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Original Article

Wnt/β-catenin pathway involved in podocyte injury is regulated by UCH-L1

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Abstract: The Wnt/β-catenin signaling pathway is re-activated in podocytes after injury *in vivo* and *in vitro,* further triggering podocyte dysfunction and albuminuria. However, the mechanisms underlying Wnt/β-catenin regulation during podocyte injury remain to be established. In this study, we investigated the activation of Wnt/β-catenin in podocytes using the adriamycin (ADR) rat nephropathy model and the regulatory role of Ubiquitin C-terminal hydrolase-L1 (UCH-L1) on β-catenin in cultured murine podocytes under conditions of inflammatory stimulation. Our results showed that UCH-L1 combines with β-catenin in podocytes and its abnormal expression is related to activation of β-catenin. Based on the collective data, we propose that β-catenin is a downstream molecule of UCH-L1, and interactions between these proteins play an important role in the development of morphological changes and dysfunction of podocytes following injury.

Keywords: β-catenin, podocyte injury, UCH-L1, proteinuria

Introduction

Podocytes, an important component of the glomerular filtration membrane, are susceptible to injury, often resulting in proteinuria and glomerular dysfunction. The Wnt/β-catenin signaling pathway is involved in the development of juvenile kidney and pathogenesis of renal diseases. The canonical Wnt pathway in which β-catenin translocation from the cytoplasm to nucleus activates downstream factors is reported to be indispensable for renal epithelial cell lineage specification and mesenchymal-epithelial transition during metanephric kidney development [1-3], while in the adult kidney, Wnt signaling is silenced [1, 4]. In many acquired proteinuric kidney diseases [5], Wnt/β-catenin signaling is re-activated in podocytes after injury *in vivo* and *in vitro* [5, 6], further triggering podocyte dysfunction and albuminuria. Downregulation of Wnt/β-catenin, either via targeted deletion of the β-catenin gene in podocytes or injection of the specific inhibitor, paricalcitol, ameliorates proteinuria induced by adriamycin in rat models [5, 7, 8]. However, the mechanisms underlying Wnt/β-catenin regulation during podocyte injury remain to be clarified.

β-Catenin is degraded to a very low level in the cytoplasm via the ubiquitin-proteasome system to maintain dynamic homeostasis in mature tissues [9]. Deubiquitination, the reverse process of ubiquitination, has been implicated in the regulation of β-catenin [10]. Ubiquitin C-terminal hydrolase-L1 (UCH-L1), also known as protein gene product 9.5 (PGP 9.5), is an important member of the deubiquitinase family [11] involved in podocyte injury and proteinuria. A previous study by our group demonstrated that UCH-L1 is upregulated in podocytes during the course of diverse immunocomplex-mediated nephritis, including acute proliferative glomerulonephritis (APGN), lupus nephritis (LN), membranous nephropathy (MN) and IgA nephropathy (IgAN) [12]. Increase in UCH-L1 levels resulted in polyubiquitin accumulation, proteasome inhibition and disease aggravation in experimental models of membranous nephritis [13].

Abnormalities in deubiquitination enzymes may influence the metabolism of important cellular proteins and cause disorders in cell function [14-17]. A recent report by Bheda and co-workers demonstrated that UCH-L1 combines with β-catenin to form a complex, resulting in increased activity of β-catenin [18], supporting a role of UCH-L1 as a regulator of Wnt/β-catenin activation. Accordingly, we hypothesized that abnormal expression of UCH-L1 promotes podocyte injury and kidney disease via regulation of the Wnt/β-catenin signaling pathway.

In this study, we detected abnormal expression of UCH-L1 and activation of β-catenin in podocytes of the adriamycin (ADR)-induced rat nephrosis model and examined whether UCH-L1 regulates β-catenin degradation in cultured murine podocytes under conditions of inflammatory stimulation. β-Catenin bound directly to and upregulated UCH-L1, resulting in increased expression of β-catenin in podocytes treated with cytokines, which may constitute a key step in the pathogenesis of podocyte injury.

Materials and methods

Rat model of ADR-induced nephrosis

ADR-induced nephrosis rat models were generated as described earlier [19]. In brief, 24 male Sprague Dawley rats, divided randomly into two groups (6 rats assigned to the control group and the other 18 rats to the ADR nephrosis group), received left unilateral nephrectomy under sodium pentobarbital (40 mg/kg intraperitoneally) anesthesia. One week later, ADR (Haizheng Co. Zhejiang, China) dissolved in 0.9% (w/v) NaCl was injected i.v. (5 mg/kg body weight) into rats of the ADR nephrosis group (twice with an interval of 7 days), while control rats received the same volume of 0.9% (w/v) NaCl. At weeks 2, 5 and 10 following the second injection, rats were anesthetized and sacrificed. Urine samples were collected during the next 24 hours (h). The excreted urine proteins were assayed by the Labway Clinical Laboratory (Shanghai, China). Kidney tissues were prepared for measurement of protein and pathological and immunostaining analyses.

The study protocol was approved by the Animal Experiment Ethics Committee of Shanghai Medical College, Fudan University, under the reference number 20100223. All experiments were performed in accordance with the approved guidelines of Shanghai Medical College, Fudan University.

Immunohistochemical staining

Deparaffinized kidney sections (4 μm thick) obtained from ADR-induced rats were treated with 0.3% H_2O_2 and boiled in a microwave(10 mM citrate buffer, pH 6.1). Sections were probed with primary antibodies against β-catenin (1:50, BD, NJ, USA) or UCH-L1 (1:200, Millipore, Eschborn, Germany) for 1 h at 37°C and overnight at 4°C respectively followed by incubation with biotinylated secondary antibodies (1 h, 37°C). DAB was used as the chromogen and hematoxylin as the nuclear counterstain. The primary antibody was replaced with PBS in the negative control.

Immunofluorescence staining

Studies were performed on selected rat deparaffinized kidney sections as above, using the same pretreatment protocol. Sections were probed with primary antibodies against podocin (1:100, Proteintech, IL, USA) for 1 h at 37°C and overnight at 4°C, and visualized using Cy-3-conjugated secondary antibodies.

Cell culture and treatments

Conditionally immortalized mouse podocyte cells (MPC5) were cultured as described previously [20]. Briefly, MPC-5 cells were maintained under growth-permissive conditions at 33°C and 5% CO_2 in RPMI-1640 with 10% (vol/vol) fetal bovine serum, and 50-10 U/ml IFN-γ (Sigma) used to maintain MPC-5 cells in proliferative conditions. To induce differentiation, podocytes were shifted to non-permissive conditions at 37°C without IFN-γ for 14 days. After serum starvation for 24 h, podocytes were treated with 20 ng/ml TNF-α (Sigma), 0.1 μg/ml ADR (Haizheng), and 10 ng/ml IL-1β (Sigma) for 45 min, 90 min, 3 h, 6 h, and 9 h. Treatment with the proteasome inhibitor, MG132 (2.5 mM), for 48 h was performed under similar conditions.

Immunofluorescence staining for F-actin

Immunofluorescence staining for F-actin was performed as described previously [21]. Briefly, mature podocytes (50%-60% density) were cultured on sterile cover slips, and subjected to

inflammatory stimulation for 9 h as described above. We used 4% polyformaldehyde-PBS as the stationary liquid for 10 min, and cells were treated with 0.1% Triton X-100-PBS for 5 min. Non-specific staining was blocked with 3% BSA for 20 min at room temperature. Cells were incubated with fluorescent phallotoxins for visualization of F-actin (1.5 IU/200 ml, Invitrogen) at 37°C for 30 min. Cover slips were examined under a fluorescence microscope (Nikon, Japan).

Western blot

Western blot was performed as described previously [12]. Primary antibodies for β-catenin (1:2000, BD, NJ, USA), active β-catenin (1:200, Millipore), UCH-L1 (1:2000, Millipore) and β-actin (1:1000, Sigma) were used. Immunoblots were developed with a peroxidase-conjugated secondary antibody (1:1000, Proteintech) and a chemiluminescence kit (ECL Plus; ThermoFisher, MA, USA). Each experiment was repeated at least three times.

Quantitative RT-PCR

Total RNA was extracted from renal cortical tissue using TRIzol® reagent (Sangon, Dalian, China). The first strand of complementary DNA was synthesized using 500 ng RNA in 10 μl reaction buffer via reverse transcription using PrimeScript™ RT Master Mix (Perfect Real Time) (Takara Bio Inc., Shiga, Japan), and subjected to qPCR with primer pairs specific for rat β-catenin (5'-ACAGGACTACAAGAAACGGC-3', 5'-GCGATATCCAAGGGCTTCTC-3'), rat UCH-L1 (5'-CTCAGTGCCATCTCCTGC-3', 5'-GTTCCCGATGGTCTGCTTC-3'), rat β-actin (5'-CACTTTCTACAATGAGCTGCG-3', 5'-CTGGATGGCTACGTACATGG-3'), mouse β-catenin (5'-GCTATTCCACGACTAGTTCAGC-3', 5'-AGCTCCAGTACACCCTTCTAC-3'), mouse UCH-L1 (5'-AAGCAGACCATCGGAAACTC-3', 5'-GCTCTATCTTCGGGGGACA-3') and mouse β-actin (5'-CATCCGTAAAGACCTCTATGCCAAC-3', 5'-ATGGAGCCACCGATCCACA-3'). Expression levels of β-catenin and UCH-L1 were determined following normalization to β-actin in the same PCR run. The specificity of amplification was confirmed using melt curve analysis.

Plasmid transfection and RNA interference

MPC5 cells were washed with Opti-MEMI (Gibco) reduced serum medium and transiently transfected with UCH-L1 plasmid or empty vector and siRNA/UCH-L1 or control siRNA (Biomics CO, Shanghai, China) using Lipofectamine® 2000 (Invitrogen), according to the manufacturer's instructions, for 60 h. Levels of β-catenin and UCH-L1 proteins were assessed via western blot.

Immunoprecipitation

Immunoprecipitation was performed according to a previously described protocol [22]. Briefly, cells were harvested and lysed in TNT buffer (20 mM Tris–HCl, pH 7.4, 200 mM NaCl, 1% Triton X-100, 10 mM iodoacetamide, 1 mM phenylmethyl sulfonyl fluoride, and 1% aprotinin). Identical amounts (1.5 mg protein) of precleared cell lysates were immunoprecipitated with 10 μg antibodies against either β-catenin or UCH-L1 by incubation at 4°C for 12 h after adjusting the volumes to 0.5 ml with NET buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P(NP)-40, 1 mM ethylene diamine tetra-acetic acid, 0.25% gelatin, 0.02% sodium azide, 1 mM PMSF, and 1% aprotinin). Immune complexes were precipitated with Protein-A Sepharose CL-4B and washed three times with TNT buffer, once with NET buffer, and once with PBS. Immunoprecipitated proteins were eluted with 5×SDS loading buffer and resolved using standard SDS-PAGE. Subsequent steps were similar as those for western blot.

Statistical analysis

All statistical analyses were performed using SPSS software. Values are expressed as means ± SD. Paired means were analyzed using Student's t-test. Statistical significance was defined as $P<0.05$, and high significance set at $P<0.01$.

Results

Activation of the Wnt/β-catenin pathway in podocytes after injury in vivo

We initially examined the Wnt/β-catenin pathway in the rat ADR model of podocyte injury and proteinuria. H & E staining showed that glomerular structures were normal in the control group, and very slight change in the 2-week (2 W) group, while a small increase in mesangial matrix was observed in the 5-week (5 W) group. Part of the capillary loop showed segmental sclerosis and adhered to the glomerular capsule in the 10-week (10 W) group (**Figure 1A**).

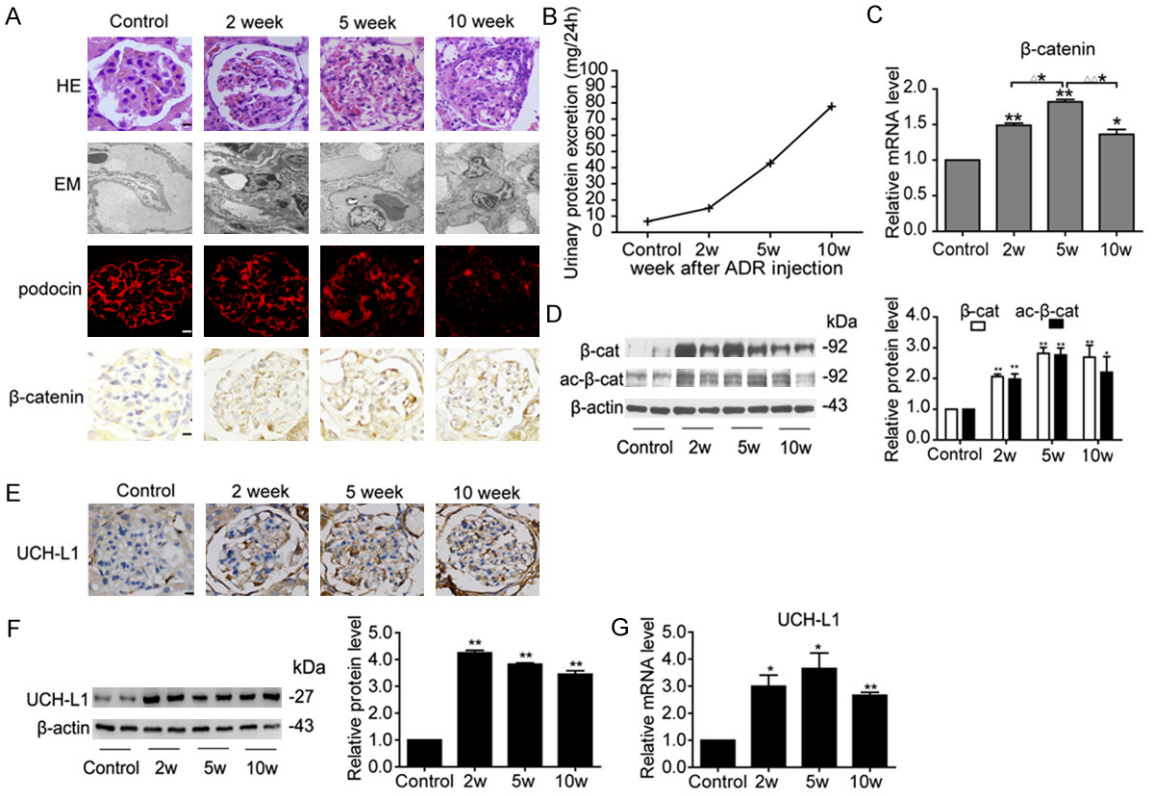

Figure 1. Activation of the Wnt/β-catenin pathway and upregulation of UCH-L1 in podocytes of the rat ADR nephrosis model. A. H & E staining, EM images of glomeruli, immunofluorescence staining for podocyte-specific markers, podocin and immunohistochemical staining for β-catenin from different groups of ADR nephrosis rats. Scale bars represent 50 μm. B. The 24 h proteinuria levels in different groups of rat ADR nephrosis. $^{*}P<0.05$, 2 weeks (w), 5 w or 10 w versus control (n=6). C. qRT-PCR measurement of β-catenin mRNA levels measured in glomeruli of the kidney cortex of rat ADR nephrosis groups ($^{*}P<0.05$, $^{**}P<0.01$ versus control, except where noted, $^{\Delta,*}P<0.05$, $^{\Delta\Delta,*}P<0.01$, n=3). D. Western blot for β-catenin and activated β-catenin in glomeruli of the kidney cortex of rat ADR nephrosis groups and the corresponding statistical pictograms obtained via densitometry analysis (cropped blots for different targets were run under the same experimental conditions, $^{*}P<0.05$, $^{**}P<0.01$ versus control, n=2 for control and 2, 5 and 10 w). E. Immunohistochemical staining for UCH-L1 from different groups of ADR nephrosis rats. Scale bars represent 50 μm. F. Western blot for UCH-L1 in glomeruli of kidney cortex from rat ADR nephrosis groups and the corresponding statistical pictograms obtained via densitometry (cropped blots for different targets were run under the same experimental conditions, $^{**}P<0.01$ versus control, n=2 for control and 2, 5, 10 w). G. qRT-PCR measurement of UCH-L1 mRNA levels measured in glomeruli of the kidney cortex of rat ADR nephrosis groups ($^{*}P<0.05$, $^{**}P<0.01$ versus control, n=3).

Electron microscopy (EM) data revealed an increase in effacements of foot processes in glomerular capillary loops from 2 W to 10 W. Immunofluorescence staining illustrated a gradually increasing reduction in podocin levelsin rat nephrosis from 2 W to 10 W. Podocin was distributed as a whole linear pattern in glomeruli in the control group, less positive in the ADR group at 2 W, and displayed agranular pattern at 5 W and 10 W. β-Catenin activation was detected in podocytes of the rat ADR model. Increased cytoplasmic and nuclear β-catenin staining were observed in glomeruli and primarily distributed at the periphery of the glomerular capillary loop where podocytes are located. From 5 W and 10 W, proteinuria was increased in ADR-induced nephrosis rats (**Figure 1B**, $P<0.05$).

Similarly, qPCR analyses disclosed increased levels of β-catenin mRNA in glomeruli of the kidney cortex of rat ADR models (**Figure 1C**). Western blot data consistently showed an increase in both β-catenin and active β-catenin (unphosphorylated β-catenin) protein levels (**Figure 1D**).

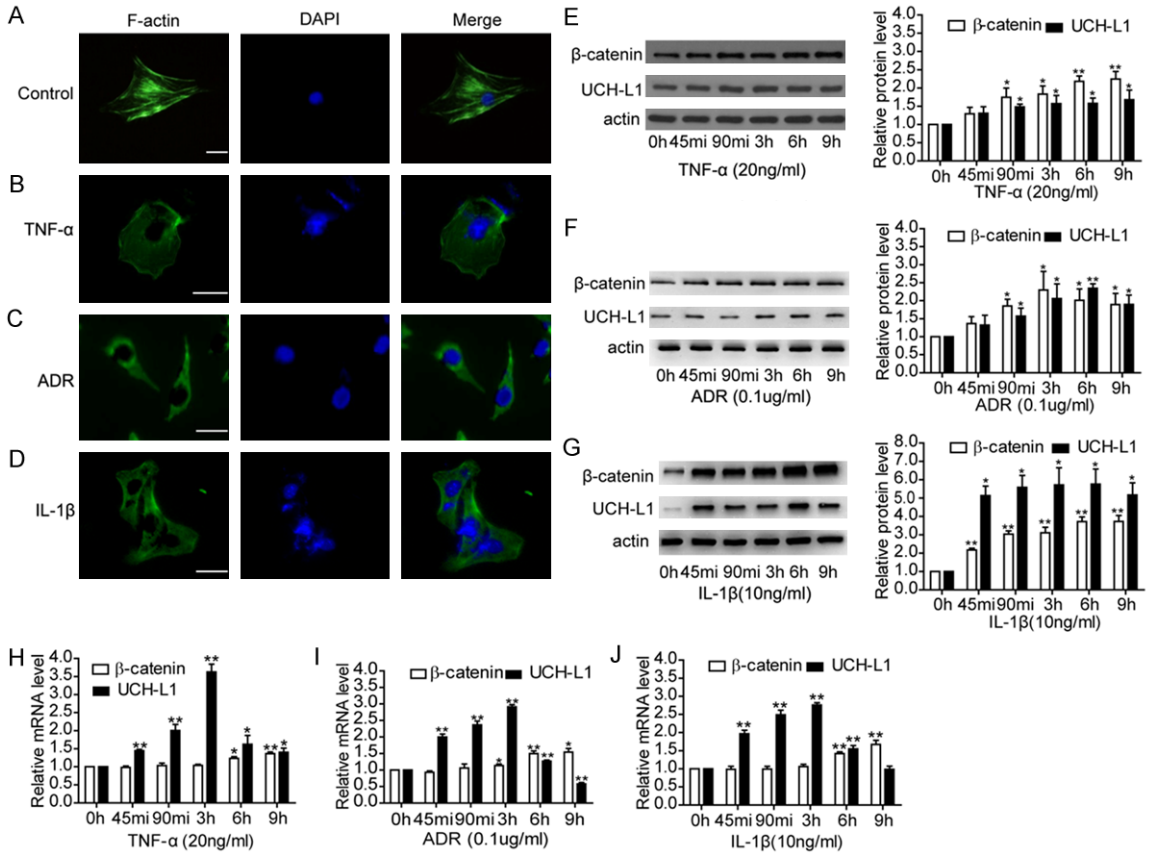

Figure 2. Upregulation of UCH-L1 and β-catenin in cultured podocytes under conditions of inflammatory stimulation. (A-D) Morphologic changes in podocytes treated with inflammatory stimulation. Actin fibers displayed in podocytes with no inflammatory stimulation (control) (A), TNF-α (B), ADR (C) and IL-1β (D). (E-G) Western blot for UCH-L1 and β-catenin in cultured podocytes treated with TNF-α (E), ADR (F) and IL-1β (G) and the corresponding statistical pictograms via densitometry (the cropped blots for different targets were run under the same experimental conditions, **P*<0.05, ***P*<0.01 versus 0h). (H-J) qRT-PCR measurement of UCH-L1 and β-catenin mRNA levels in cultured podocytes treated with TNF-α (H), ADR (I) and IL-1β (J) (**P*<0.05, ***P*<0.01 versus 0h). mi, minutes.

Upregulation of UCH-L1 in podocytes after ADR injury in vivo

Expression of UCH-L1 was also detected in the rat ADR nephrosis model. From 2 W to 10 W, immunohistochemical staining revealed an increase in cytoplasmic UCH-L1-positive cells in the glomeruli, which were primarily distributed at the periphery of the glomerular capillary loop where podocytes are located (**Figure 1E**). Both UCH-L1 protein (**Figure 1F**) and mRNA (**Figure 1G**) levels were increased in glomeruli of the kidney cortex of rat ADR model rats.

Upregulation of UCH-L1 and β-catenin in cultured podocytes under conditions of inflammatory stimulation

We further examined the protein levels of UCH-L1 and β-catenin via western blot in cultured mature podocytes treated with cytokines, including TNF-α, ADR and IL-1β.

Firstly, the structure of the cytoskeleton with F-actin was detected in cultured podocytes treated with TNF-α, ADR and IL-1β. Podocytes displayed a polygonal structure, and actin fibers were arranged in orderly radial and fasciculate patterns in the cytoplasm in the control group (**Figure 2A**). However, podocyte morphology was significantly altered in cultured podocytes treated with TNF-α, ADR and IL-1β (**Figure 2B-D**), clearly indicating the cells damage by inflammatory stimuli.

UCH-L1 expression was further analyzed via western blot. Protein levels were increased with time in podocytes receiving inflammatory stimulation at 45 min, 90 min, 3 h and 6 h, and

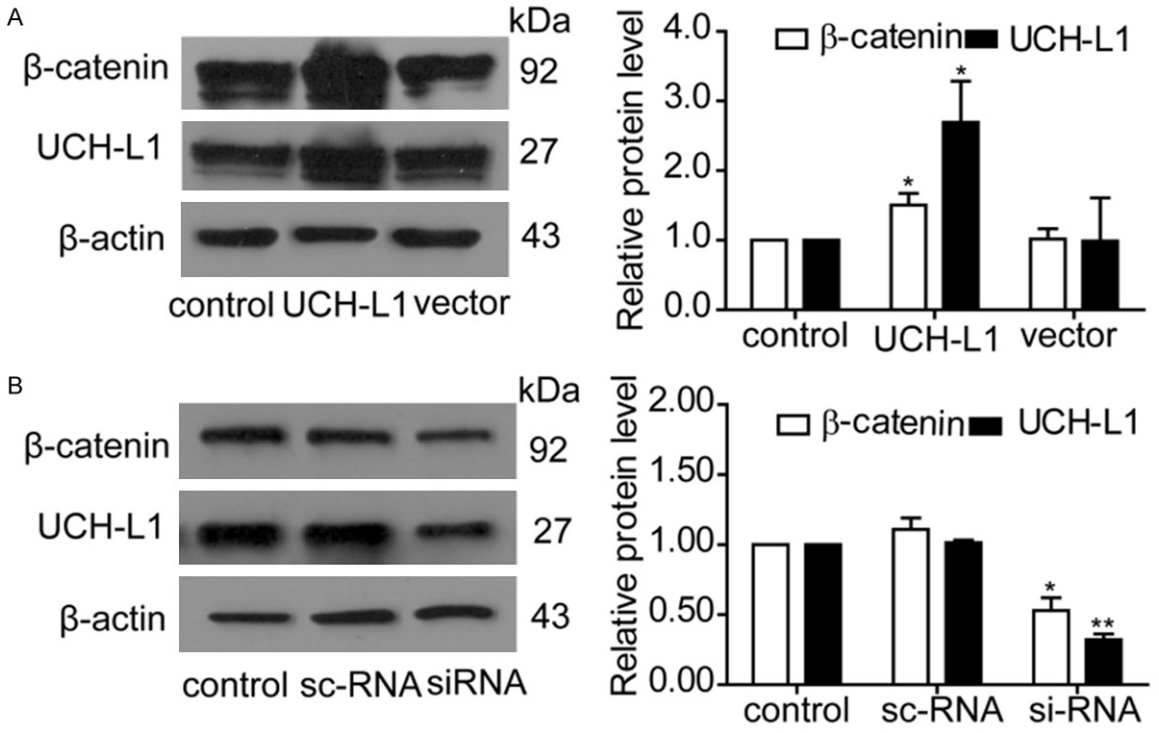

Figure 3. UCH-L1 upregulates β-catenin protein expression *in vitro*. A. Western blot for UCH-L1 and β-catenin in cultured podocytes transfected with UCH-L1-expressing or empty vector and the corresponding statistical pictograms obtained via densitometry. B. Western blot for UCH-L1 and β-catenin protein in cultured podocytes transfected with UCH-L1 siRNA or sc-RNA and the corresponding statistical pictograms obtained via densitometry. The cropped blots for different targets had been run under the same experimental conditions, **P*<0.05, ***P*<0.01 versus control.

slightly decreased at 9 h, compared with levels at 6 h (**Figure 2E-G**). The observed upregulation of UCH-L1 in cultured podocytes under inflammatory stimulation was in accordance with previous reports on UCH-L1 expression in nephritis [12]. Notably, β-catenin protein levels also increased with time in podocytes subjected to inflammatory stimulation at 45 min, 90 min, 3 h, 6 h and 9 h (**Figure 2E-G**), consistent with UCH-L1 expression.

Based on qPCR analysis, the UCH-L1 and β-catenin mRNA levels increased in a time-dependent manner in podocytes under conditions of inflammatory stimulation at 45 min, 90 min and 3 h, but decreased with time at 6 h and 9 h, relative to 3 h (**Figure 2H-J**), which was almost consistent with protein expression patterns.

Our results collectively indicate that activation of the Wnt/β-catenin signaling pathway is closely associated with UCH-L1 expression, which may play an important role in podocyte injury.

UCH-L1 upregulates Wnt/β-catenin signaling

To investigate the potential regulatory role of UCH-L1 on β-catenin levels in podocytes, we transfected podocytes with UCH-L1 plasmid and siRNA/UCH-L1 and examined β-catenin protein levels. Overexpression of UCH-L1 induced accumulation of β-catenin, while its knock down led to reduction of β-catenin (**Figure 3A**, **3B**) on a western blot, suggesting that UCH-L1 is the upstream activator of β-catenin.

Involvement of UCH-L1 in Wnt/β-catenin signaling

β-Cateninis degraded via the ubiquitin-proteasome system to maintain low levels in various tissues [23]. Upon treatment of podocytes with the proteasome inhibitor MG132 (2.5 mM), the β-catenin protein level was significantly increased, compared to those in the normal and DMSO groups (**Figure 4A**). Additionally, upon immunofluorescent co-staining of endogenous β-catenin and UCH-L1 in cultured podocytes

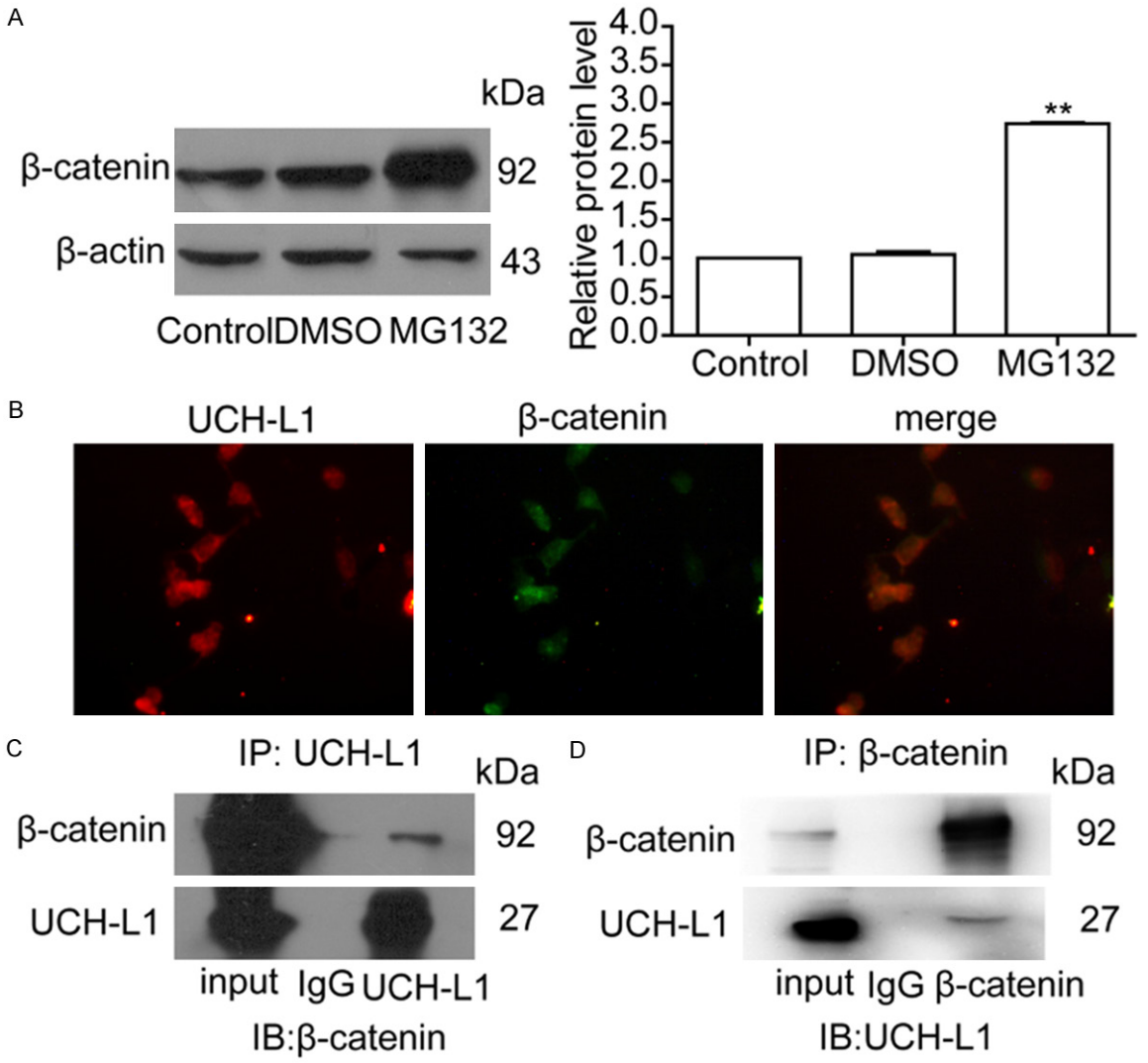

Figure 4. UCH-L1 is physically associated with β-catenin. A. Western blot for β-catenin in cultured podocytes treated with the proteasome inhibitor MG132 (2.5 mM) for 20 h and the corresponding statistical pictograms obtained via densitometry (cropped blots for different targets were run under the same experimental conditions, $^{**}P<0.01$ versus control). B. Double immunostaining for UCH-L1 (red) and β-catenin (green) to detect co-localization. C, D. Endogenous β-catenin or UCH-L1 was immunoprecipitated from cultured podocytes. Precipitants were resolved via 10%-12% PAGE and probed with the indicated antibodies. Mouse and rabbit normal immunoglobulins were used as controls for immunoprecipitates (IP).

(**Figure 4B**), both proteins were predominantly co-localized in the nucleus, although some cytoplasmic staining for UCH-L1 was also observed. Additionally, endogenous UCH-L1 and β-catenin were immunoprecipitated with specific antibodies. Western blots of immunoprecipitates (**Figure 4C**, **4D**) demonstrated that β-catenin and UCH-L1 form endogenous complexes in cultured podocytes. Based on the collective results, we propose that UCH-L1 interacts with β-catenin and blocks its degradation in cultured podocytes.

Discussion

Podocyte injury is a critical pathological feature in a variety of proteinuric kidney diseases. Recent studies indicate that Wnt/β-catenin, an essential multifunctional signaling pathway in the human body, contributes significantly to the mechanism of podocyte injury [24]. Accumulating evidence has shown that dysregulation of Wnt/β-catenin signaling promotes podocyte injury and/or dysfunction,contributing to the pathogenesis of proteinuria [5, 8]. Microarray

analysis of samples from laser capture microdissection disclosed that the Wnt/β-catenin pathway is specifically activated in glomerular podocytes from patients with human diabetic nephropathy, FSGS, and IgAN [25-27]. Activation of Wnt/β-catenin signaling was also observed in damaged podocytes from several animal nephropathy models induced by hyperglycemia, adriamycin, and puromycin [25, 28, 29]. Stable expression of β-catenin in heterozygous mice ($NPHS2^{cre}/Ctnnb1^{FloxE3/WT}$) for 20 weeks induced mild mesangial proliferation and proteinuria, and EM revealed diffuse and irregular thickening of the glomerular basement membrane, leading to massive proteinuria and glomerular sclerosis [26]. In cultured podocytes, activation of β-catenin suppressed nephrin expression, indicating that this signaling pathway specifically disrupts the slit diaphragm of the glomerular filter [25]. Genetic approaches to induce either gain or loss of Wnt/β-catenin signaling have unambiguously confirmed a crucial role of this pathway in mediating podocytopathy and proteinuria. For instance, mice with podocyte-specific expression of stabilized β-catenin developed albuminuria and exhibited increased susceptibility to glomerular injury [26, 30]. These observations highlight a role of Wnt/β-catenin signaling in mediating podocyte dysfunction and proteinuria, although the mechanisms underlying Wnt/β-catenin activation remain to be elucidated.

β-Catenin is finely and sensitively regulated by the ubiquitin-proteasome pathway for maintaining the dynamic homeostasis of expression [31]. In the pathological state, following slight changes in the processes of ubiquitination and deubiquitination, abnormal β-catenin accumulation and activation occur, potentially leading to disorders in cell morphology and function. Recent studies have shown that deubiquitinase (DUB) stabilizes β-catenin and aids in positively regulating the Wnt/β-catenin signaling pathway [18, 32]. USP4 and UCH-L1 are considered candidate DUBs for β-catenin ubiquitination [18, 33]. These data support the theory that fine control of β-catenin signaling pathways may involve multiple regulators as in the case of p53, which is modulated by USP7 and USP10 [34-36]. Among the potential regulators, the role of UCH-L1 is of particular interest.

UCH-L1 is an important member of the serine protease deubiquitin genzyme family [11]. Abnormal expression of UCH-L1 leads to dysfunction of ubiquitin-dependent degradation for many proteins, which may constitute an important mechanism in the pathogenesis of neurodegenerative diseases and cancer [14-17, 37-40]. Earlier, Zhong et al. demonstrated that UCH-L1 contributes to colorectal cancer progression by activating the β-catenin/TCF pathway through deubiquitination [41]. Data from cytology studies confirmed that UCH-L1 combines with β-catenin to form a complex and promotes its expression [18], suggesting that UCH-L1 is an important upstream promoter of Wnt/β-catenin signaling.

Interestingly, UCH-L1 has recently been shown to be involved in podocyte injury. Our group and other researchers reported that abnormal expression of UCH-L1 in renal tissue is related to podocyte injury and proteinuria. Ectopic expression of UCH-L1 was observed in diseased podocytes in a variety of nephritis, such as MN, IgAN, LN and diabetic nephropathy [12]. Expression of UCH-L1 is known to be associated with podocyte differentiation [42]. In immature podocytes, UCH-L1 expression is higher upon cell differentiation, and for mature cell development, UCH-L1 expression is significantly reduced until disappearance, accompanied by formation of foot processes [43]. Upon the occurrence of glomerular lesions, UCH-L1 expression is increased again with foot process fusion [12, 42]. Thus, elevated UCH-L1 may be a sign of podocyte injury.

We examined the hypothesis that expression of UCH-L1 and β-catenin are linked in podocyte injury. Our experiments demonstrated that UCH-L1 combines with β-catenin in podocytes, in turn, increasing and activating β-catenin in cells leading to disorders in morphology and function of podocytes. UCH-L1 overexpression or knockdown induced up- and downregulation of β-catenin in podocytes, respectively, indicating that β-catenin is a downstream molecule of UCH-L1. These findings collectively support the utility of UCH-L1 as a therapeutic target for podocyte injury and proteinuric kidney disease, in view of its role as a regulator of the β-catenin signaling pathway.

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Disclosure of conflict of interest

None.

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