

Glial Cell Line–Derived Neurotrophic Factor and Its Receptor Ret Is a Novel Ligand-Receptor Complex Critical for Survival Response during Podocyte Injury

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Glomerulosclerosis correlates with a reduction in podocyte number that occurs through mechanisms that include apoptosis. Whether glial cell line–derived neurotrophic factor (GDNF), a growth factor that is critical for neural and renal development, is a survival factor for injured podocytes was investigated. Ret, the GDNF receptor tyrosine kinase, was upregulated in podocytes in the passive Heymann nephritis and puromycin aminonucleoside (PA) nephrosis rat models of podocyte injury. In addition, Ret mRNA and protein were upregulated in mouse podocytes *in vitro* after injury that was induced by sublytic C5b-9 and PA. GDNF, which also was induced during podocyte injury, inhibited significantly the apoptosis of podocytes that was induced by ultraviolet C irradiation. Knockdown of Ret expression by small interference RNA in podocytes exacerbated apoptosis that was induced by both ultraviolet C and PA. Ret knockdown, upon injury, decreased AKT phosphorylation, suggesting that the phosphoinositol-3 kinase/AKT pathway mediated the survival effect of GDNF on podocytes. Consistent with this hypothesis, the selective phosphoinositol-3 kinase inhibitor LY294002 blocked the survival-promoting effects of GDNF. In conclusion, GDNF is a novel podocyte survival factor. Furthermore, Ret is highly upregulated during podocyte injury *in vitro* and *in vivo*, suggesting that Ret activation is a critical adaptive response for podocyte remodeling and repair.

J Am Soc Nephrol ●●: ●●●–●●●, ●●●●. doi: 10.1681/ASN.2005080835

Diabetic and nondiabetic glomerular diseases that result in proteinuria and glomerulosclerosis are the leading causes of adult chronic renal disease and ESRD in the United States. The prevalence and incidence are predicted to increase significantly during the next decade (1). Recent studies have identified an important role for the podocyte in the pathogenesis of adult proteinuric diseases. Podocytes, or glomerular visceral epithelial cells, are terminally differentiated and morphologically complex cells that form the final barrier to protein leakage in the glomerulus (2,3). In response to injury, podocytes secrete cytokines, proteases, oxidants, and matrix proteins (3).

A large body of experimental and clinical literature points to the importance of podocyte loss in the development and progression of glomerulosclerosis (4–6). This link and the relationship between podocytopenia and proteinuria have also been observed in clinical studies of types 1 and 2 diabetes (7–9) and other, nondiabetic renal disease (10). These studies raise important questions regarding the mechanisms of podocytopenia, such as apoptosis, detachment, and a lack of proliferation. It is possible that progression of glomerulosclerosis can be diverted with prevention of the podocyte loss from apoptosis. During

the injury process, there is a critical period of coordinated gene expression that determines whether podocytes survive or are lost. We postulated that survival factors are expressed in response to podocyte injury and act to support the recovery of injured podocytes. Given the similarities between the terminally differentiated podocyte and the neuron, we investigated whether glial cell line–derived neurotrophic factor (GDNF), a growth factor that is critical for neuronal development, is a survival factor for injured podocytes.

GDNF initially was identified from a rat glioma cell line as a survival factor for dopaminergic midbrain neurons (11). Since its initial discovery, the functions of GDNF in mammalian development have expanded greatly (12,13). GDNF now is known to be essential for parasympathetic, enteric, and motor neuron development; for spermatogenesis; and for kidney morphogenesis (12–14). In kidney development, GDNF acts as an inductive factor that is secreted from the nephrogenic mesenchyme, which promotes ureteric bud formation (15–17). GDNF knockout mice have kidney agenesis and die only hours after birth, making *in vivo* studies of GDNF function in the kidney limited (15,17). In addition to the kidney agenesis that is seen in the absence of GDNF, mice that lack its signal transducing receptor, the Ret receptor tyrosine kinase, or in mice that lack the co-receptor that is necessary for GDNF binding and Ret activation, GDNF family receptor- α (GFR α), also have kidney agenesis (18–21).

Because both GDNF and Ret null mice have kidney agenesis, no study has characterized whether Ret and GDNF are involved directly in glomerular or podocyte development or

Received August 9, 2005. Accepted March 23, 2006.

Published online ahead of print. Publication date available at www.jasn.org.

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whether Ret or GDNF plays a direct role in kidney disease. We report here for the first time that Ret is expressed specifically in podocytes during development and in adulthood. GDNF and Ret are induced significantly in animal models of podocyte injury. Moreover, GDNF, acting through Ret, is a potent survival factor for injured podocytes and promotes protection from injury *via* activation of the phosphoinositol-3 kinase (PI3-K)/AKT pathway.

Materials and Methods

Cell Culture

Two conditionally immortalized mouse podocyte cell lines were used for the cell culture experiments, referred to as heat-sensitive mouse podocytes (HSMP). The methods that are used to generate these cell lines have been described previously (22). All experiments were performed on differentiated cells from passages 14 to 24 and were performed on triplicate plates from two to four independent cultures. Podocytes were maintained at 10% FBS in RPMI (Invitrogen Corp., Carlsbad, CA) and replaced with fresh medium 24 h before all experiments.

C5b-9 Complement Injury of Podocytes

For determination of whether Ret expression is regulated during podocyte injury, sublytic antibody C5b-9 stimulation was used as an *in vitro* model. Differentiated HSMP were divided into three groups as described previously (23,24). For ensuring sublytic complement injury, the selection of the antibody and complement concentrations was based on the maximal amounts of antibody and complement that could be used without inducing cell lysis (defined as >5% lactate dehydrogenase release), using the LDH Kinetic Assay Kit (Sigma, St. Louis, MO). Ret expression was measured by Western blot analysis of whole-cell lysates (WCL) from these cells after 24 h, as described next.

Semiquantitative Reverse Transcription-PCR

After the treatments described in the figures, RNA was purified from podocytes using the TRIzol reagent (Sigma) and was reverse transcribed by using Superscript II Endo H (Invitrogen). This cDNA subsequently was used for PCR. Actin was amplified to control for the amount of mRNA present in each sample. Care was taken not to go beyond the linear range of the PCR reactions, and only the minimum number of cycles that were necessary to amplify a detectable product was used. For GDNF, Ret, and actin amplifications, the number of cycles used were 45, 35, and 25, respectively. PCR products were electrophoresed on agarose gels and quantified with a ChemiDoc XRS imaging system using QuantityOne software (Bio-Rad Laboratories, Inc., Hercules, CA). The primers were as follows: GDNF forward ATGAAGTTATGGGATGTCGTGGCTG and reverse ACCGTTTAGCGGAATGCTTCTTAG, Ret forward GGATGGAGAGGCCAGACAACCTGCAGC and reverse CCATAGAGTTTGTTTCAATCCATGTGG, and actin forward TATGGAGAAGATTTGGCACC and reverse GTCCAGACGCAGGATGGCAT.

Real-Time PCR

The expression of neurturin (NRTN), GFR α 1, and GFR α 2 was analyzed by using real-time PCR. Probe-based primer sets were generated using light upon extension technology (Invitrogen), and quantitative PCR was performed on a MiniOpticon System (Bio-Rad Laboratories, Inc.). NRTN, GFR α 1, and GFR α 2 expression was monitored with FAM-labeled probes and compared directly to glyceraldehyde-3-phosphate dehydrogenase by multiplex analysis with a JOE-labeled glyceralde-

hyde-3-phosphate dehydrogenase primer set (Invitrogen). The cycle conditions were as follows: 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. After 45 cycles, melting profiles were produced to confirm amplification of the intended targets. The primer sequences were as follows (* indicates the location of the fluorophore): GFR α 1 forward CGGAACAGCGAGACCATCCTTC*G and reverse CCAATGTATCGGGCAGTACACA, GFR α 2 forward CGGAGGACAACCTGCAAGAAGCTC*G and reverse GCAGCGTTCAGTGGGAGAGA, and NRTN forward CCGTCTACACGTCGGATGAGAC*G and reverse CCAGTCTAGATGCCGATG.

Experimental Design to Determine Whether GDNF Protects Podocytes

Podocytes were exposed to recombinant GDNF (25) at 50 ng/ml or vehicle alone (1 mM HCl) beginning on day 1 of restrictive conditions. GDNF was replaced every 48 h in the culture medium for 14 d. To measure podocyte number, phase contrast photos were taken 6 and 12 d after exposure to GDNF or vehicle alone at day 12. Immunocytochemistry using goat anti-actin (1:500; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was performed on methanol-fixed cells to assess the status of the actin cytoskeleton between GDNF- and vehicle-exposed podocytes at day 12. A biotinylated horse anti-goat secondary antibody was used at a 1:500 dilution, followed by streptavidin-conjugated Alexa Fluor 594 (1:200, Invitrogen), to visualize actin.

MTT Assay

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assays were used as a measurement of cell viability. HSMP cells were exposed to GDNF or vehicle alone from day 1 to day 9 under restrictive conditions, at which point the MTT assay was performed (Nonradioactive Cell Proliferation Assay; Promega, Madison, WI).

Cell-Cycle Analysis by Flow Cytometry

HSMP cells were plated at 2500 cells/cm² in 10-cm plates and treated with GDNF (50 ng/ml) or vehicle alone from day 1 of restrictive conditions. At differentiated days 8 to 10, cells were trypsinized, harvested, centrifuged, and prepared for cell-cycle analysis by flow cytometry. Cells were resuspended and fixed in 70% ethanol for 1 h at 4°C. Cells were washed twice in PBS before staining with propidium iodide (PI; 10 μ l of Igepal, 20 μ g/ml RNase A, and PI 50 μ g/ml [all from Sigma] in 1 \times PBS) for 10 min at 37°C. DNA content was analyzed by flow cytometry using a BD FACScan analyzer (BD Biosciences, Rockville, MD). The percentage of the cells in G₁, G₀, and G₂/M phases was determined. Relative cell size was determined by forward-angle light scatter. All experiments were repeated at least three times. The data were presented as the mean value with SD and represent the percentage of DNA content in the indicated cell-cycle phase(s) from three independent experiments.

Measuring Apoptosis in Cells Exposed to GDNF

Cells were exposed to GDNF (50 ng/ml) or vehicle alone for 60 min, and apoptosis then was induced by ultraviolet-C (UV-C) irradiation (25 mJ/m²). For determination of whether the PI3-K/AKT pathway mediated the effect of GDNF on podocyte survival, a selective PI3-K inhibitor (LY294002, 50 μ M; Cell Signaling Technology, Beverly, MA) or vehicle (DMSO) was given 1 h before GDNF exposure in the presence and absence of UV-C. Apoptosis was measured by counting Hoechst 33342-positive (Sigma) nuclei that were pyknotic and by measuring caspase-3 activity. For Hoechst staining, in each sample, 300 to 400 cells were randomly counted and scored as positive or negative. Apoptosis was determined 3 h after UV and expressed as the percentage of

positive cells. A colorimetric assay was used to measure caspase-3 activity (BD ApoAlert Caspase-3 colorimetric assay kit; BD Biosciences). Cells were harvested for caspase activity measurements 3 h after UV. Samples were read by a Packard SpectraCount (405 nm; Packard Bioscience, Meriden, CT).

Western Analysis of Ret

Protein expression of Ret, phospho-Y905Ret, phospho-serine 473-AKT, total AKT, and p53 was measured by Western analysis as described previously (25). Confluent HSMP were washed and denatured. Equal volumes of WCL were separated by SDS-PAGE using 4 to 12% gradient Tris-glycine minigels (Invitrogen) and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were blocked with 2% BSA (Sigma) in Tris-buffered saline (pH 7.4) that contained 0.1% Tween-20 at room temperature for 1 h. The blots were probed overnight at 4°C using rabbit polyclonal antibodies to Ret9 and Ret 51 (Santa Cruz Biotechnology, Inc.), to phospho-serine 473-AKT, to total AKT, and to p53 (Cell Signaling Technology). The Ret9 antibody was used at 1:500 dilution, and all other antibodies were used at a 1:1000 dilution. The immunoblots were visualized using a chemiluminescent substrate (Pierce Biotechnology, Inc., Rockford, IL).

Ret Knockdown Using Small Interference RNA

Ret small interference RNA (siRNA) knockdown was performed by using transient transfection of pooled functionally validated Ret siRNA (SMARTpool mouse RET siRNA; Dharmacon, Lafayette, CO). HSMP cells that were differentiated for 10 to 12 d were maintained at 10% FBS/RPMI as described above and transfected using the i-Fect siRNA transfection reagent (Neuromics, Northfield, MN). For determination of the transfection efficiency, a Texas Red–labeled siRNA (siGLO RISC-Free siRNA; Dharmacon) was co-transfected with Ret siRNA and visualized using fluorescence microscopy. For control siRNA samples, identical conditions were used with the substitution of siGLO-RISC-Free siRNA for Ret siRNA. For determination of the efficiency of Ret knockdown, Western analysis for Ret was performed on WCL from cells 24 to 96 h after the transfection. Several concentrations of Ret siRNA (40, 60, and 100 nM) were tested to determine optimal knockdown conditions. For apoptosis assays, podocytes were exposed to UV-C or PA (40 µg/ml) on days 3 to 4 after transfection of Ret siRNA or control siRNA (100 nM). Apoptosis was measured by counting podocytes with Hoechst-positive pyknotic nuclei 3 h after UV and 5 h after exposure to PA.

Embryonic Kidneys

For determination of the temporal expression of Ret during podocyte development by immunostaining, embryonic kidneys of gestational ages E18, E21, and P1 were harvested from C57/Blk6 mice (Simonsen, Gilroy, CA). Kidneys were fixed in methyl Carnoy's solution and paraffin embedded for immunoperoxidase staining.

Experimental Animal Models of Podocyte Injury

Passive Heymann nephritis (PHN) was induced in male Sprague-Dawley rats (180 to 200 g; Simonsen) by intraperitoneal injection of sheep anti-mouse podocyte IgG (26). A control group of rats received normal sheep serum. Control and PHN rats were killed on day 10 ($n = 3$ per group) after antibody injection. For puromycin aminonucleoside nephrosis (PAN) induction, male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) were given puromycin (6 mg/100 g; Sigma) by tail-vein injection. Control (saline injection alone) and PAN rats were killed and their kidneys were harvested on day 7 after the injections. All animal studies were conducted according to the animal

welfare guidelines of the University of Washington and the University of Buffalo, SUNY.

Immunostaining Analysis

Kidneys from mice or rats were fixed as described previously (26). Tissues were stained for Ret using the avidin-biotin indirect immunoperoxidase method. Sections were incubated overnight at 4°C with a goat anti-Ret antibody (1:100 to 1:200; R&D Systems, Minneapolis, MN). The specificity of this antibody for immunohistochemistry was confirmed previously in tissues from Ret knockout mice (27). This was followed by a secondary biotinylated rabbit anti-goat IgG (Zymed, San Francisco, CA) and horseradish peroxidase streptavidin (Vector, Burlingame, CA). 3,3'-Diaminobenzidine (Sigma) with nickel chloride (NiCl) was used as the chromogen with the PAN tissues and without NiCl with the PHN tissues. To determine whether Ret immunostaining co-localizes with WT-1, a podocyte-specific marker, we performed double staining by immunostaining for WT-1 using a rabbit polyclonal WT-1 antibody (1:20; Santa Cruz Biotechnology) and Ret. Anti-goat Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 (Invitrogen) were used to visualize Ret and WT-1, respectively. All antibodies were diluted in PBS with 2% BSA. Some sections were counterstained using either periodic acid-Schiff or methyl green.

Microscopy

All immunohistological stainings were imaged with an Axiovert 200M microscope (Carl Zeiss Inc., Oberkochen, Germany), and photos were generated by using Axiovision v4.5 software.

Results

GDNF and Ret Are Upregulated after Podocyte Injury In Vitro

To investigate whether GDNF and Ret are involved in podocyte injury, we measured the mRNA levels of GDNF and Ret by using reverse transcription-PCR (rt-PCR). Mouse podocytes were exposed to PA, a toxin that is known to induce PAN in rodents, for various lengths of time. A low abundance of Ret mRNA was detected in control cells that were not exposed to PA (Figure 1). In contrast, Ret was upregulated upon podocyte injury with PA by 6 h, and the level of Ret peaked within 24 h (Figure 1A). Amplification of actin confirmed the analysis of equal amounts of cDNA. Quantification of the data revealed that Ret was upregulated by 3.1-fold. Similar to Ret, GDNF mRNA was expressed at low abundance in control cells, and GDNF mRNA upregulation was not detected at either 3 or 6 h after injury with PA (data not shown) but was observed to peak within 12 h and declined thereafter (2.2-fold induction; Figure 1B).

To determine whether the level of Ret protein increased in other models of podocyte injury, sublytic complement (C5b-9) injury was used. Podocytes first were exposed to a sheep anti-mouse antibody and then exposed to serum to induce sublytic complement attack. WCL were harvested from these cells after 24 h and were analyzed by using Western blotting (Figure 2A). Densitometric analysis revealed that Ret9 protein was upregulated by at least 4.5-fold compared with control, uninjured podocytes. Ret was detected as a doublet from both podocytes and primary superior cervical ganglia neurons, which in culture express Ret in abundance, and were used as a positive control (last lane). Analysis of GDNF protein expression was

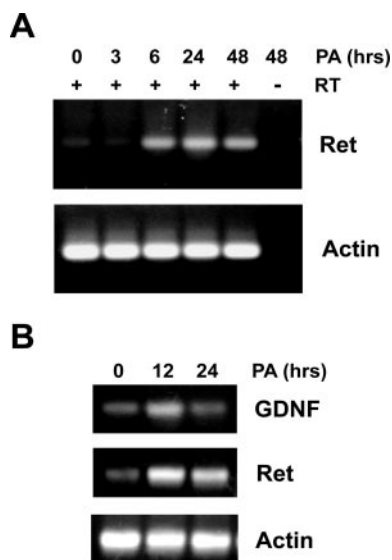


Figure 1. Ret and glial cell line–derived neurotrophic factor (GDNF) are upregulated upon injury in podocytes *in vitro* as determined by semiquantitative reverse transcription–PCR (RT-PCR). (A) Mouse podocytes in culture were exposed to puromycin aminonucleoside (PA) at 10 μ g/ml for 3, 6, 24, or 48 h or with vehicle alone, and total RNA was purified. RNA was subjected to RT-PCR analysis for Ret (top) or actin to control for the cDNA samples (bottom). To control for potential contamination of the RNA with genomic DNA, RNA that had not been reverse transcribed did not amplify PCR products (RT; right-most lane). The approximate sizes of the products amplified were 550 bp for GDNF, 280 bp for Ret, and 300 bp for actin. (B) For determination of whether GDNF was increased after PA exposure, GDNF and Ret mRNA were measured by RT-PCR 12 and 24 h after PA exposure. Densitometric analyses indicate a 3.1- and 2.2-fold induction of Ret and GDNF at 12 h, respectively. Results were obtained from three independent experiments.

not conducted because of the very low levels of expression that typically has been observed and because of the inadequate sensitivity of the antibodies that are available. Podocytes injured by PA exposure also showed increased Ret9 expression as early as 12 h and maintained this upregulation at least 48 h after injury (Figure 2B). It is interesting that, Ret51 expression was much lower than Ret9 in podocytes, and only a modest upregulation of Ret51 could be detected with PA injury (Figure 2B). Taken together, these data indicate that Ret mRNA and protein and GDNF mRNA are upregulated subsequent to podocyte injury in two independent *in vitro* models. To investigate whether Ret is activated in an autocrine manner in podocytes, we used a phospho-Ret antibody that detects Ret selectively when autophosphorylated on the catalytic tyrosine Y905. Phospho-Y905Ret immunoblotting revealed an upregulation of Ret activity upon PA injury, consistent with the autocrine production of GDNF and subsequent activation of Ret. The kinetics of Ret autophosphorylation differed significantly from the increase in Ret protein (Figure 2B), indicating that the apparent induction in Ret autophosphorylation cannot be explained simply by an increase in Ret protein alone.

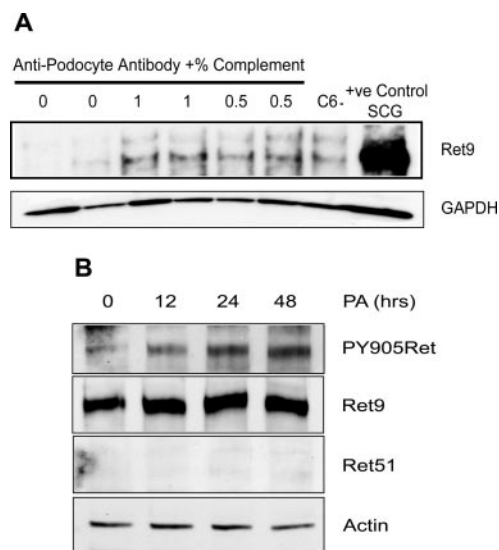


Figure 2. Upregulation of Ret protein after sublytic (C5b-9) complement and PA injuries *in vitro*. (A) Whole-cell lysates (WCL) were harvested 24 h after sublytic C5b-9 injury or control conditions, and Ret protein expression was determined by using Western blot analysis with a Ret9 antibody. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) immunoblotting served as a loading control and confirmed equal protein loading between samples. Ret protein was in low abundance in the absence of C5b-9 injury (lanes 1 and 2). In contrast, Ret increased after C5b-9 injury (lanes 3 through 6). A modest upregulation of Ret also was observed in cells treated with the sheep anti-mouse podocyte antibody in C6 serum, suggesting that there were some complement proteins in the culture medium (lane 7). WCL from superior cervical ganglia (SCG) neurons, which express very high levels of Ret, were assessed as a positive control to confirm that the molecular weight of Ret observed in podocytes corresponded with the glycosylated, mature forms of Ret (observed at 170 and 180 kD). Densitometric analyses revealed that Ret was upregulated by 4.5-fold upon C5b-9 injury at 24 h. (B) Podocytes were exposed to PA (10 μ g/ml) or vehicle alone, and WCL were harvested at 0, 12, 24, and 48 h. Ret9 and Ret51 protein expression was determined by using antibodies against Ret9 and Ret51 (second and third panels). Longer chemiluminescent exposures indicated a low abundance of Ret51 as compared with Ret9 expression in podocytes. Upregulation of both proteins was detected with PA injury. PY905Ret immunoblotting (top) revealed that Ret is activated in an autocrine manner upon PA injury in heat-sensitive mouse podocytes (HSMP), reaching a maximum within 48 h of PA treatment.

Ret Is Expressed by Developing Mouse Podocytes In Vivo

To identify cell types that express Ret in developing glomeruli, embryonic mouse kidneys were harvested from E18 mice and analyzed by immunohistochemistry. Ret was detected in cells on the outer aspect of the glomerular tufts, a location that is typical of podocytes, but not in primordial cuboidal and columnar cells in comma-shaped bodies (Figure 3A). In the cells that expressed Ret, Ret staining was localized to the membrane and not the nucleus (Figure 3A). To determine the specific cell type that expresses Ret, serial sections were stained

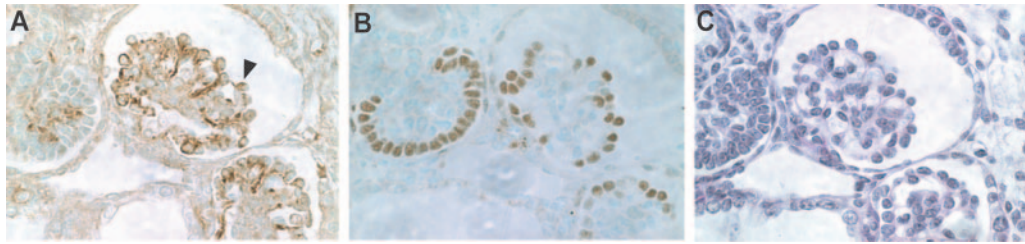


Figure 3. Ret expression in developing kidneys *in vivo*. (A) Mouse embryonic kidneys were immunostained using a pan-Ret antibody as described in Materials and Methods. Ret staining was restricted to the plasma membrane and was excluded from the nucleus (arrowhead). (B) WT-1 immunostaining of serial sections confirmed the co-localization of WT-1 with Ret in maturing podocytes. (C) Periodic acid-Schiff staining of kidney sections revealed that the morphology of glomeruli was intact.

with WT-1, a nuclear podocyte-specific protein of both primordial and developed podocytes. Cells that expressed both WT-1 and Ret were noted both from the capillary loop stage and in developed glomeruli (Figure 3, A and B). Periodic acid-Schiff staining, which allows the visualization of the various cell types and basement membranes, further indicated that Ret expression resides in podocytes within the glomeruli (Figure 3C). Similar results were observed in kidneys from E21 and P1 mice (data not shown).

In Vivo Models of Glomerular Injury Cause Ret Upregulation in Podocytes

To determine whether Ret also is regulated during podocyte injury *in vivo*, we studied PHN and PAN. A basal level of Ret staining was observed in podocytes from the kidneys of uninjured animals (Figure 4A, a and d). With PAN, the intensity of Ret staining increased in podocytes and was maximal within 7 d after injury (Figure 4A, b). Therefore, like the *in vitro* studies, Ret protein levels increase upon PAN injury in podocytes *in vivo*. To determine whether Ret upregulation also could be observed in other models of podocyte injury, we compared Ret expression in the kidneys of control *versus* PHN rats at day 10, and the results are shown in Figure 4A, d through f. There was a marked increase in Ret staining in podocytes in PHN rats at day 10. Co-staining of these sections with Ret and WT-1 indicated that Ret is expressed in the cell bodies and processes of podocytes (Figure 4B). Therefore, like the *in vitro* studies, podocyte injury that is observed in the PHN model increases the expression of Ret in podocytes. Taken together, these results indicate that Ret expression is upregulated in both immune and nonimmune models of podocyte injury *in vivo*.

GDNF Promotes Survival of Mouse Podocytes In Vitro

We used a culture system that generates podocytes that are postmitotic and differentiated and that express the appropriate podocyte-specific proteins (22). We tested whether GDNF can act as a survival factor for mouse podocytes *in vitro*. Podocytes under differentiating conditions were treated with GDNF for various lengths of time. Podocytes maintained in the presence of GDNF for the longest duration, 12 d, had the highest cell number (Figure 5A). In contrast, HSMP in the absence of GDNF had the least number of cells (Figure 5A, a). In addition, the staining pattern of actin was similar between GDNF- and ve-

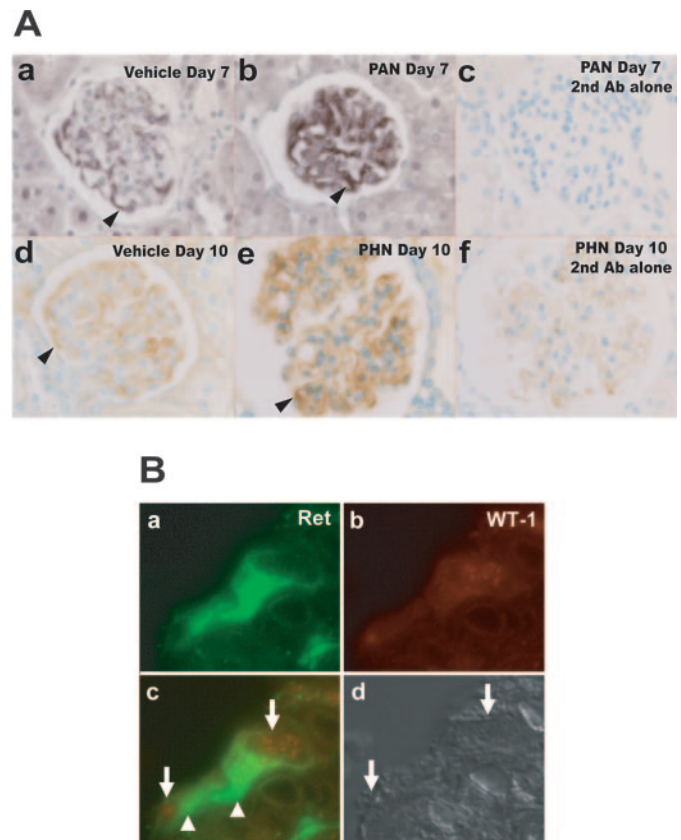


Figure 4. Ret expression is upregulated in animal models of podocyte injury *in vivo*. (A) PAN and passive Heymann nephritis (PHN) models: Immunoperoxidase staining of Ret using a pan-Ret antibody was performed on kidney sections from rodents subjected to PAN and PHN. (a and d) Basal level of Ret expression was detected in control animals at day 7, and this localized to podocytes (arrowheads). (b) Ret staining increased 7 d after PA injury compared with vehicle alone (arrowhead). (c) Absence of staining using the secondary antibody alone. (e) Ret expression was increased at day 10 of PHN in a distribution indicative of podocytes (arrowhead). (f) Secondary antibody alone showed some nonspecific capillary loop staining, which was observed to be constant between conditions (d and e). (B) Ret expression co-localizes to podocytes. (a and c) Ret expression (green) isolated to cell bodies and processes (arrowheads, composite; c). These cells also expressed WT-1 (red) in nuclei in b (arrows, composite; c). (d) Nomarski photo of these fluorescence images.

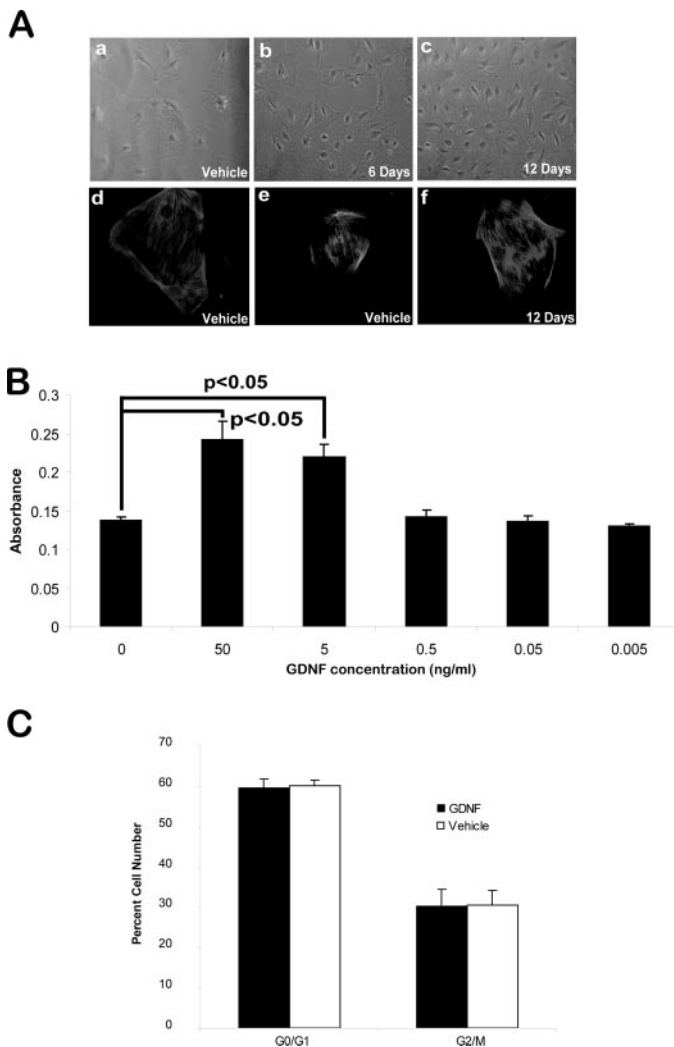


Figure 5. Exogenous GDNF increases podocyte number and enhances survival *in vitro*. (A) Mouse podocytes were exposed to vehicle alone for 12 d (a) and to GDNF at a concentration of 50 ng/ml for 6 d (b) or 12 d (c). Phase contrast microscopy revealed an increase in podocyte density with increasing lengths of time of exposure to GDNF. (d and e) Actin staining distributed throughout the cell bodies of podocytes exposed to vehicle alone for 12 d. Similar actin staining patterns were noted in GDNF-exposed cells for 12 d (f). These experiments were conducted in triplicate from three independent cultures on two different mouse podocyte cell lines. (B) For assessment of whether response to GDNF was dose dependent, podocyte viability was measured by using an MTT assay as described in Materials and Methods. Cells were treated with decreasing GDNF concentrations for 8 to 9 d, and their viability was measured and plotted. Cell viability was increased by 60 to 70% ($P < 0.05$) when GDNF was used at concentrations of 5 and 50 ng/ml. These results were done in triplicate in three independent cultures. (C) GDNF exposure did not cause proliferation or dedifferentiation of podocytes as determined by cell-cycle analysis using propidium iodide staining coupled with flow cytometry. There was no statistically significant difference in the percentage of cells in G_0/G_1 or G_2/M stages of the cell cycle in cells treated with GDNF (■) or vehicle alone (□).

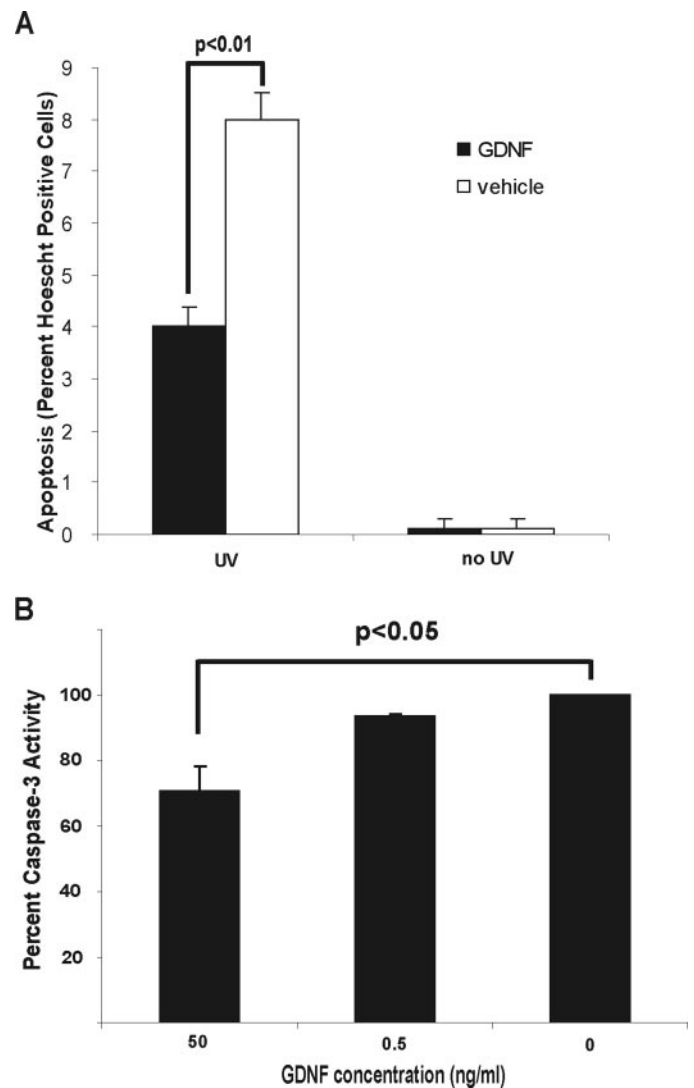


Figure 6. Exogenous GDNF protects podocytes from apoptosis. (A) Apoptosis was measured by counting Hoechst-positive, pyknotic nuclei in mouse podocytes exposed to ultraviolet-C (UV-C) irradiation (25 mJ/m²) in the presence of GDNF (■) or vehicle alone (□). GDNF decreased UV-induced apoptosis by two-fold compared with vehicle alone ($P < 0.01$). (B) Apoptosis was assessed under similar experimental conditions by measuring caspase-3 activity. GDNF (50 ng/ml) decreased caspase-3 activity by 30% ($P < 0.05$) compared with vehicle alone (expressed as 100%). These experiments were conducted in triplicate from two independent cultures.

vehicle-exposed podocytes, indicating that GDNF did not alter the actin cytoskeletal organization of podocytes *in vitro* (Figure 5A, d through f). Likewise, the viability of GDNF-treated podocytes increased in a dose-dependent manner, as determined by MTT assays (Figure 5B). Both 5 and 50 ng/ml GDNF maintained podocyte viability maximally ($P < 0.05$ compared with control), similar to the effective doses observed in other cell types. To determine whether exogenous GDNF increased the number of podocytes by stimulating their proliferation, the cell-cycle stage of GDNF-treated podocytes was examined by

using PI staining coupled with cell-cycle analysis by flow cytometry. There was no change in the number of G₀/G₁ or G₂/M cells upon GDNF treatment as compared with control cells (Figure 5C). These results demonstrated that GDNF acted as a survival factor for podocytes *in vitro* and increased significantly the number of differentiated podocytes without altering their cell-cycle stage.

GDNF Protects Mouse Podocytes from Apoptosis

To investigate whether GDNF inhibits apoptosis, we pretreated differentiated mouse podocytes with exogenous GDNF before UV-C irradiation and measured apoptosis by monitoring both nuclear condensation with Hoechst staining and caspase-3 activity. In the presence of GDNF, there was a 2.5-fold decrease in podocyte apoptosis as compared with cells without GDNF (3.7 ± 0.4 versus $7.5 \pm 0.5\%$; $P < 0.01$; Figure 6A). Likewise, GDNF significantly inhibited UV-induced caspase-3 activity in a dose-dependent manner, consistent with the notion that GDNF is protective under conditions of cellular stress ($P < 0.05$; Figure 6B).

Ret Knockdown with siRNA Exacerbates Podocyte Apoptosis

Because Ret and GDNF null mice have kidney agenesis (15,17,20) and die just after birth, we used an *in vitro* Ret knockdown system to determine whether Ret is necessary for podocyte survival. We consistently achieved close to 100% siRNA transfection efficiency in podocytes as visualized by co-transfecting a fluorescently tagged RISC-free siRNA marker with vehicle and 100 nM Ret siRNA (Figure 7A). We did not observe any morphologic changes between cells with or without Ret siRNA knockdown during the 4 d after transfection. Confocal microscopy was used to exclude the possibility that the fluorescence observed was membrane associated as a result of adsorption of the marker on the cell surface (data not shown). Knockdown of Ret expression was determined to be maximal between days 3 and 4 after transfection (Figure 7B).

For testing whether Ret is involved in the injury process of podocytes *in vitro*, podocytes subjected to Ret knockdown were injured with PA. The knockdown of Ret alone did not induce podocyte apoptosis. However, significantly more podocytes underwent apoptosis when injured with PA in the presence of Ret knockdown (8.0 ± 1.7 versus $2.7 \pm 0.6\%$; $P < 0.05$) by measuring nuclear condensation (Figure 8A). Likewise, more podocytes subjected to UV-C injury underwent apoptosis when Ret expression was knocked down, as compared with podocytes that expressed a basal level of Ret (10.9 ± 0.4 versus $2.4 \pm 0.9\%$; $P < 0.01$; Figure 8B). These studies indicate that, in the absence of Ret receptor activation, injured podocytes are considerably more likely to undergo apoptosis.

Survival Effect of GDNF and Ret Requires the PI3-K/AKT Pathway

One of the most important survival pathways that are regulated by growth factors is the PI3-K-mediated activation of the kinase AKT. To determine whether GDNF promoted survival *via* the PI3-K/AKT pathway, we used Western blot analysis to investigate whether Ret knockdown in injured podocytes al-

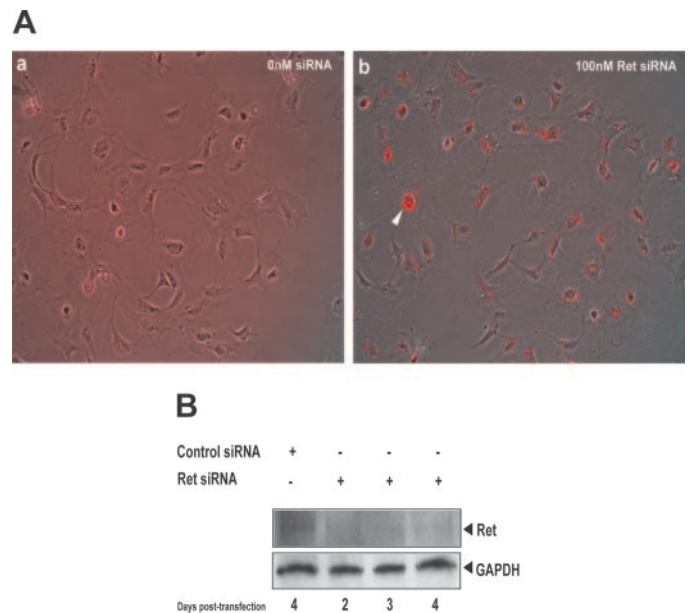


Figure 7. Ret receptor knockdown using small interference RNA (siRNA) in podocytes. (A) Transfection efficiency: mouse podocytes were transfected with 100 nM concentrations of Ret siRNA or vehicle alone. (a) When podocytes were exposed to i-Fect alone, there was no toxicity. (a and b) A transfection efficiency of nearly 100% was achieved with 100 nM concentration of Ret siRNA (b). Co-transfection with a fluorescently tagged control siRNA was used to determine the transfection efficiency, and fluorescence microscopy revealed a perinuclear localization of the tagged RNA (b, arrowhead). (B) Western blot analysis of Ret after transfection: Ret immunoblotting (top) of WCL 2, 3, or 4 d after transfection revealed that Ret was downregulated within 2 d after transfection with 100 nM Ret siRNA. Transfection of control siRNA at day 4 served as a negative control, and the maximal knockdown of Ret was observed 4 d after transfection. GAPDH immunoblotting confirmed equal protein loading (bottom).

tered the phosphorylation of AKT at Ser-473, which coincides with AKT kinase activity. Ret knockdown in podocytes dramatically decreased the level of p-Ser-473 AKT upon PA exposure (Figure 9A). However, downregulation of Ret did not affect the expression of p53, a molecule that is known to be upregulated during PA-induced podocyte apoptosis within 24 h (Figure 9A).

To test whether the PI3-K pathway is required for the protective effects of Ret on podocytes, we used the selective PI3-K inhibitor LY294002. Pretreatment of podocytes with LY294002 inhibited significantly the survival effect of GDNF against UV-C irradiation compared with vehicle alone (16.9 ± 1.1 versus $3.7 \pm 0.4\%$; $P < 0.01$; Figure 9, B and C). It is interesting that exposure of podocytes that had undergone Ret knockdown (reduces survival significantly, $P < 0.05$) to LY294002 did not cause any further increase in apoptosis, suggesting that the enhancement of apoptosis in injured podocytes upon Ret knockdown is due to the loss of AKT activation in these cells (Figure 9B). Taken together, these data indicate that the protec-

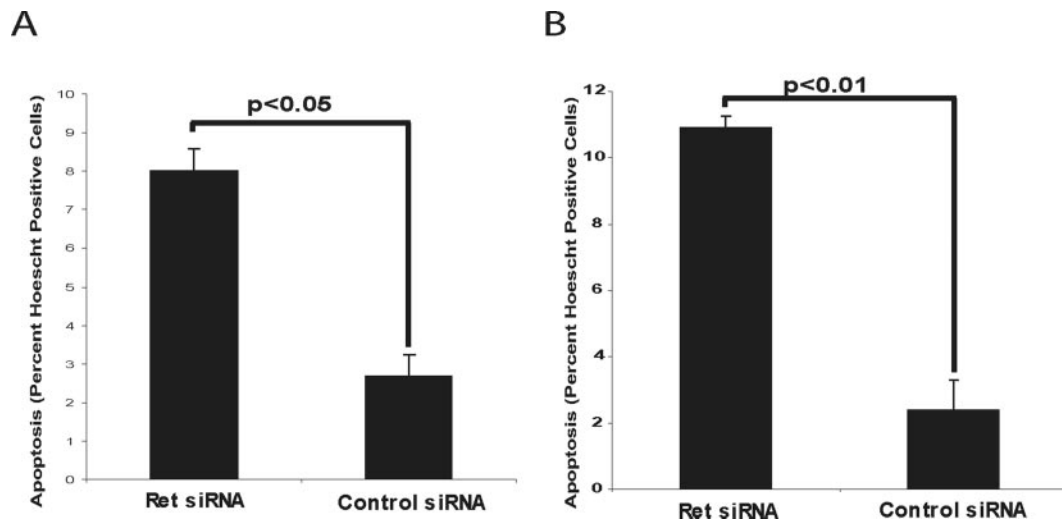


Figure 8. Knockdown of Ret expression exacerbates apoptosis after podocyte injury *in vitro*. (A) PA exposure induced apoptosis in cultured mouse podocytes transfected with control siRNA after 5 h. Apoptosis was measured by counting Hoechst-stained, condensed nuclei. In cells transfected with Ret siRNA, Ret knockdown was associated with a three-fold increase in apoptosis compared with control siRNA (8.0 ± 1.7 versus $2.7 \pm 0.6\%$; $P < 0.05$). (B) Cells transfected with Ret siRNA or control siRNA were UV irradiated. After 3 h, Ret siRNA-transfected cells had a five-fold increase in apoptosis as compared with control (10.9 ± 0.4 versus $2.4 \pm 0.9\%$; $P < 0.01$).

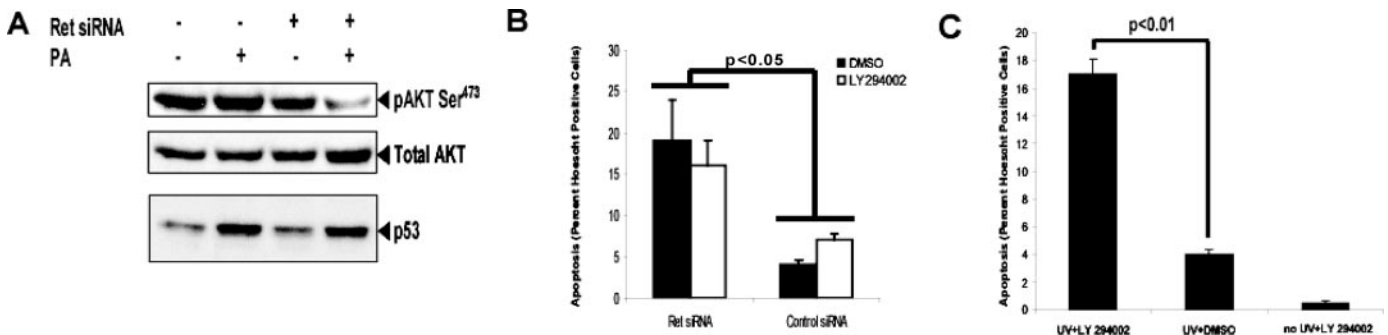


Figure 9. The survival effect of Ret requires the phosphoinositol-3 kinase (PI3-K)/AKT pathway. (A) PA exposure (24 h) decreased AKT phosphorylation on Ser-473 in cells transfected with Ret siRNA as compared with cells transfected with control siRNA (top). PA did not affect the expression of total AKT (middle) or phospho-AKT in control siRNA-transfected podocytes. Ret knockdown did not alter the upregulation of p53 that occurs during PA-induced apoptosis (bottom). (B) PA injury: A selective PI3-K inhibitor (LY294002, 50 μ M; □) did not further increase apoptosis in cells subjected to Ret knockdown. DMSO (■) was used as the vehicle control for LY294002. (C) UV irradiation: The *in vitro* survival effect of exogenous GDNF (50 ng/ml) was blocked by LY294002 treatment, as compared with cells exposed to vehicle alone, upon UV injury (16.9 ± 1.1 versus $3.7 \pm 0.4\%$; $P < 0.01$).

tive effects of GDNF and Ret activation are due to the Ret-dependent regulation of the PI3-K/AKT pathway.

Expression of NRTN, GFR α 1, and GFR α 2 Is Not Regulated with Injury in Podocytes

To determine whether the co-receptor for GDNF, GFR α 1, was also upregulated upon injury in podocytes, quantitative real-time PCR was conducted. We also examined the expression of NRTN and its co-receptor, GFR α 2, during podocyte injury, which are known to be expressed in developing kidney. Although GFR α 1, GFR α 2, and NRTN all were expressed in HSMP, their expression was not altered during PA injury of podocytes (Figure 10). Statistical analysis of these data indicated that there were no significant differences in any of the

injury conditions examined (all $P > 0.1$). Therefore, GDNF and Ret but not GFR α 1, GFR α 2, or NRTN are selectively upregulated in podocytes by injury and act in an autocrine, protective manner.

Discussion

Glomerulosclerosis is correlated with a reduction in podocyte number (6). Despite the known importance of podocytes in renal physiology and as a target of glomerular disease, the molecular mechanisms that control the survival of podocytes are incompletely understood. Ret was upregulated at the mRNA and protein level upon podocyte injury both *in vitro* and *in vivo*. Ret9 was the predominate isoform expressed in podocytes

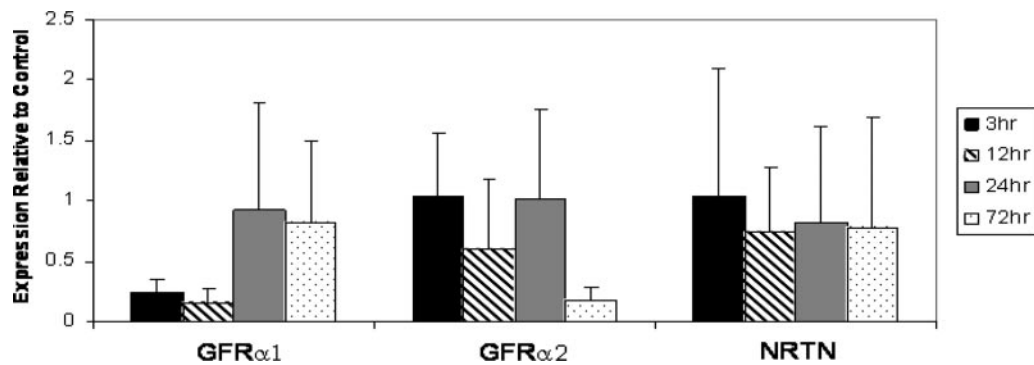


Figure 10. GDNF family receptor- α 1 (GFR α 1), GFR α 2, and neurturin (NRTN) expression is not altered upon podocyte injury. GFR α 1, GFR α 2, and NRTN expression were analyzed by using qPCR in podocytes injured with PA. HSMP were treated with vehicle alone for 72 h or with PA (10 μ g/ml) for 3, 12, 24, or 72 h. Total RNA was purified from the cells and reverse transcribed, and this product was subjected to real-time PCR analysis as described in Materials and Methods. The amount of cDNA in each sample was controlled for by multiplex analysis of GAPDH during the amplification of GFR α 1, GFR α 2, and NRTN. These data were graphed as the ratio of PA-treated cells to cells treated with vehicle alone. This experiment was conducted four to five times from two different independent cultures of HSMP, and error bars represent SEM.

cytes, and its expression was most upregulated during injury. GDNF was upregulated during podocyte injury as well. Application of exogenous GDNF to podocytes inhibited significantly their apoptosis upon injury with UV irradiation. Likewise, the selective silencing of Ret in podocytes exacerbated the proapoptotic effects of UV-C and PA. The PI3-K/AKT pathway was required for the protective effects of GDNF and Ret during podocyte injury, together implicating GDNF-dependent activation of Ret as a novel growth factor system in podocyte development and disease.

The observation that Ret was expressed in developing podocytes *in vivo* but was not detected in primordial podocytes suggests that Ret is involved in the maturation of podocytes. GDNF functions in kidney development as an inductive factor that is secreted from the nephrogenic mesenchyme and, through Ret activation, promotes ureteric bud formation (14). Mice that are heterozygous for GDNF have impaired ureteric bud formation, decreased nephron mass, and subsequent development of hypertension (28,29). The conclusion of these studies is that a reduction in GDNF, which diminishes ureteric bud branching during embryonic development, endows these mice with fewer nephrons. Our finding that Ret is highly expressed in developing glomeruli, along with the potent survival effects of GDNF on podocytes, may revise this view by suggesting that GDNF and Ret are critical for podocyte development and survival in the glomeruli and, therefore, contribute directly to nephron number independently. An important future direction will be to examine the requirement of Ret in podocyte development, which would require a conditional knockout strategy in podocytes because of kidney agenesis that occurs upon Ret deletion (20,21).

Many cell types respond to injury by enacting coordinated genetic programs. These responses can serve to modify the cell in an adaptive manner toward the noxious stimuli and promote their recovery after an injury. Alternatively, cells that are unable to recover from an injury express cell death programs to bring about their orderly demise. The upregulation of GDNF and Ret, in combination with the significant protective effects of GDNF, indicates that GDNF is part of

an adaptive, recovery response to podocyte injury. This is the first observation that GDNF, acting through Ret, functions in an injury paradigm in the kidney. These data raise the exciting therapeutic possibility of giving exogenous GDNF to patients with glomerular disease that is characterized by a loss of podocytes, such as diabetes, membranous nephropathy, and focal segmental glomerulosclerosis. Future *in vivo* studies of podocyte injury models with GDNF and other GDNF family ligands are critical to help delineate their potential in kidney disease.

These observations also raise the more general possibility that multiple growth factors may guide the development of kidneys as well as their remodeling during adult disease processes. Given that both GDNF and Ret are upregulated in injured podocytes, GDNF likely acts in an autocrine manner to support podocyte survival. Consistent with this hypothesis, Ret is activated in podocytes upon injury without the upregulation of NRTN, the other GDNF family member that is known to be expressed in kidney. GDNF may act in a paracrine manner as well, and there is evidence that mesangial cells can respond to GDNF *in vitro* (30,31). Because kidney diseases undoubtedly involve cross-talk mechanisms between cell types, growth factors such as GDNF are well poised to communicate such signals. It will be important in the future to consider other growth factor systems as a means of better understanding the complex communication and coordinated responses of various cell types during kidney disease.

Acknowledgments

This work was supported by National Institutes of Health grants to S.J.S. (DK60525, DK56799, and DK51096) and to B.A.P. (KO1-NS-045221). S.J.S. also is an Established Investigator of the American Heart Association.

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