Mutations in sarcomere protein genes can cause hypertrophic cardiomyopathy (HCM), a disorder characterized by myocyte enlargement, fibrosis, and impaired ventricular relaxation. Here, we demonstrate that sarcomere protein gene mutations activate proliferative and profibrotic signals in non-myocyte cells to produce pathologic remodeling in HCM. Gene expression analyses of non-myocyte cells isolated from HCM mouse hearts showed increased levels of RNAs encoding cell-cycle proteins, Tgf-β, periostin, and other profibrotic proteins. Markedly increased BrdU labeling, Ki67 antigen expression, and periostin immunohistochemistry in the fibrotic regions of HCM hearts confirmed the transcriptional profiling data. Genetic ablation of periostin in HCM mice reduced but did not extinguish non-myocyte proliferation and fibrosis. In contrast, administration of Tgf-β-neutralizing antibodies abrogated non-myocyte proliferation and fibrosis. Chronic administration of the angiotensin II type 1 receptor antagonist losartan to mutation-positive, hypertrophy-negative (prehypertrophic) mice prevented the emergence of hypertrophy, non-myocyte proliferation, and fibrosis. Losartan treatment did not reverse pathologic remodeling of established HCM but did reduce non-myocyte proliferation. These data define non-myocyte activation of Tgf-β signaling as a pivotal mechanism for increased fibrosis in HCM and a potentially important factor contributing to diastolic dysfunction and heart failure. Preemptive pharmacologic inhibition of Tgf-β signals warrants study in human patients with sarcomere gene mutations.
Cardiac fibrosis in mice with hypertrophic cardiomyopathy is mediated by non-myocyte proliferation and requires Tgf-β

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Mutations in sarcomere protein genes can cause hypertrophic cardiomyopathy (HCM), a disorder characterized by myocyte enlargement, fibrosis, and impaired ventricular relaxation. Here, we demonstrate that sarcomere protein gene mutations activate proliferative and profibrotic signals in non-myocyte cells to produce pathologic remodeling in HCM. Gene expression analyses of non-myocyte cells isolated from HCM mouse hearts showed increased levels of RNAs encoding cell-cycle proteins, Tgf-β, periostin, and other profibrotic proteins. Markedly increased BrdU labeling, Ki67 antigen expression, and periostin immunohistochemistry in the fibrotic regions of HCM hearts confirmed the transcriptional profiling data. Genetic ablation of periostin in HCM mice reduced but did not extinguish non-myocyte proliferation and fibrosis. In contrast, administration of Tgf-β-neutralizing antibodies abrogated non-myocyte proliferation and fibrosis. Chronic administration of the angiotensin II type 1 receptor antagonist losartan to mutation-positive, hypertrophy-negative (prehypertrophic) mice prevented the emergence of hypertrophy, non-myocyte proliferation, and fibrosis. Losartan treatment did not reverse pathologic remodeling of established HCM but did reduce non-myocyte proliferation. These data define non-myocyte activation of Tgf-β signaling as a pivotal mechanism for increased fibrosis in HCM and a potentially important factor contributing to diastolic dysfunction and heart failure. Preemptive pharmacologic inhibition of Tgf-β signals warrants study in human patients with sarcomere gene mutations.

Introduction

Dominant mutations in sarcomere protein genes cause hypertrophic cardiomyopathy (HCM), a primary myocardial disorder characterized by myocyte enlargement, increased myocardial fibrosis, and impaired ventricular relaxation that predisposes patients to develop heart failure (1–5). The population incidence of sarcomere protein gene mutations is considerable (4–6), and recent epidemiologic studies document 7-fold increased risk of heart failure in the specific mutations in sarcomere protein genes increase myocardial microscopic scars that replace dead myocytes, accrue in HCM. Increased myocardial fibrosis is maladaptive, and accumulation correlates with impaired cardiac relaxation (16) and increases the propensity for heart failure. Mechanisms by which myocyte-specific mutations in sarcomere protein genes increase myocardial fibrosis remain unknown.

To define profibrotic signals in HCM, we studied 2 mouse lines that carry different human mutations in the α-cardiac myosin heavy chain gene: Arg403Gln (referred to as α-MHC403/+ ) (17) and Arg719Trp (referred to as α-MHC719/+); Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI42028S1). These mice exhibit protean manifestations of HCM, including hypertrophy and focal fibrosis in adulthood (17, 18). The hearts from young α-MHC403/+ and α-MHC719/+ mice, like human children with HCM mutations, have normal changes (12). In addition to inducing changes within myocytes, the characteristic histopathology found in HCM hearts implies that sarcomere gene mutations also impact non-myocyte cells.

Myocardial fibrosis, the collagen-rich extracellular matrix that is presumed to derive from fibroblast-like cells within the heart is substantially increased in HCM (13–15). Both interstitial fibrosis, which surrounds individual myocytes, and focal fibrosis, the microscopic scars that replace dead myocytes, accrue in HCM. Increased myocardial fibrosis is maladaptive, and accumulation correlates with impaired cardiac relaxation (16) and increases the propensity for heart failure. Mechanisms by which myocyte-specific mutations in sarcomere protein genes increase myocardial fibrosis remain unknown.

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dimensions and histology throughout a period of clinical latency (denoted as prehypertrophic). Despite the absence of hypertrophy or HCM histopathology, our studies of RNA expression in LV tissues from prehypertrophic α-MHC<sup>403/+</sup> mouse hearts (12) demonstrated increased transcription of genes associated with fibrosis.

To characterize molecular signals that promote fibrosis in α-MHC<sup>403/+</sup> and α-MHC<sup>719/+</sup> mice, we used comprehensive transcriptional analyses of RNAs expressed in isolated myocytes and non-myocyte cells from prehypertrophic HCM hearts and from hearts with overt HCM. Guided by increased expression of RNAs in non-myocyte cells, we assessed cell proliferation in HCM hearts and targeted potential signaling molecules implicated in activating fibrosis in HCM. Using these data, we tested and report a pharmacologic strategy for extinguishing the emergence of pathologic remodeling in prehypertrophic HCM mice.

**Results**

**HCM models.** We studied 2 mouse HCM models. We used the previously described α-MHC<sup>403/+</sup> mice (17, 18), and we produced what we believe to be a new model that carries the Arg719Trp mutation in the α-cardiac myosin heavy chain gene (α-MHC<sup>719/+</sup>) mice, using homologous recombination technology (Supplemental Figure 1). Hearts from young (<20 weeks) α-MHC<sup>719/+</sup> mice, like those of juvenile α-MHC<sup>403/+</sup> mice, do not have LV hypertrophy or HCM histopathology and are designated prehypertrophic. By 35 weeks, α-MHC<sup>719/+</sup> mice exhibit both hypertrophy and HCM histopathology that is indistinguishable from that of adult α-MHC<sup>403/+</sup> mice.

Our prior studies (11) showed that cyclosporine A (CsA) administration to young α-MHC<sup>403/+</sup> mice accelerates the emergence of HCM (referred to as hypertrophic α-MHC<sup>403/+</sup>-csa mice). CsA-treated WT mice (WTcsa mice) develop neither hypertrophy nor histopathology. CsA-treated α-MHC<sup>719/+</sup> mice (referred to as hypertrophic α-MHC<sup>719/+</sup>-csa mice) showed accelerated development of increased LV wall thickness (LVWT), overt HCM histopathology, including increased myocardial fibrosis, and preserved cardiac function (Figure 1A and Table 1). The maximum LVWT was 1.8-fold increased in hypertrophic α-MHC<sup>719/+</sup>-csa mice compared with that of WTcsa mice (P = 3 × 10<sup>-4</sup>). Masson trichrome–stained sections showed approximately 80-fold greater fibrosis in hearts (n = 3 per genotype) from hypertrophic α-MHC<sup>719/+</sup>-csa mice than WTcsa mice (4.4% ± 2.8% vs. 0.05% ± 0.07%, n ≥ 27 sections per genotype; P = 1.3 × 10<sup>-11</sup>) and 40% more cardiac fibrosis than prehypertrophic α-MHC<sup>719/+</sup>-csa mice (n = 7; 3.10% ± 3.1%; n ≥ 105 sections per genotype; P = 0.012; Figure 1A).

**RNA profiles of isolated cardiac myocytes and non-myocyte cells.** To define signaling pathways that promote fibrosis in response to a sarcomere gene mutation, RNA expression was assessed by deep sequence analysis of gene expression (DSAGE; see Methods) in myocytes and non-myocyte cells isolated from hearts of 9- to 12-week-old WT, prehypertrophic α-MHC<sup>403/+</sup>-csa, WTcsa, and hypertrophic α-MHC<sup>403/+</sup>-csa mice. Of 15,579 distinct RNAs found in non-myocyte cells, 7,578 RNAs were expressed at higher levels than in myocytes. Among RNAs that were significantly enriched in non-myocyte cells (P < 0.001), 1,317 changed with hypertrophic remodeling (Supplemental Table 1); expression decreased in 272 RNAs and increased in 1,045 RNAs. Non-myocyte RNAs that were significantly increased (P < 0.0001) in hypertrophic α-MHC<sup>403/+</sup>-csa mice compared with WTcsa mice (Supplemental Tables 1 and 2) included transcripts encoding extracellular matrix proteins that have been previously identified in fibroblasts from other tissues, such as periostin (Postn), Tgfb, connective tissue growth factor (Ctgf), collagens (19), vimentin, chemokines (20), and metalloproteinases (21).

Using the DAVID (version 6.7) (22, 23) functional annotation tool, we identified 18 Gene Ontology (GO) terms (Supplemental Table 3) that were significantly overrepresented (P < 0.05 after Bonferroni correction) among differentially expressed genes in non-myocyte cells (Supplemental Table 1). This unbiased analysis corroborated our interpretation of DSAGE data, in that GO terms related to extracellular matrix, cell-cycle control, and cell proliferation were enriched in non-myocyte cell RNAs (Supplemental Table 3). Notably, 50 RNAs encoding proteins involved in cell-cycle control had significantly altered levels (P = 0.01 after Bonferroni correction; see bolded RNAs in Supplemental Tables 1 and 3). Based upon these transcriptional profiles, we concluded that non-myocyte cells in hypertrophic α-MHC<sup>403/+</sup>-csa hearts were activated.
Proteins involved in the fibrotic response to sarcomere protein gene mutations. Expression of α-smooth muscle actin and fibroblast-specific protein (Fsp1, also known as S100a4), encoded by Acta2 and S100a4, respectively, is associated with some fibroblast populations (28–30). We used antibodies to these proteins (Figure 1C) to study hearts from hypertrophic α-MHC<sup>719/+</sup>csa and WTcsa mice. The α-smooth muscle actin antibody stained perivascular smooth muscle cells but not the non-myocyte cells residing throughout the LV. In contrast, Fsp1 antibody labeled few non-myocyte cells in WTcsa hearts but substantial numbers of non-myocyte cells in hypertrophic α-MHC<sup>719/+</sup>csa LV sections, particularly in fibrotic regions in which 28% of proliferating cells were Fsp1 positive (Supplemental Figure 2).

Multiple RNAs that encode molecules involved in extracellular matrix biology were enriched in non-myocyte cells isolated from prehypertrophic and hypertrophic α-MHC<sup>403/+</sup>csa hearts (Supplemental Table 1). Among the genes that had the most significantly increased expression (27-fold) in non-myocyte cells from hypertrophic α-MHC<sup>403/+</sup>csa hearts, we identified several Tgf-β-responsive genes (Supplemental Table 2, indicated in bold), including Postn (periostin). We confirmed increased periostin protein levels by Western blots of LV extracts. Periostin levels in extracts from prehypertrophic α-MHC<sup>719/+</sup>csa, WT, and WTcsa mice were low (Figure 2A), but levels were markedly higher among age-matched hypertrophic α-MHC<sup>719/+</sup>csa mice. The amount of increase in periostin protein was variable among hypertrophic α-MHC<sup>719/+</sup>csa LV extracts, a finding that is consistent with our earlier evidence that fibroelastic load differs among identical HCM mice (18).

To determine whether periostin was essential for pathologic remodeling in HCM, we used a genetic strategy. α-MHC<sup>403/+</sup>csa mice were crossed with periostin-null mice (Postn<sup>−/−</sup> mice) (31), hypertrophy was induced with CsA, and compound mutant mice were studied by echocardiography and histopathology. WTcsa and Postn<sup>−/−</sup>csa mouse (n = 5 per genotype) hearts were indistinguishable, with comparable maximal LVWT, normal cardiac histology, and very little fibrosis (Figure 2B). Maximal LVWT was modestly but not significantly reduced in hypertrophic Postn<sup>−/−</sup>α-MHC<sup>403/+</sup>csa mice (0.97 ± 0.37 mm) compared with that of hypertrophic α-MHC<sup>403/+</sup>csa mice (1.21 ± 0.27 mm; Table 1). Masson trichrome-stained LV sections (Figure 2B) showed less myocardial fibrosis in hypertrophic Postn<sup>−/−</sup>α-MHC<sup>403/+</sup>csa hearts (2.06% ± 2.0%, n = 5) than hypertrophic α-MHC<sup>403/+</sup>csa hearts (3.12% ± 3.88%, n = 6; P = 0.03; n = 57 sections per genotype).

To determine whether periostin ablation altered non-myocyte proliferation in HCM hearts, we immunostained heart sections for nuclear Ki67 antigen (Figure 2C). Within fibrotic foci, periostin ablation reduced the number of Ki67-positive non-myocyte cells by 2.4-fold (hypertrophic Postn<sup>−/−</sup>α-MHC<sup>403/+</sup>csa mice, 0.55% ± 0.5%; n > 125,000 cells vs. hypertrophic α-MHC<sup>403/+</sup>csa mice, 1.33% ± 0.6%; n = 150,000 cells; P = 6 × 10<sup>−14</sup>). However, the fraction of Ki67-positive nuclei in the LV of Postn<sup>−/−</sup>α-MHC<sup>403/+</sup>csa mice (0.33% ± 0.27%; n > 235,000 cells) remained significantly elevated over that of control specimens (WTcsa, 0.05% ± 0.09%; n > 96,000 cells).

Table 1

<table>
<thead>
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<th>Genotype</th>
<th>Treatment&lt;sup&gt;a&lt;/sup&gt;</th>
<th>No. of mice</th>
<th>LVWTmax (mm)</th>
<th>P value</th>
<th>LVDD (mm)</th>
<th>P value</th>
<th>FS (%)</th>
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<td>5</td>
<td>0.73 ± 0.13</td>
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<td>38.79 ± 7.46</td>
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<td>4</td>
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<td>3 × 10&lt;sup&gt;−4&lt;/sup&gt;</td>
<td>2.90 ± 0.19</td>
<td>8 × 10&lt;sup&gt;−4&lt;/sup&gt;</td>
<td>37.29 ± 5.77</td>
<td>NS</td>
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<td>(0.018)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>32.85 ± 5.21</td>
<td>(NS)&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
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<td>Rb IgG</td>
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<td>1.19 ± 0.20</td>
<td>0.004</td>
<td>3.22 ± 0.46</td>
<td>NS</td>
<td>36.61 ± 4.82</td>
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<td>NS (0.009)&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>NS (NS)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>33.65 ± 2.79</td>
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<tr>
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<td>6</td>
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<td>0.97 ± 0.37</td>
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<td>43.16 ± 6.61</td>
<td>NS (NS)&lt;sup&gt;d&lt;/sup&gt;</td>
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<sup>a</sup>All mice received CsA plus the indicated antibody or drug. P values reflect comparison with WTcsa mice. Additional comparisons are shown in parentheses: Losartan-treated α-MHC<sup>719/+</sup>csa mice versus untreated α-MHC<sup>719/+</sup>csa mice; Tgf-β NAb-treated α-MHC<sup>719/+</sup>csa mice versus IgG-treated α-MHC<sup>719/+</sup>csa mice; Postn<sup>−/−</sup>α-MHC<sup>403/+</sup>csa mice versus α-MHC<sup>403/+</sup>csa mice. LVWTmax, maximum LVWT; LVDD, LV end-diastolic dimension; FS, fractional shortening at the diastole.
Based upon cardiac imaging, histopathology, and non-myocyte proliferation in periostin-null hypertrophic mice, we concluded that periostin contributed to, but was not essential for, pathologic remodeling in HCM.

To address the roles of Tgf-β signaling in HCM, we administered Tgf-β–neutralizing antibodies (Tgf-β NAb) or rabbit IgG prior to and throughout CsA treatment of α-MHC<sup>719/+</sup> mice. The maximum LVWT of Tgf-β NAb–treated α-MHC<sup>719/+</sup>-csa mice (0.77 ± 0.1 mm) was significantly less than that of IgG-treated hypertrophic α-MHC<sup>719/+</sup>-csa hearts (1.19 ± 0.2 mm; P = 0.009; Table 1).

There was also significantly less cardiac fibrosis in histological sections from Tgf-β NAb–treated mice than in those from IgG-treated hypertrophic α-MHC<sup>719/+</sup>-csa mice (n = 4 per treatment group; Figure 3A). Fibrosis areas encompassed 0.38 ± 0.4% of LV sections in Tgf-β NAb–treated α-MHC<sup>719/+</sup>-csa hearts compared with 1.77 ± 1.8% in IgG-treated hypertrophic α-MHC<sup>719/+</sup>-csa hearts (n = 60 sections per treatment group; P = 4 × 10<sup>−8</sup>). Immunohistochemical staining showed that mice treated with Tgf-β NAb but not rabbit IgG, had reduced periostin expression in hypertrophic LV sections (Figure 3B). Moreover, Tgf-β NAb–treated α-MHC<sup>719/+</sup>-csa mice had significantly less (P = 1.7 × 10<sup>−9</sup>) non-myocyte proliferation (BrdU labeling assessed in >120,000 cells per treatment group; Figure 3C) in regions with fibrosis (0.66 ± 0.06%) and regions with preserved myocardial architecture (0.13 ± 0.01%) as compared with IgG-treated mice (fibrosis, 1.87 ± 0.17%; preserved architecture, 0.62% ± 0.05%). Taken together, Tgf-β NAb attenuated hypertrophy, abrogated periostin expression, and reduced non-myocyte proliferation in HCM mice.

To identify downstream mediators of Tgf-β signaling in HCM, we assessed the location and phosphorylation of Smad2 (pSmad2) in LV sections from mice (n = 3 per genotype) (Supplemental Methods). Nuclear pSmad2 was found both in non-myocyte cells and myocytes in α-MHC<sup>719/+</sup>-csa LV sections. Immunohistochemistry (Supplemental Figure 3) detected approximately 50% (P = 9.7 × 10<sup>−17</sup>) more non-myocyte nuclei stained with anti-phosphorylated Smad2 (pSmad2-Ser465/467) in regions with fibrosis (73.2% ± 14.6%, >7,500 cells) as compared with regions with normal myocardial architecture (48.4% ± 15.9%, >6,400 cells). Based on these data, we deduced that HCM mutations activated the canonical Tgf-β signaling pathway in non-myocyte cells.

We extended these studies by harnessing a pharmacologic approach to inhibiting Tgf-β signals. Recent data demonstrate that angiotensin II promotes cardiac fibrosis in part by activating Tgf-β signals (32). Moreover, the angiotensin II type I receptor antagonist losartan has had salutary effects on animal models of human diseases, including CsA-induced nephropathy (33), Marfan syndrome (34), skeletal myopathies (35), and transgenic overexpression of a cardiac troponin T mutation (36). To assess the effects of losartan in HCM, we treated prehypertrophic α-MHC<sup>719/+</sup> mice for 2 weeks prior to and during CsA induction of HCM. There was no significant difference in the conscious blood pressures of CsA-treated α-MHC<sup>719/+</sup>-MHC<sup>719/+</sup> mice, both in fibrotic regions (treated, 0.14% ± 0.12%, >69,000 cells; untreated, 0.86% ± 0.6%, >120,000 cells; P = 5 × 10<sup>−12</sup>) and in regions with preserved myocardial architecture (treated, 0.04% ± 0.06%; >79,000 cells; untreated, 0.19% ± 0.25%; >79,000 cells; P = 2 × 10<sup>−6</sup>).
To determine whether losartan could attenuate the insidious pathologic remodeling that naturally emerges in α-MHC319/− mice after 30 weeks (without CsA), we chronically treated prehypertrophic α-MHC319/− mice with losartan, beginning at 5 weeks of age. Untreated α-MHC319/− mice developed hypertrophy by 30 weeks of age (maximal LVWT, 1.13 ± 0.02 mm; Table 2). In contrast, long-term losartan-treated α-MHC319/− mice had cardiac dimensions (maximal LVWT, 0.90 ± 0.02 mm; P = 2 × 10−10; Table 2) that were indistinguishable from those of WT mice (maximal LVWT, 0.89 ± 0.02 mm). Histopathological analysis (Figure 4C) showed little fibrosis in chronic losartan-treated α-MHC319/− mice (0.34% ± 0.04%, n = 7 mice, 105 sections) in comparison with untreated 35-week-old α-MHC319/− mice (1.34% ± 0.52%, n = 10 mice, 150 sections).

We then asked whether losartan could reverse established hypertrophy and fibrosis in hypertrophic α-MHC319/−-csa mice. Nine- to fourteen-week-old hypertrophic α-MHC319/−-csa mice (n = 4, CsA treatment for 3–4 weeks) were treated with a high dosage of losartan (2.4 mg/d; see Methods) for 4 weeks and compared with untreated age-matched α-MHC319/−-csa (n = 4) and WTcsa (n = 5) mice. The area of fibrosis in LV sections from losartan-treated mice did not differ from that of hypertrophic α-MHC319/−-csa hearts (treated, 10.99% ± 4.08%; untreated, 10.18% ± 5.59%; n ≥ 60 sections per group; P = NS; Figure 5A and data not shown). However, the percentage of BrdU-positive nuclei (Figure 5B) in fibrotic regions from untreated mice (3.27% ± 3.65%; n > 100,000 cells) was 8-fold greater than that in fibrotic regions of losartan-treated hearts (0.41% ± 0.24%; n > 100,000 cells; P = 1.4 × 10−7). Losartan treatment reduced the percentage of BrdU-positive nuclei in hypertrophic α-MHC319/−-csa hearts (0.28% ± 0.16%; n > 200,000 cells) to levels found in WTcsa hearts (0.27% ± 0.17%; n > 130,000 cells; P = NS).

**Discussion**

We demonstrate that myocyte expression of sarcomere protein mutations alters gene transcription in non-myocyte cells, inducing proliferation and expression of profibrotic molecules that produce pathologic remodeling in HCM. Tgf-β signals are essential to activating non-myocyte cells: inhibition of these signals, directly by Tgf-β NAb or indirectly by the angiotensin II type 1 receptor antagonist losartan, ameliorated hypertrophy and HCM histopathology in mice. These data indicate a potential therapeutic strategy for patients with sarcomere protein gene mutations.

Amounts of myocardial fibrosis in human HCM hearts correlate with the degree of hypertrophy (37), diminished ventricular performance (38), diastolic dysfunction (16), and energy demands (39) — factors that contribute to heart failure and may increase arrhythmic risk (40, 41). As such, the benefit from inhibiting fibrosis in HCM could be substantial.

Fibrosis accrues in HCM hearts due to premature death of mutant myocytes and expansion of the interstitial matrix. Compromised coronary flow due to hypertrophy, microvascular dysfunction (42), increased oxidative stress (43), and increased metabolic demands imposed by abnormal biophysical properties of mutant sarcomeres (7, 8) are factors that contribute to premature myocyte death and the emergence of focal fibrosis in HCM. Far less is known about mechanisms that expand the extracellular matrix in HCM hearts. We demonstrated 4- and 3-fold increased non-myocyte proliferation in fibrotic regions (with focal scarring and/or expanded interstitium) and in areas of preserved myocardial architecture, respectively, in overt HCM compared with prehypertrophic α-MHC319/− hearts. Although we used a pharmacologic strategy to accelerate the emergence of HCM, the very low proliferation rates of non-myocyte cells in CsA-treated WT mice excluded the likelihood that increased cell division was a drug-induced, mutation-independent response. Instead, we suggest that there is insidious proliferation of non-myocyte cells in untreated HCM mice and human patients. Moreover, transcriptional profiling of non-myocyte cells indicated that proliferation was coupled to increased expression of profibrotic molecules (including collagens, peristin, elastin; Supplemental Table 2), a sequence that could readily expand the extracellular matrix in HCM hearts (Figure 6) and contribute to the progressive diminution of diastolic function observed in human HCM (44).
Our studies did not address whether resident or newly recruited cells become activated in HCM. Tgf-β signals can stimulate cardiac microvascular endothelial cells to undergo endothelial-to-mesenchymal transformation, with migration into the myocardium and expression of genes such as Acta2 (14) and Fsp1 (45). Fsp1 but not α-smooth muscle actin was found in non-myocyte cells from hypertrophic hearts (Figure 1C). Activated non-myocyte cells may also be derived from circulating cells (14, 46–48). Alternatively, the enhanced biomechanical forces resulting from sarcomere protein mutations (7, 8) could also provide a local mechanism for activating resident non-myocyte cells. Lineage studies are underway to assess the source of activated non-myocyte cells in HCM hearts.

We identified periostin and Tgf-β as potentially critical molecules to non-myocyte activation. Periostin has been previously proposed to enable myocyte reentry into the cell cycle (49); however, like others (26), we found no evidence for myocyte proliferation, despite periostin expression in HCM hearts. Periostin also promotes differentiation of circulating cells into cardiac fibroblasts (50), stimulates production of collagen and extracellular matrix (50), and promotes myocardial healing and scar formation after injury (31, 52). These functions might be presumed to enable myocyte reentry into the cell cycle (53). Biophysical forces of HCM hearts can activate myocyte cells, as has been observed when non-myocyte cardiac microvascular endothelial cells to undergo endothelial-to-mesenchymal transformation, with migration into the myocardium and expression of genes such as Acta2 (14) and Fsp1 (45). Fsp1 but not α-smooth muscle actin was found in non-myocyte cells from hypertrophic hearts (Figure 1C).

Alternatively, the enhanced biomechanical forces resulting from sarcomere protein mutations (7, 8) could also provide a local mechanism for activating resident non-myocyte cells. Lineage studies are underway to assess the source of activated non-myocyte cells in HCM hearts.

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sion of these during hypertrophic remodeling. As such, we suspect that both increased Tgf-β transcription and activation contribute to pathologic remodeling in HCM.

Tgf-β signaling activates a canonical, Smad-dependent pathway as well as Smad-independent pathways (58). We found heterogeneous nuclear accumulation of pSmad2 in HCM (Supplemental Figure 3) occurring in regions with focal fibrosis and expanded interstitial matrix to a greater extent than in regions with preserved myocardial architecture. Similar activation of the canonical Tgf-β signaling has been reported in Marfan syndrome (34), inherited muscular dystrophy (35), and autosomal recessive cutis laxa type I (59).

The angiotensin II type 1 receptor inhibitor losartan can limit Tgf-β activation (60) and reduce circulating Tgf-β (61). In our studies, losartan like Tgf-β NAb prevented activation of profibrotic pathways in CsA-accelerated HCM (Figure 4A) and also prevented the spontaneous hypertrophic remodeling that gradually emerges with age in untreated mutant mice (Figure 4C). Although losartan failed to reverse hypertrophy or fibrosis in established HCM (Figure 5), it did reduce numbers of proliferating non-myocyte cells. Ongoing studies will determine whether longer treatment is more beneficial.

Collectively, these data define a mechanism by which sarcomere mutations increase myocardial fibrosis and hypertrophy in HCM through increased expression of Tgf-β and activation of the canonical signaling pathway, leading to proliferation of non-myocyte cells and expression of profibrotic molecules (Figure 6). We suggest that this mechanism implicates Tgf-β signaling in progressive diastolic dysfunction, the fundamental hemodynamic abnormality observed in HCM that accounts for patient symptoms and outcomes. Whether suppression of Tgf-β activation in HCM hearts will attenuate relaxation abnormalities remains an important question.

While these mouse models do not perfectly mimic human disease, our results indicate a new strategy for limiting HCM pathophysiology, early inhibition of Tgf-β signaling. With the availability of robust platforms for detecting HCM mutations, identification of individuals with sarcomere protein gene mutations without overt disease and preemptive reduction of Tgf-β activation is feasible and may improve outcomes in HCM. We suggest that study of this strategy is warranted.

Methods

Mouse models. All mice were maintained and studied using protocols approved by the Animal Care and Use Committee of Harvard Medical School. Studies used male heterozygous α-MHC403/+ or α-MHC719/+ mice that are in the 129/SvJ background. α-MHC403/+ mice have been extensively characterized (17, 18). α-MHC719/+ mice were generated by homologous recombination as previously described (17, 62, 63).

Figure 5

Losartan did not reverse fibrotic remodeling in established HCM but diminished non-myocyte proliferation. (A) Cardiac sections from hypertrophic α-MHC719+csa mice, treated or not treated with losartan, stained with Masson trichrome showed comparable amounts of myocardial fibrosis. Scale bar: 1 mm. (B) Non-myocyte proliferation, assessed by BrdU incorporation, was significantly reduced by losartan treatment compared with that of untreated mice in regions of fibrosis (WGA, green; DAPI, blue). Arrowheads indicate BrdU-positive nuclei. Scale bar: 75 μm.

Figure 6

A model for increasing fibrosis and diastolic dysfunction from HCM sarcomere gene mutations. Mutant myocytes have increased biophysical properties (8) and abnormal Ca²⁺ homeostasis (11), factors that trigger mechanical and/or biochemical signals that activate gene transcription, including increased Tgf-β expression. Whether by paracrine and/or autocrine signaling, Tgf-β stimulates non-myocyte proliferation and expression of profibrotic molecules. Activated non-myocyte cells secrete profibrotic molecules that expand the interstitium, increase stresses imposed on mutant myocytes, and promote myocyte death with resultant focal scarring. Tgf-β–mediated increased interstitial and focal fibrosis contributes to diastolic dysfunction in HCM hearts. Preemptive antagonism of Tgf-β signaling by a neutralizing antibody (NAb) or losartan reduces non-myocyte proliferation and profibrotic gene expression, thereby limiting cardiac fibrosis.
Hypertrophic remodeling was accelerated in prehypertrophic α-MHC{\textsuperscript{403/403}} or α-MHC{\textsuperscript{79/79}} mice (age 6–10 weeks) by subcutaneous injection of CsA (15 mg/kg body weight in PBS, twice daily) for 2–5 weeks. For all CsA studies, control mice were age-matched, male CsA-treated WT mice.

Postn{\textsuperscript{+}} mice are in the 129/SvEv background and have been previously described (31, 51, 64). Postn{\textsuperscript{+}} or α-MHC{\textsuperscript{403/403}} mice, generated by mating, were genotyped by PCR amplification and restriction enzyme digestion of genomic DNA (17, 31).

**Mouse blood pressure measurement and echocardiography.** Blood pressure was measured in conscious mice, as described previously (11). Echocardiograms were performed under 2.0% isoflurane anesthesia, using a Vevo 770 High-Resolution In Vivo Imaging System and RMV 707B Scanhead (VisualSonics Inc.), by 2 experienced researchers, without knowledge of mouse genotypes. Measurements of left parasternal long and short axes and M-mode (left parasternal short axis) images were obtained at a heart rate of 500–550 bpm, as described previously (18). LV end-diastolic diameter (LVEDD), LV end-systolic diameter (LVESD), and wall thickness were measured from M-mode tracings, and the average of 3 consecutive cardiac cycles was reported. The LV fractional shortening percentage was calculated as (LVEDD – LVESD)/LVEDD × 100.

**Losartan studies.** Losartan was administered via drinking water (110 mg/l), and estimated intake corresponded to 0.44 mg/d. For studies that assessed whether losartan prevented the emergence of HCM, prehypertrophic mice received losartan for 2 weeks before CsA treatment was initiated. For studies that assessed reversal of HCM, the losartan dose was 0.6 g/l (34).

**Tgf-β NAB studies.** Prehypertrophic mice received either high-dose Tgf-β NAB (10 mg/kg body weight in PBS) or low-dose Tgf-β NAB (5 mg/kg body weight in PBS) (R&D Systems) by intraperitoneal injection every 3 days for 2 weeks prior to CsA treatment. Data obtained from high and low doses were indistinguishable and are reported as combined. Control prehypertrophic mice were treated with rabbit IgG (R&D Systems) at identical doses as those for Tgf-β NAB for 2 weeks prior to CsA.

**Myocyte and non-myocyte isolation.** Cells were isolated from the mice after Langendorff heart preparation and enzymatic digestion, as described previously (63, 65). Following enzyme perfusion, ventricles were gently minced into small pieces and passed through a sterile pipette several times on the culture dish with transfer buffer. Cell suspensions were passed through a mesh filter into 50-ml centrifuge tubes and incubated for 15 minutes. Cells were pelleted and resuspended in gradient calcium loading buffers and sequentially transferred every 10 minutes to subsequent calcium loading buffers, using new sterile plastic pipettes to reduce cross-cell contamination. TRIzol reagent (Invitrogen) was added in the final myocyte pellet and total RNA was extracted according to manufacturer protocols. For isolation of non-myocyte cells, all supernatants from isolated cell solution and calcium loading buffers were collected in the same tube and centrifuged at 329 × g for 12 minutes. The pellet was resuspended in DMEM with 10% FBS and 1% penicillin-streptomycin and plated in sterile dishes (37°C). After a 2-hour incubation, cells attached to the dish were visible and rinsed with sterile PBS to remove nonadherent cells and debris. Attached cells were harvested and an aliquot was used for morphology and immunohistochemical staining, using vimentin and troponin I antibodies to identify non-myocyte cells and myocytes, respectively. The estimated cross-cell contamination was less than or equal to 10%. Total RNA of non-myocyte cells was extracted from harvested cells using TRIzol reagent (Invitrogen), as described above.

**DSAGE and real-time PCR analyses.** Transcriptional profiling was performed using approximately 0.5 μg RNA, pooled from total RNA isolated from cells 4 male mice matched for age and treatment. DSAGE was performed as described previously (12) but adapted to sequence 21 bp NLAIII tags using an Illumina Genome Analyzer II. More than 2 million reads were obtained from RNA preparations derived from both myocytes and non-myocyte cells. Quantitative expression of genes was computed by summing the tag counts for all of the 3’ ends detected in the DSAGE library. The entire transcriptional profile, corresponding to approximately 200,000 distinct tags, was assigned to genes using the Harvard Research Computing Cluster, Orchestra. Using specific primers for Postn, Ctg, Tgbh1, and Tgb2 genes, RNA levels were validated by quantitative real-time PCR analysis using RNA isolated from hypertrophic and non-hypertrophic ventricles as described previously (9, 12).

**GO.** Functional annotation and GO term enrichment analyses were performed using the DAVID tool, as described previously (22, 23). We compared 1,317 RNAs that were differentially expressed (P < 0.001) in non-myocyte cells (identified by DSAGE; Supplemental Table 1) to 16,500 genes expressed in the mouse LV for level 3 GO terms. GO terms that were overrepresented among these 1,317 genes reflect significant gene enrichment (Bonferroni-corrected P < 0.05; modified Fisher’s exact test) and ≥1.5-fold enrichment.

**Quantification of myocardial fibrosis.** Hearts were excised from isoflurane-euthanized mice, washed in PBS, fixed overnight in 4% paraformaldehyde, and embedded in paraffin as described previously (18, 66, 67). After serial sectioning of hearts (apex to base) 9–15, 5-μm sections were stained with Masson trichrome. Fibrosis areas within sections were measured by 2 experienced observers blinded to genotypes, by visualizing blue-stained areas, exclusive of staining that colocalized with perivascular or intramural vascular structures, the endocardium, or LV trabeculae. Using ImageJ software (http://rsweb.nih.gov/ij/), blue-stained areas and non-stained myocyte areas from each section were determined using color-based thresholding (68). The percentage of total fibrosis area was calculated as the summed blue-stained areas divided by total ventricular area, as described previously (18).

**Immunohistochemical analyses.** Cardiac tissues were harvested and processed as described above. Sections were deparaffinized in xylenes, rehydrated through ethanol gradient solutions to PBS, and permeabilized in 0.1% Tween in PBS. Heat-induced antigen retrieval was performed in citric acid buffer, pH 6.0, and/or target retrieval solution (Dako). Normal goat serum (10%) and 1% BSA in PBS were used as blocking agents. Rabbit anti-perisarcin antibody (1:500; ref. 69), rat anti-Ki67 antibody (1:100, Dako), rabbit anti–fibroblast-specific protein 1 antibody (1:100, Dako), and FITC-conjugated mouse anti–α-smooth muscle actin antibody (1:1,000, Sigma–Aldrich) were used as primary antibodies. Sections were washed in PBS and then incubated with Alexa Fluor 488– or 594–conjugated secondary antibodies. Before slide mounting, sections were stained for highly glycosylated collagen that demarcated fibrosis (70, 71) using fluorescent-conjugated WGA (Invitrogen) and for cell nuclei using DAPI (Invitrogen). Negative controls were run in parallel using isotype-specific antibodies.

**Cell proliferation.** BrdU (Sigma–Aldrich) was injected daily into the peritoneum (100 mg/kg body weight in PBS) for 3 days, with the last injection 2 hours prior to harvesting hearts. Hearts were fixed in 4% paraformaldehyde, paraffin embedded, and sectioned. BrdU-positive nuclei were detected with mouse monoclonal anti-BrdU primary antibody (Dako) and immunofluorescent staining as described above.

BrdU– or Ki67-labeled cells and DAPI-stained nuclei were counted in non-myocyte cells from 5–10 fields (×400 magnification) with focal fibrosis and/or expanded interstitium (identified by WGA staining) and areas with preserved myocardial architecture (e.g., absence of both focal fibrosis and expanded interstitium) in 9–15 sections per heart. Myocytes were identified by sarcomeres visualized with fluorescent microscopy. Cells without sarcomeres within interstitial regions were designated non-myocyte nuclei. Nuclei were quantified using ImageJ nucleus counter software (68). Percentages of proliferating cells were calculated as the number of positive BrdU– or Ki67–labeled nuclei divided by the number of DAPI-stained nuclei.
Western blots. LV tissue was dissected, washed in PBS, and homogenized in modified radioimmunoprecipitation assay buffer (50 mM Tris-HCI, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.25% sodium deoxycholate, 0.1% SDS, Protease Inhibitor Cocktail [Roche], and Halt Phosphatase Inhibitor Cocktail [Thermo Scientific]). Tissue lysates were incubated on ice for 30 minutes and clarified by centrifugation at 20817 g at 4 °C for 20 minutes. Protein concentration was determined using the BCA Protein Assay Kit, according to the manufacturer's instructions (Thermo Scientific). Tissue lysates were heated to 95°C in sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 2.5%  β-mercaptoethanol, and 0.005% bromophenol blue) for 5 minutes. For immunoblotting, lysates were resolved by 4%–20% SDS-PAGE and transferred onto PVDF membrane (Thermo Scientific). Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline/Tween 20 (TBS-T) for 1 hour at room temperature. Primary antibodies were diluted in 2.5% nonfat dried milk in TBS-T. Dilutions of peroxisome antibody (provided by S. Hoffman, Medical University of South Carolina) were 1:5,000, and dilutions of GAPDH antibody (Abcam) were 1:10,000. Both primary antibodies were incubated with membranes overnight at 4°C, and then membranes were washed in TBS-T and incubated with anti-rabbit IgG horseradish peroxidase–linked antibody (Santa Cruz Biotechnology Inc.) diluted in 2.5% nonfat dried milk in TBS-T for 1 hour at room temperature. After secondary antibody incubations, membranes were washed in TBS-T and visualized by using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific).

Statistics. All data are expressed as mean ± SD. The statistical significance of differences between experimental groups was determined using two-tailed Student's t test and Pearson's r2 test. P values of less than 0.05 are considered statistically significant.

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