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Supplemental Information

microRNA-221 Inhibits Latent TGF-β1 Activation through Targeting Thrombospondin-1 to Attenuate Kidney Failure-Induced Cardiac Fibrosis

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Supplemental Results

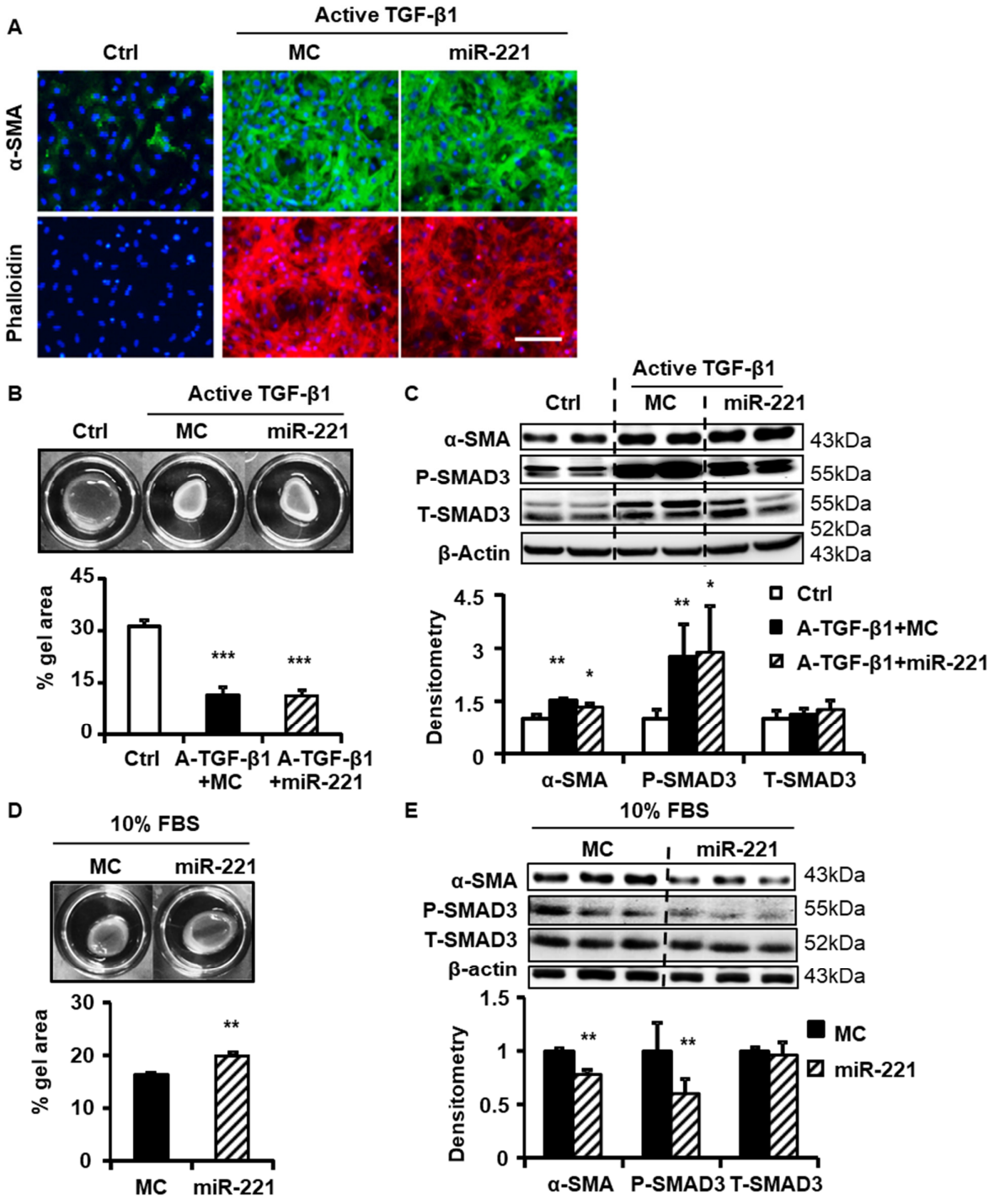

Figure S1: MiR-221 does not inhibit pro-fibrotic effects of active TGF-β1. Adult rat cardiac fibroblasts (cFB) were serum starved for 24 hr and then treated with 10 ng/ml active TGF-β1 (A-TGF-β1) for 2-day with the transfections of miR-221 mimic (miR-221) or mimic control (MC). (A) Immunofluorescence images of cFB stained with α-SMA (green), phalloidin (red) and DAPI (blue). Scale bar = 100 μm. (B) Collagen gel contraction assessment (3-day pre-stress and 2-day free contraction). Representative images (upper); percentage contraction measured as gel size relative to culture area (lower). (C) Western blot of cFB lysates for α-SMA, phosphorylated (P-) and total (T-) SMAD3, with β-Actin as loading control. (D) Collagen gel contraction in normal growth condition with 10% FBS. (E) Western blot of cFB lysates in normal growth condition. *p<0.05, **p<0.01 ***p<0.001 *vs.* Ctrl or MC by one-way ANOVA and Bonferroni post-hoc analysis. Experiments performed three times in triplicates.

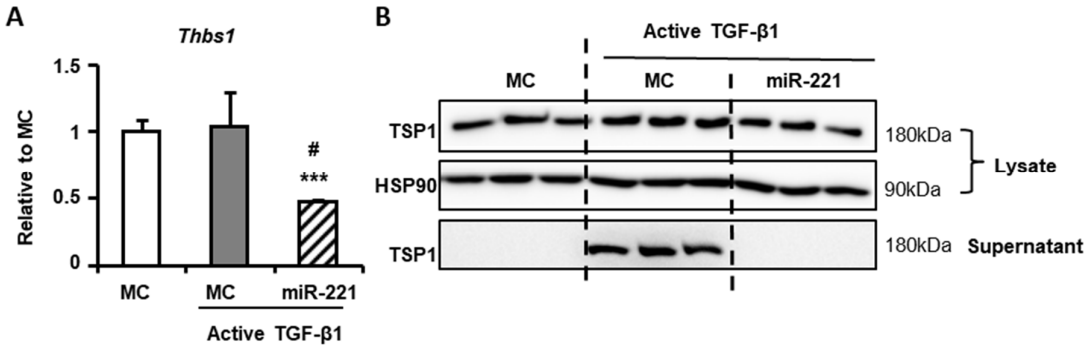

Figure S2: MiR-221 effects on active TGF-β1 induced TSP1 secretion. Adult rat cardiac fibroblasts (cFB) were serum starved for 24 hr and then treated in serum free medium with 10 ng/ml A-TGF-β1 for 5-day with transfections of miR-221 mimic (miR-221) or mimic control (MC). Expressions of (A) *Thbs1* by RT-qPCR and (B) TSP1 by Western blot. #p<0.05 *vs.* L-TGF-β1+MC; ***p<0.001 *vs.* MC by one-way ANOVA and Bonferroni post-hoc analysis. n = 3 in triplicates.

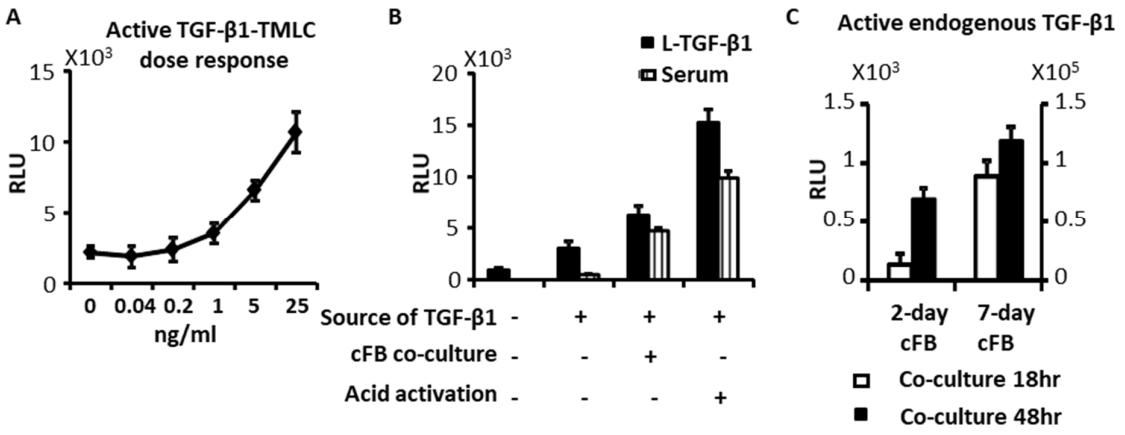

Figure S3: TGF-β1 activity and L-TGF-β1 activation assessments. (A) Transformed Mink Lung Epithelial Cells (TMLC) with Pai1-promoter driven luciferase reporter detected active recombinant TGF-β1 protein in a dose-dependent manner. (B) TMLC did not show significant luciferase activity at baseline or in the presence of L-TGF-β1 (80 ng/ml) or serum (10% FBS). Co-culture with cardiac fibroblasts (cFB) strongly increased luciferase activity in TMLC, indicating L-TGF-β1 activation. Pre-activation of L-TGF-β1 or serum with acid was performed as a positive control. (C) Endogenous L-TGF-β1 activation was increased with prolonged cFB culture, 2-day *vs.* 7-day, the activation signal was accumulated with prolonged co-culture with TMLC, 18 hr *vs.* 48 hr. RLU = relative luminescence units. n = 3 in triplicates.

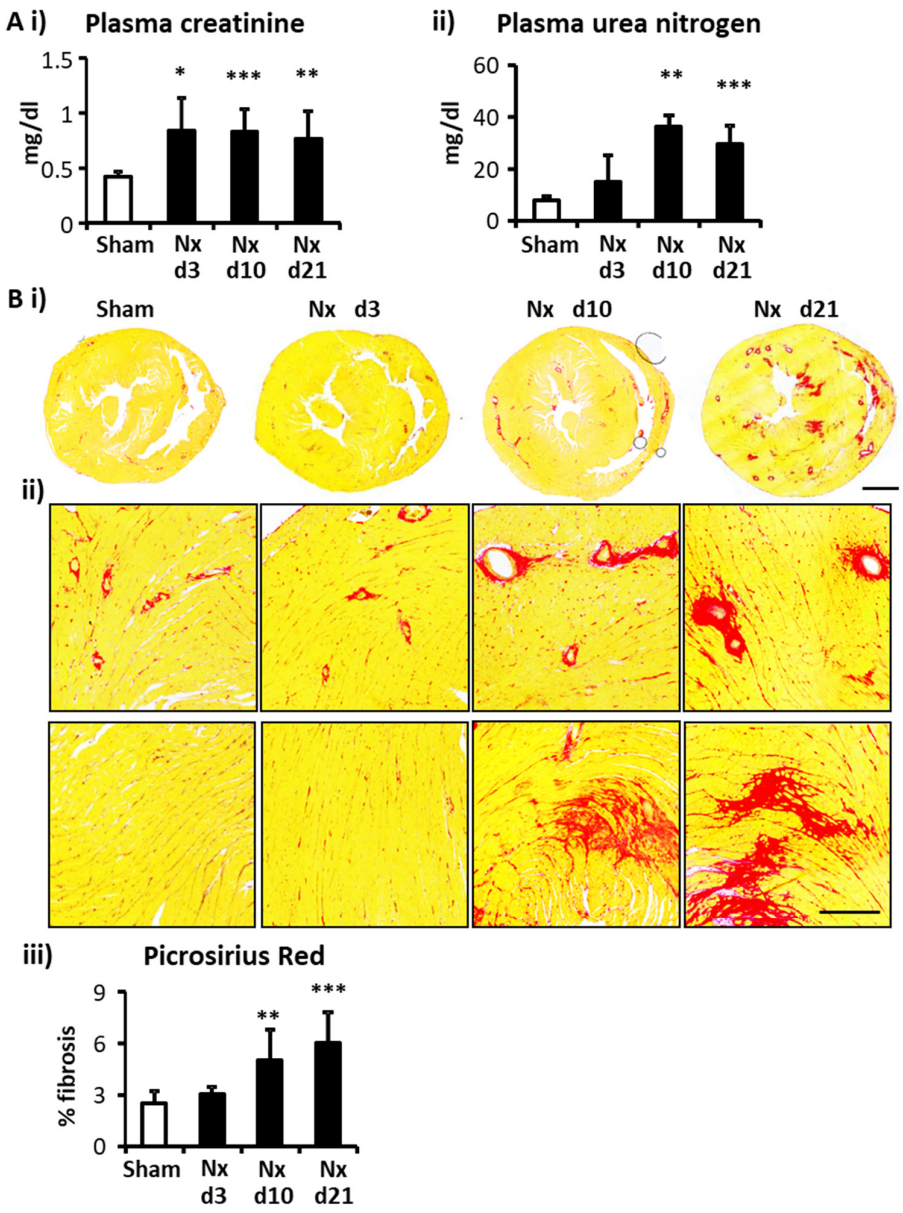

Figure S4: Time course of cardiac fibrosis development post 5/6 Nx. (A) Plasma i) creatinine and ii) urea nitrogen levels as indications of kidney function. (B) Picro-sirius red staining of the heart at day-3, -10 and -21 post-Nx. (i) Representative brightfield images of whole heart cross-sections, scale bar = 2 mm; (ii) perivascular (upper panel) and interstitial (lower panel) fibrosis, scale bar = 200 µm; (iii) Percentage of left ventricular (LV) fibrosis as quantified by ImageJ. **p< 0.01 and ***p<0.001 *vs.* Sham by one-way ANOVA and Bonferroni post-hoc analysis. n = 3-7 each group.

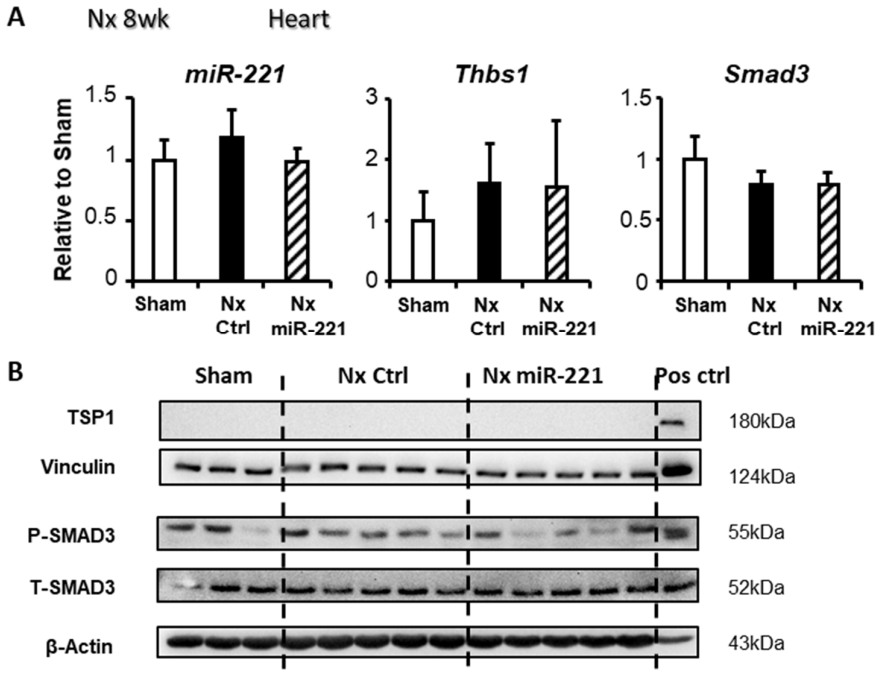

Figure S5: MiR-221, Thbs1 expression and TGF-β1 signaling in the heart at 8 weeks post-Nx. Rats received 1 mg/kg miR-221 mimics (Nx miR-221) or PBS i.v. injection (Nx Ctrl) for 3 times at day-3, -7 and -14 post-Nx. Hearts were harvested by the end of 8 weeks. (A) RT-qPCR measurements of miR-221, *Thbs1* and *Smad3* mRNA expressions. (B) Western blot measurement of protein expression for TSP1 and phosphorylated (P-) and total (T-) SMAD3. Vinculin and β-Actin served as loading control, respectively. Cultured cFB lysate was used as positive control. n=3 for sham, n=5 for both Nx control and miR-221 treated groups.

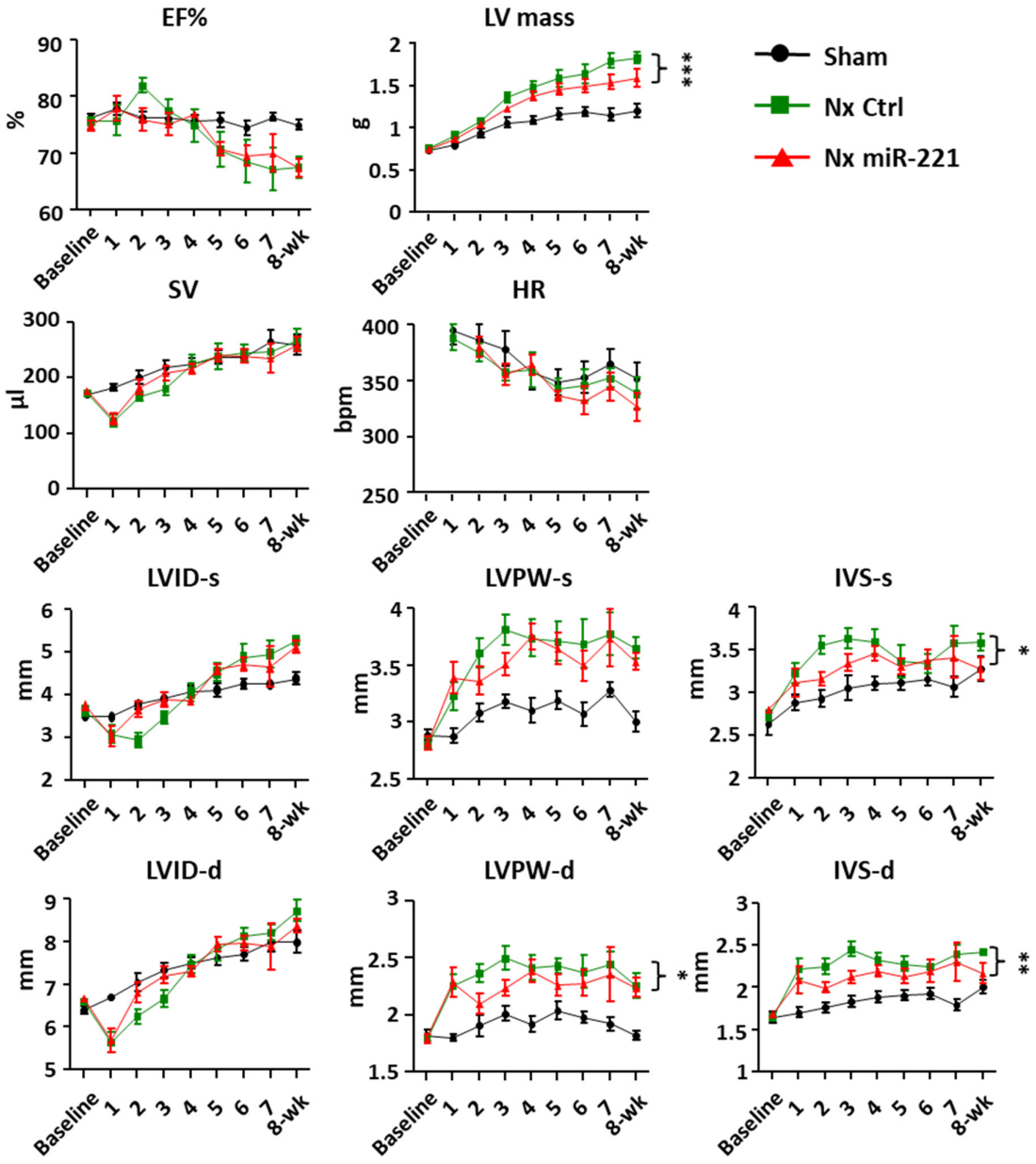

Figure S6: Echocardiography assessment of cardiac function. Nx rats received 1 mg/kg miR-221 mimics or PBS injection *i.v.* for 3 times at day-3, -7 and -14 (Nx miR-221 and Nx Ctrl). Ejection fraction (EF), left ventricular (LV) mass estimates, stroke volume (SV), heart rate (HR), LV internal dimension (LVID) at systole (s) and diastole (d), LV posterior wall (LVPW) and interventricular septum (IVS) thickness at systole (s) and diastole (d) were monitored weekly for 8 weeks. *p<0.05, **p<0.01 ***p<0.001 Nx miR-221 *vs.* Nx Ctrl. Two-way ANOVA repeated measurement with Bonferroni adjustment for analysis of serial measurement. n = 6-14 each group.

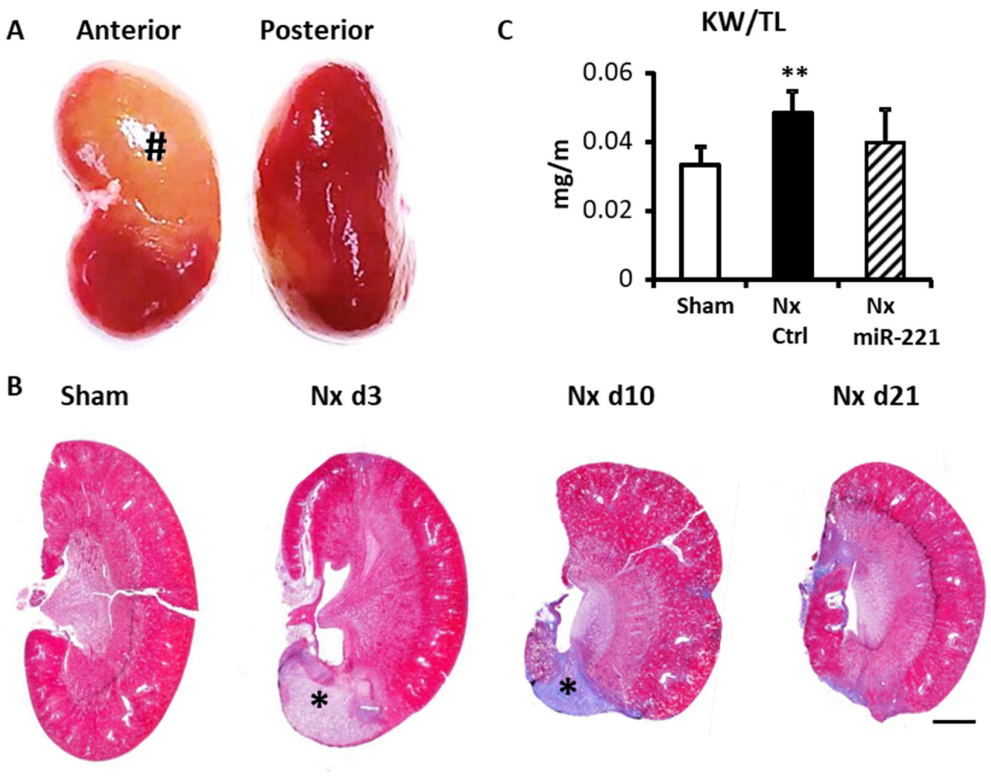

Figure S7: Kidney histology and weight. (A) Left kidney was excised right after Nx surgery and sectioned longitudinally into halves to visually confirm the proportion of nephrectomy. # remaining perfused region (yellow color, blood drained from unligated arteries); the rest was ischemia region (deep red). (B) Masson trichrome staining of longitudinal sections of the left kidney, from Sham or different time points after Nx surgery. * The infarct region could be observed as pale necrotic tissue at day-3 and blue fibrotic tissue at day-10 post-Nx. Scale bar = 2 mm. (C) Kidney weight (KW) measurement at the end of 8 weeks post-Nx, normalized by tibial length (TL). *p<0.01 *vs.* Sham by one-way ANOVA and Bonferroni post-hoc analysis. n = 3-7 each group.

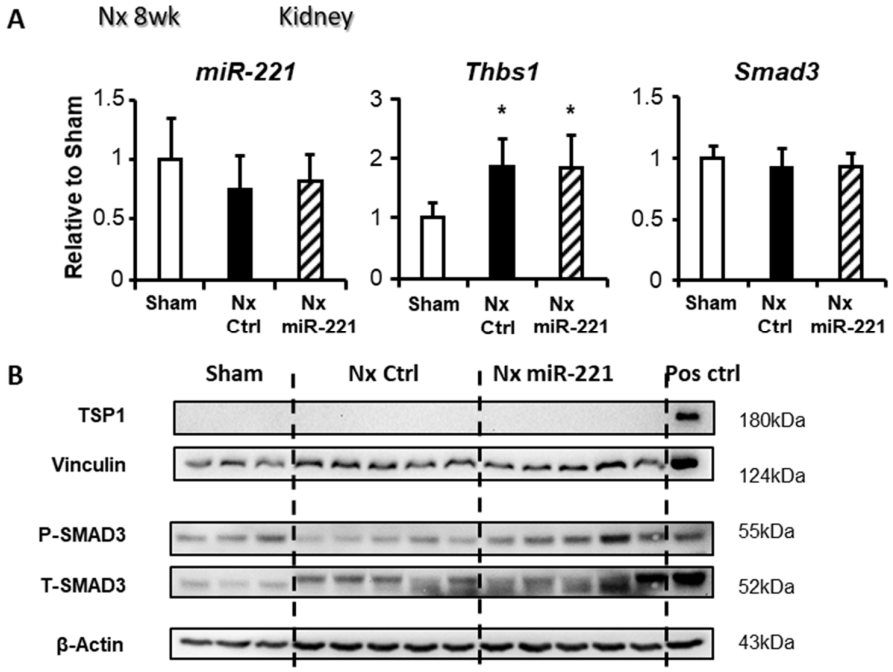

Figure S8: MiR-221, Thbs1 expression and TGF-β1 signaling in the kidney at 8 weeks post-Nx. Rats received 1 mg/kg miR-221 mimics (Nx miR-221) or PBS i.v. injection (Nx Ctrl) for 3 times at day-3, -7 and -14 post-Nx. Kidneys were harvested by the end of 8 weeks. (A) RT-qPCR measurements of miR-221, *Thbs1* and *Smad3* mRNA expressions. (B) Western blot measurement of protein expression for TSP1 and phosphorylated (P-) and total (T-) SMAD3. Vinculin and β-Actin served as loading control, respectively. Cultured cFB lysate was used as positive control. n=3 for sham, n=5 for both Nx control and miR-221 treated groups.

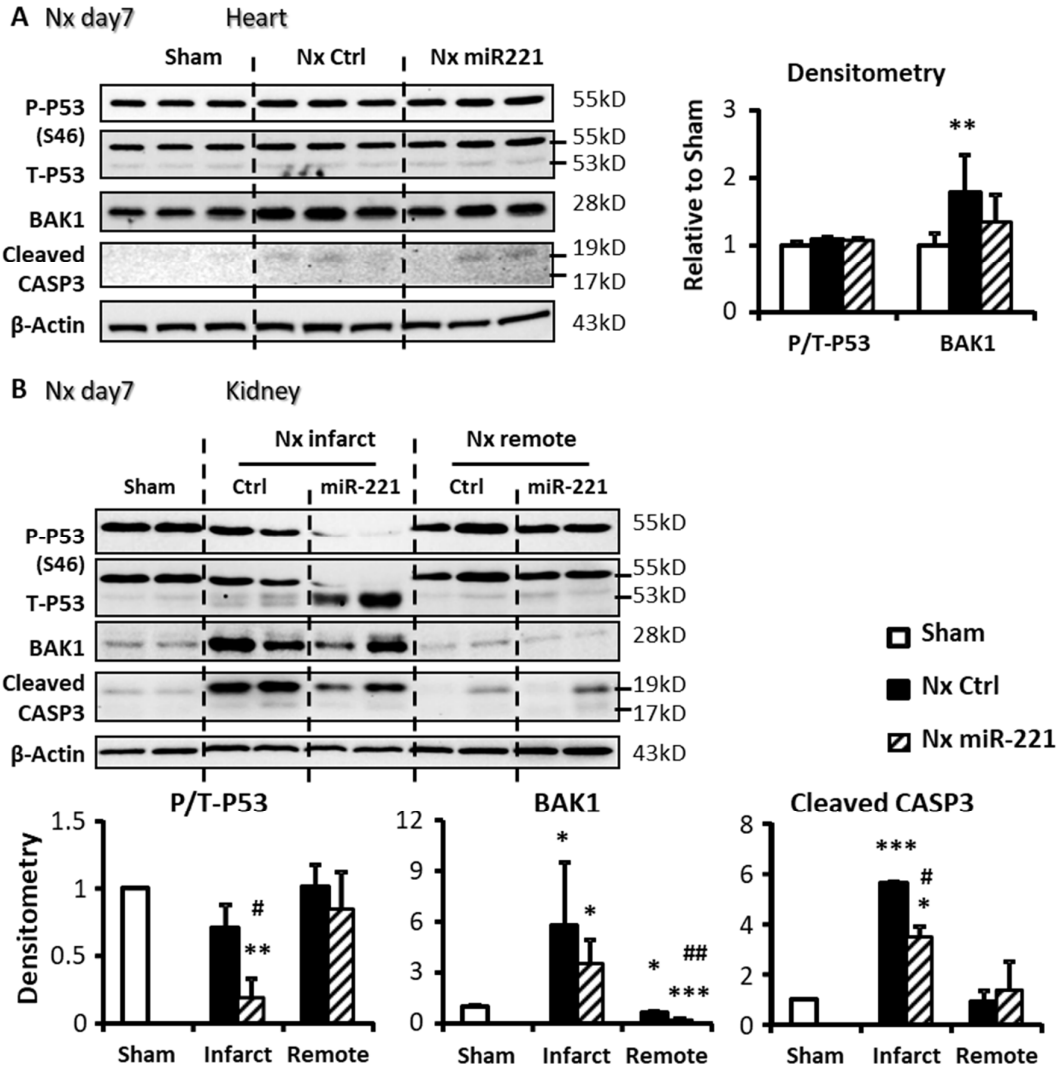

Figure S9: Apoptosis signaling in the heart and kidney and its regulation by miR-221. Rats with Nx received 1 mg/kg miR-221 mimics or PBS injection *i.v.* at day-6 post-Nx. Heart and kidney were collected 24 hr after mimics injection. Western blot of P53 (phosphorylated Ser46, P-P53 and total, T-P53), BAK1, cleaved Caspase3 (CASP3) were performed to detect apoptosis. β-Actin served as loading control. * $p<0.05$, ** $p<0.01$, ***$p<0.001$ *vs.* Sham; # $p<0.05$, ## $p<0.01$ *vs*. Nx Ctrl by one-way ANOVA and Bonferroni post-hoc analysis. n = 3 in triplicates.

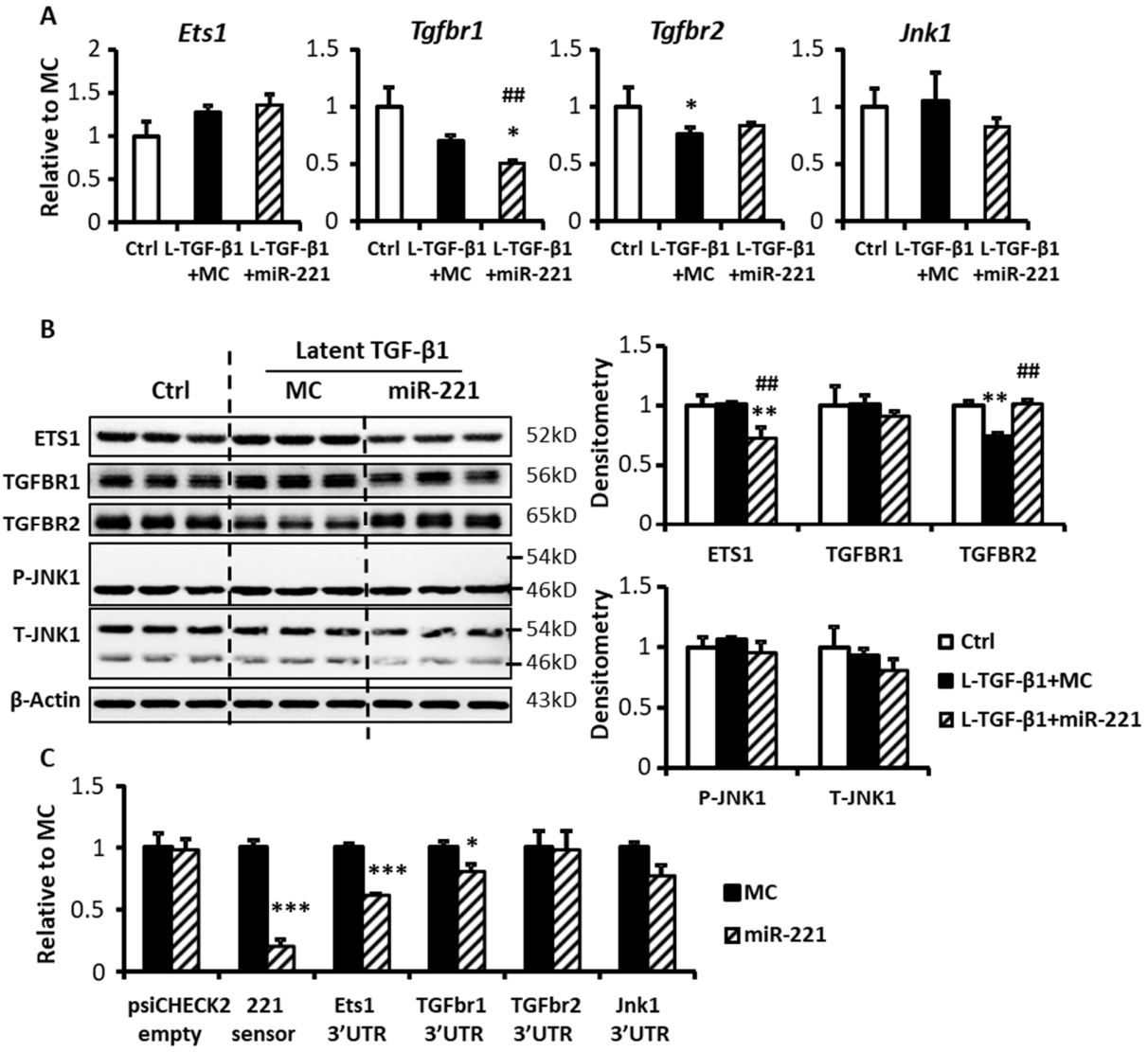

Figure S10: Other potential targets of miR-221 examined *in vitro*. Adult rat cardiac fibroblasts (cFB) were serum starved for 24 hr and then treated with 80 ng/ml L-TGF for 5 days with transfection of miR-221 mimic (miR-221) or mimic control (MC). Potential target genes *Ets1, Tgfbr1, Tgfbr2, Jnk1* expression in cFB were measured by (A) RT-qPCR and (B) Western blot. (C) 3'UTR luciferase reporter assay for measurement of interaction between the target gene 3'UTR and miR-221. Perfect complimentary sequence to miR-221 (221sensor) was used as a positive control. * p<0.05, ** p<0.01 *vs.* Ctrl; # p<0.05, ## p<0.01 *vs.* L-TGF+MC by one-way ANOVA and Bonferroni post-hoc analysis. n = 3 in triplicates.

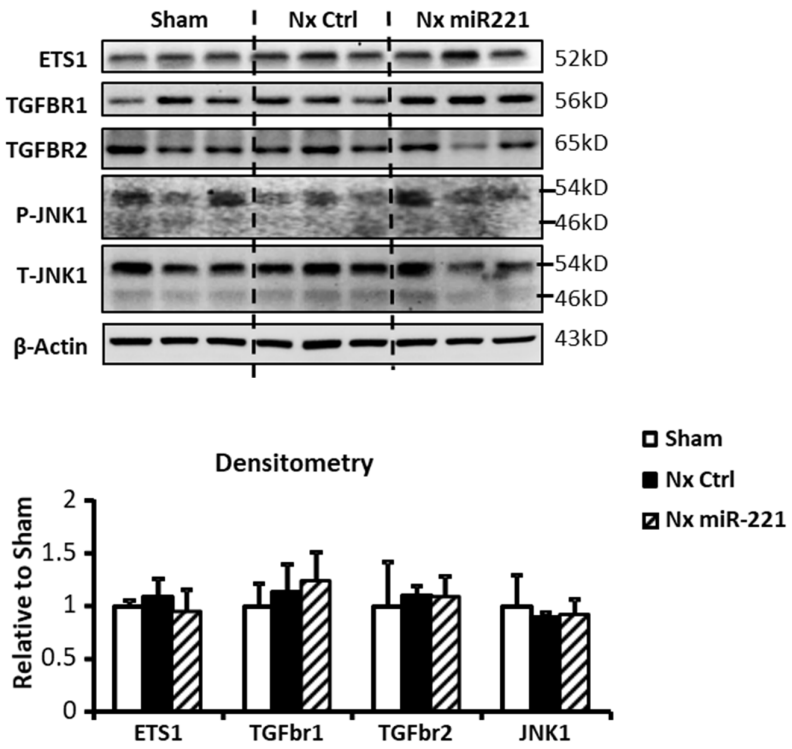

Figure S11: Other potential targets of miR-221 examined *in vivo*. Animals with Nx received 1 mg/kg miR-221 mimics or PBS injection *i.v.* at day-7 post-Nx. Heart tissues were collected 24 hr after mimics injection. Western blot was performed to assess protein expression of potential targets ETS1, TGFBR1, TGFBR2, JNK1 (phosphorylated, Thr183/Tyr185, P-JNK1 and total, T-JNK1). β-Actin served as loading control. No significant differences were observed by one-way ANOVA and Bonferroni post-hoc analysis. n = 3 in triplicates.

Supplemental methods

Table S1: Primers used for mRNA RT-qPCR.

Gene symbol	Forward primer (5'->3')	Reverse primer (5'->3')
Actb (β-actin)	GTACAACCTTCTTGCAGCTCCTC	TGACCCATACCCACCATCAC
Ets1	GCATCTAGAGATCCTGCAGAA	GCTCGATACCATAGCTAATGAAGT
Mapk8 (Jnk1)	GAGGTCATGGATTTGGAGGA	TCGGATCTGTGGACATTGAAG
Thbs1 (Tsp1)	GAATGTGAGGTTTGTCTTTGGAA	GATGTAGTTGGTGCGGATGG
Tgfb1	ATGACATGAACCGACCCTTC	CGTACACAGCAGTTCTTCTCT
Tgfbr1	TGAATCCTTCAAACGTGCTG	CTCTGCCATCTGTTGGGAAT
Tgfbr2	GATCTAACCTGTTGCCTGTGT	CCATGTATCTCGCTGTTCCC

Table S2: Primers used for cloning.

Construct name	Forward primer (5'->3')	Reverse primer (5'->3')
psiCHECK2-Tsp1-1-3'UTR	GCCGCTCGAG AATCACCACCCCGATCATAA	GGTGGTGCGGCCGC CCACGCATCCTGTTTCAATAAC
psiCHECK2-Tsp1-2-3'UTR	GCCGCTCGAG ATTGAAACAGGATGCGTGG	AAGGAAAAAAGCGGCCGC CCTAGAACACAGGGCATTGG
psiCHECK2-Ets1-3'UTR	GGTGGTCTCGAG GAGCGGACTTGACTTACAG	GGTGGTGCGGCCGC GACACTTACATGGCTACATCTC
psiCHECK2-TGFbr1-3'UTR	AATAAGCGATCGC ATCTGCTCCTGGGTGTTTTG	ATAAGAATGCGGCCGC AGGGCAGAGATCACACCAAC
psiCHECK2-TGFbr2-3'UTR	ACCGCTCGAG GGCTCCCTGAACACTACCAA	ATAAGAATGCGGCCGC AAGCCACAGTAACATGACACCA
psiCHECK2-Jnk1-3'UTR	GGTGGTCTCGAG CTACTTGCCAATCCCATCTTAG	GGTGGTGCGGCCGC GCACTTCACACACTTACTGG

Added nucleotides for restriction digestion are underlined.