

## Original Article

# Sodium-dependent phosphate co-transporter type IIb associates with glomerular podocyte apoptosis and injury through regulating p38 MAPK/JNK signaling pathway in mice diabetic nephropathy

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**Abstract:** Objective: During the past decades, emerging experimental evidences had shown the important role of the sodium-dependent phosphate co-transporter type IIb (Npt2b) in phosphate absorption and hyperphosphatemia. This study was designed to evaluate the role of Npt2b in podocyte apoptosis induced glomerular injury in diabetic nephropathy. Methods: Male wild type and Npt2b knockout C57BL/6L mice were fed with high fat food, kidney change were detected, cell apoptosis related proteins and podocyte apoptosis percentage was evaluated to investigate the effect of Npt2b deletion on pathogenesis of glomerular injury in diabetic nephropathy. Results: Upregulation of Npt2b expression was confirmed in animal models fed with high fat food and primary podocytes incubated with high glucose, as compared with control, respectively. Npt2b knockout attenuated glomerular injury in mice model. Moreover, high glucose medium treated podocytes showed lower apoptosis percentage, accompanied with decrease in apoptosis related proteins (i.e. AIF, cleaved Caspase-3, Bax, p-p38, and p-JNK) and upregulation of Bcl-2. Conclusion: In conclusion, we confirmed that Npt2b expression in small intestine contributed to glomerular injury in diabetic nephropathy via modulating podocyte apoptosis through modulation of p38 MAPK/JNK signaling pathway. Npt2b siRNA in small intestine might be used as a potential management for preventing diabetic nephropathy.

**Keywords:** Kidney disease, podocyte, Npt2b, phosphate

## Introduction

Chronic kidney disease (CKD) is a group of heterogeneous disorders and characterized by impaired kidney structure and function [1], i.e. diabetic nephropathy, which is also characterized by glomerular hypertrophy, decreased glomerular filtration, proteinuria, renal fibrosis and loss of renal function [2]. The emerging prevalence and limit management strategies results it to be an increasing public health issue.

CKD and diabetic nephropathy tubulointerstitial injury is associated with elevations in serum phosphate [3], and hyperphosphatemia might be an independent risk factor of end-stage renal disease, and diabetic nephropathy [4]. Important role of a sodium-dependent phosphate co-transporters, Npt2b, in maintain-

ing phosphate homeostasis [5] and in CKD [6, 7]. Npt2b is one of sodium-dependent phosphate (Na/Pi) co-transporters and plays important role in phosphate metabolism [8, 9]. Using different agents to modulate Npt2b, potential therapeutic target of Npt2b for hyperphosphatemia management have been evidenced by several studies [7]. Additionally, glucose-induced podocytes apoptosis and podocyte injury initiate diabetic nephropathy [10]. Taken together, we speculated that the apoptosis protective effect of Npt2b against hyperphosphatemia might associate with podocytes apoptosis.

This study was aimed to investigate the effect of Npt2b on podocytes apoptosis, and the potential of targeting Npt2b for diabetic nephropathy therapeutic management. *In vivo*

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and *in vitro* diabetic nephropathy model was established using high fat food treated male wild type C57BL/6L mice and high glucose treated primary mouse podocytes, respectively. We evaluated podocytes apoptosis and glomerular injury in response to Npt2b modulation. This present study would provide us with more information on associations of Npt2b with podocytes apoptosis and the diabetic nephropathy pathogenesis.

### Materials and methods

#### *Animal model and experimental groups*

All protocols of animal experiments were reviewed and approved by the Institutional Animal Care and Use Committee at Central Hospital of Minhang district of Shanghai, China. Forty wild type C57BL/6L mice (8-week old, male) were acclimated for 7 days under a 12 h light/dark cycle with free access to food and water, followed by randomly divided into four groups: Control group (n = 10) fed with normal food, Diabetic group (n = 10) induced by high fat food for 14 weeks, accompanied with four times junction (ip.) of streptozotocin (STZ, 30 mg/kg BW, Sigma-Aldrich, St Louis, MO, USA) every two weeks, Npt2b silencing group (lentivirus-siRNA-Npt2b, tail vein injection, n = 10) fed with normal food, and siRNA-Npt2b + Diabetic group (n = 10). At the eighth weeks, animals were sacrificed and kidneys were separated for the immunohistochemistry and western blot analysis assay.

#### *Cells, cell culture conditions and transfection*

Primary podocytes was isolated from wild type C57BL/6L mice kidney as described previously [11]. Mouse glomeruli were isolated, incubated with trypsin solution (Sigma-Aldrich), sieved using a 30- $\mu$ m-pore-size filter (BD Biosciences, Discovery Labware, Bedford, MA), and cells were then precipitated by centrifugation. Next, cells were resuspended using PBS and incubated with RPMI 1640 medium supplemented with 10% FBS, 50 U/mL interferon- $\gamma$  (INF- $\gamma$ , Sigma-Aldrich, down to 50 U/mL during successive passages), 100 units per ml of penicillin/streptomycin [11]. Cells were then harvested by trypsin and subcultured in nonpermissive conditions (without INF- $\gamma$ ) for 10-day differentiation [12]. Cells were then treated with high glucose (20 mmol/L glucose) for 48 h and harvested for

protein. For Npt2b silencing, cells placed at 24-well plates were transfected with lentivirus-siRNA-Npt2b plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) 24 h prior to glucose stimuli.

#### *Lentivirus siRNA gene transduction*

SiRNAs oligoes targeting Npt2b and scramble control were synthesized by GenePharma (Shanghai, China), and lentivirus siRNAs were constructed using lentivirus vector pLKO.1 puro (Agel and EcoR, Sigma). PLKO.1-puro lentiviral vector without shRNA was used as a control (Mock transfection).

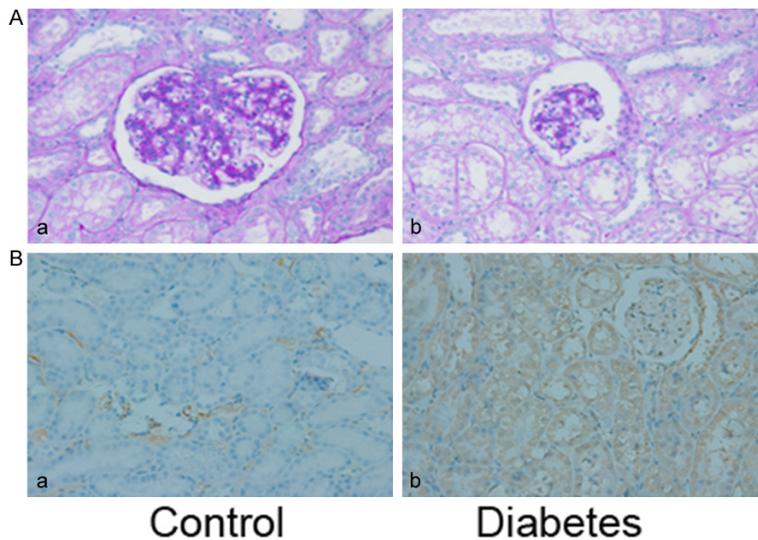
#### *Histology analysis and immunostaining*

Isolated mouse glomeruli were fixed in 10% formalin, processed and embedded with Paraplast Plus medium (Sigma). Six-micrometer sections were cut and stained with periodic acid Schiff's (PAS) reagent to analysis the pathological variation induced by high fat food and siRNA-Npt2b. Immunohistochemistry was performed on Paraplast Plus-embedded tissue sections as described [13]. The primary antibody against Npt2b (H-14, 1:200 dilutions, Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used according to the manufacturers' instruction.

#### *Immunoblot analysis*

For western blot, tissue and cellular proteins were separated, quantified (Bio-Rad DC protein assay, Bio-Rad Laboratories Inc, Hercules, CA, USA), separated (10% SDS-PAGE), and then subsequently transblotted onto Millipore polyvinylidene difluoride (PVDF, Millipore, Billerica, MA, USA) membranes. Millipore membranes were then blocked and incubated with the specific primary antibodies at 4°C overnight, and HRP-conjugated secondary antibodies for 1 h. Antibodies against AIF, cleaved Caspase-3, phospho-p38 (p-p38 MAPK), p-JNK, total p38 MAPK, and total JNK were purchased from Cell Signaling Technology (1:1000 dilution, CST, Danvers, MA, USA). Immunoblot antibodies against Npt2b (1:500 dilution) and Nephtrin (1:500 dilution) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Immunoreactive protein bands were visualized by a chemiluminescence reaction, and data were analyzed using a Bio-Rad Quantity

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**Figure 1.** Renal pathology and immunohistochemistry analysis of mouse glomerular. (A) Representatives of PAS staining for renal pathology of control (a) and mice induced by high fat food and STZ junction (b). (B) Immunohistochemistry analysis for Npt2b expression in normal (a) and diabetic mouse glomerular (b). Original magnification,  $\times 400$ .

One software (Bio-Rad) normalizing to the band with GAPDH control (1:2000 dilution, Sizhengbo, Beijing, People's Republic of China).

### Apoptosis assay

Apoptosis analysis of primary podocytes cells was detected by flow cytometry with PE-labeled annexin V Apoptosis Detection Kit (BD Biosciences). After being transfected for 72 h, cells were harvested, and incubated with Annexin V and propidium iodide for 10 min. Then cells were analyzed using a FACS Calibur flow cytometer (BD Biosciences). Early apoptotic cells percentage was counted by Annexin V-PE positive and PI-negative cells (Annexin V+/PI-).

### Statistical analysis

Each experiment was performed in triplicate. Statistical analysis for quantitative data was performed using SPSS 19.0 (SPSS Inc., USA). Statistical graphs were made using Graphpad Prism 6.0 (GraphPad Software Inc, La Jolla, CA). All data were expressed as the mean  $\pm$  standard deviation (SD). Differences between groups were analyzed by Tukey's test, and among four groups were analyzed using ANOVA, respectively.  $P < 0.05$  was considered statistically significant.

## Results

### *Mouse glomerular change and Npt2b expressed in diabetic mice*

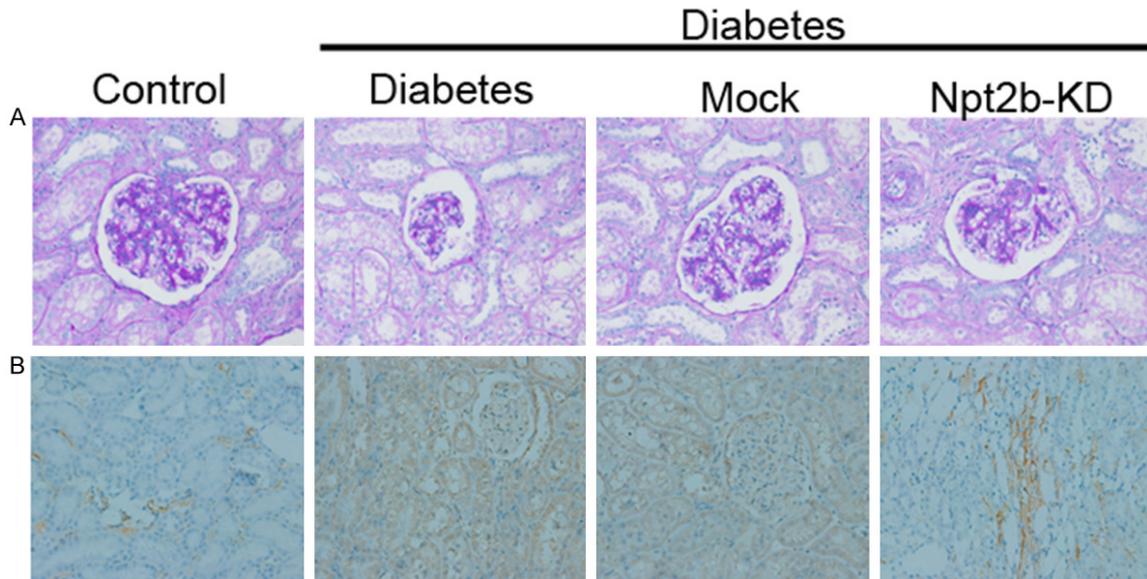
High fat food accompanied by junction (ip.) of low dose STZ result in diabetes, corneal nerve sensitivity, and diabetic nephropathy [14-17]. We performed PAS staining for renal pathology analysis and the results showed that high fat food plus STZ treated mice developed severe glomerular lesions and tubular injury, as compared to control (**Figure 1A**), demonstrating the successful induction of diabetic nephropathy. Moreover, immunohistochemistry for Npt2b expression showed Npt2b was increased in diabetic mouse glomerular (**Figure 1B**), suggesting an Npt2b upregulation in diabetic nephropathy.

### *Npt2b siRNA repaired glomerular injury in diabetic mice*

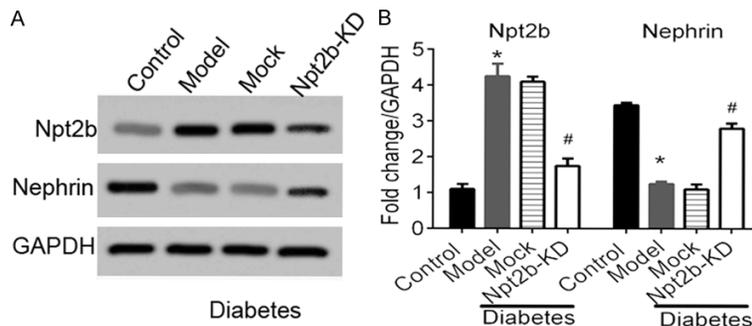
To investigate whether Npt2b contributes to diabetic nephropathy, we knocked out (KD) the expression of Npt2b in mice by tail vein injecting lentivirus-siRNA-Npt2b plasmids, and confirmed Npt2b-KD attenuated high fat food induced tubular injury (**Figure 2A**). Impaired mouse glomerular in diabetic mice were partially repaired by Npt2b-KD, as compared to those of mock and diabetic mice (**Figure 2**).

### *Nephrin decreased in diabetic nephropathy and enhanced by Npt2b-KD*

As it has been evidenced that diabetic nephropathy is characterized by podocytes dedifferentiation and apoptosis [18, 19]. Nephrin is a transmembrane protein and is protective and essential for podocyte function [18, 20]. Then we detected the nephrin expression of established diabetic nephropathy using immunoblotting assays. Western blot analysis demonstrated the downregulation of nephrin expression, as compared to control, in contrast to Npt2b expression (**Figure 3A**). In mice transfected with lentivirus-siRNA-Npt2b plasmids,



**Figure 2.** Renal pathology and immunohistochemistry analysis of mouse glomerular repaired by Npt2b-KD. A. Representatives of PAS staining for renal pathology of diabetic mice injected with injecting lentivirus-siRNA-Npt2b plasmids. B. Immunohistochemistry analysis for Npt2b expression mouse glomerular of diabetic mice injected with injecting lentivirus-siRNA-Npt2b plasmids. Original magnification,  $\times 400$ .



**Figure 3.** Expression of nephrin. Expression of nephrin in mouse glomerulus was detected using Western blotting analysis and contrary expression pattern to Npt2b was determined. \*and #indicates difference at  $P < 0.05$  level in comparison with control and diabetes mice, respectively.

cytes was increased and decreased, respectively, in comparison with controls (**Figure 4A**). This demonstrated that we successfully established the podocytes diabetic model. Notably, siRNA-Npt2b significantly upregulated expression while inhibited expression of Npt2b in transfected podocytes. In addition, high-glucose induced podocytes cell apoptotic percentage was attenuated by siRNA-Npt2b transfection (**Figure 4B**).

in some extent enhancement of nephrin expression was determined (**Figure 3B**). This demonstrated that podocyte function was dysregulated in our mice model of diabetic nephropathy.

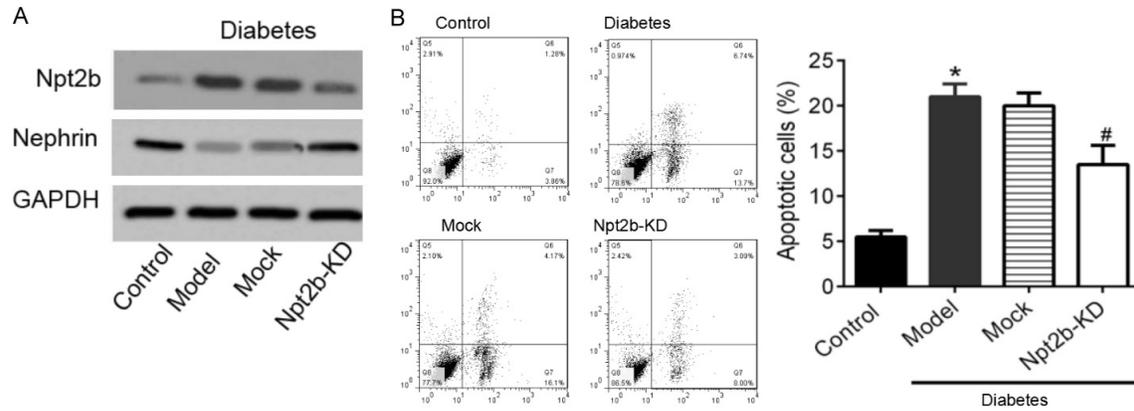
*Npt2b-KD inhibited high-glucose induced podocytes apoptosis*

We isolated the primary podocytes from normal mouse glomeruli, and treated with podocytes with high-glucose medium (20 mmol/L glucose) to establish an *in vitro* model of diabetic nephropathy. Npt2b and nephrin expression in high-glucose treated dedifferentiated podocytes

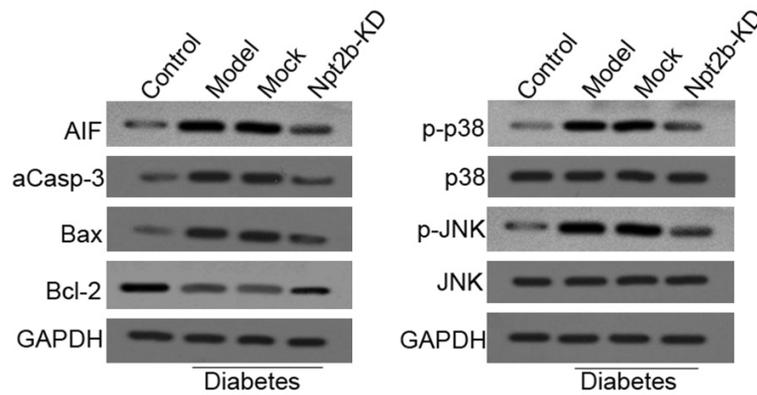
*Npt2b expression promoted podocytes apoptosis partially through p38/JNK/Bcl-2 pathway*

Cell apoptosis is complex and associates with number of apoptosis related factors, such as Bcl-2 family members (antiapoptotic Bcl-2 protein and proapoptotic Bax protein) [21], Caspase-3 [22, 23], the apoptosis inducing factor (AIF) [24], and p38/JNK signaling pathways [25, 26]. As expected, upregulated expression of AIF, cleaved Caspase-3, Bax, p-p38, and p-JNK proteins were detected in high-glucose treated podocytes, as compared to controls (**Figure 5**). In contrast, upregulation of these proteins in

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**Figure 4.** Effect of siRNA-Npt2b on cell apoptosis transfected podocytes. A. Expression of nephrin and Npt2b in transfected podocytes. B. Apoptotic cell percentage of transfected podocytes. \*and #indicates difference at P < 0.05 level in comparison with control and diabetes mice, respectively.



**Figure 5.** Effect of siRNA-Npt2b on cell apoptosis related proteins.

cell model were restricted by siRNA-Npt2b transfection. Reverse expression pattern of Bcl-2 contrary to the above proteins was observed, demonstrating siRNA-Npt2b inhibited podocytes partially through p38 MAPK/JNK, Bcl-2, and Caspase-3.

### Discussion

Diabetic nephropathy induced by high fat food and low dose STZ is a frequent *in vivo* animal for diabetic nephropathy pathogenesis and therapeutic strategies exploring. Using this model, we confirmed in this present study that overexpression Npt2b contributes to diabetic nephropathy and knock out of Npt2b successfully inhibited glomerular injury and podocytes apoptosis.

Based on the contribution of Npt2b to maintaining phosphate homeostasis, Npt2b effect on lung tumorigenesis and hyperphosphatemia

associated with CKD had been evidenced [5, 6]. Knockout of Npt2b had been reported to reduce serum phosphate level, and the combination of Npt2b-deletion and sevelamer, a dietary phosphorus binders, showed higher therapeutic efficiency to CKD than sevelamer alone, demonstrating Npt2b deletion benefits to CKD treatment [6]. In this present study, we confirmed that Npt2b knock out could attenuate high fat food induced diabetic nephropathy

and podocytes apoptosis, revealing Npt2b contributed to diabetic nephropathy pathogenesis and knock out of Npt2b might be explored as a diabetic nephropathy treatment.

Podocytes apoptosis induced loss is an early feature of diabetic nephropathy [27, 28], and high glucose-induced podocytes apoptosis had been reported diabetic nephropathy [27]. Glucose-induced enrichment of reactive oxygen species (ROS) stressed podocytes functions and initiated podocytes apoptosis [29]. In this present study, we confirmed the proanti-apoptotic effect of high glucose on podocytes apoptosis and diabetic nephropathy *in vitro* and *in vivo* is accompanied with increased expression of Npt2b. On the contrary, knockout of Npt2b attenuated glucose-induced podocytes apoptotic percentage, and inhibited glucose-induced expression of proapoptotic proteins (i.e. AIF, cleaved Caspase-3, Bax, p-p38, and p-JNK), suggesting Npt2b deletion partially

restricted podocytes apoptosis via inhibiting signaling pathways (e.g. p38 MAPK/JNK/Bcl-2 pathway and Caspase-3 signaling).

Npt2b is one Na/Pi co-transporter and plays important role in phosphate metabolism, and in Npt2b-deletion significantly decreased serum phosphate levels of uremic mice [6]. In this present study, we demonstrated that Npt2b was upregulated in high fat food induced diabetic nephropathy *in vivo* and high glucose induced diabetic nephropathy *in vitro*. Npt2b knockout in this present study significantly attenuated podocytes apoptosis and diabetic nephropathy induced by higher glucose. Reduced serum phosphate level in Npt2b knockout mice had been evidenced [6], Taken together, these suggested the association of Npt2b with glucose-phosphate isomerism, and attenuating glucose transferring to phosphate might be useful for preventing diabetic nephropathy.

### Conclusion

In conclusion, we demonstrated that overexpression of Npt2b induced by high glucose contributed to high fat food and STZ induced podocytes apoptosis and diabetic nephropathy, and Npt2b knockout attenuated these processes, demonstrating the crucial role of Npt2b in diabetic nephropathy pathogenesis and the potential of using Npt2b to be a diabetic nephropathy treatment. However, more experiments should be done to explore the Npt2b-mediated glucose-phosphate isomerism and the related mechanism.

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### Disclosure of conflict of interest

None.

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