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ORIGINAL ARTICLE

ERK1/2 directly acts on CTGF/CCN2 expression to mediate myocardial fibrosis in cardiomyopathy caused by mutations in the lamin A/C gene

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Abstract

Cardiomyopathy caused by lamin A/C gene mutations (LMNA cardiomyopathy) is characterized by increased myocardial fibrosis, which impairs left ventricular relaxation and predisposes to heart failure, and cardiac conduction abnormalities. While we previously discovered abnormally elevated extracellular signal-regulated kinase 1/2 (ERK1/2) activities in heart in LMNA cardiomyopathy, its role on the development of myocardial fibrosis remains unclear. We now showed that transforming growth factor (TGF)- β /Smad signaling participates in the activation of ERK1/2 signaling in LMNA cardiomyopathy. ERK1/2 acts on connective tissue growth factor (CTGF/CCN2) expression to mediate the myocardial fibrosis and left ventricular dysfunction. Studies *in vivo* demonstrate that inhibiting CTGF/CCN2 using a specific antibody decreases myocardial fibrosis and improves the left ventricular dysfunction. Together, these findings show that cardiac ERK1/2 activity is modulated in part by TGF- β /Smad signaling, leading to altered activation of CTGF/CCN2 to mediate fibrosis and alter cardiac function. This identifies a novel mechanism in the development of LMNA cardiomyopathy.

Introduction

One cause of dilated cardiomyopathy is dominant mutations in LMNA, the gene encoding A-type nuclear lamins, intermediate filament proteins of the nuclear envelope (1,2). Cardiomyopathy

caused by LMNA mutations (i.e. LMNA cardiomyopathy) is characterized by increased myocardial fibrosis that impairs left ventricular relaxation and predisposes to heart failure, and cardiac conduction abnormalities (3–6). The onset of symptoms in LMNA

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cardiomyopathy, although variable, occurs most frequently in the third decade and the disease has a more aggressive course than most other inherited dilated cardiomyopathies (7). While sudden death from arrhythmias may be prevented by implantation of a pacemaker and/or defibrillator, the progressive heart failure eventually becomes resistant to treatment and heart transplantation is often the last therapeutic option (4).

We previously discovered abnormally elevated activity of extracellular signal-regulated kinase 1/2 (ERK1/2) in the hearts of *Lmna*^{H222P/H222P} mice that develop cardiomyopathy (8). These mice recapitulate the cardiac disease in patients with LMNA mutations and therefore serve as a useful animal model. Inhibition of ERK1/2 signaling in *Lmna*^{H222P/H222P} mice by an inhibitor of mitogen-activated protein kinase 1/2 (MEK1/2), the enzyme that activates ERK1/2, improved left ventricular ejection fraction and prolonged survival (9–11). MEK1/2 inhibitor treatment of these mice further slowed progression of myocardial fibrosis, which is prominent in humans with LMNA cardiomyopathy (10,11). Myocardial fibrosis consists of the replacement of functional cells with accumulation of collagen-rich extracellular matrix (ECM). Cardiomyocytes are tethered within the ECM, consisting of collagen, elastin, proteoglycans and glycoproteins. ECM provides a scaffold for myofiber alignment protects against sarcomere overstretching and plays a role in electrical behavior of the myocardium. Therefore, ECM stiffness and deposition of fibrous tissue have dramatic effects on heart function (12). Indeed, myocardial fibrosis contributes to diastolic and systolic dysfunctions and conduction defects in the heart (13,14).

Pro-fibrotic changes during cardiac remodeling are mainly driven by cytokines such as transforming growth factor- β (TGF- β) and the matricellular protein connective tissue growth factor (CTGF/CCN2) (15). TGF- β -dimers bind to type II receptors, which recruit and phosphorylate type I receptors such as activin receptor-like 5 (ALK5). Ligand binding to this type I receptor recruits and phosphorylates Smad2/3, which is then translocated to the nucleus and activates target gene transcription. We therefore assessed the modulation of TGF- β /Smad signaling implicated in activating fibrosis in *Lmna*^{H222P/H222P} mice and its interaction with ERK1/2 signaling. We found that TGF- β /Smad signaling participates in the activation of ERK1/2 signaling in LMNA cardiomyopathy and that ERK1/2 acts on CTGF/CCN2 expression to mediate the myocardial fibrosis and left ventricular dysfunction.

Results

Myocardial fibrosis in *Lmna*^{H222P/H222P} mice

Consistent with previous results (16), hearts of *Lmna*^{H222P/H222P} mice at 20 weeks of age, when left ventricles function is altered (10), had an increase in fibrotic tissue compared with *Lmna*^{+/+} mice (WT mice) (Supplementary Material, Fig. S1). At 20 weeks of age, hearts from *Lmna*^{H222P/H222P} mice also had significantly increased expression of mRNAs from the *Col1a1* and *Col1a2* encoding type I collagens of the ECM and *Fn1*, *Scd1*, *Nid1* and *Dcn1*, respectively, encoding the basal lamina proteins fibronectin, syndecan, nidogen and decorin (Fig. 1). Our data confirmed that there is increased myocardial fibrosis in *Lmna*^{H222P/H222P} mice compared with WT mice.

TGF- β /Smad signaling is enhanced in hearts from *Lmna*^{H222P/H222P} mice

Given that TGF- β signaling is an important mediator of fibrosis, we assessed its activity in hearts and isolated cardiomyocytes

of *Lmna*^{H222P/H222P} mice. We observed enhanced *Tgf-b1* and *Tgf-b2* mRNA expression in hearts from *Lmna*^{H222P/H222P} mice at 20 weeks of age compared with hearts from WT mice (Fig. 2A). In hearts of *Lmna*^{H222P/H222P} mice, *Tgf-b1* and *Tgf-b2* mRNAs were increased, respectively, by 2- and 20-fold compared with WT mice. We then further assessed the modulation of TGF- β signaling by studying the abundance of phosphorylated Smad2/3 (p-Smad2/3) in protein extracts from left ventricles and isolated ventricular cardiomyocytes from *Lmna*^{H222P/H222P} and WT mice. There was a significant increase of cardiac p-Smad2/3 in *Lmna*^{H222P/H222P} mouse heart (Fig. 2B). Immunofluorescence microscopy also revealed a nuclear localization of p-Smad2/3 in the heart from *Lmna*^{H222P/H222P} mice (Fig. 2C), which was occurring to a lesser extent in cardiac endothelial cells (Fig. 2D). This increased nuclear translocation of p-Smad2/3 was confirmed by western blot analysis of fractionated cells (Fig. 2E). These results demonstrated abnormal activation of TGF- β signaling in hearts from *Lmna*^{H222P/H222P} mice at an age when myocardial fibrosis occurs. These data extend a previous report showing activation of TGF- β /Smad2/3 signaling in failing hearts from *Lmna*^{H222P/H222P} mice at 24 weeks of age (16).

Inhibiting TGF- β /Smad signaling improves cardiac dysfunction

Given the enhanced TGF- β /Smad signaling in hearts of *Lmna*^{H222P/H222P} mice, we hypothesized that reducing this activity would attenuate myocardial fibrosis and improve cardiac function. We inhibited ALK5, a receptor for TGF- β that recruits and phosphorylates Smad2/3 (17), with daily intra-peritoneal injections of SB-431542 (5 mg/kg) starting at 16 weeks of age (Fig. 3A). SB-431542 specifically targets the TGF- β signaling (18,19). After 4 weeks of treatment, mice were analyzed by echocardiography and sacrificed for biochemical and histological analysis. SB-431542 treatment reduced Smad2/3 phosphorylation in hearts of *Lmna*^{H222P/H222P} mice compared with DMSO placebo treatment (Fig. 3B). After the treatment with SB-431542, myocardial fibrosis was reduced in treated *Lmna*^{H222P/H222P} mice compared with placebo-treated mice (Fig. 3C). SB-431542 also reduced the levels of *Col1a1* and *Col1a2* mRNAs in heart (Fig. 3C). M-mode echocardiography showed that left ventricular end diastolic and end systolic diameters in *Lmna*^{H222P/H222P} mice treated with SB-431542 were significantly smaller and fractional shortening was significantly increased compared with the placebo-treated mice (Fig. 3D and Table 1). SB-431542 also reduced the mRNA levels of *NppA* and *NppB*, genes that encode natriuretic peptide precursors that are synthesized in response to left ventricular dilatation, as well as *Myl4*, the gene encoding the light chain of myosin (Fig. 3E). Hence, TGF- β /Smad signaling inhibition that reduces fibrosis concomitantly improves left ventricular function in *Lmna*^{H222P/H222P} mice.

Decreased ERK1/2 activation lowers myocardial fibrosis

We previously showed that reducing ERK1/2 signaling in *Lmna*^{H222P/H222P} mice, either genetically or using chemical inhibitors, reduces myocardial fibrosis (9–11,20). We confirmed this using PD325901, an inhibitor of MEK1/2, to reduce ERK1/2 signaling in *Lmna*^{H222P/H222P} mice from 16 to 20 weeks of age. Treating mice for 4 weeks with PD325901 reduced ERK1/2 signaling, reduced left ventricular diameters and improved function (Supplementary Material, Fig. S2A, Supplementary Material, Table S1). Inhibition of ERK1/2 signaling further decreased the

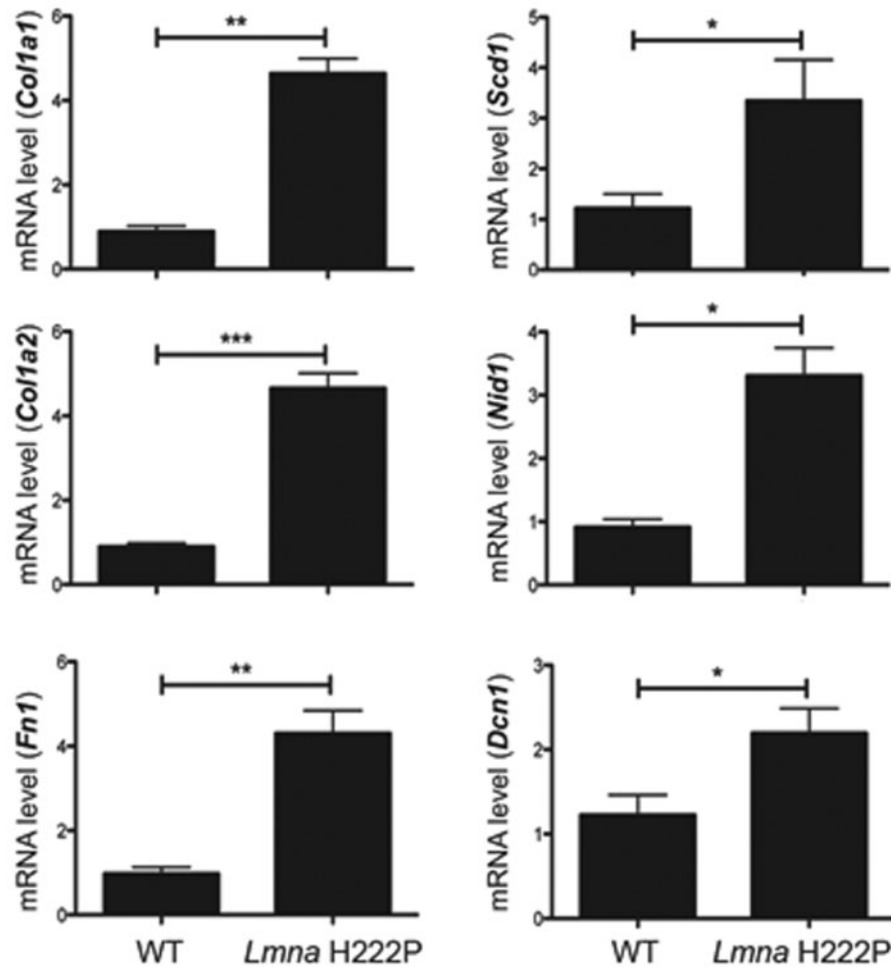


Figure 1. Quantification of myocardial fibrosis in *Lmna*^{H222P/H222P} mice. Cardiac expression of genes encoding proteins of the ECM in *Lmna*^{H222P/H222P} mice. Bar diagrams represent relative mean mRNA (as well as standard errors of means) expression of *Col1a1*, *Col1a2*, *Sdc1*, *Nid1*, *Fn1* and *Dcn1* in hearts from WT mice ($n = 4$) and *Lmna*^{H222P/H222P} mice (*Lmna* H222P) ($n = 8$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

degree of fibrosis in hearts from *Lmna*^{H222P/H222P} mice (Supplementary Material, Fig S2B). Immunohistochemistry also revealed decreased nuclear translocation of p-Smad2/3 when *Lmna*^{H222P/H222P} mice were treated with PD325901, compared with untreated or DMSO-treated animals (Fig. 4). In PD325901-treated *Lmna*^{H222P/H222P} mice, *Col1a1* and *Col1a2* mRNAs were significantly decreased compared with DMSO-treated mice (Supplementary Material, Fig. S2C). We similarly assessed expression of *Col1a1* and *Col1a2* mRNAs in *Lmna*^{H222P/H222P}/*Erk1*^{-/-} mice lacking ERK1 (20). The level of *Col1a2* mRNA was significantly decreased in hearts from these mice compared with *Lmna*^{H222P/H222P}/*Erk1*^{+/+} mice (Supplementary Material, Fig. S2C). These results suggest that ERK1/2 hyperactivation contributes to the enhancement of myocardial fibrosis in the hearts of *Lmna*^{H222P/H222P} mice.

We next assessed the impact of inhibition of TGF- β signaling on ERK1/2 activity in *Lmna*^{H222P/H222P} mice. After treatment with SB-431542 for 4 weeks, expression of p-ERK1/2 was lower in protein extracts from hearts of *Lmna*^{H222P/H222P} mice compared with placebo-treated mice (Fig. 5A). Smad2/3 phosphorylation paralleled activation of ERK1/2 in hearts of *Lmna*^{H222P/H222P} mice at 12 and 24 weeks of age (Fig. 5B). This was confirmed in C2C12 cells stimulated with TGF- β . When cells were treated for 6 h with 2

nM TGF- β 1, an increase of both p-Smad2/3 and p-ERK1/2 was observed (Fig. 5C). This demonstrated the role played by TGF- β on ERK1/2 signaling in LMNA cardiomyopathy.

Cardiac fibrosis is mediated by CTGF/Ccn2

As ERK1/2 has been shown to mediate up-regulation of CTGF/Ccn2, which may be involved in myocardial fibrosis, we sought to determine if CTGF/Ccn2 expression is altered in hearts of *Lmna*^{H222P/H222P} mice. CTGF/Ccn2 expression was measured in hearts at 20 weeks of age. Compared with WT mice, hearts and isolated cardiomyocytes from *Lmna*^{H222P/H222P} mice exhibited increased CTGF/Ccn2 protein and mRNA expression (Fig. 6A and B). We next assessed the expression of *Ctgf* mRNA level in hearts of *Lmna*^{H222P/H222P} mice between 4 weeks of age and 16 weeks of age concurrent with the progression of the left ventricular dysfunction. A significant increase of *Ctgf* mRNA was observed in hearts of *Lmna*^{H222P/H222P} mice as early as 12 weeks of age, when compared with hearts from WT mice (Fig. 6C). This increase corresponded to the beginning of fibrosis, as we did not observe any increase of *Col1a1* mRNA up to 12 weeks of age and of *Col1a2* mRNA up to 16 weeks of age (Fig. 6C). To test whether

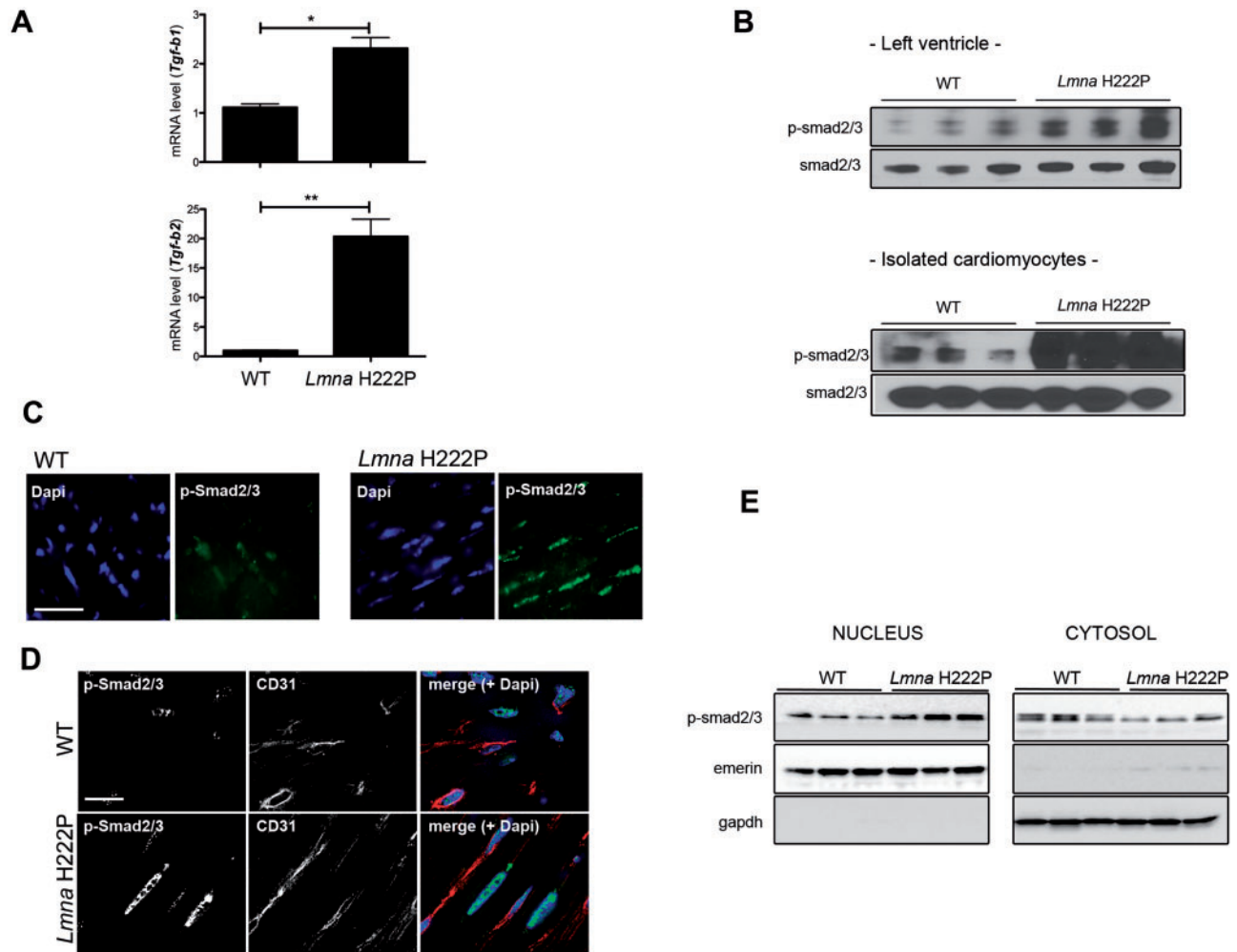


Figure 2. Activity of Tgf- β signaling in *Lmna*^{H222P/H222P} mice. (A) Expression of genes encoding proteins of the Tgf- β signaling in heart from *Lmna*^{H222P/H222P} mice. Bar graphs indicate the expression of *Tgf-b1* and *Tgf-b2* from WT mice ($n = 3$) and *Lmna*^{H222P/H222P} mice (*Lmna* H222P) ($n = 8$). * $P < 0.05$, ** $P < 0.01$. (B) Immunoblots showing phosphorylated Smad2/3 (p-smad2/3) and total smad2/3 in protein extracts of hearts (upper panel) and isolated cardiomyocytes (lower panel) from WT and *Lmna*^{H222P/H222P} mice (*Lmna* H222P). Each lane contains protein extracts from a different mouse. (C) Micrographs showing p-smad2/3 labeling (upper part, Scale bar: 25 μ m) of cross sections of hearts from WT compared with *Lmna*^{H222P/H222P} mice (*Lmna* H222P). Nuclei counter-stained with 4',6-diamidino-2-phenylindole (dapi) are also shown. (D) Micrographs showing p-smad2/3 and CD31 staining (lower part, Scale bar: 25 μ m) of cross sections of hearts from WT compared with *Lmna*^{H222P/H222P} mice (*Lmna* H222P). Nuclei counter-stained with 4',6-diamidino-2-phenylindole (dapi) are also shown. (E) Immunoblots showing p-smad2/3 and total smad2/3 in nuclear and cytosolic extracts from hearts from WT and *Lmna*^{H222P/H222P} mice (*Lmna* H222P). Each lane contains protein extracts from a different mouse.

the changes we observed in hearts of *Lmna*^{H222P/H222P} mice also occur in humans, we examined CTGF/CCN2 levels in left ventricular tissue from human subjects with cardiomyopathy caused by mutations in LMNA. Compared with controls, elevated CTGF/CCN2 expression was observed in left ventricles tissue of human subjects with LMNA cardiomyopathy (Fig. 6D). These observations established that enhanced CTGF/CCN2 expression is a feature of cardiac remodeling in LMNA cardiomyopathy.

CTGF/CCN2 expression influences myocardial fibrosis and left ventricular function

We next hypothesized that reducing CTGF/CCN2 activity would reduce myocardial fibrosis and improve left ventricular function. To test this hypothesis, an inhibitory human monoclonal antibody against CTGF (FG-3019) was administered to *Lmna*^{H222P/H222P} mice for 2 weeks, starting at 16 weeks of age. As a control group, *Lmna*^{H222P/H222P} mice were injected with

non-specific human immunoglobulin G (Fig. 7A). There was less myocardial fibrosis in hearts from *Lmna*^{H222P/H222P} mice treated with FG-3019 relative to mice treated with control human immunoglobulin G (Fig. 7B). Consistently, there was a decreased *Col1a1* mRNA expression in hearts from *Lmna*^{H222P/H222P} mice treated with FG-3019 compared with mice treated with control human immunoglobulin G (Fig. 7B). Two weeks of treatment with FG-3019 significantly improved both the left ventricular end systolic diameter and fractional shortening (Fig. 7C and Table 2). There was also a significant decrease in *Myh7* and *NppA* mRNA expression after the treatment (Fig. 7D).

ERK1/2 signaling modulates CTGF/CCN2 expression

Given the enhanced ERK1/2 signaling that is observable in hearts of *Lmna*^{H222P/H222P} mice as early as 4 weeks of age (21), which is before detectable myocardial fibrosis, we hypothesized that ERK1/2 may drive expression of CTGF/CCN2. Treatment of 16 week-old *Lmna*^{H222P/H222P} mice with a MEK1/2 inhibitor

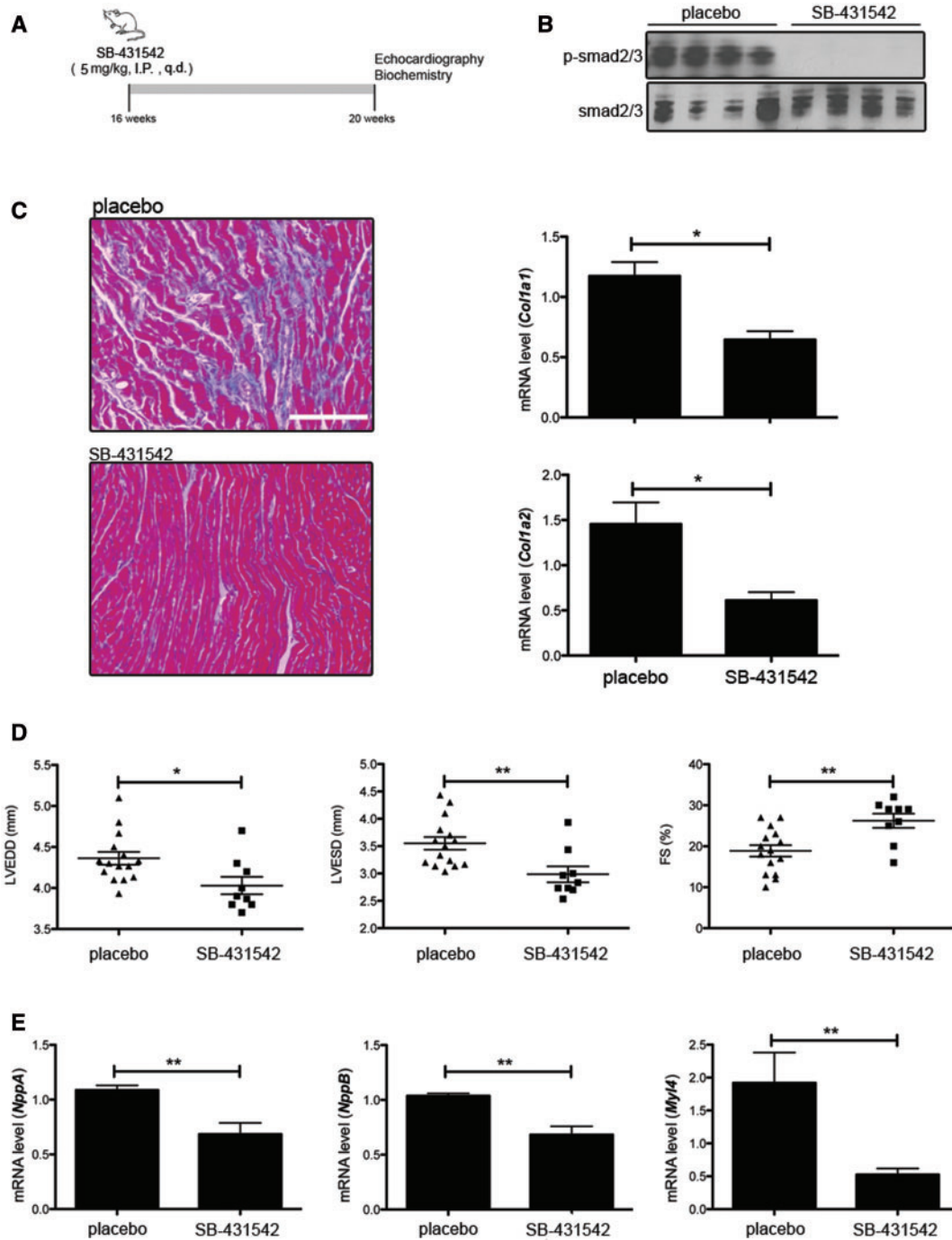


Figure 3. Pharmacological inhibition of Tgf- β signaling improves left-ventricular function. (A) Schematic representation of the treatment protocol of *Lmna*^{H222P/H222P} mice with SB-431542. (B) Representative immunoblots using antibodies against p-smad2/3 and total smad2/3 to probe proteins extracted from hearts from *Lmna*^{H222P/H222P} mice (*Lmna* H222P) treated with placebo or SB-431542. Each lane contains protein extracts from a different mouse. (C) Mason trichrome staining of cross sections of hearts from *Lmna*^{H222P/H222P} mice (*Lmna* H222P) treated with placebo or SB-431542. Scale bar: 50 μ m. Bar diagrams indicate the expression of Col1a1 and Col1a2 from *Lmna*^{H222P/H222P} mice (*Lmna* H222P) treated with placebo ($n = 3$) or SB-431542 ($n = 5$). * $P < 0.05$. (D) Graph showing mean left ventricle end-diastolic diameter (LVEDD), mean left ventricle end-systolic diameter (LVESD) and fraction shortening (FS) in 20-week-old male *Lmna*^{H222P/H222P} mice (*Lmna* H222P) treated with placebo ($n = 15$) or SB-431542 ($n = 9$). Values for each individual mouse receiving placebo or SB-431542 as well as standard errors of means (bars) are shown. * $P < 0.05$, ** $P < 0.01$. (E) Relative expression of mRNAs from genes encoding myosin light chain (*Myl4*), atrial natriuretic peptide A (*Nppa*) and brain natriuretic peptide B (*Nppb*) in *Lmna*^{H222P/H222P} mice (*Lmna* H222P) treated with placebo ($n = 3$) or SB-431542 ($n = 5$). Values shown are means \pm standard errors. ** $P < 0.01$.

(PD325901) for 1 month significantly decreased *Ctgf* mRNA in heart compared to that in DMSO-treated mice (Fig. 8A). Similarly, *Ctgf* mRNA was significantly decreased in hearts of *Lmna*^{H222P/H222P}/*Erk1*^{-/-} mice lacking ERK1 compared with hearts

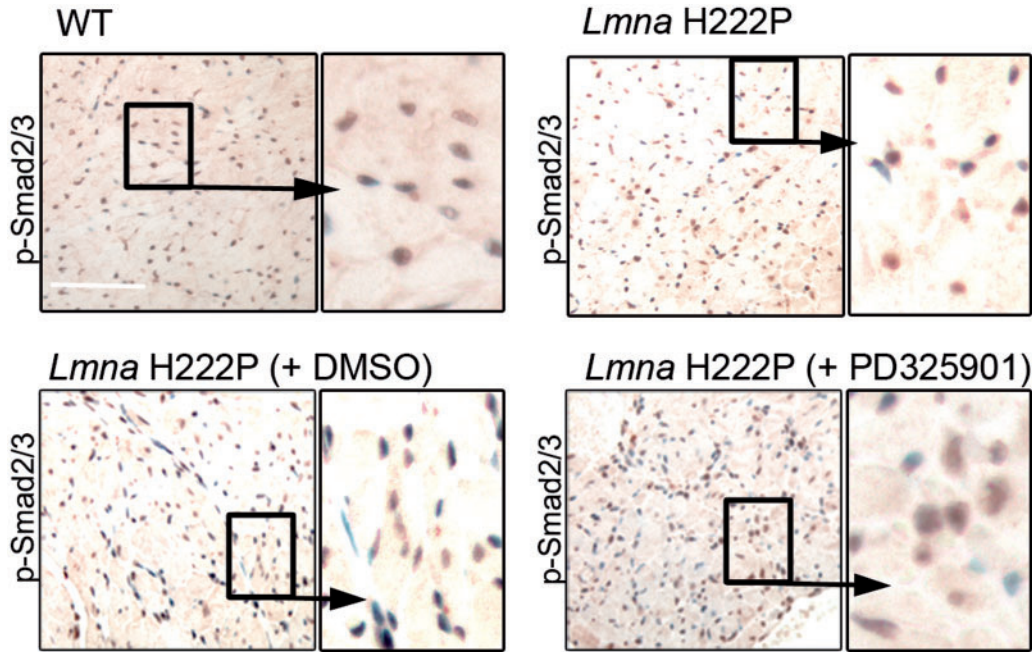
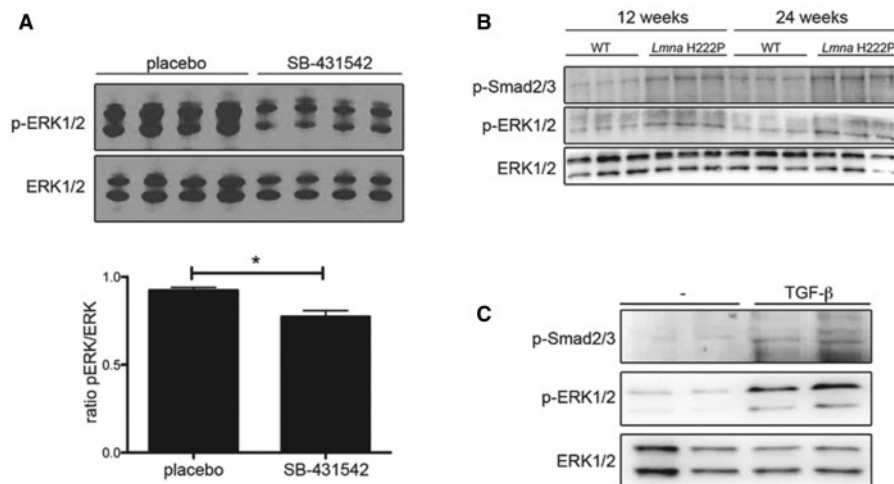
of *Lmna*^{H222P/H222P}/*Erk1*^{+/+} mice (Fig. 8A). To further demonstrate the link between ERK1/2 signaling and enhanced expression of CTGF/CCN2, we used C2C12 cells. When we treated C2C12 cells with different doses of PD325901 (10, 5 and 1 μ M), we observed

Table 1. Quantification of left ventricular function in *Lmna* H222P mice treated with SB-431542

Genotype	Treatment	n	Heart rate (beats/min)	LVEDD (mm)	LVESD (mm)	FS (%)
<i>Lmna</i> WT	None	10	532.0 ± 21.1	4.2 ± 0.2	2.4 ± 0.1	42.9 ± 1.5
<i>Lmna</i> H222P	DMSO	15	500.7 ± 4.6	4.4 ± 0.3	3.6 ± 0.4	18.9 ± 5.3
<i>Lmna</i> H222P	SB-431542	9	496.3 ± 13.5	4.0 ± 0.3*	3.0 ± 0.5**	26.2 ± 5.0**

*P < 0.05.

**P < 0.005 between DMSO-treated and SB-431542-treated mice.

**Figure 4.** Pharmacological inhibition of ERK1/2 signaling acts on Tgf- β signaling in *Lmna*^{H222P/H222P} mice. Micrographs showing p-smad2/3 labeling (immunohistochemistry, Scale bar: 50 μ m) of cross sections of hearts from WT compared with *Lmna*^{H222P/H222P} mice (*Lmna* H222P) treated with placebo or PD325901. Nuclei are counterstained with 4',6-diamidino-2-phenylindole (dapi).**Figure 5.** Tgf- β signaling acts on ERK1/2 signaling in *Lmna*^{H222P/H222P} mice. (A) Immunoblots showing pERK1/2 and ERK1/2 in protein extracts of hearts from *Lmna*^{H222P/H222P} mice (*Lmna* H222P) treated with placebo or SB-431542. Each lane contains protein extracts from a different mouse. The bar graph shows means \pm standard errors values of the ratio pERK1/2 normalized to ERK1/2 from scan band densities of three immunoblots from $n = 4$ different mice per groups. *P < 0.05. (B) Immunoblots showing p-smad2/3, pERK1/2 and ERK1/2 in protein extracts of hearts from *Lmna*^{H222P/H222P} mice (*Lmna* H222P) at 12 and 24 weeks of age. Each lane contains protein extracts from a different mouse. (C) Immunoblots showing p-smad2/3, pERK1/2 and ERK1/2 in protein extracts of C2C12 cells treated with or without (–) TGF- β 1 for 6 h (2 nM).

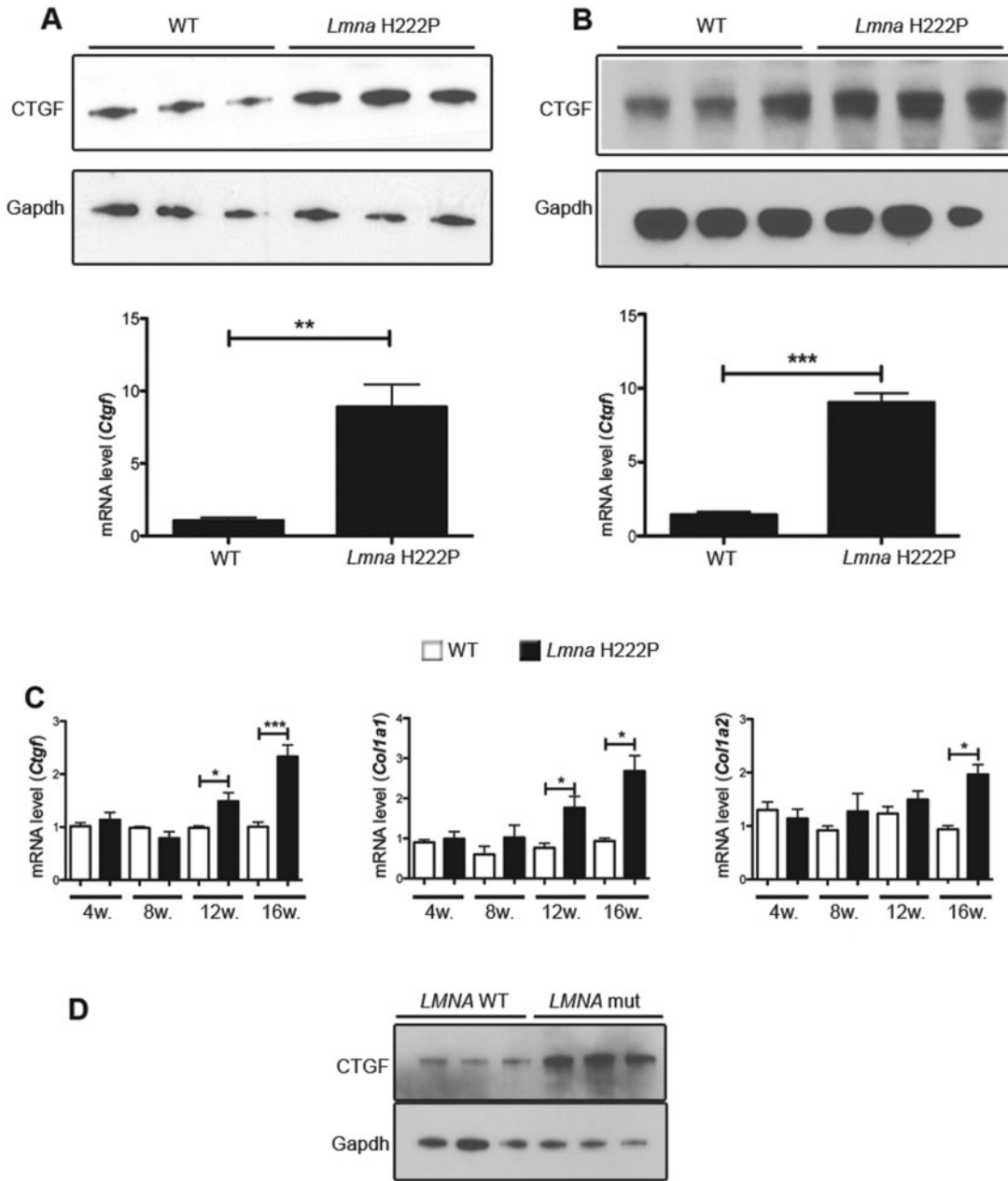


Figure 6. Abnormal CTGF expression in hearts of *Lmna*^{H222P/H222P} mice and human subjects with LMNA cardiomyopathy. (A) Immunoblots showing CTGF and GAPDH protein expression in hearts from WT and *Lmna*^{H222P/H222P} mice. Each lane contains protein extracts from a different mouse. The bar graph represents mRNA relative expression (means \pm standard errors of means) of *Ctgf* in hearts from WT ($n=3$) and *Lmna*^{H222P/H222P} (*Lmna* H222P) ($n=3$) mice. ** $P < 0.01$. (B) Immunoblots showing CTGF and GAPDH protein expression in isolated cardiomyocytes from WT and *Lmna*^{H222P/H222P} mice. Each lane contains protein extracts from cardiomyocytes isolated from a different mouse. The bar graph represents mRNA relative expression (means \pm standard errors of means) of *Ctgf* in cardiomyocytes from WT ($n=3$) and *Lmna*^{H222P/H222P} (*Lmna* H222P) ($n=3$) mice. *** $P < 0.001$. (C) Validation of differential mRNA expression of *Ctgf*, *Col1a1* and *Col1a2* in hearts from WT and *Lmna*^{H222P/H222P} mice. RNA was obtained from hearts of mice at 4, 8, 12, 16 weeks of age. White bars show relative RNA expression levels in hearts of WT mice and black bars in hearts of *Lmna*^{H222P/H222P} mice. Values are means \pm standard errors for $n=4$ samples per group. * $P < 0.05$, *** $P < 0.001$. (D) Immunoblots showing CTGF and GAPDH in protein extracts of heart tissue from three human control (LMNA WT) and three individuals with dilated cardiomyopathy caused by LMNA mutations (LMNA mut).

decreased expression of CTGF/CCN2 (Fig. 8B). Conversely, the overexpression of ERK2 triggered an increase in CTGF/CCN2 expression (Fig. 8B).

To test whether expression of the lamin A H222P variant was correlated with CTGF/CCN2 activation, we assessed CTGF/CCN2 promoter activity in stably transfected C2C12 cells overexpressing wild type or H222P lamin A (22). CTGF/CCN2 promoter activity was increased in transfected cells expressing lamin A H222P compared with cells expressing wild type lamin

A (Fig. 8C). These results demonstrated a correlation between H222P lamin A expression and CTGF/CCN2 expression, recapitulating the expression pattern in hearts from *Lmna*^{H222P/H222P} mice. We next assessed if the modulation of ERK1/2 signaling caused by H222P lamin A altered CTGF/CCN2 expression. Treatment of transfected C2C12 cells expressing H222P lamin A with PD325901 (10, 5 and 1 μ M) showed a dose-dependent decrease in activity of the CTGF/CCN2 promoter (Fig. 8C). To address whether ERK1/2 activation drove the enhanced CTGF/

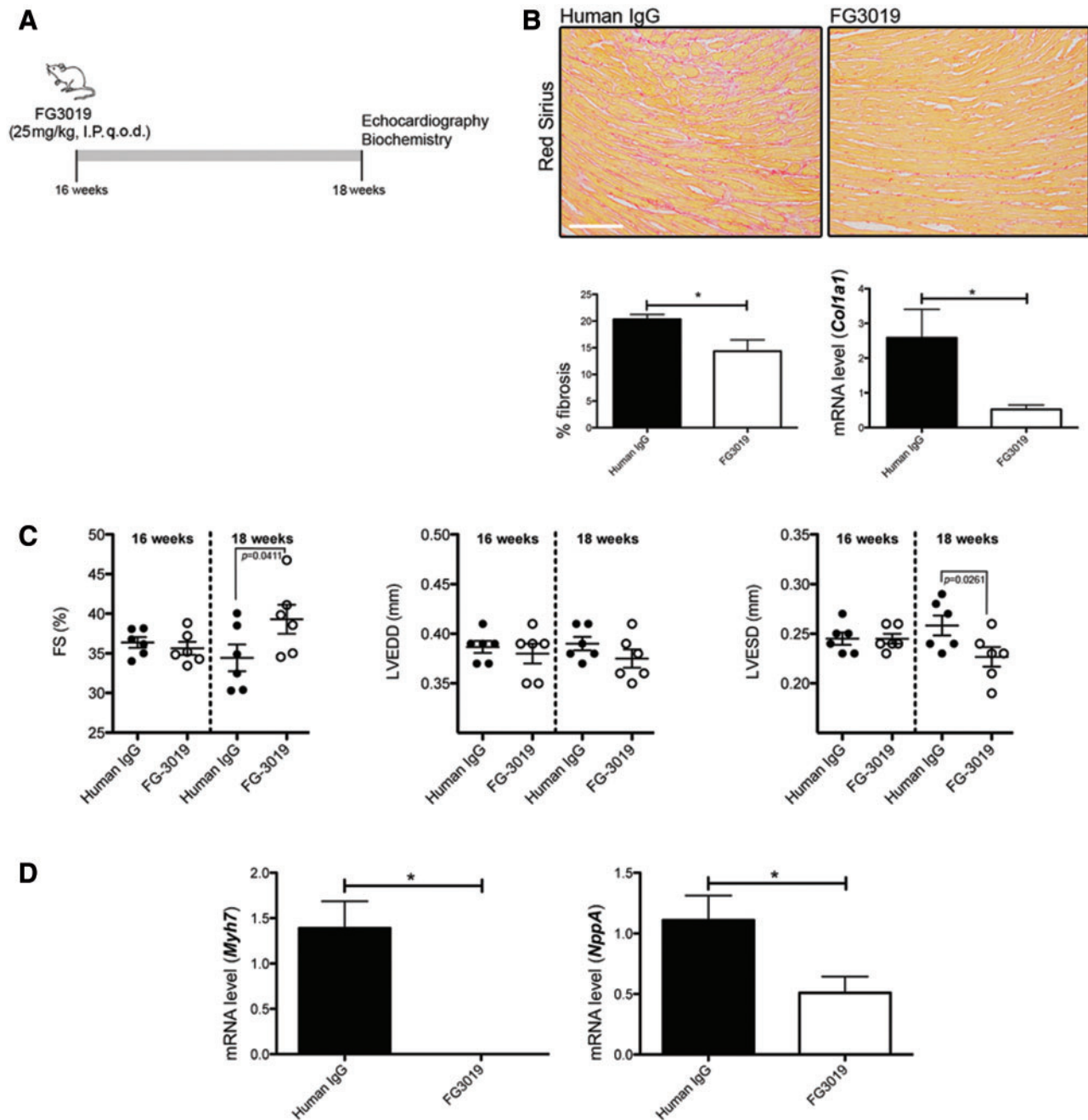


Figure 7. Pharmacological inhibition of CTGF/CCN2 improves left-ventricular function in *Lmna*^{H222P/H222P} mice. (A) Schematic representation of the treatment protocol of *Lmna*^{H222P/H222P} mice with FG-3019. (B) Sirius Red staining of cross sections of hearts from *Lmna*^{H222P/H222P} mice (*Lmna* H222P) treated with IgG or FG-3019. Scale bar: 50 μ m. Left panel bar graph shows the quantification of myocardial fibrosis (means \pm standard errors of means) from *Lmna*^{H222P/H222P} mice (*Lmna* H222P) treated with immunoglobulin G (IgG) or FG-3019. * $P < 0.05$. Right panel shows expression of *Col1a1* gene (means \pm standard errors of means) in heart from *Lmna*^{H222P/H222P} mice treated with IgG ($n = 3$) or FG-3019 ($n = 3$). * $P < 0.05$. (C) Graph showing mean LVEDD, mean LVESD and FS in 18-week old male *Lmna*^{H222P/H222P} mice (*Lmna* H222P) treated with IgG ($n = 6$) or FG-3019 ($n = 6$). Values for each individual mouse receiving placebo or FG-3019 as well as standard errors (bars) are shown. (D) Expression of *Myh7* and *NppA* genes in heart from *Lmna*^{H222P/H222P} mice (*Lmna* H222P) treated with IgG ($n = 3$) or FG-3019 ($n = 3$). * $P < 0.05$, n.s. non-significant.

CCN2 expression, ERK2 was overexpressed in C2C12 cells expressing lamin A H222P. CTGF/CCN2 expression increased in proportion to the amount of transfected ERK2 while cells transfected with a dominant negative variant of ERK2 did not exhibit elevated CTGF/CCN2 expression (Fig. 8C). These data demonstrate that cardiac CTGF/CCN2 expression in *Lmna*^{H222P/H222P} mice was ERK1/2-dependent.

Crosstalk between ERK1/2 and TGF- β signaling in the hearts from *Lmna*^{H222P/H222P} mice

Feedback control could exist between ERK1/2 and TGF- β /Smad signaling. To address this possibility, we examined expression of p-Smad2/3 in hearts of *Lmna*^{H222P/H222P} mice treated with MEK1/2 inhibitor. The amount of p-Smad2/3 from left ventricular tissue was lowered in hearts of PD325901-treated *Lmna*^{H222P/}

Table 2. Quantification of left ventricular function in *Lmna* H222P mice treated with FG3019

2 weeks treatment						
Genotype	Treatment	n	Heart rate (beats/min)	LVEDD (mm)	LVESD (mm)	FS (%)
<i>Lmna</i> WT	None	4	350.2 ± 34.5	3.7 ± 0.4	2.1 ± 0.3	43.4 ± 1.6
<i>Lmna</i> H222P	Human IgG	6	317.3 ± 51.5	3.9 ± 0.2	2.6 ± 0.2	34.4 ± 4.1
<i>Lmna</i> H222P	FG3019	6	329.5 ± 65.9	3.7 ± 0.2*	2.2 ± 0.2**	39.3 ± 4.5*

*P < 0.05.

**P < 0.005 between IgG-treated and FG3019-treated mice.

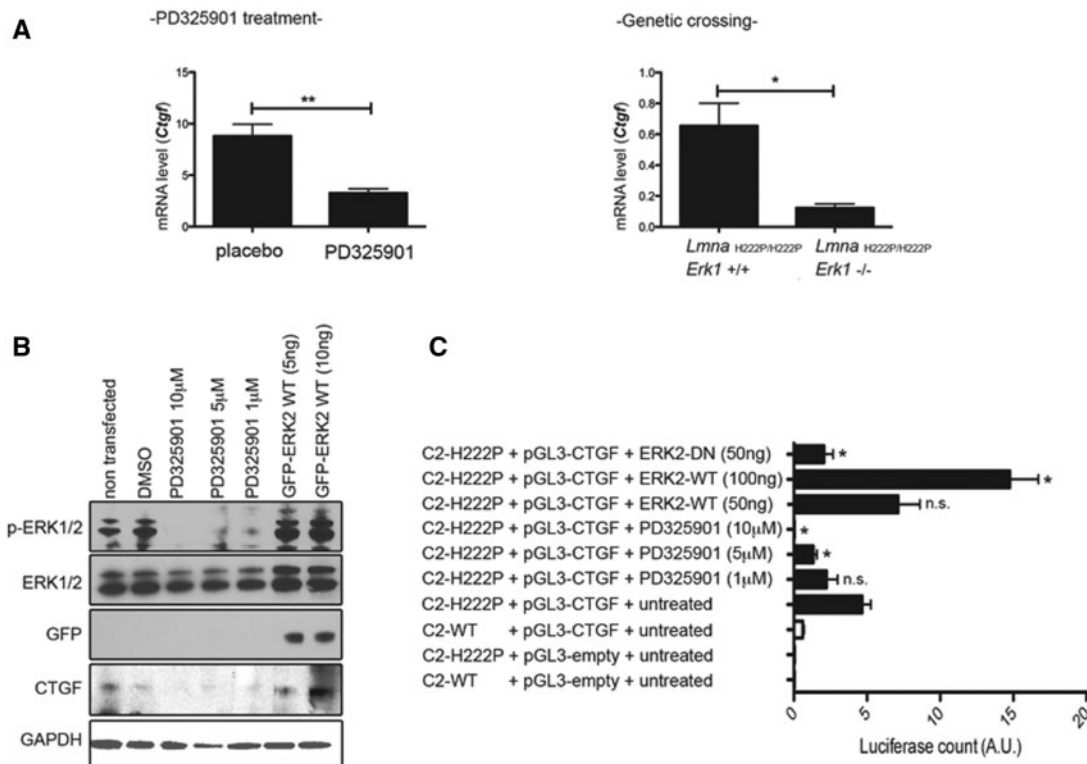


Figure 8. Effect of ERK1/2 on CTGF/CCN2 in hearts of *Lmna*^{H222P/H222P} mice and C2C12 cells. (A) Expression of *Ctgf* gene in heart from *Lmna*^{H222P/H222P} mice (*Lmna* H222P) treated with PD325901 (left panel) and *Lmna*^{H222P/H222P} mice crossed with *Erk1*^{-/-} mice (right panel). Bar diagrams indicate the expression of *Ctgf* (means ± standard errors of means) from *Lmna*^{H222P/H222P} mice treated with placebo ($n = 8$) or PD325901 ($n = 8$) and from *Lmna*^{H222P/H222P}/*Erk1*^{+/+} mice ($n = 4$) or *Lmna*^{H222P/H222P}/*Erk1*^{-/-} mice ($n = 3$). *P < 0.05, **P < 0.01. (B) C2C12 were treated with DMSO or PD325901 (10, 5 or 1 µM) or transfected with plasmid encoding ERK2-WT (5, 10 nM). Immunoblots showing phosphorylated ERK1/2 (p-ERK1/2), total ERK1/2, GFP and CTGF in protein extracts of C2C12 cells treated or transfected. (C) C2C12 cells stably expressing FLAG-tagged H222P-lamin A (C2-H222P) were transfected with the plasmid encoding the promoter of CTGF alone or in association with ERK2-WT (5, 10 nM) or ERK2-DN (5 nM). C2-H222P transfected with the plasmid encoding the promoter of CTGF were also treated with DMSO or PD325901 (10, 5 or 1 µM). After 48 h, luciferase activities induced by expression of CTGF were measured in cell lysates and normalized to β-gal activities obtained from a protein encoded by a co-transfected plasmid. Results are means ± standard errors of means of three experiments. *P < 0.05, n.s. non-significant (compared to C2-H222P transfected with the plasmid encoding the promoter of CTGF alone).

H222P mice compared with hearts of placebo-treated *Lmna*^{H222P/H222P} mice (Supplementary Material, Fig. S3A). To determine if CTGF affects TGF-β/Smad or ERK1/2 signaling, the hearts of *Lmna*^{H222P/H222P} mice treated with FG-3019 were examined. FG-3019 had no effect on Smad2/3 signaling in these mice (Supplementary Material, Fig. S3B). However, compared to mice treated with control human immunoglobulin G, FG-3019 appeared to decrease the extent of ERK1/2 activation (Supplementary Material, Fig. S3B). These results suggested that in hearts of *Lmna*^{H222P/H222P} mice TGF-β signaling mediates the activation of ERK1/2, which modulates myocardial fibrosis through up-regulation of CTGF (Fig. 9).

Discussion

Cardiac fibrosis exacerbates the clinical progression of heart failure (23,24). Therefore, early expression of mediators of fibrosis may contribute to the progressive development of cardiac dysfunction in primary cardiomyopathy. In this study, *Lmna*^{H222P/H222P} mice were used to explore mechanisms of cardiac fibrosis in dilated cardiomyopathy. At 20 weeks, *Lmna*^{H222P/H222P} mice had elevated levels of mRNA for ECM proteins, elevated expression of TGF-β and activation of TGF-β/Smad signaling. Up-regulation of TGF-β/Smad signaling was observed as early as 12 weeks of age in *Lmna*^{H222P/H222P} mice, which is a time that precedes development of both cardiac fibrosis and the

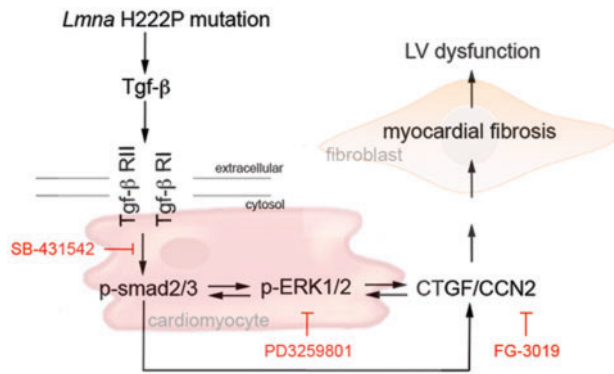


Figure 9. Proposed mechanism of myocardial fibrosis and cardiac dysfunction caused by *Lmna* mutation. Schematic representation of the sequential events of our hypothesis leading to left ventricular dysfunction in LMNA cardiomyopathy.

onset of overt cardiac dysfunction. Inhibition of TGF- β /Smad signaling suppressed cardiac fibrosis and attenuated cardiac dysfunction. These observations indicate that TGF- β is a mediator of the cardiomyopathy that develops as a result of LMNA mutations.

The mechanism by which LMNA mutations lead to TGF- β expression and activation remains to be elucidated. However, our study demonstrated some of the pathways by which TGF- β signaling promotes fibrosis. The canonical TGF- β /Smad signaling pathway involves phosphorylation of Smad2 and/or Smad3, which facilitates their interaction with Smad4 and nuclear translocation to induce transcriptional activation of target genes (25,26). *Lmna*^{H222P/H222P} mice exhibited elevated cardiac p-Smad2/3, its enrichment in the nucleus and a concomitant depletion in the cytosol, which are all characteristic of canonical TGF- β /Smad signaling. TGF- β also has several non-canonical functions, one of which involves activation of the ERK1/2 signaling pathway (27). We have previously reported that ERK1/2 is activated in the hearts of *Lmna*^{H222P/H222P} mice (8) and that reducing its activity ameliorates development of cardiomyopathy (9–11,20). We have demonstrated that at least one path to ERK1/2 activation in the hearts of *Lmna*^{H222P/H222P} mice is via TGF- β /Smad signaling, since the ALK5 kinase inhibitor SB-431542 decreased activated ERK1/2. This is in line with a recent finding by Huang *et al.* (28), showing that ERK1/2 inhibition resulted in significant decreased of Smad2/3-dependent myocardial fibrosis. The same group also showed that another member of ALK (i.e. ALK7) attenuates cardiac fibrosis by blocking ERK1/2 signaling (28).

One of the well-known TGF- β target genes is CTGF. TGF- β can induce CTGF/CCN2 production in cardiac fibroblasts and cardiomyocytes (29), endothelial cells (30,31) and pericytes (32). The regulation of CTGF/CCN2 expression is complex (33). A TGF- β response element located in the promoter region of the CTGF/CCN2 gene was first reported by Grotendorst *et al.* (34) and regulation by Smad2/3 binding to the promoter was subsequently confirmed (35). Our data demonstrate that cardiac CTGF/CCN2 expression is elevated in *Lmna*^{H222P/H222P} mice, as early as 12 weeks after birth. Inhibition of ERK1/2 activation or genetic depletion of ERK1/2 decreased the expression of CTGF/CCN2 mRNA. This confirmed that CTGF/CCN2 mRNA expression is mediated in part by ERK1/2 signaling (36–38). CTGF/CCN2 was reported to be essential for the sustained fibrotic activity of TGF- β (39) and to promote fibrosis in multiple organs (40–43). There are some reports showing that CTGF/CCN2 expression is increased in cardiac fibrosis (44) and failing hearts (23,45,46).

However, CTGF/CCN2's mechanism of action is incompletely understood (47). While it is broadly recognized that TGF- β induces the expression of CTGF/CCN2 (34), there are also data indicating that CTGF/CCN2 promotes the expression of TGF- β (45,48–50). Thus, CTGF/CCN2 appears to promote TGF- β /Smad signaling by inducing expression of TGF- β in a positive feedback loop. This may involve TGF- β -induced ERK1/2 signaling, since inhibition of ERK1/2 strongly reduced CTGF/CCN2 expression and transcription from the CTGF/CCN2 promoter.

The effect of CTGF/CCN2 overexpression in cardiac fibroblasts, which are the major cell type responsible for collagen synthesis in the heart, has not yet been examined. Genetic manipulation of CTGF/CCN2 in cardiomyocytes has resulted in a variety of apparently contradictory effects. The first group to generate transgenic mice reported that overexpression of CTGF/CCN2 in cardiomyocytes did not lead to cardiac fibrosis but the mice developed age-dependent cardiomyopathy that was concordant with cardiac hypertrophy (51). Soon after, Yoon *et al.* (52) generated transgenic mice overexpressing CTGF/CCN2 in cardiomyocytes. These mice had no cardiac hypertrophy but increased fibrosis upon pressure overload (52). A contradictory report by others showed that overexpressing CTGF/CCN2 in cardiomyocytes lead to mice with smaller myocytes, enhanced collagen and fibronectin expression and greater blood vessel density but normal left ventricular function (45). These mice were protected against ischemia/reperfusion injury (45), myocardial infarction (53) and pressure overload (54). Most recently, transgenic mice were generated that only overexpressed CTGF in cardiomyocytes (55). After a month of CTGF/CCN2 expression, these mice exhibited no signs of cardiac fibrosis or hypertrophy. It remains unclear if the cause of the discrepant results between these studies is the result of the promoters used to drive CTGF/CCN2 expression, the background mouse strains or some other reasons.

In the context of the heart, fibrosis can cause increased tissue stiffness, cardiomyocyte atrophy, arrhythmias and hypoxia. Although targeting TGF- β has shown promise as an anti-fibrotic therapy, it also functions in other biological processes (56). Hence, broad targeting of TGF- β /Smad signaling may be problematic. Inhibiting CTGF/CCN2 may therefore be a more specific target for blocking fibrosis. Similarly, it may be a more specific anti-fibrotic therapy than the anti-protozoal drug, halofuginone, which was reported to attenuate cardiac fibrosis and dysfunction in the *mdx* mouse model via inhibition of TGF- β /Smad signaling (57,58). While some of the data from transgenic mouse models could be interpreted as indicating that inhibition of CTGF/CCN2 might be detrimental to cardiac structure and function, this has not been observed when CTGF/CCN2 was pharmacologically inhibited with antibodies. In a cardiac allograft rejection model, FG-3019 administration for 3 weeks decreased fibrosis in the transplanted heart and suppressed cardiac hypertrophy (59). In a rat model of bronchopulmonary dysplasia, inhibition of CTGF/CCN2 with FG-3149, a mouse chimera of FG-3019, inhibited hyperoxia-induced increases of right ventricular systolic pressure and prevented right ventricular hypertrophy (60). And in a transverse aortic constriction-induced mouse model of pressure overload, FG-3149 inhibited cardiac hypertrophy, improved cardiac function and exhibited a trend toward reduced fibrosis that was not significant for the cohort size used (61). Treating *mdx* mice with FG-3019 leads also to an improvement in tissue morphology, less fibrosis, a decrease in damage and an improvement in strength of skeletal muscle (62). These data suggest that CTGF/CCN2 function in the fibrotic process and inhibiting it prevents or reverses fibrosis. In our study, 2 weeks of

FG-3019 administration inhibited collagen deposition. Hence, inhibiting CTGF/CCN2 could be beneficial for patients with LMNA cardiomyopathy by reducing cardiac fibrosis.

Established management of LMNA cardiomyopathy included the use of angiotensin-converting enzyme (ACE) inhibitors or the combination of an ACE inhibitor with β -adrenergic receptor blockade (63). Treatment with ACE inhibitors improves cardiac function in patients and mice with dilated cardiomyopathy; however, ACE inhibition does not attenuate cardiac fibrosis (64,65). Modulation of ECM function may prove beneficial in attenuating disease progression, as shown by the success of anti-fibrotic therapy in the *mdx* mouse (57,58). Future studies will better define the role of CTGF/CCN2 in LMNA cardiomyopathy, as well as determine if CTGF/CCN2 can be a therapeutic target more generally in dilated cardiomyopathy.

Materials and Methods

Animals

Lmna^{H222P/H222P} mice (16) were fed chow and housed in a disease-free barrier facility with 12 h/12 h light/dark cycles. For treatments, PD0325901, SB-431542 and FG-3019 were delivered (intraperitoneal injection) daily at a dose of 1, 5 and 25 mg/kg, respectively. All animal experiments were carried out according to Ministère de l'Éducation Nationale de l'Enseignement Supérieur et de la Recherche at Center for Research in Myology and The Institutional Animal Care and Use Committee at Columbia University Medical Center for the care and use of experimental animals.

Human heart tissue

Sections of explanted hearts from human subjects with LMNA mutations were obtained from Myobank-AFM de l'Institut de Myologie (Paris, France). Myobank-AFM received the authorizations from the French Ministry of Health and from the Comity for Protection of Patient to share tissues and cells of human origin for scientific purposes, ensuring the donors the maintenance of anonymity, respect of their volition, and consent according to the legislation. Control human heart samples were obtained from the National Disease Research Interchange (Philadelphia, PA, USA); information regarding donor confidentiality and consent can be found at <http://www.ndriresource.org>.

Histology

Hearts from *Lmna*^{H222P/H222P} mice were fixed in 4% formaldehyde for 48 h, embedded in paraffin, sectioned at 5 μ m and stained with Masson trichrome. Representative stained sections were photographed using a Microphot SA (Nikon) light microscope attached to a Spot RT Slide camera (Diagnostic Instruments). Images were processed using Adobe Photoshop CS (Adobe Systems).

Immunohistochemistry and immunofluorescence microscopy

Immunofluorescence staining was performed on frozen 8 μ m thick sections of transversal cardiac muscles by fixing them in 3.7% formaldehyde in PBS for 15 min, then blocking in 5% fetal goat serum in PBS/Triton X-100 for 1 h. Sections were incubated in blocking solution with anti-p-smad2/3 antibody (Cell Signaling Technology), CD31 (Abcam) or Col1a1 (Santa Cruz)

antibody overnight at 4 °C followed by PBS washing and incubation with Texas red-conjugated goat anti-mouse IgG secondary antibody (Invitrogen) and counterstained with 0.1 μ g/ml DAPI (Sigma-Aldrich).

Quantitative polymerase chain reaction analysis

Total RNA was extracted using the Rneasy isolation kit (Qiagen). cDNA was synthesized using Superscript first strand synthesis system according to the manufacturer's instructions (Invitrogen) on total RNA. For each replicate in each experiment, RNA from tissue samples of different animals was used. Primers for *Col1a1*, *Col1a2*, *Fn1*, *Sdc1*, *Nid1*, *Dcn1*, *Tgf-b1*, *Tgf-b2* and *Ctgf* were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Real-time quantitative polymerase chain reaction (qPCR) reactions containing HotStart-IT SYBR green qPCR Master Mix (Affymetrix), 200 nM of each primer and 0.2 μ l of template in a 25 μ l reaction volume were amplified using the ABI 7300 Real-Time PCR System (Applied Biosystems). Relative levels of mRNA expression calculated using the $\Delta\Delta C_T$ method were normalized by comparison to housekeeping mRNA.

Protein extraction and immunoblotting

Mouse heart tissue and cultured cells were homogenized in sample extraction buffer (Cell Signaling) then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and blotted with primary antibodies against Smad2/3 (Santa Cruz Biotechnology), phosphorylated Smad2/3 (Cell Signaling), CTGF (Santa Cruz), ERK1/2 (Santa Cruz Biotechnology), phosphorylated ERK1/2 (Cell Signaling) and GFP, GAPDH (Ambion). Secondary antibodies were horseradish peroxidase-conjugated (GE Healthcare). Recognized proteins were visualized by enhanced chemiluminescence.

Isolation of mouse cardiomyocytes

Wild type and *Lmna*^{H222P/H222P} mice were anesthetized with pentofurane and ventricular cardiomyocytes were isolated as described previously (8). Briefly, hearts were removed and the aorta cannulated. After Ca²⁺-free buffer was perfused for 2 min, 0.25 mg/ml collagenase I/II solution was perfused through the coronary arteries for 6 min with 12.5 mM Ca²⁺. Left ventricular tissue was teased apart and pipetted to release individual cells. After enzymatic dispersion, Ca²⁺ concentration in the buffer containing bovine serum albumin was elevated in three steps up to 500 mM.

Luciferase assays

C2C12 cells (American Type Culture Collection) were maintained in a humidified 37 °C/5% CO₂ incubator and subcultured at 80% confluency. Unmodified and stable C2C12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Invitrogen). Stable C2C12 cells were plated at 1 \times 10⁶ cells/9.5 cm² DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin. Twenty-four hours after plating, the cells were co-transfected with 500 ng of the pGL3-CTGF-Luc, 50 ng of the pGFP-ERK2-DN and 50–100 ng of pGFP-ERK2-WT in OptiMEM medium using Lipofectamine Reagent (Invitrogen). Forty-eight hours after transfection, the luciferase substrate was applied using

Luciferase Assay system (Promega). For cell normalization, CellTiterGlo[®] Luminescent Cell Viability Assay (Promega) was used.

Thansthoracic echocardiography

Lmna^{H222P/H222P} mice were anesthetized with 1.5% isoflurane in O₂ and placed on a heating pad (37°C). Echocardiography was performed using a Visualsonics Vevo 770 ultrasound with a 30 MHz transducer applied to the chest wall. Cardiac ventricular dimensions and fractional shortening were measured in 2D mode and M-mode three times for the number of animals indicated. 'Blinded' echocardiographers, unaware of the treatment, performed the examinations.

Statistics

Values for real-time qPCR were compared using an unpaired Student t-test. Comparisons of echocardiographic parameters between PD0325901 and SB-431542-treated and placebo-treated *Lmna*^{H222P/H222P} mice were performed using a Welch t-test; to validate these results, a non-parametric test (Mann-Whitney) was performed and concordance checked. Statistical analyses were performed using GraphPad Prism software.

Supplementary Material

Supplementary Material is available at HMG online.

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Conflict of Interest statement. K.E.L. is a FibroGen employee. All other authors have declared no conflicts of interest.

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