Therapeutic Potential of Progranulin in Hyperhomocysteinemia-Induced Cardiorenal Dysfunction

Yi Fu,* Yu Sun,* Meng Zhou, Xiaojie Wang, Ziyong Wang, Xinbing Wei, Yan Zhang, Zeyu Su, Kaili Liang, Wei Tang, Fan Yi

Abstract—Hyperhomocysteinemia (hHcys) is an important independent risk factor for the development of cardiovascular disease and end-stage renal disease. Although multiple approaches lowering the levels of homocysteine have been used in experimental studies and clinical trials, there is no effective therapy available to fully prevent homocysteine-induced injury. Therefore, identifying key molecules in the pathogenic pathways may provide clues to develop new therapeutic strategies for the treatment of hHcys-associated injury beyond lowering the plasma homocysteine levels. In this study, we found that the levels of progranulin (PGRN), an autocrine growth factor, were significantly reduced in the kidney and heart from a mouse model of hHcys. We further observed that in hHcys, PGRN-deficient mice significantly exacerbated cardiorenal injury as evidenced by higher levels of urinary albumin excretion, more severe renal morphological injuries, including pronounced glomerular basement membrane thickening and podocyte foot process effacement, and adverse myocardial remodeling versus wild-type mice. Mechanistically, we found that PGRN-mediated Wnt/β-catenin signaling was one of the critical signal transduction pathways that links homocysteine to cardiorenal injury. Importantly, we finally provided direct evidence for the therapeutic potential of PGRN in mice with hHcys by pretreatment with recombinant human PGRN. Collectively, our results suggest that PGRN may be an innovative therapeutic strategy for treating patients with hHcys. (Hypertension. 2017;69:259-266. DOI: 10.1161/HYPERTENSIONAHA.116.08154.) • Online Data Supplement

Key Words: cardiac hypertrophy ■ glomerular filtration barrier ■ homocysteine ■ PGRN ■ Wnt pathway

Clinical and epidemiological studies have demonstrated that hyperhomocysteinemia (hHcys) is an important independent risk factor for the development of cardiovascular disease and end-stage renal disease. Although multiple approaches lowering the levels of homocysteine (Hcys) have been used in experimental studies and clinical trials, there is no effective therapy available to fully prevent Hcys-induced cardiac and renal injury. Therefore, identifying key molecules in the pathogenesis of Hcys will provide new therapeutic strategy for treating patients with Hcys. Progranulin (PGRN), an autocrine growth factor, has been identified in various tissues and is involved in a diversity of physiological and pathological processes, including tissue development, host-defense response, insulin resistance, and modulation of inflammation. Recent studies highlight the protective role of PGRN in chronic inflammation. Tang et al have reported that PGRN directly binds to tumor necrosis factor receptors and disturbs the tumor necrosis factor-α—tumor necrosis factor receptor interaction. Administration of recombinant human PGRN (rPGRN) significantly alleviates inflammatory responses in rheumatoid arthritis animal models. In cardiovascular system, PGRN deficiency exacerbates atherosclerosis in apolipoprotein E knockout mice by modulation of local and systemic inflammation. Recently, we also demonstrate that PGRN serves as a negative regulator of immunity by regulation of nucleotide-binding oligomerization domain containing 2—mediated immune responses in acute kidney injury. However, it keeps unknown the role of PGRN in the pathogenesis of hHcys. In this study, we found that PGRN levels were significantly reduced in the kidney and heart in mice with hHcys. Importantly, we further provided direct evidence for the therapeutic potential of PGRN in hHcys-induced cardiorenal dysfunction, which was associated with the negative regulation of Wnt/β-catenin signaling.

Materials and Methods

An extended Materials and Methods section can be found in the online-only Data Supplement.

Animals

Twelve-week-old male PGRN-deficient (Grn−/−) mice and wild-type C57BL/6 mice were purchased from the Jackson laboratory (Bar Harbor, ME).
Isolation of Glomeruli

Isolation of glomeruli was performed and confirmed as described, and the purity of glomeruli was estimated to be >98%. (Representative isolated glomeruli are shown in Figure S1 in the online-only Data Supplement.)

Echocardiography

Echocardiography was performed with a Vevo770 imaging system (VisualSonics, Toronto, Canada) using a 30-MHz high-frequency transducer.

Immunofluorescence Staining and Confocal Microscopy

Immunofluorescent staining and images obtained by an LSM780 laser scanning confocal microscope (ZEISS, Oberkochen, Germany) system were performed as described.

Statistics

Data are expressed as means±SE. The significance of the differences in mean values between and within multiple groups was examined by 1-way analysis of variance followed by Duncan’s multiple range test. *P<0.05 was considered statistically significant.

Results

PGRN Was Reduced in the Kidney and Heart From a Mouse Model of hHcys

Compared with controls, the levels of PGRN were reduced in the renal cortex, isolated glomeruli, and renal tubules from mice with hHcys; a more significant PGRN decrease was observed in glomeruli than in tubules from mice with hHcys (Figure 1A). To define the expression patterns of PGRN, we used double immunofluorescent staining for PGRN (green) and various markers for major renal parenchymal cells (red).

Although PGRN was expressed in all these cells, a significant decrease in the expression of PGRN was observed in podocytes, glomerular endothelial cells (GECs), and distal tubules, and there were no obvious changes in other tubular...
areas, including proximal tubules and collecting ducts in mice with hHcys (Figure 1B). We also found that PGRN was downregulated in the heart from mice with hHcys (Figure 1C). Interestingly, unlike previous studies showing an increase in the plasma levels of PGRN in mice with acute renal injury\textsuperscript{8} or diabetic nephropathy,\textsuperscript{10} there were no significant changes in the plasma in mice with hHcys (Figure 1D). In vitro, PGRN was significantly reduced in podocytes, GECs, cardiomyocytes, and human cardiac microvascular endothelial cells in response to Hcys rather than proximal tubule epithelial cells and mesangial cells (Figure 1E), indicating the tissue- or cell-specific expression patterns of PGRN under different stress conditions, and a local rather than systemic effect of PGRN contributes to the regulation of cardiorenal function.

**PGRN Deficiency Exacerbated Cardiorenal Dysfunction in Mice With hHcys**

PGRN-deficient (Grn\textsuperscript{−/−}) mice were fed with folate-free diets for 10 weeks to induce hHcys. As shown in Table, although PGRN deficiency had no effects on the levels of Hcys, glucose, and blood pressure compared with wild-type controls, PGRN deficiency aggravated renal injuries in mice with hHcys as evidenced by higher levels of urine albumin-to-creatinine ratio (Table) and glomerulosclerosis (Figure 2A). Transmission electron microscopy analyses further revealed more severe glomerular basement membrane (GBM) injuries, including pronounced GBM thickening and podocyte foot process effacement (Figure 2B). At the molecular levels, the reduced expression of podocyte markers, including nephrin, podocin, and synaptopodin (Figure 2C; Figure S2), and the loss of tight-junction proteins, including ZO-1 and occludin, in the kidney were further exacerbated by PGRN deficiency (Figure 2D). In addition, PGRN deficiency enhanced the levels of proinflammatory mediators (Figure 2E) in renal cortex, and a more significant increase was observed in isolated glomeruli than in tubular areas from mice with hHcys (Figure S3). In the heart, mice with hHcys showed more spherical left ventricle (LV) chamber shape, LV dilatation, and myocyte hypertrophy with increased cardiomyocyte width. PGRN deficiency exacerbated the altered LV shape and the enlarged cardiomyocytes (Figure 2F). At the end of week 10 after the mice were fed with folate-free diets, we examined cardiac functions by echocardiography. As shown in Table, Hcys significantly decreased cardiac function in wild-type mice as evidenced by decreases in ejection fraction \(\%\) and fractional shortening \(\%\), which were aggravated in Grn\textsuperscript{−/−} mice. We also measured cardiac remodeling–related parameters and found that PGRN deficiency induced more LV enlargement and wall thinning.

**Administration of rPGRN Protected Against Cardiorenal Dysfunction in Mice With hHcys**

The level of endogenous plasma PRGN in hHcys-treated groups was \(\approx 0.6 \mu g/mL\), which had no obvious difference compared with control mice. According to pharmacokinetic profile, Hcys-treated mice were administrated intraperitoneally rPGRN (5 mg/kg body weight) twice per week. The plasma PGRN levels could reach to \(>5 \mu g/mL\) after exogenous administration at 6 hours and keep the relative high level for at least 72 hours (Figure 3A). Although we found that administration of rPGRN after 12 hours slightly increased

<table>
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<th>Variables</th>
<th>WT Mice</th>
<th>FF Mice</th>
<th>Normal Diet</th>
<th>FF Diet</th>
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<tr>
<td>Body weight, g</td>
<td>29.55±1.60</td>
<td>27.12±0.52</td>
<td>28.55±1.14</td>
<td>26.18±0.83</td>
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<td>Kidney weight, g</td>
<td>0.27±0.02</td>
<td>0.28±0.01</td>
<td>0.31±0.03</td>
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<td>Plasma total Hcys, (\mu mol/L)</td>
<td>4.65±0.42</td>
<td>14.22±1.21*</td>
<td>5.04±0.14</td>
<td>13.86±1.02*</td>
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<td>Glucose, mmol/L</td>
<td>5.96±0.68</td>
<td>5.71±0.45</td>
<td>6.35±0.89</td>
<td>5.86±0.39</td>
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<tr>
<td>UACR, mg/g</td>
<td>34.48±5.49</td>
<td>65.83±3.23*</td>
<td>42.75±7.05</td>
<td>100.42±11.29*†</td>
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<tr>
<td>Heart rate, bpm</td>
<td>468.54±9.03</td>
<td>476.25±14.7</td>
<td>493.60±9.94</td>
<td>469.67±12.10</td>
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<td>Blood pressure, mm Hg</td>
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<td>Systolic</td>
<td>122.64±5.48</td>
<td>112.26±5.31</td>
<td>120.32±4.75</td>
<td>121.25±3.59</td>
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<td>Diastolic</td>
<td>76.03±4.96</td>
<td>67.60±4.27</td>
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<td>LVIDd, mm</td>
<td>3.11±0.15</td>
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<td>3.51±0.11</td>
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<tr>
<td>LVPWd, mm</td>
<td>0.88±0.04</td>
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<td>0.83±0.06</td>
<td>0.60±0.03†</td>
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<tr>
<td>EF, %</td>
<td>78.60±2.83</td>
<td>53.89±2.17*</td>
<td>69.75±3.13</td>
<td>42.69±0.63†</td>
</tr>
<tr>
<td>FS, %</td>
<td>45.81±3.44</td>
<td>31.83±1.2*</td>
<td>40.42±2.27</td>
<td>26.71±0.92†</td>
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*\(P<0.05\) vs WT normal diet mice.
†\(P<0.05\) vs WT mice with hHcys.
the plasma glucose levels and came back to the normal level after 72 hours (Figure S4), rPGRN significantly attenuated the increase in proteinuria (Figure S4) accompanied by reversed mesangial expansion and ameliorated podocyte injury in mice with hHcys (Figure 3B; Figure S5), as well as the decreased production of proinflammatory mediators in the kidney (Figure 3C). In addition, the protective role of rPGRN in dilated cardiomyopathy was also verified. Treatment with rPGRN prevented the loss of cardiac function as reflected by the recovery of attenuated LV ejection fraction and LV fractional shortening, as well as improved LV enlargement and posterior wall thinning (Figure 3D through 3F; Figure S6). Meanwhile, we also found that rPGRN ameliorated diastolic dysfunction as indicated by decreased A wave and Aa velocity and increased E/A and Ea/Aa ratio in mice with hHcys (Figure S6). Collectively, our study demonstrated that rescue therapy with rPGRN promoted kidney and cardiac repairs in mice with hHcys.

**Figure 2.** Progranulin (PGRN) deficiency exacerbated cardiorenal dysfunction in mice with hyperhomocysteinemia (hHcys). A, Photomicrographs showing typical glomerular structure changes in different groups of mice. B, Representative photomicrographs and quantifications of mean glomerular basement membrane (GBM) thickness, mean foot process width, and the number of foot processes in different groups of mice by transmission electron microscopy (TEM) analyses. C, Representative immunofluorescent staining for podocyte markers, including nephrin, podocin, and synaptopodin in the kidney from different groups of mice. D, Representative Western blot gel documents and summarized data showing tight-junction protein levels, including ZO-1 and occludin in the kidney from different groups of mice. E, Relative levels of proinflammatory mediators in renal cortex from different groups of mice. F, Heart size (1 mm/unit on the scale ruler), representative histological cross-sectional anatomy at the papillary muscle level, and hematoxylin and eosin (H&E) staining of left ventricular (LV) longitudinal and transverse sections showing altered LV chamber and the cardiomyocytes. *P<0.05 vs control, †P<0.05 vs WT mice with hHcys (n=10). FF indicates folate free.
PGRN Negatively Regulated Wnt/β-Catenin Signaling Pathways

Among Wnt genes that are closely associated with renal injury, including Wnt1, Wnt2b, Wnt3, Wnt3a and Wnt4, we found that Wnt1 was upregulated in the kidney from mice with hHcys by mRNA (Figure 4A) and Western blot analyses (Figure 4B), which was further enhanced by PGRN deficiency. We also found that the upregulation of Wnt1 was mainly in glomeruli as observed by Western blot and immunofluorescent analyses (Figure S7). To examine the biological consequence of Wnt1 induction, we next investigated the activation of β-catenin. The amount of Hcys-induced dephosphorylated β-catenin was dramatically enhanced, as well as the total β-catenin expression levels. Because the phosphorylation of β-catenin by GSK-3β leads to its degradation via the ubiquitin/proteasome pathway, we next examined the cellular activity of GSK-3β. Our results showed that the increase in β-catenin levels was associated with an increase in phospho-GSK-3β (Ser9) levels, which inactivated GSK-3β. PGRN deficiency enhanced the effect of Hcys on phospho-GSK-3β, β-catenin in the kidney (Figure 4C). Consistently, administration of rPGRN inhibited Hcys-induced Wnt/β-catenin signaling in the kidney from mice with Hcys (Figure 4D).

PGRN Ameliorated Hcys-Induced Podocyte Dysfunction Through Inhibition of Wnt/β-Catenin Signaling Pathways

In consistent with the results from animal studies, in vitro, we found that Hcys-enhanced levels of active β-catenin were blockaded in podocytes by rPGRN treatment (Figure S8). As well as rPGRN (Figure 5A), Dkk1, an unique inhibitor of the canonical Wnt signaling pathway, recovered the expressions of nephrin and podocin in podocytes under Hcys treatment (Figure 5B). In addition, we further observed that both Dkk1 and rPGRN improved podocyte cytoskeleton rearrangement (Figure 5C) and inhibited Hcys-induced apoptosis (Figure 5D). The protective effects of rPGRN were also observed in GECs (Figure S9) and cardiomyocytes (Figure S10).

Discussion

This study for the first time demonstrates that PGRN protects against cardiorenal injury in mice with hyperhomocysteinemias (hHcys). A, The levels of plasma PRGN in mice with hHcys after exogenous administration rPGRN (5 mg/kg body weight) at different time points. B, Representative photomicrographs showing typical glomerular structure changes by periodic acid–Schiff (PAS) staining and morphological changes in the podocyte foot process by transmission electron microscopy (TEM) in different groups of mice. C, Relative levels of proinflammatory mediators in renal cortex from different groups of mice. D, Representative echographic images of the mouse hearts obtained from M-mode, pulsed-wave Doppler of mitral inflow, and tissue Doppler showing the changes in the systolic and diastolic cardiac function in different groups of mice. E, Ejection fraction (EF%) in different groups of mice. F, Fractional shortening (FS%) in different groups of mice. *P<0.05 vs control, †P<0.05 vs WT mice with hHcys (n=10). FF indicates folate free; and WT, wild-type.
providing direct evidence for the essential role of PGRN in maintaining renal function. In addition, studies have demonstrated that cardiovascular-related mortality and morbidity are associated with Hcys. Ventricular enlargement, myocardial hypertrophy, and adverse myocardial remodeling are principal features in the development of heart failure. Experimental animal studies have also demonstrated that the increased levels of Hcys contribute to cardiac hypertrophy, which is further confirmed in human heart. In this study, we found that PGRN deficiency exacerbated Hcys-induced LV dilatation and hypertrophy in mice with hHcys, indicating that PGRN is a central target molecule for maintaining cardiorenal functions.

Mechanistically, we found that PGRN negatively regulated Wnt/β-catenin signaling, which is an evolutionarily conserved developmental signaling cascade that exhibits a pivotal function in the regulation of a variety of biological processes in the tissue development and in the pathogenesis of human diseases. Although in the kidney, Wnt/β-catenin signaling is indispensable for nephron formation and becomes functionally silent after differentiation in the adult kidney, emerging evidence has indicated that Wnt/β-catenin is reactivated after renal injury and plays a critical role in promoting renal injury through effects on regulatory molecules, such as Snail1, TRPC6 (transient receptor potential channel 6), and angiotensin II type I receptor. In particular, both Wnt and β-catenin are specifically activated in podocytes from patients with focal segmental glomerulosclerosis and diabetic nephropathy, suggesting the clinical relevance of Wnt pathway to human proteinuric kidney diseases. In cardiovascular system, Wnt signaling is centrally involved in myocardial remodeling after pathological injuries. Studies from Nakagawa et al suggest that the sustained activation of Wnt/β-catenin signaling in endothelial cells might be a cause of heart failure. Although functional genomic analyses have shown the involvement of Wnt signaling pathways in PGRN deficiency in human fetal neural progenitors, the contributions of Wnt signaling and the association between PGRN and Wnt cascade in hHcys-induced cardiorenal dysfunction keep unknown. In this study, we found that PGRN negatively regulated Wnt1/β-catenin signaling pathways in hHcys, which had multiple functions in different cell types by regulation of cell fate determination, the expression of podocyte differentiation markers, and endothelial cell permeability, and so on. Collectively, these data clearly indicate that PGRN serves as a protective factor in hHcys, at least in part, by negative regulation of Wnt/β-catenin signaling (Figure 5E).

Figure 4. Progranulin (PGRN) negatively regulated Wnt/β-catenin signaling pathways. A, Relative mRNA levels of Wnt1, Wnt2b, Wnt3, Wnt3a, and Wnt4 in the renal cortex from different groups of mice. B, Representative Western blot gel documents and summarized data showing the relative protein levels of Wnt1 in the renal cortex from mice. C, Representative Western blot gel documents and summarized data showing the levels of phospho-GSK-3β (Ser9), total GSK-3β, active and total β-catenin in the kidney from mice. D, Representative Western blot gel documents and summarized data showing the effect of recombinant progranulin (rPGRN) on the levels phospho-GSK-3β (Ser9), total GSK-3β, active, and total β-catenin in the kidney from mice with hyperhomocysteinemia (hHcys). *P<0.05 vs control, †P<0.05 vs WT mice with hHcys (n=10). FF indicates folate free; and WT, wild-type.

Considering that administration of rPGRN significantly alleviated inflammatory responses in rheumatoid arthritis animal models and both pretreatment with and delayed administration of rPGRN protected against acute kidney injury in mice, we further detected the effect of rPGRN in mice with hHcys.
One of the most striking findings was the therapeutic efficacy of rPGRN for the treatment of mice with hHcys. Administration of rPGRN attenuated disease progression and ameliorated Hcys-induced cardiorenal injury, indicating that PGRN is essential for conferring cardiorenal protection and may be an innovative therapeutic strategy for treating patients with hHcys.

**Perspectives**

Although several multicenter, prospective, case–control studies have demonstrated that Hcys is an independent risk factor of cardiovascular disease and end-stage renal disease, and a decrease in the plasma level of Hcys reduces the risk of coronary heart disease, the beneficial effects of Hcys-lowering therapy by daily folic acid or some B vitamins are not conclusive. The current findings indicate that PGRN-mediated Wnt/β-catenin signaling is one of the critical signal transduction pathways that links Hcys to cardiorenal injury, suggesting that PGRN may be an innovative therapeutic strategy for the treatment of hHcys-associated end-organ damage. In addition, endothelial dysfunction tends to be the initial event in macrovascular complications, such as peripheral arterial disease, coronary artery disease, and stroke, and in microvascular complications, such as nephropathy and retinopathy. In this study, we found that PGRN protected against endothelial injury not only in GECs but also in human cardiac microvascular endothelial cells, suggesting that PGRN may even have therapeutic potential in treating these diseases in a vast range of medical specialties.

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**Disclosures**

None.
References


Novelty and Significance

What Is New?

- The present study explored a novel molecular mechanism indicating that progranulin (PGRN)-medicated Wnt/beta-catenin signaling is one of the critical signal transduction pathways that links homocysteine to cardiovascular injury and further provided direct evidence for therapeutic potential of PGRN in hyperhomocysteinemia.

What Is Relevant?

- A better understanding of the mechanisms responsible for homocysteine-induced cardiorenal injury and identifying the protective role of PGRN in the pathogenic pathways may provide clues to develop new therapeutic strategies for the treatment of hyperhomocysteinemia-associated end-organ damage below lowering the plasma hyperhomocysteinemia levels.

Summary

These findings for the first time demonstrate that PGRN protects against cardiorenal injury in mice with hyperhomocysteinemia. Such beneficial effects of PGRN are associated with, at least in part, the negative regulation of Wnt/beta-catenin signaling. Modulation of this pathway may have important therapeutic implications to treat patients with hyperhomocysteinemia.
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Therapeutic potential of PGRN in hyperhomocysteinemia-induced cardiorenal dysfunction

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Running title: Progranulin and homocysteine

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Materials and Methods

**Animal studies:** Twelve-week-old male PGRN deficient mice (B6(Cg)-Grntm1.1Aidi/J, 20-25g) and wild-type (WT) C57BL/6 mice were purchased from the Jackson laboratory (Bar Harbor, ME, USA). To accelerate the damaging effect of Hcys on the kidney, the mice were uninephrectomized. After one week recovery period from uninephrectomy, the mice were maintained on a normal diet or a folate-free (FF) diet purchased from Dyets Inc. (Bethlehem, PA, USA) for 10 weeks. To further implicate the therapeutic effects of PGRN in hHcys-induced cardiorenal dysfunction, mice on a FF diet, meanwhile, were administrated intraperitoneally recombinant human PGRN (rPGRN, 5mg/kg body weight) or vehicle (0.1% bovine serum albumin/PBS 1X) twice per week. The plasma levels of exogenous rPGRN were measured at various time points after injection by using human PGRN enzyme-linked immunosorbent assay (ELISA) Kit. During the 10 weeks period, blood pressure and plasma glucose were detected intermittently. At the end of the study, blood and 24 hours urine samples were collected. Plasma total Hcys levels were measured by high-performance liquid chromatograph (HPLC) method as described. Urinary albumin excretion was detected by using a mouse albumin ELISA quantitation kit (Bethyl Laboratories, Montgomery, TX, USA). Simultaneously, the mice were euthanized and the kidney and heart tissue samples were harvested for histopathological and biochemical analysis. All protocols were approved by institutional animal care and use committee of Shandong University.

**Isolation of glomeruli:** Glomeruli was isolated by using a modified method as described previously. Mice were anesthetized and the surgical procedures were performed as follows: the distal abdominal aorta, distal inferior cava vein, the superior mesenteric and coeliac arteries were ligated; the proximal abdominal aorta was ligated above the coeliac arteries; the abdominal aorta together with inferior cava vein were clipped with vessel clamps below the renal artery and vein; polyethylene tubing was inserted into the middle of the abdominal aorta and fixed in place; the vessel clamp was removed; a hole was cut in the inferior cava vein to give a outlet in order to make a complete circuit. Following operation, the kidney was perfused with Dynabeads (Invitrogen, M-450 Tosylactivated) diluted in Hanks’ balanced salt solution (HBSS) through the polyethylene tubing. Kidneys were removed, minced into small pieces and digested with collagenase A, proteinase E and deoxyribonuclease I at 37°C for 30 minutes with gentle agitation. The digested tissue was then gently pressed through a 100 μm cell strainer, followed by ice-cold HBSS flushing. The cell suspension was centrifuged at 200×g at 4°C for 5 minutes. Finally, the pellet was resuspended and glomeruli containing Dynabeads were gathered by a magnetic particle concentrator after three times washing. The residul containing renal tubules were also collected. Glomeruli purity was determined by inspecting 20 μl aliquots of glomeruli suspensions on glass slides using a light microscope. Purity was estimated by the number of glomeruli divided by the total number of particles counted, including tubular fragments, and averaged over 10 fields. In our studies, the purity of glomeruli was estimated to be above 98%. Only preparations showing minimal tubular contamination were used for the experiment. Lastly, the extracted glomeruli and tubules were put aside respectively for mRNA and protein analyses and stored at -80°C until further examination.

**Cell culture and treatments:** podocytes, glomerular endothelial cells (GECs), rat H9c2 cardiomyocytes, human cardiac microvascular endothelial cells (HCMECs), proximal tubule epithelial cells (HK-2) and Human renal glomerular mesangial cells (HMCs) were cultured as
described\textsuperscript{3,4}. L-Hcys (50 and 100 μmol/l) were used to treat cells for 24 hours in this study. In addition, 500 ng/ml rPGRN and of 200nmol/l Dkk1 was used to study the effect of rPGRN and Dkk1 on the cell function.

**Transmission electron microscopy (TEM):** Electron microscopic sample handling and detection were performed by the electron microscopic core lab of Shandong University as described\textsuperscript{5}. TEM images were analyzed using Image J (National Institutes of Health, NIH, Bethesda, MD, USA) and analysis was based on previous studies.\textsuperscript{6,7} The GBM thickness, foot process width and the number of foot processes per μm of GBM were calculated using a curvimeter (SAKURA CO., LTD, Tokyo, Japan) as described.\textsuperscript{8} Five glomeruli were randomly selected from each mouse and 10 electron micrographs were taken in each glomerulus.

**Echocardiography:** Mice were lightly anesthetized with 1.5% isoflurane inhalation to maintain their heart rates around 500 beats/min. Echocardiography was performed with a Vevo770 imaging system (VisualSonics, Toronto, Canada), using a 30-MHz high-frequency transducer. M-mode, two-dimensional (2-D), pulse wave (PW) Doppler and tissue Doppler imaging (TDI) were used to measure left ventricular ejection fraction (LVEF), fractional shortening (FS), the ratio of early to late mitral inflow velocity (E/A), the ratio of diastolic mitral annulus velocities (Ea/Aa) and left ventricular end internal diameter at diastolic phase (LVIDd), LV internal in ternal diameter at systolic phase(LVIDs), LV internal posterior wall thickness at diastolic phase(LVPWd), left ventricular posterior wall thickness at systolic phase(LVPWs) from the long-axis view at the level of chordae tendineae. Peak velocity of early (E) and late (A) ventricular filling velocity were obtained by the apical four-chamber view at the level of mitral inflow. Tissue Doppler echocardiography was used for grading the severity of LV diastolic dysfunction.

**Immunofluorescence staining and confocal microscopy:** Immunofluorescent staining was performed using a modified protocol as previously described\textsuperscript{9} and images were obtained by a LSM780 laser scanning confocal microscope (ZEISS, Germany) equipped with a Plan-Apochromat 63×/1.4 objective. Sections were incubated with different primary antibodies, and were subsequently incubated with secondary Alexa 488 or 555 conjugated antibody (Invitrogen). Nuclei were counterstained with DAPI (Roche, Mannheim, Germany). Moreover, double immunofluorescence staining for PGRN (green) and glomeruli, glomerular endothelial cells and various tubular markers (red) in the kidney. Segment-specific tubular markers and glomeruli markers were used based on previous studies: podocytes, synaptopodin; glomerular endothelial cell, endomucin; distal tubule, calbindin D28K; proximal tubule, aquaporin-1 (AQP1); and collecting duct, aquaporin-3 (AQP3)\textsuperscript{9,10}.

**Flow cytometry:** Cell apoptosis was determined by propidium iodide (PI)-Annexin V staining, as described\textsuperscript{3}.

**RNA extraction and real time RT-PCR:** Total RNA was isolated from mouse kidney or cells using TRIzol reagent (Invitrogen) as described previously\textsuperscript{11}. The mRNA expression levels for target genes were analyzed by real-time quantitative RT-PCR using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA).The sequences of specific primers for target genes in this study are listed in Table S1. Levels of the housekeeping gene β-actin were used as an internal control for the normalization of RNA quantity and quality differences among the samples.

**Western blot analysis:** Total tissue or cellular lysates preparation and Western blot analysis were performed as described previously\textsuperscript{12}, Antibodies used in this study were summarized in Table S2.
To document the loading controls, the membrane was reprobed with a primary antibody against housekeeping protein β-actin and GAPDH (ProteinTech Group).

**Cell monolayer permeability assay:** GECs were cultured on the upper chamber of Transwell™ system (0.4-μm pore size of polycarbonate membrane coated with poly-L-lysine, Corning, NY). A total of 500 μl of the appropriate media were added to the lower chambers. When cells reached to confluence 48 hours later, they were stimulated by L-Hcys for 24 hours, the media were removed from both chambers without disturbing the cell monolayer; 150 μl of FITC-dextran (1 mg/ml) was added to the upper chamber, and 500 μl culture medium (without phenol red) was added to the lower chamber. The plates were left to stand at RT for 20 min, and then 100 μl of the medium was removed from the lower chamber to measure the fluorescence intensity using a fluorescence spectrometer. The relative permeability of cells in each treatment group was calculated as the ratio of the mean intensity of the experimental group to the mean intensity of the control group.

**Statistics:** Data are expressed as means ± S.E. The significance of the differences in mean values between and within multiple groups was examined by one-way ANOVA followed by Duncan's multiple range test. *P* < 0.05 was considered statistically significant.
References


Table S1. Primer pairs of target genes used for real time RT-PCR in this study

<table>
<thead>
<tr>
<th>Genes</th>
<th>Accession No.</th>
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Table S2. Antibodies used in this study

<table>
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<th>Application</th>
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<td>WB</td>
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<td>GAPDH</td>
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Figure S1. Assessing the isolation of glomeruli. A. Mice glomeruli isolated by Dynabeads together with sieving visualized by light microscopy. Representative sample showing >98% purity (at ×10 and ×20 magnification) and glomeruli samples were not contaminated with tubular fragments. B. Representative Western blot gel documents showing the expression of nephrin, a specific podocyte marker in the same amount of isolated glomeruli and renal tubules tissue lysates.
Figure S2. PGRN deficiency exacerbated podocyte dysfunction in the kidney of mice with hHcys. Representative Western blot gel documents and summarized data showing the protein levels of podocyte markers including nephrin, podocin and synaptopodin in the kidney from different groups of mice. *P<0.05 vs.control (Normal diet WT mice), †P<0.05 vs. WT mice with hHcys (n=10).
Figure S3. PGRN deficiency enhanced the level of proinflammatory mediators mainly observed in glomeruli. A. Relative levels of proinflammatory mediators in isolated glomeruli from different groups of mice. B. Relative levels of proinflammatory mediators in renal tubules from different groups of mice. *P<0.05 vs. control (Normal diet WT mice), † P<0.05 vs. WT mice with hHcys (n=8).
Figure S4. A. The effect of administrated recombinant PGRN (rPGRN) on the levels of plasma glucose, blood pressure and proteinuria. A. The levels of plasma glucose at different time points in mice following exogenous administration of rPGRN (5mg/kg body weight). B. Blood pressure including systolic and diastolic blood pressure in different groups of mice by tail-cuff blood pressure measurements. C. Urine albumin-to-creatinine (Cr) ratio in different groups of mice. *$P<0.05$ vs. control (Normal diet WT mice), †$P<0.05$ vs. WT mice with hHcys (n=10).
Figure S5. Administration of rPGRN significantly ameliorated podocyte injury in mice with hHcys. A-C, Transmission electron microscopy analyses showing indices for glomerular filtration barrier integrity, including glomerular basement membrane (GBM) thickness (A), podocyte foot process width (B) and the number of foot processes/μm GBM (C). *P<0.05 vs.control (Normal diet WT mice), †P<0.05 vs. WT mice with hHcys (n=8).
Figure S6. Administration of rPGRN protected against cardiac dysfunction in mice with hHcys. A-B. LVId, LVpWd were used to evaluate the LV remodeling; C. Pulse Doppler of the early to late transmitral inflow (E/A) was used for evaluation of the LV diastolic function. D. Pulse tissue Doppler waveform was obtained from the LV posterior wall (Ea/Aa) and used to assess the abnormality of the regional wall motion. * $P<0.05$ vs. control (Normal diet WT mice), †$P<0.05$ vs. Hcys treated mice (n=10).
Figure S7. Upregulation of Wnt1 in the kidney from mice with hHcys mainly in glomeruli.
A. Representative Western blot gel documents and summarized data showing the expression of Wnt1 in isolated glomeruli and renal tubules in the kidney from different groups of mice.* P<0.05 vs. control (Normal diet WT mice), †P<0.05 vs. Hcys treated mice (n=10). B. Representative immunofluorescent staining for Wnt1 in the kidney from different groups of mice (arrowheads indicate glomeruli).
Figure S8. Administration of rPGRN inhibited hHcy-induced Wnt/β-catenin signaling in the kidney from mice with hHcy. Representative Western blot gel documents and summarized data showing the expression level of Wnt1, phosphorylated and total GSK-3β, active and total β-catenin levels in podocytes with different treatments. *P<0.05 vs. control, †P<0.05 vs. vehicle of L-Hcys treatment (n=8).
Figure S9. Administration of rPGRN ameliorated Hcy-induced glomerular endothelial cell dysfunction accompanied by the inhibition of Wnt/β-catenin signaling pathways. A. Representative Western blot gel documents and summarized data showing active and total β-catenin levels in glomerular endothelial cells (GECs) with different treatments. B. GECs monolayer permeability was assayed by FITC-dextran fluorescence levels leaked through the transwell membrane after different treatments. C. Representative Western blot gel documents and summarized data showing TJP levels in GECs after different treatments. D. Representative confocal microscopic images showing that ZO-1 protein changes in GECs with different treatments. E. Representative dot plots and summarized data showing the levels of apoptosis determined by flow cytometric analysis in GECs with different treatments. *$P<0.05$ vs. control, †$P<0.05$ vs. vehicle of L-Hcys treatment ($n=8$).
Figure S10. Administration of rPGRN inhibited Hcys-induced cardiomyocytes apoptosis. A. Representative Western blot gel documents and summarized data showing active and total β-catenin levels in cardiomyocytes (H9c2) with different treatments. B. Summarized data showing the levels of apoptosis determined by flow cytometric analysis in H9c2 with different treatments. *P<0.05 vs. control, †P<0.05 vs. vehicle of L-Hcys treatment (n=8).