Supplementary material

Supplementary figures and legends

Fig S1

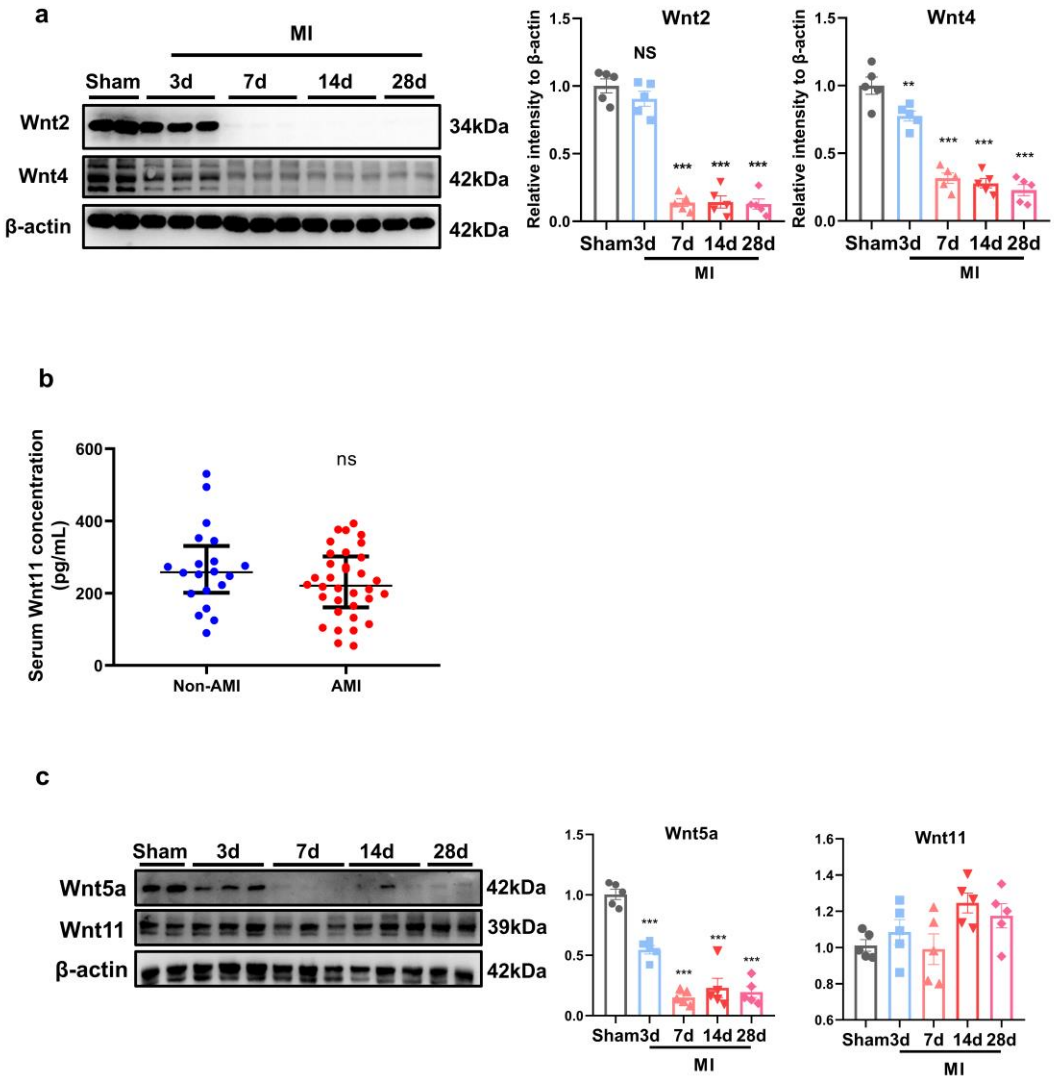


Fig. S1: Cardiac Wnts protein levels are changed in mice following MI. a: the expression of Wnt2 and Wnt4 of mice in the infarction area post MI at different time points. Values were expressed as means±S.E.M; **p<0.01, ***p<0.001.vs sham group. n=5/group. Statistics: One-way ANOVA with post-hoc Tukey test. b:ELISA analysis of serum Wnt11 level in a total of 34 patients with AMI and 20 non-AMI patients. Data are expressed as median. c: Western blot analysis of the expression of Wnt5a and Wnt11 in the border zone of infarcted area at different time-points (3d, 7d, 14d, 28d) following MI. Values were expressed as means±S.E.M; *p<0.05, **p<0.01, ***p<0.001 vs sham group, n=5/group. Statistics: One-way ANOVA with post-hoc Tukey test.

Fig S2

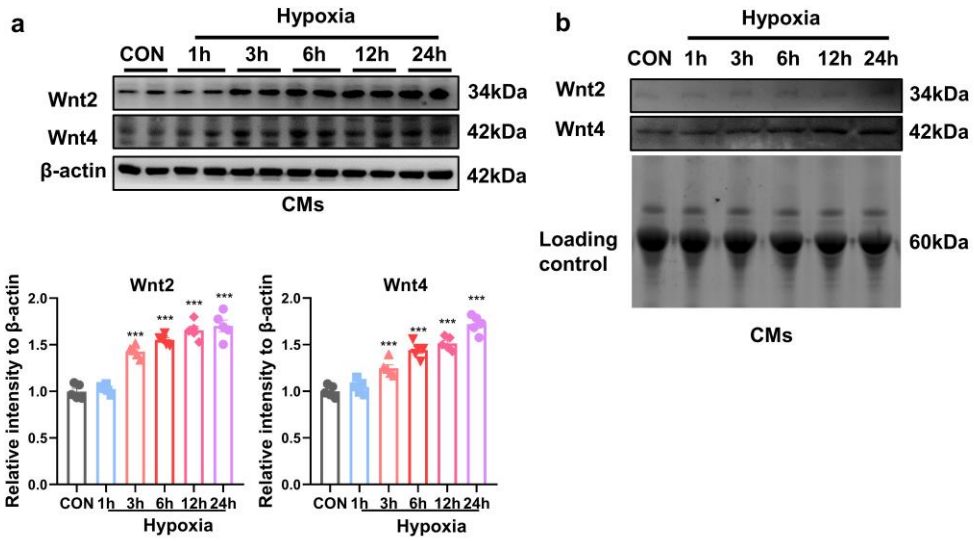


Fig. S2: Hypoxia promotes the expression and secretion of Wnt2 or Wnt4 in cultured neonatal rat cardiomyocytes (NRCMs). a: Wnt2 and Wnt4 expression were analyzed by Western blot analysis in cultured NRCMs at different time-points (1h,3h,6h,12h,24h) in response to hypoxia. Values were expressed as means±S.E.M; *p<0.05, **p<0.01, ***p<0.001 vs control group. n=4/group. Statistics: One-way ANOVA with post-hoc Tukey test. b: The representative images of Wnt2 and Wnt4 expression with Western blot analysis in conditional medium from NRCMs at different time-points (1h,3h,6h,12h,24h) after hypoxia. The experiment was repeated for three times.

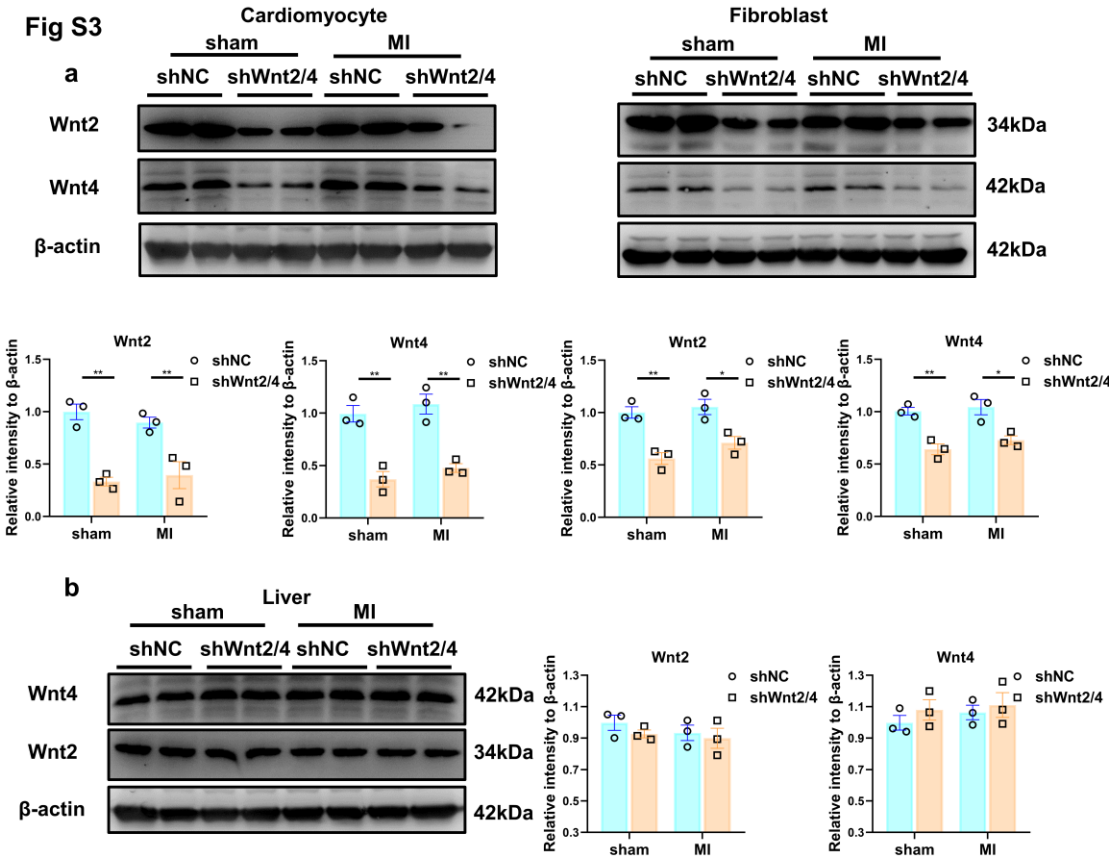


Fig. S3: The AAV9-shWnt2/4 specifically inhibits the expression of wnt2/4 in cardiomyocytes and fibroblasts in adult mice. a: Wnt2 and Wnt4 expression were analyzed by Western blot analysis in cardiomyocytes and fibroblasts isolated from shNC and shWnt2/4 mice.
b: The representative images of Wnt2 and Wnt4 expression with Western blot analysis in liver of adult mice. Values were expressed as means±S.E.M; *p<0.05, **p<0.01, ***p<0.001.vs shNC group. n=3/group. Statistics: Two-way ANOVA with a Bonferroni post hoc test.

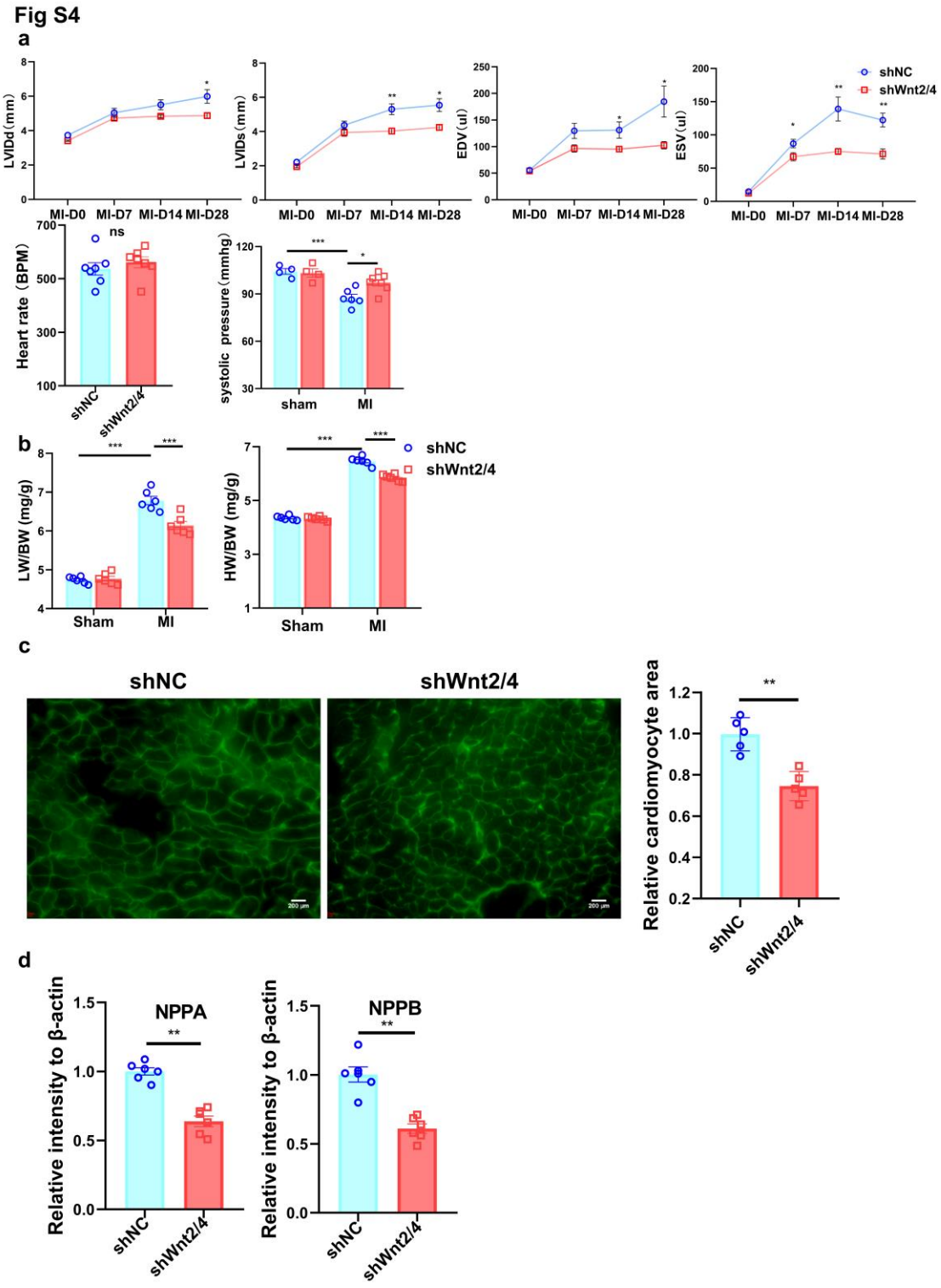


Fig. S4: Knockdown of Wnt2/Wnt4 suppresses cardiac hypertrophy post-MI. a: Echocardiographic analysis of mice at day 7,14 and 28 post MI. Quantitative analysis of ESV, EDV, LVIDs, LVIDd. ESV, End-systolic volume; EDV, End-diastolic volume; LVIDs, Left ventricular end systolic diameter; LVIDd, Left ventricular end diastolic diameter. Values were expressed as means±S.E.M; *p<0.05, **p<0.01, ***p<0.001.vs shNC group. n=6/group. Statistics: Student's t test. Hemodynamics analysis of systolic pressure after MI in mice. Values

were expressed as means±S.E.M. *p<0.05;**p<0.01;***p<0.001, n=4-8 in each group. Statistics: Two-way ANOVA with a Bonferroni post hoc test. b: statistical analysis of heart and lung weight, adjusted for body weight (HW/BW and LW/BW) at day 28 post-MI. Values were expressed as means±S.E.M; *p<0.05, **p<0.01, ***p<0.001. n=6/group. Statistics: Two-way ANOVA with a Bonferroni post hoc test. c: Analysis of cross-sectional area (CSA) of cardiomyocytes by Wheat germ agglutinin (WGA) staining in left ventricles from shWnt2/4 mice and shNC group after MI. Values were expressed as means±S.E.M; **p<0.01*vs* shNC group. n=5/group. Statistics: Student's t test. d: mRNA levels of NPPA and NPPB in remote area of mice from shWnt2/4 mice and shNC group at day 28 post MI. Values were expressed as means±S.E.M; **p<0.01 *vs* shNC group. n=6/group. Statistics: Two-way ANOVA with a Bonferroni post hoc test.

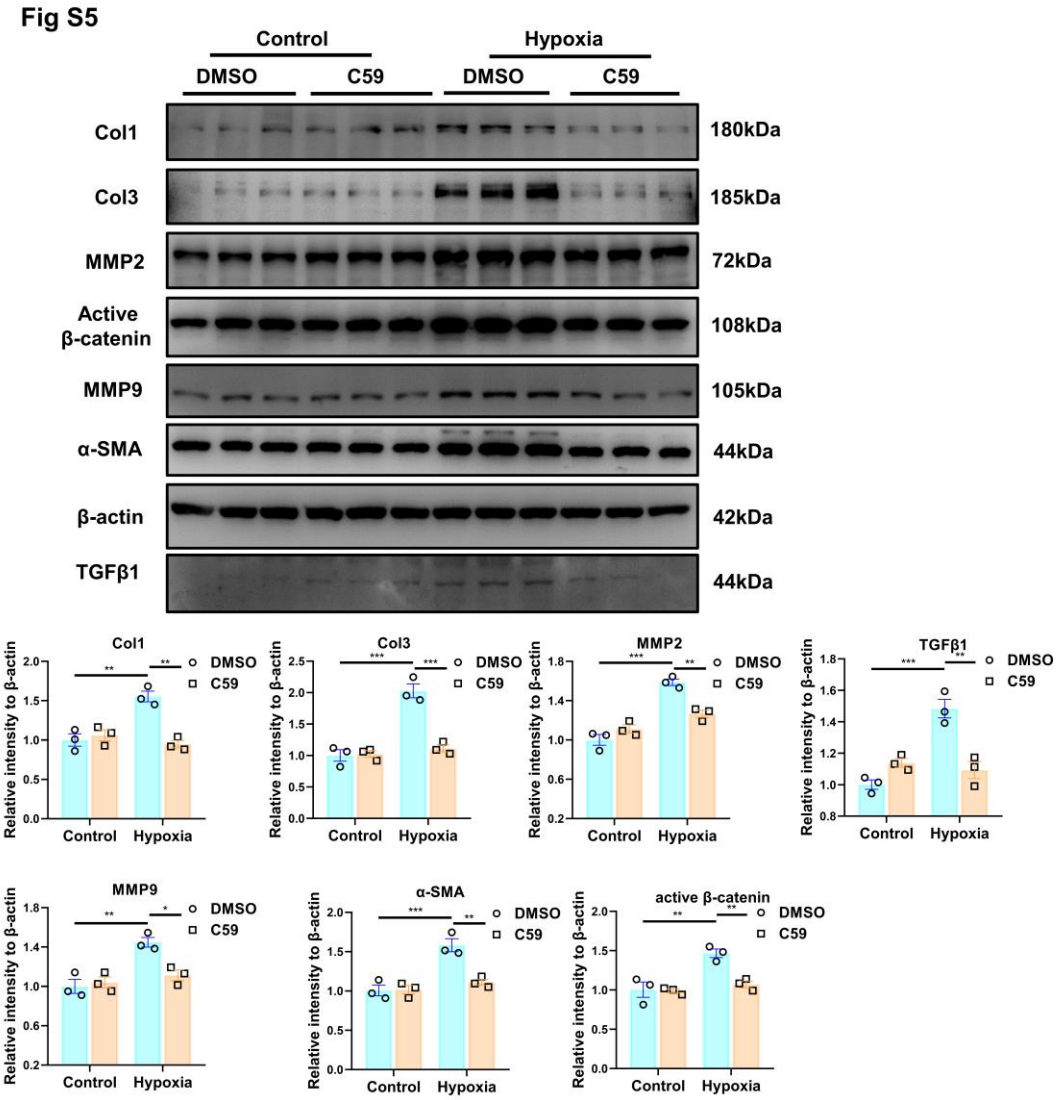
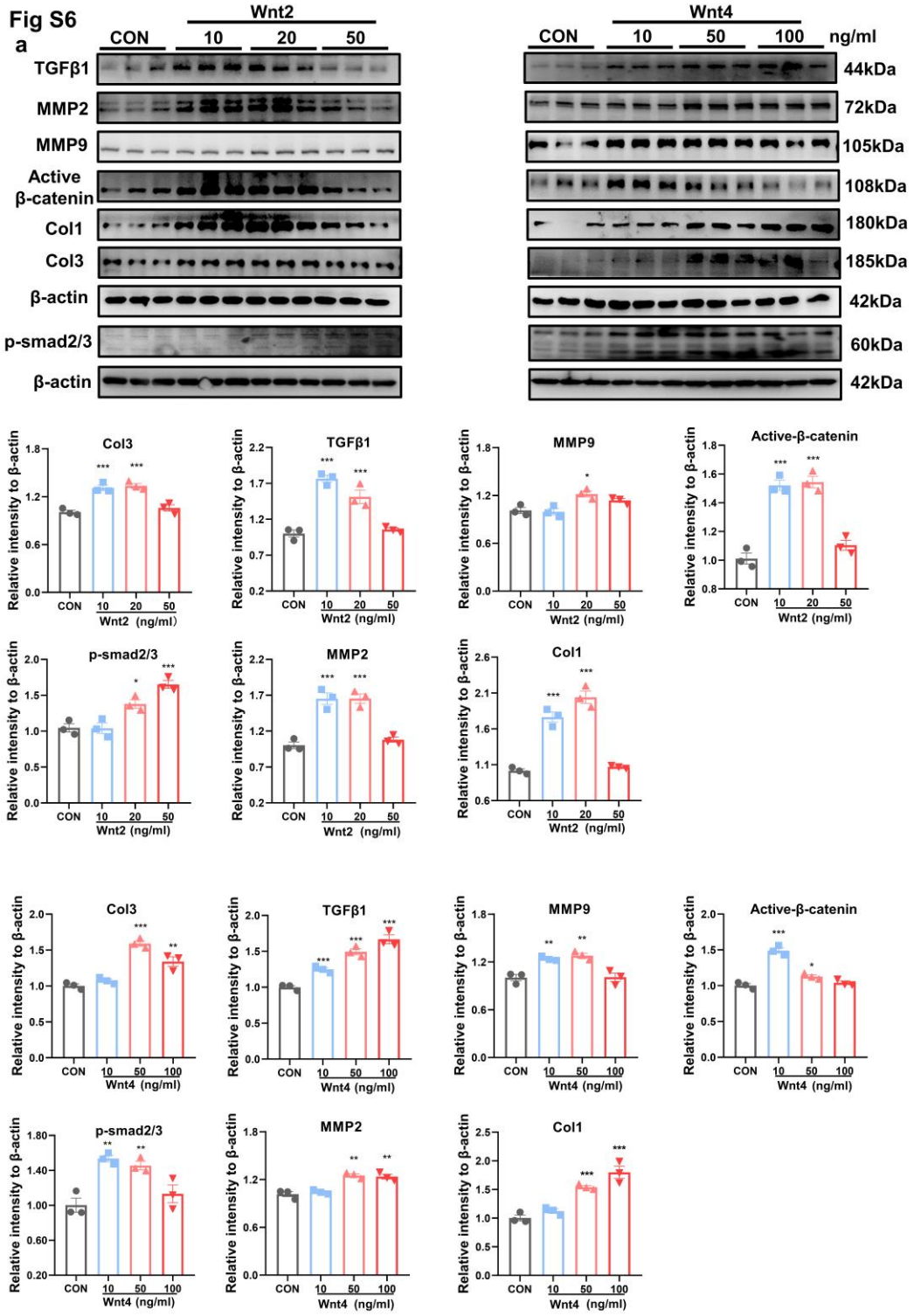


Fig. S5: Wnt-C59(C59), a PORCN inhibitor ameliorates hypoxia-induced pro-fibrotic effects in neonatal rat cardiac fibroblasts (NRCFs). The expressions of Col1, Col3, active β-catenin, MMP2, MMP9, α-SMA, TGFβ1 were measured in NRCFs by Western blot analysis. Values

were expressed as means±S.E.M; *p<0.05, **p<0.01, ***p<0.001vs DMSO control group. n=3/group. Statistics: Two-way ANOVA with a Bonferroni post hoc test.



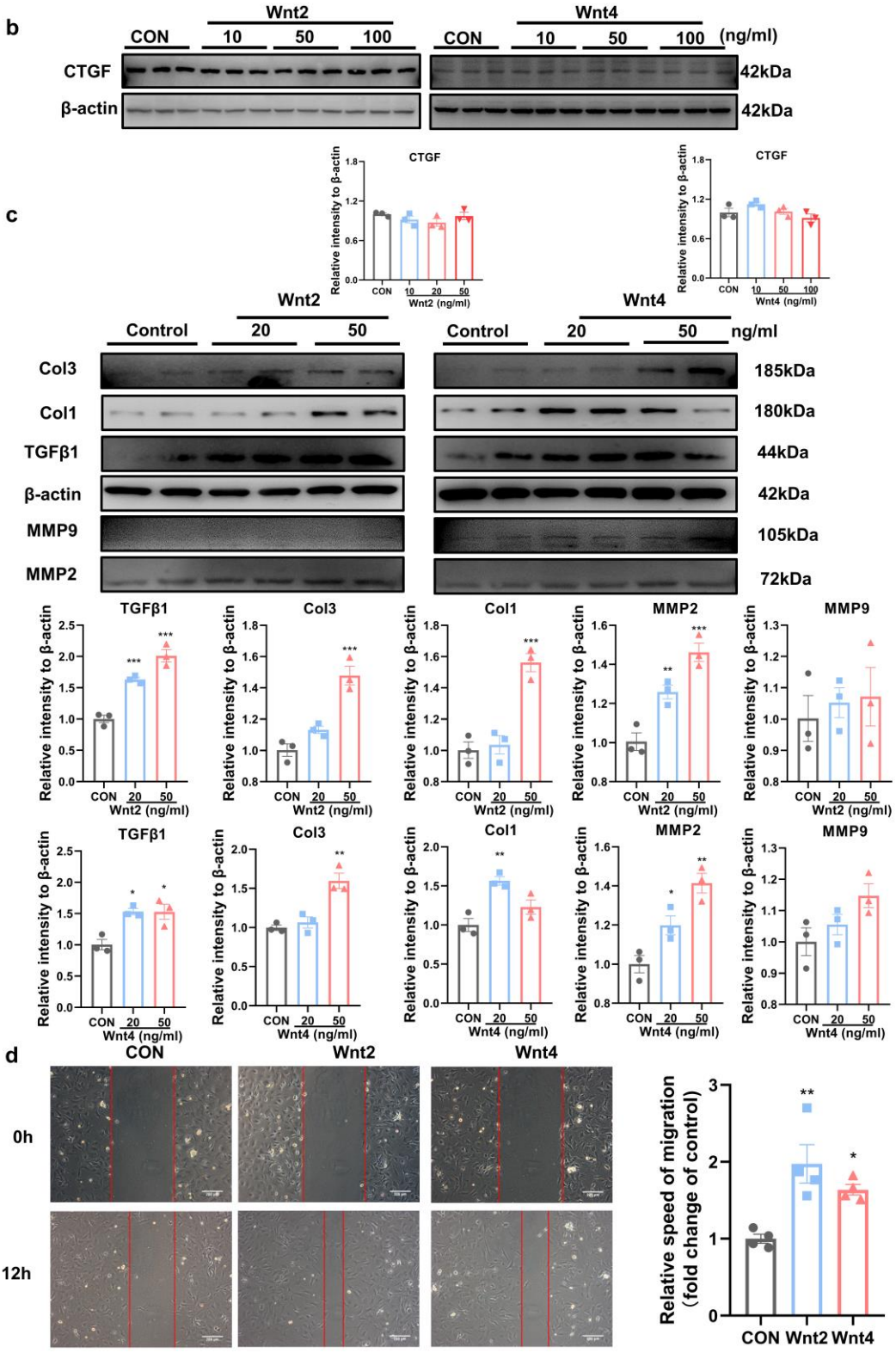


Fig. S6 Wnt2 or Wnt4 induces pro-fibrotic effects in cultured cardiac fibroblasts. a: The expression of Col1, Col3, TGF-β1, active β-catenin, MMP2, MMP9 and p-smad2/3 were determined by Western blot analysis in cultured neonatal rat cardiac fibroblasts (NRCFs) treated with human recombinant Wnt2 (0, 10ng, 20ng, 50ng/ml) or Wnt4 (0, 10ng, 50ng, 100ng/ml). The same volume of PBS was treated at the same time as control (CON). Values were presented as means±S.E.M. *p<0.05, **p<0.01, ***p<0.001 vs control group; n=3/group.

Statistics: One-way ANOVA with post-hoc Tukey test. b: Western blot analysis of the expression of CTGF in NRCFs treated with Wnt2(0, 10, 20ng, 50ng/ml) or Wnt4(0, 20, 50ng, 100ng/ml). n=3/each group. Statistics: One-way ANOVA with post-hoc Tukey test. c: The expression of Col1, Col3, TGF-β1, MMP2 and MMP9 were determined by Western blot analysis in cardiac fibroblasts isolated from adult mice treated with Wnt2 (0, 20ng, 50ng/ml) or Wnt4 (0, 20ng, 50ng/ml). N=3 per group. Statistics: One-way ANOVA with post-hoc Tukey test. d: Scratch assay demonstrated migratory ability of cardiac fibroblasts treated with PBS(CON), Wnt2 at 20 ng/ml or Wnt4 at 50ng/ml over 12h. The representative images were showed (Left). Values were quantitified and expressed as means±S.E.M (Right). *p<0.05, **p<0.01 vs control group; n=4/group. Statistics: One-way ANOVA with post-hoc Tukey test. bar=200um

Fig S7

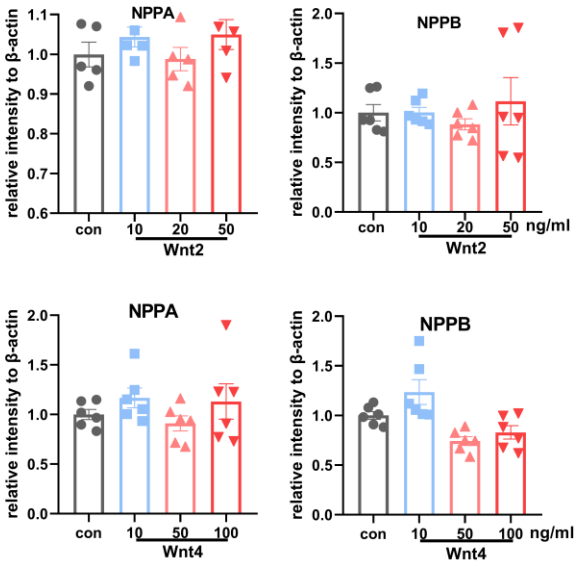


Fig. S7: Wnt2 or Wnt4 didn't affect hypertrophic genes expression. mRNA levels of NPPA and NPPB expression in neonatal rat ventricular myocytes treated with human recombinant Wnt2(0,10,20,50ng/ml) or Wnt4(0,20,50,100ng/ml) for 24h. Values were expressed as means±S.E.M; Statistics: One-way ANOVA with post-hoc Tukey test.

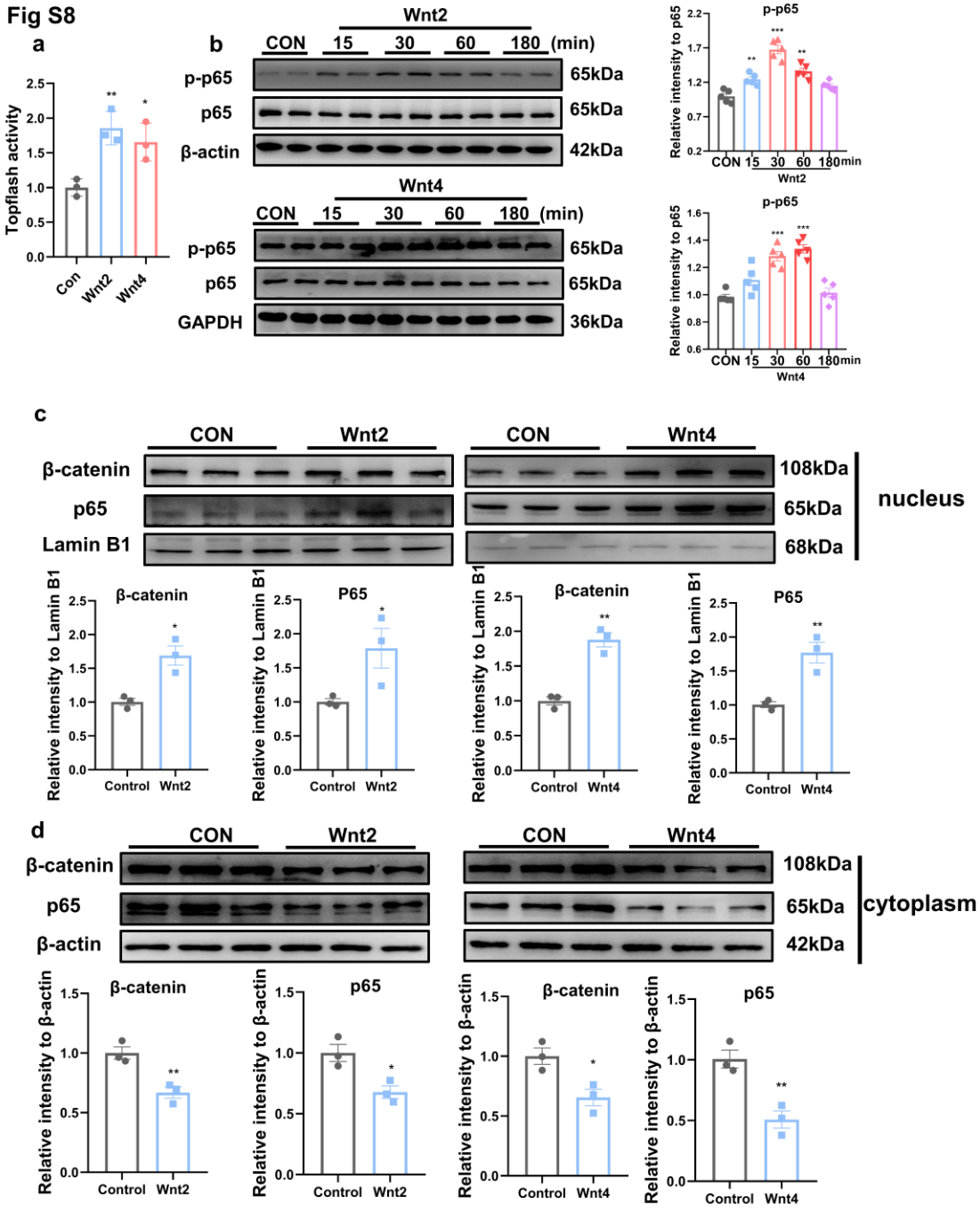


Fig S8
a
Topflash activity
Con
Wnt2
Wnt4
b
Wnt2
CON 15 30 60 180 (min)
p-p65 65kDa
p65 65kDa
β-actin 42kDa
Wnt4
CON 15 30 60 180 (min)
p-p65 65kDa
p65 65kDa
GAPDH 36kDa
p-p65
Relative intensity to p65
CON 15 30 60 180min
Wnt2
p-p65
Relative intensity to p65
CON 15 30 60 180min
Wnt4
c
CON Wnt2 CON Wnt4
β-catenin 108kDa
p65 65kDa
Lamin B1 68kDa
nucleus
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β-actin 42kDa
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Fig S8

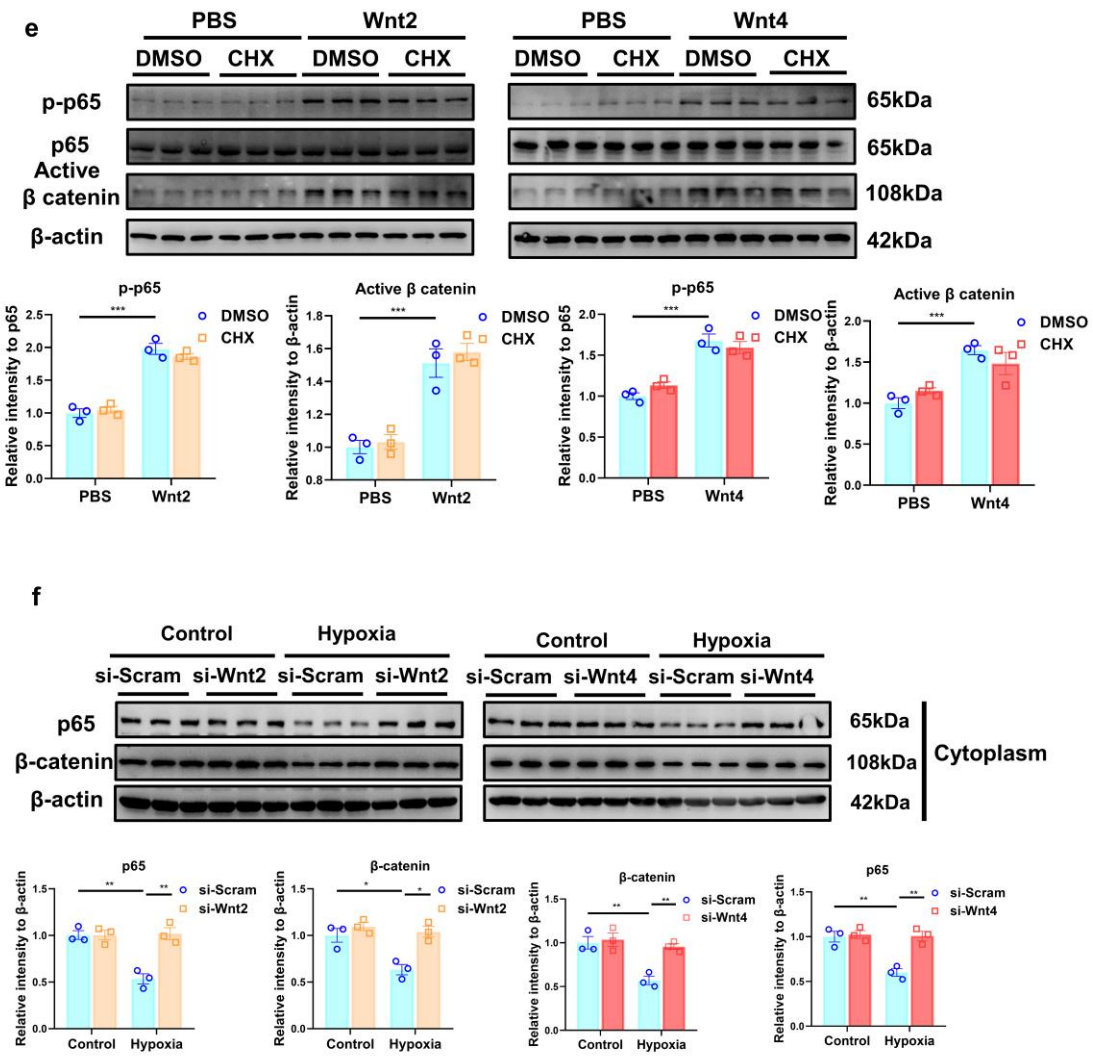

Fig. S8 Wnt2 or Wnt4 promotes the activation of β-catenin and NF-κB in neonatal rat cardiac fibroblasts (NRCFs) under hypoxia. a: Luciferase reporter assay for canonical Wnt signaling in COS-7 cells transfected with TOPFlash plasmid and treated with human recombinant Wnt2(20ng/ml) and Wnt4(50ng/ml) N=3 per group. Statistics: One-way ANOVA with post-hoc Tukey test. b: Western blot analysis of the expressions of NF-κB/p65 in NRCFs treated with human recombinant Wnt2 protein (20ng/ml) or Wnt4 protein(50ng/ml) at different time points (0, 15, 30, 60, 180min). The same volume of PBS was treated as control (CON). n=3/each group. Statistics: One-way ANOVA with post-hoc Tukey test. c: The expression of p65 and β-catenin in nucleus were determined by Western blot analysis in cultured NRCFs treated with human recombinant Wnt2 (20ng/ml) or Wnt4 (50ng/ml) for 12h. The same volume of PBS was treated at the same time as control (CON). n=3/each group. Statistics: Student's t test. d: The expression of p65 and β-catenin in cytoplasm were determined by Western blot analysis in cultured NRCFs treated with human recombinant Wnt2 (20ng/ml) or Wnt4 (50ng/ml) for 12h. The same volume of PBS was treated at the same time as control (CON). n=3/each group.

Statistics: Student's t test. e: The expression of p-p65 and active β-catenin were determined by Western blot analysis. n=3/each group. Statistics: Two-way ANOVA with a Bonferroni post hoc test. NRCFs were pretreated with Cycloheximide(CHX) 50um/ml for 0.5h and then Wnt2 (20ng/ml) or Wnt4 (50ng/ml) were added for another 1.5h. The same volume of DMSO was treated at the same time as control. f: The expression of p65 and β-catenin in cytoplasm were analyzed by Western blot. NRCFs were pretreated with siRNA targeted Wnt2 or Wnt4 (si-Wnt2 or si-Wnt4) or si-Scramble (si-Scram) for 24 hours, and then exposed to hypoxia or normoxia (Control) for 3 hours. n=3/each group. The data were expressed as means±S.E.M. *p<0.05, **p<0.01, ***p<0.001 vs control group. Statistics: Two-way ANOVA with a Bonferroni post hoc test.

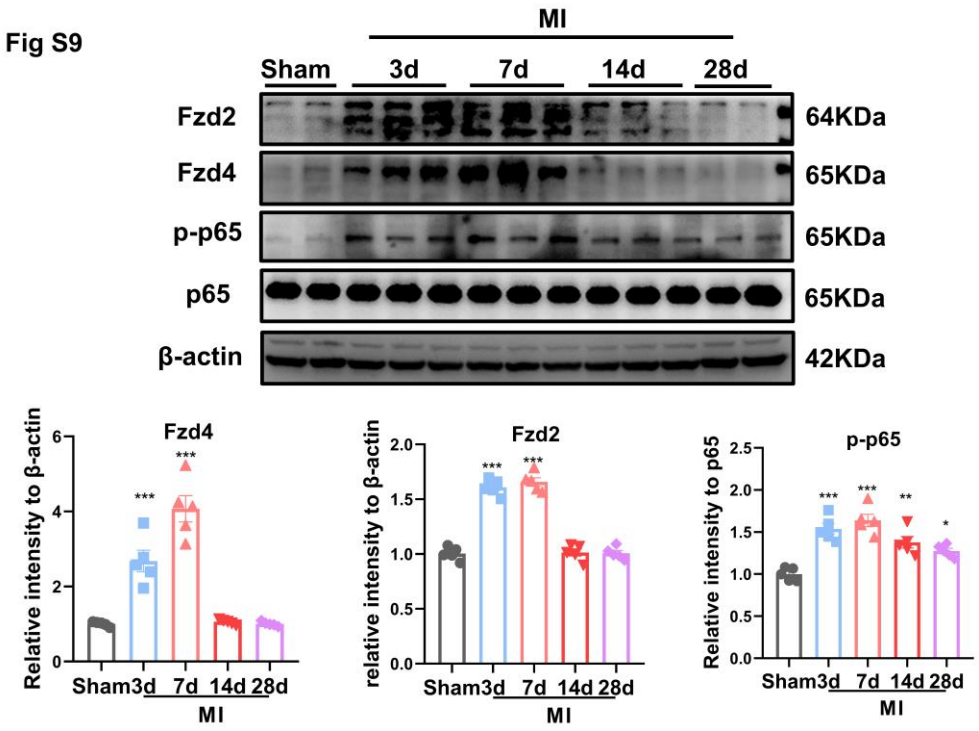

Fig. S9: Fzd2, Fzd4, p-p65 protein levels were altered in mice after MI. Western blot analysis of the protein levels of Fzd2, Fzd4 and p-p65 in border area of the infarcted tissue at the different time-points following MI. n=5 in each group. *p<0.05, **p<0.01, ***p<0.001 compared with sham group. Statistics: One-way ANOVA with post-hoc Tukey test.

Fig S10

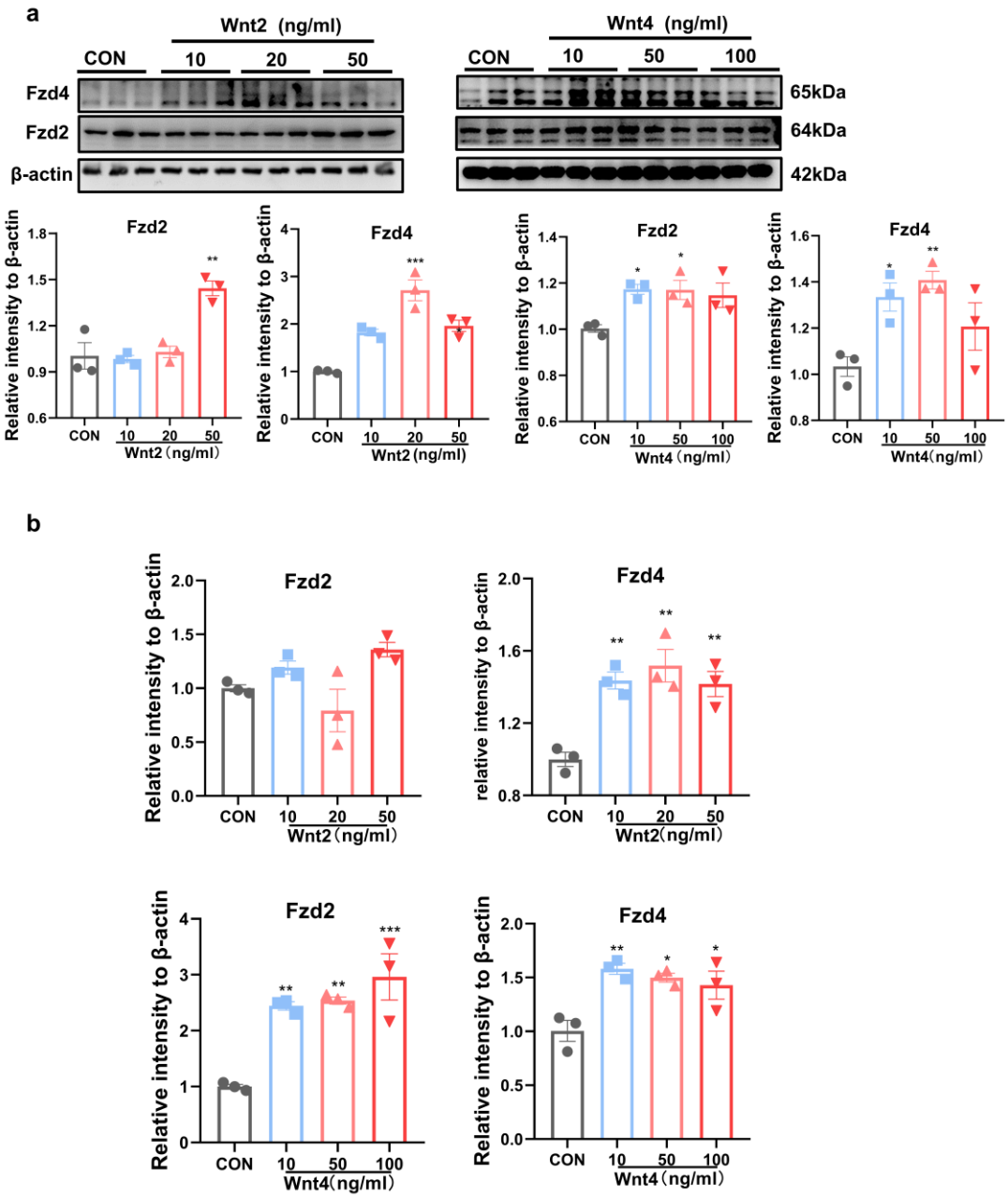

Fig. S10: The effects of Wnt2 or Wnt4 on Fzd2 or Fzd4 expression in neonatal rat cardiac fibroblasts (NRCFs). a,b Western blot and RT-PCR analysis of Fzd2/Fzd4 proteins and mRNA expression in NRCFs treated with human recombinant Wnt2 protein (0, 10ng, 20ng, 50ng/ml) or Wnt4 protein (0, 10ng, 50ng, 100ng/ml) for 24 hours. Values were expressed as means±S.E.M. *p<0.05, **p<0.01, ***p<0.001 vs control group; n=3 in each group. Statistics: One-way ANOVA with post-hoc Tukey test.

Fig S11

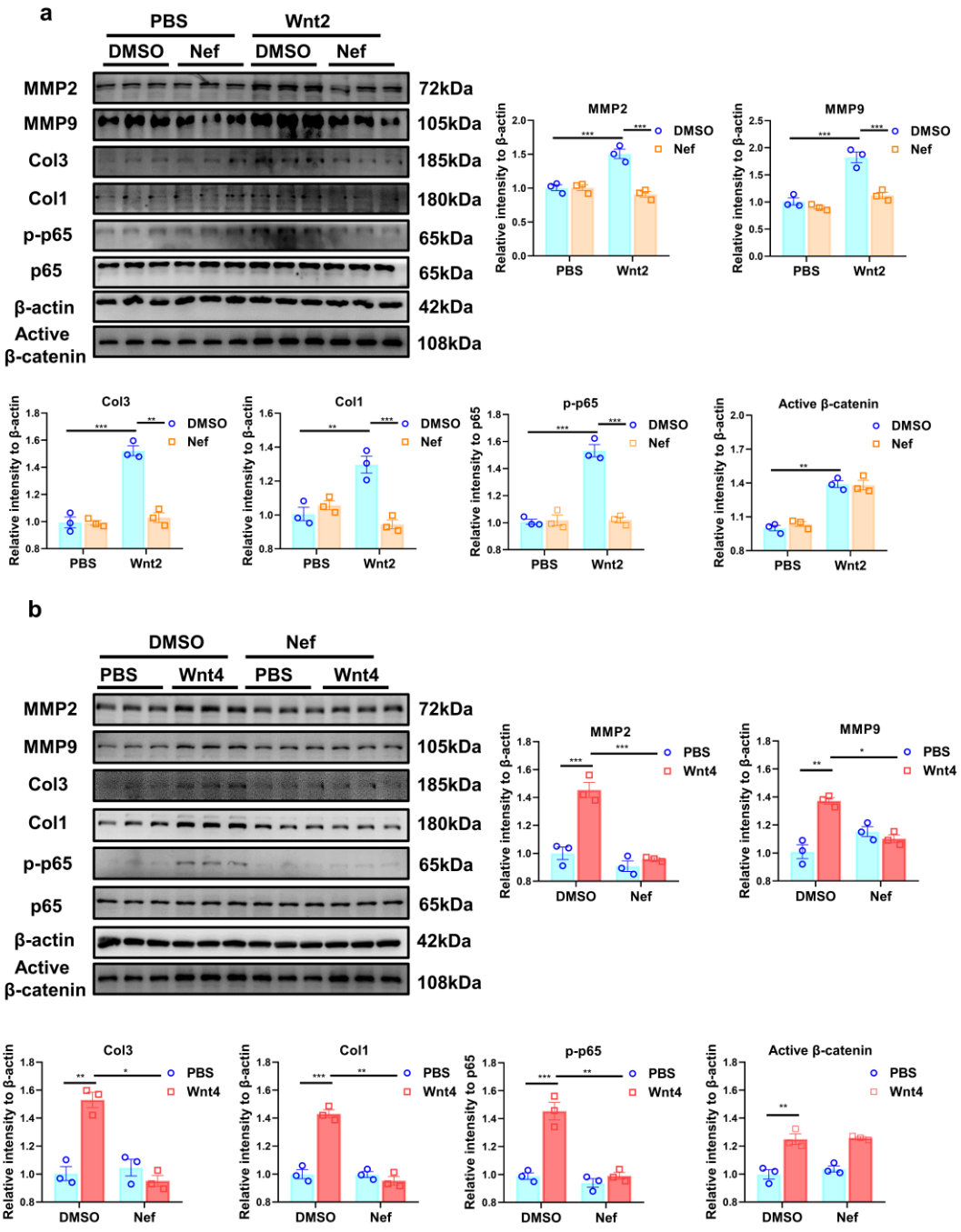

Fig. S11: NF-κB inhibitor ameliorates Wnt2/Wnt4-induced pro-fibrotic effects in neonatal rat cardiac fibroblasts (NRCFs). a,b The expressions of Col1, Col3, active β-catenin, MMP2, MMP9, p65 and p-p65 were measured in NRCFs by Western blot analysis. These cardiac fibroblasts were pretreated with neferine (Nef, NF-κB inhibitor, 5μM) or the same volume of DMSO for 1 h, followed by PBS, human recombinant Wnt2 (20ng/ml) or Wnt4 (50ng/ml) treatment for 24 hours. Values were expressed as means±S.E.M. *p<0.05, **p<0.01,

***p<0.001, n=3 in each group. Statistics: Two-way ANOVA with a Bonferroni post hoc test.

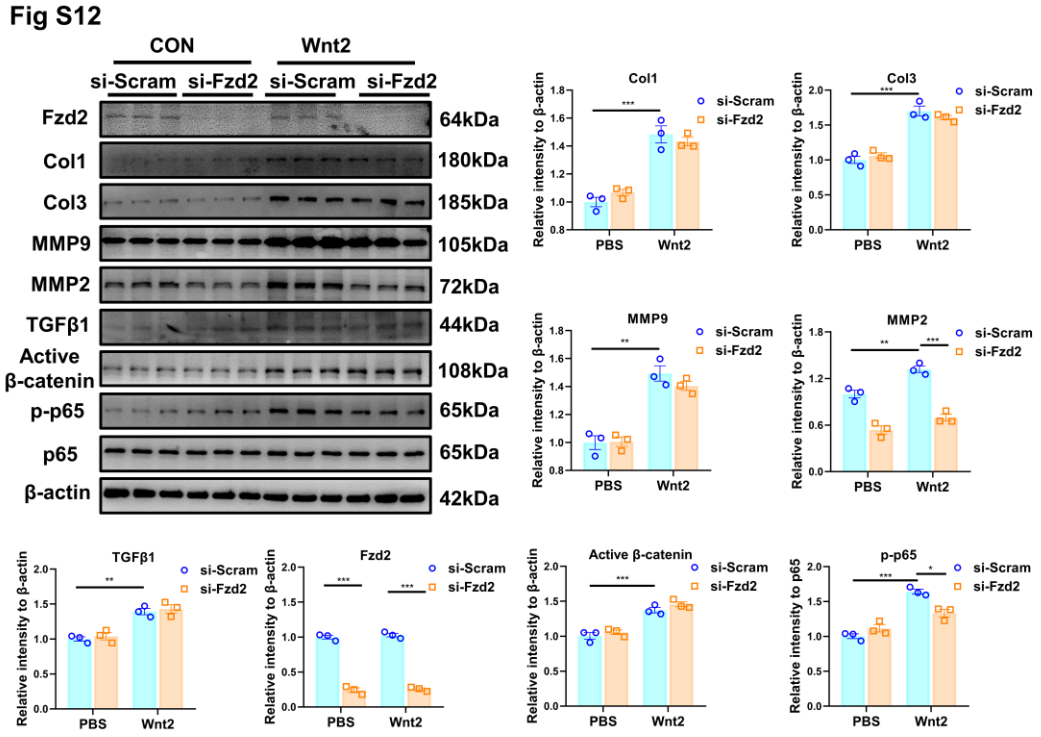

Fig. S12: Si-Fzd2 has few effects on the activation of fibroblasts induced by Wnt2. Western blot analysis of the expressions of Fzd2, Col1, Col3, active β-catenin,, MMP9, and TGFβ1 in neonatal rat cardiac fibroblasts (NRCFs). These NRCFs were pretreated with siRNA targeted Fzd2 (si- Fzd2) for 24 hours, followed by PBS, human recombinant Wnt2 (20ng/ml) treatment for another 24 hours. Values were expressed as means±S.E.M. *p<0.05, **p<0.01, ***p<0.001, n=3 in each group. Statistics: Two-way ANOVA with a Bonferroni post hoc test.

Fig S13

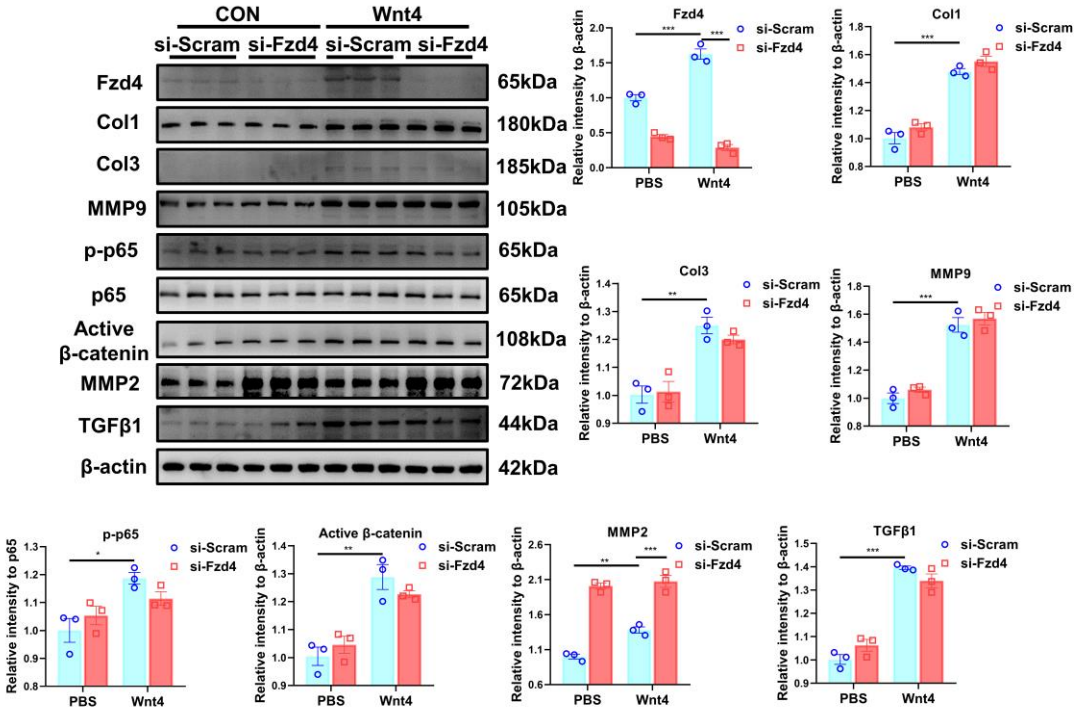

Fig. S13: Si-Fzd4 doesn't affect the pro-fibrotic effects caused by Wnt4. Western blot analysis of the expressions of Fzd4, Col1, Col3, active β-catenin, MMP9, and TGFβ1 in neonatal rat cardiac fibroblasts (NRCFs). These NRCFs were pretreated with siRNA targeted Fzd4 (si- Fzd4) or si-Scramble (si-Scram) for 24 hours, followed by PBS, human recombinant Wnt4 (50ng/ml) treatment for another 24 hours. Values were expressed as means±S.E.M. *p<0.05, **p<0.01, ***p<0.001, n=3 in each group. Statistics: Statistics: Two-way ANOVA with a Bonferroni post hoc test.