ORIGINAL RESEARCH

MMI-0100 Inhibits Cardiac Fibrosis in a Mouse Model Overexpressing Cardiac Myosin Binding Protein C

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***Background*—**Cardiac stress can trigger production of a 40-kDa peptide fragment derived from the amino terminus of the cardiac myosin-binding protein C. Cardiac stress, as well as cMyBP-C mutations, can trigger production of 1 such truncated protein fragment, a 40-kDa peptide fragment derived from the amino terminus of cMyBP-C. Genetic expression of this 40-kDa fragment in mouse cardiomyocytes (cMyBP-C^{40k}) leads to cardiac disease, fibrosis, and death within the first year. Fibrosis can occur in many cardiovascular diseases, and mitogen-activated protein kinase—activated protein kinase-2 signaling has been implicated in a variety of fibrotic processes. Recent studies demonstrated that mitogen-activated protein kinase—activated protein kinase-2 inhibition using the cell-permeant peptide inhibitor MMI-0100 is protective in the setting of acute myocardial infarction. We hypothesized that MMI-0100 might also be protective in a chronic model of fibrosis, produced as a result of cMyBP-C^{40k} cardiomyocyte expression.

***Methods and Results*—**Nontransgenic and cMyBP-C^{40k} inducible transgenic mice were given MMI-0100 or PBS daily for 30 weeks. In control groups, long-term MMI-0100 was benign, with no measurable effects on cardiac anatomy, function, cell viability, hypertrophy, or probability of survival. In the inducible transgenic group, MMI-0100 treatment reduced cardiac fibrosis, decreased cardiac hypertrophy, and prolonged survival.

***Conclusions*—**Pharmaceutical inhibition of mitogen-activated protein kinase—activated protein kinase-2 signaling via MMI-0100 treatment is beneficial in the context of fibrotic cMyBPC^{40k} disease. **(*J Am Heart Assoc*. 2017;6:e006590. DOI: 10.1161/JAHA.117.006590.)**

Key Words: fibrosis • hypertrophy/remodeling • transgenic mice • transgenic model

Cardiovascular disease remains the leading cause of death in the United States. It is estimated that more than a third of American adults have at least 1 type of cardiovascular disease.[1] Our previous studies identified a 40-kDa fragment of cMyBP-C (cMyBP-C^{40k}) in stressed mouse hearts,[2,3] and the fragment is also present in human heart failure patients' plasma and hearts.[4,5] Transgenic mice expressing the cMyBP-C^{40k} fragment in cardiomyocytes developed cardiac disease, fibrosis, and heart failure by 30 to 50 weeks.[4]

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A pathogenic fibrotic response is generally characterized by the accumulation of excess connective extracellular matrix, eventually leading to a loss of cellular homeostasis and subsequent tissue remodeling. This can result in scar formation and, in the heart, thickening and decreased chamber compliance.[6] The major cell type contributing to extracellular matrix deposition is the myofibroblast, a specialized cell type derived from the quiescent fibroblasts.[7,8] During activation, the myofibroblast expresses characteristic proteins such as α-smooth muscle actin (αSMA) and periostin that, in the mouse, are encoded by the *acta2* and *postn* genes, respectively.[9] While a physiological fibrotic response may be beneficial, and plays critical roles in wound healing and reparative processes, persistent fibrosis usually is detrimental to the organism.[10] Many studies have focused on understanding the underlying mechanisms driving pathological fibrosis and numerous data now suggest that pathways across evolution and between different organ systems are conserved during this chronic reactive process.[6,10,11]

A critical profibrotic cytokine is transforming growth factor β (TGFβ), and numerous studies suggest that both the canonical and noncanonical TGFβ pathways are involved in

Clinical Perspective

What Is New?

- Testing of the cell-permeant peptide inhibitor MMI-0100 efficacy in ameliorating cardiac fibrosis in a well-defined model of sarcomere protein caused cardiac disease.
- Provides proof of principle that MMI-0100 treatment reduced cardiac fibrosis, decreased cardiac hypertrophy, and prolonged survival under conditions where the primary disease-causing peptide was continually made.
- The probability of survival at 40 weeks was significantly increased in the mice treated with MMI-0100.

What Are the Clinical Implications?

- Cardiovascular disease can be caused by and is often accompanied by alterations in cardiac fibrosis.
- Numerous approaches to impacting on pathogenic fibrosis in the heart are currently being investigated with the goal being to move new therapeutics into the clinic.
- MMI-0100 appears to be benign when administered over long periods of time but effectively reduced pathogenic fibrosis even if the primary etiologic stimulus is not changed.

cardiac fibrosis.[6] TGFβ signaling is initiated when the extracellular TGFβ ligand binds to its heteromeric receptor complex formed by TGFβ receptor type I (TβRI) and II (TβRII).[12] The SMAD2/3-dependent canonical pathway is activated through TβRI.[12] While still controversial, studies in which SMAD3 is inhibited pharmaceutically or genetically suggest that SMAD3 is involved in regulating myofibroblast differentiation either directly or indirectly.[6] On the other hand, TβRII-activated noncanonical TGFβ signaling is also a critical player in myofibroblast differentiation.[13] Several signaling cascades have been implicated as downstream targets of TβRII, including mitogen-activated protein kinases, Rho-GTPase, phosphoinositide 3 kinase, and tumor necrosis factor receptor–associated factors 4 and 6.[13] Cardiomyocyte-specific genetic ablation of TβRI or TβRII in a pressure overload model suggested that the cardiomyocyte-based noncanonical signaling cascade involving TAK1-p38 may also play a critical role in cardiac fibrosis.[14] The intracellular serine/threonine kinase substrate mitogen-activated protein kinase-activated protein kinase-2 or MK2 is the major downstream target of p38 signaling in the TGFβ noncanonical pathway involved in the fibrotic response.[15] TGFβ induces αSMA expression, but fibroblast differentiation is blocked in mouse embryonic fibroblasts lacking MK2,[16] confirming the critical nature of the pathway.

Pathogenic myocardial fibrosis is associated with the development of ventricular arrhythmias, left ventricular dysfunction, sudden cardiac death, cardiac remodeling, and heart failure.[17–20] MK2 is a critical downstream target within the TGFβ pathway. MK2 ablation blocks TGFβ-induced αSMA expression and fibroblast differentiation.[15] In a mouse model, MK2 gene ablation rescued the cardiac remodeling that ensued as a result of chronic p38 activity,[21] suggesting that MK2 may be a potential target for intervention in pathological fibrosis.

Recent studies identified a 22 amino-acid cell-permeant peptide, MMI-0100, which inhibits MK2 activity and significantly reduces fibrosis subsequent to vascular grafts,[17] abdominal surgeries,[18] and myocardial infarction[19] as well as bleomycin-induced idiopathic pulmonary fibrosis.[11] While significant effort has been expended towards development of small molecule ATP-competitive MK2 inhibitors, these molecules have been plagued by low solubility, inadequate specificity, and limited ability to cross the cell membrane.[22] To circumvent these limitations, a family of rationally designed, non-ATP-competitive, cell-permeating peptide inhibitors of MK2, including MMI-0100, was made, utilizing a consensus sequence of the native MK2 substrate HSP27.[23]

The studies testing the antifibrotic effects of MMI-0100 all used an acute, surgical intervention to induce a fibrotic response. In light of those data, we wished to explore whether MMI-0100 had therapeutic value when directed to the cMyBP-C^{40k} cardiac disease model, in which a chronic fibrotic stimulus is present before, during, and after drug administration, and is caused by a pathogenic peptide fragment that is known to be present in human heart failure.[4,5] Long-term MMI-0100 treatment alleviated cMyBP-C^{40k}-induced mouse cardiac fibrosis, decreased hypertrophy, and prolonged survival.

Methods

Animals

The bi-transgenic system that allows inducible expression of the selected transgene in cardiomyocytes has been described.[20] Both the driver construct (α-myosin heavy-chain-tTA) and the responder construct that encoded the 40-kDa fragment of cMyBP-C (α- myosin heavy-chain-tetO-40-kDa) were previously described as well and are schematically depicted in Figure 1A.[4] Constructs were injected into C57/B6 mice to generate the founder lines and maintained on the C57/B6 background. Doxycycline treatment results in the absence of cMyBP-C^{40k} expression in double transgenic (Dtg) cardiomyocytes. Two cohorts of mice (n=51) were used in this study. Cohort #1: n values were Ntg_PBS (n=7), Ntg_MMI (n=8), Dtg_PBS (n=10), and Dtg_MMI (n=11). Cohort #2: n values were Ntg_PBS (n=3), Ntg_MMI (n=4), Dtg_PBS (n=5), and Dtg_MMI (n=3). Animals were handled in accordance with the principles and procedures of the *Guide for the Care and Use of Laboratory Animals*. All proposed procedures were

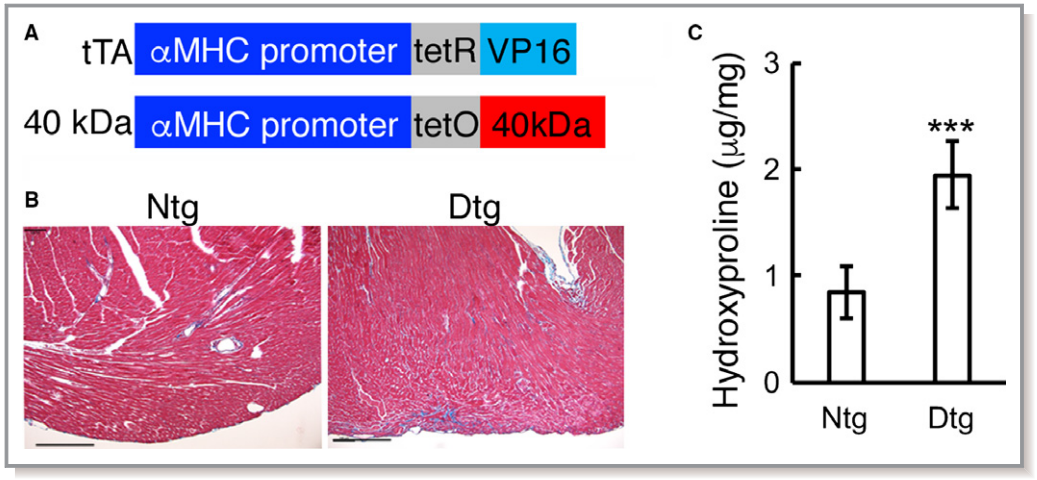

Figure 1. Inducible cardiomyocyte expression of cMyBP-C^{40k} results in cardiac fibrosis. A, Transgenic (tg) constructs used in this study. The "tet-off" transgenic system was used to control cardiomyocyte-specific 40-kDa expression. B, Representative Masson's trichrome staining in 10-week-old mouse hearts (6 weeks after MyBP-C^{40k} induction). Transgene expression was silenced during embryonic development and until after weaning by doxycycline treatment of the mothers' food (4 weeks postbirth). Scale bar: 250 μm. C, Hydroxyproline levels were also determined to quantitate fibrosis at the apex. n=3, ***P<0.001. 40 kDa indicates the MyBP-C^{40k} cDNA sequences; Ntg, Dtg, nontransgenic, double transgenic, respectively; tetR/tetO, tetracycline repressor/operator sequences, respectively; tTA, tetracycline-sensitive transactivator sequence; VP16, virus protein 16; αMHC, α myosin heavy chain promoter sequence.

approved by the Institutional Animal Care and Use Committee at Cincinnati Children's Hospital.

Peptide Preparation and Treatment

The MMI-0100 peptide (YARAAARQARAKALARQLGVAA, MW=2283.67 g/mol) was synthesized using standard fluorenylmethyloxycarbonyl group chemistry (Moerae Matrix, Inc.) as described.[17] For daily intraperitoneal injection at a dose of 50 μg/kg,[19] MMI-0100 was dissolved in sterilized phosphate buffered saline (PBS) to a final concentration of 20 μg/mL. Daily aliquots of the drug were stored at −80°C and gently thawed immediately before use.

Cardiac Function

To determine left ventricular function, 1% isoflurane-anesthetized mice were subjected to 2-dimensional guided M-mode echocardiography with a VisualSonics Vevo 2100 Imaging System using a 30-MHz transducer. Cardiac function was evaluated every 6 to 12 weeks beginning at 10 weeks of age when drug treatment began. Cardiac systolic function was analyzed by PVAN software as described.[4]

Antibodies and Other Reagents

Anti-αSMA and heterogeneous nuclear ribonucleoprotein A0 (HNRNPA0) antibodies were from Sigma-Aldrich (St. Louis, MO). Anti-periostin antibody was from Novus Biologicals (Littleton, CO). Anti-GAPDH antibody was from EMD Millipore (Darmstadt, Germany). Recombinant human TGFβ1 (CHO cell derived) was from PeproTech (Rocky Hill, NJ). Wheat germ agglutinin was from Roche Diagnostics (Indianapolis, IN).

Histology and Immunofluorescent Histochemistry

Hearts were perfused, fixed with 10% formalin, and then treated with a graded series of alcohol dehydrations before being embedded in paraffin blocks for histology. Cardiac fibrosis was determined by Masson's trichrome staining. Cardiomyocyte size was determined by wheat germ agglutinin staining and the cellular surface area was quantified using NIS-Elements software (Nikon, Tokyo, Japan).

RNA Extraction, Western Blots, and Hydroxyproline Assays

Heart homogenates were lysed in RNAzol-RT reagent (Molecular Research Center, Cincinnati, OH) and mRNA isolated following the manufacturer's protocol. Two micrograms of mRNA was used to generate the cDNA pool using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA) following the manufacturer's protocol. Heart homogenates were lysed in buffer containing CellLytic™ Cell Lysis buffer (Sigma, St. Louis, MO) with 1:100 dilution of protease inhibitor and phosphatase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). Equal amounts of protein were analyzed via SDS gel electrophoresis and Western blots. Ventricular tissue

was analyzed using the hydroxyproline assay to quantitate cardiac fibrosis as described.[21]

Luciferase Assay

Neonatal cardiac fibroblasts were plated on 24-well plates and transfected with α-SMA-luciferase together with a cytomegalovirus-promoter-driven renilla-luciferase reporter.[24] Twenty-four hours after transfection, cells were pretreated with DMSO or 100 μmol/L MMI-0100 for 3 hours followed by PBS or 10 ng/mL TGFβ treatment for 48 hours. Luciferase activity was determined by using the Dual-luciferase Assay Kit (Promega, Madison, WI). Each well's αSMA-luciferase (firefly) activity was first normalized to renilla-luciferase activity and the control group was set=1. One-way ANOVA was used to analyze the data.

Wound Healing Assay

Neonatal cardiac fibroblasts were plated on 24-well plates and the "wound" was created by inducing a measured scratch on the plate when cells were 100% confluent. Cells were either treated with DMSO or 100 μmol/L MMI-0100 for 3 hours followed by PBS or 10 ng/mL TGFβ. Wound size was measured at 0 and 24 hours post–wound creation. Cell migration distance was measured for each well and statistical significance was calculated using 1-way ANOVA.

Statistical Analysis

All statistical analyses were performed with SigmaPlot v.13 unless otherwise stated and data are expressed as mean±SD. Shapiro–Wilk and Brown–Forsythe tests were performed to examine data normality and variance equality. For data passing normality and equal variance, 1-way ANOVA with the Holm–Šídák post hoc test was performed. Otherwise, Welch's ANOVA with Tukey's post hoc analysis was performed using R v3.4.0. Kaplan–Meier curves using the log-rank test were generated to detect changes in survival probabilities. Statistical significance was defined as $*P<0.05$, $**P<0.01$, and $***P<0.001$.

Results

Cardiomyocyte Expression of cMyBP-C^{40k} Results in Cardiac Fibrosis

To investigate the potential cardiac pathological consequences of MyBP-C^{40k} expression, 2 Tg mouse lines were generated previously.[4] The driver line produces a protein, tTA, under the control of the α-myosin heavy-chain promoter that, when combined with doxycycline, renders the promoter on the responder line inactive. The responder line therefore produces cMyBP-C^{40k} under the control of the responder promoter only in the absence of the drug (Figure 1A). In the drug's absence, the bi-transgenic animals (Dtg) produce cMyBP-C^{40K} in cardiomyocytes, leading to cardiac disease, compromised cardiac function, and premature death.[4] Cardiac fibrosis could be detected 4 weeks after cMyBP-C^{40k} expression and increased over the next 12 to 16 weeks after cMyBP-C^{40k} induction.[4] Thus, the Dtg mice represent a model of chronic fibrosis brought about by a primary genetic lesion.

To avoid potential developmental effects of cMyBP-C^{40k} expression, the breeding pairs were fed doxycycline chow. Dtg mice and their nontransgenic (Ntg) control littermates were then switched to regular chow after weaning; cMyBP-C^{40k} expression was induced 4 weeks after birth. Collagen deposition, which was examined using Masson's trichrome staining, could be detected as early as 6 weeks after cMyBP-C^{40k} induction at the cardiac apex (Figure 1B). Fibrosis was also quantitated using the hydroxyproline assay and showed a 2-fold increase at this time (Figure 1C).

As recent studies have shown, MK2 signaling is involved in fibrosis after myocardial infarction,[19] and expression of MK2's downstream target, HNRNPA0, accurately reflects cellular MK2 activity in the heart.[19,25,26] We therefore examined HNRNPA0 levels (Figure 2). Comparing with Ntg littermates, a steady induction of HNRNPA0 expression was detected in the Dtg hearts over time (Figure 2). At the same time, we measured the increase in fibrosis over the first 12 weeks of life and showed that increased fibrosis is mirrored by the increased HNRNPA0 levels. The data show that expression of cMyBP-C^{40k} leads to a slow but steady increase in fibrotic area in the heart (Figure 2).

Chronic MMI-0100 Treatment Reduces Cardiac Fibrosis in cMyBP-C^{40k} Mice

To investigate whether administering MMI-0100 could reduce cardiac fibrosis, 6 weeks after cMyBP-C^{40k} induction, Dtg mice and their Ntg littermates were given either PBS or MMI-0100 (50 μg/kg) via daily intraperitoneal injections (Figure 3A). We first wished to determine whether the treatment inhibited MK2 activity. After >30 weeks of cMyBP-C^{40k} expression, HNRNPA0 expression was significantly elevated in the cMyBP-C^{40k} hearts (Figure 3B and 3C). This induction was abolished in the 24-week MMI-0100-treated samples.

Cardiac fibrosis was then determined at the same 24-week treatment time point. Collagen deposition was examined using Masson's trichrome staining (Figure 3D). Treatment with MMI-0100 dramatically reduced the interstitial fibrosis, although perivascular fibrosis was relatively unaffected. Enlarged atria and heavy collagen depositions were also present in the untreated Dtg hearts, while these phenotypes

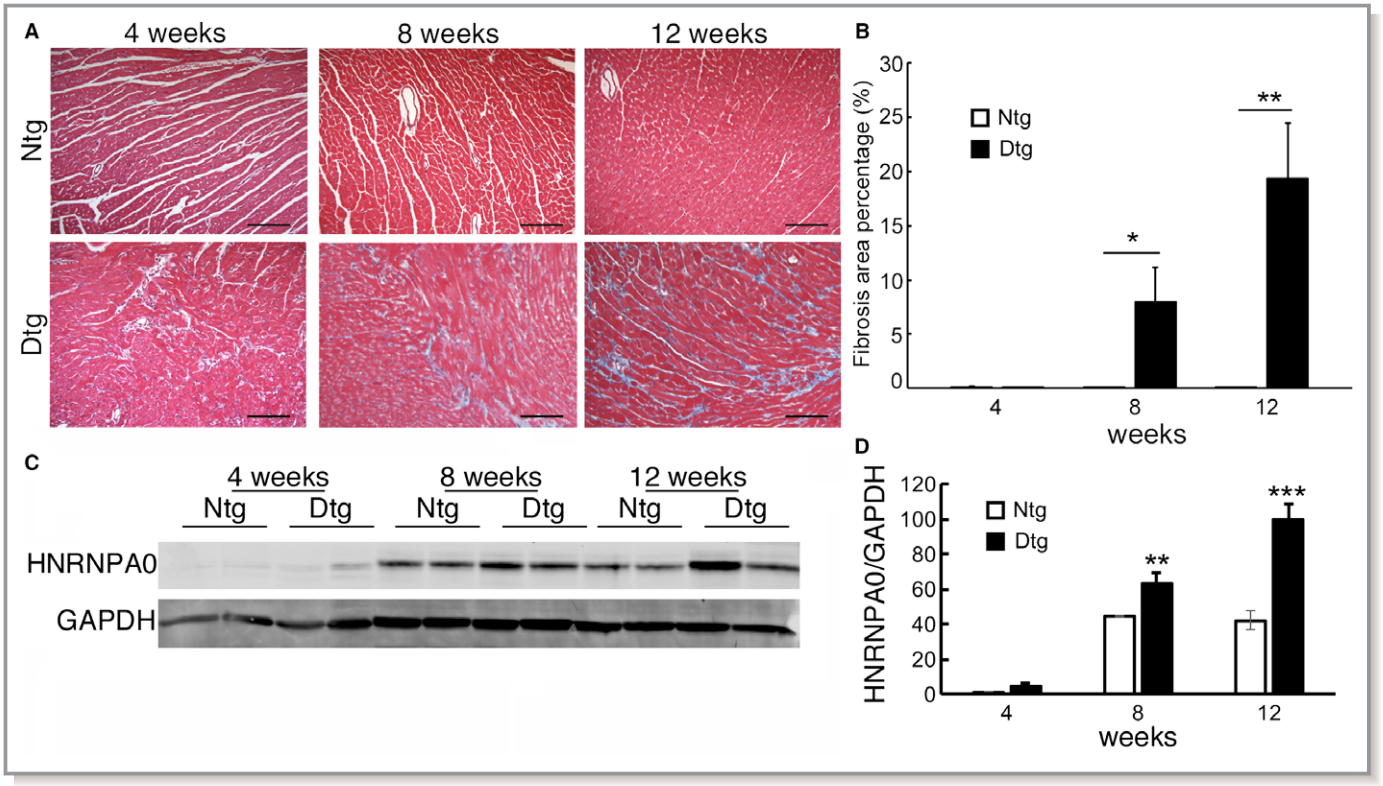

Figure 2. A, Fibrosis progression after cMyBP-C^{40k} expression was examined by trichrome staining. Scale bar: 200 μm. B, Quantification of fibrosis area, n=4. C, Activation of fibrosis-related signaling was detected using Western blot analyses of total cardiac protein. D, Quantitation of the Western blot, analyzed using comparisons between the Ntg and Dtg groups at each time point. *P<0.05, **P<0.01, ***P<0.001. Dtg indicates double-transgenic; HNRNPA0, heterogeneous nuclear ribonucleoprotein A0; Ntg, nontransgenic.

were drastically alleviated in the MMI-0100-treated Dtg samples. Reduced ventricular fibrosis in the MMI-0100-treated Dtg hearts was confirmed by the hydroxyproline assay (Figure 3E). The 2-fold induction of hydroxyproline content in the PBS-treated Dtg hearts was completely blocked by MMI-0100 treatment. Also, no obvious cardiac fibrosis was detected in MMI-0100 treated Ntg hearts (Figure 3C through 3E), suggesting that long-term MMI-0100 treatment does not lead to cardiac fibrosis.

MMI-0100 Reduces Cardiac αSMA Expression and Blocks Fibroblast Differentiation

Activated myofibroblasts are a major source of the cardiac extracellular matrix.[22] In response to extracellular stimuli, the cells express αSMA during fibrosis, contributing to the fibroblasts' stress fiber formation and differentiation into myofibroblasts.[23] Recent studies have identified the secreted matricellular protein periostin as a specific marker of the cardiac myofibroblast,[27] and, during fibroblast differentiation, the periostin promoter is activated.[28] To determine whether MMI-0100 treatment inhibited these early molecular markers of cardiac fibrosis, we measured cardiac αSMA and periostin expression. αSMA and periostin transcript levels were determined using quantitative reverse transcription–polymerase chain reaction and were elevated 3- and 7-fold, respectively, in the cMyBP-C^{40k} hearts (Figure 4A and 4B). αSMA and periostin proteins were also determined by Western blot analyses (Figure 4C). The densitometric analysis showed that there was ≈3-fold induction of αSMA in the PBS-treated Dtg hearts (Figure 4D), while periostin was elevated ≈4-fold (Figure 4E). Immunostaining showed that αSMA was apparent in the perivascular region but also scattered throughout the cardiomyocytes (Figure 4F). The appearance of periostin was also detected in the tissue using immunohistochemical analyses (Figure 4G). For both fibrotic markers, MMI-0100 treatment significantly attenuated expression at both the mRNA (Figure 4A and 4B) and protein levels (Figure 4C through 4G).

We further investigated the underlying mechanism of MMI-0100's inhibition of αSMA synthesis. Neonatal rat cardiac fibroblasts were transfected with an αSMA promoter-luciferase reporter and a fibrotic response initiated via TGFβ treatment (Methods). MMI-0100 pretreatment of the culture effectively abolished TGFβ induction of αSMA transcription (Figure 5A). Because a primary role of αSMA is in stress fiber formation, we examined the effects of MMI-0100 treatment on this cytoplasmic marker. As expected, TGFβ-induced

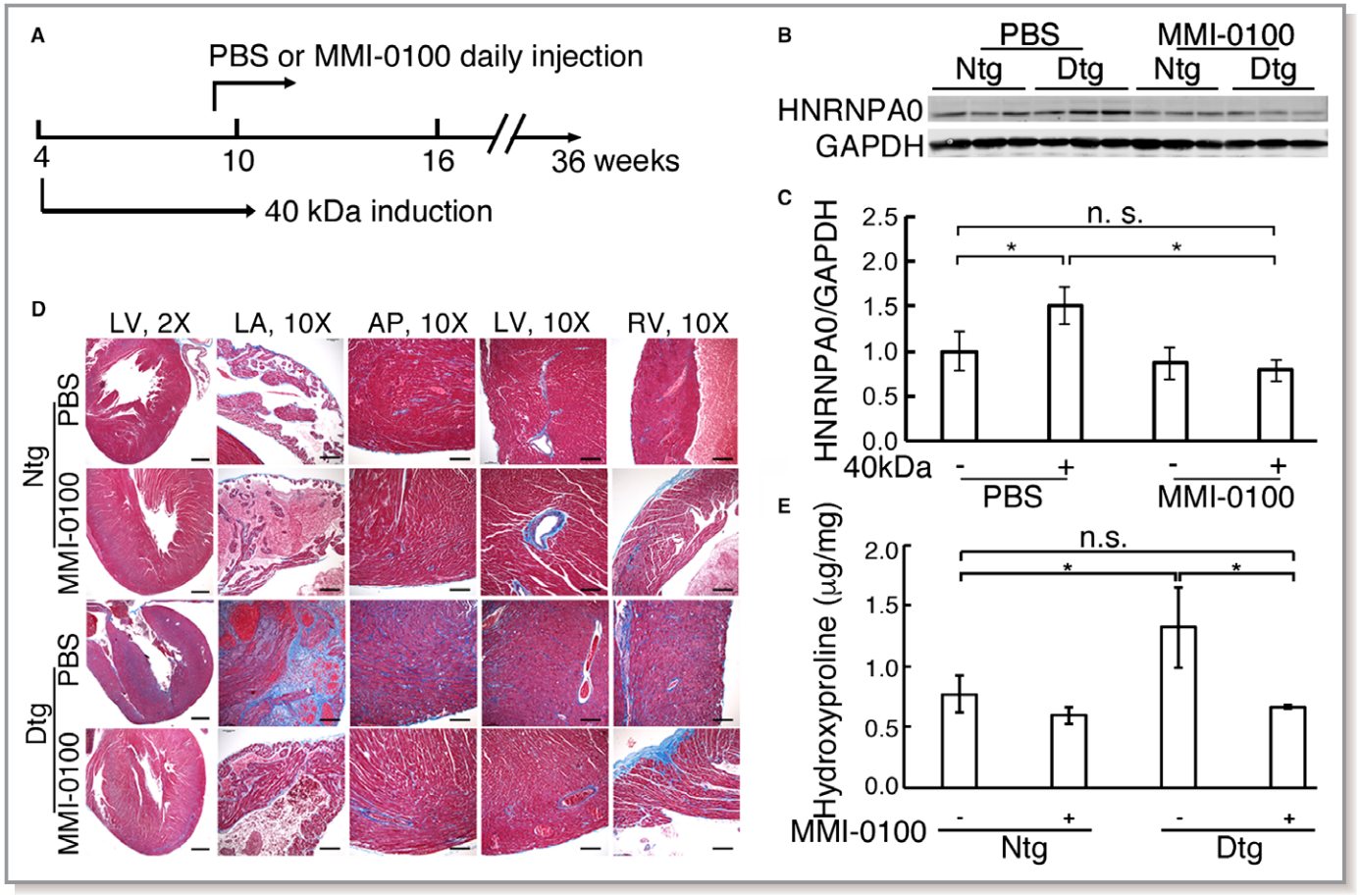

Figure 3. Chronic MMI-0100 treatment reduces cardiac fibrosis in cMyBP-C^{40K} hearts. A, Outline of the experimental design. Four weeks after transgene induction, control and cMyBP-C^{40K} mice were given phosphate buffered saline (PBS) or MMI-0100 (50 μg/kg per day) intraperitoneally for up to 30 weeks. B, C, HNRNPA0 levels were quantitated. D, Masson's trichrome staining in 34 weeks (24-week treatment) hearts. Scale bar: ×2, 2 mm; ×10, 500 μm. E, Hydroxyproline levels were determined to quantitate left ventricle fibrosis. n=3, *P<0.05. AP indicates apex; Dtg, double-transgenic cMyBP-C^{40K}; GAPDH, glyceraldehyde phosphate dehydrogenase; HNRNPA0, heterogeneous nuclear ribonucleoprotein A0; LA, left atrium; LV, left ventricle; Ntg, nontransgenic; n.s., not significant; RV, right ventricle.

stress fiber formation was blocked by MMI-0100 treatment (Figure 5B and 5C). We then further explored the consequences of MMI-0100 treatment at this functional level. Because stress fiber formation is critical for fibroblast migration during wound healing and fibrosis progression,[23] a "wound" was created on fully confluent fibroblast cell layers by scratching the plate substrate. Cell migration over the scratch was subsequently determined as a measure of fibrotic wound repair. TGFβ-induced cell migration was blocked by MMI-0100 treatment (Figure 5D and 5E). Taken together, these data indicate that MMI-0100 treatment inhibits αSMA expression, stress fiber functionality, and progression of a fibrotic response.

MMI-0100 Treatment Alleviates cMyBP-C^{40K}-Induced Cardiac Hypertrophy

Cardiac hypertrophy is an important pathological condition associated with cMyBP-C^{40k} expression. Previous data showed that pharmaceutically inhibiting hypertrophic signaling mediated through the ERK pathway significantly decreased cardiac hypertrophy in the mice.[4] We wished to determine whether cardiac fibrosis was also a hypertrophic stimulus in cMyBP-C^{40k} hearts. Heart weight to body weight ratios were first determined as an indicator of cardiac hypertrophy (Figure 6A). The PBS-treated Dtg mice demonstrated severe cardiac hypertrophy after 24 weeks of injection and this was significantly reduced in the MMI-0100-injected Dtg mouse group. Cardiomyocyte size was determined using wheat germ agglutinin staining (Figure 6B). Quantitative analysis clearly demonstrated increased cardiomyocyte cross-sectional areas in the PBS-treated Dtg mice, but MMI-0100 treatment severely blunted the hypertrophic response as indicated by decreased cross-sectional areas (Figure 6B and 6C) and decreased activation of the molecular markers for hypertrophy, atrial natriuretic peptides A and B (Figure 6D). No significant differences in the heart weight/body weight ratios, cardiomyocyte cross-sectional

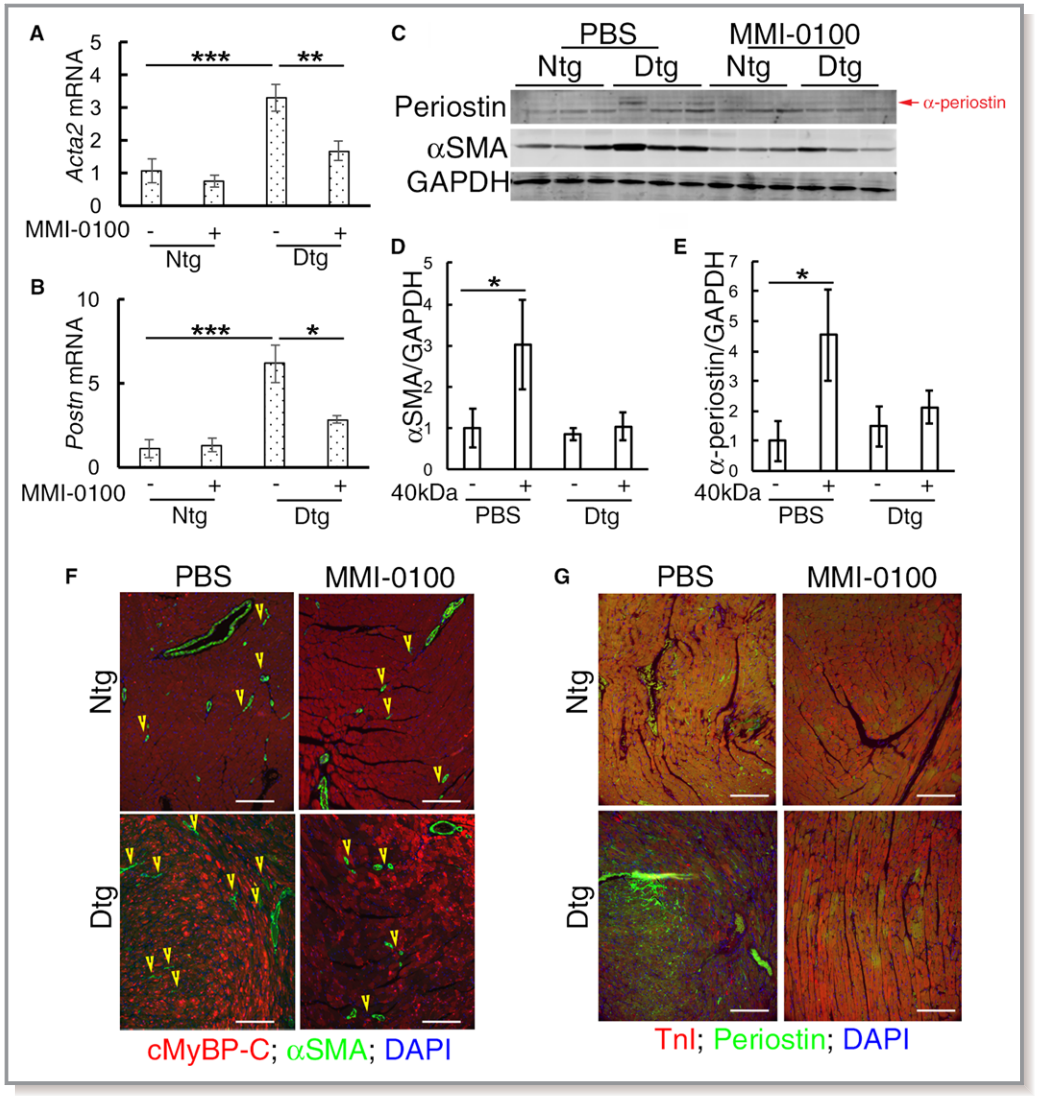

Figure 4. MMI-0100 treatment reduces periostin and α-smooth muscle actin (αSMA) expression in cMyBP-C^{40k} hearts. All mice received 24 weeks of MMI-0100 treatment and were 34 weeks old when samples were obtained. A, αSMA and (B) Periostin mRNA expression levels were normalized to GAPDH and then to the phosphate buffered saline (PBS)–treated, nontransgenic (Ntg) group. C, Periostin and αSMA protein was also detected by using Western blots of cardiac protein. D and E, Quantitation of the Western analyses, **P*<0.05, ***P*<0.01, ****P*<0.001. F, αSMA positive cells were detected via immunofluorescence. Arrowhead: nonperivascular αSMA-positive cells. G, Upregulation of periostin was detected via immunofluorescence. Scale bar: 250 μm. Double transgenic (Dtg), 4′,6-diamidino-2-phenylindole (DAPI, nuclear staining; blue). αSMA indicates α-smooth muscle actin; *postn*, periostin; TnI, troponin I.

areas, or cardiac hypertrophy markers' expression were observed when the Ntg PBS-treated and MMI-0100-treated animals were compared, showing that long-term, 24-week MMI-0100 treatment does not lead to cardiac hypertrophy or overt remodeling.

MMI-0100 Treatment Decreases Development of Hypertrophy and Prolongs Survival

Cardiac hemodynamics and structural parameters were determined using echocardiography (Methods) (Figure 7). Baseline cardiac systolic function was measured immediately before treatment started, and was repeatedly measured with echocardiography throughout the 30-week treatment period as indicated (Figure 7). No changes were detected in the Ntg groups that were treated with MMI-0100, suggesting that long-term treatment does not adversely affect cardiac hemodynamics. We did note statistically significant increases in left ventricular mass, interventricular septal thickness at end diastole, and left ventricular posterior wall thickness at end diastole. These increases did not occur in the MMI-0100-treated animals.

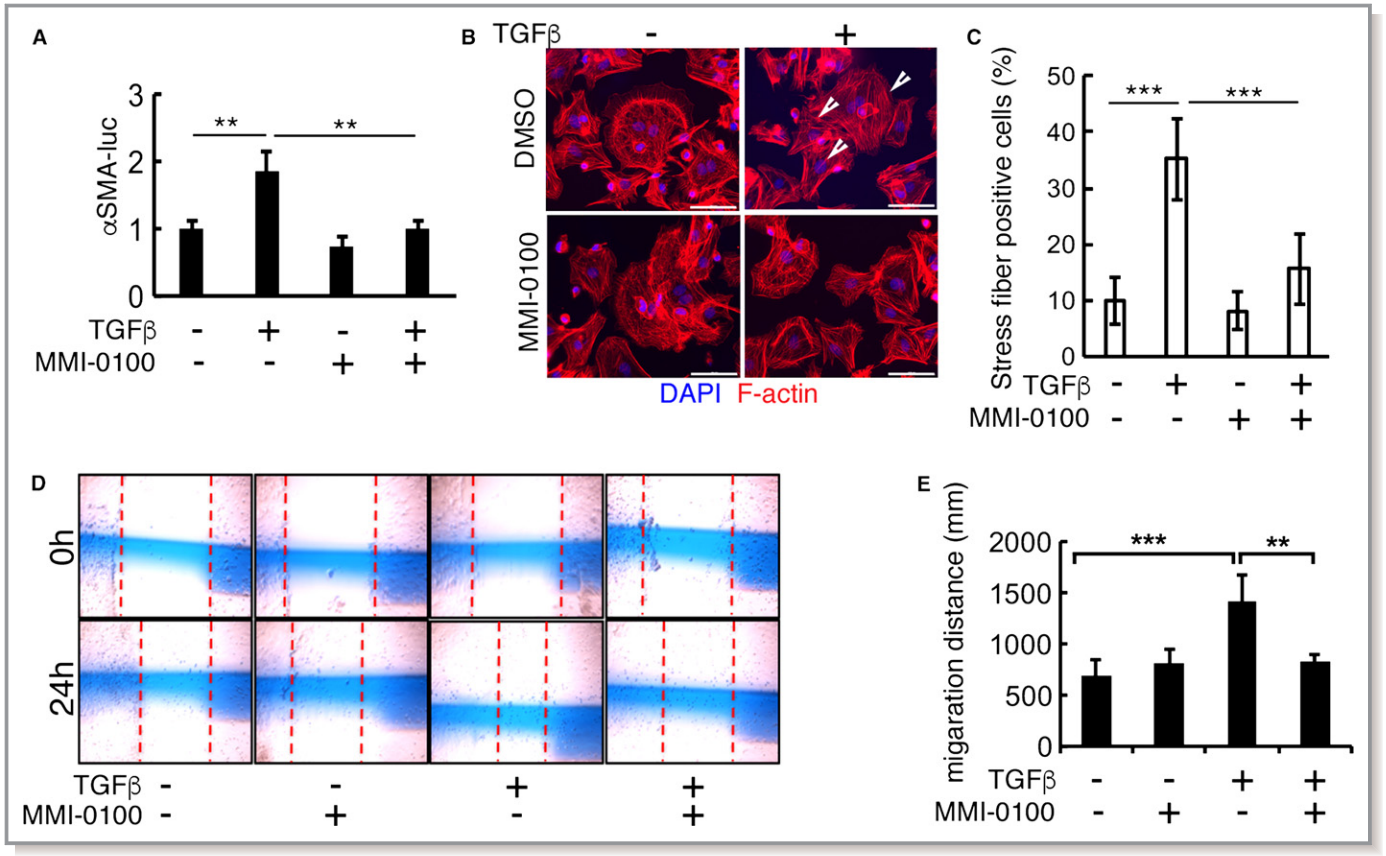

Figure 5. MMI-0100 treatment inhibits αSMA-driven luciferase (αSMA-luc) activity and transforming growth factor β1 (TGFβ) induced cardiac fibroblast migration in vitro. A, Neonatal rat cardiac fibroblasts were transfected with an αSMA promoter-driven luciferase reporter (αSMA-luc) construct and a CMV driven renilla luciferase reporter was co-transfected as a control. Luciferase activity was measured 24 hours after TGFβ treatment with or without MMI-0100 pre-treatment. B, Stress fibers were visualized by F-actin staining using phalloidin. White arrow: stress fiber positive cells. Scale bar: 100 μm. C, The percentage of cells containing stress fibers was calculated. More than 200 cells/treatment were examined for each treated culture. D, Representative image of in vitro cell migration assay. Neonatal rat cardiac fibroblasts were plated, grown to confluence and the plate subsequently scratched. Images were taken at 0 and 24 hours. E, Cell layer migration distance was measured. $^{**}P<0.01$, $^{***}P<0.001$.

Kaplan–Meier curves were constructed for the experimental groups (Figure 8). The curve obtained with Dtg mice treated with PBS began to diverge from the MMI-0100 group at ≈28 weeks and, by 40 weeks, had achieved statistical significance, showing that MMI-0100 treatment prolonged cMyBP-C^{40k} mouse survival. The dead mice were invariably found at the start of the work day and were not analyzed further because of rapid decay/cannibalization. No significant differences occurred between the PBS and MMI-0100 treated Ntg groups' survival rates, a result consistent with long-term MMI-0100 treatment not leading to adverse effects at the whole animal level, at least in terms of survival.

Discussion

Although mutations in cMyBP-C are one of the most frequent causes of hypertrophic cardiomyopathy on a per gene basis with >150 individual mutations being documented, the majority of these mutations (≈60%) result not in a full-length, mutated protein, but in a truncated peptide and these mutated alleles exhibit autosomal dominance.[29,30] We have shown that a truncated form of cMyBP-C is produced from endogenous, normal cMyBP-C as a result of ischemia–reperfusion injury and/or general cardiovascular stress and is generated from Ca^{2+} activated μ-calpain activity.[2] This fragment is stable, can be expressed inducibly in cardiomyocytes and causes cardiac disease, fibrosis, and eventually heart failure and death.[4] This model displays pathology that is often seen in human cardiac fibrosis and myocardial disease: the hearts develop hypertrophy and show extensive interstitial fibrosis and perivascular fibrosis while maintaining systolic function. Thus, in terms of a fibrotic response, the model represents a chronic, ongoing fibrotic stimulus that is suitable for testing the efficacy of an antifibrotic agent in terms of prevention.

MK2 inhibition by the cell permeant peptide MMI-0100 can alleviate acute fibrotic injury in the lung and heart.[11,19] The present study tested the concept that MMI-0100 inhibition of

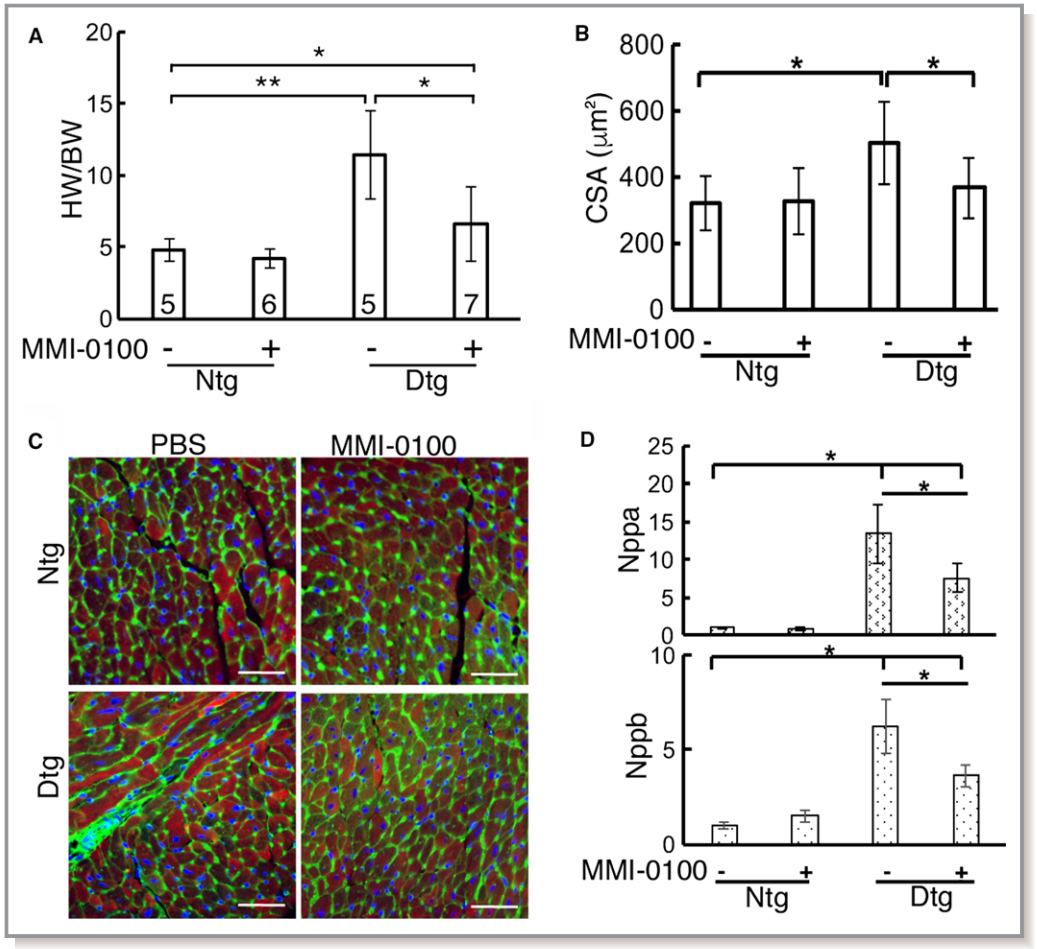

Figure 6. MMI-0100 treatment alleviates cardiac hypertrophy. Mice were 34 weeks old when samples were obtained. A, Ratios of heart weight (HW) to body weight (BW) of 34-week-old nontransgenic (Ntg) and double transgenic (Dtg) mice treated with phosphate buffered saline (PBS) or MMI-0100. **P*<0.05, ***P*<0.01. B, Wheat germ agglutinin (green) staining was used to determine the cross-sectional area (CSA) of the cardiomyocytes. Scale bar=50 μm. C, Cardiomyocyte CSA. Averaged data from 500 cells per heart, 3 hearts per group. D, Natriuretic peptides A and B (Nppa and Nppb, respectively) mRNA expression normalized to GAPDH and then to Ntg treated with PBS set arbitrarily to a value of 1. n=3, **P*<0.05.

the MK2 cascade will reduce cardiac fibrosis and benefit the animal in a chronic model of fibrosis induced by cardiomyocyte-specific expression of cMyBP-C^{40k}. The data demonstrated that long-term MMI-0100 treatment inhibited αSMA expression, blocked fibroblast differentiation, reduced extracellular collagen deposition, and alleviated cardiac fibrosis in cMyBP-C^{40k} hearts. No adverse effects on cardiac structure or function were observed during the 30 weeks of drug treatment.

While significant effort has been expended towards development of small-molecule ATP-competitive MK2 inhibitors, these molecules have been plagued by low solubility, inadequate specificity, and limited ability to cross the cell membrane.[31] Accordingly, a family of rationally designed, non-ATP-competitive, cell-permeating peptide inhibitors of MK2, including MMI-0100, was synthesized utilizing a consensus sequence of the native MK2 substrate HSP27.[32]

MMI-0100, administered via inhalation, is currently in clinical development for the treatment of fibrotic and obstructive pulmonary disease. Three Phase I clinical trials testing MMI-0100 have been successfully completed, following studies in multiple in vitro, ex vivo, and in vivo models of fibrosis and inflammation, demonstrating MK2 target engagement and anti-inflammatory and antifibrotic efficacy, in both prevention and treatment settings.[17,19,29,30] Perhaps most relevant to the current report is the demonstration that MMI-0100 reduced development of cardiac fibrosis and/or improved cardiac function in a murine postmyocardial infarction model of cardiac fibrosis.[19,33] In another model of organ fibrosis, bleomycin-induced pulmonary fibrosis, MMI-0100—whether systemically delivered (intraperitoneal injection) or administered locally to the lung via inhalation (nebulized)—reversed previously established fibrosis, significantly decreased circulating IL-6 levels, and modulated lung matrix remodeling gene

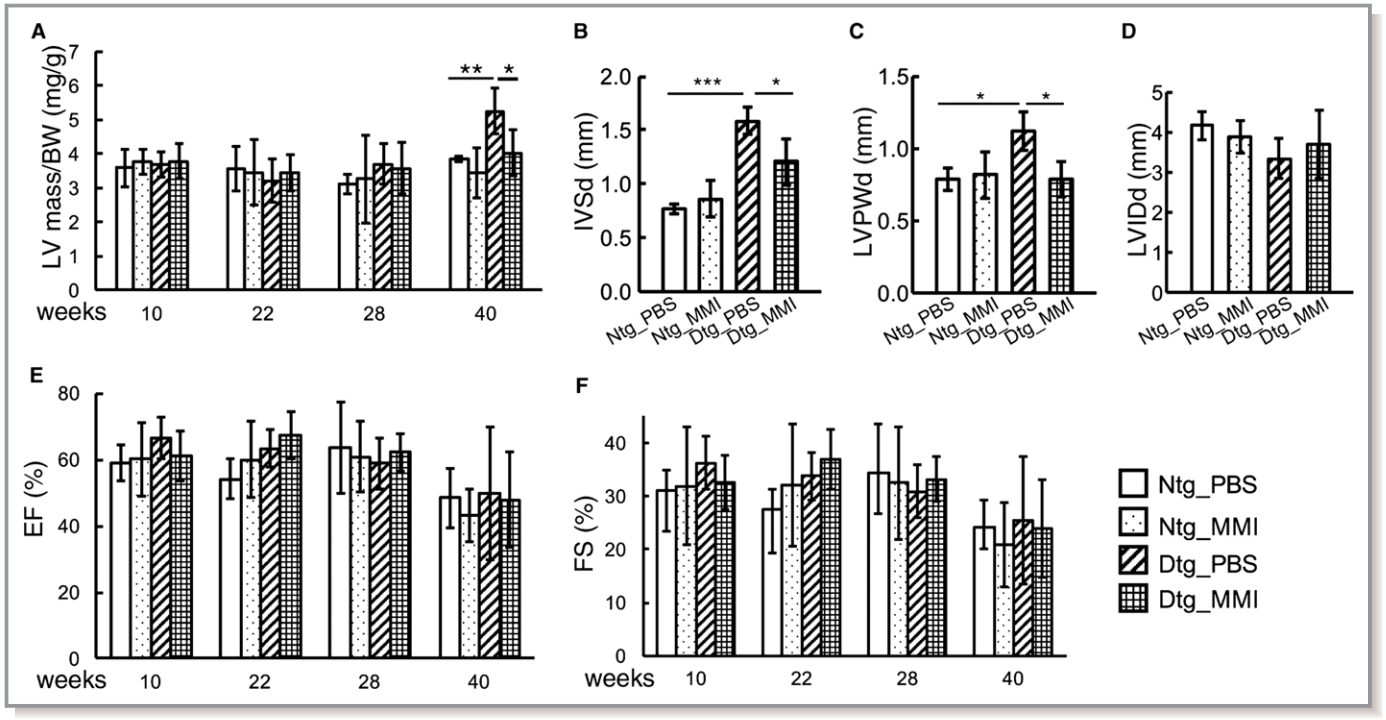

Figure 7. Thirty-week MMI-0100 treatment alleviates cardiac hypertrophy but has no effect on cardiac hemodynamics. A through F, Echocardiographic analysis of Ntg and Dtg cMyBP-C^{40k}-expressing mice after long-term PBS or MMI-0100 treatment. n=7 to 11. Left ventricular mass (LV mass), body weight (BW), interventricular septum thickness end diastole (IVSd), left ventricular posterior wall thickness end diastole (LVPWd), left ventricular internal diameter end diastole (LVIDd), ejection fraction (EF), fractional shortening (FS). Groups at each time point were subjected to statistical analyses to determine if any differences were present. **P*<0.05, ***P*<0.01, ****P*<0.001. Dtg indicates double transgenic; Ntg, nontransgenic; PBS, phosphate buffered saline.

expression, including the TGFβ-inducible SMAD 3, 6, and 7 as well as serpine 1.[11] In addition, MMI-0100 reduced TGFβ-stimulated myofibroblast differentiation and extracellular matrix deposition (α-SMA, fibronectin, collagen Type 1) in cultured normal human fetal fibroblasts[29] and inhibited both hyaluronan synthesis and fibroblast invasiveness in fibroblasts harvested from bleomycin-treated murine and human idiopathic pulmonary fibrosis patient lung.[34]

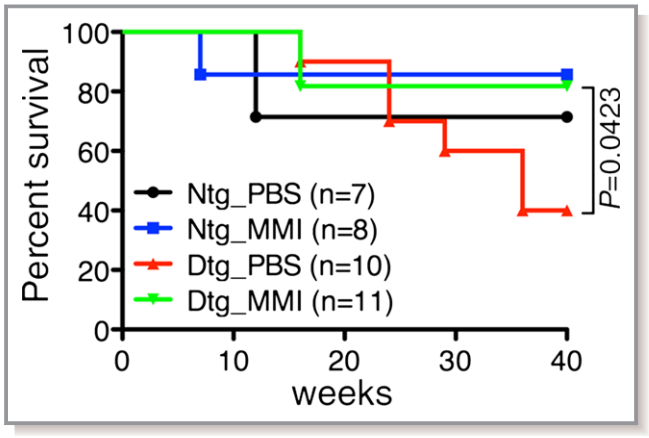

Figure 8. MMI-0100 treatment prolongs survival of cMyBP-C^{40k} mice. Survival curve of nontransgenic (Ntg) and double transgenic (Dtg) mice treated with phosphate buffered saline (PBS) or MMI-0100.

Because we focused on fibrosis, we purposely chose an interval in the pathogenic process where acute heart failure did not present, but fibrosis did. We did not determine the physiological effects after stopping administration as this would have carried us into the acute heart failure stage of the process and complicating morbidities would then cloud our ability to interpret the drug's particular effects on fibrosis and probability of survival.

MMI-0100 treatment decreased cardiac hypertrophy in this model, as might be expected if the drug blunted the pathogenic fibrotic response. Despite the hypertrophy and fibrosis occurring in the cMyBP-C^{40k} hearts, cardiac function was conserved in the mice, a result consistent with the conserved systolic function that normally is seen in human hypertrophic cardiomyopathy.[35] Indeed, normal ejection fractions and fractional shortening values were maintained in all experimental cohorts throughout the study's duration in all experimental cohorts (Figure 7).

MMI-0100 treatment also prolonged cMyBP-C^{40k} mouse survival. While the primary insult—cardiomyocyte expression of cMyBP-C^{40k}—continued, inhibiting fibrosis significantly increased the probability of survival in the 40-week-old

animals treated with MMI-0100. These data suggest that the peptide-mediated inhibition of MK2 via MMI-0100 may represent a novel therapeutic approach to the treatment of chronic cardiac fibrosis and the cardiac disease that sometimes results.

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