Expression of Mutant p193 and p53 Permits Cardiomyocyte Cell Cycle Reentry After Myocardial Infarction in Transgenic Mice

Hidehiro Nakajima, Hisako O. Nakajima, Shih-Chong Tsai, Loren J. Field

Abstract—Previous studies have demonstrated that expression of p193 and p53 mutants with dominant-interfering activities renders embryonic stem cell–derived cardiomyocytes responsive to the growth promoting activities of the E1A viral oncoproteins. In this study, the effects of p53 and p193 antagonization on cardiomyocyte cell cycle activity in normal and infarcted hearts were examined. Transgenic mice expressing the p193 and/or the p53 dominant-interfering mutants in the heart were generated. Transgene expression had no effect on cardiomyocyte cell cycle activity in uninjured adult hearts. In contrast expression of either transgene resulted in a marked induction of cardiomyocyte cell cycle activity at the infarct border zone at 4 weeks after permanent coronary artery occlusion. Expression of the p193 dominant-interfering mutant was also associated with an induction of cardiomyocyte DNA synthesis in the interventricular septa of infarcted hearts. A concomitant and marked reduction in hypertrophic cardiomyocyte growth was observed in the septa of hearts expressing the p193 dominant-interfering transgene, suggesting that cell cycle activation might partially counteract the adverse ventricular remodeling that occurs after infarction. Collectively these data suggest that antagonization of p193 and p53 activity relaxes the otherwise stringent regulation of cardiomyocyte cell cycle reentry in the injured adult heart. (Circ Res. 2004;94:000-000.)

Key Words: cardiomyocyte proliferation ■ apoptosis ■ DNA synthesis ■ heart regeneration

Many forms of cardiac disease are accompanied by cardiomyocyte death. Although there is evidence indicating the presence of limited adult cardiomyocyte cell cycle reentry,1 as well as the presence of stem cells with angiogenic and/or cardiomyogenic capabilities,2 these activities by themselves are insufficient to promote functional restoration of severely diseased hearts. Considerable effort has thus been invested to augment these activities with the hope that therapeutic efficacy can be achieved.

A variety of gene transfer approaches have been used to identify proteins that can induce cardiomyocyte cell cycle activity.3 For example, it is well established that expression of the SV40 large T-antigen oncprotein is sufficient to induce cardiomyocyte cell cycle activity in transgenic animals.4–6 It was hypothesized that cardiomyocyte proliferation in these models resulted in part from T-antigen binding to, and altering the activity of, endogenous cell cycle regulatory proteins. Subsequent studies identified three predominant cardiomyocyte T antigen binding proteins, namely p53, p107, and p193.7 p53 is a tumor suppressor protein that regulates cell cycle activity and apoptosis through its activity as a transcription factor.8 p107 is a member of the retinoblastoma gene family that regulates G1/S transit.9 p193 is a large protein that induces apoptosis when transiently expressed in NIH-3T3 cells; apoptosis is dependent on the presence of a short 16 amino acid motif that encodes a BH3 motif.10 Other studies have shown that p193 (also called cul7 and p185)11,12 has E3 ligase activity, indicating that it is involved in targeting protein(s) for ubiquination and subsequent proteosome-mediated degradation. The physiological substrate(s) of p193-mediated ubiquination are currently under investigation.

Recent observations have underscored the importance of p193 and p53 in regulating cardiomyocyte cell cycle activity in response to expression of DNA tumor virus oncoproteins. For example, E1A expression induced cell cycle reentry followed by apoptosis in neonatal13 and embryonic stem cell (ESC)–derived14 cardiomyocytes. Coexpression of dominant-interfering p193 and p53 mutants blocked E1A-induced apoptosis, resulting in robust cardiomyocyte proliferation.14 It was hypothesized that expression of the dominant-interfering molecules mimicked the effects resulting from T antigen binding to (and thereby altering the activity of) p193 and p53 in transgenic cardiac tumors. In support of this, expression of a T antigen mutant lacking the p53 binding sites (but retaining the p193 binding site)
resulted in cardiomyocyte apoptosis in transgenic mice and in ESC-derived cultures. Coexpression of a dominant-interfering p53 mutant blocked mutant T antigen–induced cardiomyocyte apoptosis.

The profound roles of p193 and p53 in DNA tumor virus-induced cardiomyocyte proliferation raised the possibility that these proteins might also exert a cell cycle regulatory role in the adult heart. To directly test this hypothesis, transgenic mice were produced wherein the α-cardiac myosin heavy chain (MHC) promoter was used to target expression of the p193 and/or the p53 dominant-interfering mutants to cardiomyocytes. No effect on cardiomyocyte DNA synthesis was observed in uninjured adult hearts expressing the p193 and p53 transgenes alone or in combination. In contrast, robust cardiomyocyte DNA synthesis was observed at 4 weeks after permanent coronary artery occlusion in mice expressing either the p193 or the p53 dominant-interfering transgenes. Antagonization of p193 and p53 activity had a differential impact on cardiomyocyte DNA synthesis: although expression of either transgene resulted in cardiomyocyte DNA synthesis at the infarct border zone, cardiomyocyte cell cycle induction in the interventricular septum was only seen in mice expressing the p193 transgene. A concomitant reduction in the degree of cardiomyocyte hypertrophy was also observed in the septa of mice expressing the p193 dominant-interfering transgene. These data suggest that antagonization of p193 and p53 activity relaxes the otherwise stringent regulation of cardiomyocyte cell cycle reentry in the injured adult heart.

Materials and Methods

Generation of MHC-1152stop and MHC-CB7 Transgenic Mice

The MHC-1152stop transgene used the mouse MHC promoter to target expression of a dominant-interfering p193 mutant (premature stop codon engineered at amino acid residue 1153; Figure 1A). The SV40 early region transcription terminator/polyadenylation site (nucleotide residues nos. 2586 to 2452) was inserted downstream from the p193 sequences. The MHC-CB7 transgene used the MHC promoter and genomic sequences comprising an 8-kb fragment of a p53 CB7 genomic clone (Figure 1B). The CB7 p53 allel harbors an arg to pro substitution at amino acid residue no. 193 that results in a dominant-interfering phenotype. Transgenic mice were generated and screened as described using standard techniques. Male mice were used for all studies.

Western Blot Analyses

Hearts were homogenized and processed as described previously. Anti-p193 monoclonal antibody was generated using a recombinant p193; the antibody recognizes p193 amino acid residues nos. 830 to 847. Anti-p53 monoclonal antibody PC35 was obtained from Oncogene. Signal was visualized by the ECL method according to the manufacturer’s protocol (Amersham).

Myocardial Injury Models

Animals were intubated and ventilated (2% isoflurane with supplemental oxygen), and the left coronary artery was occluded at the inferior border of the left auricle with an 8-0 Prolene ligature. The interventricular septum is spared after ligation of the left coronary artery in mice. Isoproterenol infusion was performed as described previously. There was no difference in the acute infarction size in the different transgenic models at 24 hours after injury as measured by tetrazolium staining.

Cardiomyocyte DNA Synthesis, Apoptosis, and Minimal Diameter Assays

Cardiomyocyte DNA synthesis was monitored using a tritiated thymidine incorporation assay (200 µCi IP at 28 Ci per mmol/L,
Amersham) in combination with the MHC-nLAC reporter transgenic strain.\textsuperscript{24,25} Standard methods were used for cryosectioning.\textsuperscript{26} β-Galactosidase activity was detected by staining with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-GAL) and autoradiography was performed as described.\textsuperscript{27} Antivertically caspase 3 (Promega) and phosphorylated histone H3 (Upstate, Lake Placid, NY) immune assays were performed according to the manufacturer’s specifications. The infarct border zone was defined as myocardial tissue residing within 0.5 mm of fibrous scar tissue. To measure cardiomyocyte minimal diameter, images were captured, digitized, and analyzed with NIH Image 1.61 software as described.\textsuperscript{28}

**Statistics**

Results are presented as mean±SEM. Statistical significance \((P<0.05)\) among multiple groups was determined by ANOVA and the Tukey method.

**Results**

**Generation of Transgenic Mice Expressing Dominant-Interfering p193 and/or p53 in the Heart**

To antagonize p193 activity in the heart, transgenic mice were generated expressing a mutant p193 cDNA encoding apparent dominant-interfering activity (1152stop cDNA) under the regulation of the cardiac MHC promoter (MHC-1152stop mice; Figure 1A). A total of 14 transgenic lineages were generated. Transgene expression was initially stratified by Northern blot analysis in F1 generation mice (not shown), and MHC-1152stop line no. 9 was selected for subsequent analyses based on its level of expression. To antagonize p53 activity in the heart, transgenic mice that expressed a genomically encoded dominant-interfering p53 activity (CB7 clone)\textsuperscript{18} under the regulation of the MHC promoter were generated (MHC-CB7 mice, Figure 1B). A total of 5 transgenic lineages were generated. Transgene expression was stratified initially via Northern blotting, and MHC-CB7 line no. 3 was selected for further analyses. MHC-1152stop, MHC-CB7, and MHC-nLAC mice were intercrossed and transgene expression in the progeny was analyzed. The MHC-nLAC transgene encoded a nuclear localized β-galactosidase reporter transgene, which facilitates quantitation of cardiomyocyte DNA synthesis\textsuperscript{24,25} (see later). Western blot analyses revealed expression of the endogenous p193 gene in all hearts, whereas expression of the 1152stop protein was only detected in mice inheriting the MHC-1152stop transgene (Figure 1C, top). Expression of CB7 protein was seen in mice inheriting the MHC-CB7 transgene, whereas expression of the endogenous p53 gene was not detected under the conditions used (Figure 1C, bottom). Inheritance of multiple MHC-promoted transgenes (including the MHC-nLAC reporter gene) had no effect on the level of 1152stop and CB7 protein expression.

**Baseline Cardiac Attributes in Normal and Injured Mice Expressing the MHC-1152stop and/or the MHC-CB7 Transgenes**

Gross cardiac parameters were analyzed in uninjured transgenic animals and their nontransgenic controls. MHC-1152stop, MHC-CB7, and MHC-nLAC mice were intercrossed. At 12 weeks of age, the mice were euthanized and heart weight (HW), heart weight/body weight (HW/BW), and heart weight/tibia length (HW/TL) were determined. There were no statistically significant differences in these basic attributes between the control mice and mice inheriting the MHC-1152stop transgene and/or the MHC-CB7 transgene (Table 1), suggesting that transgene expression had little impact on normal postnatal cardiac growth.

Basic cardiac attributes were also determined after myocardial injury. MHC-nLAC, MHC-1152stop, and MHC-CB7 mice were intercrossed, and at 12 weeks of age the resulting mice were anesthetized and intubated, and the heart was exposed via thoracotomy. The left coronary artery was permanently occluded at the inferior border of the left atrial appendage, the chest was closed, and the mice were allowed to recover for 4 weeks. The mice were euthanized and basic cardiac attributes were determined. Basic cardiac parameters

### TABLE 1. Cardiac Attributes in Adult Transgenic Mice (Some Mice Also Carried the MHC-nLAC Transgene)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Body Weight, g</th>
<th>Tibia Length, mm</th>
<th>Heart Weight, mg</th>
<th>HW/BW</th>
<th>HW/TL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninjured*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)/(-) (9 mice)</td>
<td>25.4±0.70</td>
<td>17.6±0.14</td>
<td>137.7±3.62</td>
<td>5.4±0.09</td>
<td>7.8±0.17</td>
</tr>
<tr>
<td>(-)/MHC-CB7 (6 mice)</td>
<td>27.9±0.71</td>
<td>17.8±0.16</td>
<td>154.1±10.02</td>
<td>5.5±0.26</td>
<td>8.6±0.52</td>
</tr>
<tr>
<td>MHC-1152stop/(-) (7 mice)</td>
<td>24.8±1.21</td>
<td>17.6±0.20</td>
<td>137.1±3.56</td>
<td>5.5±0.15</td>
<td>7.8±0.23</td>
</tr>
<tr>
<td>MHC-1152stop/MHC-CB7 (5 mice)</td>
<td>25.3±0.87</td>
<td>17.6±0.12</td>
<td>151.0±6.90</td>
<td>6.0±0.22</td>
<td>8.6±0.36</td>
</tr>
<tr>
<td>Mice with myocardial infarcts, 4 weeks after injury*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)/(-) (9 mice)</td>
<td>25.0±0.66</td>
<td>17.9±0.07</td>
<td>201.3±8.22</td>
<td>8.2±0.56</td>
<td>11.3±0.46</td>
</tr>
<tr>
<td>(-)/MHC-CB7 (5 mice)</td>
<td>25.0±1.28</td>
<td>17.4±0.20</td>
<td>197.1±8.53</td>
<td>7.9±0.39</td>
<td>11.3±0.45</td>
</tr>
<tr>
<td>MHC-1152stop/(-) (10 mice)</td>
<td>25.5±1.25</td>
<td>17.9±0.12</td>
<td>216.0±0.15</td>
<td>8.8±0.80</td>
<td>12.1±0.53</td>
</tr>
<tr>
<td>MHC-1152stop/MHC-CB7 (12 mice)</td>
<td>26.4±0.86</td>
<td>18.0±0.14</td>
<td>232.3±15.27</td>
<td>8.8±0.52</td>
<td>13.0±0.80</td>
</tr>
<tr>
<td>Mice with 7-day isoproterenol infusion*</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(-)/(-) (23 mice)</td>
<td>24.7±0.57</td>
<td>17.7±0.09</td>
<td>191.4±5.75</td>
<td>7.8±0.15</td>
<td>10.8±0.29</td>
</tr>
<tr>
<td>(-)/MHC-CB7 24.8±0.62 (20 mice)</td>
<td>17.7±0.13</td>
<td>194.4±4.43</td>
<td>7.9±0.16</td>
<td>11.0±0.25</td>
<td></td>
</tr>
<tr>
<td>MHC-1152stop/(-) (14 mice)</td>
<td>25.4±0.87</td>
<td>17.6±0.15</td>
<td>188.9±7.30</td>
<td>7.5±0.21</td>
<td>10.7±0.38</td>
</tr>
<tr>
<td>MHC-1152stop/MHC-CB7 (13 mice)</td>
<td>24.6±0.69</td>
<td>17.5±0.18</td>
<td>194.8±6.06</td>
<td>7.9±0.09</td>
<td>11.1±0.37</td>
</tr>
</tbody>
</table>

\*\(P<0.05\) between all experimental groups.
were similar in transgenic and nontransgenic mice at 4 weeks after MI, indicating that expression of the MHC-1152stop and/or the MHC-CB7 transgenes had minimal effect on gross hypertrophic cardiac growth (Table 1). Basic cardiac attributes were also similar between nontransgenic and transgenic mice after 7 days of isoproterenol infusion (Table 1), indicating that transgene expression did not interfere with the ability to respond to β-agonist-induced hypertrophy.

Reactivation of Cardiomyocyte DNA Synthesis in Mice Expressing the MHC-1152stop and/or MHC-CB7 Transgenes After Myocardial Infarction

The crosses performed incorporated MHC-nLAC mice to facilitate quantitation of cardiomyocyte DNA synthesis. Cardiomyocyte nuclei in mice carrying the MHC-nLAC reporter transgene are readily identified in X-GAL stained histological sections. To monitor cardiomyocyte DNA synthesis, the mice were given a single injection of tritiated thymidine and euthanized 4 hours later. The hearts were harvested and sectioned, and the sections were stained with X-GAL and processed for autoradiography. The presence of silver grains over blue nuclei was indicative of cardiomyocyte DNA synthesis. Because tritiated thymidine incorporation is quantitative in nature, this approach can discriminate between DNA synthesis from genomic duplication versus that resulting from repair processes.1,24 No DNA synthesis was detected in mice inheriting the MHC-nLAC transgene alone when 30 048 ventricular cardiomyocyte nuclei were counted, indicating that the level of cardiomyocyte DNA synthesis in the uninjured adult heart is less than 0.003% (ie, less than 1 per 30 048 nuclei). This result is in agreement with most assessments of cardiomyocyte DNA synthesis rates in uninjured adult hearts.1 Expression of the MHC-1152stop and/or the MHC-CB7 transgenes had no overt impact on cardiomyocyte DNA synthesis in the uninjured hearts (Table 2). Interestingly, when the levels of DNA synthesis between the 4 genotypes were combined, only 1 positive nucleus was observed in a total of 156 135 screened; this rate is remarkably similar to that observed previously in uninjured hearts using the MHC-nLAC reporter system (0.0005%.24).

Cardiomyocyte DNA synthesis was also monitored in infarcted hearts. Very low levels of cardiomyocyte DNA synthesis were seen at the border zone of mice inheriting the MHC-nLAC reporter gene alone at 4 weeks after myocardial infarction, consistent with our previous results (Table 2).24 In marked contrast, comparably high levels of cardiomyocyte DNA synthesis were seen at the infarct border zone in mice inheriting either the MHC-1152stop or the MHC-CB7 transgene (Figure 2A; Table 2). Cardiomyocyte DNA synthesis was also monitored in the interventricular septa of the infarcted hearts. No cardiomyocyte DNA synthesis was detected in the septa of mice inheriting the MHC-nLAC transgene alone or in mice inheriting the MHC-CB7 transgene (Table 2). In contrast, comparatively high levels of cardiomyocyte DNA synthesis were detected in the septa of mice inheriting the MHC-nLAC transgene alone or in mice inheriting the MHC-CB7 transgene (Table 2). The infarcted MHC-1152stop hearts were also screened for the presence of cardiomyocyte-restricted phosphorylated histone H3 immune reactivity (a marker of mitotic cells).27 Colocalization of X-GAL activity and histone H3 immune reactivity were detected, suggesting that these cells are able to undergo cytokinesis and/or karyokinesis.

Reduction of Cardiomyocyte Hypertrophy in the Interventricular Septa of Mice Expressing the MHC-1152stop Transgene After Myocardial Infarction

Examination of X-GAL stained sections from hearts processed at 4 weeks after myocardial infarction revealed that there were fewer cardiomyocyte nuclei per microscopic field in the interventricular septa of hearts from mice inheriting the MHC-nLAC transgene alone as compared with mice inheriting the MHC-1152stop transgene (Figure 3A and 3B). Car-

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Uninjured Vent</th>
<th>MI Border Zone</th>
<th>MI Septum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute cardiomyocyte tritiated thymidine labeling index</td>
<td>(&lt;-/-) 0.003%</td>
<td>0.008%</td>
<td>&lt; 0.002 %</td>
</tr>
<tr>
<td></td>
<td>0/30 048 nuclei</td>
<td>3/38 292</td>
<td>0/44 233</td>
</tr>
<tr>
<td></td>
<td>4 mice</td>
<td>9 mice</td>
<td>9 mice</td>
</tr>
<tr>
<td>(-)/MHC-CB7</td>
<td>0.002%</td>
<td>0.6%*</td>
<td>&lt; 0.003%</td>
</tr>
<tr>
<td></td>
<td>1/37 231 nuclei</td>
<td>26/4158</td>
<td>0/31 753</td>
</tr>
<tr>
<td></td>
<td>4 mice</td>
<td>5 mice</td>
<td>5 mice</td>
</tr>
<tr>
<td>MHC-1152stop/(-)</td>
<td>&lt;0.003%</td>
<td>0.4%*</td>
<td>0.05%*</td>
</tr>
<tr>
<td></td>
<td>0/43 236 nuclei</td>
<td>12/3102</td>
<td>10/20 782</td>
</tr>
<tr>
<td></td>
<td>4 mice</td>
<td>6 mice</td>
<td>6 mice</td>
</tr>
<tr>
<td>MHC-1152stop/MHC-CB7</td>
<td>&lt;0.002%</td>
<td>0.5%*</td>
<td>0.06%*</td>
</tr>
<tr>
<td></td>
<td>0/45 620</td>
<td>23/4213</td>
<td>18/29 626</td>
</tr>
<tr>
<td></td>
<td>4 mice</td>
<td>5 mice</td>
<td>5 mice</td>
</tr>
</tbody>
</table>

*P<0.05 vs (<-/-) mice.
diomyocyte hypertrophy could produce an apparent reduction in the number of cardiomyocyte nuclei per unit area of histological section (ie, as cardiomyocyte volume increases, the likelihood that a cardiomyocyte nucleus would be present in the plane of section decreases). Because gross analyses failed to demonstrate an overt reduction in hypertrophic growth in the MHC-1152stop hearts after myocardial infarction (Table 1), additional analyses were performed to determine if differential hypertrophy was present at the cellular level. Previous studies have shown that minimal cardiomyo-

Figure 2. Cardiomyocyte cell cycle progression in infarcted transgenic mice. A, Cardiomyocyte DNA synthesis at the infarct border zone and (B) in the interventricular septum of an MHC-nLAC/MHC-1152stop/MHC-CB7 transgenic heart at 4 weeks after permanent coronary artery occlusion. Presence of silver grains over blue nuclei is indicative of cardiomyocyte DNA synthesis. C, Phosphorylated histone H3 immune reactivity (black signal, horseradish peroxidase-conjugated secondary antibody) in a cardiomyocyte nucleus (blue signal, X-GAL staining) in the same heart depicted in A. Bar=10 μm.
Cyte diameter measurements in histological sections provide a reliable index of cardiomyocyte hypertrophy. Accordingly, minimal cardiomyocyte diameter was measured in sections prepared from sham-operated and infarcted mice inheriting the MHC-1152stop and/or MHC-CB7 transgenes at 4 weeks after injury (Figure 3C). As expected, infarcted mice carrying the MHC-nLAC or the MHC-nLAC and MHC-CB7 transgenes exhibited a marked and similar increase in minimal fiber diameter, consistent with hypertrophic cardiomyocyte growth. In contrast, the increase in cardiomyocyte minimal fiber diameter was markedly and significantly less in mice inheriting the MHC-1152stop transgene. Given that the volume of a cylinder is related to the square of the radius, and given that hypertrophic growth occurs largely along the short axis of the cell, the impact of MHC-1152stop transgene expression on cardiomyocyte volume after coronary artery ligation was pronounced.

Similar Levels of Cardiomyocyte Apoptosis and Cardiomyocyte DNA Synthesis in the Interventricular Septa of Mice Expressing the MHC-1152stop Transgene After Myocardial Infarction

Antiactivated caspase 3 immune histology was used to measure the baseline rate of cardiomyocyte apoptosis in control and transgenic mice. Because activated caspase 3 is distributed in the cytoplasm, cardiomyocytes at early stages of apoptosis were easily identified by morphological criteria (ie, rod-shaped cells; see Figure 4). Using this criterion, only one activated caspase 3 immune reactive cardiomyocyte was detected when 21 heart sections from uninjured mice inheriting the MHC-nLAC reporter gene only were screened. Low rates of activated caspase 3 activity were also detected in uninjured hearts from mice inheriting the MHC-nLAC transgene alone. Screens of multiple coronal sections at a position midway between the apex and base of the heart revealed approximately 3 cardiomyocytes with activated caspase 3 immune reactivity per section (Table 3), indicating that antagonization of p193 and/or p53 activity had little effect on cardiomyocyte apoptosis in the absence of cardiac injury.

Cardiomyocyte activated caspase 3 immune reactivity was also monitored in the infarcted hearts. Activated caspase 3 immune reactivity was detected in cardiomyocytes at the infarct border zone of mice inheriting the MHC-nLAC reporter gene only. Screens of multiple coronal sections at a position midway between the apex and base of the heart revealed approximately 3 cardiomyocytes with activated caspase 3 immune reactivity per section (Table 3), indicating that antagonization of p193 and/or p53 activity had little effect on cardiomyocyte apoptosis in the absence of cardiac injury.

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inheriting the MHC-CB7 transgene (Table 3). Activated caspase 3 immune reactivity was also monitored in the interventricular septum. On average, approximately 3 immune reactive cardiomyocytes were observed per section in the septa of hearts from mice inheriting the MHC-nLAC reporter gene alone (Table 3). Similar levels of cardiomyocyte activated caspase 3 immune reactivity were seen in the interventricular septa of hearts from mice inheriting the MHC-1152stop and/or the MHC-CB7 transgenes (Table 3).

The cardiomyocyte apoptosis data were presented as the average number of cardiomyocytes with activated caspase 3 immune reactivity per coronal heart section (Table 3). In contrast the cardiomyocyte DNA synthesis levels were determined by counting individual X-GAL stained nuclei (Table 2). In order to more directly compare the levels of cardiomyocyte apoptosis and DNA synthesis in the interventricular septum, the number of tritiated thymidine-positive cardiomyocyte nuclei per section was determined for mice with the various genotypes at 4 weeks after MI. No cardiomyocyte DNA synthesis was seen in the interventricular septa of hearts from mice inheriting the MHC-nLAC reporter gene or the MHC-CB7 transgene (Table 4), in agreement with the data obtained when individual cardiomyocyte nuclei were counted (Table 2). In contrast, cardiomyocyte DNA synthesis was

**TABLE 3. Activated Caspase Immune Reactivity in Normal and Injured Transgenic Hearts (Mice Also Carried the MHC-nLAC Reporter Transgene)**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Uninjured Vent</th>
<th>MI Border Zone</th>
<th>MI Septum</th>
</tr>
</thead>
<tbody>
<tr>
<td>(−)/(-)</td>
<td>0.05 ± 0.05/section</td>
<td>3.1 ± 0.34/section</td>
<td>2.6 ± 0.57/section</td>
</tr>
<tr>
<td>(-)/MHC-CB7</td>
<td>0.06 ± 0.06/section</td>
<td>1.3 ± 0.57/section</td>
<td>1.9 ± 0.51/section</td>
</tr>
<tr>
<td>MHC-1152stop(-)</td>
<td>0.06 ± 0.06/section</td>
<td>2.4 ± 0.56/section</td>
<td>1.6 ± 0.47/section</td>
</tr>
<tr>
<td>MHC-1152stop/MHC-CB7</td>
<td>&lt;0.07/section</td>
<td>1.5 ± 0.27/section</td>
<td>1.7 ± 0.37/section</td>
</tr>
</tbody>
</table>

*P<0.05 between all experimental groups.*
ready seen in the interventricular septa of hearts from mice inheriting the MHC-1152stop transgene (Table 4). Direct comparison of cardiomyocyte DNA synthesis and apoptosis levels required a correction to account for the fraction of cardiomyocytes whose nuclei were not present in the plane of section (see for example, Figure 3A and 3B). Accordingly, adjacent coronal heart sections prepared from infarcted mice carrying the MHC-nLAC and MHC-1152stop transgenes were stained with X-GAL, and the total number of cardiomyocyte cell bodies and total number of cardiomyocyte nuclei (ie, nLAC positive nuclei) were determined. Cardiomyocyte nuclei were present in 27.9±1.21% of the cardiomyocyte cell bodies screened (19 mice, 19 sections). Thus, an additional correction factor of 3.4 was needed to directly compare the apoptosis and DNA synthesis data in coronal sections. When analyzed in this manner, the number of tritiated thymidine-positive cardiomyocytes in the interventricular septum of hearts from mice expressing the MHC-1152stop transgene at 4 weeks after infarction was roughly comparable to the number of activated caspase 3 immune-reactive cardiomyocytes (Table 4).

### Discussion

The data presented in this study indicates that antagonization of p193 and p53 activity relaxes cell cycle checkpoints that would otherwise prevent reactivation of cardiomyocyte DNA synthesis after injury. Importantly, transgene expression had no detectable impact on cardiomyocyte DNA synthesis in uninjured hearts. Although activation of border zone cardiomyocyte DNA synthesis occurred in mice expressing either the MHC-1152stop or the MHC-CB7 transgene, cardiomyocyte DNA synthesis in the interventricular septum only occurred in MHC-1152stop mice. The differential effects observed with the 1152stop and CB7 transgenes suggest that cardiomyocytes at the border zone and in the interventricular septum are subject to markedly different physiological stimuli after myocardial infarction. This view is supported by the observation that cardiomyocyte DNA synthesis in mice carrying the MHC-nLAC reporter gene alone was detected in the border zone but not in the interventricular septum (Tables 2 and 4). The unique albeit overlapping patterns of cardiomyocyte DNA synthesis seen in the different transgenic mice also suggest that expression of the p193 and p53 dominant-interfering mutants may antagonize partially overlapping sets of cell cycle checkpoints. Previous studies demonstrating that expression of both the MHC-1152stop and MHC-CB7 transgenes was required for sustained E1A-induced cell cycle activation14 in ESC-derived cardiomyocytes support the argument for the antagonization of distinct checkpoints.

The ability of p53 to regulate cell proliferation and apoptosis in a variety of cell types is well established, and the studies described earlier support a central role for p53 in DNA tumor virus oncoprotein-induced cardiomyocyte proliferation.14,15 Although cardiac p53 expression is known to increase in several in vivo injury models (ie, myocardial infarction, reperfusion injury, pacing-induced heart failure, and isoproterenol-induced hypertrophy), these studies suggested that p53 induction is part of a proapoptotic signal transduction pathway that ultimately leads to the elimination of injured cells. In contrast, the observation that blockade of p53 activity enables border zone cardiomyocytes to reenter the cell cycle (this study) suggests that p53 induction engages a cardiomyocyte cell cycle checkpoint. This view is supported by the fact that failure to abrogate p53 activity resulted in apoptosis (rather than cell cycle activation) in response to DNA tumor virus oncoprotein expression.13-15 This interpretation is indirectly supported by the observation that tritiated thymidine incorporation at the infarct border zone in MHC-CB7 mice was accompanied by a decrease in the level of activated caspase 3 immune reactivity (Table 3), although this decrease failed to reach statistical significance. The low rates of cell cycle reentry seen in hearts of mice inheriting only the MHC-nLAC transgene presumably results from subthreshold levels of p53 induction in those cardiomyocytes.

The recent observation that p193 has E3 ligase activity11,12 suggests a potential mechanism by which expression of the p193 dominant-interfering mutant relaxes cardiomyocyte cell cycle control. E3 ligases are multiprotein complexes that impart substrate specificity for ubiquitination and subsequent degradation via the 26S proteasome. The observations that expression of a p193 dominant-interfering mutant renders ESC-derived cardiomyocytes14 and transgenic cardiomyocytes (this study) amenable to cell cycle activation suggest that p193 may target the degradation of cell cycle regulatory proteins. Studies are currently underway to determine how expression of the 1152stop molecule relaxes this cell cycle control. The discovery of Parc, a large protein that is thought to provide a cytoplasmic anchor for p53, provides a clue that might explain the apparent partial overlap of 1152stop and CB7 activity at the infarct border zone. We have recently shown that Parc, p193, and p53 form a multiprotein complex in the cytoplasm of U2OS cells (Dowell and Field, unpublished data, 2004). This suggests that expression of 1152stop may alter some aspect of p53 regulation. If this hypothesis holds true, then antagonization of p53 activity might underlie the induction of infarct border zone cardiomyocyte DNA synthesis in both the MHC-1152stop and MHC-CB7 trans-
genic animals. In contrast, the presence of cardiomyocyte DNA synthesis in the interventricular septa of MHC-1152stop mice (but not MHC-CB7 mice) likely results from the ability of the p193 dominant-interfering mutant to alter the processing of some other checkpoint protein(s).

Expression of the MHC-1152stop transgene, but not the MHC-CB7 transgene, had a profound effect on remodeling of the interventricular septum after myocardial infarction. Hypertrophic cardiomyocyte growth was greatly reduced in the MHC-1152stop mice, whereas the effect of MHC-CB7 transgene expression was minimal (Figure 3), despite similar rates of cardiomyocyte apoptosis (Table 3). This differential response did not result from an intrinsic resistance to hypertrophic stimuli, as isoproterenol infusion had similar effects on heart weight (Table 1) and minimal fiber diameter (H. Nakajima, unpublished observation, 2004) in MHC-1152stop and MHC-CB7 animals.

The observation that cardiomyocyte DNA synthesis was only detected in the septa of the MHC-1152stop mice raises the possibility that cell cycle activation might underlie the differential hypertrophy. Moreover, these data are consistent with the notion that injury-induced hypertrophy (as opposed to cell cycle progression) may be a consequence of a G1/S (and perhaps a G2/M) checkpoint that is activated on cardiomyocyte terminal differentiation. This view is further supported by the presence of phosphorylated histone H3 immune reactivity in the interventricular septum of infarcted MHC-1152stop hearts (Figure 2C). The frequency of cardiomyocyte phosphorylated histone H3 immune reactivity was approximately 1/10th of that observed for cardiomyocyte DNA synthesis, in good agreement with the previously reported ratio of mitotic figure to DNA synthesis in fetal and neonatal hearts with active cardiomyocyte cell cycle activity.35 The marked induction in atrial cardiomyocyte DNA synthesis and cell content observed in infarcted MHC-1152stop mice with left atrial thrombi (see online data supplement, available at http://circres.ahajournals.org) further supports the notion that antagonization of p193 activity renders cardiomyocytes more permissive to cell cycle reentry.

In light of these observations, it is of interest to note that the rate of cardiomyocyte cell cycle reentry in the interventricular septa of infarcted MHC-1152stop mice was roughly comparable to the level of cardiomyocyte apoptosis in the septa of infarcted nontransgenic hearts. Assuming that the duration of S-phase and activated caspase 3 immune reactivity are not greatly different, these data are consistent with the notion that cell cycle reactivation as mediated by expression of the 1152stop transgene results in partial replacement of cardiomyocyte loss via apoptosis in the interventricular septum. Partial replacement of the lost cells could in turn reduce the need for hypertrophic growth in the remaining cardiomyocytes. At present, the potential contribution of infarct border zone cardiomyocyte cell cycle activity to the reduced hypertrophic growth in the interventricular septum cannot be assessed because both transgenic models have similar activity. The presence of septal cardiomyocyte hypertrophy in the MHC-CB7 mice indicates that infarct border zone cell cycle activity by itself is insufficient to impact on cardiomyocyte hypertrophy in the interventricular septum. Finally, the absence of induced cardiomyocyte DNA synthesis after isoproterenol infusion (0/30 447 labeled nuclei in nontransgenic mice versus 1/31 379 in MHC-1152stop mice, n = 4 mice per group; P < 0.05) suggests that only a subset of hypertrophic signal transduction pathways can activate ventricular cardiomyocyte cell cycle reentry in the MHC-1152stop mice.

In summary, the data presented in this study indicate that expression of the MHC-CB7 transgene allows infarct border zone cardiomyocytes to reenter the cell cycle, whereas expression of the MHC-1152stop transgene allows both border zone and interventricular septum cardiomyocytes to reenter the cell cycle. Transgene expression in MHC-1152stop mice is associated with a favorable effect on postinfarct remodeling of the interventricular septum. It will be informative to determine if the remodeling effect is accompanied with improved cardiac function. If so, pharmacological inhibition of the p193 pathway might facilitate a viable intervention to promote regenerative cardiac growth.

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**References**


Expression of Mutant p193 and p53 Permits Cardiomyocyte Cell Cycle Reentry After Myocardial Infarction in Transgenic Mice
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ON LINE SUPPLEMENTARY DATA

(1) Gene expression in the hearts of non-transgenic and MHC-1152stop transgenic mice.

Protein isolated from non-injured and infarcted non-transgenic and MHC-1152stop transgenic mice (4 weeks post MI) was subjected to Western blot analyses as described in the manuscript. ANF (Peninsula Laboratories, Belmont CA) and c-fos (Oncogene, San Diego CA) expression were monitored. In non-transgenic mice, a slight induction in ANF expression was observed in infarcted hearts (see figure). Interestingly, a repression of ANF expression in both control and infarcted MHC-1152 hearts was observed. No change in c-fos levels was observed in the chronic MIs with either genotype.

Western blot analysis of gene expression:

<table>
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<tr>
<th>Genotype:</th>
<th>Non-Txg</th>
<th>MHC-1152stop</th>
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<tr>
<td>MI:</td>
<td>-</td>
<td>+</td>
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<tr>
<td>Anti-ANF</td>
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<td>Anti-c-fos</td>
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(2) Atrial cardiomyocyte phenotype in MHC-1152stop hearts following myocardial infarction

Previous studies have reported low levels of left atrial cardiomyocyte DNA synthesis following myocardial infarction in rats (reviewed by Rumyantsev PP. In: Carlson BM, ed. Reproduction of Growth and hyperplasia of cardiac muscle cells. Chur, Switzerland: Harwood Academic Publishers;1991:239-268). DNA synthesis was monitored in MNC-nLAC / (-) and MHC-nLAC / MHC-1152stop mice under base line conditions and following myocardial infarction using the tritiated thymidine incorporation assay described in the manuscript. No left atrial cardiomyocyte DNA synthesis was detected in either the MNC-nLAC / (-) or the MHC-nLAC / MHC-1152stop mice under base line conditions. Low rates of cell cycle activity were seen in infarcted MNC-nLAC / (-) hearts (0.04 ± 0.027%, n = 4 mice), in agreement with previous work from the Rumyanstev lab. A rather robust cardiomyocyte DNA synthesis response was observed in the left atria of the infarcted MHC-nLAC / MHC-1152stop mice (0.37 ± 0.17%, n = 5 mice, see figure; arrows demark cardiomyocytes synthesizing DNA). Interestingly, these high rates of cardiomyocyte DNA synthesis were accompanied by a large increase in the number of blue nuclei, supporting the notion of transgene expression plus injury results in cell cycle progression. Moreover, the high rates of DNA synthesis were observed only in mice with overt left atrial distension due to the presence of thrombi, suggesting that hypertrophic stimuli is again required for the phenotype.
Left atrial cardiomyocyte DNA synthesis in a MHC-nLAC/ MHC-1152stop heart at 4 weeks post MI: