

Supplemental Appendix

Neuregulin-1 β improves uremic cardiomyopathy and renal dysfunction in rats

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Table of content

Supplemental methods	2
Supplemental Table 1	4
Supplemental Table 2	5
Supplemental Table 3	6
Supplemental Table 4	7
Supplemental Table 5	8
Supplemental Table 6	3
Supplemental Figure 1	9
Supplemental Figure 2	10
Supplemental Figure 3	11
Supplemental Figure 4	12
Supplemental Figure 5	13
Supplemental Figure 6	14

Supplemental Methods

Immunostainings for CD68-positive macrophages and type I collagen

Deparaffinized cardiac tissue sections were immunostained to detect CD68-positive macrophages and type I collagen (COL1). Briefly, dewaxed slides were incubated with monoclonal mouse anti-CD68 antibodies (1:100, ED1, Abcam, Cambridge, MA, USA), then sequentially detected with biotinylated goat anti-mouse secondary antibody (Vector Laboratories, Burlingame, CA, USA) and horseradish peroxidase (HRP) conjugated streptavidin (Dako, Glostrup, Denmark), and finally visualized using 3,3'-diaminobenzidine/hydrogen peroxidase chromogen substrate kit (DAB; Vector Laboratories, Burlingame, CA, USA).²³ Images were captured by a Nikon Eclipse 80i (Tokyo, Japan) microscope.

Type 1 collagen (COL1) was detected using a Ventana Benchmark Ultra automated immunostainer (Roche Diagnostics, Tucson, AR, USA). After antigen retrieval was done in a high pH CC1 buffer for 40 min, and incubation with anti-human type-I collagen IgG (1:1000, #PA5- 95137, ThermoFisher/Invitrogen, Waltham, MA, USA) for 60 min, the detection was performed using the Ultraview system for 40 min and visualized with DAB/hydrogen peroxide kit.¹⁹ Immunostained slides were counterstained with hematoxylin, dehydrated, mounted using DPX (Merck, Darmstadt, Germany), and digitalized with a Panoramic 250 scanner (3DHitech, Budapest, Hungary).

SiRNA interference with ErbB3

SiRNA interference with ErbB3 was applied to clarify the receptor's role in response to TGF- β and rhNRG-1 β stimuli in HVCFs. Lyophilized siRNA for ErbB3 was diluted under aseptic conditions to a final concentration of 5 nmol in 5x siRNA buffer (Dharmacon). To evaluate the ideal concentrations of both siRNA and transfection agent (Dharmacon), trial experiments with 6 groups of different concentrations were performed using the fluorescent control siRNA (Supplementary Table 6). The most efficient mixture with the highest fluorescence consisting of 2 μ l transfection agent and 5 μ l siRNA was used in each well in 24-well plates. 480 μ l of antibiotic-free growth medium were added and again mixed carefully. If mediators were applied, they were added to this antibiotic-free growth medium. The final solution was then added to the respective well and incubated for 24 hours.

Supplemental Table 1 Primer sequences used for RT-qPCR

<i>Ace1</i>	rat	CCCGGCAACTTTTCTGCTGAC	GATGTTGGTGTCTGTCGCC
<i>Acta2</i>	rat	ACCATCGGGAATGAACGCTT	CTGTCAGCAATGCCTGGGTA
<i>Agtr1</i>	rat	TGCCTCCTCGCCAATGATTC	CCAGACGTCCTGTCACCTCG
<i>Col 1</i>	rat	TGTTTGGGTCATTTCCACATGC	AGCACAAAGCAGTTTTTCCCC
<i>Col 3</i>	rat	GGCTGAGTTTTATGACGGGC	GAGCGAGAAGTAGCCAGCTC
<i>ErbB2</i>	rat	TCAGATTGCCAAGGGGATG	AACCGGCGTCTGAGAATAG
<i>ErbB3</i>	rat	CGAGATGGGCAACTCTCAGGC	AGGTTACCCATGACCACCTCACAC
<i>ErbB4</i>	rat	GGAATATTTGGTCCCCCAGGCTTC	GAGGAGGGCTGTGTCCAATTTCA
<i>Gapdh</i>	rat	GACAGTCAGCCGCATCTTCT	GCGCCAATACGACCAAATC
<i>Il6</i>	rat	TGAACTCCTTCTCCACAAGCG	TGGAATCTTCTCCTGGGGGTA
<i>Mmp2</i>	rat	AATGCCATCCCCGATAACC	TCCAAACTTCACGCTCTTCAG
<i>Mmp9</i>	rat	AACCAATCTCACCGACAGGC	CACCACCAACACACCCCTAA
<i>Acta2</i>	human	GCAGCTCCAGCTATGTGTGAAG	TTGTCCCATTCCCACCATCACC
<i>Col1</i>	human	AGTCGAGGGCCAAGACGAAG	ACAACACCTTGCCGTTGTCTG
<i>Col3</i>	human	GTGGACGTTGGCCCTGTTTG	TGTCGGTCACTTGCACTGGT
<i>ErbB3</i>	human	GGCAACTCTCAGGCAGGTAA	CTGAGACCGTGCCCATACC
<i>Lox1</i>	human	ACCTCATTATCACCTTCCCC	TGTCTGTCTGTCTGTCTGTC
<i>Gapdh</i>	mouse	ACTCCCACTCTTCCACCTTC	TCTTGCTCAGTGTCTTGC
<i>Nox2</i>	mouse	CGGAGGGGCTATTCAATGCT	CACTGGCTGTACCAAAGGGT
<i>Nox4</i>	mouse	TTTAGCATTCCCTGCAGCCTC	AAAGGTTATCTTGCCGCCCA
<i>Nppa</i>	mouse	GGTACTGGGTCCATTCCTGAG	GACCTCATCTTCTACCGGCAT
<i>Nppb</i>	mouse	GGGAGAACACGGCATCATTG	TCCCAGAGGATAGGAGTGACC
<i>Tnf</i>	mouse	CCACGTCTGAGCAAACCACC	GTACAACCCATCGGCTGGCA

Supplemental Table 2 The effect of NRG-1 treatment on serum and urine parameters at week 4 and the endpoint.

Parameter (unit)	week 4			week 10		
	Sham	CKD	CKD+rhNRG-1 β	Sham	CKD	CKD+rhNRG-1 β
Urine volume (mL)	27 \pm 1.9	36 \pm 3.1	31 \pm 2.7	29 \pm 4.2	31 \pm 3	34 \pm 3.6
Serum carbamide (mmol/L)	5.6 \pm 0.2	15 \pm 1.4*	13 \pm 0.5*	7.2 \pm 0.36	18 \pm 3.4*	14 \pm 1.1*
Serum creatinine (μ mol/L)	30 \pm 1.8	67 \pm 4.6*	57 \pm 1.6* [#]	25 \pm 1.3	78 \pm 11* ^{\$}	47 \pm 5.4* [#]
Urine creatinine (mmol/L)	4844 \pm 258	3467 \pm 353*	4177 \pm 313	5655 \pm 603	4649 \pm 495 ^{\$}	4790 \pm 548
Urine protein (mg/dL)	77 \pm 8	131 \pm 27	110 \pm 15	61 \pm 10	371 \pm 90* ^{\$}	191 \pm 44
Creatinine clearance (mL/min)	2.94 \pm 0.14	1.28 \pm 0.11*	1.51 \pm 0.04*	4.04 \pm 0.2 ^{\$}	1.46 \pm 0.24*	2.5 \pm 0.44*
Serum total cholesterol (mmol/L)	1.49 \pm 0.04	2.21 \pm 0.16*	1.95 \pm 0.08*	1.31 \pm 0.08	2.60 \pm 0.29* ^{\$}	1.90 \pm 0.06*
Serum HDL-cholesterol (mmol/L)	1 \pm 0.04	1.59 \pm 0.13*	1.38 \pm 0.07*	0.88 \pm 0.05	1.73 \pm 0.2*	1.28 \pm 0.04*
Serum LDL-cholesterol (mmol/L)	0.33 \pm 0.03	0.47 \pm 0.04*	0.39 \pm 0.04	0.32 \pm 0.06	0.69 \pm 0.09* ^{\$}	0.47 \pm 0.04 [#]
Serum triglyceride (mmol/L)	0.93 \pm 0.14	0.73 \pm 0.1	0.92 \pm 0.16	0.54 \pm 0.04	0.89 \pm 0.12*	0.76 \pm 0.07

Values are presented as mean \pm SEM, * p < 0.05 vs. sham-operated group and # p < 0.05 vs. CKD group (n=7-10, one-way ANOVA, Holm-Sidak *post hoc* test), ^{\$} p < 0.05 vs. week 4 value in the same group (n=7-10, Repeated-measures two-way ANOVA, Holm-Sidak *post hoc* test). Creatinine clearance was calculated according to the standard formula (urine creatinine concentration [μ M] \times urine volume for 24 h [mL]) / (serum creatinine concentration [μ M] \times 24 \times 60 min). Sham: sham-operated group, CKD: chronic kidney disease group, CKD+rhNRG-1 β : recombinant human neuregulin-1 β -treated chronic kidney disease group.

Supplemental Table 3 Left ventricular morphological and functional parameters assessed by transthoracic echocardiography at week 2, before the NRG-1 β treatment.

Parameter (unit)	week 2		
	Sham	CKD	CKD+rhNRG-1 β
Anterior wall thickness - systolic (mm)	3.03 \pm 0.13	3.20 \pm 0.09	3.22 \pm 0.05
Anterior wall thickness - diastolic (mm)	1.69 \pm 0.09	1.83 \pm 0.06	1.89 \pm 0.05
Inferior wall thickness - systolic (mm)	2.89 \pm 0.13	3.22 \pm 0.08	3.18 \pm 0.13
Inferior wall thickness - diastolic (mm)	1.73 \pm 0.06	1.82 \pm 0.04	1.66 \pm 0.04
Posterior wall thickness - systolic (mm)	3.14 \pm 0.17	3.36 \pm 0.09	3.4 \pm 0.11
Posterior wall thickness - diastolic (mm)	1.71 \pm 0.06	1.85 \pm 0.06	1.88 \pm 0.09
Septal wall thickness - systolic (mm)	3.19 \pm 0.11	3.29 \pm 0.11	3.25 \pm 0.03
Septal wall thickness - diastolic (mm)	1.78 \pm 0.07	2 \pm 0.08	1.9 \pm 0.04
Left ventricular end-diastolic diameter (mm) (cross-sectional)	7.23 \pm 0.12	6.81 \pm 0.12	6.65 \pm 0.19
Left ventricular end-systolic diameter (mm) (cross-sectional)	3.72 \pm 0.20	2.88 \pm 0.14	2.59 \pm 0.18
Heart rate (beats/min)	379 \pm 10	398 \pm 6	392 \pm 14
Ejection fraction (%)	55 \pm 3	60 \pm 3	61 \pm 3
E-velocity (m/s)	1.07 \pm 0.05	1.02 \pm 0.04	1.08 \pm 0.05
E'-velocity (m/s)	0.04 \pm 0.003	0.04 \pm 0.002	0.04 \pm 0.003
E/E'	29 \pm 2.41	25.92 \pm 1.46	28.28 \pm 2.41

Values are presented as mean \pm SEM, * $p < 0.05$ vs. sham-operated group, and # $p < 0.05$ vs. CKD group (n=7-10, one-way ANOVA, Holm-Sidak *post hoc* test). Sham: sham-operated group, CKD: chronic kidney disease group, CKD+rhNRG-1 β : recombinant human neuregulin-1 β -treated chronic kidney disease group.

Supplemental Table 4 Left ventricular morphological and functional parameters assessed by transthoracic echocardiography at week 4 and the endpoint.

Parameter (unit)	Week 4			Week 10		
	Sham	CKD	CKD+rhNRG-1 β	Sham	CKD	CKD+rhNRG-1 β
Anterior wall thickness - systolic (mm)	3.06 \pm 0.1	3.1 \pm 0.05	3.19 \pm 0.08	2.94 \pm 0.12	3.79 \pm 0.2 ^s	3.5 \pm 0.11
Anterior wall thickness - diastolic (mm)	1.75 \pm 0.07	1.84 \pm 0.05	1.79 \pm 0.06	1.78 \pm 0.08	2.41 \pm 0.16 ^{*s}	1.93 \pm 0.08 [#]
Inferior wall thickness - systolic (mm)	3.26 \pm 0.15	3.51 \pm 0.22	3.51 \pm 0.15	2.93 \pm 0.13	3.77 \pm 0.20	3.45 \pm 0.14
Inferior wall thickness - diastolic (mm)	1.89 \pm 0.07	2.23 \pm 0.27	1.94 \pm 0.08	1.66 \pm 0.07	2.22 \pm 0.13 [*]	1.91 \pm 0.1 [#]
Posterior wall thickness - systolic (mm)	3.22 \pm 0.15	3.32 \pm 0.13	3.4 \pm 0.13	3.02 \pm 0.06	3.99 \pm 0.15 ^{*s}	3.57 \pm 0.15
Posterior wall thickness - diastolic (mm)	1.92 \pm 0.08	1.92 \pm 0.08	1.85 \pm 0.08	1.77 \pm 0.05	2.33 \pm 0.14 ^{*s}	2.08 \pm 0.08
Septal wall thickness - systolic (mm)	3.44 \pm 0.14	3.32 \pm 0.10	3.41 \pm 0.09	3.33 \pm 0.07	4.03 \pm 0.15 ^{*s}	3.62 \pm 0.13
Septal wall thickness - diastolic (mm)	1.94 \pm 0.11	1.88 \pm 0.08	1.9 \pm 0.08	1.89 \pm 0.04	2.40 \pm 0.11 ^{*s}	2.12 \pm 0.08 ^{#s}
Left ventricular end-diastolic diameter (mm) (cross sectional)	7.09 \pm 0.31	6.59 \pm 0.22	7.02 \pm 0.20	7.51 \pm 0.18	6.50 \pm 0.18 [*]	7.15 \pm 0.18 [#]
Left ventricular end-systolic diameter (mm) (cross sectional)	3.30 \pm 0.31	2.96 \pm 0.24	3.09 \pm 0.28	4.11 \pm 0.21	2.64 \pm 0.23	3.06 \pm 0.27
Heart rate (1/min)	390 \pm 12	391 \pm 8	379 \pm 9	358 \pm 19 ^s	370 \pm 10 ^s	368 \pm 14
Ejection fraction (%)	61 \pm 2	58 \pm 4	58 \pm 2	53 \pm 1 ^s	56 \pm 2	54 \pm 1
E-velocity (m/s)	1.01 \pm 0.06	1.04 \pm 0.04	1.05 \pm 0.04	0.88 \pm 0.07	0.99 \pm 0.03	0.93 \pm 0.02
E'-velocity (m/s)	0.05 \pm 0.003	0.04 \pm 0.001	0.04 \pm 0.003	0.050 \pm 0.0024	0.033 \pm 0.0016 ^{*s}	0.041 \pm 0.0030 [#]
E/E'	22.70 \pm 1.75	26.38 \pm 1.82	26.85 \pm 1.89	18.14 \pm 1.77	29.08 \pm 1.70 [*]	23.56 \pm 1.95

Values are presented as mean \pm SEM, *p < 0.05 vs. sham-operated group and #p < 0.05 vs. CKD group (n=7-10, one-way ANOVA, Holm-Sidak *post hoc* test), ^sp < 0.05 vs. week 4 value in the same group (n=7-10, Repeated-measures two-way ANOVA, Holm-Sidak *post hoc* test). Sham: sham-operated group, CKD: chronic kidney disease group, CKD+rhNRG-1 β : recombinant human neuregulin-1 β -treated chronic kidney disease group.

Supplemental Table 5 Body weight, tibia length, and organ weights at week 10

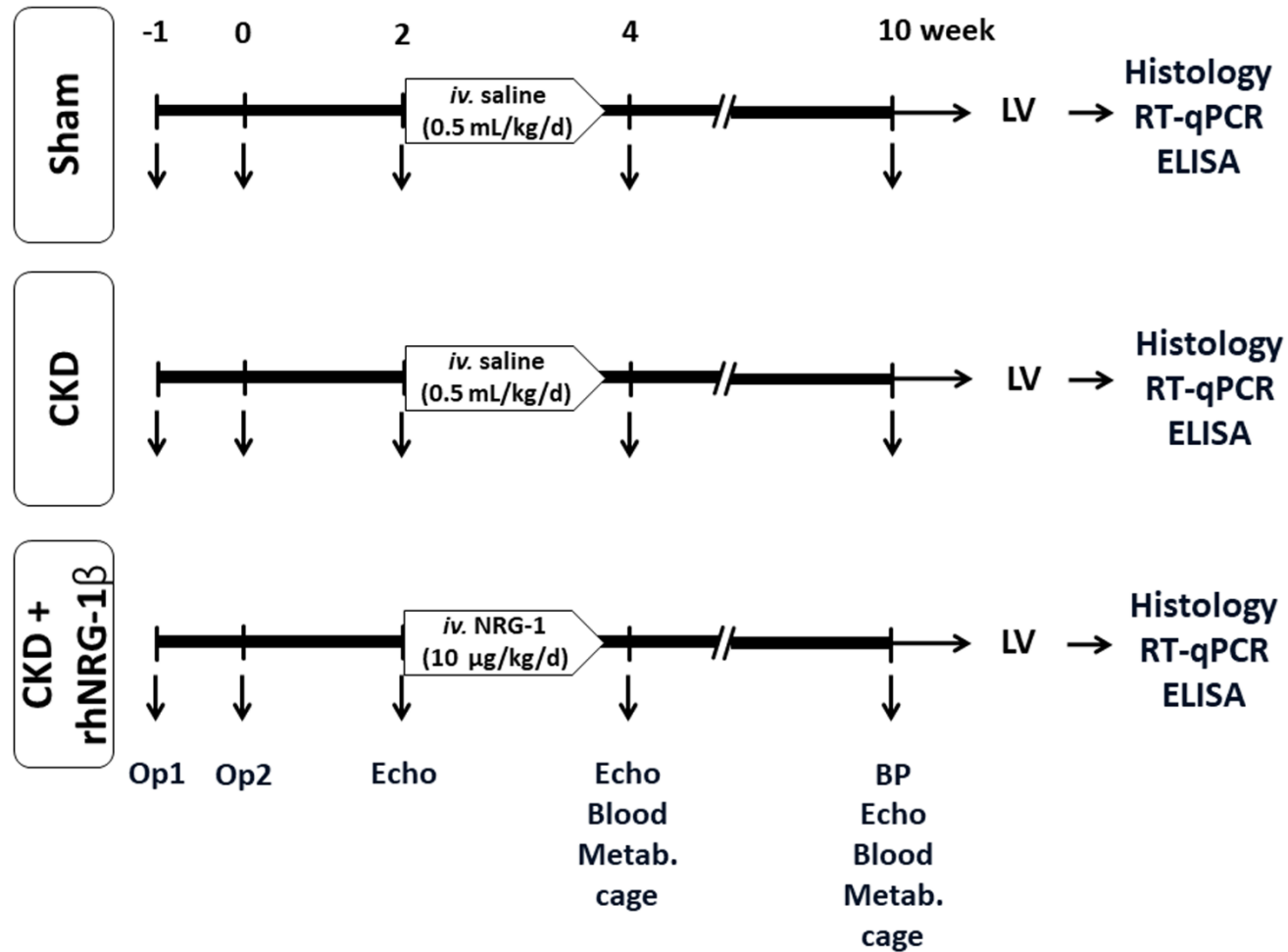
Parameter (unit)	Groups		
	Sham	CKD	CKD+rhNRG-1 β
Body weight (g)	446 \pm 17	444 \pm 9	452 \pm 6
Tibia length (cm)	4.2 \pm 0.03	4.3 \pm 0.04	4.3 \pm 0.03
Left ventricular weight (mg)	934 \pm 30	1037 \pm 32*	926 \pm 18 [#]
Left kidney weight (mg)	1491 \pm 72	1669 \pm 97	1720 \pm 103*
Lung weight (mg)	1676 \pm 88	1768 \pm 44	1740 \pm 49

Values are presented as mean \pm SEM, * $p < 0.05$ vs. sham-operated group, and # $p < 0.05$ vs. CKD group (n=7-10, one-way ANOVA, Holm-Sidak *post hoc* test). Sham: sham-operated group, CKD: chronic kidney disease group, CKD+rhNRG-1 β : recombinant human neuregulin-1 β -treated chronic kidney disease group.

Supplemental Table 6 Groups to evaluate ideal transfection mix and description of the siRNA transfection.

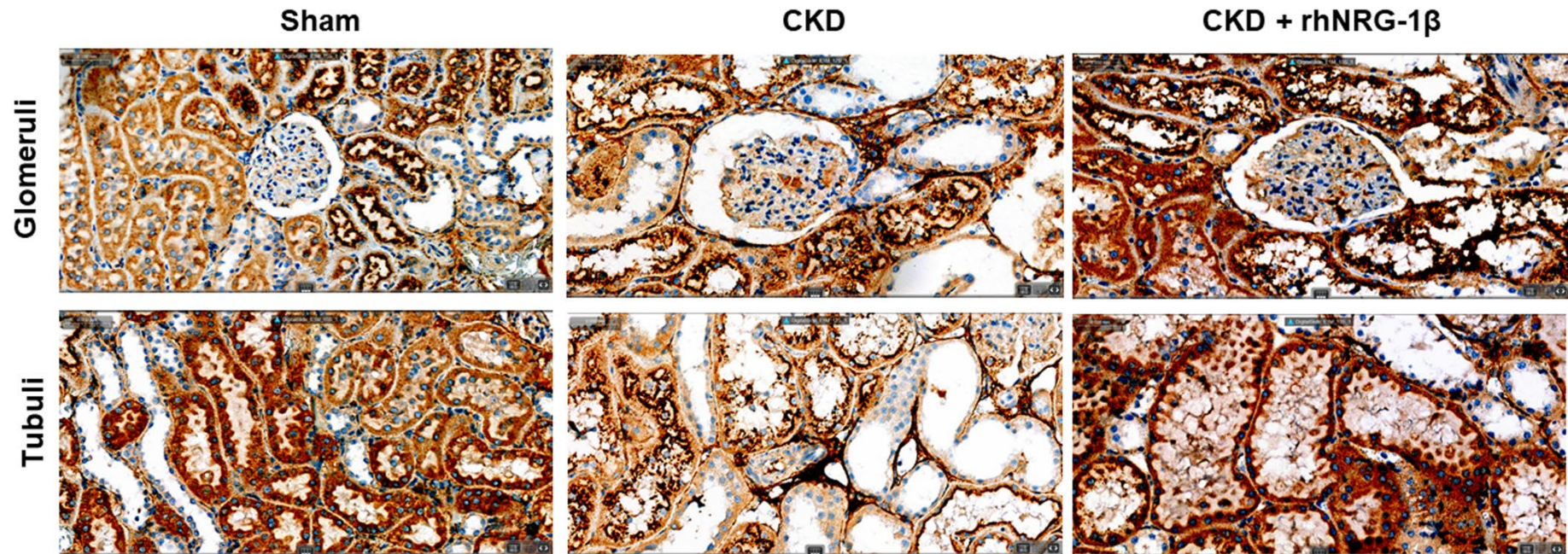
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2	3	2
3	3	3
4	5	1
5	5	2
6	5	3

Fig. S1



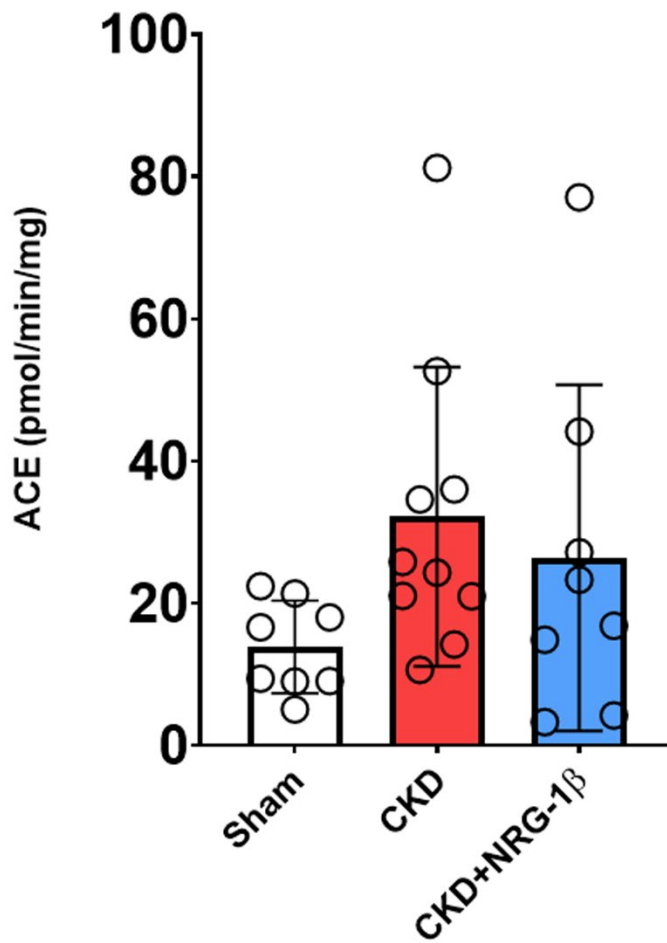
Supplemental Figure 1 Experimental setup. Blood: blood sampling, BP: blood pressure recording, CKD: chronic kidney disease, Echo: echocardiography, d: day, ELISA: enzyme-linked immunosorbent assay, LV: left ventricle, Metab. cage: metabolic cage, Op: operation, rhNRG-1 β : recombinant human neuregulin-1 β , RT-qPCR: reverse transcription-quantitative polymerase chain reaction.

Fig. S2



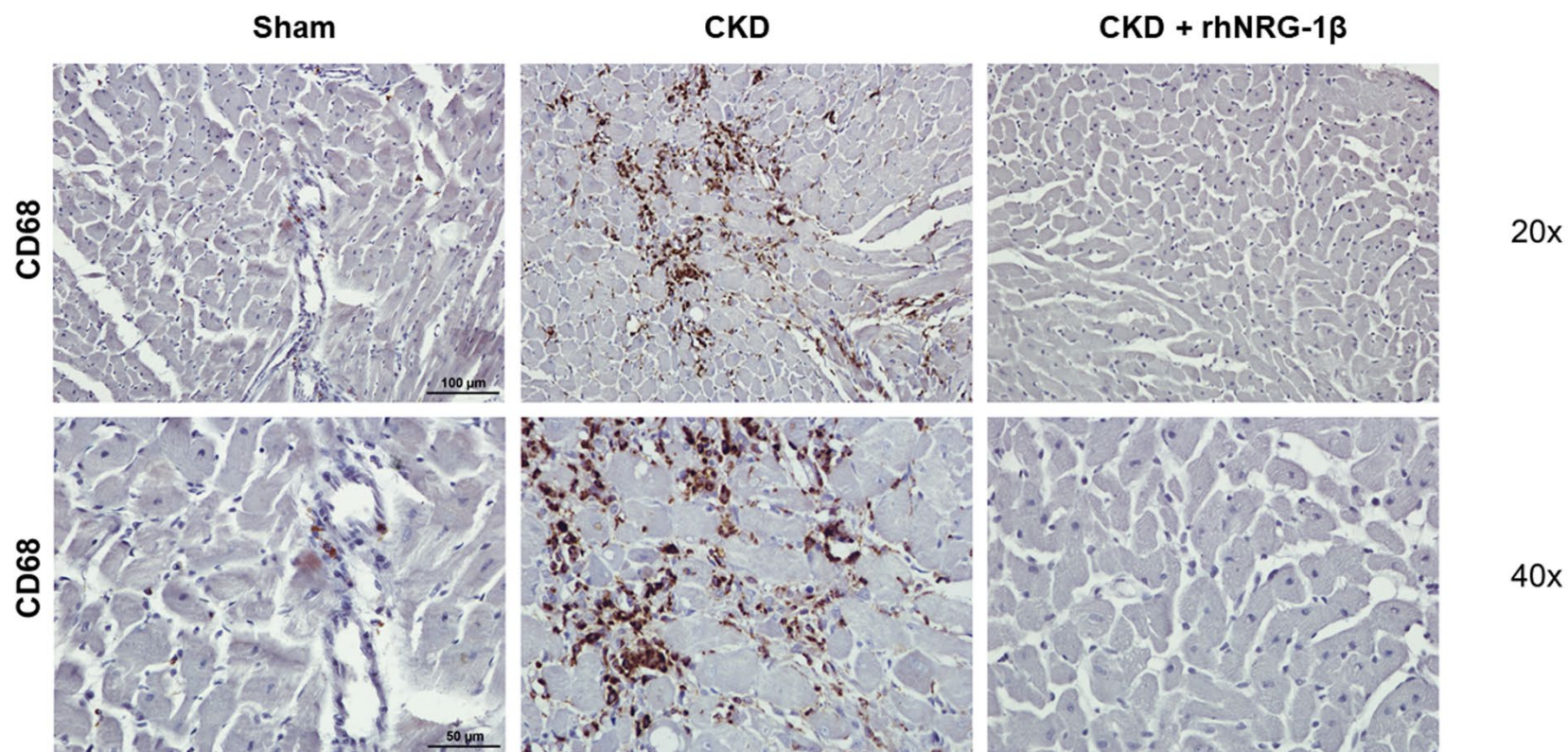
Supplemental Figure 2 CKD substantially increased the renal expression of collagen. Representative images of collagen type 1 (COL1)-immunostained kidney samples (magnification 30x, scale bar: 50 μ m). Sham: sham-operated group, CKD: chronic kidney disease group, CKD+rhNRG-1 β : recombinant human neuregulin-1 β -treated chronic kidney disease group.

Fig. S3



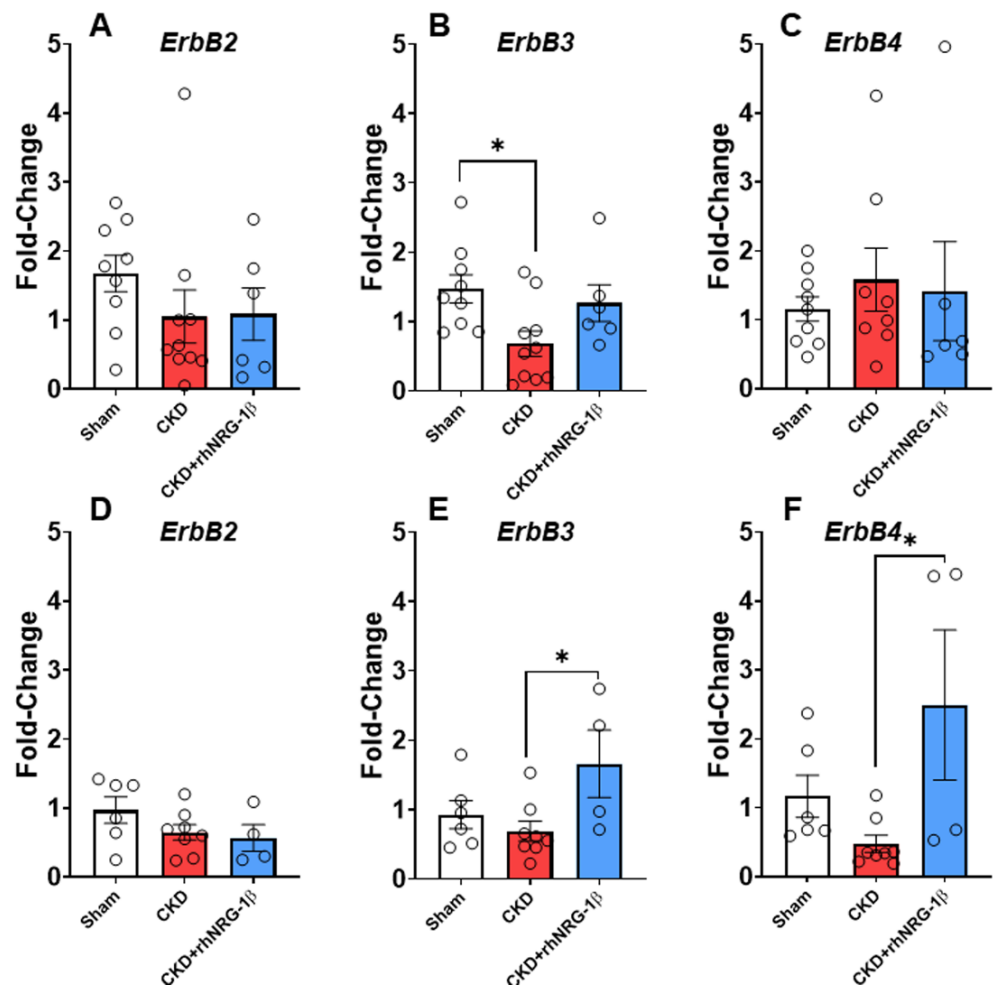
Supplemental Figure 3 The effects of rhNRG-1 β on the renal angiotensin-converting enzyme-1 (ACE1) activity in chronic kidney disease (CKD) after 10 weeks. Data are expressed as mean \pm SEM, n=8-10 replicates/group; one-way ANOVA, Tukey post hoc test. Sham: sham-operated group, CKD: chronic kidney disease group, CKD+rhNRG-1 β : recombinant human neuregulin-1 β -treated chronic kidney disease group.

Fig. S4



Supplemental Figure 4 Immunohistochemical assessment have shown a reduced number of CD-68 positive macrophages in the cardiac tissue of CKD-rats following rhNRG-1 β treatment. Representative images of CD68-positive macrophages (brown-stained cells) in left ventricular tissue section (objectives 20x and 40x, scale bar: 100 and 50 μ m, respectively). Sham: sham-operated group, CKD: chronic kidney disease group, CKD+rhNRG-1 β : recombinant human neuregulin-1 β -treated chronic kidney disease group.

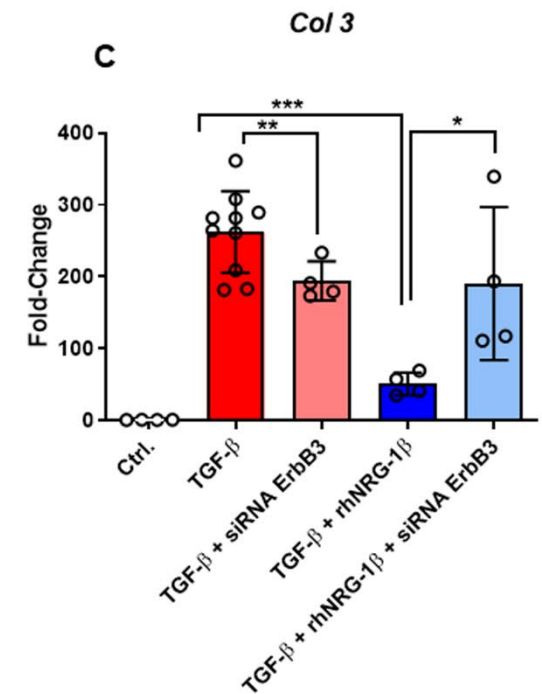
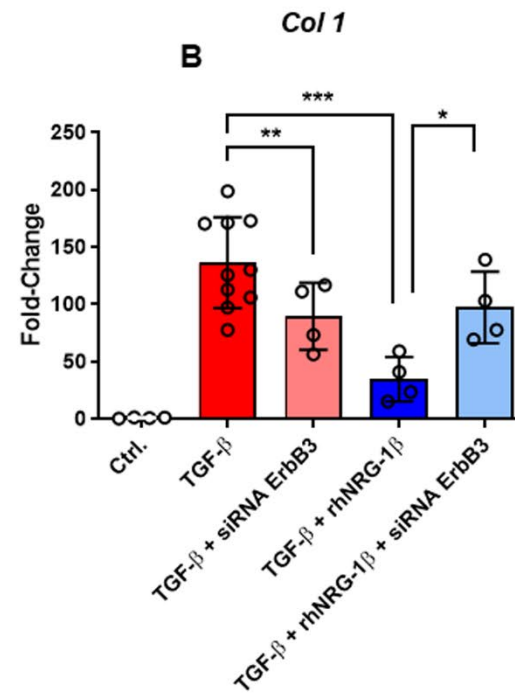
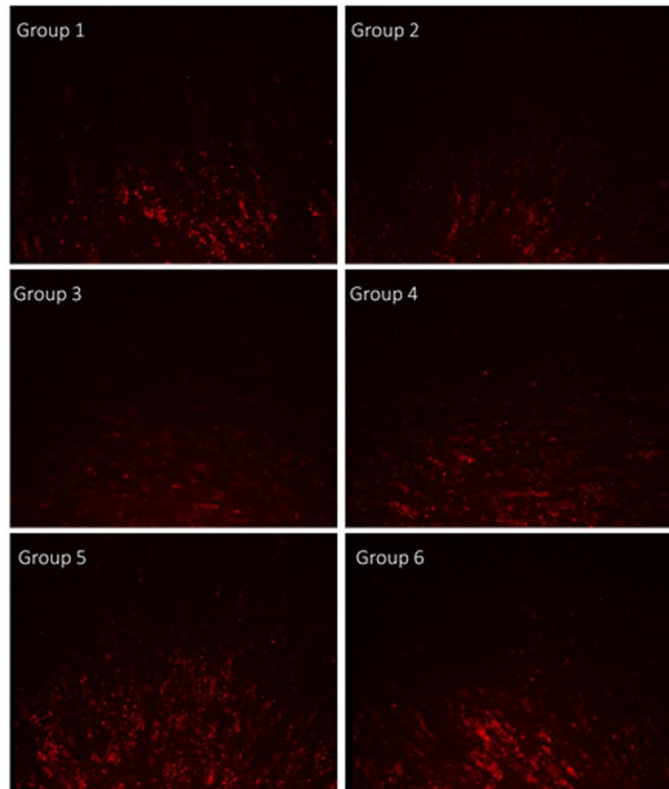
Fig. S5



Supplemental Figure 5 The effects of rhNRG-1β on mRNA expressions of ErbB2-4 receptors in the kidney (A-C) and left ventricular samples (D-E). mRNA expressions of (A and D) *ErbB2*: human epidermal growth factor receptor (HER) 2; (B and E) *ErbB3*: human epidermal growth factor receptor (HER) 3; (C and F) and *ErbB4*: human epidermal growth factor receptor (HER) 4. Data are expressed as mean±SEM.; n=4-8, *p<0.05, one-way ANOVA, Tukey *post hoc* test. Sham: sham-operated group, CKD: chronic kidney disease group, CKD+rhNRG-1β: recombinant human neuregulin-1β-treated chronic kidney disease group.

Fig. S6

A



Supplemental Figure 6 Specific siRNA-based interaction with ErbB3 receptor transcripts counteracted the NRG1 β -mediated suppression of TGF- β -induced collagen expression in human ventricular cardiac fibroblasts. (A) Representative images of siRNA transfection in human ventricular cardiac fibroblasts. Cells

were treated with 3 μL (Group 1-3) or 5 μL of siRNA (5 nmol/L) in combination with 1 μL (Group 1 and 4), 2 μL (Group 2 and 5), or 3 μL (Group 3 and 6) of transfection reagents. (B) The effect of siRNA-ErbB3 with/without TGF- β or/and rhNRG-1 β on Col 1 and Col 3 mRNA expression in human ventricular cardiac fibroblasts. Data are expressed as mean \pm SEM, n=4-8 replicates/group; *p<0.05, **p<0.01, and ***p<0.001, one-way ANOVA, Tukey post hoc test. Col 1: collagen 1; Col 3: collagen; rhNRG-1 β : recombinant human neuregulin-1 β ; TGF- β : Transforming growth factor-beta; siRNA-ErbB3: Small interfering RNA-ErbB3.