Cellular Signalling xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Cellular Signalling

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

Down-regulation of Wt1 activates Wnt/β-catenin signaling through modulating endocytic route of LRP6 in podocyte dysfunction in vitro

Zhou Jing, Yuan Wei-jie *, Zhu-ge Yi-feng

Department of Nephrology, Shanghai First People's Hospital, Shanghai Jiao Tong University, Shanghai, People's Republic of China

ARTICLE INFO

Article history:
Received 24 May 2015
Accepted 27 May 2015
Available online xxxx

Keywords:
Wt1
Wnt/β-catenin signaling
LRP6
Podocyte dysfunction

ABSTRACT

Podocyte dysfunction plays important roles in the pathogenesis of chronic kidney disease, and Wt1 has long been considered to be a marker of podocyte, whereas its roles and mechanisms in podocyte injury are still unclear though Wt1 mutations are reported to be involved in the development of glomerular disease in human and mice. Here we show that down-regulation of Wt1 could induce podocyte dysfunction and apoptosis through activating Wnt/β-catenin signaling. Podocytes treated with adriamycin demonstrated decreased expression of Wt1, coupled with activated Wnt/β-catenin signaling in vitro. Reduced expression of Wt1 in podocytes transfected with Wt1 siRNA is correlated with activated Wnt/β-catenin signaling, increased podocyte apoptosis, as well as suppressed expression of nephrin. Blockade of Wnt/β-catenin signaling with Dickkopf-1 ameliorated podocyte injury and apoptosis induced by Wt1 siRNA. We also found that membrane LRP6 was increased dramatically in podocytes transfected with Wt1 siRNA compared with control siRNA, while no significant change was found in total LRP6. Caveolin- and clathrin-dependent endocytosis were both involved in the regulation of β-catenin signaling. And we found that down-regulation of Wt1 in podocytes mediates activation of Wnt/β-catenin signaling by recruiting LRP6 to the caveolin-mediated endocytosis route, thereby sequestering it from clathrin-dependent endocytosis. As a result, we concluded that Wt1 expression levels in podocytes regulate Wnt/β-catenin signaling through modulating the endocytic fate of LRP6, and this indicates a potential target for the therapy of CKD.

1. Introduction

Chronic kidney disease (CKD), one of the leading factors determining human morbidity and mortality, has become a common problem affecting human health, and it will inevitably progress into end-stage renal disease when renal replacement therapy including hemodialysis and peritoneal dialysis is necessary [1]. The prevalence and incidence of CKD have also posed an enormous challenge for health resources worldwide [2,3]. As a result, it is increasingly important that we must study the kidney, not just as they develop, but also as they age and respond to injurious stimuli.

Glomerular filtration barrier is essential for the maintenance of normal renal function, and it is composed of three layers: the fenestrated glomerular endothelial cells, podocytes, and glomerular basement membrane [4]. Increasing evidence indicates that podocyte dysfunction, described as foot process effacement and podocyte depletion, play critical roles in the pathogenesis of CKD [5–7]. However, the molecular mechanisms underlying podocyte dysfunction remain poorly understood. Besides, podocytes are highly differentiated and specialized cells with few ability to regenerate [8]. Hence, it is of great significance to clarify the molecular mechanism underlying podocyte dysfunction, thus providing new targets for the therapy of CKD.

Wnt/β-catenin signaling, also called canonical Wnt signaling, was first discovered in Drosophila. It is a conserved signaling pathway and involved in diverse physiological and pathological processes ranging from embryogenesis to development of disease [9–11]. Over the years, Wnt/β-catenin signaling is getting more and more attention in the field of nephrology as a result of its effects in kidney development and renal injury. Reportedly, activated Wnt/β-catenin signaling could induce podocyte dysfunction and proteinuria [12–14].

The human *Wnt* gene encodes a large family of ligand molecules composed of 19 secreted lipid-modified proteins. Upon binding to the cell membrane receptor Frizzled and its co-receptors LRP5/6 (LRP, low density lipoprotein receptor-related protein), Wnts induce a series of downstream gene expression. In the absence of Wnt ligand, β-catenin is phosphorylated by binding to a multiprotein complex consisting of the scaffold proteins axis inhibition protein (Axin), the tumor suppressor adenomatosis polyposis coli (APC), and glycogen synthase kinase-

Abbreviations: CKD, Chronic kidney disease; LRP, Low density lipoprotein receptor-related protein; Axin, Axis inhibition protein; APC, Adenomatosis polyposis coli; GSK-3β, Glycogen synthase kinase-3β; TCF/LEF, T-cell factor/lymphocyte enhancer factor; Wt1, Wilms' tumor suppressor gene; ADR, Adriamycin; MDC, Monodansyl-cadaverine.

* Corresponding author at: Department of Nephrology, Shanghai First People's Hospital, Shanghai Jiao Tong University, Shanghai, People's Republic of China, 100 HaiNing Road, Shanghai 200080, People's Republic of China. Fax: +86 21 55061090.
E-mail addresses: ywj4169@163.com, zhou_jing_2010@126.com (Y. Wei-jie).

http://dx.doi.org/10.1016/j.cellsig.2015.05.018
0898-6568/

3β (GSK-3β). Phosphorylated β-catenin can be easily degraded by proteasome, thus blocking downstream gene expression. With the presence of Wnt ligand, β-catenin is stabilized, accumulated in the cytoplasm, translocated into nucleus and interacted with T-cell factor/lymphocyte enhancer factor (TCF/LEF) to induce Wnt responsive gene expression [15–17]. As a result, β-catenin, especially active β-catenin (or unphosphorylated β-catenin), could be regarded as a switch controlling "on and off" of Wnt/β-catenin signaling.

The Wilms' tumor suppressor gene (Wt1), well-known for its role in Wilms' tumor, is essential for kidney development. Wt1 knocked-out mice have no kidney because the nephric duct fail to grow out and apoptosis of the metanephric blastema [18,19]. Wt1 mutations is also reported to be involved in the development of glomerular disease in human and mice [20,21]. In the glomerular of adult kidney, Wt1 expression is limited to podocytes, and it has long been used as a marker for the location of podocytes [22]. However, the role of Wt1 and its mechanisms in the maintenance of podocyte biology is poorly understood. Reportedly, mutations in Wt1 and consequent activation of Wnt/β-catenin signaling have been found in the development of Wilms' tumor [23,24]. We suppose that reduced expression of Wt1 in podocytes after injurious stimuli may induce podocyte dysfunction through activating Wnt/β-catenin signaling. As a result, our study is designed aiming to explore the effect of down-regulation of Wt1 in podocyte dysfunction and its related mechanism.

In the current study, we demonstrated that down-regulation of Wt1 could activate Wnt/β-catenin signaling, induce podocyte dysfunction and apoptosis. We also found that loss of Wt1 not only mediated internalization of LRP6 based on increased membrane LRP6 expression in podocytes transfected with Wt1 siRNA but also activates Wnt/β-catenin signaling by recruiting LRP6 to the caveolin-mediated endocytic route, thus sequestering it from clathrin-dependent endocytosis. In detail, with the presence of Wt1, LRP6 interacts with clathrin, while in the absence of Wt1, LRP6 is sequestered by caveolin. Collectively, our results suggest that down-regulation of Wt1 in podocytes regulate Wnt/β-catenin signaling through modulating the endocytic fate of LRP6, and this indicates a potential target for the therapy of CKD.

2. Materials and methods

2.1. Cell culture and treatment

The conditionally immortalized mouse podocyte cells (MPCs) were kindly provided by Dr. Peter Mundel (Mount Sinai School of Medicine, New York, NY), as described previously [25]. MPCs were cultured at 33 °C for propagation in RPMI 1640 medium supplemented with 10% FBS and 10 U/mL recombinant mouse IFN-r. To induce differentiation, MPCs were then cultured in RPMI 1640 medium supplemented with 10% FBS at 37 °C in the absence of IFN-r. Differentiated podocytes were treated with adriamycin(ADR) at different time points with different dosages as indicated. In order to inhibit Wnt/β-catenin signaling in vitro, podocytes were also treated with recombinant mouse DKK1 protein as indicated.

2.2. Western blotting analysis

Cultured mouse podocytes were lysed in RIPA buffer. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and then electrophoretically transferred to a nitrocellulose membrane (Millipore). Membrane protein was extracted using the Membrane and Cytosol Protein Extraction Kit (Beyotime technology, Shanghai, China). As for the housekeeping gene for the analysis of membrane protein LRP6, we applied β-actin as loading control based on the article written by Nakayama et al. [26], who used tubulin as loading control for the analysis of membrane protein. The primary antibodies used were as follows: anti-dephosphorylated, active β-catenin (05-665, Upstate), anti-β-catenin(ab6302, Abcam), anti-LRP6 (sc-25317, Santa Cruz), anti-Nephrin (ab58968, Abcam), anti-PAI-1(ab66705, Abcam), anti-Snail (ab180714, Abcam), anti-Caveolin-1 (3267, CST), anti-Clathrin (4796, CST), anti-GAPDH (5174, CST), anti-Actin (1854-1, Epitmics).

2.3. RNA extraction and real-time RT-PCR

For the analysis of mRNA relative expression levels, total RNA were isolated, and RT-PCR were carried out based on routine procedures. Briefly, first-strand cDNA was synthesized using a reverse transcription system kit based on the instructions of the manufacturer (Thermo). Real-time RT-PCR was performed on ABI Prism 7300, and quantification of relative mRNA levels was calculated using the $2^{-\triangle\triangle Ct}$ method. Sequences of the primers were as follows: Wt1,5′-CTA-CCA-TCC-GCA-ACC-AAG- 3′ (sense) and 5′-GGG-TCC-TCG-TGT-TTG-AAG-3′ (antisense); β-catenin, 5′-TCA-CGC-AAG-AGC-AAG-TAG-3′ (sense) and 5′-CTG-GAC-ATT-AGT-GGG-ATG-AG-3′ (antisense); Nephrin, 5′-GGA-CCC-ACA-CTA-CTA-CTC-3′ (sense) and 5′-CTC-TCC-ACC-TCG-TCA-TAC-3′ (antisense); LRP6, 5′-TCC-TGG-TCT-TCC-ACT-CTT-C-3′ (sense) and 5′-GGT-CCT-GTT-GTC-AGC-ATT-C-3′ (antisense); PAI-1, 5′-TTA-CTG-GGT-GAG-TCA-GAG-3′ (sense) and 5′-GGC-CTG-CTA-GGA-AAT-TAC-3′ (antisense); Snail, 5′-TTT-GCT-GAC-CGC-TCC-AAC-3′ (sense) and 5′-GGG-TAC-AAA-GGC-ACT-CCA-TCA-3′ (antisense); GAPDH, 5′-ATC-ACT-GCC-ACC-CAG-AAG-3′ (sense) and 5′-TCC-ACG-ACG-GAC-ACA-TTG-3′ (antisense).

2.4. Immunofluorescence staining

Podocytes cultured on glass slides were fixed in 4% paraformaldehyde for 15 minutes at room temperature and washed with PBS containing 0.05% Tween 20, then these slides were blocked with 5% bovine serum albumin for 2 hours at room temperature. The prepared slides were stained with LRP6 antibody overnight at 4 °C (anti-LRP6, sc-25317, Santa cruz). After washing, the slides were then stained with FITC-conjugated secondary antibody (Beyotime biotechnology). Slides were visualized using microscopy (Olympus CX 41), and photographed with a digital camera (Nikon D5100).

2.5. Preparation of siRNA and transient transfection

The Wt1 siRNA was synthesized by JRDUN biotechnology (Shanghai, China). The sequence of mouse Wt1 siRNA was 5′-GGC-GAU-UGU-UAA-AGC-UCA-UUU-3′, and the control siRNA was 5′-UUG-UAC-UAC-ACA-AAA-GUA-CUG-3′. A total of about 5 × 105 cells were seeded into each well of a six-well cell culture plate. And the Wt1, siRNA and control siRNA were transfected into podocytes by Lipofectamine 2000 transfection reagent following the instructions (Invitrogen).

2.6. Immunoprecipitation

Immunoprecipitation was used to study whether LRP6 was internalized through the caveolin- or clathrin-dependent endocytosis when Wt1 was down-regulated in podocytes. Cell lysates were immunoprecipitated with anti-Caveolin-1 (3267, CST), anti-Clathrin (4796, CST), and protein A/G plus agarose (sc-2003, Santa cruz). The precipitated complexes were washed with lysis buffer, then immunoblotting with anti-LRP6 antibody (ab134146, Abcam).

2.7. Statistical analyses

All data are presented as mean ± SEM with at least three separate repetitions. Statistical analysis of the data was performed using the SPSS statistical software package (standard version 17.0; SPSS).

Please cite this article as: Z. Jing, et al., Down-regulation of Wt1 activates Wnt/β-catenin signaling through modulating endocytic route of LRP6 in podocyte dysfunction in v..., Cell. Signal. (2015), http://dx.doi.org/10.1016/j.cellsig.2015.05.018