2.5. Wound healing assay

Cultured differentiated podocytes (1×10^5/ml) under different conditions were sowned overnight on vitronectin-coated coverslips in six-well plates. After 24 h, each coverslip was scratched with a sterile 200-μl pipette tip, and then washed with PBS. Cells were fixed with cold methanol, permeabilized with 0.5% Triton X-100 in PBS and cell nuclei were stained with DAPI (Roche Diagnostics). Images were captured by phase-contrast microscopy under a x4 objective on a Leica SP5-FCS microscope (Leica Microsystems). The number of cells that had migrated into the same-sized square fields were counted. Results were presented as the mean ± standard deviation (SD) of six independent experiments.

2.5.1. Flow cytometric analysis

After different treatment conditions, an Annexin V-APC/PI apoptosis detection kit (Nanjing Key GEN Biotech, China) was used to assay cell apoptosis. In brief, using 200μLof binding buffer to resuspend, cells (10ˆ5) were treated with 5 μL Annexin V (conjugated with APC) and protected from light for 15 min. PI was incubated at room temperature for 15 min, followed by detection using a FACS can flow cytometry and cell quest software (BD).

2.6. Statistical analysis

All data were presented as mean ± SEM. Statistical analysis of the data was carried out using Ver.20.0 (SPSS, USA). Comparisons between groups were made using one-way analysis of variance with Bonferroni

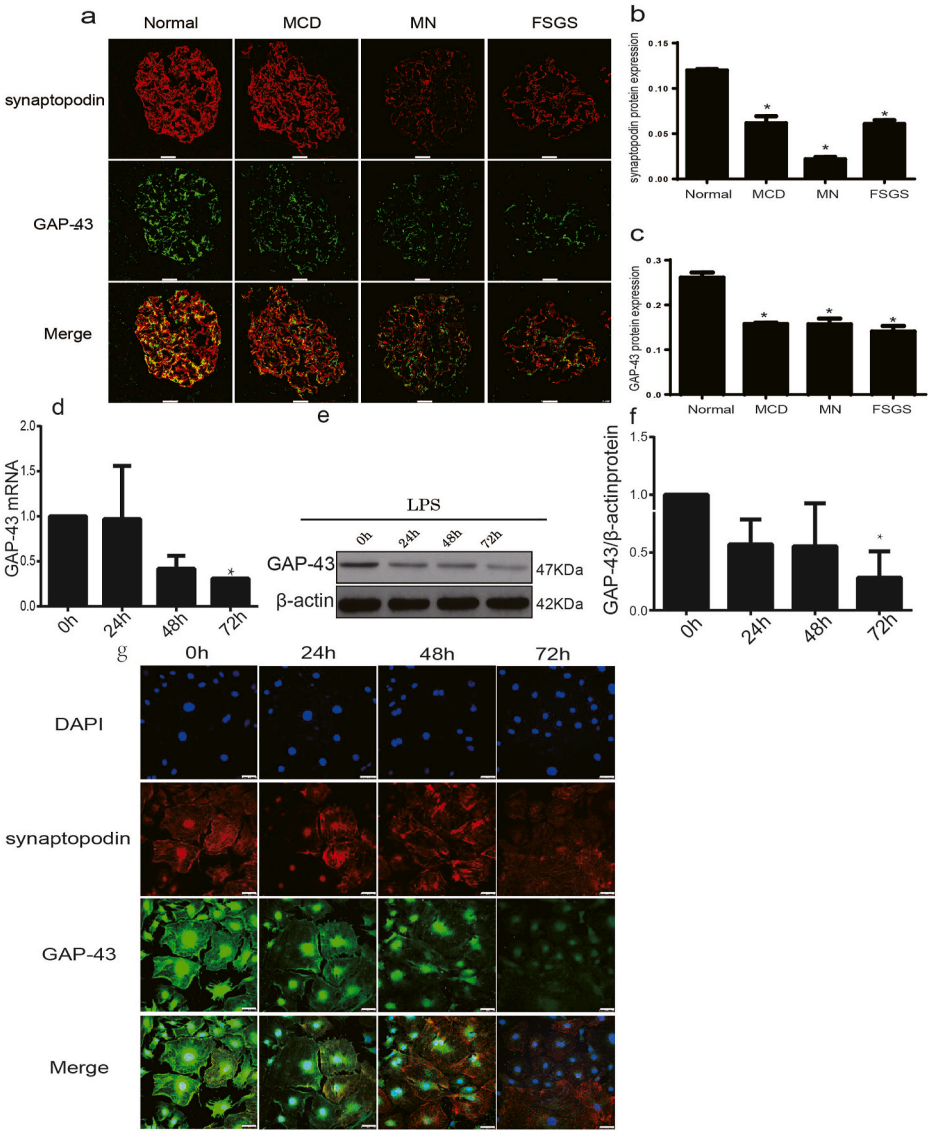

Fig. 1. GAP-43 is down-regulated in podocytes in proteinuric patients, and GAP-43 was decreased in lipopolysaccharide (LPS)-treated podocytes *in vitro*. **(a,b,c)** The podocyte-specific marker synaptopodin (synpo, red) and GAP-43 protein (green) were identified in renal biopsy samples from patients and normal kidney tissues acquired from patients who suffered from surgical nephrectomies. As shown by double immunofluorescence, GAP-43 expression in normal glomeruli was high, which was in contrast to the glomeruli from patients. However, the expression of GAP-43 was significantly reduced in human glomerular *injury* and *disease* like the patients with minimal change disease (MCD, n = 3), focal segmental glomerulosclerosis (FSGS, n = 3), and membranous nephropathy (MN, n = 3). Bars = 50 μm **(d,e,f)** GAP-43 mRNA and protein are decreased in podocytes with LPS treatment for 24, 48, and 72 h (n = 3). **(g)** Similarly, immunofluorescence staining showed GAP-43 protein was decreased after 72 h treatment of LPS. Data were from at least three independent experiments. Bars = 50 μm*P < 0.05 versus controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

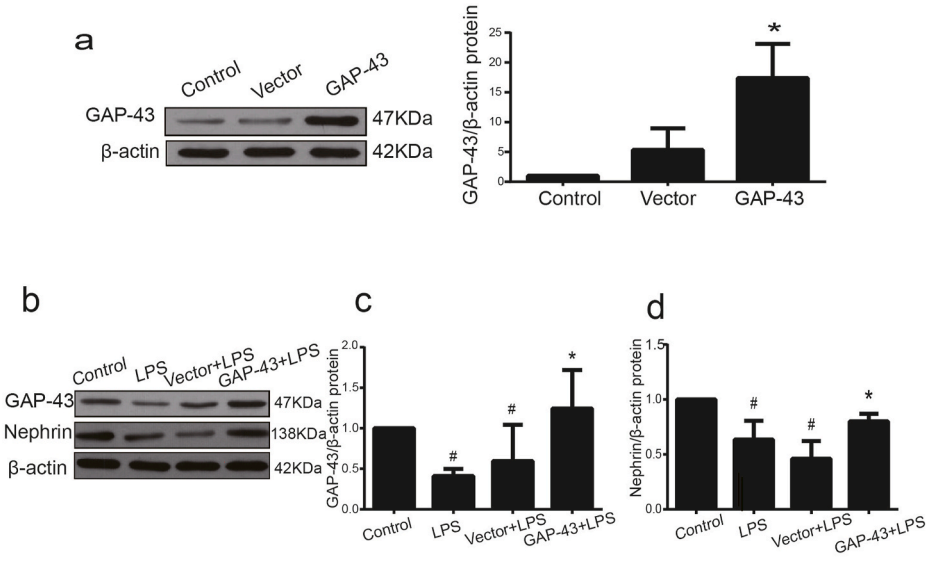

Fig. 2. Overexpression of GAP-43 *in vitro* increased podocyte nephrin expression. **(a)** To investigate overexpression GAP-43 in podocytes, the adenovirus containing GFP-GAP-43 was added to cells. After treatment of adenovirus packing GFP-GAP-43 clone, GAP-43 protein was obviously increased in podocytes (n = 3). **(b,c)** Similar results were observed for GAP-43 protein expression in GAP-43- overexpressed podocytes treated with LPS (n = 3). **(b,d)** Consistent with GAP-43 protein expression, the expression of nephrin was increased in GAP-43- overexpressed podocytes treated with LPS (n = 3). #P < 0.05, LPS vs Control; *P < 0.05, Vector + LPS vs GAP-43 + LPS.

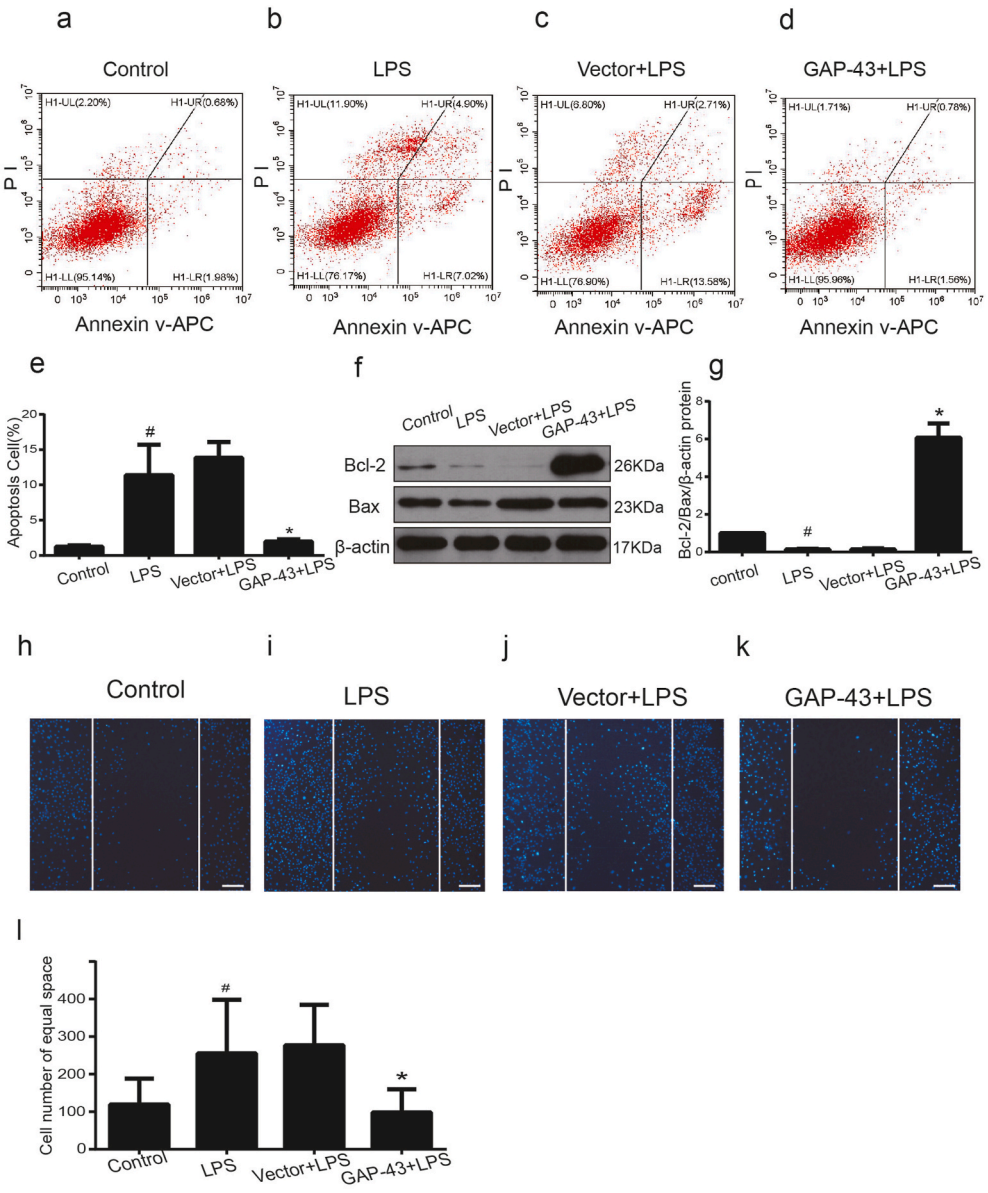

Fig. 3. Overexpression of GAP-43 attenuated *in vitro* podocyte apoptosis and inhibited podocyte motility induced by LPS. (**a–e**) Podocytes were stained with Annexin V-APC/PI for flow cytometry analysis. Apoptosis was significantly increased in LPS-treated podocytes. By contrast, overexpression of GAP-43 reduced lipopolysaccharide-induced podocyte apoptosis (n = 3). (**f**) Bax and Bcl-2 are specific indicators of apoptosis. Here, the pro-apoptotic Bax was decreased. On the contrary, the anti-apoptotic Bcl-2 was increased after overexpression of GAP-43. (**g**)The ratio of Bcl-2/Bax was up-regulated (n = 3). Data were from at least three independent experiments. Overexpression of GAP-43 inhibits podocyte motility induced by LPS. (**h**) Control group. (**i**) Overexpression of GAP-43 significantly reduced LPS-induced podocyte motility. (**j,k**) By contrast, treatment with LPS increased podocyte motility. Bars = 100 μm. (**l**) Quantified numbers of migrated podocytes. All Values are expressed as the mean ± standard deviation. Magnification, × 40. #P < 0.05, LPS vs Control; *P < 0.05, Vector + LPS vs GAP-43 + LPS.

adjustment/Tukey's test or the Dunnett's T3 test. P-values <0.05 were considered significant.

3. Results

3.1. GAP-43 is markedly decreased in human proteinuric diseases

Using synaptopodin labels to identify podocytes, we found that GAP-43 was expressed in glomeruli podocytes (Fig. 1a). As shown in Fig. 1, there was high expression of GAP-43 in normal glomeruli podocytes (Fig. 1a, c). In contrast, GAP-43 was significantly reduced in human glomerular diseases, including MCD, FSGS, and MN (Fig. 1a, c).

3.2. GAP-43 expression decreased in LPS-treated cultured podocytes in vitro

Compared with the control-group, podocytes with LPS treatment resulted in a marked decrease in GAP-43 in a time-dependent manner (Fig. 1d). Similar results were observed in GAP-43 protein using western blotting analysis (Fig. 1e and f). Consistent with mRNA levels, a reduction in the expression of GAP-43 and synaptopodin was observed in podocytes with LPS treatment using immunofluorescent staining (Fig. 1g).

3.3. Overexpression of GAP-43 increased podocyte nephrin expression

To explore whether GAP-43 impacted podocyte injury, we built a podocyte model of GAP-43 overexpression using adenovirus containing GFP-GAP-43 (Ad-GAP-43). Compared with Ad-GFP podocytes (vector), Ad-GAP-43 podocytes increased GAP-43 protein expression (Fig. 2a). Moreover, there was a noticeable increase in GAP-43 protein in LPS treated podocytes (Fig. 2b and c), and the expression of nephrin also significantly increased in the Ad-GAP-43 group. By contrast nephrin protein, a specific marker of podocytes, decreased compared with the control group after LPS-treatment. Therefore, these results suggest that GAP-43 impacted podocyte injury, and that GAP-43 may contribute to podocyte protection.

3.4. Overexpression of GAP-43 attenuated podocyte apoptosis and inhibited podocyte motility induced by LPS in vitro

To elucidate the influence of GAP-43 on podocytes, we investigated

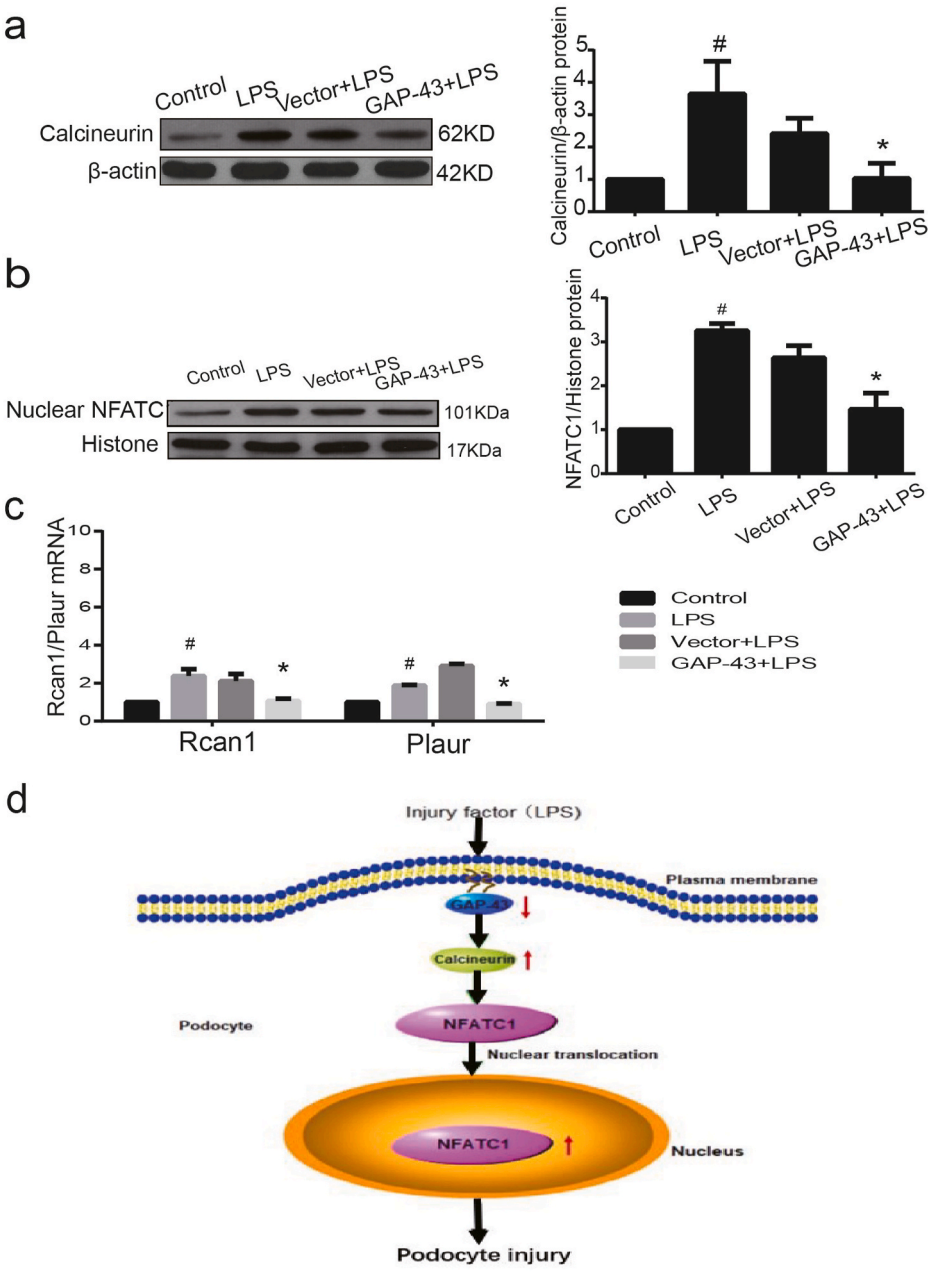

Fig. 4. Overexpression of GAP-43 decreased calcineurin expression and nuclear localization of NFATc1 in injured podocytes. (a,b) The expression of calcineurin and nuclear NFATc1 were decreased in GAP-43-overexpression podocytes with LPS treatment.(**c**)The expression of Rcan1 and Plaur were reduced in podocytes overexpressing GAP-43 (n = 3). #P < 0.05, LPS vs Control; *P < 0.05, Vector + LPS vs GAP-43 + LPS.(**d**)The mechanism of GAP-43 deficiency aggravates podocyte injury. Black arrows indicate positive regulation.

podocytes apoptosis and motility using wound-healing and flow cytometry assay. The apoptosis of podocytes with high expression of GAP-43 decreased, as assessed by flow cytometry analysis, in contrast to LPS treated podocytes (Fig. 3a–e). Meanwhile, two specific indicators of apoptosis, Bax, and Bcl-2, were also investigated in this study. We found that overexpression of GAP-43 reduced the pro-apoptotic Bax protein and increased the anti-apoptotic Bcl-2 protein (Fig. 3f), and the ratio of Bcl-2/Bax was also up-regulated (Fig. 3g). The spatial mobility of podocytes was analyzed using scrape-wound assay (Fig. 3h-l). Compared with the LPS-treated podocytes, the migration of podocytes was remarkably alleviated in the GAP-43 overexpression group. The data indicated that GAP-43 overexpression inhibited the apoptosis and motility of podocytes *in vitro*.

3.5. Overexpression of GAP-43 decreased calcineurin expression and nuclear localization of NFATc1 in injured podocytes

To further explore the effect of GAP-43 on calcineurin/NFATC1 signaling. As shown in Fig. 4a and b, we found that the expression of calcineurin protein and nuclear NFATc1 increased with LPS-treatment. Conversely, calcineurin protein and nuclear NFATc1 were reduced after 72 h-treatment with LPS in GAP-43 overexpression podocytes (Fig. 4a and b). Two downstream target genes of NFAT signaling, Rcan1 and Plaur, mediated podocyte injury by NFAT activation. The mRNA of Rcan1 and Plaur was a marked reduction in podocytes overexpressing GAP-43, but significantly increased in podocytes with LPS treatment (Fig. 4c). The data suggested that GAP-43 ameliorated podocyte injury by inhibiting calcineurin-NFATc1 signaling pathway.

4. Discussion

Proteinuria, renal function deterioration, and glomerular diseases are often attributed to podocyte injury [20]. Although numerous signal pathways have been implicated in chronic kidney disease, the exact mechanism still needs more study. Previous studies have pointed out that the podocyte and the neuron share many molecular cell biological

features, including nephrin and synaptopodin expression [21]. GAP-43 is expressed in neurons. However, the role of GAP-43 in podocyte injury remains unclear. In this study, we found that GAP-43 had a protective action in podocyte injury by mediating the calcineurin-NFATc1 signaling pathway.

GAP-43 is a substrate for protein kinase C and serves as a marker of axonal and synaptic growth [22]. The up-regulation of GAP-43 is often followed by stroke and traumatic brain injury [16–18]. As an example, during the injury regeneration process, decrease of GAP-43 is recovered in motor neurons [23]. Many studies suggested that axonal structural remodeling was led by the enhanced phosphorylation status of GAP-43. Importantly, these studies showed that GAP-43 played a critical role in the injury-induced regeneration process [24,25]. We found that GAP-43 was abundantly expressed in human glomerular cells, especially in podocytes. GAP-43 expression in normal glomeruli was high, which was in contrast to the glomeruli from MN, MCD, or FSGS. Besides, qRT-PCR and western blotting analysis showed that both GAP-43 mRNA and protein of GAP-43 were downregulated in LPS-treated podocytes. One of the novel findings was that the expression of the pro-apoptotic protein, Bax, was decreased in the present study. On the contrary, that of the anti-apoptotic protein, Bcl-2, was significantly increased after overexpression of GAP-43. The ratio of Bcl-2/Bax was up-regulated in podocytes with LPS treatment. Meanwhile, the migration ability of cells and down-regulation of nephrin were recovered by overexpression of GAP-43 in podocytes with LPS treatment. These data indicated that GAP-43 was reduced in podocyte injury *in vivo* and *in vitro* and had a critical role in alleviating damage.

The protective mechanism of podocyte injury mediated by GAP-43 is still unclear. Gerendasy et al. [15] implied that GAP-43 constrained Ca^{2+} signal transduction system in the neuron. Meanwhile, Noredn et al. [26] found that GAP-43 could affect calcium mediated events and play a role in phospholipid metabolism. Many studies have demonstrated that Ca^{2+}/calcineurin/NFATc1 signaling activation is an essential cause of podocyte injury, apoptosis, and glomerulosclerosis [10–12]. NFAT is the substrate of calcineurin, representing a family of Ca^{2+} dependent transcription factors [14]. It consists of five members: NFAT1, NFAT2, NFAT3, NFAT4, and NFAT5 [27]. In this study, we demonstrated that both calcineurin and nuclear NFATc1 were downregulated in GAP-43 overexpression podocytes. Another important finding was that podocytes with overexpression of GAP-43 reduced both Rcan1 and Plaur expression. Based on this finding, we presume that the possible protection mechanism of GAP-43 in podocytes might rely on the fact that GAP-43 blocks calcineurin activation, thereby inhibitings NFATc1 translocation into the nucleus.

Recent data showed that calcineurin (CaN) inhibitors, such as cyclosporine A and FK506, partially restored the decrease of nephrin expression, and improved albuminuria [28–30]. In this study, we found that overexpression of GAP-43 decreased calcineurin expression and increased podocyte nephrin expression. The data suggested that GAP-43 may improve podocyte nephrin expression by reducing calcineurin to protect podocytes.

In summary, we firstly demonstrated that GAP-43 was decreased in human podocytopathies and in LPS-treated podocytes. Besides, the reduced nephrin was significantly recovered in GAP-43-overexpressed podocytes with LPS treatment. Moreover, the mRNA expression of Rcan1 and Plaur were reduced in GAP-43 overexpressed podocytes. Finally, GAP-43 has anti-apoptotic effects on podocytes. The data indicated that GAP-43 ameliorated podocyte injury by suppressing calcineurin-NFATc1 signaling (Fig. 4).

Statement of ethics

These investigations were conducted in accordance with the Second Helsinki Declaration. All of the participants signed a written informed consent form for participation in this study.

Declaration of competing interest

The authors declared that they have no conflicts of interest to this work.

We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted. Some of the data of this article have been reported in Chinese.

Acknowledgements

This study was supported by National Natural Science Foundation (81870508, 81670656, 81970625 and 81900658), Guangdong Province High-level Hospital Construction Project (No. DFJH201901), Guangdong Provincial Science Foundation (No. 2018A030313260), Sichuan Science and Technology Program (2021YFS0159), Guangzhou City Science and Technology Project (No. 201707010009), Guangdong-Hong Kong Joint Laboratory on Immunological and Genetic Kidney Diseases (No. 2019B121205005).

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