**Inhibition of Endogenous Mst1 Prevents Apoptosis and Cardiac Dysfunction Without Affecting Cardiac Hypertrophy After Myocardial Infarction**

Mari Odashima,* Soichiro Usui,* Hiromitsu Takagi, Chull Hong, Jing Liu, Mitsuhiro Yokota, Junichi Sadoshima

Abstract—Mammalian sterile 20–like kinase-1 (Mst1) plays an important role in mediating cardiac myocyte apoptosis in response to ischemia/reperfusion. Whether or not Mst1 is also involved in the long-term development of heart failure after myocardial infarction (MI) is unknown. We addressed this issue using transgenic mice with cardiac specific overexpression of dominant negative Mst1 (Tg-DN-Mst1). The left coronary artery was permanently ligated, and the size of MI was similar between Tg-DN-Mst1 and nontransgenic controls (NTg). After 4 weeks, Mst1 was significantly activated in the remodeling area in NTg, but not in Tg-DN-Mst1. Although left ventricular (LV) enlargement was significantly attenuated in Tg-DN-Mst1 compared with NTg, neither LV weight/body weight nor myocyte cross sectional area was statistically different between Tg-DN-Mst1 and NTg. LV ejection fraction was significantly greater in Tg-DN-Mst1 than in NTg (53 versus 38%, $P<0.01$), whereas LV end-diastolic pressure (6 versus 12 mm Hg, $P<0.05$) and lung weight/body weight (9.8 versus 12.2 $P<0.05$) were significantly smaller in Tg-DN-Mst1 than in NTg. The number of TUNEL-positive myocytes (0.17 versus 0.28%, $P<0.05$) and amount of interstitial fibrosis (5.0 versus 7.1%, $P<0.05$) in the remodeling area were significantly less in Tg-DN-Mst1 than in NTg. Upregulation of matrix metalloproteinase 2 and proinflammatory cytokines was significantly attenuated in Tg-DN-Mst1. These results indicate that endogenous Mst1 plays an important role in mediating cardiac dilation, apoptosis, fibrosis, and cardiac dysfunction, but not cardiac hypertrophy, after MI. Inhibition of Mst1 improves cardiac function without attenuating cardiac hypertrophy. Thus, Mst1 may be an important target of heart failure treatment. (*Circ Res. 2007;100:1344-1352.*)

**Key Words:** apoptosis ■ hypertrophy ■ myocardial infarction ■ signal transduction

A diverse remodeling after myocardial infarction (MI) has a significant impact on global cardiac function.1 The presence of a nonfunctional area in left ventricular (LV) myocardium resulting from MI increases mechanical loading in the surviving myocardium and local production of autocrine/paracrine factors, such as angiotensin II and tumor necrosis factor (TNF)-α, which induce global histopathological changes in the LV myocardium, including hypertrophy, inflammation, apoptosis, and fibrosis. This, in turn, leads to chamber dilation and LV dysfunction.2 Although the signaling mechanisms involved in this process have been gradually elucidated during the past few years, our knowledge still falls short of the ability to translate observations made in basic research into effective treatment for patients with chronic MI. Mammalian sterile 20–like kinase 1 (Mst1) is a ubiquitously expressed serine/threonine kinase3 that belongs to the mammalian sterile 20–like (STE 20–like) kinase family.4 Mst1 is activated not only by environmental stresses and cytokines5 but also by pathologically relevant stimuli, such as hypoxia/reoxygenation.6 Mst1 and other STE 20–like family kinases play an important role in mediating apoptosis (reviewed elsewhere6). Phosphorylation/activation of downstream targets, including histone H2B,7 DAP4,8 c-Jun N-terminal kinases /p38 mitogen-activated protein kinase (p38-MAPK),9,10 FOXOs,11 and Lats1/2,12 play an important role in mediating the pro-apoptotic function of Mst1. We have previously shown that cardiac-specific overexpression of Mst1 causes dilated cardiomyopathy in mice.6 Inhibition of endogenous Mst1 prevents cardiac myocyte apoptosis and reduces the size of MI in response to ischemia/reperfusion (I/R) in an acute setting.6 It remains to be elucidated whether or not Mst1 chronically mediates cardiac myocyte apoptosis during cardiac remodeling and contributes to the development of congestive heart failure long-term.
the present study, we hypothesized that inhibition of Mst1 attenuates cardiac myocyte apoptosis after MI, thereby inhibiting the development of heart failure. To test this hypothesis, we evaluated apoptosis and cardiac function in transgenic mice with cardiac-specific overexpression of dominant negative Mst1 (Tg-DN-Mst1), which effectively prevents activation of endogenous Mst1 by upstream stimuli.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Transgenic Mice
Tg-DN-Mst1 were generated on a C57BL/6 background, using a cDNA of human myc-Mst1 K59R driven by the α-myosin heavy chain promoter.6 These mice have no obvious baseline cardiac phenotype as described.6 All experiments involving animals were approved by the Institutional Animal Care and Use Committee at New Jersey Medical School.

MI Surgery
Transgenic or nontransgenic male mice, 4 to 5 months old, were used in this study. The mice were anesthetized by intraperitoneal injection of pentobarbital sodium (60 mg/kg). The mice were ventilated via tracheal intubations connected to a rodent ventilator with 65% oxygen during the surgical procedure. The left thoracotomy was performed through the third intercostal space. The pericardial sac was opened, and the left anterior descending branch of the coronary artery (LAD) was visualized. Under direct microscopic control, an 8-0 nylon suture was placed around the vessel and ligated ~1.0 to 2.0 mm from the tip of the normally positioned left auricle. The chest wall was then closed in layers using 5-0 nylon sutures, and the pneumothorax was reduced.

Additional age-matched sham-operated control animals underwent a similar surgery without placement of the ligature. The mice were extubated and allowed to recover in a cage with the temperature maintained at 31°C overnight. All mice were subsequently fed oxygen during the surgical procedure. The left thoracotomy was performed through the third intercostal space. The pericardial sac was opened, and the left anterior descending branch of the coronary artery (LAD) was visualized. Under direct microscopic control, an 8-0 nylon suture was placed around the vessel and ligated ~1.0 to 2.0 mm from the tip of the normally positioned left auricle. The chest wall was then closed in layers using 5-0 nylon sutures, and the pneumothorax was reduced.

Assessment of Myocyte Number and Proliferation
The number of myocyte (N) in the sampled area (A) was measured as described by Gundersen,14 and the myocyte density was calculated as N/A. Using the histologically determined thickness of the LV free wall and the myocyte density per unit area of myocardium, the average number of myocytes across the ventricular wall was computed.15 Proliferation of cardiac myocytes was detected by double staining with anti-serine 10 phosphorylated histone H3 antibody (Cell Signaling Technology), anti-Ki-67 antibody (Dako), and antitroponin I antibody (Sigma).

Statistics
Data are reported as mean±SEM. Statistical analyses between groups were done by 1-way ANOVA, and differences among group means were evaluated using the Student–Newman–Keuls multiple comparison test. A probability value less than 0.05 was considered significant.

Results
Mortality Ratio and Size of MI Were Similar Between Tg-DN-Mst1 and NTg
Three-month-old Tg-DN-Mst1 and NTg mice were subjected to MI. There were no significant differences in systolic blood pressure and heart rate between Tg-DN-Mst1 and NTg mice at 4 to 5 months of age. There was no mortality in sham-operated mice. In mice subjected to MI, 6 of 27 Tg-DN-Mst1 mice died, whereas 5 of 24 NTg mice died (P>0.05) between days 2 and 12. We have previously reported that there was no difference in the area at risk between Tg-DN-Mst1 and NTg.6 The size of MI on days 2 and 28 was similar between Tg-DN-Mst1 and NTg (Figure 1A and 1B). These results suggest that Mst1 is not essential in determining the size of MI after permanent coronary ligation.

Activation of Mst1 in the Remodeling Area Was Abolished in Tg-DN-Mst1
Activation of Mst1 was evaluated by immune complex kinase assays. LV homogenates were prepared from the risk area and the nonrisk area at 15 minutes of ischemia or the remodeling area of LV in mice subjected to MI at 1 and 4 weeks. Corresponding areas of the LV myocardium were harvested from sham-operated mice. The total kinase activity of Mst1 was determined by immune complex kinase assays, using histone H2B as a substrate. In NTg, the homogenate from the risk area and the nonrisk area after 15 minutes of ischemia showed increases in the kinase activity of Mst1 (Figure 1C).

LV Dilation Was Attenuated in Tg-DN-Mst1
After 4 weeks of coronary ligation, both LV weight (LVW)/body weight (BW) and LVW/tibial length (TL) were significantly elevated in NTg hearts subjected to MI compared with sham, suggesting that cardiac remodeling took place (Table 1). Increases in LVW/BW and LVW/TL were also observed in Tg-DN-Mst1 after MI. Neither LVW/BW nor LVW/TL in Tg-DN-Mst1 hearts subjected to MI differed significantly from those in NTg hearts after MI (Table 1). LV cardiac myocyte cross sectional area was similar.
between Tg-DN-Mst1 and NTg subjected to MI (Figure 2A). Expression of atrial natriuretic factor (ANF), a fetal type gene, was significantly elevated in the remodeling area in both NTg and Tg-DN-Mst1. The level of ANF expression was not significantly different between the 2 groups (Figure 2B). These results suggest that cardiac hypertrophy develops almost equally in Tg-DN-Mst1 and NTg mice after MI.

Echocardiographic analyses were conducted on day 28. In NTg hearts, both LVEDD and LVESD after MI were greater than in sham, suggesting that the LV chamber was dilated after MI, another important sign of LV remodeling (Table 2).

Figure 1. A and B, The size of MI was not significantly different between NTg and Tg-DN-Mst1 hearts at early and chronic stages of LV remodeling. The animals were subjected to either MI or sham for 2 days (day 2) or 4 weeks (day 28). A, Representative Masson trichrome stainings of LV horizontal sections are shown. B, The size of MI was determined as described in Materials and Methods. Each column represents the mean of 6 animals. C (left), Activation of Mst1 in the risk and nonrisk areas at 15 minutes of coronary ligation. C (right), Activation of Mst1 in the remodeling area 1 and 4 weeks after coronary ligation or sham. The activity of Mst1 was determined by immune complex kinase assays, using histone H2B as a substrate. The Mst1 activity of sham NTg was set to 1. Note that Mst1 in the remodeling area was significantly activated in NTg but not in Tg-DN-Mst1 mouse hearts. Each column represents the mean of 6 animals. *P<0.05 vs sham D (top), Activation of p38-MAPK in the risk and nonrisk areas at 15 minutes of coronary ligation. D (bottom), Activation of p38-MAPK in the remodeling area 1 and 4 weeks after coronary ligation or sham. Activation of p38-MAPK was evaluated using immunoblotting with anti–phospho-p38-MAPK and anti–total p38-MAPK antibodies. The data are representative of 4 samples each.
TABLE 1. Postmortem Pathologic Measurement of Tg-DN-Mst1 and NTg Mice Four Weeks After Coronary Artery Ligation or Sham Operation

<table>
<thead>
<tr>
<th></th>
<th>NTg (n=7)</th>
<th>MI (n=6)</th>
<th>Tg-DN-Mst1 (n=6)</th>
<th>MI (n=8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBL, mm</td>
<td>17.71±0.18</td>
<td>17.67±0.21</td>
<td>17.33±0.21</td>
<td>17.88±0.23</td>
</tr>
<tr>
<td>LW, mg</td>
<td>98.57±2.46</td>
<td>143.60±4.71</td>
<td>94.67±2.23</td>
<td>130.75±5.49</td>
</tr>
<tr>
<td>RW, mg</td>
<td>28.00±1.51</td>
<td>34.00±3.16</td>
<td>25.00±2.24</td>
<td>32.50±2.87</td>
</tr>
<tr>
<td>LW/TBL</td>
<td>5.57±0.17</td>
<td>8.06±0.22</td>
<td>5.47±0.15</td>
<td>7.33±0.36</td>
</tr>
<tr>
<td>LUW/TBL</td>
<td>8.56±0.26</td>
<td>12.20±1.17 †</td>
<td>8.26±0.17</td>
<td>9.76±0.77</td>
</tr>
<tr>
<td>LW/TBL</td>
<td>81.83±5.39</td>
<td>91.16±5.13</td>
<td>80.50±4.56</td>
<td>88.10±2.61</td>
</tr>
</tbody>
</table>

*P<0.001, †P<0.01 vs sham. TBL indicates tibia length; LW, left ventricular weight; RW, right ventricular weight; LUW, lung weight; LIW, liver weight.

Increases in LVEDD and LVESD after MI were significantly blunted in Tg-DN-Mst1 (Table 2), suggesting that the enlargement of the LV chamber was significantly attenuated in Tg-DN-Mst1.

LV Dysfunction Was Attenuated in Tg-DN-Mst1

Echocardiographically determined LV systolic function 4 weeks after MI was significantly reduced in both Tg-DN-Mst1 and NTg. However, both LVEF and percentage fractional shortening were significantly greater in Tg-DN-Mst1 than in NTg, suggesting that LV systolic dysfunction was significantly attenuated in Tg-DN-Mst1 during cardiac remodeling. Furthermore, hemodynamic measurement indicated that NTg showed significantly elevated LV end-diastolic pressure (LVEDP), whereas LVEDP was not significantly elevated in Tg-DN-Mst1 (Table 3). Consistent with these observations, lung weight/body weight, an index of lung congestion, was significantly greater in NTg 4 weeks after MI than in Tg-DN-Mst1 (Table 1). Taken together, the extent of cardiac dysfunction after MI was significantly less in Tg-DN-Mst1 than in NTg at least under the conditions of anesthesia we used.

Cardiac Fibrosis Was Attenuated in Tg-DN-Mst1 Hearts

The extent of myocardial fibrosis was determined using picric acid/Sirius red staining. The ratio of the collagen volume fraction in the LV remodeling area was significantly elevated in NTg subjected to MI (P<0.01). However, upregulation of collagen accumulation after MI was not statistically significant in Tg-DN-Mst1 (Figure 3A and 3B).

Myocardial upregulation of metalloproteinase plays an important role in mediating cardiac remodeling.16 We examined expression of matrix metalloproteinase (MMP)2, a known mediator of extracellular matrix remodeling and LV dilation.17 RT-PCR analyses indicated that expression of MMP2 in the LV remodeling area was upregulated after 4 weeks of MI in both NTg and Tg-DN-Mst1, whereas expression of MMP2 was significantly greater in NTg than in Tg-DN-Mst1 (Figure 3C). Zymographic measurements indicated that the enzymatic activity of MMP2 in the LV remodeling area was significantly elevated in NTg but not in Tg-DN-Mst1 (Figure 3D). Taken together, upregulation of MMP2 during cardiac remodeling was significantly attenuated in Tg-DN-Mst1 hearts.

Inflammatory cytokines, including TNF-α, interleukin (IL)-1, and IL-6, play an important role in mediating cardiac remodeling.16 Expression of these cytokines was significantly upregulated after 4 weeks of MI in NTg. In contrast, upregulation of these cytokines was significantly attenuated in Tg-DN-Mst1 (Figure 4A through C), suggesting that Mst1 mediates upregulation of inflammatory cytokines in the location.
remodeling heart. Because nuclear factor (NF)-κB plays an important role in mediating expression of inflammatory cytokines, we examined the effect of Mst1 on NF-κB–mediated transcription. We conducted NF-κB reporter gene assays, using neonatal rat cardiac myocytes. Stimulation of myocytes with TNF-α (40 ng/mL) for 48 hours significantly increased the activity of NF-κB/luciferase, which has 5 tandem NF-κB–binding sites. TNF-α–induced activation of NF-κB transcriptional activity was significantly suppressed in the presence of DN-Mst1, suggesting that endogenous Mst1 plays an important role in regulating the transcriptional activity of NF-κB (Figure 4D).

**Apoptosis Was Attenuated in Tg-DN-Mst1**

LV remodeling is associated with increased apoptosis in the myocardium. To determine the extent of apoptosis, TUNEL-positive nuclei were counted among more than 5000 nuclei in the remodeling area of each animal (Figure 5A). In NTg, the number of TUNEL-positive myocytes was significantly greater in animals subjected to MI than in sham-operated animals, suggesting that apoptosis is enhanced during cardiac remodeling. The number of TUNEL-positive myocytes in the remodeling area was also higher in Tg-DN-Mst1 after coronary artery ligation. However, the number of TUNEL-positive myocytes in the remodeling area was significantly less in Tg-DN-Mst1 than in NTg. Thus, inhibition of endogenous Mst1 partially but significantly suppresses apoptosis in the remodeling heart.

**Myocyte Density Was Maintained in Tg-DN-Mst1**

Because increases in apoptosis in the remodeling area were significantly suppressed in Tg-DN-Mst1 compared with NTg, we examined whether this could lead to differences in the myocyte density in the surviving myocardium. The myocyte density in the remodeling area was significantly greater in Tg-DN-Mst1 than in NTg (Figure 5B).

Hpo, a *Drosophila* homolog of mammalian Mst1, not only stimulates apoptosis but also inhibits cell proliferation. Although the fact that Tg-DN-Mst1 hearts after MI have more myocytes than NTg hearts may be explained by partial suppression of apoptosis by DN-Mst1, myocyte proliferation may also be enhanced. To test this hypothesis, we conducted immunostaining with anti-phosphorylated histone H3 (serine 10) antibody. The number of phosphorylated histone H3 positive nuclei was not significantly different between Tg-DN-Mst1 and NTg on days 2 or 28 after MI (Figure 5C and 5D and data not shown). Similar results were obtained using Ki67 staining (Figure II in the online data supplement). These

### TABLE 2. Echocardiographic Analyses of Tg-DN-Mst1 and NTg Mice Four Weeks After Coronary Artery Ligation or Sham Operation

<table>
<thead>
<tr>
<th></th>
<th>NTg (n=7)</th>
<th>MI (n=6)</th>
<th>Tg-DN-Mst1 (n=5)</th>
<th>MI (n=8)</th>
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<tr>
<td>Age, mo</td>
<td>4.14±0.26</td>
<td>4.33±0.21</td>
<td>4.33±0.21</td>
<td>4.28±0.16</td>
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<tr>
<td>LVEDD, mm</td>
<td>4.12±0.10</td>
<td>6.02±0.27*</td>
<td>3.89±0.07</td>
<td>5.25±0.23*†</td>
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<tr>
<td>LVESD, mm</td>
<td>2.83±0.08</td>
<td>5.13±0.33*</td>
<td>2.62±0.09</td>
<td>4.08±0.25*‡</td>
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<tr>
<td>LVEF, %</td>
<td>67.86±0.88</td>
<td>38.33±4.12*</td>
<td>69.40±1.40</td>
<td>53.14±2.58§</td>
</tr>
<tr>
<td>%FS, %</td>
<td>31.56±0.60</td>
<td>15.05±1.88*</td>
<td>32.74±1.04</td>
<td>22.54±1.40*§</td>
</tr>
<tr>
<td>DSEP WT, mm</td>
<td>0.81±0.03</td>
<td>0.60±0.05§</td>
<td>0.74±0.02</td>
<td>0.64±0.03</td>
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<tr>
<td>DPW WT, mm</td>
<td>0.77±0.01</td>
<td>0.80±0.03</td>
<td>0.72±0.03</td>
<td>0.82±0.04</td>
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<tr>
<td>HR, bpm</td>
<td>397±15</td>
<td>415±30</td>
<td>425±23</td>
<td>364±19</td>
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</table>

*P<0.001, †P<0.01 vs sham; ‡P<0.05, §P<0.01 vs NTg. LVEDD indicates LV end-diastolic dimension; LVESD, LV end-systolic dimension; LVEF, LV ejection fraction; %FS, percentage of fractional shortening; DSEP WT, end-diastolic septal wall thickness; DPW WT, end-diastolic posterior wall thickness; HR, heart rate.

### TABLE 3. Hemodynamic Measurement of Tg-DN-Mst1 and NTg Mice Four Weeks After Coronary Artery Ligation or Sham Operation

<table>
<thead>
<tr>
<th></th>
<th>NTg (n=7)</th>
<th>MI (n=6)</th>
<th>Tg-DN-Mst1 (n=6)</th>
<th>MI (n=8)</th>
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<tr>
<td>LVSP, mm Hg</td>
<td>94±3</td>
<td>86±3</td>
<td>94±3</td>
<td>86±2</td>
</tr>
<tr>
<td>LVEDP, mm Hg</td>
<td>3±1</td>
<td>12±3*†</td>
<td>3±1</td>
<td>6±2†</td>
</tr>
<tr>
<td>+LV dP/dt</td>
<td>6417±397</td>
<td>4767±191</td>
<td>6283±448</td>
<td>5554±496</td>
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<tr>
<td>−LV dP/dt</td>
<td>6343±446</td>
<td>4467±251‡</td>
<td>6466±463</td>
<td>4994±380</td>
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<tr>
<td>SBP</td>
<td>93±2</td>
<td>81±3‡</td>
<td>90±4</td>
<td>81±2</td>
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<tr>
<td>DBP</td>
<td>66±3</td>
<td>52±3‡</td>
<td>59±2</td>
<td>54±3</td>
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<tr>
<td>MBP</td>
<td>75±2</td>
<td>62±3*‡</td>
<td>69±3</td>
<td>63±3</td>
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<tr>
<td>HR, bpm</td>
<td>431±15</td>
<td>412±11</td>
<td>428±17</td>
<td>489±40</td>
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</tbody>
</table>

*P<0.01, †P<0.05 vs sham; ‡P<0.05 vs NTg. LVSP indicates LV systolic pressure; LVEDP, LV end-diastolic pressure; SBP, systolic blood pressure; DSP, diastolic blood pressure; MBP, mean blood pressure; HR, heart rate.
results suggest that, unlike in the case of Drosophila Hpo, inhibition of Mst1 is not sufficient to stimulate myocyte proliferation after MI in the mouse heart in vivo and that inhibition of apoptosis could be the primary mechanism for maintaining the myocyte number during cardiac remodeling in the Tg-DN-Mst1 heart.

**Discussion**

Our results suggest that Mst1 is activated during cardiac remodeling and that inhibition of Mst1 inhibits cardiac dilation, cardiac myocyte apoptosis, and upregulation of proinflammatory cytokines and attenuates cardiac dysfunction without inhibiting compensatory hypertrophy. Thus, Mst1 could be an important target of heart failure treatment.

We have previously shown that inhibition of Mst1 significantly reduces the number of cardiac myocytes undergoing apoptosis and the size of MI in response to 20 minutes of ischemia and 24 hours of reperfusion. However, using the same transgenic mouse line, permanent coronary ligation caused similar levels of MI/risk area in Tg-DN-Mst1 and NTg mice at acute (2 days) and chronic (4 weeks) phases. This suggests that Mst1 is not a critical determinant of the size of MI caused by permanent ischemia without reperfusion, most likely mediated by necrotic cell death. Inhibition of Mst1, however, partially but significantly reduced apoptosis in the surviving myocardium, suggesting that Mst1 plays a significant role in mediating apoptosis in the remodeling heart. Because the extent of MI created by permanent ligation...
of the coronary artery was similar between Tg-DN-Mst1 and NTg at 2 days and 4 weeks, a reduced level of apoptosis in the remodeling area of Tg-DN-Mst1 is not attributable to a difference in the initial size of MI. The fact that inhibition of apoptosis in the cardiac remodeling area was accompanied by attenuation of LV dysfunction in Tg-DN-Mst1 is consistent with the notion that cardiac myocyte apoptosis plays an important role in mediating cardiac dysfunction during cardiac remodeling. Our preliminary results suggest that chronic MI induced enhancement of apoptosis in transgenic mice with mild cardiac specific overexpression of Mst1 (Tg-Mst1, line no. 8), which was accompanied by the enhancement of cardiac dysfunction (supplemental Figure III). Thus, regulation of apoptosis by Mst1 during cardiac remodeling was paralleled by changes in cardiac function. Increasing lines of evidence suggest that even a small increase in the number of apoptotic cells in a given time period leads to significant impairment of LV function, and that antiapoptotic treatment improves cardiac function during cardiac remodeling. Thus, Mst1 could be an important target for prevention of heart failure after MI.

Inhibition of apoptosis in Tg-DN-Mst1 was partial, despite the fact that activation of Mst1 is nearly completely inhibited. Thus, other mechanisms, such as p38α-MAPK (Figure 1D), may mediate the remaining apoptosis during cardiac remodeling. Loss-of-function studies, using either knock out or dominant negatives, have shown that several signaling molecules, including p38α-MAPK and Ask1, mediate cardiac myocyte apoptosis during cardiac remodeling. The contributions of these molecules to the cardiac remodeling process are not identical. For example, inhibition of p38α-MAPK reduces the size of MI 3 days after permanent coronary ligation and subsequently suppresses post-MI LV hypertrophy. In fact, because p38α-MAPK is activated in the risk area during ischemia, it is possible that it may be involved in necrotic cell death in the MI area. Genetic deletion of Ask1 also inhibits LV hypertrophy after MI. Thus, one can argue that suppression of apoptosis by inhibition of p38α-MAPK or Ask1 could be secondary to either smaller MI or improvement of LV remodeling. In contrast, suppression of Mst1 did not affect the size of MI or post MI cardiac hypertrophy. Consistent with this notion, activation of p38α-MAPK and Ask1 in the mouse heart resulted predominantly in extracellular matrix remodeling or a hypertrophy phenotype, respectively, but not in a proapoptotic phenotype in cardiac myocytes. This is in contrast to the predominantly apoptotic phenotype in transgenic mice with cardiac specific overexpression of Mst1.

Inhibition of apoptosis is accompanied by suppression of interstitial fibrosis and upregulation of inflammatory cytokines. Because interstitial fibrosis occurs in part attributable to the loss of myocytes by apoptosis, suppression of apoptosis in Tg-DN-Mst1 may secondarily cause inhibition of fibrosis. However, inhibition of Mst1 may also have a direct effect on the progression of interstitial fibrosis, possibly by attenuating upregulation of proinflammatory cytokines, such as TNF-α, IL-1, and IL-6, or matrix metalloproteinases, such as MMP-2, in the surviving myocardium. For example, recent evidence
suggests that Mst1 regulates lymphocyte functions through protein–protein interaction with RAPL.\textsuperscript{24} Alternatively, Mst1 is translocated into the nucleus on activation and affects transcription through phosphorylation of nuclear transcription factors, such as FOXO\textsuperscript{15} and YAP.\textsuperscript{25}

Another interesting finding in this report is that cardiac hypertrophy is little affected in Tg-DN-Mst1 during cardiac remodeling, despite the fact that inhibition of Mst1 significantly reduced LV dilation, cell death, LV fibrosis, and inflammatory cytokines and improved LV function. This property of Mst1 inhibition seems unique in that the improvement of LV function after MI by many interventions is usually accompanied by attenuation of LV remodeling.\textsuperscript{28} The fact that LV function was significantly improved without attenuation of LV hypertrophy in Tg-DN-Mst1 suggests not only that cardiac hypertrophy and LV dysfunction are not necessarily mediated by the same signaling mechanism but also that hypertrophy alone may not necessarily be detrimental during cardiac remodeling, whereas the extent of apoptosis and fibrosis may be more critical. Together with suppression of LV dilation, compensatory hypertrophy would reduce the LV wall stress and hence oxygen consumption.\textsuperscript{2} It has recently been shown that other modalities of intervention in post-MI hearts, including hepatocyte growth factor treatment\textsuperscript{26} and genetic deletion of S100B,\textsuperscript{27} are also beneficial without affecting compensatory hypertrophy. We have shown previously that dilated cardiomyopathy in Tg-Mst1 (line no. 28) is not accompanied by compensatory hypertrophy. Thus, it is expected that Mst1 has anti-hypertrophic actions and, by inference, one would expect that hypertrophy would be enhanced in Tg-DN-Mst1 during cardiac remodeling. However, this was not the case. We speculate that hypertrophy after MI may be mediated by a signaling mechanism which is not inhibited by Mst1, so that inhibition of Mst1 would not enhance cardiac hypertrophy in Tg-DN-Mst1.

The myocyte density in the remodeling myocardium of Tg-DN-Mst1 was significantly greater than that in NTg. Although this observation can be explained by suppression of apoptosis in Tg-DN-Mst1, considering the fact that both Tg-DN-Mst1 and NTg exhibited a similar extent of cardiac hypertrophy, the greater myocyte density could be attributable to increases in myocyte proliferation in Tg-DN-Mst1. Hpo, a Drosophila homolog of mammalian Mst1, inhibits cell proliferation by affecting cyclins.\textsuperscript{12} Thus, it is possible that myocyte proliferation in the remodeling area could be suppressed by activation of endogenous Mst1. However, the rate of myocyte prolifera-
tion was not enhanced in the remodeling area in Tg-DN-Mst1 compared with that in NTg. Thus, Mst1 may not be a critical regulator of myocyte proliferation in the remodeling heart.

Mst1 is activated by pathologically relevant stimuli in cardiac myocytes, such as hypoxia/reoxygenation in vitro and ischemia/reperfusion and pressure overload in vivo (our unpublished observation, 2006). Mst1 is also activated by cytokines, such as Fas ligand, and oxidative stress. Thus, increases in mechanical loading, cytokine production, or oxidative stress might be involved in activation of Mst1 in the cardiac remodeling area. Mst1 is activated by caspase 3- and/or phosphorylation-dependent mechanisms, the latter of which involves Rassf1A, a Ras-associating scaffold protein. The molecular mechanism mediating activation of Mst1 in the remodeling area requires further investigation.

In conclusion, suppression of endogenous Mst1 improves cardiac function after MI in the mouse heart through inhibition of cardiac dilation, apoptosis, extracellular matrix remodeling, and upregulation of proinflammatory cytokines, without affecting compensatory hypertrophy. Thus, Mst1 mediates heart failure through multiple mechanisms, which makes Mst1 an attractive target for drug therapies in post MI patients.

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Disclosures

None.

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Inhibition of Endogenous Mst1 Prevents Apoptosis and Cardiac Dysfunction Without Affecting Cardiac Hypertrophy After Myocardial Infarction
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Expanded methods and Data supplement

Transgenic mice

Tg-DN-Mst1 were generated on a C57BL/6 background, using a cDNA of human myc-Mst1 K59R driven by the α-myosin heavy chain promoter. These mice have no obvious baseline cardiac phenotype as described. For experiments described in the supplemental data, we used transgenic mice with cardiac specific overexpression of Mst1 (line #8). These mice have mild overexpression of Mst1 and have negligible baseline cardiac phenotype described. All experiments involving animals were approved by the Institutional Animal Care and Use Committee at New Jersey Medical School.

Echocardiography

Echocardiography was performed under anesthesia with an intraperitoneal injection of 2.5% Avertine (12μL/g body weight), as described.

Mst1 Assay

Tissue homogenates were prepared in MAP kinase buffer, containing 25 mmol/L NaCl, 25 mmol/L Tris (pH7.5), 1 mmol/L Na3VO4, 10 mmol/L NaF, 10 mmol/L Na-pyrophosphate, 0.5 mmol/L EGTA, 1 mmol/L AEBSF, 0.5 μg/mL leupeptin, and 0.5 μg/mL aprotinin. Samples (300μg) were immunoprecipitated using anti-Mst1 antibody (BD Transduction Laboratories, San Diego, California, USA), and incubated with 10 μL of assay buffer (400 mmol/L HEPES (pH 7.6), 200mmol/L MgCl2, 100 mmol/L ATP, 0.5 μg of Histone H2B (Sigma) for 30 minutes at 30°C. Samples were subjected to SDS-
PAGE and phosphorylated histone H2B was detected by immunoblotting with anti-Serine 14 phosphorylated histone H2B antibody (UBI).

**Hemodynamic measurement**

Hemodynamic measurement was performed using a 2F micromanometer-tipped catheter (Miller Instruments, Houston, TX, USA) as described 1.

**Cross sectional area**

Myocyte cross sectional area was measured from images captured from wheat germ agglutinin staining sections as described 1.

**Collagen morphometry**

Morphometric analysis of collagen was performed with 6 μm thick paraffin sections stained with Picric acid Sirius red (PASR) as described 3.

**Evaluation of apoptosis**

DNA fragmentation was detected in situ using TUNEL, as described 1. Nuclear density was determined by counting of DAPI-stained nuclei in six fields for each animal 1. Double staining with anti-α-sarcomeric actin was conducted in order to identify the apoptotic cell as a cardiac myocyte 1.

**Zymography**
Matrix metalloproteinase (MMP) abundance was measured by gelatin zymography. Whole-heart extract was prepared as described. Twenty to 25 mg frozen heart tissue was smashed and agitated for 48 hours at 4°C in 0.5 mL ice-cold buffer containing 10 mmol/L cacodylic acid, 150 mmol/L NaCl, 1 µmol/L ZnCl₂, 20 mmol/L CaCl₂, 3 mmol/L NaN₃, and 0.01% Triton X-100. The supernatant was collected after mixing with 100 µL of 0.1 mol/L Tris-HCl buffer (ph 7.4) and centrifugation at 13 000g at 4°C for 15 minutes. Equal amounts of protein from each sample were mixed with Tris-glycine SDS sample buffer without reducing agents and separated in gelatin zymography gel by electrophoresis. The gel was incubated in 2.5% Triton X-100 for 30 minutes, followed by incubation in 10 mmol/L Tris-HCl (ph 7.4), 0.2 mol/L NaCl, 5 mmol/L CaCl₂, and 0.02% Brij 35. The gel was stained with 0.5% Coomassie blue R250 and destained in 30% MeOH and 10% acetic acid to reveal the lytic bands.

RT-PCR

PCR primers for TNF-α, IL-1, IL-6 and MMP2, and glyceraldehyde-3-phosphate dehydrogenase were selected using Vector NTI (Invitrogen). Primer sequences are described in the online data supplement. Methods of quantitative RT-PCR have been described.

The following PCR primers were used:

IL-1:
sense 5’-GACCTTCCAGGATGATTACA-3’
anti-sense 5’-AGGCCACAGGTATTTTGTCG-3’

IL-6
sense 5’-TGGAGTCACAGAAGGGAGTGGCTAA-3’
antisense 5’-TCTGACCACAGTGAGGATGTCCAC-3’
TNFα
sense 5’-GGCAGGTCTACTTTGGAGTCATTGC-3’
antisense 5’-ACATTCGAGGCTCCAGTGAATTCGG-3’

GAPDH
sense 5’-TTCTTGTGCAGTGCCAGCCTCGTC -3’
antisense 5’-TAGGAACACGGAAGGCCATGCCAG -3’

**NF-κB reporter gene assays**

To determine whether NF-κB is activated, the PathDetect *cis*-reporting system (Stratagene) was used. An SV40-β galactosidase construct was cotransfected to determine the transfection efficiency.
Figure S1  To estimate expression levels of endogenous Mst1, PCR primers were selected in the 3′-UTR of mouse Mst1 cDNA, using Vector NTI. RT-PCR was performed with the following pair of primers: Mouse Mst1 (forward): 5’-tacacagggcctgcataaca-3’, Mouse Mst1 (reverse): 5’-gacagtttgctcccttctgc -3’. A, Semi-quantitative PCR, B, quantitative PCR. n=3 in each column.
Cardiac myocyte proliferation in the remodeling area was quantitated using immunostaining with anti-Ki-67 and anti-troponin I antibodies as described in Methods. Upper panel shows an example of Ki-67 positive myocytes in Tg-DN-Mst1 hearts. Scale bar = 10 μm. Lower panel shows the result of quantitative analysis. Note that Ki-67 staining generally showed greater sensitivity than phospho histone H3 staining in our hands. n=3 each.
The effect of permanent coronary ligation on cardiac dilation, lung weight/tibial length and TUNEL positive myocytes in transgenic mice with cardiac specific overexpression of Mst1 (Tg-Mst1 line #8, mild overexpression line). Since it was expected that increased expression of Mst1 would cause more severe cardiac dysfunction and increased mortality in Tg-Mst1 (line #8), we applied coronary ligation more distally in this experiment than in the experiments using Tg-DN-Mst1 described in the main text. All data were obtained at 4 weeks of myocardial infarction. A, the size of MI at 4 weeks; B, echocardiographically determined LV end diastolic dimension; C, Lung weight/tibial length, D, TUNEL positive myocytes. NTg, n=6; Tg-Mst1, n=5.

