Original Paper

Netrin-1 Attenuates the Progression of Renal Dysfunction by Inhibiting Peritubular Capillary Loss and Hypoxia in 5/6 Nephrectomized Rats

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Key Words

Netrin-1 • Peritubular capillary • Vascular endothelial cell • Renal interstitial fibrosis • HIF-1 α

Abstract

Background/Aims: The aim of this study was to investigate the effect of netrin-1 on peritubular capillary (PTC) loss and hypoxia in 5/6 nephrectomized (Nx) rats. **Methods:** Male Sprague-Dawley rats were divided into three groups (n = 10 rats/group): sham-operated rats treated with control adenovirus; 5/6 Nx rats treated with control adenovirus; and 5/6 Nx rats treated with recombinant adenovirus mediated netrin-1 gene (Ad-netrin-1) therapy. Rats were killed 12 weeks after surgery. Blood urea nitrogen (BUN), serum creatinine (Scr) and 24-h urinary albumin excretion rates were measured. Pathological changes in renal tissues were analyzed histologically. The concentration of netrin-1, CD34, and hypoxia-inducible factor-1 α (HIF-1 α) were analyzed by immunohistochemistry, Western blotting and real-time PCR. **Results:** Renal function and histopathological damage were significantly improved in Ad-netrin-1 treated 5/6 Nx rats, compared with rats treated with the control adenovirus in the 5/6 Nx group. Furthermore, Ad-netrin-1 treatment induced a significant increase in renal PTC density, accompanied by a significant decrease in HIF-1 α expression. **Conclusion:** Adenovirus mediated netrin-1 treatment attenuates PTC damage, relieves tissues hypoxia and improves renal function, thus alleviating renal pathological changes and interstitial fibrosis in 5/6 Nx rats.

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Introduction

Chronic kidney disease has a major impact on human health; it is characterized by an irreversible scarring of the renal parenchyma leading to end-stage renal disease [1]. Recent studies have shown that the loss of peritubular capillaries (PTC) and the resultant chronic tissue hypoxia play critical roles in tubulointerstitial damage and fibrosis, which is a common pathway for various chronic diseases leading toward end-stage renal failure [2]. Therefore, increasing the density of PTC by enhancing their repair *in vivo* is an important approach to prevent renal interstitial fibrosis. In this regard, studies have reported that stimulating angiogenesis in the kidney can provide therapeutic benefits against renal fibrosis [3].

Netrin-1 is an important guidance cue for growing axons, guiding their growth and inducing the migration of axonal growth cones [4]. Studies have found that netrin-1 is an endothelial mitogen, stimulating the proliferation and migration of vascular endothelial cells and accelerating angiogenesis [5]. Furthermore, a recent study found that administration of recombinant netrin-1 significantly reduced ischemia-induced renal dysfunction in an *in vitro* model of acute kidney injury [6]; however, the role of netrin-1 in chronic kidney diseases is still unknown. As such, the goal of this study was to investigate the role of netrin-1 in protecting against renal dysfunction in hypertensive 5/6 nephrectomized (Nx) rats.

Materials and Methods

Construction of recombinant adenovirus netrin-1

Adenovirus-expressing netrin-1 was created using the AdEasy Vector system (Qbiogene, Nottingham, UK). The pcDNA 3.0-netrin-1 plasmid used in this study was a generous gift from Patrick Mehlen (Apoptosis, Cancer, and Development Laboratory, Equipe Labellisée la Ligue, University of Lyon, France). Netrin-1 sequences were subcloned into the adenovirus shuttle vector pAdTrack-CMV to create pAdTrack-CMV-netrin-1. The resultant plasmid was linearized by digesting with the restriction endonuclease *PmeI*, and subsequently cotransformed into *E. coli* BJ5183 cells with an adenoviral backbone plasmid pAdEasy-1 by electroporation. Colonies were then selected on kanamycin LB plates to obtain Ad-netrin-1 plasmids. Correct recombination was verified by restriction digestion. The *PacI*-linearized recombinant plasmid was then transfected into HEK293 cells (ATCC, Manassas, VA, USA) to package the viral vector. Adenovirus titers were determined by plaque-forming assay, and expressed as plaque-forming units (PFU). Virus stocks were amplified by culturing HEK293 cell with low-passaged virus stocks, and amplification continued until the titer reached 10^{10} PFU/ml. Aliquots of recombinant adenoviruses were then frozen at -80°C until further use.

Animals and experimental protocol

All animal procedures were conducted according to the animal care and ethics legislation and were approved by the Animal Care Committee of the Chinese Medical University. Chronic renal failure was studied using 5/6 Nx rats, induced according to the method published by Ghosh et al. [7]. Thirty male Sprague-Dawley rats (8 to 10 weeks old; 250 to 300 g) were obtained from the Chinese Medical University Animal Centre (Shenyang, China). They were then randomly divided into one of three groups ($n = 10$ rats/group): sham-operated rats treated with control adenovirus; 5/6 Nx rats treated with control adenovirus; and 5/6 Nx rats treated with recombinant adenovirus mediated netrin-1 gene expression (Ad-netrin-1). The rats in the sham-operated group were anesthetized by intraperitoneal injection of 10% chloral hydrate (3 ml/kg) followed by surgical exposure and removal of the renal capsule. The 5/6 Nx rats first underwent a partial nephrectomy of their left kidney (removal of 1/3 of the superior pole and 1/3 of the inferior pole) under anesthesia. The animals were then returned to the vivarium to recover. One week later, they underwent another nephrectomy to remove the whole right kidney, thus accomplishing a 5/6 nephrectomy. During the second surgery, 1×10^8 PFU of control adenovirus (empty vector) or Netin-1-encoding adenovirus was injected via the vena caudalis, as reported in previous studies [8]. The rats were followed-up for 12 weeks, and all animals were killed on the 13th week of the experiment. At death, blood and kidney tissues were collected and kept at -80°C for further analysis.

Biochemical analyses

Blood samples were collected from all animals on weeks 1, 7, and 12 postsurgery, for the analysis of blood urea nitrogen (BUN), serum creatinine (Scr) and urinary albumin. BUN and Scr concentrations were assayed with an Olympus 400 clinical chemistry analyzer, and 24-hour urinary albumin excretion rates were measured using the sulfosalicylic acid method.

Histological examinations

Renal tissue samples were fixed by immersion in 4% neutral formaldehyde overnight. They were then dehydrated, cleared, embedded in paraffin, sectioned into 4- μ m tissue slices, and stained with hematoxylin and eosin (H&E) and Masson's trichrome. The H&E stained slices were examined by optical microscopy for pathological changes, and the Masson's trichrome stained slides were analyzed for interstitial fibrosis as previously described [9]. To determine the percent area of interstitial space in the kidney, five consecutive fields were randomly selected in the renal cortex and evaluated at 400 $\times$ on a 10 $\times$ 10 grid-imprinted reticule. All points not counted within tubular cells, lumen, glomerulus, or vascular spaces were considered interstitial. This fraction represented the relative interstitial volume. Results were expressed as percentage of the measured area, which represented the interstitial space and was determined as the relative volume of the interstitium.

Immunohistochemical staining

First, tissue slices were dewaxed and rehydrated, and then endogenous peroxidase activity was blocked by immersion in 3% methanol/hydrogen peroxide in water. Next, nonspecific antibody binding was blocked with goat serum for 20 min at room temperature. The slices were then incubated for 1 h at RT with a rabbit polyclonal anti-netrin-1 primary antibody (1:200; Abcam, Cambridge, UK), a mouse monoclonal anti-CD34 primary antibody (1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), or rabbit polyclonal anti-hypoxia-inducible factor-1 α (HIF-1 α ; 1:400; Santa Cruz Biotechnology). Following washing, tissue sections were then incubated with an anti-rabbit/mouse secondary antibody for 30 min and staining was visualized using a streptavidin-biotin complex kit (SABC, Boster, Wuhan, Hubei, China), followed by reaction with 3,3'-diaminobenzidine (Vector Labs Inc, California, USA). After washing in tap water the sections were counterstained with hematoxylin, and dehydrated through an ascending series of ethanol, cleared with xylene, and mounted in neutral balsam. The resulting slices were analyzed by light microscopy. Staining of netrin-1, CD34 and HIF-1 α were measured using computerized morphometry by Image Pro Plus ver. 6.0.0 (Media Cybernetics, Silver Spring, MD, USA) [10]. Stained areas of 20 randomly selected fields in the renal cortex were quantified at a magnification of 400 $\times$ and expressed as percentage of total measured area. Capillary density was quantified in capillaries per square millimeter via immunohistochemical (IHC) staining for CD34 [11].

RNA extraction and quantitative real-time PCR

Frozen tissue samples were lysed in TRIzol reagent (Invitrogen, Carlsbad, CA), and total RNA was extracted according to the manufacturer's instructions. RNA was quantified using a spectrophotometer (Beckman-Coulter, Fullerton, CA). Isolated RNA was then diluted to a 100 ng/ μ L stock and reverse transcribed using the M-MLV Reverse transcriptase (Invitrogen). Real-time PCR was performed using SYBR[®]Premix Ex Taq[™] II (TaKaRa, Shiga, Japan) on an ABI 7901HT series PCR cycler (Applied Biosystems, Foster City, CA, USA). The data were normalized to GAPDH expression and further normalized to the negative control. Primers were obtained from Sangon Biological Engineering Technology and Services (Shanghai, China), specific primers were designed as follows: GAPDH 5'-CCC ACT CTT CCA CCT TTG-3' (sense), 5'-TAG CCA TAT TCA TTG TCA TAC C-3' (antisense); netrin-1 5'-TCC GAT CCC AAG AAA GCG-3' (sense), 5'-TGC CGA GCG ACA GAG TGA-3' (antisense); HIF-1 α CAT CTC CAC CTT CTA CCC-3' (sense), 5'-TCT TTC CTG CTC TGT CTG-3' (antisense).

Western blotting

Total protein was extracted from tissue samples with a commercial kit (GE healthcare Biosciences, Pittsburgh, PA, USA). Renal cortex samples were transferred into test tubes and mixed with phenylmethyl sulfonylfluoride (Sigma-Aldrich Corporation, St. Louis, MO), 300 to 500 μ L of lysis buffer, 2 μ L of leupeptin, and 2 μ L of aprotinin. After maintaining the tubes in an ice bath for 15 min, the protein concentrations in

the lysates were assayed by BCA assay using commercial kits (Invitrogen). According to the assay results, the lysates were adjusted to a protein concentration of 1 g/l and kept in Eppendorf tubes (35 µl/tube) at -70°C before subsequent use. A fixed amount of total protein was collected from each sample and separated by electrophoresis on polyacrylamide gels containing 8% sodium dodecyl sulfate. The separated fractions were blotted onto a polyvinylidene fluoride membrane, treated with skim milk for 2 h to block unspecific antibody binding sites, rinsed with tris buffered saline (TBS), and incubated with a rabbit polyclonal anti-rat netrin-1 primary antibody (1:500; Abcam) and a rabbit polyclonal anti-rat HIF-1α (1:400; Santa Cruz Biotechnology) overnight at 4°C. After rinsing with TBS (5 min × 3), the membrane was incubated with a horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (1:5,000, Sigma-Aldrich Co.) at room temperature for 1 h, followed by rinsing with TBST (Tris buffered saline with 10% Tween-20) and development with enhanced chemiluminescence kits (ECL, GE Healthcare Biosciences). Equal protein loading was confirmed by immunostaining against β-actin (1:4,000; Sigma-Aldrich Co.). The membrane was imaged and analyzed with a GIS-2020 image processing system (Bio-Rad Hercules, CA, USA). The concentration of netrin-1 and HIF-1α were quantified based on their optical densities with reference to the β-actin band on the same membrane.

Statistical analyses

Data are expressed as the mean ± standard error of the mean (SEM). Results were analyzed with an unpaired t-test using Welch's correction or ANOVA for multiple comparisons. All analyses were performed with SPSS 13.0 (SPSS, Chicago, IL, USA). BUN and Scr values were analyzed using two-way ANOVA with time as the repeated factor. The results were considered significant at a *P*-value < 0.05.

Results

Effect of netrin-1 on renal function and pathological changes in 5/6 Nx rats

In order to assess the success of Ad-netrin-1 expression, we analyzed netrin-1 expression by IHC. In the sham-operated group (Fig. 1A.a), netrin-1 was abundantly expressed in the tubular cytoplasm but was scarce in the interstitial space. The 5/6 Nx group treated with the control adenovirus (Fig. 1A.b) had substantially reduced netrin-1 expression compared with the sham-operated group, and netrin-1 was not detectable in the interstitial space. Importantly, the Ad-netrin-1-treated 5/6 Nx group (Fig. 1A.c) showed a substantially increased netrin-1 expression compared with 5/6 Nx group, though it was still significantly lower than the sham-operated group (Fig. 1B). Next, we examined the mRNA and protein levels of netrin-1 to support our IHC analysis, generating complementary data. As shown in Fig. 1C and D, the sham operated group showed a relatively high expression level of netrin-1; this level was significantly decreased in 5/6 Nx rats treated with the control adenovirus, but the Ad-netrin-1-treated 5/6 Nx group demonstrated an increased expression of netrin-1 compared with 5/6 Nx alone group (*P* < 0.05).

A significant increase in Scr, BUN and urine albumin level were observed in the 5/6 Nx groups with and without Ad-netrin-1 treatment, from the seventh week postsurgery, compared with the sham-operated group (Table 1; *P* < 0.05). However, in those rats receiving Ad-netrin-1, the BUN and Scr levels were significantly lower than the 5/6 Nx group administered the control adenovirus at week 7 and 12 postsurgery (*P* < 0.05). Furthermore, in the Ad-netrin-1-treated 5/6Nx group, 24-h urinary albumin levels were significantly reduced compared with the control adenovirus 5/6Nx group at each time-point (*P* < 0.05).

Our histological examination with H&E stained kidney sections revealed that the sham-operated group (Fig. 2a) had no noticeable changes in renal glomeruli, tubules, or interstitia, with normal morphology and distribution patterns observed. However, the 5/6 Nx group (Fig. 2b) showed proliferation of glomerular mesangial cells, an increase in the mesangial matrix, dilation or obstruction of capillary vessels, and thickening of glomerular walls. Some glomeruli showed focal or total sclerosis, tubules were dilated or atrophic, and the interstitial space was widened, with multiple sites of focal fibrosis and infiltration of inflammatory cells. In contrast, the Ad-netrin-1-treated 5/6 Nx group (Fig. 2c) showed substantially attenuated

Fig. 1. The expression of netrin-1 in the kidney. A) Representative micrographs of netrin-1 staining in the renal tissue of sham-operated rats treated with control adenovirus at week 13 postoperatively (a), 5/6 Nx rats treated with the control adenovirus (b) and 5/6 Nx rats treated with Ad-netrin-1 (c); original magnification: 400 $\times$. B) Quantification of IHC staining for netrin-1 (d) in the renal tissue of sham-operated rats treated with control adenovirus at week 13 postoperatively (sham), 5/6 Nx rats treated with the control adenovirus (5/6 Nx), and 5/6 Nx rats treated with Ad-netrin-1 (5/6 Nx + netrin-1). *, $P < 0.05$ vs. sham; #, $P < 0.05$ vs. 5/6 Nx; $n = 10$ for each group. All data is presented as the mean $\pm$ SEM. C) Quantification of netrin-1 mRNA expression by real-time RT-PCR and normalized to GAPDH mRNA in the renal tissue of sham-operated rats treated with control adenovirus at week 13 postoperatively (sham), 5/6 Nx rats treated with the control adenovirus (5/6 Nx), and 5/6 Nx rats treated with Ad-netrin-1 (5/6 Nx + netrin-1). Results are expressed as the mean $\pm$ SEM; *, $P < 0.05$ vs. sham; #, $P < 0.05$ vs. 5/6 Nx; $n = 10$ for each group. D) A representative Western blot of protein extracted from the three experimental groups indicated above, which indicates that the level of netrin-1 protein expression is relative to β -actin.

Table 1:
Time course
analysis
of renal
function
in rats fol-
lowing 5/6
Nx

Group	Week	BUN (mmol/L)	Scr (μ mol/L)	Urine albumin (mg/24 h)
Sham + vehicle	1	5.76 $\pm$ 0.86	54 $\pm$ 12.35	8.91 $\pm$ 0.74
	7	5.83 $\pm$ 0.64	52 $\pm$ 10.90	9.43 $\pm$ 0.52
	12	5.95 $\pm$ 0.91	57 $\pm$ 16.74	9.52 $\pm$ 1.28
5/6 Nx + vehicle	1	6.01 $\pm$ 0.93	55 $\pm$ 15.82	9.30 $\pm$ 1.43
	7	18.61 $\pm$ 3.52 ^a	243 $\pm$ 25.67 ^a	32.58 $\pm$ 4.32 ^a
	12	25.37 $\pm$ 5.03 ^a	422 $\pm$ 37.24 ^a	59.31 $\pm$ 6.61 ^a
5/6 Nx + netrin-1	1	6.21 $\pm$ 1.04	53 $\pm$ 13.75	9.74 $\pm$ 0.84
	7	10.85 $\pm$ 2.42 ^{a,b}	128 $\pm$ 36.54 ^{a,b}	18.94 $\pm$ 2.75 ^{a,b}
	12	13.15 $\pm$ 2.89 ^{a,b}	186 $\pm$ 39.15 ^{a,b}	28.75 $\pm$ 3.45 ^{a,b}

Values are expressed as mean $\pm$ SEM; $n = 10$. BUN, blood urea nitrogen; Scr, serum creatinine; ^a $P < 0.05$ versus the sham group and ^b $P < 0.05$ versus the 5/6 Nx + vehicle group, respectively

pathological changes compared with the 5/6 Nx group which only received the control adenovirus.

Similarly, analysis of trichrome stained kidney sections revealed no significant histological abnormality or fibrosis in the sham-operated group (Fig. 3A.a). Whereas in the 5/6 Nx group with control adenovirus treatment (Fig. 3A.b), almost all of the tubules were

Fig. 2. Hematoxylin and eosin staining in the renal tissue of sham-operated rats treated with control adenovirus at week 13 postoperatively (a), 5/6 Nx rats treated with control adenovirus (b) and 5/6 Nx rats treated with Ad-netrin-1 (c; original magnification: 400×).

Fig. 3. Interstitial fibrosis in the kidney. A) Masson's trichrome staining of the renal tissue of sham-operated rats treated with control adenovirus at week 13 postoperatively (a), 5/6 Nx rats treated with control adenovirus (b) and 5/6 Nx rats treated with Ad-netrin-1 (c; original magnification: 400×). B) Quantification of the percentage area of interstitial space in the kidney is presented in a bar chart, the relative interstitial volume was quantified by morphometric analysis in Masson trichrome-stained kidney sections in each group; all results are expressed as the mean $\pm$ SEM; *, $P < 0.05$ vs. sham; #, $P < 0.05$ vs. 5/6 Nx; $n = 10$ for each group.

destroyed and significant renal lesions were observed, including marked interstitial fibrosis. In agreement with our H&E results, the Ad-netrin-1-treated 5/6 Nx group (Fig. 3A.c) showed less stromal fibrosis than the sham-operated and 5/6 Nx group with control adenovirus treatment. Quantitative image analysis (Fig. 3B) revealed that the interstitial space of the image fields in the sham-operated group was significantly lower than in the 5/6 Nx group ($P < 0.05$). Furthermore, the interstitial space in the Ad-netrin-1-treated 5/6 Nx group was significantly lower than in the 5/6 Nx group ($P < 0.05$).

PTC distribution and density

The CD34 antigen is normally found to be expressed in the endothelial cells of blood vessels. The number of CD34-positive capillaries is standardly used to evaluate PTC Number

Fig. 4. The expression of CD34 in the kidney. A) Representative micrographs of CD34 staining in the renal tissue of sham-operated rats treated with control adenovirus at week 13 postoperatively (a), 5/6 Nx rats treated with the control adenovirus (b) and 5/6 Nx rats treated with Ad-netrin-1 (c; original magnification: 400×). B) Quantification of IHC staining for CD34 in the renal tissue of sham-operated rats treated with control adenovirus at week 13 postoperatively (sham), 5/6 Nx rats treated with the control adenovirus (5/6 Nx), and 5/6 Nx rats treated with Ad-netrin-1 (5/6 Nx + netrin-1). *, $P < 0.05$ vs. sham; #, $P < 0.05$ vs. 5/6 Nx; $n = 10$ for each group. All data is presented as the mean $\pm$ SEM.

Fig. 5. The expression of HIF-1 α in the kidney. A) Representative micrographs of HIF-1 α staining in the renal tissue of sham-operated rats treated with control adenovirus at week 13 postoperatively (a), 5/6 Nx rats treated with the control adenovirus (b) and 5/6 Nx rats treated with Ad-netrin-1 (c; original magnification: 400×). B) Quantification of IHC staining for HIF-1 α in the kidney tissue of sham-operated rats treated with control adenovirus at week 13 postoperatively (sham), 5/6 Nx rats treated with the control adenovirus (5/6 Nx), and 5/6 Nx rats treated with Ad-netrin-1 (5/6 Nx + netrin-1). *, $P < 0.05$ vs. sham; #, $P < 0.05$ vs. 5/6 Nx; $n = 10$ for each group. All data is presented as the mean $\pm$ SEM. C) Quantification of HIF-1 α mRNA expression by real-time RT-PCR and normalized to GAPDH mRNA in the kidney tissue of sham-operated rats treated

with control adenovirus at week 13 postoperatively (sham), 5/6 Nx rats treated with the control adenovirus (5/6 Nx), and 5/6 Nx rats treated with Ad-netrin-1 (5/6 Nx + netrin-1). Results are expressed as the mean $\pm$ SEM; *, $P < 0.05$ vs. sham; #, $P < 0.05$ vs. 5/6 Nx. D) A representative Western blot of protein extracted from the three experimental groups indicated above is shown, indicating the level of HIF-1 α protein expression relative to β -actin.

[12]. In the sham-operated group (Fig. 4A.a), PTC with similar sizes and morphology were regularly distributed in the interstitial space. In the 5/6 Nx group without Ad-netrin-1 treatment (Fig. 4A.b), PTC were focally distributed with a substantially reduced spatial density, whereas regions with severe fibrosis or tubular atrophy contained no PTC. In the Ad-netrin-1-treated 5/6 Nx group (Fig. 4A.c), the PTC density was slightly reduced, but the PTC still showed similar sizes and morphology and retained a regular arrangement. Quantitative image analysis revealed that the administration of recombinant netrin-1 increased the score for CD34 staining; specifically, it was shown that the PTC density was significantly lower in the sham-operated group than in the control 5/6 Nx group and Ad-netrin-1-treated 5/6 Nx group ($P < 0.05$; Fig. 4B). Furthermore, PTC density was significantly lower in the Ad-netrin-1-treated 5/6 Nx group than in the 5/6 Nx group treated with the control adenovirus ($P < 0.05$).

Netrin-1 ameliorated hypoxia in 5/6 Nx rats

HIF-1 α is a sensitive hypoxia indicator since it is stabilized in a low oxygen cellular milieu, constituting a nuclear transcription factor normally upregulated under hypoxic conditions [13]. In order to observe the development of hypoxia, we investigated expression levels of HIF-1 α in renal tissues. No HIF-1 α expression was detected in the sham-operated group (Fig. 5A.a). In contrast, the 5/6 Nx group without Ad-netrin-1 treatment (Fig. 5A.b) had strong HIF-1 α expression in the nuclei of both renal tubular epithelial cells and interstitial cells; however, HIF-1 α expression was markedly decreased in the Ad-netrin-1-treated 5/6 Nx group (Fig. 5A.c) when compared with 5/6 Nx only group; this effect was statistically significant when assessed by quantitative image analysis ($P < 0.05$; Fig. 5B).

Similarly, the mRNA and protein levels of HIF-1 α (Fig. 5C and D) were only minimally expressed in the sham-operated group, but was found to be significantly increased in both groups following 5/6 Nx ($P < 0.05$). Importantly however, HIF-1 α expression decreased significantly in the Ad-netrin-1-treated 5/6 Nx group compared with 5/6 Nx group treated with the control adenovirus ($P < 0.05$).

Discussion

The data presented in this study provides evidence that the administration of recombinant netrin-1, a laminin-related neuronal guidance cue, can attenuate renal dysfunction and the development of tubulointerstitial fibrosis. Furthermore, our data also suggests that netrin-1 plays a role in the prevention of PTC loss and hypoxia in the remnant kidney. Taken together, this study provides novel insights regarding the roles of netrin-1 in the process of renal fibrosis. This conclusion is in agreement with the general concept that netrin-1 not only plays a key role in neuronal development, but also has an important role outside the nervous system.

Endogenous netrin-1, predominantly expressed by floor plate cells in the central nervous system, induces axonal outgrowth and orientation, and neuronal migration during neuronal development [4, 14]. Recent studies have indicated a role for netrin-1 beyond the nervous system including the development of the lung and blood vessels [15, 16]. In the context of the current work, the kidneys are among the highest netrin-1-expressing organs studied. Furthermore, research has shown that netrin-1 expression is highly induced in renal tubular epithelial cells and down-regulated in endothelial cells in vivo after ischemia-

reperfusion injury of the kidney, and that netrin-1 can protect against ischemia-reperfusion injury of the kidney [6]. With regard to kidney disease, netrin-1 is used as an early diagnostic biomarker and has also been shown to play an important role in the pathophysiology of acute and chronic kidney diseases [17, 18]; however, the pathways that are activated by netrin-1 and their role in chronic kidney diseases are largely unknown. To our knowledge, this is the first study to document the role of netrin-1 in an animal model of chronic kidney disease.

The 5/6 Nx rat model is a well-established model of chronic kidney disease [19]. In agreement with this, we observed significant renal dysfunction in our 5/6 Nx rats, as indicated by increased urine albumin secretion, serum BUN and Scr levels. We found that the administration of recombinant netrin-1 into the remnant kidney suppressed the increase in urine albumin, BUN and Scr observed in the control rats. As indicated above, netrin-1 expression is dysregulated in the kidney after ischemia-reperfusion injuries. In this model, the administration of recombinant netrin-1 attenuated renal dysfunction, possibly by reducing the expression of chemokines and inhibiting leukocyte infiltration. Others have speculated on the beneficial effects of netrin-1, positing that the protective effect of netrin-1 overexpression is likely due to the marked suppression of apoptosis in the kidney [20]. Netrin-1 can also protect renal tubular epithelial cells against cisplatin-induced kidney injury by suppressing apoptosis and inflammation [21], which suggests that netrin-1 may also be a useful therapeutic molecule for treating cisplatin nephrotoxicity and other inflammatory diseases.

Although PTCs are essential for maintaining the normal structure and function of the renal tubules, little is known about the role of PTC in the development of interstitial fibrosis. PTCs are essential for supplying oxygen to the tubular system, and their role has recently been emphasized both in renal diseases and chronic allograft nephropathy [22, 23]. It is thought that hypoperfusion and injury to the PTC may result in interstitial hypoxia and fibrosis, as well as tubular damage. An increasing number of studies have also shown that PTC loss may be involved in chronic ischemia and hypoxia in the tubulointerstitium, which could stimulate the renal scarring process, and that hypoxia itself can initiate the inflammatory process and eventually lead to tissue fibrosis [23]. In our model, we found loss of PTC in the remnant kidney occurring 12 weeks post-Nx. To confirm the development of hypoxia following PTC loss we investigated tubulointerstitial expression levels of HIF-1 α . HIF-1 α is the master hypoxia response regulator, the expression of which is determined by oxygen-dependent degradation. Our results showed that there was no detectable expression of HIF-1 α in the normal kidney cortex (sham-operated group), which is consistent with previous reports [13]. Furthermore, the administration of recombinant netrin-1 into remnant kidneys attenuated PTC damage and the induction of HIF-1 α in 5/6 Nx rats.

Recent studies have also shown that netrin-1 promotes blood perfusion after skeletal muscle ischemia, acting by promoting revascularization and inhibiting leukocyte migration in sepsis [5]. The vascular system shares many striking similarities with the peripheral nervous system, as many of the molecular players originally uncovered as regulators of axon path-finding have recently been found to affect branching and development of blood vessels [24]. Based on these findings, the role of netrin-1 in the vascular system has gained extensive attention. Previous studies have found that netrin-1 is a potent vascular mitogen that can enhance the migration and proliferation of primary vascular endothelial cells and stimulate angiogenesis [25]. By using the gain-of-function/loss-of-function approach, Wilson et al [5] demonstrated that Netrins accelerate angiogenesis in mammals and zebrafish, and that netrin-1 stimulates the proliferation, migration, and tubular formation of endothelial cells. Additionally, they showed that Netrins accelerate neovascularization and reperfusion in an in vivo model of ischemia and restore nerve conduction velocity.

Other studies have indicated that HIF-1 α dependent induction of netrin-1 attenuates hypoxia-elicited inflammation at mucosal surfaces [26]. The hypothesis that hypoxia can induce inflammation has gained general acceptance from studies of the hypoxia signaling pathway [27]. The accumulation of inflammatory cells in multiple organs and elevated serum levels of cytokines occur in mice after their short-term exposure to low oxygen concentrations

[26]. Hypoxia-induced changes in gene expression by epithelial cells help to increase the production of anti-inflammatory signaling molecules, such as adenosine, by the epithelium [28]. Previous studies have implicated that adenosine is involved in tissue adaptation to hypoxia [29]. HIF enhances adenosine receptor signaling by increasing the cell surface expression of adenosine receptors (AR), which attenuates immune responses, vascular fluid leakage, and neutrophil accumulation in the presence of renal damage [30]. Adora2b is the most insensitive AR, requiring adenosine concentrations in the micromolar range, whereas the activation of Adora1a or Adora2a occurs at much lower adenosine concentrations [31]. Additional transcriptionally regulated pathways that enhance extracellular adenosine signaling during hypoxic conditions involve the HIF-dependent repression of adenosine kinase (AK) or the HIF-dependent induction of the neuronal guidance molecule, netrin-1 [26]. Indeed, netrin-1 has been implicated in an alternative pathway of Adora2b activation and was previously shown to protect the kidneys during ischemic AKI [32]. Taken together, it is likely that netrin-1 stimulates angiogenesis and attenuates hypoxia-elicited inflammation *in vivo* through adenosine signaling and augments the response to vascular endothelial growth factor.

Conclusion

The angiogenic factor, netrin-1, was found to stabilize renal function and reduce renal fibrosis in the experimental 5/6 Nx rat model of kidney disease. This beneficial effect of netrin-1 appears to be mediated by preservation of PTC endothelium and is associated with partial reversal of impaired angiogenesis. This effect is also independent of proteinuria changes. Our results suggest that the axonal attractant netrin-1 may represent a novel therapeutic strategy to combat progressive renal disease. Further studies are being performed in different models of renal diseases to confirm this beneficial and promising effect.

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