Published in final edited form as:

Exp Cell Res. 2017 March 15; 352(2): 193–201. doi:10.1016/j.yexcr.2017.01.019.

Hedgehog pathway plays a vital role in HIV-induced epithelial-mesenchymal transition of podocyte

Xiqian Lan*, Hongxiu Wen, Kang Cheng, Andrei Plagov, Seyedeh Shadafarin Marashi Shoshtari, Ashwani Malhotra, Pravin C Singhal**

Renal Molecular Research Laboratory, Feinstein Institute for Medical Research, Hofstra Northwell Medical School, New York, USA

Abstract

HIV-associated nephropathy (HIVAN) is characterized by heavy proteinuria, rapidly progressive renal failure, and distinct morphological features in the kidney. HIV-induced epithelial-mesenchymal transition (EMT) is critically important for the progression of kidney injury. In this study, we tested the role of hedgehog pathway in the HIV-induced EMT and fibrosis of kidney. We used the Tg26 mice, the abundantly used HIVAN mouse model, to investigate the activation of hedgehog pathway by HIV. Western blotting and real time PCR results showed that renal tissue expression of hedgehog pathway related molecules, including hedgehog homologous (Shh, Ihh, Dhh), PTCH, and Gli1, were increased in HIVAN (Tg26) mice; while immunofluorescent staining displayed localization PTCH expression in podocytes. For *in vitro* studies, we used recombinant sonic hedgehog (Shh) and HIV for their expression by podocytes. Both the methods activated the hedgehog pathway, enhanced the expression of EMT markers, and decreased impermeability. Overexpression of Gli1 by human podocytes also augmented their expression of EMT markers. On the other hand, the blockade of hedgehog pathway with Gant 58, a specific blocker for Gli1-induced transcription, dramatically decreased HIV-induced podocyte EMT and permeability. These results indicate that hedgehog pathway plays an important role in HIV-induced podocyte injury. The present study provides mechanistical insight into a new target for therapeutic strategy.

Keywords

HIV-associated nephropathy; podocyte; hedgehog pathway; epithelial-mesenchymal transition

Introduction

HIV-associated nephropathy (HIVAN) predominantly occurs in patients with African-American descent. It is characterized by heavy proteinuria, rapidly progressive renal failure, and distinct morphological features in the form of collapsing focal segmental

*Correspondence to: Feinstein Institute for Medical Research, 225 Community Drive, Great Neck, NY 11021. Tel.: +516 918 4863; fax: +516 918 4384. xlan@northwell.edu. **Correspondence to: Division of Kidney Diseases and Hypertension, 100 Community Drive, Great Neck, NY 11021. Tel.: +516 465 3010; fax: +516 465 3011. psinghal@northwell.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

glomerulosclerosis (FSGS) and microcystic tubular dilatation [1]. Although a variety of cytokines are released during HIV infection, HIV infection of kidney cells including both glomerular and tubular epithelial cells plays a critical role of for the development and progression of HIVAN [2, 3].

Podocytes are specialized, terminally differentiated visceral epithelial cells in the Bowman's capsule in the kidney. They wrap around capillaries of the glomerulus, and extend foot processes to form the blood urine filtration barrier, and are directly implicated in the integrity of the glomerular basement membrane [4, 5]. Podocytes play a key role in maintaining the filtration barrier in kidney, and their injury or damage has been linked to the development of proteinuria as well as the progression of chronic kidney disease [6]. We and other investigators have shown that HIV could infect podocytes leading to kidney cell injury in the form of dedifferentiation and proliferation [7–13].

Epithelial mesenchymal transition (EMT) is a process in which epithelial cells lose their epithelial phenotype and attain characteristic features of mesenchymal cells such as proliferation and migration. It has been widely recognized that renal tubular epithelial cells are able to undergo EMT under pathological conditions [14–19]. *In vitro* and *in vivo* studies show that after treatment with fibrogenic cytokines such as TGF- β , tubular epithelial cells usually lose epithelial markers, including E-cadherin and ZO-1, and acquire mesenchymal markers such as vimentin, α -SMA, FSP1, and fibronectin [20]. Our previous studies also displayed occurrence of EMT in tubular cells in HIVAN (Tg26) mice [21, 22]. Besides tubular cells, recent evidences have shown that podocytes can also undergo EMT in some pathological conditions [20, 23–26]. For example, Li *et al* reported that EMT was observed in the podocytes in human diabetic kidneys [23]. They also found that TGF- β 1 treatment not only suppress epithelial P-cadherin and ZO-1 expression in podocytes but also impairs the filtration barrier function of podocytes [23]. The occurrence of EMT in glomerular epithelial cells manifests in the form of proliferation, migration, matrix synthesis, and loss of their original function and eventually contributing to fibrosis.

The Hedgehog signaling pathway (hedgehog pathway) is a evolutionarily conserved pathway, which is essential for regulating a diverse array of biologic processes, such as embryonic development, tissue homeostasis, injury repair, and tumorigenesis [27, 28]. This pathway starts from hedgehog homologues binding to their special receptor Patched (PTCH) in the plasma membrane, resulting in the de-repression of smoothened (SMO) [29, 30]. Activated smoothened changes its position from an intracellular vesicle to the cell membrane where the Gli family of transduction factors are activated. Subsequently, the activated Gli proteins enter the nucleus and initiate the transcription of their target genes. Several of its own major components, including Gli1, PTCH, and hedgehog interacting protein 1 (Hhip 1), are also the direct transcriptional targets of hedgehog pathway, which provides both positive and negative feedback to ensure the delicate regulation of this system. Among these molecules, Gli1 is the principal downstream target and mediator of hedgehog signaling. Mammals have three hedgehog homologues, Dhh, Ihh, and Shh, of which Sonic (Shh) is the most widely studied and best characterized.

Hedgehog pathway has been reported to regulate EMT in cancer cells [31, 32]. It also mediates the EMT in renal tubular cells and promotes fibrosis [32, 33]. Whether hedgehog pathway is involved in the podocyte injury or not is not yet clear. Since HIV has been reported to modulate hedgehog pathway in T lymphocyte [34], we speculate that it may have similar effects in podocyte. We hypothesize that HIV enhances podocyte EMT through hedgehog pathway activation.

Materials and Methods

Reagents

Recombinant hedgehog (Shh) was purchased from R&D System (Minneapolis, MN), while Gant 58 was purchased from Tocris Bioscience (Ballwin, MO).

Animal experiments

All animal experiments complied with Institutional Animal Care and Use Committee (IACUC)-approved protocols (approval #2009–012). FVB/N and Tg26 mice (FVB/N background) purchased from Jackson Laboratory (Bar Harbor, ME, USA), were housed within the rodent holding facilities in the Feinstein Institute for Medical Research (Northwell Health) in Manhasset, New York. Three male and three female mice at 8 weeks old were used in this study.

Culture of human podocytes

Human podocytes were cultured as previously reported [35–37]. Briefly, immortalized human podocytes proliferated in the growth medium containing RPMI 1640 supplemented with 10% fetal bovine serum, 1 X penicillin- streptomycin, 1 mM L-glutamine, and 1 X insulin, transferrin, and selenium (ITS) (Invitrogen, Grand Island, NY) at permissive temperature (33°C). When the cells reached about 80% confluence, they were transferred to 37°C for differentiation in a medium without ITS for 7 days.

Real time-PCR

Total RNA was isolated from mouse kidney tissue or human podocytes using Trizol reagent (Invitrogen). Five micrograms of total RNA were reverse transcribed using the first-strand synthesis system (Invitrogen). Real time-PCR was performed in a Prism 7900HT sequence-detection system (Applied Biosystems, Foster City, CA, USA). Relative mRNA levels were determined and standardized with a GAPDH internal control using comparative CT method. Sequences of primers for the targeting genes are listed in Table 1.

Gli1 overexpression

Human podocytes (5×10^5) were cultured in 100 mm dish. After differentiation for 7 days, the cells were transfected with 3 µg TAL2271-Gli1 plasmid (Addgene, Cambridge, MA) by using the Effectene Transfection Reagent (Qiagen, Germantown, MD) following the manufacturer's instruction.

Western blot analysis

Western blotting was performed using established methodology [36–41]. Briefly, cells were washed with PBS and lysed in RIPA buffer (1 X PBS, pH7.4, 0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 1.0 mM sodium orthovanadate, 10 μ l of protease inhibitor cocktail (100 $\times$, Calbiochem) per 1 ml of buffer, and 100 μ g/ml PMSF). Proteins (20–30 μ g) were separated by 12% SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred on an Immuno-Blot polyvinylidene fluoride (PVDF) membrane (Bio-Rad, Hercules, CA). After blocking in PBS/Tween (0.1%) with 5% nonfat milk, the membrane was incubated with primary antibodies overnight at 4°C followed by horseradish peroxidase-conjugated secondary antibodies (Santa Cruz, 1:3000) and then developed using Enhanced Chemiluminescent (ECL) solution (Pierce). Primary antibodies used were rabbit anti-Shh, (Cell Signaling Technology, 1:1000), rabbit anti-PTCH (Santa Cruz, 1:1000), rabbit anti-Gli1 (Santa Cruz, 1:1000), rabbit anti-P-Cadherin (Santa Cruz, 1:1000), rabbit anti-Vimentin (Santa Cruz, 1:1000), rabbit anti- α -SMA (Santa Cruz, 1:1000), mouse anti-Fibronectin (Santa Cruz, 1:1000), rabbit anti-Snail (Santa Cruz, 1:1000), and goat anti-actin (Santa Cruz, 1:3000). For protein expression quantification, the films were scanned with a CanonScan 9950F scanner and the acquired images were then analyzed using the public domain NIH image program (<http://rsb.info.nih.gov/ni-image/>).

Pseudo-types HIV transduction

Replication defective HIV viral supernatants were prepared and transduced into human podocyte as published previously [39–41]. In brief, GFP reporter gene was substituted for *gag/pol* genes in HIV-1 proviral construct pNL4–3. This parental construct (pNL4–3: G/P-GFP) was used to produce VSV. G pseudotyped viruses to provide pleiotropism and high-titer virus stocks. Infectious viral supernatants were produced by the transient transfection of 293T cells using effectene (Qiagen Inc., Valencia, CA) according to the manufacturer's instructions. The HIV-1 *gag/pol* and VSV.G envelope genes were provided *in trans* using pCMV R8.91 and pMD.G plasmids, respectively. As a negative control, virus was also produced from pHR-CMV-IRES2-GFP- B, which contained HIV-1 *LTR* and GFP empty expression vector. The viral stocks were titrated by infecting HeLa cells with tenfold serial dilution as reported previously [39]. Differentiated human podocytes were transduced with these pseudo-typed HIV (titrated as 0.8 pg HIV p24 protein per cell) for 3 h, followed by culture in fresh medium.

Albumin Influx Assay

A simple albumin influx assay was used to evaluate the filtration barrier function of podocyte monolayer, as described by Li *et al* with some modifications [23]. Briefly, podocytes (5×10^4) were seeded onto the collagen-coated transwell filters (1- μ m pore; Corning, New York, NY) in the top chamber and cultured under differentiating conditions. After 7 days differentiation, podocytes were serum-starved overnight and treated without or with SHH or HIV for a certain period. Cells were washed twice with PBS supplemented with 1 mM MgCl₂ and 1 mM CaCl₂. The top chamber was then refilled with 0.5 ml of RPMI 1640 and the bottom chamber with 2 ml of RPMI 1640 supplemented with 40 mg/ml

of bovine serum albumin and incubated at 37°C. A small aliquot of media from the top chamber was collected at different time intervals and albumin concentration was determined.

Statistical analyses

Data were presented as means $\pm$ standard deviation (SD) unless otherwise noted. All experiments were repeated at least three times with duplicate or triplicate samples in each assay. All data were evaluated statistically by the analysis of variance (ANOVA), followed by Newman-Keuls multiple comparison tests using software (Prism 4.0, GraphPad Software). In the case of single mean comparison, data were analyzed by t test. P values < 0.05 were considered as statistically significant.

Results

HIV activates hedgehog pathway in podocytes

We examined whether HIV infection activates hedgehog pathway in human podocytes. We transduced differentiated human podocytes with pseudo type HIV for 12, 24, 48, and 72 h, and then extracted total RNA for real time-PCR. Results showed that the mRNA levels of Shh, Dhh, Ihh, Gli1, and PTCH, were dramatically increased in a time course effect (Figure 1A). For protein expression, cellular lysates (collected after 48 hours) were subjected to Western blotting. HIV-transduced podocytes displayed enhanced expression of Shh, Gli1, and PTCH (Figs 1B–1D) indicating that hedgehog pathway was activated by HIV in human podocytes.

We then evaluated the expression of these factors in the kidneys of Tg26 as well as control (FVB/N) mice. Renal tissues were used for real time-PCR and Western blot analysis. mRNA expressions of hedgehogs (Dhh, Ihh, Shh), Gli1, and PTCH were significantly increased in Tg26 mice when compared with control mice (Fig. 2A); Western blot analysis also revealed increased expression of Gli1 and PTCH in renal tissues of Tg26 mice (Fig. 2B–C). These results indicated that hedgehog pathway was activated in the kidney of Tg26 mice.

To further examine the activation of hedgehog pathway in HIV milieu, we performed immunofluorescent staining in renal cortical sections of control and Tg26 mice. Results showed enhanced podocyte expression of PTCH in Tg26 mice when compared FVB/N (control) mice (Figures 2E and 2F). The results also showed that some PTCH co-localized with synaptopodin, a protein marker for podocytes. Taken together, these results clearly demonstrate that hedgehog pathway in podocytes is activated by HIV.

Hedgehog pathway activation enhances EMT in podocytes

To determine the effect of hedgehog pathway activation on EMT in podocytes, we overexpressed Gli1 in human podocytes. Results showed that Gli1 was successfully expressed, which subsequently decreased the epithelial protein P-cadherin, but significantly increased the mesenchymal proteins fibronectin, vimentin, and α -SMA (Figure 3). Overexpression of Gli1 also increased the expression of Snail, a protein robustly promotes EMT. These results suggest that increasing up-regulating Gli1 enhances EMT in podocytes.

To verify this observation, we treated human podocytes with 100 ng/ml recombinant Shh for 6, 12, 24, and 48 h. The expression of Gli1 increased as a function of time course intervals, indicating that hedgehog pathway was activated (Figure 4). Meanwhile, Shh also increased vimentin and α -SMA indicating that EMT was induced by addition of Shh.

To further confirm this observation, we treated human podocytes with 5, 10, 50, or 100 ng/ml recombinant Shh for 48 h. We found that the expression of PTCH and Gli1 increased following the increasing concentration of Shh (Figure 5). Correspondingly, the expression of vimentin and α -SMA also increased with the increasing Shh concentrations; however, P-cadherin expression decreased. These results indicate that Shh activates hedgehog signaling pathway and induces podocyte EMT in a dose-dependent manner.

Taken together, these results clearly demonstrated that activating hedgehog pathway causes EMT in podocyte.

HIV induces EMT of in podocytes via hedgehog pathway

In our previous studies, we have found that HIV can induce EMT in kidney proximal tubular cells [21, 22]. To determine its effect on podocyte EMT, we transduced human podocytes with pseudo type HIV. We also added Gant 58, an inhibitor of hedgehog pathway, in the medium to examine whether hedgehog pathway is involved in this process. Results showed that HIV dramatically increased the expression of mesenchymal markers, including fibronectin, vimentine, and α -SMA; while addition of Gant 58, significantly decreased their expression (Figure 6). These results indicate that hedgehog pathway plays an important role in HIV-induced EMT in podocytes.

Hedgehog pathway activation increases the permeability of podocytes

Li *et al* reported that enhanced EMT in podocyte impairs the filtration barrier function of podocytes [23]. To investigate the role of hedgehog pathway in the dysfunction of podocytes, we treated human podocytes with recombinant Shh for 48 h, and then examined the permeability of podocytes by determining the albumin flux. Results showed that Shh-treated podocytes had higher albumin flux at 2 hours and 4 hours intervals (Figure 7), indicating an increase in their permeability.

To detect the role of hedgehog pathway in HIV-induced alteration in protein permeability, we added hedgehog blocker Gant 58 to the podocytes immediately after the HIV transduction. Albumin flux assay results showed that addition of Gant 58 significantly attenuated HIV-enhanced podocyte permeability as the albumin flux decreased (Figure 8).

Discussion

Hedgehog pathway activation has been reported in renal tubular and mesangial cells [33, 42–44], and it plays an important role in kidney fibrosis; however, its role in the maintenance of podocyte integrity has not been investigated. In this study, we performed both *in vitro* and *in vivo* experiments, and found that HIV increases the expression of Gli1 and PTCH in podocytes, demonstrating that hedgehog pathway is activated in podocytes in HIV milieu. Addition of Shh or overexpression of Gli1 significantly decreased the expression of P-

cadherin, while increased fibronectin, vimentin, Snail, and α -SMA, indicating that activation of hedgehog pathway stimulates EMT in podocytes. Addition of Shh or transduction of HIV also increased the permeability of podocytes, while hedgehog pathway inhibitor Gant 58 partially attenuated this impairment. All these findings demonstrate that HIV promoted EMT in podocytes via activation of hedgehog pathway. This is the first report to demonstrate the role of hedgehog pathway in podocyte injury.

Although it has been demonstrated that hedgehog signaling pathway can be activated by various viruses such as hepatitis B virus [45], hepatitis C virus [46], Epstein-Barr virus [47, 48], and human papillomavirus [49], there is hardly any data on the role of HIV in the activation of this pathway [34]. In this study, HIV-infected podocytes displayed enhanced expression of hedgehog homologues (Dhh, Ihh, and Shh), Gli1, and PTCH. Moreover, renal tissues from HIV-transgenic (Tg26) mice also exhibited enhanced expression of all these molecules. These results strongly suggest that HIV also activates hedgehog signaling pathway. Since HIV increases the expression of hedgehog homologues in podocytes, it indicates that an autocrine hedgehog secretion and hedgehog signaling activation loop exists in podocytes. Since tubule-derived Shh has been reported to mediate epithelial-mesenchymal communication by targeting interstitial fibroblasts after kidney injury [42], we speculate that podocyte-secreted Shh may also act on adjacent renal fibroblasts and mesenchymal cells, leading to the proliferation/fibrosis of these cells. It will be interesting to investigate the role of hedgehog pathway in HIV-induced kidney fibrosis in future studies.

Hedgehog signaling pathway plays an important role in the EMT of cancer cells as well as renal tubular cells [31–33]. In this study, we observed that overexpressing Gli1, or stimulating human podocytes with Shh significantly activated hedgehog pathway, and an increase in the expression of vimentin, α -SMA, and Snail, but decreased expression of P-cadherin. These results suggest that activation of hedgehog pathway may also cause EMT in podocytes.

The consequence of enhanced EMT in renal tubular cells is to increase their fibrotic potential [14–20, 50, 51]; for podocytes, EMT increases its impermeability and compromises filtration barrier [23–26]. In this study, we found that Shh treatment significantly increased the permeability of human podocytes in the form of higher albumin flux. These results suggest that hedgehog pathway regulated podocyte EMT also mediates their permeability.

HIV infection has been demonstrated to cause podocyte injury through many factors, such as vascular endothelial cell growth factors (VEGFs), fibroblast growth factor-2 (FGF-2), IL-1 β , TNF- α , angiotensin II (Ang II), Interferon (IFN)- α and IFN- β [52–58]. These factors impair podocyte homeostasis through activating different signaling pathways. Additionally, we demonstrate a new signaling pathway. We found that in podocytes, HIV significantly increased the production of hedgehog homologues including Shh, Dhh, and Ihh, which subsequently activate the hedgehog signaling pathway. The hedgehog pathway inhibitor Gant 58 obviously attenuated HIV-induced podocyte EMT, as well as decreased impermeability. All these results clearly demonstrated that HIV induces podocyte EMT at least partially through activating hedgehog pathway.

In conclusion, we have demonstrated that HIV activates hedgehog signaling pathway in podocytes, which further enhances EMT, increases its impermeability, and compromises filtration barrier (see schematic in Figure 9). Our study provides insight into new mechanisms involved in HIV-induced podocyte damage, and highlights some new therapeutic targets for HIVAN.

Acknowledgement

This work was supported by grant RO1DK084910, RO1 DK083931, and 1R01DK098074 (PCS) from National Institutes of Health, Bethesda, MD.

References

1. Kimmel PL, Barisoni L, Kopp JB. (2003). Pathogenesis and treatment of HIV-associated renal diseases: lessons from clinical and animal studies, molecular pathologic correlations, and genetic investigations. *Annals of internal medicine*, 139 (3): 214–226. [PubMed: 12899589]
2. Bruggeman LA, Ross MD, Tanji N, Cara A, Dikman S, Gordon RE, Burns GC, D'Agati VD, Winston JA, Klotman ME, Klotman PE. (2000). Renal epithelium is a previously unrecognized site of HIV-1 infection. *J Am Soc Nephrol*, 11: 2079–2087. [PubMed: 11053484]
3. Ross MJ, Bruggeman LA, Wilson PD, Klotman PE. (2001). Microcyst formation and HIV-1 gene expression occur in multiple nephron segments in HIV-associated nephropathy. *J Am Soc Nephrol*, 12: 2645. [PubMed: 11729233]
4. Pavenstädt H, Kriz W, Kretzler M. (2003). Cell biology of the glomerular podocyte. *Physiol Rev*, 83 (1): 253–307. [PubMed: 12506131]
5. Benzing T. Signaling at the slit diaphragm. (2004). *J Am Soc Nephrol*, 15 (6): 1382–1391. [PubMed: 15153549]
6. Huby AC, Rastaldi MP, Caron K, Smithies O, Dussaule JC, Chatziantoniou C. (2009). Restoration of podocyte structure and improvement of chronic renal disease in transgenic mice overexpressing renin. *PLoS One*, 4 (8): e6721. [PubMed: 19696925]
7. Chandel N, Sharma B, Husain M, Salhan D, Singh T, Rai P, Mathieson PW, Saleem MA, Malhotra A, Singhal PC. (2013). HIV Compromises Integrity of Podocyte Actin Cytoskeleton through down regulation of Vitamin D receptor. *Am J Physiol Renal Physiol*, 304 (11): F13471357.
8. Cheng K, Rai P, Plagov A, Lan X, Subrati A, Husain M, Malhotra A, Singhal PC. (2013). MicroRNAs in HIV-associated nephropathy (HIVAN). *Exp Mol Pathol*, 94 (1): 65–72. [PubMed: 23085312]
9. Lu TC, He JC. (2007). Klotman PE. Podocytes in HIV-associated nephropathy. *Nephron Clin Pract*, 106 (2): c67–71. [PubMed: 17570932]
10. Doublier S, Zennaro C, Spatola T, Lupia E, Bottelli A, Deregibus MC, Carraro M, Conaldi PG, Camussi G. (2007). HIV-1 Tat reduces nephrin in human podocytes: a potential mechanism for enhanced glomerular permeability in HIV-associated nephropathy. *AIDS*, 21 (4): 423–432. [PubMed: 17301560]
11. Tandon R, Levental I, Huang C, Byfield FJ, Ziembicki J, Schelling JR, Bruggeman LA, Sedor JR, Janmey PA, Miller RT. (2007). HIV infection changes glomerular podocyte cytoskeletal composition and results in distinct cellular mechanical properties. *Am J Physiol Renal Physiol*, 292 (2): F701–710 [PubMed: 17047167]
12. Zhong J, Zuo Y, Ma J, Fogo AB, Jolicœur P, Ichikawa I, Matsusaka T. (2005). Expression of HIV-1 genes in podocytes alone can lead to the full spectrum of HIV-1-associated nephropathy. *Kidney Int*, 68 (3): 1048–1060. [PubMed: 16105035]
13. Husain M, Gusella GL, Klotman ME, Gelman IH, Ross MD, Schwartz EJ, Cara A, Klotman PE. (2002). HIV-1 Nef induces proliferation and anchorage-independent growth in podocytes. *J Am Soc Nephrol*, 13 (7): 1806–1815. [PubMed: 12089376]

14. Xiao Y, Liu J, Peng Y, Xiong X, Huang L, Yang H, Zhang J, Tao L. (2016) GSTA3 Attenuates Renal Interstitial Fibrosis by Inhibiting TGF-Beta-Induced Tubular Epithelial-Mesenchymal Transition and Fibronectin Expression. *PLoS One*, 11 (9): e0160855. [PubMed: 27602565]
15. Torsello B, Bianchi C, Meregalli C, Di Stefano V, Invernizzi L, De Marco S, Bovo G, Brivio R, Strada G, Bombelli S, Perego RA. (2016). Arg tyrosine kinase modulates TGF- β 1 production in human renal tubular cells under high-glucose conditions. *J Cell Sci*, 129 (15): 2925–2936. [PubMed: 27298228]
16. Zhao L, Chi L, Zhao J, Wang X, Chen Z, Meng L, Liu G, Guan G, Wang F. (2016). Serum response factor provokes epithelial-mesenchymal transition in renal tubular epithelial cells of diabetic nephropathy. *Physiol Genomics*, 48 (8): 580–588. [PubMed: 27260841]
17. Yang J, Liu Y. (2001). Dissection of key events in tubular epithelial to myofibroblast transition and its implications in renal interstitial fibrosis. *Am J Pathol*, 159 (4): 1465–1475. [PubMed: 11583974]
18. Iwano M, Plieth D, Danoff TM, Xue C, Okada H, Neilson EG. (2002). Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J Clin Invest*, 110 (3): 341–350. [PubMed: 12163453]
19. Tang O, Chen X, Shen S, Hahn M, Pollock CA. (2013). miRNA-200b represses transforming growth factor beta1-induced EMT and fibronectin expression in kidney proximal tubular cells. *Am J Physiol Renal Physiol*, 304 (10): F1266–1273. [PubMed: 23408168]
20. Liu Y New insights into epithelial-mesenchymal transition in kidney fibrosis. (2010). *J Am Soc Nephrol*, 21 (2): 212–222. [PubMed: 20019167]
21. Yadav A, Kumar D, Salhan D, Rattanavich R, Maheshwari S, Adabala M, Ding G, Singhal PC. (2012). Sirolimus modulates HIVAN phenotype through inhibition of epithelial mesenchymal transition. *Exp Mol Pathol*, 93 (1): 173–181 [PubMed: 22579465]
22. Yadav A, Vallabu S, Kumar D, Ding G, Charney DN, Chander PN, Singhal PC. (2010). HIVAN phenotype: consequence of epithelial mesenchymal transdifferentiation. *Am J Physiol Renal Physiol*, 298 (3): F734–744. [PubMed: 20015943]
23. Li Y, Kang YS, Dai C, Kiss LP, Wen X, Liu Y. (2008). Epithelial-to-mesenchymal transition is a potential pathway leading to podocyte dysfunction and proteinuria. *Am J Pathol*, 172 (2): 299–308. [PubMed: 18202193]
24. Yamaguchi Y, Iwano M, Suzuki D, Nakatani K, Kimura K, Harada K, Kubo A, Akai Y, Toyoda M, Kanauchi M, Neilson EG, Saito Y. (2009). Epithelial-mesenchymal transition as a potential explanation for podocyte depletion in diabetic nephropathy. *Am J Kidney Dis*, 54 (4): 653–664. [PubMed: 19615802]
25. Boini KM, Xia M, Xiong J, Li C, Payne LP, Li PL. (2012). Implication of CD38 gene in podocyte epithelial-to-mesenchymal transition and glomerular sclerosis. *J Cell Mol Med*, 16 (8): 1674–1685. [PubMed: 21992601]
26. Kang YS, Li Y, Dai C, Kiss LP, Wu C, Liu Y. (2010). Inhibition of integrin-linked kinase blocks podocyte epithelial-mesenchymal transition and ameliorates proteinuria. *Kidney Int*, 78 (4): 363–373. [PubMed: 20505657]
27. Briscoe J (2009). Making a grade: Sonic Hedgehog signalling and the control of neural cell fate. *EMBO J*, 28 (5): 457–465. [PubMed: 19197245]
28. Jenkins D (2009). Hedgehog signalling: Emerging evidence for non-canonical pathways. *Cell Signal*, 21 (7): 1023–1034. [PubMed: 19399989]
29. Lee RT, Zhao Z, Ingham PW. (2016). Hedgehog signalling. *Development*, 143 (3): 367–372. [PubMed: 26839340]
30. Gorojankina T (2016). Hedgehog signaling pathway: a novel model and molecular mechanisms of signal transduction. *Cell Mol Life Sci*, 73 (7): 1317–1332. [PubMed: 26762301]
31. Liu X, Yun F, Shi L, Li ZH, Luo NR, Jia YF. (2015). Roles of Signaling Pathways in the Epithelial-Mesenchymal Transition in Cancer. *Asian Pac J Cancer Prev*, 16 (15): 6201–6206. [PubMed: 26434817]
32. Flemban A, Qualtrough D. (2015). The Potential Role of Hedgehog Signaling in the Luminal/Basal Phenotype of Breast Epithelia and in Breast Cancer Invasion and Metastasis. *Cancers (Basel)*, 7 (3): 1863–1884 [PubMed: 26389956]

33. Bai Y, Lu H, Lin C, Xu Y, Hu D, Liang Y, Hong W, Chen B. (2016). Sonic hedgehog-mediated epithelial-mesenchymal transition in renal tubulointerstitial fibrosis. *Int J Mol Med*, 37 (5): 1317–1327. [PubMed: 27035418]
34. Lee-Huang S, Zhang L, Huang PL, Chang YT, Huang PL. (2003). Anti-HIV activity of olive leaf extract (OLE) and modulation of host cell gene expression by HIV-1 infection and OLE treatment. *Biochem Biophys Res Commun*, 307 (4): 1029–1037. [PubMed: 12878215]
35. Saleem MA, O'Hare MJ, Reiser J, Coward RJ, Inward CD, Farren T, Xing CY, Ni L, Mathieson PW, Mundel P. (2002). A conditionally immortalized human podocyte cell line demonstrating nephrin and podocin expression. *J Am Soc Nephrol*, 13: 630–638. [PubMed: 11856766]
36. Lan X, Rai P, Chandel N, Cheng K, Lederman R, Saleem MA, Mathieson PW, Husain M, Crosson JT, Gupta K, Malhotra A, Singhal PC. (2013). Morphine induces albuminuria by compromising podocyte integrity. *PLoS One*, 8: e55748. [PubMed: 23555556]
37. Lan X, Jhaveri A, Cheng K, Wen H, Saleem MA, Mathieson PW, Mikulak J, Aviram S, Malhotra A, Skorecki K, Singhal PC. (2014). APOL1 risk variants enhance podocyte necrosis through compromising lysosomal membrane permeability. *Am J Physiol Renal Physiol*, 307 (3): F326–336. [PubMed: 24899058]
38. Lan X, Lederman R, Eng JM, Shoshtari SS, Saleem MA, Malhotra A, Singhal PC. (2016). Nicotine Induces Podocyte Apoptosis through Increasing Oxidative Stress. *PLoS One*, 11 (12): e0167071. [PubMed: 27907022]
39. Husain M, Meggs LG, Vashistha H, Simoes S, Griffiths KO, Kumar D, Mikulak J, Mathieson PW, Saleem MA, Del Valle L, Pina-Oviedo S, Wang JY, Seshan SV, Malhotra A, Reiss K, Singhal PC. (2009). Inhibition of p66ShcA longevity gene rescues podocytes from HIV-1-induced oxidative stress and apoptosis. *J Biol Chem*, 284 (24): 16648–16658. [PubMed: 19383602]
40. Chandel N, Ayasolla KS, Lan X, Sultana-Syed M, Chawla A, Lederman R, Vethantham V, Saleem MA, Chander PN, Malhotra A, Singhal PC. (2015). Epigenetic modulation of human podocyte vitamin D receptor in HIV milieu. *J Mol Biol*, 427 (20): 3201–15. [PubMed: 26210663]
41. Salhan D, Husain M, Subrati A, Goyal R, Singh T, Rai P, Malhotra A, Singhal PC. (2012). HIV-induced kidney cell injury: role of ROS-induced downregulated vitamin D receptor. *Am J Physiol Renal Physiol*, 303 (4): F503–514. [PubMed: 22647636]
42. Ding H, Zhou D, Hao S, Zhou L, He W, Nie J, Hou FF, Liu Y. (2012). Sonic hedgehog signaling mediates epithelial-mesenchymal communication and promotes renal fibrosis. *J Am Soc Nephrol*, 23 (5): 801–813. [PubMed: 22302193]
43. Kramann R (2016). Hedgehog Gli signalling in kidney fibrosis. *Nephrol Dial Transplant*, (12): 1989–1995.
44. Fabian SL, Penchev RR, St-Jacques B, Rao AN, Sipilä P, West KA, McMahon AP, Humphreys BD. (2012). Hedgehog-Gli pathway activation during kidney fibrosis. *Am J Pathol*, 180 (4): 1441–1453. [PubMed: 22342522]
45. Arzumanyan A, Sambandam V, Clayton MM, Choi SS, Xie G, Diehl AM, Yu DY, Feitelson MA. (2012). Hedgehog signaling blockade delays hepatocarcinogenesis induced by hepatitis B virus X protein. *Cancer Res*, 72 (22): 5912–5920. [PubMed: 22986746]
46. Port RJ, Pinheiro-Maia S, Hu C, Arrand JR, Wei W, Young LS, Dawson CW. (2013). Epstein-Barr virus induction of the Hedgehog signalling pathway imposes a stem cell phenotype on human epithelial cells. *J Pathol*, 231 (3): 367–377. [PubMed: 23934731]
47. Deb Pal A, Banerjee S. (2015). Epstein-Barr virus latent membrane protein 2A mediated activation of Sonic Hedgehog pathway induces HLA class Ia downregulation in gastric cancer cells. *Virology*, 484: 22–32. [PubMed: 26057149]
48. Granato M, Zompetta C, Vescarelli E, Rizzello C, Cardi A, Valia S, Antonelli G, Marchese C, Torrisi MR, Faggioni A, Cirone M. (2016). HCV derived from sera of HCV-infected patients induces pro-fibrotic effects in human primary fibroblasts by activating GLI2. *Sci Rep*, 6: 30649. [PubMed: 27476557]
49. Samarzija I, Beard P. (2012). Hedgehog pathway regulators influence cervical cancer cell proliferation, survival and migration. *Biochem Biophys Res Commun*, 425 (1): 64–69. [PubMed: 22820185]

50. Lovisa S, Zeisberg M, Kalluri R. (2016). Partial epithelial-to-mesenchymal transition and other new mechanisms of kidney fibrosis. *Trends Endocrinol Metab*, 27 (10): 681–695. [PubMed: 27372267]
51. Fragiadaki M, Mason RM. (2011). Epithelial-mesenchymal transition in renal fibrosis - evidence for and against. *Int J Exp Pathol*, 92 (3): 143–150. [PubMed: 21554437]
52. Das JR, Gutkind JS, Ray PE. (2016). Circulating Fibroblast Growth Factor-2, HIV-Tat, and vascular endothelial cell growth factor-A in HIV-Infected children with renal disease activate Rho-A and Src in cultured renal endothelial cells. *PLoS One*, 11 (4): e0153837. [PubMed: 27097314]
53. Haque S, Lan X, Wen H, Lederman R, Chawla A, Attia M, Bongu RP, Husain M, Mikulak J, Saleem MA, Popik W, Malhotra A, Chander PN, Singhal PC. (2016). HIV Promotes NLRP3 Inflammasome Complex Activation in Murine HIV-Associated Nephropathy. *Am J Pathol*, 186 (2): 347–358. [PubMed: 26683666]
54. Abkhezr M, Dryer SE. (2014). Angiotensin II and canonical transient receptor potential-6 activation stimulate release of a signal transducer and activator of transcription 3-activating factor from mouse podocytes. *Mol Pharmacol*, 86 (2): 150–158. [PubMed: 24850910]
55. Xie X, Colberg-Poley AM, Das JR, Li J, Zhang A, Tang P, Jerebtsova M, Gutkind JS, Ray PE. (2014). The basic domain of HIV-tat transactivating protein is essential for its targeting to lipid rafts and regulating fibroblast growth factor-2 signaling in podocytes isolated from children with HIV-1-associated nephropathy. *J Am Soc Nephrol*, 25 (8): 1800–1813. [PubMed: 24578133]
56. Migliorini A, Angelotti ML, Mulay SR, Kulkarni OO, Demleitner J, Dietrich A, Sagrinati C, Ballerini L, Peired A, Shankland SJ, Liapis H, Romagnani P, Anders HJ. (2013). The antiviral cytokines IFN- α and IFN- β modulate parietal epithelial cells and promote podocyte loss: implications for IFN toxicity, viral glomerulonephritis, and glomerular regeneration. *Am J Pathol*, 183 (2): 431–440. [PubMed: 23747509]
57. Bruggeman LA, Drawz PE, Kahoud N, Lin K, Barisoni L, Nelson PJ. (2011). TNFR2 interposes the proliferative and NF- κ B-mediated inflammatory response by podocytes to TNF- α . *Lab Invest*, 91 (3): 413–425 [PubMed: 21221075]
58. Korgaonkar SN, Feng X, Ross MD, Lu TC, D'Agati V, Iyengar R, Klotman PE, He JC. (2008). HIV-1 upregulates VEGF in podocytes. *J Am Soc Nephrol*, 19 (5): 877–883. [PubMed: 18443354]

Highlights

- Podocytes/renal tissues in HIV mice revealed activation of hedgehog pathway.
- Hedgehog pathway-induced epithelial mesenchymal transition (EMT) in HIV-podocytes.
- Blockade of hedgehog pathway attenuated activation of EMT in HIV-podocytes.

Figure 1. HIV activates Hedgehog pathway in human podocyte.

A. Differentiated human podocytes were transduced with pseudo-typed HIV for different time periods, and RNAs were extracted for real time-PCR to examine the changes of hedgehog pathway molecules. * $p < 0.05$ when compared with the control of starting time (0 h). **B-E.** Differentiated human podocytes were transduced with pseudo-typed HIV for 48 h, and cell lysates were collected for Western blotting to examine the changes of hedgehog pathway molecules. * $p < 0.05$ when compared with the controls at starting time (0 h). Representative gels are displayed (**B**). **C-E**, Quantification of the expression of Shh (**C**), PTCH (**D**), Gli1 (**E**) in **B**, and the results (mean $\pm$ SD) represent three independent samples. * $p < 0.05$ compared with their respective control empty vector.

Figure 2. Hedgehog pathway is activated in Tg26 mice kidney.

A. RNA was extracted from the kidney of FVB/N control and Tg26 mice, and real time-PCR was performed to examine the changes of hedgehog pathway molecules. * $p < 0.05$ when compared with the FVB/N control mice. **B-D.** Protein lysate was prepared from the kidney of FVB/N control and Tg26 mice, and Western blotting was performed to examine the changes of hedgehog pathway molecules. Representative gels are displayed (**B**). **C-D**, Quantification of the expression of PTCH (**C**), Gli1 (**D**) in **B**, and the results (mean $\pm$ SD) represent three independent samples. * $p < 0.05$ when compared with the FVB/N control mice. **E-F.** Immunofluorescence staining was performed to detect the expression of PTCH and podocyte marker synaptopodin in the paraffin section from the kidneys of FVB/N control and Tg26 mice. Representative pictures are displayed (**E**). The average fluorescence intensity was measured per region of interest (ROI) for synaptopodin and PTCH. * $p < 0.05$ when compared with the FVB/N control mice.

Figure 3. Overexpression of Gli1 enhances EMT in podocyte.

Human podocytes were transfected with TAL2271-Gli1 plasmid or empty vector (control) and were then cultured fresh medium. Cells were harvested after 24 h, lysates were subjected to Western blotting to examine the changes of hedgehog pathway and EMT markers. **A.** Representative gels are displayed. **B.** Quantification of the expression of Gli1, Fibronectin, P-cadherin, vimentine, α -SMA, and Snail, in **A**, and the results (mean $\pm$ SD) represent three independent samples. * $p < 0.05$ when compared with the control vector.

Figure 4. Shh activates hedgehog pathway and induces EMT at different time points.

Human podocytes were treated with recombinant Shh (100 ng/ml) for 6, 12, 24, and 48 h, and the cell lysates were collected for Western blotting to examine the changes of hedgehog pathway and EMT markers. **A.** Representative gels are displayed. **B.** Quantification of the expression of Gli1, vimentine, and α -SMA in **A**, and the bar graphs (mean $\pm$ SD) represent three independent samples. * $p < 0.05$ when compared with the control of starting time (0 h).

Figure 5. Shh activates hedgehog pathway and induces EMT in a dose-dependent manner. Human podocytes were treated with 5, 10, 50, or 100 ng/ml of recombinant Shh for 48 h, then the cell lysates were subjected to Western blotting to examine the changes of hedgehog pathway and EMT markers. **A.** Representative gels are displayed. **B.** Quantification of the expression of PTCH, Gli1, P-cadherin, vimentine, and α-SMA in **A**, and the bar graphs (mean ± SD) represent three independent samples. * $p < 0.05$ when compared with the control (0 ng/ml).

Figure 6. Blocking hedgehog pathway attenuates HIV-induced EMT in podocyte.

Human podocytes were transduced with pseudo-typed HIV for 3 h, and cultured in the medium containing hedgehog inhibitor Gant 58 (10 μ M) for 48 h. Cell lysates were collected for Western blotting to examine the changes of EMT markers. **A**. Representative gels are displayed. **B**. Quantification of the expression of fibronectin, vimentine, and α -SMA in **A**, and the bar graphs (mean $\pm$ SD) represent three independent samples. * $p < 0.05$ when compared with the vector control without the addition of Gant 58; while # $p < 0.05$ when compared with the HIV transduction without the addition of Gant 58.

Figure 7. Shh increases podocyte permeability.

Human podocytes were cultured on the membrane of transwell with 3 μ m pores, and were treated with 100 ng/ml Shh for 48 h. Then the permeability of the membrane was analyzed by testing the albumin influx. * $p < 0.05$.

Figure 8. Hedgehog pathway inhibitor decreases HIV-increased podocyte permeability. Human podocytes were cultured on the membrane of transwell with 3 μ m pores for 7 days. Pseudo-typed HIV was then transduced for 3 h, and then hedgehog inhibitor Gant 58 (10 μ M) was added. After another 3 days, the permeability of the membrane was determined by testing the albumin influx. * $p < 0.05$.

Figure 9. Schematic for the role of hedgehog pathway in HIV-induced podocyte EMT.

HIV infection increases the production of hedgehog homologues (Dhh, Ihh, and Shh) in podocyte. The hedgehogs were released out of the cell, and bind to their receptor PTCH in the plasma membrane, which further leads to the de-repression of smoothened (SMO). The activated SMO changes its position from an intracellular vesicle to the cell membrane, where it activates the Gli family of transduction factors such as Gli1. The activated Gli proteins subsequently enter the nucleus and start the transcription of their object genes, causing the epithelial mesenchymal transition (EMT) of podocyte. The consequence of podocyte EMT is to increase its permeability and to decrease its filtration function.

Table 1.

Primers for real time-PCR

Gene	Forward primer	Reverse Primer
Human Dhh	CGTCCACGTGTCGGTCAAAG	AGGCCACTCGGTCTCCACAG
Human Ihh	CGGACGCTATGAAGGCAAGAT	CACCGAGATAGCCAGCGAGTT
Human Shh	CGCACGGGGACAGCTCGGAAGT	CTGCGCGGCCCTCGTAGTGC
Human Gli1	TCTCAAAGTGGGAGGCACAA	CCCTTAGGAAATGCGATCTG
Human PTCH	TGGGATTAAAAGCAGCGAAC	TCTCCAATCTTCTGGCGAGT
Human GAPDH	CCATGGAGAAGGCTGGGC	CAAAGTTGTCATGGATGA
Mouse Dhh	CTACGAGTCCCGCAACCACA	TCCAGGAAGAGCAGCACTGG
Mouse Ihh	TTCAAAGAGCTCACCCCAAC	GTCACCCGCAGTTTCACACC
Mouse Shh	GTGGAAGCAGGTTTCGACTG	CGGGACGTAAGTCCTTCACC
Mouse Gli1	AGACCAGCAGCTGCACTGAA	TGGCAGGTTGCACGTGGTC
Mouse PTCH	ACGGTGCTGTGTCCACTCTG	AGAGGACAGGCAGCAGAACC
Mouse GAPDH	CGTCTTCACCACCATGGAGA	CGGCCATCACGCCACAGTTT