Myofibroblast Specific TGFβ Receptor II Signaling in the Fibrotic Response to Cardiac Myosin Binding Protein C-induced Cardiomyopathy

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***Running Title*:**
Myofibroblast ablation of TGFβ signaling

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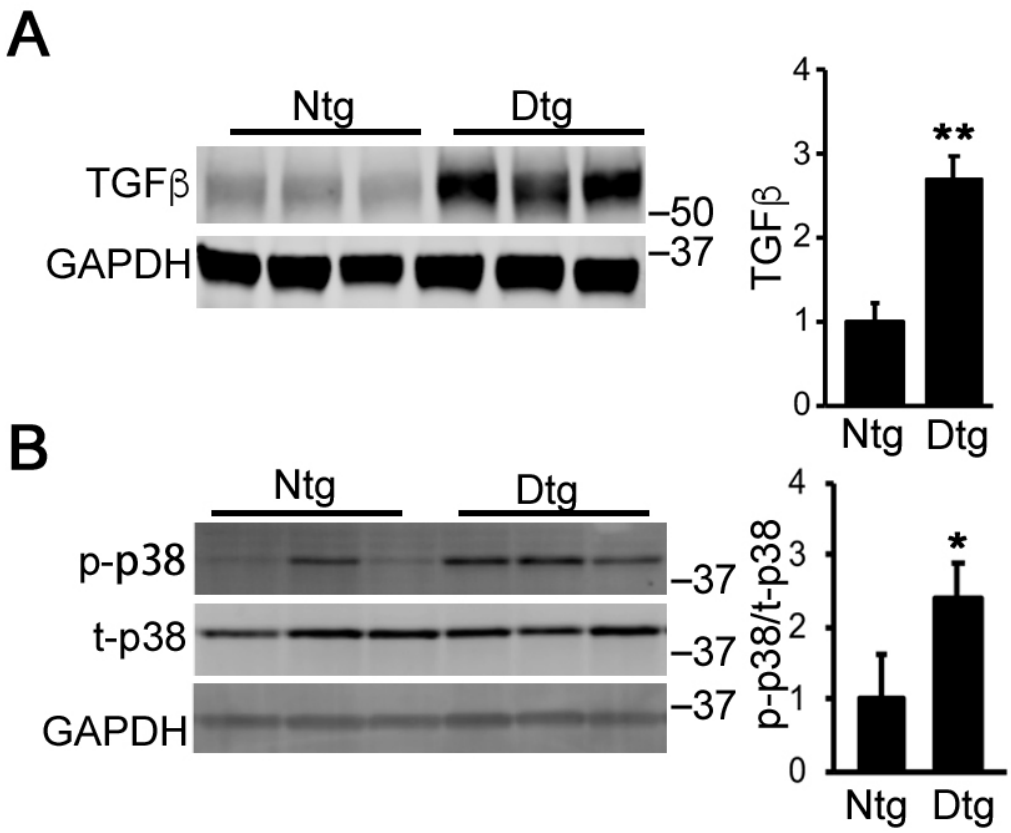

Figure l. Western blot analyses of TGFβ signaling in *Mybpc3*^{40kDa} hearts. Samples were derived from 4-month old nontransgenic (Ntg)- and *Mybpc3*^{4kDa}-expressing (Dtg) hearts. **A,** TGFβ expression: the antibody used detects TGFβ1, 2 and 3. **B,** p38 signaling. Data normalization and between group differences analysis were performed as described in **Methods**. Data are expressed as mean ± SD, n=4. **P*<0.05, ***P*<0.01. p-p38; phosphorylated p38, t-p38; total p38.

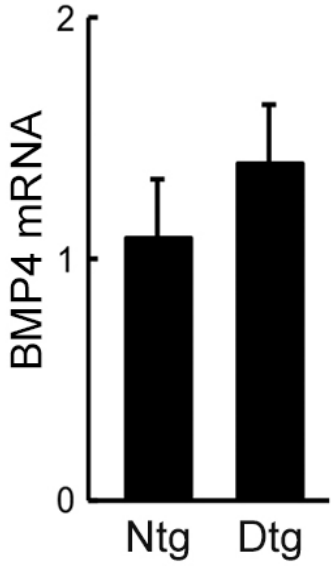

Figure II. BMP 4 expression in *Mybpc3*^{40kDa} hearts. Samples were derived from 4-month old nontransgenic (Ntg)- and *Mybpc3*^{4kDa}-expressing (Dtg) hearts. n=4, *P*=0.3814. Data normalization and between group differences analysis were performed as described in **Methods**.

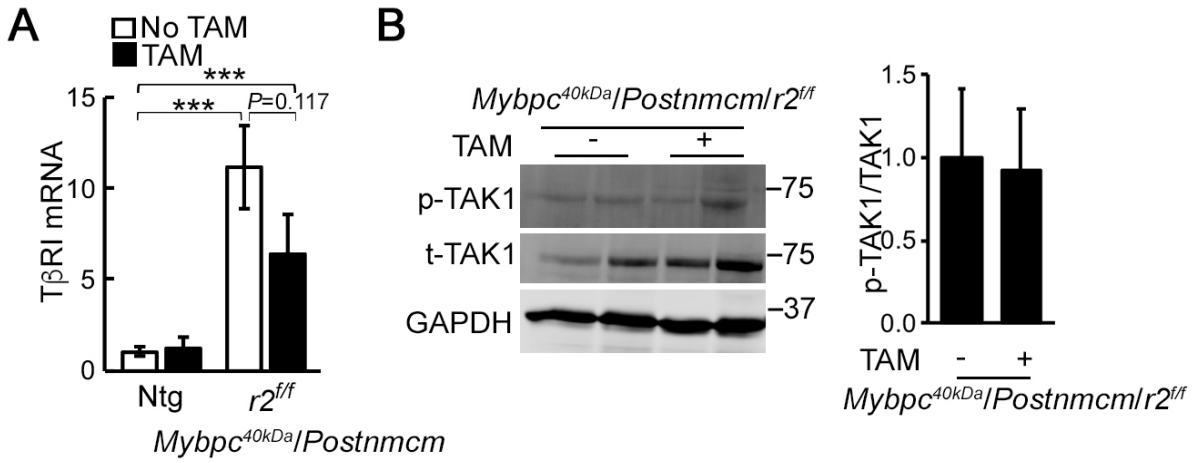

Figure III. Myofibroblast specific ablation of Tgfbr2 (r2). **A,** No significant changes in *Tgfbr1* expression were detected in cardiac fibroblasts isolated from the *Mybpc^{40KDa}/Postnmcm/r2$^{f/f}$* mice fed with TAM or normal chow, n=4. One-way ANOVA analysis with Tukey's post hoc test, ****P*<0.001, compared to the Ntg, No TAM group. **B,** TAK1 protein in cardiac fibroblasts isolated from the *Mybpc^{40KDa}/Postnmcm/r2$^{f/f}$* mice fed with TAM (+) or normal chow (-). Data normalization and between group differences analysis were performed as described in **Methods**. All samples were derived from 6-month-old mice. p-TAK1; phosphorylated TAK1, t-TAK1; total TAK1.

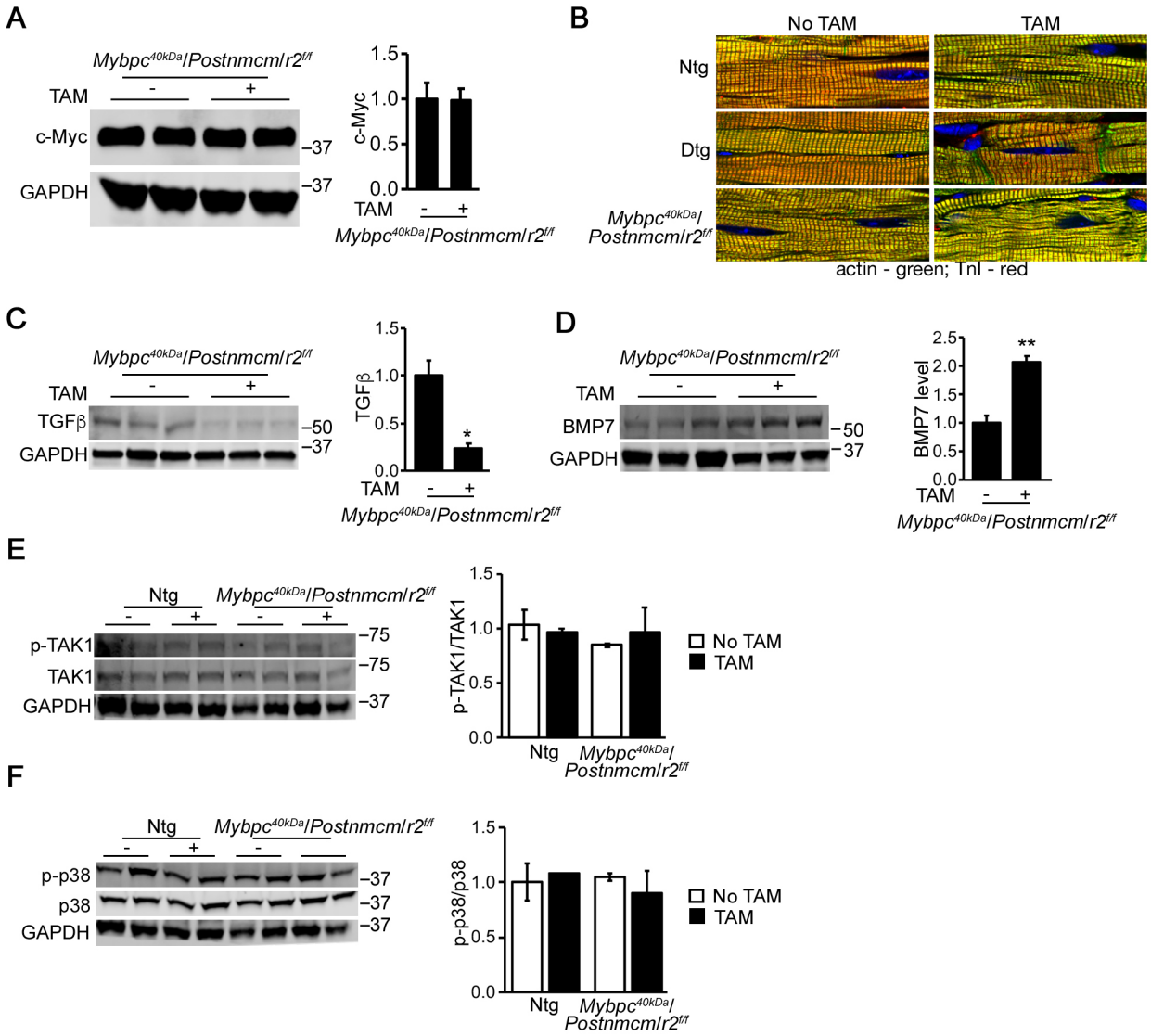

Figure IV. **A,** Mybpc3^{40KDa} expression in selected experimental groups was determined using Western blot analyses. Between group differences were analyzed as described in **Methods**, n=4, *P*=0.9391. **B**, Sarcomere organization in selected experimental groups. **C,** Western blot analysis of TGFβ expression in tamoxifen (TAM)-fed cohort compared to the normal chow cohort. The antibody used detects TGFβ1, 2 and 3. **D,** Western blot analysis of BMP7 expression in TAM-fed cohort compared to the normal chow cohort. Between group comparison, n=4, **P*<0.05, ***P*<0.01. **E, F,** Western blot analyses of TAK1 (**E**) and p38 (**F**) signaling in heart lysates isolated from Ntg and *Mybpc^{40kDa}/Postnmcm/r2$^{f/f}$* TAM-fed cohorts compared to normal chow cohorts. One-way ANOVA analysis, n=4, *P*=0.1956 for Panel E and *P*=0.8047 for Panel F. Data normalization and between group differences analysis were performed as described in **Methods**. All samples were derived from 6-month-old mice. p-p38; phosphorylated p38, t-p38; total p38, p-TAK1; phosphorylated TAK1, t-TAK1; total TAK1.

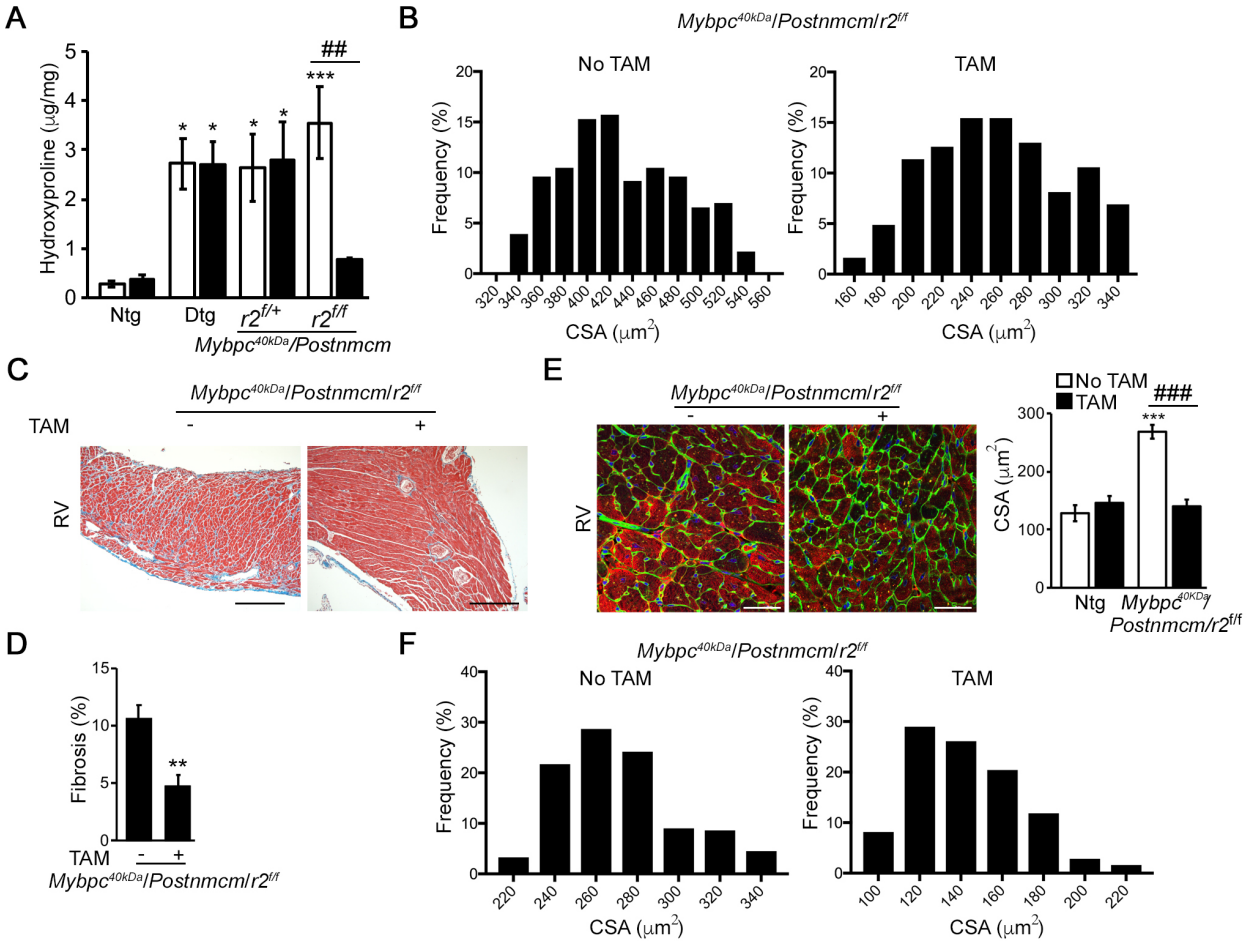

Figure V. Myofibroblast specific ablation of *Tgfbr2* (*r2*) reduces cardiac fibrosis and hypertrophy in the *Mybpc3*^{40kDa} hearts. **A**, Hydroxyproline levels as a measure of the fibrotic response. Samples are derived from both the left and right ventricles. One-way ANOVA analysis with Tukey's post hoc test, **P*<0.05, ****P*<0.001, comparing the different experimental groups to the nontransgenic, normal chow group (Ntg), $^{\#\#}$*P*<0.01, between group comparison, n=4. **B,** The distribution of cardiomyocyte cross-sectional area (CSA) in the left ventricle; n>1000/experimental group. **C** and **D**, Representative sections derived from the right ventricles subjected to Masson's trichrome staining. The *Mybpc*^{40kDa}*/Postnmcm/r2*$^{f/f}$ right ventricles derived from the groups fed regular (-) or tamoxifen (TAM) supplemented (+) chow were examined. Scale bar: 10X, 500 μm. Between group differences were analyzed as described in **Methods,** n=4, ***P*<0.01. **E,** Wheat germ agglutinin (green) staining was used to determine the cross-sectional area of right ventricle cardiomyocytes. Scale bar=20 μm. One-way ANOVA analysis with Tukey's post hoc test, ****P*<0.001, comparing to Ntg, no tamoxifen group; $^{\#\#\#}$*P*<0.05, between group comparison, n=4. No significant differences were detected between the TAM-fed Ntg and *Mybpc*^{40kDa}*/Postnmcm/r2*$^{f/f}$ groups; adjusted *P*=0.9906. **F**, The distribution of the cardiomyocyte cross-sectional area (CSA) in the right ventricle; n>1000/experimental group. All samples were derived from 6-month-old mice.

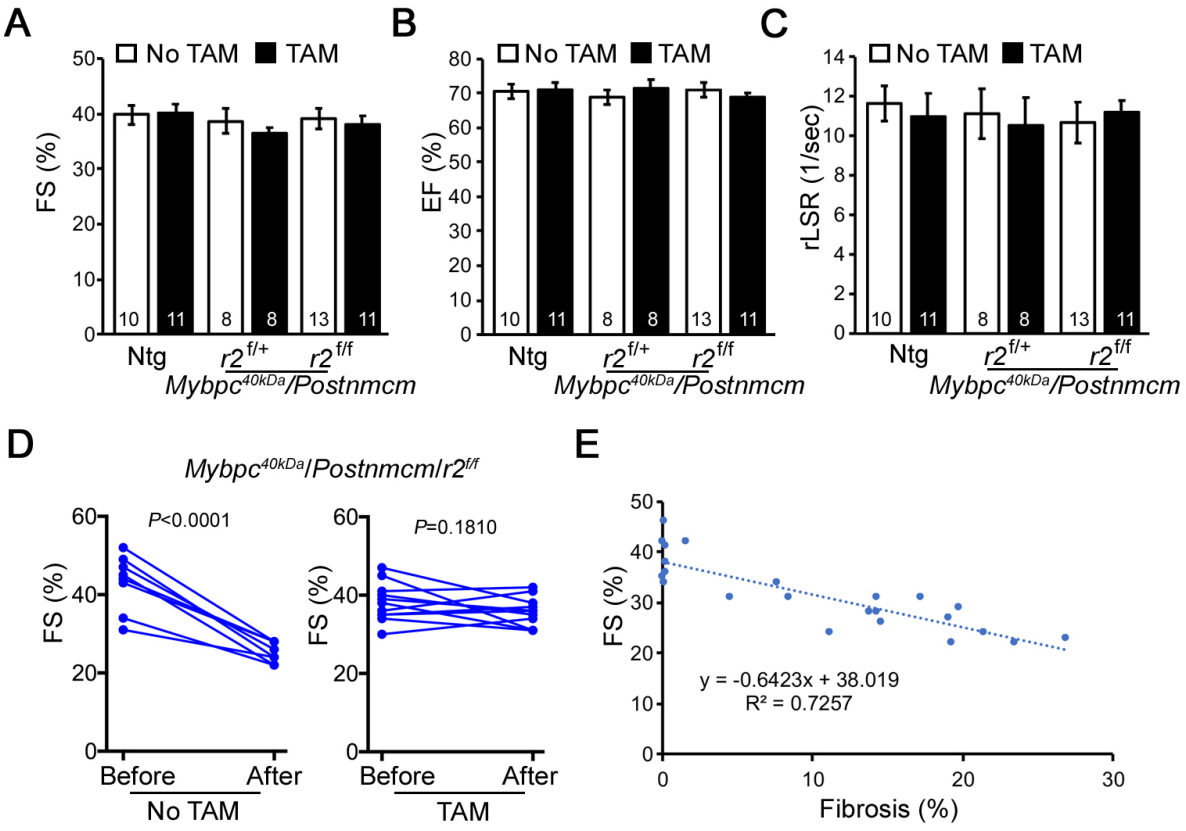

Figure VI. Myofibroblast specific ablation of *Tgfbr2* (*r2*) preserves *Mybpc3*^{40kDa} mouse cardiac function **A-C,** Echocardiographic analyses of nontransgenic (Ntg)- and *Mybpc3*^{40kDa}-expressing (Dtg) hearts as well as heterozygote (*r2*$^{f/+}$) and homozygote (*r2*$^{f/f}$) nulls of *Tgfbr2* crossed into the Dtg background before tamoxifen (TAM) treatment beginning at 2 months of age. No significant differences were identified among experimental groups. One-way ANOVA analysis, *P*=0.7099 for Panel A, *P*=0.9467 for Panel B, *P*=0.9807 for Panel C. **D**, Fractional shortening (FS) of the *Mybpc*^{40kDa}*/Postnmcm/r2*$^{f/f}$ mice before (2-months-old) and after (6-months-old) TAM or normal chow feeding. Paired t test, n=8 for the No TAM group, n=11 for the TAM group. **E**, Percent fibrosis versus FS was plotted and a Pearson correlation analysis done. n=24. FS; fractional shortening, EF; ejection fraction, rLSR; reverse peak longitudinal strain rate.

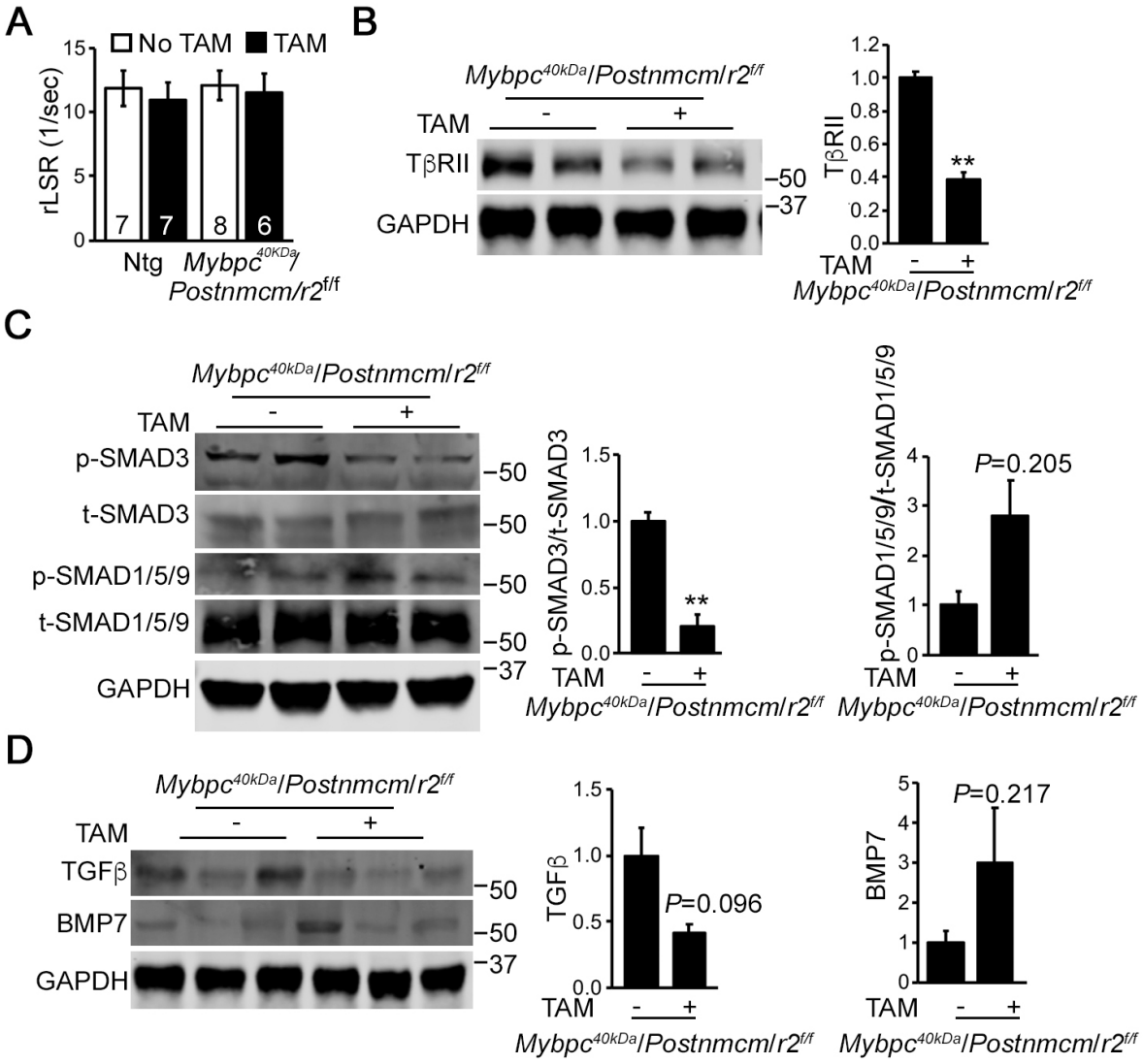

Figure VII. Late stage myofibroblast specific ablation of *Tgfbr2* (*r2*). **A**, Echocardiographic analyses of 5-month old nontransgenic (Ntg)- and homozygote ($r2^{f/f}$) nulls of *Tgfbr2* crossed into the $Mybpc3^{40kDa}$-expressing (Dtg) background. One-way ANOVA analysis, *P*=0.9511, n=6-8. rLSR; reverse peak longitudinal strain rate. **B**, Reduced expression of TβRII occurred in cardiac fibroblasts isolated from the 8-month-old $Mybpc^{40kDa}/Postnmcm/r2^{f/f}$ mice fed with tamoxifen (TAM) chow. Between group comparisons, n=4, ***P*<0.01. **C**, Reduced p-SMAD3 signaling and no-changed p-Smad1/5/9 signaling were detected were detected in cardiac fibroblasts isolated from the 8 months old $Mybpc^{40kDa}/Postnmcm/r2^{f/f}$ mice fed with tamoxifen (TAM) chow (+). Between group comparison, n=4, ***P*<0.01. **D**, TGFβ and BMP7 expression levels were detected in heart lysates isolated from $Mybpc^{40kDa}/Postnmcm/r2^{f/f}$ mice fed with TAM chow and compared to samples obtained from animals fed normal chow. The antibody used detects TGFβ1, 2 and 3. n=4. Data normalization and between group differences analysis were performed as described in **Methods**.

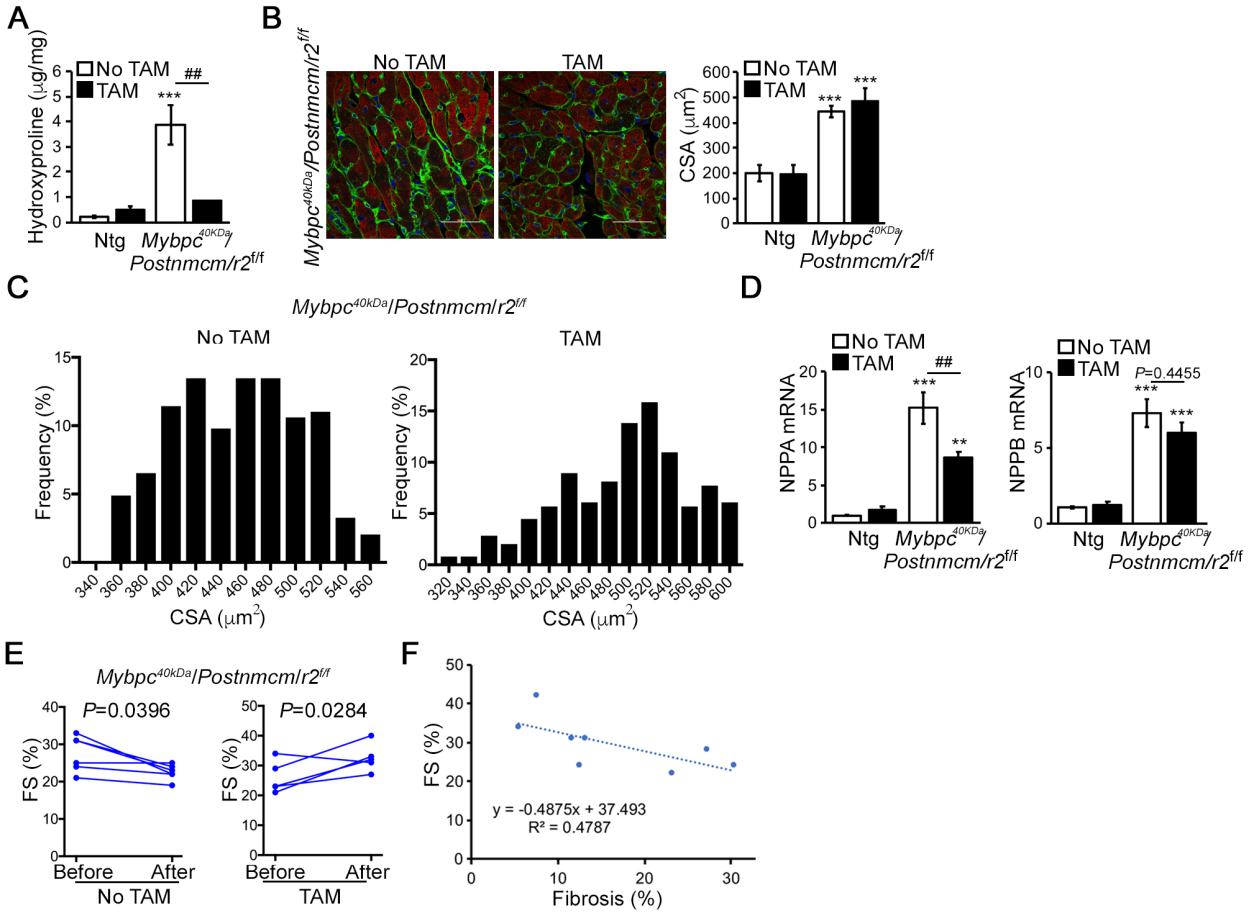

Figure VIII. Late stage myofibroblast specific ablation of *Tgfbr2* (*r2*). **A**, Hydroxyproline levels in the ventricles at 8 months. One-way ANOVA analysis with Tukey's post hoc test, comparing the different experimental groups to the Ntg, normal chow group, n=4, ***P<0.001; $^{\#\#}P$<0.01, between group comparison. **B**, Wheat germ agglutinin (green) staining was used to determine cardiomyocyte cross-sectional area (CSA). Scale bar=20 μm. One-way ANOVA analysis with Tukey's post hoc test, ***P<0.001, compared to Ntg, No tamoxifen (TAM) group; No significant changes were detected between the normal chow and TAM fed *Mybpc^{40kDa}/Postnmcm/r2$^{f/f}$* groups, adjusted P=0.9416, n=4. **C**, The distribution of cardiomyocyte cross-sectional area (CSA) in the left ventricle; n>1000/experimental group **D**, Natriuretic peptide A and B (Nppa and Nppb, respectively) mRNA expression levels. One-way ANOVA analysis with Tukey's post hoc test, **P<0.01, ***P<0.001, comparing the indicated genotypes to the Ntg, No TAM group. $^{\#\#}P$<0.01, between group comparison, n=4. **E**, Fractional shortening (FS) of the *Mybpc^{40kDa}/Postnmcm/r2$^{f/f}$* mice before (at 5 months) and after (8-month-old) TAM or normal chow feeding. Paired t test, n=6. **F**, Percent fibrosis versus FS was plotted and Pearson correlation analysis performed, n=8. Data normalization and between group differences analysis were performed as described in **Methods**.

Table I: TGFβ Signaling DNA Array

Gene name	Fold change	S.E.M	*P* value
Acta2	1.798540499	0.322753166	0.07071278
Bcl2	6.087137817	0.850573103	0.00763072
Ccl3	1.667364104	0.580979535	0.318644005
Ccr2	1.167967006	0.179478583	0.418663706
Col1a2	1.170331644	0.321983297	0.626484161
Col3a1	1.409549267	0.251282183	0.211351287
Ctgf	7.606501008	1.426370627	0.009810724
Fasl	0.397997886	0.005472134	0.14024283
Grem1	1.561406494	0.652454442	0.456596226
Hgf	1.432698105	0.185283837	0.183196972
Ilk	1.458875868	0.101433622	0.028967826
Itga3	0.874719496	0.180157466	0.613998126
Itgb3	1.916653597	0.122131153	0.021332423
Itgb5	1.95549053	0.280897856	0.028782827
Lox	3.41479917	0.604010527	0.01776082
Mmp14	2.851457476	0.480556953	0.022953163
Mmp3	8.477328393	2.995392206	0.067135509
Myc	2.370525927	0.41870515	0.031098847
Serpine1	2.347624607	0.923549924	0.227348
Smad7	2.197400882	0.260867709	0.013446329
Sp1	1.696164983	0.174908689	0.02260103
Tgfb1	0.914397469	0.029424608	0.282754346
Tgfb2	3.951699757	0.661386479	0.011249811
Tgfb3	3.209497513	0.773295477	0.046551467
Tgfbr2	1.987590627	0.372564851	0.057968811
Tgif1	2.669198301	0.379973722	0.012122713
Thbs1	2.838619836	0.195723195	0.001051126
Tnf	2.005860483	0.211840661	0.012023426

Transcript levels of the indicated species. Four-month old *Mybpc3*^{40kDa} hearts compared to Ntg hearts (n=3). GAPDH was used as internal control. Fold change was calculated by dividing each *Mybpc3*^{40kDa}-expressing (Dtg) value with the average of the Ntg values.