Krüppel-like Factor 15 (KLF15) Is a Key Regulator of Podocyte Differentiation*

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Podocyte injury resulting from a loss of differentiation is the hallmark of many glomerular diseases. We previously showed that retinoic acid (RA) induces podocyte differentiation via stimulation of the cAMP pathway. However, many podocyte maturity markers lack binding sites for RA-response element or cAMP-response element (CREB) in their promoter regions. We hypothesized that transcription factors induced by RA and downstream of CREB mediate podocyte differentiation. We performed microarray gene expression studies in human podocytes treated with and without RA to identify differentially regulated genes. In comparison with known CREB target genes, we identified Krüppel-like factor 15 (KLF15), a kidney-enriched nuclear transcription factor, that has been previously shown to mediate cell differentiation. We confirmed that RA increased KLF15 expression in both murine and human podocytes. Overexpression of KLF15 stimulated expression of differentiation markers in both wild-type and HIV-1-infected podocytes. Also, KLF15 binding to the promoter regions of nephrin and podocin was increased in RA-treated podocytes. Although KLF15+/− mice at base line had minimal phenotype, lipopolysaccharide- or adriamycin-treated KLF15−/− mice had a significant increase in proteinuria and podocyte foot process effacement with a reduction in the expression of podocyte differentiation markers as compared with the wild-type treated mice. Finally, KLF15 expression was reduced in glomeruli isolated from HIV transgenic mice as well as in kidney biopsies from patients with HIV-associated nephropathy and idiopathic focal segmental glomerulosclerosis. These results indicate a critical role of KLF15 in mediating podocyte differentiation and in protecting podocytes against injury.

In normal mature kidneys, podocytes are regarded as highly differentiated and growth-arrested cells with a limited capacity for replication. Podocyte injury manifests clinically as proteinuria and structurally as foot process effacement. Significant podocyte injury leads to proliferation, apoptosis, and a loss of podocyte differentiation markers. For example, in minimal change disease, nephrotic range proteinuria is associated with significant but reversible effacement of the foot process. However, in diabetic nephropathy, the podocyte number is reduced because of either cell detachment or apoptosis (1, 2). In collapsing focal segmental glomerulosclerosis (FSGS)3 of HIV-associated nephropathy (HIVAN), proliferation and dedifferentiation of podocytes are prominent features (3, 4). Specifically, the viral infection of the podocytes is responsible for podocyte proliferation and dedifferentiation in HIVAN (5–8). Because podocyte dedifferentiation is a major mechanism in the pathogenesis of podocyte injury, it is critical to identify targets for therapy that can prevent or reverse this disease process.

Retinoic acids (RA) are derivatives of vitamin A and have multiple cellular functions, including inhibition of proliferation, induction of cell differentiation, and inhibition of inflammation (9). In addition to their established benefits in the treat-

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3 The abbreviations used are: FSGS, focal segmental glomerulosclerosis; RA, retinoic acid; KLF, Krüppel-like factor; HIVAN, HIV-associated nephropathy; all-trans-retinoic acid; RARE, retinoic acid-response element; CREB, cAMP-response element-binding protein; WT-1, Wilms tumor 1; R, reverse; GDNF, glial cell-derived neurotrophic factor; GBM, glomerular basement membrane.

Significance: Identification of KLF15 in mediating podocyte differentiation provides new insight into kidney disease.
Retinoic Acid Treatment of Podocytes in Culture—Wild-type and HIV-1-infected murine podocytes were treated with atRA (1 µM) or control vehicle as described previously (20). Similarly, human podocytes infected with and without HIV-1 were treated with or without atRA (1 µM).

KL15 Construct—A KL15 cDNA clone purchased from Thermo Scientific (Huntsville, AL) was inserted into a gag-, pol-, and env-deficient lentivirus construct, VVE/BBW (a gift of Dr. G Luca Gusella, Mount Sinai School of Medicine). Lentiviral particles were generated and used for infection of podocytes. Podocytes with stable KL15 expression were selected using blasticidin. Cultured murine and human podocytes infected with the empty VVE/BBW lentivirus served as controls.

Real Time PCR—Total RNA was extracted by using TRIzol (Invitrogen). First strand cDNA was prepared from total RNA (1.5 µg) using the SuperScript™ III first strand synthesis kit (Invitrogen), and cDNA (1 µl) was amplified in triplicate using SYBR GreenER qPCR Supermix on an ABI PRISM 7900HT Applied Biosystems, Foster City, CA). Pre-designed primer sets were obtained from Sigma for synaptopodin, nephrin, podocin, WT-1, podocin, GAPDH, and KL15 (mouse F, 5'-GAGACCT-TCCTGTCACCGAAA-3', and R, 5'-GCTGGAGACATCG-CTGTCCT-3'), (human F, 5'-GTTGGGATCTGGAATAAGCC-3', and R, 5'-GAGAGTCGGGACTGAAACAG-3'). Light Cycler analysis software was used to determine crossing points using the second derivative method. Data were normalized to housekeeping genes (GAPDH) and presented as fold increase compared with RNA isolated from WT animals using the 2^−ΔΔCT method.

Chromatin Immunoprecipitation Assay (ChIP)—The ChIP assay was performed using a kit from Upstate Biotechnologies, Inc. (Lake Placid, NY), as described previously (21). Briefly, 3 × 10^6 cultured murine podocytes per experimental condition were serum-starved for 16 h and then treated with either atRA (1 µM) or control vehicle for 4 h. Cells were cross-linked with formaldehyde for 10 min, followed by the addition of 1/20 volume of 2.5 M glycine to quench unreacted formaldehyde. Cells were lysed using a series of non-SDS-containing buffers as described previously (21). Chromatin extracted from the lysed cells was sonicated using a Misonix 3000 sonicator with microtip to generate chromatin fragments of between 300 and 1000 bp. Immunoprecipitation of KL15-cross-linked chromatin was carried out using M-280 Dynabeads (Invitrogen) with sheep anti-rabbit immunoglobulin G (IgG) preincubated with goat anti-KL15 (Abcam) antibody. To control for nonspecific IgG binding, rabbit IgG (Sigma) was used. After incubation of chromatin with antibody-coupled Dynabeads, the beads were washed several times, and immunoprecipitated chromatin complexes were eluted from the beads. DNA-protein cross-links were reversed by incubation at 65 °C for 6 h, and then RNase A and protein kinase K were added sequentially to remove RNA and proteins. DNA was purified using the
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QIAquick® PCR purification kit (Qiagen) by following the manufacturer’s instructions. Purified DNA was used for the analysis of the nephrin and podocin proximal promoter by real time PCR on an ABI PRISM 7900HT using SYBR GreenER qPCR supermix. PCR primers for murine nephrin and podocin promoters were derived from available sequence (GenBank™ accession numbers AAK38483 and AY050309, respectively) and were the following: nephrin F, 5’-CCCAGCCACACAGGCTAGC-3’, and R, 5’-TTCTCTGAGTCTGCACCGGC; podocin F, 5’-AGAGAACCCCAAGCACGCTA-3’, and R, 5’-AGCAGGGCTTCTAGTGGCT-3’. The relative amplification of the promoter sequence of each gene was calculated using the 2-ΔΔCt method, and normalization was performed against the 1:100 diluted input of DNA.

Genotyping of Tg26 Mice—Mount Sinai School of Medicine Animal Institute Committee approved all animal studies, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals was strictly followed. Derivation of a transgenic mouse line (Tg26 mice) that bears a defective HIV-1 provirus lacking gag-pol (Tg26) has been described (22). Tg26 mice are in the FVB/N background. Mice generated from the same virus lacking gag-pol (Tg26) has been described (22). Tg26 mice were viable, fertile, and born in the Mendelian ratios as expected in C57BL/6 background. Mice generated from the same

Genotyping by tail preparation and PCR was performed at 2 litter of Tg26 mice were used as the controls in the studies.

Laboratory Animals was strictly followed. Derivation of a trans-

expected in C57BL/6 background.

mice were administered low dose LPS (10^6/g) intraperitone-

mice were viable, fertile, and born in the Mendelian ratios as expected in C57BL/6 background.

LPS Murine Model—Transient nephrotic syndrome with podocyte EF effacement was induced by LPS injection as described previously (24). Base-line urine was collected in wild-type and KLF15−/− mice (C57BL/6 at 8–9 weeks of age). All mice were administered low dose LPS (10 μg/g) intraperitoneally at 0 and 24 h. Urine was collected at 12-h increments with 1 ml of normal saline boluses intraperitoneally. Mice were sacrificed at 48 h.

Adriamycin Murine Model—In the adriamycin model, wild-type and KLF15−/− mice (C57BL/6 at 8–9 weeks of age) were administered adriamycin (20 mg/kg) intravenously by tail vein injection (25). Urine was collected weekly to assess for proteinuria, and mice were sacrificed 4 weeks post-treatment.

AtRA Treatment of Mice—Administration of atRA was performed as described previously (14). 12 h prior to treatment with LPS, mice were administered intraperitoneal injections of atRA at 16 mg/kg or vehicle alone (DMSO). Following the initial injection, two additional injections were given at 24-h intervals at the same dose. Mice were sacrificed at 48 h.

Measurement of Urine Albumin and Creatinine—Urine albumin was quantified by ELISA using a kit from Bethyl Laboratories, Inc. (Houston, TX). Urine creatinine levels were measured in the same samples using QuantiChrom™ creatinine assay kit (DICT-500) (BioAssay Systems) according to the manufacturer’s instruction. The urine albumin excretion rate was expressed as the ratio of albumin to creatinine.

Histopathology by Transmission Electron Microscopy—Mice were perfused with PBS and then immediately fixed in glutar-aldehyde for EM. Sections were mounted on a copper grid and photographed under a Hitachi H7650 microscope. Briefly, negatives were digitized, and images with a final magnification of approximately ×15, 000 were obtained. ImageJ 1.26t software (National Institutes of Health, rsb.info.nih.gov) was used to measure the length of the peripheral GBM, and the number of slit pores overlying this GBM length was counted. The arithmetic mean of the foot process width (WFp) was calculated as shown in Equation 1,

\[
WF_p = \frac{\pi}{4} \times \frac{\Sigma_{GBM\ length}}{\Sigma_{slits}}
\]

where \( \Sigma_{slits} \) indicates the total number of slits counted; \( \Sigma_{GBM\ length} \) indicates the total GBM length measured in one glomerulus, and \( \pi/4 \) is the correction factor for the random orientation by which the foot processes were sectioned (26).

Isolation of Glomeruli from Mice for RNA Extraction—Mouse glomeruli were isolated as described (27). Briefly, animals were perfused with Hanks’ buffered salt solution containing 2.5 mg/ml iron oxide and 1% bovine serum albumin. At the end of perfusion, kidneys were removed, decapsulated, minced into 1-mm³ pieces, and digested in Hanks’ buffered salt solution containing 1 mg/ml collagenase A and 100 units/ml deoxyribonuclease I. Digested tissue was then passed through a 100-μm cell strainer and collected by centrifugation. The pellet was resuspended in 2 ml of Hanks’ buffered salt solution, and glomeruli were collected using a magnet. The purity of glomerular was verified under microscopy. Total RNA was isolated from kidney glomeruli of mice using TRIzol (Invitrogen).

Isolation of Primary Podocytes—After glomerular isolation, primary podocytes were isolated as described previously (28). In brief, isolated glomeruli were initially cultured on collagen I-coated culture dishes in RPMI 1640 medium containing 10% fetal bovine serum (Cansera International, Canada) supplemented with 1% insulin/transferrin/selenium A liquid media supplement (Invitrogen) and 100 units/ml penicillin. Cultures were incubated in a 37 °C humidified incubator. Subculture of primary podocytes was performed after 5 days of culture of isolated glomeruli. Cellular outgrowths were detached with trypsin/EDTA solution (Sigma) and passed through a 25-mm sieve to remove the remaining glomerular cores. The filtered cells were cultured on collagen I-coated dishes and processed for RNA or protein preparation.

Western Blot—Glomeruli were lysed with a buffer containing 1% Triton, a protease inhibitor mixture and tyrosine and serine/threonine phosphorylation inhibitors. Lysates were subjected to immunoblot analysis using goat anti-KLF15 (Abcam), rabbit anti-synaptopodin (Sigma), rabbit anti-nephin (a gift from Dr. Tomoko Takano), and rabbit anti-GAPDH antibodies (Sigma). Densitometry analysis for quantification was performed as described previously (29).

Immunofluorescence—Kidney sections from these mice were prepared in an identical fashion. Immunostaining was performed using rabbit anti-synaptopodin (Fitzgerald), rabbit anti-nephin (a gift from Dr. Larry Holzman), and mouse anti-WT-1
**TABLE 1**

Genes regulated by RA and targeted by CREB in mouse podocytes

Significant criteria are as follows: 1.5-fold change and 0.1 false discovery rate.

| Gene symbol | Description |
|-------------|-------------|
| **Up-regulated genes** | |
| GSTA4 | Glutathione S-transferase α4 |
| PER2 | Period homolog 2 |
| IFRD1 | Interferon-related developmental regulator 1 |
| HIVEP1 | Human immunodeficiency virus type 1 enhancer binding protein 1 |
| LIPE | Lipase, hormone-sensitive |
| METTL1 | Methyltransferase-like 1 |
| RFX1 | Regulatory factor X, 1 (influences HLA class II expression) |
| SNX5 | Sorting nexin 5 |
| PLAC8R | Plasminogen activator, urokinase receptor |
| CLCN3 | Chloride channel 3 |
| SFXN4 | Sideroflexin 4 |
| DMD | Dystrophin |
| FOXC1 | Forkhead box C1 |
| KLIF15 | Kruppel-like factor 15 |
| GDNF | Gial cell-derived neurotrophic factor |
| GABA, | GABA receptor-associated protein like 1 |

| **Down-regulated genes** | |
| GATA3 | GATA-binding protein 3 |
| CCND2 | Cyclin D2 |
| IGF3 | Signal transducer and activator of transcription 2 |
| MCAM | Melanoma cell adhesion molecule |
| SIAF1 | ST6 β-galactosamide α-2,6-sialyltransferase 1 |
| FGF1 | Fibroblast growth factor 18 |
| PTK2 | Paired-like homeodomain 2 |
| GNBP4 | Guanine nucleotide-binding protein (G protein), β-polypeptide 4 |
| WISP1 | WNT1-inducible signaling pathway protein 1 |
| YWHAZ | Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, ζ polypeptide |
| CACNA1C | Calcium channel, voltage-dependent, L-type, α1C subunit |

**FIGURE 1.** RA stimulates KLF15 mRNA expression by real time PCR. KLF15 mRNA expression was measured in atRA (1 μM) treated and untreated wild-type (WT) and HIV-1-infected murine (A) and human (B) podocytes in culture (n = 3, **, p < 0.05 versus control cells without RA treatment). C, KLF15 protein expression was measured in atRA (1 μM)-treated and untreated wild-type (WT) and HIV-1-infected human podocytes in culture. The representative blots of three independent experiments are shown. The densitometry analyses of these blots are shown in the lower panel (n = 3, *, p < 0.05 versus cells without atRA treatment). Human podocytes were treated with atRA (1 μM) for the indicated time intervals, and cells were collected for KLF15 expression by real time PCR (D) and Western (E) (n = 3, *, p < 0.05 versus control cells without atRA treatment). The representative blots of three independent experiments are shown. The densitometry analyses of these blots are shown in the lower panel (n = 3, *, p < 0.05 versus cells without atRA treatment).
antibodies (Santa Cruz Biotechnology). After washing, sections were incubated with or without atRA for 4 h, and nuclear proteins were extracted for ChIP assay as described. Binding of KLF15 to the putative KLF15-binding sites in the promoter of nephrin and podocin was measured in atRA-treated and untreated WT murine podocytes. Primary podocytes were isolated from wild-type and KLF15−/− mice and then stimulated with or without atRA for 4 h, and nuclear proteins were extracted for ChIP assay ([n = 3, *p < 0.05 versus atRA 0 μM]. Binding of KLF15 to the putative KLF15-binding sites in the promoter of nephrin and podocin was measured in atRA-treated and untreated primary podocytes from WT mice and KLF15−/− mice.

**Immunohistochemistry**—Archival human biopsy specimens of healthy donor nephrectomies, HIVAN, and idiopathic FSGS for immunohistochemistry were collected at Columbia University under a protocol approved by its Institutional Review Board. Specimens were initially baked for 20 min in 55–60 °C oven and then processed as described previously below. Briefly, formalin-fixed and paraffin-embedded sections were deparaffinized, and endogenous peroxidase was inactivated with H2O2. Sections were then blocked in 2% goat serum in phosphate-buffered saline (PBS) for 1 h at room temperature and then incubated with a rabbit anti-KLF15 antibody (1:1000, GenScript) at 4 °C overnight. The next day, sections were washed three times with PBS and then incubated with secondary antibody for 30 min. Positive staining was revealed by peroxidase-labeled streptavidin and diaminobenzidine substrate. The control included a section stained with only secondary antibody.

**Quantification of Immunostaining**—After sections were stained with anti-KLF15 antibody, negatives were digitized, and images with a final magnitude of approximately ×40 were obtained. ImageJ 1.26t software was used to measure the level of immunostaining in the glomeruli. First, the images were converted to 8-bit grayscale. Then, the background intensity was measured by selecting three distinct areas in the background with no staining. The corrected optical density (COD) was determined as shown in Equation 2.

\[
COD = ID - (A \times MGV)
\]

(Eq. 2)

where ID is the integrated density of the selected glomerular region; A is the area of the selected glomerular region, and MGV is the mean gray value of the background readings (30).

**Statistical Analysis**—Data were expressed as mean ± S.D. The unpaired t test was used to analyze data between two groups. The analysis of variance followed by Bonferroni correc-
FIGURE 3. KLF15 mRNA and protein expression is increased in differentiated human podocytes. Immortalized human podocytes in culture were either incubated and 33 or 37 °C. Cells were collected 1 week after incubation for real time PCR and Western blot. KLF15 mRNA (A) and protein expression (B) were measured in human podocytes in culture at 37 °C compared with 33 °C (n = 3, *, p < 0.01 versus 33 °C; representative blot of three independent experiments is shown). The densitometry analysis is shown in the lower panel (n = 3, *, p < 0.01 versus cells at 33 °C).

FIGURE 4. Overexpression of KLF15 increases expression of podocyte differentiation markers in both control and HIV-1-infected cells. A, murine podocytes were transiently transfected with control vector or KLF15 construct, and protein was extracted for Western blot. Cells were collected 24 h after transfection for determining mRNA levels of podocyte differentiation markers by real time PCR. Synaptopodin (B), podocin (C), and nephrin (D) were measured in control and HIV-1-infected cells with and without KLF15 overexpression (n = 3, *, p < 0.05 compared with the control cells; n = 3, **, p < 0.05 compared between cells with or without KLF15 overexpression).
tion was used when more than two groups were present. All experiments were repeated at least three times, and representative experiments are shown. Statistical significance will be considered when at \( p < 0.05 \).

**RESULTS**

Identification of KLF15 as Potential Downstream Transcription Factor Mediating RA-induced Podocyte Differentiation—Our previous studies suggest that RA-induced podocyte differentiation occurs through activation of the cAMP/PKA/CREB pathway (14). However, many podocyte-specific genes lack CREB-binding sites. To identify genes downstream of CREB that may potentially mediate RA-induced podocyte differentiation, we performed GeneChip expression analysis on podocytes stimulated with either atRA (1 \( \mu \)M) or with dimethyl sulfoxide (DMSO) (control) at 6 h (\( n = 4 \)). A total of 111 genes (90 up and 21 down) were selected based on \( t \) test with a false discovery rate of 0.1, indicating that 11 genes on the list may be picked at random. The functions of these genes include antioxidant, anti-inflammatory processes, and inhibition of reactive oxidant species generation. Interestingly, only two of the identified targets were directly implicated in cellular differentiation, glial cell-derived neurotrophic factor (GDNF), and KLF15. Previous studies have already demonstrated that GDNF plays a role in podocyte differentiation (31). KLF15, which was originally identified in kidneys (14), has been shown to promote differentiation of adipocytes under metabolic stress (16), but its role in podocyte differentiation was to date unknown. In addition, recent studies suggest that many of podocyte-specific genes share four common binding sites in their promoter regions with one of the most common binding domains being for Kruppel-like factor (19). Thus, we chose to further explore the role of KLF15 in regulation of podocyte differentiation.

Retinoic Acid Stimulates KLF15 Expression in Cultured Podocytes—To confirm whether atRA increased KLF15 expression, wild-type and HIV-1-infected murine podocytes were exposed to atRA for 6 h. atRA-treated wild-type (WT) and KLF15 \(-/-\) mice (\( n = 6, *, p < 0.01 \) versus LPS-treated WT mice). B, Coomassie stain revealed that the change in proteinuria was mainly albuminuria. The representative gel of three mice in each group is shown. C, podocyte foot process effacement was compared between LPS-treated WT and KLF15 \(-/-\) mice (\( \times 5000 \)). The representative images are shown. D, quantification of foot process effacement is shown (\( n = 6, *, p < 0.01 \)).
1 h after atRA addition, suggesting that KLF15 is an early inducible gene (Fig. 1D). Additionally, these findings were confirmed by Western blot (Fig. 1, C and E). Combined, these findings confirm the microarray gene expression analysis that atRA stimulates KLF15 expression in murine and human podocytes.

Retinoic Acid Increases Binding of KLF15 in Promoter Regions of Podocyte-specific Genes—atRA has been shown to induce the expression of nephrin and podocin in murine podocytes (14). Because we observed that atRA enhanced the expression of KLF15, we examined KLF15 binding to the promoter regions of podocyte-specific genes in response to atRA treatment by ChIP followed by quantitative real time PCR. Binding of KLF15 to the putative KLF15-binding sites in the promoter of nephrin and podocin was significantly increased in atRA-treated murine podocytes compared with the untreated cells (Fig. 2, A and B). In contrast, this finding was not observed in atRA-treated podocytes isolated from KLF15−/− mice (Fig. 2, C and D). These findings suggest that KLF15 likely mediates RA-induced expression of podocyte-specific genes.

KLF15 Expression Is Increased in Differentiated Human Podocytes—Because atRA has been previously shown to induce podocyte differentiation (14) and KLF15 is an atRA-induced gene (Fig. 1), KLF15 expression was measured in a known in vitro model of podocyte differentiation. The conditionally immortalized human podocyte cell line harbors a gene encoding a temperature-sensitive T antigen, where T antigen is stable at a permissive temperature of 33 °C and becomes unstable and degraded at a nonpermissive temperature of 37 °C. At 33 °C, this podocyte cell line proliferates and remains in an undifferentiated state, whereas at 37 °C it undergoes differentiation. We observed that KLF15 mRNA and protein expression was significantly increased in the differentiated and nonproliferative state as compared with undifferentiated cells in culture (Fig. 3). These findings indicate that KLF15 expression is increased in a cell culture model of podocyte differentiation.

Effects of KLF15 on Expression of Differentiated Podocyte Markers—To determine the role of KLF15 in podocyte differentiation, KLF15 was overexpressed in cultured murine podocytes (Fig. 4A), and the expression of differentiated podocyte markers (nephrin, synaptopodin, and podocin) was measured (Fig. 4, B–D). KLF15 overexpression significantly increased the expression of differentiated podocyte markers, suggesting that KLF15 is a key regulator of podocyte differentiation.
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**FIGURE 7.** Adriamycin (AD)-treated KLF15**−/−** mice had increased albuminuria with podocyte effacement. Urine was collected prior to treatment and 4 weeks after treatment. All mice were sacrificed and renal cortex fixed for histology at 4 weeks. A, proteinuria (urine protein/creatinine) was measured in adriamycin-treated wild-type (WT) and KLF15**−/−** mice (n = 6, *p* < 0.01 versus all other groups). B, Coomassie stain revealed that the change in proteinuria was mainly albuminuria. The representative gel from two mice in each group is shown. C, podocyte foot process effacement was compared between AD-treated WT and KLF15**−/−** mice (×5000). The representative images are shown. D, quantification of foot process effacement is shown (n = 6, *p* < 0.01).

**KLF15 Null (KLF15**−/−**) Mice Are More Susceptible to Murine Models of Podocyte-specific Injury**—To validate the role of KLF15 in vivo, we initially studied KLF15**−/−** mice at ages ranging from 6 to 30 weeks. By 30 weeks of age, KLF15**−/−** mice did not develop significant proteinuria or glomerulosclerosis, suggesting that KLF15 is likely not essential for maintaining podocyte differentiation at base line in the unperturbed state.

To determine the role of KLF15 on podocyte differentiation after injury, two different experimental models of podocyte-specific injury, lipopolysaccharide-induced podocyte injury and adriamycin-induced nephropathy, were examined in KLF15**−/−** mice. LPS is known to cause significant podocyte injury and podocyte injury in a BALB/c background (25). A higher dose of adriamycin (20 mg/kg of body weight) was used to induce proteinuria in KLF15**−/−** mice, which are on a C57BL/6 background. We observed that the KLF15**−/−** mice developed a significant increase in albuminuria 4 weeks after adriamycin treatment, whereas wild-type C57BL/6 littermates were resistant to adriamycin.

To further confirm that a loss of KLF15 increases the susceptibility to kidney injury, we treated KLF15**−/−** and wild-type mice with adriamycin. Adriamycin-induced nephropathy has been previously shown to cause significant glomerular disease and podocyte injury in a BALB/c background (25). Ultrastructural examination of the kidney revealed significant podocyte effacement in the adriamycin-treated KLF15**−/−** mice as compared with adriamycin-treated wild-type littermates (Fig. 7, A–C). The expression of podocyte-specific genes (synaptopodin, nephrin, and WT-1) in isolated glomeruli of KLF15**−/−** mice was significantly reduced compared with adriamycin-injected control littermates as determined by real time PCR analysis of isolated glomeruli and by immunofluorescence staining of kidney sections (Fig. 6, A–C).

PCR analysis of isolated glomeruli and by immunofluorescence staining of kidney sections (Fig. 6, A–C).

To further confirm that a loss of KLF15 increases the susceptibility to kidney injury, we treated KLF15**−/−** and wild-type mice with adriamycin. Adriamycin-induced nephropathy has been previously shown to cause significant glomerular disease and podocyte injury in a BALB/c background (25). A higher dose of adriamycin (20 mg/kg of body weight) was used to induce proteinuria in KLF15**−/−** mice, which are on a C57BL/6 background. We observed that the KLF15**−/−** mice developed a significant increase in albuminuria 4 weeks after adriamycin treatment, whereas wild-type C57BL/6 littermates were resistant to adriamycin (1689 ± 392 versus 137 ± 56, p < 0.05; Fig. 7, A and B). Ultrastructural examination of the kidney revealed significant podocyte effacement in the adriamycin-treated KLF15**−/−** mice as compared with adriamycin-treated wild-type littermates (Fig. 7, A–C). The expression of podocyte-specific genes (synaptopodin, nephrin, and WT-1) in isolated glomeruli of KLF15**−/−** mice was significantly reduced compared with adriamycin-injected control littermates as determined by real time PCR and immunofluorescence staining (Fig. 8, A–C). Combined, these findings highlight a critical role for KLF15 in regulating podocyte differentiation and response to kidney injury in both in vitro and in vivo models of the disease.
Retinoic Acid Does Not Increase Podocyte Differentiation Markers in Podocytes Lacking KLF15 Expression—Primary podocytes were isolated from wild-type and KLF15−/− mice and confirmed by Western blot (Fig. 9A). As published previously (14), atRA treatment increased the expression of nephrin, podocin, and synaptopodin in podocytes isolated from wild-type mice (Fig. 9, B–D). This finding was not observed in podocytes isolated from KLF15−/− mice (Fig. 9, B–D).

Retinoic Acid Is Unable to Attenuate LPS-induced Proteinuria in KLF15−/− Mice—To characterize that atRA-induced podocyte differentiation is dependent on induction of KLF15, wild-type and KLF15−/− mice were treated concurrently with atRA and LPS. Within 24 h, LPS treated wild-type mice had an attenuation in proteinuria with atRA treatment. This finding was not observed in LPS treated KLF15−/− mice (Fig. 10).

KLF15 Expression Is Reduced in HIV Transgenic Mice—Because the HIV transgenic mouse line (Tg26 mice) is a known model of podocyte dedifferentiation (20) and our studies indicate that KLF15 regulates podocyte differentiation, the expression of KLF15 was determined in this murine model. Glomeruli isolated from Tg26 mice revealed a significant decrease in KLF15 mRNA and protein expression as compared with wild-type mice (Fig. 11, A and B). Immunohistochemistry confirmed the reduced KLF15 expression in Tg26 mice as compared with the wild-type mice (Fig. 11C). Combined, these findings indicate that KLF15 expression is suppressed in Tg26 mice and may contribute to the podocyte dedifferentiation in this HIVAN model.

Reduction of KFL15 Expression in Human Glomerular Disease—To ascertain the role of KFL15 in human kidney disease, immunostaining for KFL15 was performed on a renal biopsy specimen from healthy donor nephrectomies, HIVAN, and idiopathic FSGS. We observed that the staining for KFL15 had a nuclear distribution in normal podocytes, parietal cells, and tubular cells from healthy donors (Fig. 12A). Quantification of immunostaining in the glomeruli confirmed the changes in KFL15 expression (Fig. 12D). Combined with the rest of our findings, these data suggest that KFL15 expression is reduced in human glomerular disease and may lead to increased susceptibility to podocyte injury in these conditions.

DISCUSSION

A large body of evidence suggests that RA ameliorates kidney injury in several animal models of kidney disease, including...
HIVAN. However, the transcriptional regulators mediating the effects of RA on podocyte differentiation have not been well characterized. We had previously shown that RA induces podocyte differentiation by activating the cAMP/PKA/CREB pathway (14). Here, we identify that KLF15 is likely one of the mediators for RA-induced podocyte differentiation, and KLF15 deficiency increases the susceptibility of podocytes to injury. This was demonstrated by the following lines of evidence: 1) overexpression of KLF15 induces podocyte differentiation, and RA did not induce podocyte differentiation in podocytes lacking KLF15 expression; 2) RA did not induce podocyte differentiation in podocytes lacking KLF15 expression; 3) an exaggerated proteinuric response and a significant reduction in expression of podocyte differentiation markers occur in KLF15−/− mice subjected to two experimental models of podocyte injury (LPS and adriamycin); 4) RA was unable to attenuate LPS-induced proteinuria in KLF15−/− mice; and 5) reduced KLF15 expression occurs in the murine HIVAN model and in renal biopsy specimens from human subjects with podocyte disease.

Transcriptional regulators of podocyte differentiation have remained largely unknown. Notch signaling, Wilms tumor 1..
FIGURE 11. **KLF15 expression is reduced in HIV transgenic (Tg26) mice.** Glomeruli were isolated, and RNA was extracted for real-time PCR. A, KLF15 mRNA expression was measured in wild-type (WT) and Tg26 mice (n = 4, *p < 0.05 versus control). B, Western blot analysis was performed in glomerular lysates from WT and Tg26 mice for KLF15 and GAPDH. The representative blot of three independent experiments is shown. The densitometry analysis of these blots is shown in the lower panel (n = 3, *p < 0.01). C, representative images from the immunostaining of KLF15 in kidney sections from WT and Tg26 mice are shown.

FIGURE 12. **Reduced KLF15 expression in human glomerular disease.** A, immunostaining for KLF15 performed on healthy donor nephrectomy specimens shows a nuclear distribution in normal podocytes, parietal cells, and tubular cells. In comparison with biopsy specimen from healthy donor subjects (A), KLF15 expression in the podocytes is shown in biopsy specimens from patients with diagnosed HIVAN (B) and idiopathic FSGS (C). The representative images of three subjects in each group are shown. D, glomerular region was selected, and optical density (OD) was measured and quantified as a relative fold change to healthy donor specimens (n = 3, *p < 0.0001).
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(WT1), and Forkhead box protein C2 (FOXC2) are key regulators in the Xenopus podocyte gene network that specify the expression of podocyte terminal differentiation markers (33). WT1 is known to mediate podocyte differentiation and kidney development in mice as well as in humans (34). Although RA stimulates the expression of several podocyte differentiation markers (14) and CREB is a key transcription factor that mediates the effects of RA on podocyte differentiation (35), most markers/genes of podocyte terminal differentiation lack CREB-binding elements and RA-response elements in their promoters. This was the impetus for our approach to identify additional transcriptional regulators of podocyte differentiation that are RA-induced and CREB-mediated. We found that KLF15 is able to bind directly to the promoter of nephrin and podocin and stimulate expression of these two key podocyte differentiation markers. Consistent with this, a comparative promoter analysis by Cohen et al. (19) revealed that KLF motif is one of the four most common bindings sites in the promoter region of many podocyte-specific genes. We recognize that other factors may also mediate RA-induced podocyte differentiation. For example, our data suggest that GDNF is also CREB-targeted and highly regulated by RA. Previous studies have already shown that GDNF is implicated in podocyte differentiation (31). Although the KLF15−/− mice developed minimal podocyte injury at base line, the murine models of podocyte injury used in our study revealed that the KLF15 deficiency increased the susceptibility of KLF15−/− mice to LPS- or adriamycin-induced albuminuria and foot process effacement. These data indicate that KLF15 is dispensable for normal podocyte development and maintenance of the glomerular filtration barrier in health, but KLF15 is required for protection against podocyte injury. We speculate a classic “two-hit” model for podocyte injury, the loss of KLF15 expression and the insult from either LPS or Adriamycin. Additionally, as with other members of the KLF family (36), the lack of significant podocyte injury in the KLF15−/− mice may be attributed to the compensation by the other factors in the KLF family. Further studies will be required to fully characterize the role of KLF15 in this two-hit model.

We also confirmed that KLF15 expression is reduced in Tg26 mice and in human glomerular diseases, such as HIVAN and idiopathic FSGS, where podocyte injury is prominent. Podocyte dedifferentiation is a hallmark of collapsing FSGS in HIVAN. KLF15 expression was markedly reduced in Tg26 mice as well as in human HIVAN kidneys suggesting that a loss of KLF15 expression may contribute to podocyte dedifferentiation in HIVAN kidneys. Therefore, knock-out of KLF15 in Tg26 mice may not yield a significant change in an already severely diseased phenotype. Consequently, further studies are needed to determine whether overexpression of KLF15 protects against kidney injury in Tg26 mice. However, the KLF15 gene was recently found to be associated with the HIVAN2 gene. Although this finding alone does not establish causality between the lack of KLF15 and human kidney disease, our animal and in vitro studies suggest that KLF15 may have a protective role in human kidney disease.

Regulation of KLF15 in human kidney disease is not known. Promoter analysis of KLF15 revealed transcription factor-binding sites for NF-κB. Because HIV-1 infection of kidney cells is known to transactivate NF-κB (37) in Tg26 mice (38) and patients with HIVAN (39), future studies are required to examine whether NF-κB mediates regulation of KLF15 in kidney disease such as HIVAN.

Here, we report that KLF15 is a novel transcriptional regulator of podocyte differentiation. Our studies indicate that the loss of KLF15 leads to increased susceptibility to podocyte injury. In addition, KLF15 expression is suppressed in human kidney disease. This study provides new insight into podocyte biology and pathology, as well as a potential new target for therapy of kidney diseases with podocyte injury.

REFERENCES

1. Wolf, G., Chen, S., and Ziyadeh, F. N. (2005) From the periphery of the glomerular capillary wall toward the center of disease. Podocyte injury comes of age in diabetic nephropathy. *Diabetes* **54,** 1626–1634
2. Kriz, W., Gretz, N., and Lemley, K. V. (1998) Progression of glomerular diseases. Is the podocyte the culprit? *Kidney Int.** **54,** 687–697
3. Barisoni, L., Kriz, W., Mundel, P., and D’Agati, V. (1999) The dysregulated podocyte phenotype. A novel concept in the pathogenesis of collapsing idiopathic focal segmental glomerulosclerosis and HIV-associated nephropathy. *J. Am. Soc. Nephrol.* **10,** 51–61
4. Barisoni, L., Bruggeman, L. A., Mundel, P., D’Agati, V. D., and Klotman, P. E. (2000) HIV-1 induces renal epithelial dedifferentiation in a transgenic model of HIV-associated nephropathy. *Kidney Int.** **58,** 173–181
5. Bruggeman, L. A., Dikman, S., Meng, C., Quaggan, S. E., Coffman, T. M., and Klotman, P. E. (1997) Nephropathy in human immunodeficiency virus–1 transgenic mice is due to renal transgene expression. *J. Clin. Invest.* **100,** 84–92
6. Schwartz, E. J., Cara, A., Snoeck, H., Ross, M. D., Sunamoto, M., Reiser, I., Mundel, P., and Klotman, P. E. (2001) Human immunodeficiency virus-1 induces loss of contact inhibition in podocytes. *J. Am. Soc. Nephrol.* **12,** 1677–1684
7. Husain, M., Gusaella, G. L., Klotman, M. E., Gelman, I. H., Ross, M. D., Schwartz, E. J., Cara, A., and Klotman, P. E. (2002) HIV-1 Nef induces proliferation and anchorage-independent growth in podocytes. *J. Am. Soc. Nephrol.* **13,** 1806–1815
8. He, J. C., Husain, M., Sunamoto, M., D’Agati, V. D., Klotman, M. E., Iyengar, R., and Klotman, P. E. (2004) Nef stimulates proliferation of glomerular podocytes through activation of Src-dependent Stat3 and MAPK1/2 pathways. *J. Clin. Invest.* **114,** 643–651
9. Evans, T. R., and Kaye, S. B. (1999) Retinoids. Present role and future potential. *Br. J. Cancer* **80,** 1–8
10. Xu, Q., Lucio-Cazaña, J., Kitamura, M., Ruan, X., Fine, L. G., and Norman, I. T. (2004) Retinoids in nephrology. Promises and pitfalls. *Kidney Int.** **66,** 2119–2131
11. Lehrke, I., Schaier, M., Schade, K., Morath, C., Waldherr, R., Ritz, E., and Wagner, J. (2002) Retinoid receptor–specific agonists alleviate experimental glomerulonephritis. *Am. J. Physiol. Renal Physiol.* **282,** F741–F751
12. Suzuki, A., Ito, T., Imai, E., Yamato, M., Iwataki, H., Kawachi, H., and Hori, M. (2003) Retinoids regulate the repairing process of the podocytes in puromycin aminonucleoside–induced nephrotic rats. *J. Am. Soc. Nephrol.* **14,** 981–991
13. Pérez de Lema, G., Lucio-Cazaña, F. I., Molina, A., Luckow, B., Schmid, H., de Wit, C., Moreno-Manzano, V., Banas, B., Mampaso, F., and Schrödl, D. (2004) Retinoic acid treatment protects MRL/lpr lupus mice from the development of glomerular disease. *Kidney Int.** **66,** 1018–1028
14. He, J. C., Lu, T. C., Fleet, M., Sunamoto, M., Husain, M., Fang, W., Neves, S., Chen, Y., Shankland, S., Iyengar, R., and Klotman, P. E. (2007) Retinoic acid inhibits HIV-1-induced podocyte proliferation through the cAMP pathway. *J. Am. Soc. Nephrol.* **18,** 93–102
15. Vaughan, M. R., Pippin, J. W., Griffin, S. V., Kroff, R., Fleet, M., Haselar, A. G. (1999) KLF motif is one of the four most common bindings sites in the promoter region of many podocyte-specific genes. We recognize that other factors may also mediate RA-induced podocyte differentiation. For example, our data suggest that GDNF is also CREB-targeted and highly regulated by RA. Previous studies have already shown that GDNF is implicated in podocyte differentiation (31). Although the KLF15−/− mice developed minimal podocyte injury at baseline, the murine models of podocyte injury used in our study revealed that the KLF15 deficiency increased the susceptibility of KLF15−/− mice to LPS- or adriamycin-induced albuminuria and foot process effacement. These data indicate that KLF15 is dispensable for normal podocyte development and maintenance of the glomerular filtration barrier in health, but KLF15 is required for protection against podocyte injury. We speculate a classic “two-hit” model for podocyte injury, the loss of KLF15 expression and the insult from either LPS or Adriamycin. Additionally, as with other members of the KLF family (36), the lack of significant podocyte injury in the KLF15−/− mice may be attributed to the compensation by the other factors in the KLF family. Further studies will be required to fully characterize the role of KLF15 in this two-hit model.

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*4* Ali G. Gharavi, personal communication.
