The Krüppel-like factor 15-NFATc1 axis ameliorates podocyte injury: a novel rationale for using glucocorticoids in proteinuria diseases

Caoshuai Dou*, Hong Zhang*, Guibao Ke, Li Zhang, Zhiwen Lian, Xueqin Chen, Xingchen Zhao, Yuanhan Chen, Ruizhao Li, Jianchao Ma, Zhuo Li, Wenjian Wang, Zhi ming Ye, Xinling Liang, Wei Shi, Bin Zhang and Shuangxin Liu

Department of Nephrology, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Guangdong Provincial Institute of Geriatrics, Guangzhou, 510080, China

Correspondence: Bin Zhang (13925056339@139.com) or Shuangxin Liu (13543456446@139.com)

Podocyte injury and loss contribute to proteinuria, glomerulosclerosis and eventually kidney failure. Recent studies have demonstrated that the loss of Kruppel-like factor 15 (KLF15) in podocytes increases the susceptibility to injury; however, the mechanism underlying the protective effects on podocyte injury remains incompletely understood. Herein, we showed that KLF15 ameliorates podocyte injury through suppressing NFAT signaling and the salutary effects of the synthetic glucocorticoid dexamethasone in podocyte were partially mediated by the KLF15–NFATc1 axis. We found that KLF15 was significantly reduced in glomerular cells of proteinuric patients and in ADR-, LPS- or HG-treated podocytes in vitro. Overexpression of KLF15 attenuated podocyte apoptosis induced by ADR, LPS or HG and resulted in decreased expression of pro-apoptotic Bax and increased expression of anti-apoptotic Bcl-2. Conversely, the flow cytometry analysis and TUNEL assay demonstrated that loss of KLF15 accelerated podocyte apoptosis and we further found that 11R-VIVIT, a specific NFAT inhibitor, and NFATc1–siRNA rescued KLF15-deficient induced podocyte apoptosis. Mechanistically, ChIP analysis showed that KLF15 bound to the NFATc1 promoter region -1984 to -1861 base pairs upstream of the transcription start site and the binding amount was decreased after treatment with LPS. The dual-luciferase reporter assay indicated that NFATc1 was a direct target of KLF15. In addition, we found that in vitro treatment with dexamethasone induced a decrease of NFATc1 expression in podocytes and was abrogated by knockdown of KLF15. Hence, our results identify the critical role of the KLF15–NFATc1 axis in podocyte injury and loss, which may be involved in mediating the salutary effects of dexamethasone in podocytes.

Introduction

Chronic kidney disease (CKD) is a worldwide public health burden with a high socioeconomic cost to health systems and is growing in incidence and prevalence. The progression of CKD eventually develops to end-stage renal disease (ESRD) requiring renal replacement therapy [1,2]. Therefore, it is highly important to elucidate the mechanisms underlying CKD progression. Podocytes are terminally differentiated cells, located in the outer surface of the glomerular basement membrane (GBM), and play a vital role in maintaining the structural and functional integrity of the glomerular filtration barrier [3]. Ongoing
Podocyte injury has been proven to be a prominent feature of CKD and is a center stage for the development of potential therapeutic interventions for CKD progression [4,5]. Notably, accumulating evidence suggests that podocyte injury and loss play a key role in the pathogenesis of many human kidney diseases including minimal change disease (MCD) [6], focal segmental glomerulosclerosis (FSGS) [7,8], membranous nephropathy (MN) [9], diabetic nephropathy (DN) [10,11] and others. Therefore, to investigate the mechanism underlying podocytes injury becomes increasingly urgent.

Kruppel-like factors (KLFs), a subclass of zinc finger family of DNA-binding transcriptional factors containing KLF1–KLF17, are implicated in a diverse range of cellular processes including proliferation, apoptosis and differentiation [12]. KLFs share high homology with Sp1-like transcription factors, one of the first mammalian transcription factors to be identified and classified [13]. KLFs are widely expressed, but their expressions also showing a difference according to the type of tissue. Krüppel-like factor 15 (KLF15), a kidney-enriched transcription factor, is a key regulator of podocyte differentiation and the loss of KLF15 in podocytes increases the susceptibility to injury [14]. Specifically, the transcription of podocyte specific genes, such as podocin and nephrin, is regulated by KLF15 [14]. Low-protein diets have shown to induce the KLF15 expression and protect against renal fibrosis [15]. In addition, treatment with dexamethasone in vitro directly enhances KLF15 expression. Accordingly, the protective effect of dexamethasone on podocyte injury is abrogated in podocyte-specific KLF15 KO mice [16]. However, the mechanisms underlying the protective effects of KLF15 on podocyte injury remain largely unexplored.

A recent promoter analysis combined with enrichment analysis and the Fisher exact test have identified representative genes with KLF15-binding sites, including nuclear factor of activated T cells c1 (NFATc1) [16]. NFATc1, also known as NFAT2, is a member of the NFAT transcription factors family, which was originally characterized as an important mediator in immune responses [17]. NFAT proteins are the most widely studied substrates for calcineurin and the major regulators of transcription in response to Ca²⁺/calcineurin signals [18]. In resting cells, NFAT proteins are highly phosphorylated and located in the cytoplasm. Upon stimulation, NFAT proteins are dephosphorylated by calcineurin and then translocate into the nucleus, becoming transcriptionally active [19,20]. Accumulating evidences now indicate that activation of NFATc1 has identified as a central pathological mechanism underlying podocyte injury and glomerulosclerosis [21,22]. Meanwhile, our previous studies showed that activation of NFATc1 exacerbates podocyte injury, including motility, effacement of foot processes, apoptosis and deletion [23–25]. Here, we found that KLF15 plays a protective role in podocyte injury by directly regulating NFATc1, and the KLF15–NFATc1 axis may be involved in mediating the salutary effects of dexamethasone in podocytes.

Materials and methods

Patients
The study of patients was complied with the Second Helsinki Declaration received full approval from the Ethics Committee of Guangdong Provincial People’s Hospital (NO.GDREC20160140H). Renal biopsies tissues from patients with minimal change disease (MCD), focal segmental glomerulosclerosis (FSGS), diabetic nephropathy (DN) and renal cell carcinoma (adjacent normal tissues) were respectively obtained after patients signed written informed consent.

Cell culture and treatments
The conditionally immortalized mouse podocyte cell line was a kind gift provided by Dr Jochen Reiser (Rush University Medical Center, Chicago, IL, U.S.A.) and cultured as previously described [26]. Differentiated podocytes were treated with HG (30 mM) and LPS (100 µg/ml) for 48 h, ADR (0.25 µg/ml) for 24 h or dexamethasone (10 µM, Sigma) for 6, 12, 24 or 48 h. Inhibition experiments on NFATc1 activity, podocytes were exposed to 11R-VIVIT at concentrations of 100 nM for 48 h.

siRNA transfection and infection of adenovirus
KLF15 knockdown in podocyte was performed using KLF15-siRNA designed and synthesized by RiboBio Co. Ltd (Guangzhou, China). Transfection experiments were performed with siRAN (50 nM) using Lipofectamine 2000 reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA) following the manufacturer’s protocol. Adenovirus packing ORF–KLF15 clone was purchased from Hanbio (Shanghai, China) and transfected podocytes to achieve KLF15 overexpression according to instructions provided by the manufacturer.
Real-time quantitative-PCR

The total RNA from cultured podocytes was extracted using Trizol reagent (Invitrogen) according to the supplier's protocols. The cDNAs were prepared from total RNA (1 μg) by using PrimerScript RT reagent kit (Takara Biotechnology, Dalian, China) and then the cDNA (1 μl) was amplified using Power SYBR Green PCR Master Mix (Takara Biotechnology, Dalian, China) on a Bio-Rad CFX96. The 2−ΔΔCt method was used to calculate the relative expression levels of mRNA and the data were normalized to GAPDH (housekeeping gene). The primers used are listed as follows: GAPDH, forward 5′-AGGTCGCGTGAAGCGAATTG-3′, reverse 5′-TGTA GACCATTGAGTGGTCA-3′; KLF15, forward 5′-GAGAACTTCTCGTCA CGAAA-3′, reverse 5′-GCTGGAGACATCCTGTCTCAT-3′; NFATc1, forward 5′-GGAGAGTGCGAA TCGAGAT-3′, reverse 5′-TTGCA GCTGAGAATGCTGT-3′; Bax, forward 5′-AGAC AGGCCCTTTTGTCTAC-3′, reverse 5′-AATTCCCGCGAGACACTG-3′; Bcl-2, forward 5′-GC TACCCTGACTGCCCTCG-3′, reverse 5′-CCCA CCGCAAATCAGAAAGG-3′; Plaur, forward 5′-GA CTACCGTGCTGCGAAATG-3′, reverse 5′-ATGGTCTGTTGGTCCTT GCG-3′; Fzd9, forward 5′-GCGAC GACCTCTG ATGGAG-3′, reverse 5′-GGC CGAGACCA GAAACACCTC-3′; Rcan1, forward 5′-CTCCCTCGGTTTGCTGAA A-3′, reverse 5′-CTGGGA GTGGTGTCTGT CG-3′.

Western blotting

Whole and nuclear protein extraction from podocytes under diverse experimental conditions was prepared as previously described [24]. Protein concentration was determined with a protein assay reagent kit (Invitrogen). Equal amount (60 μg) of proteins were separated by 10% SDS–PAGE and then transferred onto polyvinylidene fluoride membranes (Millipore, Billerica, MA, U.S.A.). The electroblotted membranes were blocked in 5% non-fat dry milk for 1 h at room temperature and then incubated overnight at 4°C with the following primary antibodies: mouse anti-KLF15 (Santa Cruz, Dallas, TX, U.S.A.), rabbit anti-NFATc1 (Abcam), rabbit anti-Bcl-2 (Cell Signaling Technology), rabbit anti-Histone (Cell Signaling Technology, Danvers, MA, U.S.A.), rabbit anti-Bax (Santa Cruz, Dallas, TX, U.S.A.), rabbit anti-GAPDH (Bioworld Technology, Nanjing, China). After washing, HRP-conjugated secondary anti-mouse IgG or anti-rabbit IgG (Cell Signaling Technology) were added for 1 h at room temperature. Finally, the membranes were immersed in ECL reagent (Advansta, Menio Park, CA, USA) and detected with Image Quant LAS 500 (GE Healthcare Life Sciences). ImageJ software was used to analysis the band intensity and the data were standardize to GAPDH or Histone (nuclear fractions).

Immunofluorescent staining and TUNEL staining

Frozen tissue samples from proteinuria patients or cultured podocytes were subjected to immunofluorescent staining according to a standard immunofluorescence protocol as previously described [26]. The primary antibodies are listed as follow: mouse anti-KLF15 (Santa Cruz); goat anti-synaptopodin (Santa Cruz); rabbit anti-WT1 (Abcam); Secondary antibodies purchased from Cell Signaling Technology (goat anti-rabbit Alexa Fluor 555; goat anti-mouse Alexa Fluor 488) and Protein Tech Group, Inc (donkey anti-goat IgG 488; donkey anti-mouse IgG 546) were used. Apoptotic cells were detected by using TUNEL kit (Roche) according to the manufacturer's instructions. TUNEL-positive cells were represented by green fluorescence. The laser confocal microscopy (LCSM, Zeiss KS 400, Postfach, Gernany) was used to capture the images.

Flow cytometric analysis

For cell apoptosis assay, an Annexin V-FITC/PI apoptosis detection kit or Annexin V-APC/PI (Nanjing KeyGEN Biotech, Nanjing, China) was used according to manufacturer's protocol. Concisely, the podocytes were collected and then were resuspended with 200 μl 1 × binding buffer following by incubation with 5 μl Annexin V-FITC or Annexin V-APC (adenovirus packing ORF-KLF15 clone treated podocytes) in the dark for 15 min at room temperature. After incubation with PI for 5 min in the dark, the samples were detected with a FACSscan flow cytometer (BD).

Chromatin immunoprecipitation (ChIP)-quantitative PCR assay

The ChIP assay was performed using Thermo ChIP Kit (Invitrogen) as previously described [25]. Briefly, chromatin extracted from the lysed podocytes was sonicated to shear chromatin fragments of 500 base pairs in length. Immunoprecipitation of KLF15-crosslinked chromatin was performed using mouse anti-KLF15 antibody (Santa Cruz). Normal rabbit anti-IgG (Millipore) was used as a negative control. After incubation of protein G magnetic beads, DNA protein cross-links were reversed and then the DNA was purified. Purified DNA samples were subsequently amplified in ABI PRISM® 7500 Sequence Detection System (Applied Biosystems, Carlsbad, CA) using

Attribution License 4.0 (CC BY).

© 2020 The Author(s). This is an open access article published by Portland Press Limited on behalf of the Biochemical Society and distributed under the Creative Commons Attribution License 4.0 (CC BY).
SYBR Green qPCR (TAKARA). The primer sequences targeted mouse NFATc1 promoter are listed as follows: forward 5′TCCCCAAGGTCTCGGGTGGG3′, reverse 5′TTCCGTCCCCGCCCCTTCC CT3′. The 2−ΔΔCT method was used to analyze the data.

**Dual-luciferase reporter assay**

For mouse NFATc1 promoter luciferase assay, the promoter plasmids were constructed by GeneCopoeia (Guangzhou, China). Luciferase assay was performed as per manufacturer’s protocol. After 48 h of co-transfection, the cell culture medium was collected and detected with the Secretre-Pair Dual Luminescence Assay Kit (GeneCopoeia). For data analysis, Gaussia luciferase was normalized to secreted Alkaline Phosphatase, respectively. Each experiment was repeated in triplicate.

**Statistical analysis**

Data were presented as the means ± SEM. All statistical analyses were conducted using the SPSS for Windows Ver. 20.0 (Inc., Chicago, IL, U.S.A.). Student’s t-test was used to compare data between two groups and one-way ANOVA followed by Bonferroni adjustment/Tukey test or the Dunnett T3 test was used to compare data among more than two groups. All experiments were repeated at least three times and P < 0.05 was considered statistically significant.

**Results**

**KLF15 was markedly decreased in human proteinuric diseases**

We first evaluated the expression of KLF15 in the kidney from patients with proteinuria and normal controls (renal cell carcinoma patients). As shown in Figure 1, KLF15 protein was abundantly expressed in human glomerular cells, including podocytes, which were characterized by co-labeling with WT1, a specific maker of this cell type [27]. In contrast, we found low expression of KLF15 in glomeruli from patients with diabetic nephropathy (DN), minimal change disease (MCD) and focal segmental glomerulosclerosis (FSGS).

KLF15 was reduced in adriamycin (ADR)-, lipopolysaccharide (LPS)- or high glucose (HG)-treated podocytes in vitro

To further assess the change of KLF15 in injured podocytes, we treated cultured podocytes with ADR for 24 h, LPS for 48 h and HG for 48 h, respectively. First, the mouse podocyte cell line was identified by immunofluorescent staining with the podocyte specific marker synaptopodin (Supplementary Figure S1). RT-qPCR analysis showed a significantly decrease of KLF15 mRNA expression in ADR-, LPS- or HG-treated podocytes (Figure 2A). Consistent with mRNA expression, ADR, LPS or HG induced an obvious decrease of KLF15 protein expression in cultured podocytes (Figure 2B).

**Induction of KLF15 attenuates podocyte apoptosis in vitro**

A previous study showed that KLF15 plays a protective role in podocyte injury [28]; however, the role of KLF15 involed in podocyte apoptosis remains understood. Therefore, we generated a cell model of KLF15-overexpressed podocytes using adeno virus packing ORF–KLF15 clone. To evaluate the efficiency of adeno virus packing ORF–KLF15 clone, RT-qPCR analysis and Western blotting were performed on Ad-KLF15 and Ad-GFP (control) podocytes. We observed that Ad-KLF15 cells exhibited a markedly increase of KLF15 mRNA and protein expression compared with Ad-GFP cells (Figure 3A,B).

Bax and Bcl-2 are the core regulators of the intrinsic pathway of apoptosis [29]. Here, we found that the mRNA and protein expression of Bax, a well-known indicator of apoptosis, were significantly reduced in KLF15 overexpressed podocytes. Meanwhile, overexpression of KLF15 in podocytes was significantly up-regulated the mRNA and protein expression of anti-apoptotic Bcl-2 (Figure 3C,D). Furthermore, the flow cytometry analysis demonstrated that the podocyte apoptosis was decreased in Ad-KLF15 cells (Figure 3E,F). In addition, we further demonstrated that over-expression of KLF15 in podocytes alleviated the cell apoptosis induced by ADR, LPS or HG (Figure 3G–M). Taken together, these data indicated that overexpression of KLF15 attenuates podocyte apoptosis in vitro.

**KLF15 knockdown increased podocyte apoptosis in vitro**

To further confirm the role of KLF15 in podocyte apoptosis, we investigated the effect of silencing KLF15 in podocytes using KLF15-siRNA. Western blotting and RT-qPCR analysis confirmed an efficient siRNA-mediated KLF15 gene knocking down in podocytes especially KLF15-siRNA#2 (Figure 4A,B). The flow cytometry analysis determined...
Figure 1. KLF15 is down-regulated in podocytes in proteinuric patients

Immunostaining for the podocyte specific maker WT1 (green) and KLF15 (red) was performed in glomeruli of proteinuric patients and normal samples from patients with renal cell carcinoma. Representative confocal images showed that KLF15 was enriched in normal glomeruli and was obviously reduced in glomeruli of patients with diabetic nephropathy (DN, n=4), minimal change disease (MCD, n=3) or focal segmental glomerulosclerosis (FSGS, n=3). The arrows mark KLF15 expression in podocyte; scale bars: 50 μm.

that the cell apoptosis rate was markedly elevated after loss of KLF15 in podocytes (Figure 4C–E,H). Consistently, the TUNEL assay further demonstrated that podocyte apoptosis was increased in KLF15-siRNA#2 group (Figure 4I). These results further confirmed that KLF15 had a protective action in podocyte apoptosis.

**NFATc1 was a direct target of KLF15**

Recently studies predicted that KLF15 possess potential binding sites on the promoter region of NFATc1, which is an important transcription factor contributed to podocyte injury and glomerulosclerosis [16,21]. To explore the mechanism underlying the protective effects of KLF15 on podocyte injury, we determined whether NFATc1 was a direct target of KLF15. We initially transfected cultured podocytes with adenovirus packing ORF–KLF15 clone and measured
NFATc1 expression. RT-qPCR analysis showed that NFATc1 mRNA was down-regulated in KLF15 overexpressed podocytes (Figure 5A). Consistent with mRNA expression, overexpression of KLF15 decreased NFATc1 protein expression in podocytes in vitro (Figure 5B). In contrast, silencing of KLF15 largely elevated the mRNA and protein expression of NFATc1 in podocytes (Figure 5C,D). These data supported a role for KLF15 in regulating the NFATc1 expression.

To further explore the potential of KLF15 to modulate expression of NFATc1 gene, we performed a chromatin immunoprecipitation (ChIP)-quantitative PCR assay to identify the NFATc1 promoter region binding sites for KLF15. DNA electrophoretogram confirmed that KLF15 bound to the NFATc1 promoter region -1984 to -1861 base pairs upstream of the transcription start site (Figure 5E). Furthermore, ChIP-qPCR assay revealed a decreased level of KLF15 in the promoter of NFATc1 in podocytes after treatment with LPS (Figure 5F). More importantly, direct interaction between KLF15 and the promoter region of NFATc1 was clearly determined by the dual-luciferase reporter assay in vitro, in which overexpression of KLF15 significantly down-regulated the transcription activity of NFATc1 (Figure 5G). Fzd9, Rcan1 and plaur are the downstream target genes of NFAT signaling in podocytes and other cells, and mediate podocyte injury as a result of NFAT activation [18,24,30,31]. Our results further confirmed that the mRNA expression of Fzd9, Rcan1 and plaur were significantly decreased in KLF15 overexpressed podocytes and were markedly increased in KLF15 knockdown podocytes respectively (Figure 5H,I). These results indicated that KLF15 directly suppressed the NFATc1 expression by binding to the promoter region.

Salutary effects of dexamethasone are partially mediated by the KLF15–NFATc1 axis

Glucocorticoids still remain an initial therapeutic option for primary glomerulopathies, such as MCD and FSGS [32]. Previous studies have reported that dexamethasone (DEX) induced a rapid increase of KLF15 in podocytes [16], prompting us to explore the effect of dexamethasone on regulating the expression of NFATc1. We treated cultured podocytes with DEX for 6, 12, 24 and 48 h respectively and then analyzed the KLF15 and NFATc1 expression. RT-qPCR analysis demonstrated that KLF15 mRNA expression was obviously increased accompanied by down-regulation of NFATc1 mRNA expression in DEX-treated podocytes (Figure 6A,B). A time course revealed that NFATc1 mRNA expression was minimal at 24 h in podocytes after DEX stimulation (Figure 6B). Consistent with mRNA expression, NFATc1 protein expression was significantly reduced in response to DEX stimulation in podocytes (Figure 6C). Interestingly, RT-qPCR analysis and Western blotting for NFATc1 further confirmed that the decreased expression of NFATc1 induced by DEX was markedly restored in podocytes treated with KLF15–siRNA, indicating that the DEX-induced NFATc1 decrease was mediated by KLF15 (Figure 6D,E). Furthermore, we found that 11R-VIVIT, a specific NFAT inhibitor, and NFATc1-siRNA rescued KLF15-deficient induced podocytes apoptosis, implying that KLF15–NFATc1 axis played a vital role in podocytes injury (Figure 4F–I). These above data demonstrated that KLF15-NFATc1 axis was involved in mediating the salutary effects of dexamethasone in podocytes.
Figure 3. Induction of KLF15 attenuates podocyte apoptosis in vitro

(A) A cell model of KLF15-overexpressed podocyte was established. After treatment of adenovirus packing ORF–KLF15 clone, KLF15 mRNA expression was obviously increased in podocyte. (B) Consistent with mRNA expression, the protein expression of KLF15 was obviously increased in adenovirus packing ORF–KLF15 clone treated podocytes. (C) In KLF15 overexpressed podocytes, the mRNA expression of Bax, a well-recognized indicator of apoptosis, was significantly decreased as the anti-apoptotic Bcl-2 mRNA was increased. (D) Consistently, Bax protein expression was down-regulated while Bcl-2 protein expression was up-regulated in KLF15 overexpressed podocytes. (E and F) Podocytes were stained with APC/PI for flow cytometry analysis. Compared with Ad-GEP podocytes, the cell apoptosis rate was significantly reduced in Ad-KLF15 podocytes. (G–L) The flow cytometry analysis showed apoptosis induced by ADR, LPS or HG was decreased in Ad-KLF15 podocytes. (M) Quantitative results of apoptosis rate. Data were from at least three independent experiments; *P<0.05 and **P<0.01 vs. controls.

Discussion

Although a large body of evidence has demonstrated that podocyte injury and loss directly contribute to proteinuria, glomerulosclerosis and eventually kidney failure [33,34], the current understanding of the underlying mechanisms is limited and remains to be elucidated. Therefore, exploring the mechanisms underlying podocyte injury, especially...
identifying the targets to ameliorate podocyte injury, is urgent and imperative. Here, we described that KLF15 ameliorated podocyte injury through suppressing NFAT signaling and the salutary effects of dexamethasone in podocytes were partially mediated by the KLF15–NFATc1 axis.

Previous studies have shown that KLF15, a kidney-enriched transcription factor, is highly expressed in podocytes and regulate the transcription of podocyte specific genes, such as podocin and nephrin. Mutations and abnormalities of the slit diaphragm proteins podocin and nephrin are representative markers of podocyte injury [35,36]. After injury treatment, KLF15 expression is reduced in WT and CCR5-/- (KO) mice [37]. In ADR-treated mice, podocyte specific KLF15 induction ameliorates podocyte injury [28]. In LPS-induced mouse model for proteinuric kidney disease, loss of KLF15 increased podocyte foot process effacement and proteinuria [14]. Here, we found that KLF15 protein was...
Figure 5. KLF15 directly mediated the regulation of NFATc1 expression in podocytes

(A) NFATc1 mRNA expression was decreased in KLF15 overexpressed podocytes. (B) Consistent with mRNA expression, the protein expression of NFATc1 was reduced in KLF15 overexpressed podocytes. (C) In contrast, silencing of KLF15 elevated NFATc1 mRNA expression in podocytes. (D) Similarly, NFATc1 protein expression was increased in KLF15 silenced podocytes. (E and F) Chor-matin immunoprecipitation (ChIP) was performed using an antibody to KLF15, followed by qPCR using the NFATc1 gene promoter specific primer designed at the region -1984 to -1861 base pairs upstream of the transcription start site. DNA electrophoretogram demonstrated that KLF15 bound to NFATc1 promoter region (E) and ChIP-qPCR further demonstrated that the binding amount was decreased in LPS-treated podocytes (F). IgG was used as negative control and input fraction was used as positive control. Fold enrichment = [% (ChIP/Input)] / [% (Negative control/Input)].

(G) KLF15 directly mediated the regulation of NFATc1 gene promoter activity. Dual-luciferase reporter assay showed that the transcriptional activity of NFATc1 was decreased in KLF15 overexpressed podocytes. The secreted alkaline phosphatase (SEAP) was used as an internal control and Gaussia luciferase (Gluc) was normalized to SEAP, respectively. (H) Fzd9, Rcan1 and plaur, the downstream target genes of NFAT signaling, were decreased in KLF15 overexpressed podocyte. (I) In contrast, silencing of KLF15 increased the mRNA expression of Fzd9, Rcan1 and plaur in podocytes. All data were shown as mean ± SEM. *P < 0.05, **P < 0.01 and ***P < 0.001 vs. controls.
Figure 6. Dexamethasone (DEX) induced decrease expression of NFATc1 and was abrogated in KLF15 silenced podocytes

(A) Dexamethasone (DEX) induced a fast increase of KLF15 mRNA expression. (B) After treatment of DEX, the mRNA expression of NFATc1 was reduced and was minimal at 24 h. (C) Representative Western blots and quantitative band intensity analysis for NFATc1 protein expression levels in con- or DEX–treated podocytes. NFATc1 protein expression was decreased in DEX treated podocytes. (D) Compared with DEX-treated groups, the mRNA expression of NFATc1 was up-regulated in KLF15 silenced podocytes under DEX stimulation. (E) Western blot analysis also verified that the protein expression of NFATc1 was increased in KLF15 knockdown podocytes after DEX treatment. Data were from at least three independent experiments. *P<0.05, **P<0.01 vs. controls.

abundantly expressed in human glomerular cells, including podocytes, and was markedly decreased in patients with DN, MCD or FSGS. In addition, we further confirmed that KLF15 mRNA and protein expression were obviously reduced in ADR-, LPS- or HG-treated podocytes in vitro. Although previous studies have demonstrated that KLF15 knockdown in podocytes increased the susceptibility to injury, the role of KLF15 involved in podocyte apoptosis remained to be elucidated. In the present study, we found that the mRNA and protein expression of pro-apoptotic Bax were significantly reduced, while anti-apoptotic Bcl-2 mRNA and protein expression were up-regulated in KLF15 overexpressed podocytes. Meanwhile, the cell apoptosis induced by ADR, LPS or HG was alleviated in KLF15 overexpressed podocytes. In contrast, KLF15 knockdown increased podocyte apoptosis in vitro. These data indicate that KLF15 has a protective action in podocyte apoptosis.

The mechanism underlying the KLF15-mediated protective effect during podocyte injury under chronic kidney disease remains incompletely understood. A recent promoter analysis combined with enrichment analysis and the Fisher exact test identified that KLF15 has binding sites on NFATc1 gene promoter region, promoting us to determine
Figure 7. Schematic depicting KLF15 ameliorates podocyte injury by suppressing NFAT signaling

Under normal conditions, KLF15 inhibits the transcription of NFATc1 gene by directly binding to the promoter region of NFATc1. Under pathological conditions, KLF15 reduction leads to the decrease level of KLF15 in the promoter region of NFATc1, thereby enhancing the transcription of NFATc1 gene. Finally, the activation of NFAT signaling contributes to podocyte injury.

Clinical Science (2020) 134 1305–1318
https://doi.org/10.1042/CS20200075

the interaction between KLF15 and NFATc1. NFATc1, also known as NFAT2, is a member of the NFAT transcription factors family, which activation has been proven to be a central pathological mechanism underlying podocyte injury and glomerulosclerosis [21,22]. We previously also showed that NFATc1 activation leads to podocyte injury through targeting uPAR-mediated beta3 integrin signaling [24]. Recent studies showed the important role of KLF15 as a central regulator involved in diverse cellular processes by regulating transcription including activating transcription or repressing transcription. For instance, knockdown of KLF15 in 3T3-L1 cells leads to a failure of expression of Pparγ and inhibition adipogenesis [12]. Nonetheless, KLF15 controls cardiac progenitor cell homeostasis in the adult heart by inhibiting β-catenin/TCF-transcriptional activity [38]. In the present study, we found that KLF15 directly suppressed NFATc1 transcriptional activity. First, we demonstrated that NFATc1 mRNA and protein expression were down-regulated in KLF15 overexpressed podocytes. In contrast, KLF15 silencing significantly up-regulated NFATc1 mRNA and protein expression. Mechanistically, ChIP analysis showed that KLF15 bound to the NFATc1 promoter region -1984 to -1861 base pairs upstream of the transcription start site and the binding amount was decreased after treatment with LPS. The dual-luciferase reporter assay further confirmed that overexpression of KLF15 inhibited the transcription activity of NFATc1. Furthermore, our results showed that the mRNA expression of Fzd9, Rcan1 and plaur, the downstream target genes of NFAT signaling in podocytes, were reduced in KLF15 overexpressed podocytes and increased in KLF15 knockdown podocytes, respectively. These data suggest that KLF15 directly suppressed the NFATc1 expression by binding to the promoter region.

Clinically, glucocorticoids are widely used for the treatment of human glomerular diseases including minimal change disease, FSGS and membranous nephropathy, which are all characterized by podocyte injury and proteinuria [39]. Mechanistically, previous studies described that glucocorticoids have a direct effect in ameliorating podocyte injury [40]. Considering that recent study investigated that dexamethasone, a synthetic glucocorticoid, induced a rapid increase of KLF15 in podocytes [16], we speculated that dexamethasone had a regulation effect on NFATc1 expression.
As expected, we found that the KLF15 mRNA expression was obviously increased accompanied by NFATc1 mRNA expression down-regulated in podocytes after dexamethasone treatment. Accordingly, we showed that NFATc1 mRNA and protein expression was minimal at 24 h in podocytes after DEX stimulation. Interestingly, we further found that the decreased expression of NFATc1 induced by dexamethasone was markedly restored in podocytes treated with KLF15-siRNA, implying that KLF15 was involved in dexamethasone induced the decreased expression of NFATc1. In addition, the flow cytometry analysis revealed that 11R-VIVIT, a specific NFAT inhibitor, and NFATc1–siRNA rescued KLF15-deficient induced podocytes apoptosis. Consistently, the TUNEL assay further confirmed that the cell apoptosis was decreased in KLF15 silenced podocytes after loss of NFATc1. These results indicating that the KLF15–NFATc1 axis plays a vital role in mediating the salutary effects of dexamethasone in podocytes.

Collectively, the present study demonstrated that KLF15 has anti-apoptotic effects in podocytes and the protective effects on podocyte injury was at least in part through regulation of NFAT signaling pathways. Furthermore, our study showed that the salutary effects of dexamethasone were partially mediated by the KLF15–NFATc1 axis (Figure 7). Our findings identify an insight into the molecular mechanisms underlying the protective effects of KLF15 on podocyte injury and provide a novel rationale for the treatment of glomerular diseases characterized by podocyte injury using glucocorticoids in clinical practice.

Clinical perspectives

- Glucocorticoids exhibit notable efficacy in primary glomerulopathies, such as MCD and FSGS, where a proinflammatory milieu is not readily apparent, but the underlying mechanism remains largely unexplored.
- Our results identify the critical role of the KLF15–NFATc1 axis in podocyte injury and loss and the salutary effects of dexamethasone were partially mediated by the KLF15–NFATc1 axis.
- Our findings provide a novel rationale for the treatment of glomerular diseases characterized by podocyte injury using glucocorticoids in clinical practice.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Funding

This work was supported by grants from National Natural Science Foundation of China [grant numbers 81900658, 81670656 and 81870508]; Scientific research project of Guangdong Provincial People’s Hospital-Summit plan [grant number DFJH201916] and in part by grants from the Fundamental Research Funds for the Central Universities [grant number D2180530].

Author Contribution

C.-S.D., H.Z., and S.-X.L. designed research, performed the experiments, analyzed data, and wrote and edited the paper. B.Z. helped to design the research. G.-B.K., L.Z., Z.-W.L., X.-Q.C., and X.-C.Z. helped to perform the experiments. Y.-H.C., R.-Z.L., J.-C.M., Z.L., and T.L. helped to analyze data. W.-J.W., Z.-M.Y., X.-L.L. and W.S. helped to review and edited the manuscript.

Abbreviations

Ad, adenovirus; ADR, adriamycin; ChIP, chromatin immunoprecipitation; CKD, chronic kidney disease; DN, diabetic nephropathy; ESRD, end-stage renal disease; FSGS, focal segmental glomerulosclerosis; GBM, glomerular basement membrane; HG, high glucose; KLF15, Kruppel-like factor 15; LPS, lipopolysaccharide; MCD, minimal change disease; MN, membranous nephropathy; NFAT, nuclear factor of activated T cells; siRNA, small interfering RNA; WT1, Wilms Tumor 1.

References

1. Webster, A.C., Nagler, E.V., Morton, R.L. and Masson, P. (2017) Chronic Kidney Disease. Lancet 389, 1238–1252, https://doi.org/10.1016/S0140-6736(16)32064-5
2. Hill, N.R., Fatoba, S.T., Oke, J.L., Hirst, J.A., O’Callaghan, C.A., Lasserson, D.S. et al. (2016) Global Prevalence of Chronic Kidney Disease - A Systematic Review and Meta-Analysis. PLoS ONE 11, e0158765, https://doi.org/10.1371/journal.pone.0158765

© 2020 The Author(s). This is an open access article published by Portland Press Limited on behalf of the Biochemical Society and distributed under the Creative Commons Attribution License 4.0 (CC BY).
3. Pavenstadt, H., Kriz, W. and Kretzler, M. (2003) Cell biology of the glomerular podocyte. *Physiol. Rev.* **83**, 253–307, https://doi.org/10.1152/physrev.00020.2002

4. Assaf, S., Wanner, N., Skorecki, K.L. and Huber, T.B. (2017) New Insights into Podocyte Biology in Glomerular Health and Disease. *J. Am. Soc. Nephrol.* **28**, 1707–1715, https://doi.org/10.1681/ASN.20171001027

5. Nagata, M. (2016) Podocyte injury and its consequences. *Kidney Int.* **89**, 1221–1230, https://https://doi.org/10.1016/j.kint.2016.01.012

6. Vivarelli, M., Massella, L., Ruggiero, B. and Emma, F. (2017) Minimal Change Disease. *Clin. Am. Soc. Nephrol.* **12**, 332–345, https://doi.org/10.2215/CJN.0500516

7. Fogo, A.B. (2015) Causes and pathogenesis of focal segmental glomerulosclerosis. *Nat. Rev. Nephrol.* **11**, 76–87, https://doi.org/10.1038/nrneph.2014.216

8. Rosenberg, A.Z. and Kopp, J.B. (2017) Focal Segmental Glomerulosclerosis. *Clin. J. Am. Soc. Nephrol.* **12**, 502–517, https://doi.org/10.2215/CJN.0506016

9. Francis, J.M., Beck, Jr., L.H. and Salant, D.J. (2016) Membranous Nephropathy: A Journey From Bench to Bedside. *Am. J. Kidney Dis.* **68**, 138–147, https://doi.org/10.1053/j.ajkd.2016.01.030

10. Dai, H., Liu, Q. and Liu, B. (2017) Research Progress on Mechanism of Podocyte Depletion in Diabetic Nephropathy. *J. Diabetes Res.* **2017**, 2615286, https://doi.org/10.1155/2017/2615286

11. Tagawa, A., Yasuda, M., Kume, S., Yamahara, K., Nakazawa, J., Chin-Kanasaki, M. et al. (2016) Impaired Podocyte Autophagy Exacerbates Proteinuria in Diabetic Nephropathy. *Diabetes* **65**, 755–767, https://doi.org/10.2337/db15-0473

12. Pearson, R., Fleetwood, J., Eaton, S., Crossley, M. and Bao, S. (2008) Krüppel-like transcription factors: a functional family. *Int. J. Biochem. Cell Biol.* **40**, 1996–2001, https://doi.org/10.1016/j.biocel.2007.07.018

13. Rane, M.J., Zhao, Y. and Cai, L. (2019) Krüppel-like factors (KLFs) in renal physiology and disease. *EBioMedicine* **40**, 743–750, https://doi.org/10.1016/j.ebiom.2019.01.021

14. Mallipattu, S.K., Liu, R., Zheng, F., Narla, G., Ma’ayan, A., Dikman, S. et al. (2012) Kruppel-like factor 15 (KLF15) is a key regulator of podocyte differentiation. *J. Biol. Chem.* **287**, 1922–1932, https://doi.org/10.1074/jbc.M111.345983

15. Wang, Y. and Mitch, W.E. (2011) Proteins and renal fibrosis: low-protein diets induce Kruppel-like factor 15 (KLF15) expression. *Kidney Int.* **79**, 933–944, https://doi.org/10.1038/ki.2011.8

16. Mallipattu, S.K., Guo, Y., Revelo, M.P., Roa-Peña, L., Miller, T., Ling, J. et al. (2017) Krüppel-Like Factor 15 Mediates Glucocorticoid-Induced Restoration of Podocyte Differentiation Markers. *J. Am. Soc. Nephrol.* **28**, 166–184, https://doi.org/10.1681/ASN.2015060672

17. Hogan, P.G. (2017) Calcium-NFAT transcriptional signalling in T cell activation and T cell exhaustion. *Cell Calcium* **63**, 66–69, https://doi.org/10.1016/j.ceca.2017.01.014

18. Wang, Y., Jarad, G., Tripathi, P., Pan, M., Cunningham, J., Martin, D.R. et al. (2010) Activation of NFAT signaling in podocytes causes glomerulosclerosis. *J. Am. Soc. Nephrol.* **21**, 1657–1666, https://doi.org/10.1681/ASN.2009121253

19. Hogan, P.G., Chen, L., Nardone, J. and Rao, A. (2003) Transcriptional regulation by calcium, calcineurin, and NFAT. *Genes Dev.* **17**, 2205–2232

20. Li, S.Z., McBill, B.W., Kovach, P.A., Ding, L., Go, W.Y., Ho, S.N. et al. (2007) Calcineurin-NFATc signaling pathway regulates AQP2 expression in response to calcium signals and osmotic stress. *Am. J. Physiol.-Cell Physiol.* **292**, C1606–C1616, https://doi.org/10.1152/ajpcell.00586.2005

21. Pedigo, C.E., Ducasa, G.M., Leclercq, F., Sloan, A., Mitrofanova, A., Hashmi, T. et al. (2016) Local TNF causes NFATc1-dependent cholesterol-mediated podocyte injury. *J. Clin. Invest.* **126**, 3336–3350, https://doi.org/10.1172/JCI85939

22. Nijenhuis, T., Sloan, A.J., Hoenderop, J.G., Flesche, J., van Goor, H., Kistler, A.D. et al. (2011) Angiotensin II contributes to podocyte injury by increasing TRPC6 expression via an NFAT-mediated positive feedback signaling pathway. *Am. J. Pathol.* **179**, 1719–1732, https://doi.org/10.1016/j.ajpath.2011.06.033

23. Zhang, L., Li, R., Shi, W., Liang, X., Liu, S., Ye, Z. et al. (2013) NFAT2 inhibitor ameliorates diabetic nephropathy and podocyte injury in db/db mice. *Br. J. Pharmacol.* **170**, 426–439, https://doi.org/10.1111/bph.12392

24. Zhang, B., Shi, W., Ma, J., Sloan, A., Fauil, C., Wei, C. et al. (2012) The calcineurin-NFAT pathway allows for urokinase receptor-mediated beta3 integrin signaling to cause podocyte injury. *J. Mol. Med. (Berl.)* **90**, 1407–1420, https://doi.org/10.1007/s00109-012-0960-6

25. Zhang, H., Liang, S., Du, Y., Li, R., He, C., Wang, W. et al. (2016) Inducible ATF3-NFAT axis aggravates podocyte injury. *J. Mol. Med. (Berl.)* **96**, 53–64, https://doi.org/10.1007/s00109-017-1601-x

26. Liang, S., Zhang, H., Du, Y., Dou, C., Liu, S., Zhang, L. et al. (2018) RANK Deficiency Ameliorates Podocyte Injury by Suppressing Calcium/Calcineurin/ NFATc1 Signaling. *Kidney Blood Press. Res.* **43**, 1149–1159

27. Turk, T., Leeuwis, J.W., Gray, J., Torti, S.V., Lyons, K.M., Nguyen, T.Q. et al. (2009) BMP signaling and podocyte markers are decreased in human diabetic nephropathy in association with CTGF overexpression. *J. Histochem. Cytochem.* : Off. J. Histochem. Soc. **57**, 623–631, https://doi.org/10.1369/jhc.2009.953224

28. Guo, Y., Pace, J., Li, Z., Ma’ayan, A., Wang, Z., Revelo, M.P. et al. (2018) Podocyte-Specific Induction of Kruppel-Like Factor 15 Restores Differentiation Markers and Attenuates Kidney Injury in Proteinuric Kidney Disease. *J. Am. Soc. Nephrol.* **29**, 2529–2545, https://doi.org/10.1681/ASN.2018030324

29. Pena-Blanco, A. and Garcia-Saez, A.J. (2018) Bax, Bak and beyond - mitochondrial performance in apoptosis. *FEBS J.* **285**, 416–431, https://doi.org/10.1111/febs.14186

30. Lee, M.Y., Garvey, S.M., Baras, A.S., Lemmon, J.A., Gomez, M.F., Schoppee Bortz, P.D. et al. (2010) Integrative genomics identifies DSCR1 (RCAN1) as a novel NFAT-dependent mediator of phenotypic modulation in vascular smooth muscle cells. *Hum. Mol. Genet.* **19**, 468–479, https://doi.org/10.1093/hmg/ddp511

31. Zhang, B. and Shi, W. (2012) Is the antiproteinuric effect of cyclosporine a independent of its immunosuppressive function in T cells? *Int. J. Pediatr. Nephrol.* **2012**, 809456
32 Mallipattu, S.K. and He, J.C. (2016) The podocyte as a direct target for treatment of glomerular disease? Am. J. Physiol.-Renal Physiol. 311, F46–F51, https://doi.org/10.1152/ajprenal.00184.2016
33 Huang, Z., Zhang, L., Chen, Y., Zhang, H., Zhang, Q., Li, R. et al. (2016) Cdc42 deficiency induces podocyte apoptosis by inhibiting the Nwasp/stress fibers/HAP pathway. Cell Death. Dis. 7, e2142
34 Raij, L., Tian, R., Wong, J.S., He, J.C. and Campbell, K.N. (2016) Podocyte injury: the role of proteinuria, urinary plasminogen, and oxidative stress. Am. J. Physiol.-Renal Physiol. 311, F1308–F1317, https://doi.org/10.1152/ajprenal.00162.2016
35 Roselli, S., Gribouval, O., Boute, N., Sich, M., Benesey, F., Attie, T. et al. (2002) Podocin localizes in the kidney to the slit diaphragm area. Am. J. Pathol. 160, 131–139, https://doi.org/10.1016/S0002-9440(10)64357-X
36 Putaala, H., Soininen, R., Kilpelainen, P., Wartiovaara, J. and Tryggvason, K. (2001) The murine nephrin gene is specifically expressed in kidney, brain and pancreas: inactivation of the gene leads to massive proteinuria and neonatal death. Hum. Mol. Genet. 10, 1–8, https://doi.org/10.1093/hmg/10.1.1
37 Han, S.S., Yu, M.Y., Yoo, K.D., Lee, J.P., Kim, D.K., Kim, Y.S. et al. (2018) Loss of KLF15 accelerates chronic podocyte injury. Int. J. Mol. Med. 42, 1593–1602
38 Noack, C., Zafiriou, M.P., Schaeffer, H.J., Renger, A., Pavlova, E., Dietz, R. et al. (2012) Krueppel-like factor 15 regulates Wnt/β-catenin transcription and controls cardiac progenitor cell fate in the postnatal heart. EMBO Mol. Med. 4, 992–1007, https://doi.org/10.1002/emmm.201101043
39 Wada, T., Pippin, J.W., Marshall, C.B., Griffin, S.V. and Shankland, S.J. (2005) Dexamethasone prevents podocyte apoptosis induced by puromycin aminonucleoside: role of p53 and Bcl-2-related family proteins. J. Am. Soc. Nephrol. 16, 2615–2625, https://doi.org/10.1681/ASN.2005020214
40 Xing, C.Y., Saleem, M.A., Coward, R.J., Ni, L., Witherden, I.R. and Mathieson, P.W. (2006) Direct effects of dexamethasone on human podocytes. Kidney Int. 70, 1038–1045