Cardiomyocyte Cell Cycle Activation Ameliorates Fibrosis in the Atrium

Hidehiro Nakajima, Hisako O. Nakajima, Klaus Dembowsky, Kishore B.S. Pasumarthi, Loren J. Field

Abstract—MHC-TGFcys\(^{33\text{ser}}\) transgenic mice have elevated levels of active transforming growth factor (TGF)-\(\beta\) in the myocardium. Previous studies have shown that these animals develop atrial, but not ventricular, fibrosis. Here we show that atrial fibrosis was accompanied with cardiomyocyte apoptosis. Although similar levels of cardiomyocyte apoptosis were present in the right and left atria of MHC-TGFcys\(^{33\text{ser}}\) hearts, the extent of fibrosis was more pronounced in the right atrium. Thus, additional factors influence the degree of atrial fibrosis in this model. Tritiated thymidine incorporation studies revealed cardiomyocyte cell cycle activity in left atrial cardiomyocytes, but not in right atrial cardiomyocytes. These observations suggested that cardiomyocyte cell cycle activation ameliorated the severity of atrial fibrosis. To directly test this hypothesis, MHC-TGFcys\(^{33\text{ser}}\) mice were crossed with MHC-cycD2 mice (which have constitutive cardiomyocyte cell cycle activity in the right atrium). Mice inheriting both transgenes exhibited right atrial cardiomyocyte cell cycle activity and a concomitant reduction in the severity of right atrial fibrosis, despite the presence of a similar level of cardiomyocyte apoptosis as was observed in mice inheriting the MHC-TGFcys\(^{33\text{ser}}\) transgene alone. These data support the notion that cardiomyocyte cell cycle induction can antagonize fibrosis in the myocardium. (Circ Res. 2006;98:141-148.)

Key Words: cardiomyocyte proliferation ■ cardiomyocyte apoptosis ■ heart regeneration

Progressive necrotic, apoptotic, and/or oncotic cardiomyocyte death underlies many forms of cardiovascular disease.\(^1\) Pathophysiological cardiomyocyte loss is often accompanied with fibrosis, which is characterized by pronounced changes in the extracellular matrix including interstitial collagen deposition.\(^3\)–\(^5\) Fibrosis increases myocardial stiffness and induces electrical heterogeneity, both of which contribute to the progressive loss of systolic and diastolic function, as well as susceptibility to cardiac arrhythmia and sudden death. Fibrosis is thought to be regulated in part by the activities of several approaches have been used to attempt to control progressive cardiomyocyte loss and in turn reduce fibrosis. In addition, replacement of lost cardiomyocytes might inhibit the progression of, and perhaps even promote the regression of, cardiac fibrosis. Potential approaches to promote cardiomyocyte replacement include cell cycle activation,\(^1\) direct replacement of lost cardiomyocytes via cellular transplantation,\(^1\) and recruitment and/or activation of cardiomyogenic stem cells.\(^1\)

We have previously generated transgenic mice which overexpress TGF-\(\beta_1\) in the myocardium to directly examine the role of this cytokine in the genesis of myocardial fibrosis.\(^1\) The stringent posttranslational regulation of TGF-\(\beta_1\) necessitated the use of cDNAs encoding site-directed mutations that increased the level of active cytokine. Accordingly, mice were generated which carried a transgene comprising the cardiomyocyte-restricted \(\alpha\) cardiac myosin heavy chain (MHC) promoter and a cDNA encoding a mutant TGF-\(\beta_1\) molecule harboring a cysteine to serine substitution at amino acid residue 33. This mutation blocked covalent tethering of the TGF-\(\beta_1\) latent complex to the extracellular matrix,\(^2\) resulting in an approximately 30-fold increase in the level of active TGF-\(\beta_1\) in the adult transgenic hearts.\(^1\) Increased TGF-\(\beta_1\) activity in these animals, which were designated MHC-TGFcys\(^{33\text{ser}}\), did not induce ventricular fibrosis, as evidenced by histochemical and gene expression.
analyses. A delay in ventricular wound healing, which appeared to be secondary to cytokine-induced apoptosis of proliferating cardiac fibroblasts, was observed following myocardial injury in MHC-TGFcys33ser transgenic hearts. These observations suggested that TGF-β1 activity in itself was insufficient to promote fibrosis in the ventricles. Given the absence of overt fibrosis in the transgenic ventricles, it was surprising that age-dependent fibrosis was observed in the atria of these mice.19

In this study, the atrial phenotype in MHC-TGFcys33ser transgenic mice was further characterized. The extent of fibrosis was observed to be more severe in the right atrium as compared with the left. Cardiomyocyte apoptosis was present at similar levels in the left and right atria, suggesting that factors in addition to cell death contributed to the differential atrial fibrosis. Surprisingly, tritiated thymidine incorporation studies revealed cardiomyocyte cell cycle activity in left atrium of MHC-TGFcys33ser hearts, but not in the right atrium. These data suggested that the presence of cardiomyocyte cell cycle activity could ameliorate the severity of atrial fibrosis. To directly test this hypothesis, MHC-TGFcys33ser mice were intercrossed with MHC-cycD2 mice,21 which exhibit constitutive right atrial cell cycle activity. Mice inheriting both transgenes exhibited right atrial cardiomyocyte cell cycle activity and a concomitant reduction in the severity of right atrial fibrosis, despite having a similar level of right atrial cardiomyocyte apoptosis as compared with mice inheriting the MHC-TGFcys33ser transgene alone. Collectively, these data suggest that cardiomyocyte cell cycle activation can antagonize fibrosis and support the notion that the controlled proliferation of cardiomyocytes can have a beneficial impact on diseased hearts.

Materials and Methods

Transgenic Mouse Models

The generation and initial analyses of the MHC-TGFcys33ser,19 MHC-cycD2,21 and MHC-nLAC22 transgenic mice have been described previously. Mice were maintained in a DBA/2J genetic background. All animal manipulations were performed according to NIH and Institutional Animal Care and Use Committee Guidelines.

Histology

Paraffin sections were prepared from Bouin’s fixed samples using standard procedures.23 Cryosections (10 μm) were generated using standard histologic techniques.24 Sections were stained with picrosirius solution (0.1% Sirius Red in picric acid, Sigma Diagnostics, St Louis, Mo) and counter-stained with Fast green (Fischer Scientific, Fair Lawn, NJ). To quantitate atrial collagen content, images were captured with a digital camera and the red pixel content of the images for Windows Beta 4.0.2 software as described.19

Apoptosis Assays

For anti–activated caspase 3 immune assay, cryosections were processed according to the specifications of the manufacturer (Promega, Madison, Wis). For in situ end labeling (ISEL) analysis, cryosections were processed using the KLENOW-FragEL DNA Fragmentation Detection Kit (no. QIA21, Oncogene Research, Boston, Mass). Signal was developed using biotinylated nucleotides and fluorescein isothiocyanate–conjugated streptavidin (Roche Molecular Biochemicals, Indianapolis, Ind). β-Galactosidase activity was detected in the same section via immune reactivity using a polyclonal anti-β-galactosidase antibody (no. 55976, ICN, Aurora, Ohio) and rhodamine-conjugated secondary antibody (no. AQ301R, Chemicon, Temecula, Calif).

Cardiomyocyte DNA Synthesis Assay

To monitor cardiomyocyte DNA synthesis, mice received a single injection of tritiated thymidine (200 μCi IP at 28 Ci per mmol/L; Amersham, Arlington Heights Ill) and were euthanized 4 hours or 2 weeks later. Hearts were cryosectioned as described above and cardiomyocyte DNA synthesis was identified by colocalization of β-galactosidase activity (dark blue signal following X-GAL staining) and silver grains as described.25

Statistical Analysis

Results are expressed as mean±SEM. The differences between right and left atrial parameters were calculated with Student’s paired t test. The differences among MHC-TGFcys33ser/(-), (-)/MHC-cycD2, and MHC-TGFcys33ser/MHC-cycD2 mice were evaluated by ANOVA followed by Bonferroni post hoc test.

Results

Differential Atrial Fibrosis in MHC-TGFcys33ser Mice

Previous studies demonstrated that MHC-TGFcys33ser transgenic mice developed age-dependent atrial fibrosis.19 Histologic analysis of aged transgenic mice revealed that collagen deposition (red signal, Sirius Red stain) in the interstitial spaces between cardiomyocytes was markedly greater in the right atrium as compared with the left atrium (Figure 1a; heart is from an 18-month-old transgenic mouse). To further characterize the atrial fibrosis, MHC-TGFcys33ser mice were crossed with MHC-nLAC mice (the presence of the MHC-nLAC reporter transgene facilitated the identification of cardiomyocyte nuclei in subsequent experiments; see below). Sections of hearts from 2-, 4-, 6-, 8-, and 12-week-old MHC-TGFcys33ser/MHC-nLAC double transgenic mice were stained with Sirius Red and Fast Green (Figure 1b). Interstitial collagen content was similar in left and right atria at 2 weeks of age. By 4 weeks of age, collagen content appeared more pronounced in the right atrium, and this asymmetry in atrial collagen content was apparent at all subsequent ages examined. Quantitative image analysis confirmed similar collagen content in right and left transgenic atria at 2 weeks of age. Collagen content progressively increased with age in the right atria. In contrast, collagen content in the left atria remained relatively constant over the time points studied (Figure 1c).

Cardiomyocyte Apoptosis in MHC-TGFcys33ser Atria

Activated caspase 3 immune assays were performed to determine whether increased levels of cardiomyocyte apoptosis accompanied the increased fibrosis observed in the right atria of MHC-TGFcys33ser mice. Because activated caspase 3 is distributed in the cytoplasm, cardiomyocytes at early stages
of apoptosis were easily identified by morphological criteria (i.e., rod-shaped cells; see Figure 2a). Similar levels of cardiomyocyte activated caspase 3 immune reactivity were observed in the right and left atria of the MHC-TGFcys^{33}ser hearts (0.45 ± 0.2 and 0.58 ± 0.18 activated caspase 3 positive cardiomyocytes per atrial section, respectively, n = 8 mice; paired t test, NS). No apoptosis was detected when similar numbers of atrial cardiomyocytes were screened in age-matched nontransgenic littermates.

ISEL and TUNEL analyses were performed to confirm these results. These analyses took advantage of the presence of the MHC-nLAC transgene, which targets expression of a nuclear localized β-galactosidase reporter to cardiomyocytes. In the case of ISEL analyses, sections prepared from MHC-TGFcys^{33}ser/MHC-nLAC double transgenic mice were stained with X-GAL (blue signal over cardiomyocyte nuclei) and then processed for ISEL activity using biotinylated nucleotides and HRP-conjugated streptavidin (dark brown signal over apoptotic nuclei). ISEL-positive cardiomyocytes, which were identified by the superimposition of dark brown and blue signals, were readily seen (Figure 2b, arrows). In the case of TUNEL analysis, sections from MHC-TGFcys^{33}ser/MHC-nLAC double transgenic mice were processed simultaneously for β-galactosidase immune reactivity (Figure 2c, left, red signal) and TUNEL activity (Figure 2c, middle, green signal). Merging the images confirmed that the TUNEL-positive nucleus was from a cardiomyocyte (Figure 2c, right, yellow signal). In agreement with the activated caspase 3 immune reactivity assay, similar levels of cardiomyocyte ISEL activity and TUNEL signal were observed in the left and right atria in MHC-TGFcys^{33}ser hearts. These data suggested that additional factors were likely to

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**Figure 1.** Atrial fibrosis in MHC-TGFcys^{33}ser transgenic hearts.

a. Sections from the right and left atria of an 18 month old MHC-TGFcys^{33}ser transgenic mouse, stained with Sirius Red and Fast Green. Bar = 20 μm. b. Sections of right and left atria from individual MHC-TGFcys^{33}ser/MHC-nLAC transgenic hearts at 2, 4, 6, 8, and 12 weeks of age, stained with Sirius Red and Fast Green. Bar = 20 μm. c. Quantification of collagen content in the right (RA) (filled squares) and left (LA) (open squares) atria of MHC-TGFcys^{33}ser/MHC-nLAC mice at the indicated ages. Data are from 5 mice per time point. *P < 0.05, right atria vs left atria.

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**Figure 2.** Atrial cardiomyocyte apoptosis in MHC-TGFcys^{33}ser/MHC-nLAC double transgenic mice. a. Activated caspase 3 immune assay for cardiomyocyte apoptosis. Cardiomyocytes at early stages of apoptosis were identified by the presence of activated caspase 3 immune reactivity (cytoplasmic brown signal in rod-shaped cells). Bar = 20 μm. b. ISEL assay for cardiomyocyte apoptosis. Sections were stained with X-GAL to identify cardiomyocyte nuclei (blue signal) and for ISEL activity to identify apoptotic cells (HRP-conjugated reaction, dark brown signal). Apoptotic cardiomyocytes have brown and blue signals over the nucleus (arrows). Bar = 10 μm. c. TUNEL assay for cardiomyocyte apoptosis. Sections were stained for β-galactosidase immune reactivity to identify cardiomyocyte nuclei (rhodamine conjugated secondary antibody, red signal, left panel) and for TUNEL activity to identify apoptotic cells (fluorescein isothiocyanate–conjugated secondary antibody, green signal (middle). Apoptotic cardiomyocytes are identified in the merged image (yellow signal) (right). Bar = 10 μm.
contribute to the differential levels of fibrosis seen in the left and right atria of the transgenic mice.

Cardiomyocyte Cell Cycle Activity in the Left Atria of MHC-TGFcys\textsuperscript{33}ser Mice

Tritiated thymidine incorporation assays were performed to monitor cell cycle activity in the atria of MHC-TGFcys\textsuperscript{33}ser mice. Once again, the experiments made use of the presence of the MHC-nLAC reporter transgene. MHC-TGFcys\textsuperscript{33}ser/MHC-nLAC double transgenic mice received a single injection of tritiated thymidine. The animals were euthanized 4 hours later, and the hearts were sectioned, stained with X-GAL, and processed for autoradiography. Cardiomyocyte DNA synthesis was then quantified by screening for the presence of silver grains over blue nuclei.\textsuperscript{22,25} Surprisingly, cardiomyocyte DNA synthesis was readily detected in the left atria of 12-week-old MHC-TGFcys\textsuperscript{33}ser/MHC-nLAC double transgenic mice, but not in the right atria (Figure 3a and Table 1).

To establish the time course of atrial cardiomyocyte DNA synthesis, thymidine incorporation analyses were performed on 2-, 4-, 6-, 8-, and 12-week-old MHC-TGFcys\textsuperscript{33}ser/MHC-nLAC double transgenic mice. Cardiomyocyte DNA synthesis was detected in both the right and left atria of the MHC-TGFcys\textsuperscript{33}ser mice at 2 weeks of age (Figure 3b). Cardiomyocyte DNA synthesis was markedly down regulated in both atria by four 4 of age. These observations are in agreement with the marked decrease in cardiomyocyte cell cycle activity that occurs during neonatal life.\textsuperscript{26–28} At 6 weeks of age, cardiomyocyte DNA synthesis was again apparent in the left atrium of MHC-TGFcys\textsuperscript{33}ser mice and persisted at similar levels for the remainder of the experiment; in contrast, DNA synthesis did not resume in right atrial cardiomyocytes (Figure 3b).

Pulse chase experiments were performed to determine the stability of cardiomyocytes which synthesized DNA. Six 12-week-old MHC-TGFcys\textsuperscript{33}ser/MHC-nLAC double transgenic animals were injected with tritiated thymidine. Three mice were euthanized 4 hours later, and 3 were euthanized 14 days later. The hearts were sectioned, processed for X-GAL staining and autoradiography, and the number of radiolabeled cardiomyocyte nuclei was determined for each time point (Table 1). The increased tritiated thymidine labeling observed in the chase group indicated that left atrial cardiomyocytes that synthesized DNA were stable and also suggested that a portion of these cells underwent karyokinesis and perhaps cytokinesis. This view was supported by the observation that phosphorylated histone H3 immune reactivity (a marker for mitosis)\textsuperscript{29} was detected in left atrial cardiomyocytes of MHC-TGFcys\textsuperscript{33}ser mice (Figure 3c, arrow). The frequency of cardiomyocyte phosphorylated histone H3 immune reactivity was approximately 1/15 of that observed for cardiomyocyte thymidine incorporation; similar ratios of cardiomyocyte DNA synthesis and mitosis have been reported in neonatal hearts\textsuperscript{30} and in the ventricle of adult transgenic mice with elevated cardiomyocyte cell cycle activity.\textsuperscript{21,31} DNA synthesis was also monitored in older MHC-TGFcys\textsuperscript{33}ser transgenic mice. Similar levels of left atrial cardiomyocyte DNA synthesis were observed in 12- and 26-week-old animals (Table 1).

Cell Cycle Activation Ameliorates Fibrosis in the Right Atria of MHC-TGFcys\textsuperscript{33}ser Mice

The data presented above raised the possibility that cardiomyocyte cell cycle activation ameliorated the extent of fibrosis in the left atria of MHC-TGFcys\textsuperscript{33}ser transgenic mice. To directly examine the consequences of cardiomyocyte cell cycle activation on atrial fibrosis, MHC-TGFcys\textsuperscript{33}ser mice were crossed with MHC-cycD2 mice. Previous studies have shown that expression of the MHC-cycD2 transgene was sufficient to drive cardiomyocyte DNA synthesis and prolif-
eration in normal and diseased ventricles.\textsuperscript{21} MHC-TGF\textsuperscript{cys33ser}(\textendash ), (\textendash )MHC-cycD2, and MHC-TGF\textsuperscript{cys33ser}/MHC-cycD2 transgenic mice were identified and sequestered. To facilitate quantitation of cardiomyocyte DNA synthesis, these mice also carried the MHC-nLAC reporter gene, indicating that targeted expression of cyclin D2 had marked reduction in right atrial collagen content (Figure 4). Right atrial cardiomyocyte DNA synthesis was detected in the right atria of mice inheriting both the MHC-cycD2 transgene and MHC-TGF\textsuperscript{cys33ser} transgenes and was accompanied with a marked reduction in right atrial collagen content (Figure 4 and Table 2). Importantly, similar rates of cardiomyocyte apoptosis (as evidenced by activated caspase 3 immune reactivity) were observed in the right atria of mice inheriting the MHC-TGF\textsuperscript{cys33ser} transgene alone and in mice inheriting both the MHC-TGF\textsuperscript{cys33ser} and MHC-cycD2 transgenes. Analysis of adjacent sections revealed that the level of cardiomyocyte DNA synthesis (thymidine-positive, nLAC-positive nuclei per section) was very similar to the level of cardiomyocyte apoptosis (cell bodies with activated caspase-3 immune reactivity per section) in the right atria of MHC-TGF\textsuperscript{cys33ser}/MHC-cycD2 transgenic hearts (Table 2).

If the presence of cyclin D2–induced cardiomyocyte DNA synthesis culminated in cell division, the data presented above would predict that there would be an increased number of right atrial cardiomyocytes in the MHC-TGF\textsuperscript{cys33ser}/MHC-cycD2 double transgenic mice as compared with MHC-TGF\textsuperscript{cys33ser} single transgenic animals. Accordingly, direct counts of cardiomyocyte cell bodies per unit area were performed. As expected, expression of cyclin D2 resulted in a statistically significant increase in the number of cardiomyocytes per unit area of right atrial tissue in the MHC-TGF\textsuperscript{cys33ser}/MHC-cycD2 mice as compared with MHC-TGF\textsuperscript{cys33ser} mice (Table 2).

**Discussion**

Transgene expression in the right atria of MHC-TGF\textsuperscript{cys33ser} mice resulted in cardiomyocyte apoptosis as evidenced by activated caspase 3, ISEL, and TUNEL assays. Cardiomyocyte loss was accompanied by increased extracellular collagen deposition. Thus the progressive histopathology observed in the MHC-TGF\textsuperscript{cys33ser} transgenic mice was consistent with the process of reparative fibrosis in response to cardiomyocyte death.\textsuperscript{1} Although a similar level of cardiomyocyte apoptosis was apparent in MHC-TGF\textsuperscript{cys33ser} left and right atria at all ages examined, the extent of reparative fibrosis was markedly less in the left atrium. The presence of cell cycle activation in the left atrium suggested that cardiomyocyte proliferation could effectively ameliorate fibrosis.

Other observations argued against alternative mechanisms for the differential fibrosis in the MHC-TGF\textsuperscript{cys33ser} atria. For example, the levels of fibroblast DNA synthesis as measured by tritiated thymidine incorporation were very similar in right atria of MHC-TGF\textsuperscript{cys33ser}/MHC-cycD2 mice; previous studies have shown that the presence of multiple transgenes using the MHC promoter did not result in a reduction of expression levels.\textsuperscript{31,32} At 12 weeks of age, the mice received an injection of tritiated thymidine and the direct counts of cardiomyocyte cell bodies per unit area were performed. As expected, expression of cyclin D2 resulted in a statistically significant increase in the number of cardiomyocytes per unit area of right atrial tissue in the MHC-TGF\textsuperscript{cys33ser}/MHC-cycD2 mice as compared with MHC-TGF\textsuperscript{cys33ser} mice (Table 2).

**TABLE 1. Cardiomyocyte DNA Synthesis Levels in Atria From MHC-TGF\textsuperscript{cys33ser}/MHC-nLAC Double Transgenic Mice**

<table>
<thead>
<tr>
<th>Right Atrium</th>
<th>Left Atrium</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Thymidine + nuclei</td>
<td>0.10±0.03</td>
</tr>
<tr>
<td>Nuclei screened</td>
<td>38,381</td>
</tr>
<tr>
<td>Mice screened</td>
<td>8</td>
</tr>
</tbody>
</table>

**TABLE 2. Cardiomyocyte Apoptosis, Cardiomyocyte DNA Synthesis, Collagen Content, and Cardiomyocyte Number per mm\textsuperscript{2} in Right Atria From MHC-TGF\textsuperscript{cys33ser}/(\textendash ), (\textendash )MHC-cycD2, and MHC-TGF\textsuperscript{cys33ser}/MHC-cycD2 Transgenic Mice**

<table>
<thead>
<tr>
<th>MHC-TGF/ (\textendash )</th>
<th>MHC-cycD2</th>
<th>MHC-cycD2</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Thymidine + nuclei</td>
<td>0.081±0.018*</td>
<td>0.055±0.010*</td>
</tr>
<tr>
<td>Nuclei screened</td>
<td>26,493</td>
<td>12,340</td>
</tr>
<tr>
<td>Mice screened</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Collagen content (%)</td>
<td>42±1.8</td>
<td>6.8±1.8*</td>
</tr>
<tr>
<td>Mice screened</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Activated caspase 3+ cardiomyocytes/section</td>
<td>0.60±0.31</td>
<td>0.83±0.14</td>
</tr>
<tr>
<td>Mice screened</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Total thymidine + nuclei/section</td>
<td>1.3±0.10*</td>
<td>1.0±0.31*</td>
</tr>
<tr>
<td>Mice screened</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Cardiomyocyte no.</td>
<td>12.4±1.0</td>
<td>21.8±4.8#</td>
</tr>
<tr>
<td>Mice screened</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>

All values are mean±SEM; statistical significance was determined by ANOVA followed by Bonferroni post hoc test. *P<0.05 vs MHC-TGF/ (\textendash ) mice; †P<0.05, (\textendash )MHC-cycD2 vs MHC-TGF/MHC-cycD2; ‡P<0.01, MHC-TGF\textsuperscript{cys33ser}/(\textendash ) vs (\textendash )MHC-cycD2; ‡P<0.001, MHC-TGF\textsuperscript{cys33ser}/(\textendash ) vs MHC-TGF\textsuperscript{cys33ser}/MHC-cycD2.
and left atria of the MHC-TGF\textsubscript{cys33ser} transgenic mice (0.28\textpm0.039% and 0.36\textpm0.048%, respectively; n=5 mice; paired t test, NS). The differential atrial collagen content was thus not attributable to differences in fibroblast cell cycle activity. In addition, the presence of similar levels of transgene-encoded mRNA in the right and left atria suggested that differential TGF-\(\beta_1\) expression did not underlie the asymmetrical atrial fibrosis (see the online data supplement available at http://circres.ahajournals.org); unfortunately the lack of species-specific antibodies, and the presence of a marked induction of the endogenous TGF-\(\beta_1\) gene,\textsuperscript{19} prohibited direct quantitation of transgene-encoded TGF-\(\beta_1\) protein in mice carrying the MHC-TGF\textsubscript{cys33ser} transgene alone or in combination with the MHC-cycD2 transgene. Collectively, these data suggested that cardiomyocyte cell cycle activity could ameliorate atrial fibrosis. This hypothesis was directly supported by the observation that cyclin D2–mediated cardiomycyte proliferation markedly reduced the severity of fibrosis in the right atria of MHC-TGF\textsubscript{cys33ser}/MHC-cycD2 double transgenic mice without impacting the level of cardiomycyte apoptosis.

The mechanism by which expression of the MHC-TGF\textsubscript{cys33ser} transgene induced atrial cardiomycyte apoptosis, as well as left atrial cardiomycyte cell cycle activity, was not clear. In vitro studies with cultured fetal or neonatal ventricular cardiomycocytes suggested that TGF-\(\beta_1\) administration antagonized the mitogenic effects of other growth factors but, by itself, has little effect on cardiomyocyte cell cycle activity.\textsuperscript{33,34} In light of this, the hypoplastic heart phenotype in mice expressing an activated TGF type 1 receptor (Alk5) under the regulation of the cardiac MHC promoter\textsuperscript{19} likely resulted from interplay between intrinsic (ie, transgene expression) and extrinsic (ie, cytokines) factors. In contrast, targeted expression of a constitutively active variant of the TGF-\(\beta_1\)-activated kinase (TAK-1, a major effector of the TGF-\(\beta_1\) signal transduction pathway) with the same MHC promoter did not induce a hypoplastic myocardial response.\textsuperscript{36} Rather these animals developed cardiac hypertrophy and exhibited cardiomycyte apoptosis at an early age that progressed rapidly to overt heart failure and death. Moreover, this proapoptotic effect of TAK1 activation on ventricular myocardium in vivo contrasted with the apparent cardioprotective effect of TGF-\(\beta_1\) administration during reperfusion injury of cultured adult cardiomycocytes.\textsuperscript{37}

Thus, varying outcomes have been obtained following stimulation of the TGF-\(\beta_1\) signal transduction pathway, depending on the extent to which the pathway was activated as well as the degree to which pathway cross-talk occurred; this was particularly evident when a given effector molecule participated in multiple signaling pathways. The situation in MHC-TGF\textsubscript{cys33ser} mice was further complicated as cardiomycocytes from different chambers of the heart exhibited strikingly different phenotypes. Indeed, with respect to cell cycle activity, TGF-\(\beta_1\) expression induced cardiomycyte DNA synthesis in left atrial cardiomycocytes, but not right atrial cardiomycocytes. Moreover, transgene expression did not influence the rate of ventricular cardiomycyte DNA synthesis in normal adult hearts.\textsuperscript{19} In addition, experimental injury did not induce ventricular cardiomycyte DNA synthesis in injured hearts.\textsuperscript{19} The presence of cardiac injury per se was probably not an overriding factor in the differential induction of atrial cardiomycyte cell cycle activity in the MHC-TGF\textsubscript{cys33ser} transgenic mice, as similar levels of apoptosis were present in left and right atria. Of interest, previous studies suggested a greater propensity for cell cycle induction in left atrial cardiomycocytes as compared other cardiomycocytes in the heart,\textsuperscript{39} a phenomenon that may be reflected in the results observed here.

Although direct evidence indicating that the MHC-TGF\textsubscript{cys33ser} left atrial cardiomycocytes that synthesize DNA ultimately divided is lacking, the results of the pulse-chase experiment suggested that at least karyokinesis occurred, a view supported by the presence of phosphorylated histone H3 immune. The failure to detect enlarged cardiomycocytes in histologic sections of the left atrium provided additional, albeit circumstantial, support of this viewpoint. Given that the MHC promoter used in this study was only expressed in differentiated cardiomycocytes, the observed results were most consistent with the notion that expression of the MHC-TGF\textsubscript{cys33ser} transgene resulted in cell cycle activation in differentiated left atrial cardiomycocytes. Although the experiments as performed did not rule out the possibility that transgene-induced cardiac injury resulted in the activation of resident cardiomycogenic stem cell populations,\textsuperscript{37} the absence of cardiomycyte cell cycle activity in the right atrium did not support this argument.

The mechanistic underpinning of cardiomycyte cell cycle activation in MHC-cycD2 transgenic mice was much more

![Figure 4. Reduced right atrial fibrosis in mice carrying both the MHC-TGF\textsubscript{cys33ser} and the MHC-cycD2 transgenes. Right atria from MHC-TGF\textsubscript{cys33ser}(-/−) (left), (−/−)/MHC-cycD2 (middle), or MHC-TGF\textsubscript{cys33ser}/MHC-cycD2 (right) mice, stained with Sirius Red and Fast Green. Bar=20 μm.](http://circres.ahajournals.org/)}
straightforward. D-type cyclins (cyclin D1, D2, and D3) are induced in response to growth factors and are obligate cofactors for the activity of cyclin dependent kinase. The active kinase complex phosphorylates members of the retinoblastoma protein family, resulting in the release of E2F transcription factors. E2F transcription factors induce expression of genes required for S-phase entry, thereby triggering cell cycle progression. Previous studies have shown that targeted expression of cyclins D1, D2, or D3 induced cell cycle activity in ventricular cardiomyocytes in uninjured hearts from transgenic mice. Interestingly, cardiomyocyte cell cycle activity persisted following myocardial infarction in mice that expressed cyclin D2 (but not cyclins D1 or D3) and was accompanied by a reduction in infarct size.

In the current study, cyclin D2–induced right atrial cardiomyocyte cell cycle activity reduced the level of TGF-β–mediated fibrotic injury in mice inheriting both transgenes. Cardiomyocyte cell cycle activity was evidenced by tritiated thymidine incorporation. Although the absence of increased ploidy and multinucleation in MHC-cycD2 right atrial cardiomyocytes (see the online data supplement) suggested that cyclin D2–induced cardiomyocyte DNA synthesis was associated with cell division, the sensitivity of these analyses cannot preclude the possibility that the observed DNA synthesis contributed in part to multinucleation and/or endoreduplication. However, the observed increase in the number of cardiomyocytes per unit area right atrial tissue supports the notion that cell division occurred. Because transgene-mediated cyclin D2–induced cell cycle activation occurred independently of the transgene-mediated TGF-cy53ser–induced fibrosis, the increase in cardiomyocyte cell number and concomitant reduction of atrial fibrosis in the right atria of MHC-TGF-cy53ser/MHC-cycD2 double transgenic mice strongly supports the hypothesis that cell cycle activation can ameliorate structural damage to the heart.

The data presented here join a growing number of studies in which cardiomyocyte cell cycle induction was observed in response to cardiac-restricted gene expression in transgenic mice. The results from the current study indicating that cell cycle induction ameliorated reparative fibrosis in the atrium complement and extend recent observations that cell cycle induction in the ventricle promotes favorable postinfarction remodeling and infarct regression. It is thus becoming apparent that cardiomyocyte cell cycle induction can promote myocardial repair in response to a variety of injuries. Successful therapeutic translation of these observations will likely require the development of small molecules that are able to mimic key aspects of transgene expression.

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A. Transgene expression in left and right atria

For assessment of transgene expression in the MHC-TGFcys\(^{33}\)ser mice, we were unable to discriminate between transgene-encoded and endogenous TGF-beta 1 via Western blot analysis (previous studies have shown that the endogenous TGF-beta gene is markedly induced in the atria of these mice, see CircRes 86:571-579, Figure 3). Accordingly, RT-PCR analysis was used to demonstrate that there are similar levels of transgene encoded TGF-beta1 mRNA in the left atria, right atria and ventricles of the MHC-TGFcys\(^{33}\)ser transgenic hearts (Supplemental Figure 1). In this experiment, one microgram of RNA was reverse-transcribed, and used to program a PCR reaction with primers specific for the TGFcys\(^{33}\)ser transcript and the endogenous connexin 43 transcript. The reactions were run for 20, 25 or 30 cycles, and then displayed on a agarose gel, stained with Ethidium Bromide and visualized under a UV lamp. Similar levels of transgene mRNA were detected in the right atrial, left atrial and ventricular samples. In other controls, no signal was seen in the absence of RT, confirming that there was no genomic DNA in the samples.

For assessment of transgene expression in MHC-cycD2 transgenic mice, Western blots analysis was performed on protein isolated from the ventricle, right atrium and left atrium from two individual mice. Protein was run on standard SDS-PAGE, transferred to support matrix and probed with an anti-cyclin D2 antibody. Signal was developed with the ECL method as described (see CircRes 96:110-118). Similar levels of cyclin D2 protein were observed in the right atrial, left atrial and ventricular samples.

B. Assessment of right atrial endoreduplication in MHC-cycD2 transgenic mice

To determine if transgene expression promoted right atrial cardiomyocyte endoreduplication, dispersed cell preparations from non-transgenic and MHC-
cycD2 right atria were subjected to flow cytometric analysis as described previously by our group (see JClinInvest 99:2644-2654.). Briefly, dispersed cardiomyocyte preparations were generated by digesting atrial appendages with collagenase. The cells were suspended into 1 ml of PBS, incubated with 500 µl of RNase (12µg/ml in PBS) and 500 µl propidium iodine (50 µg/ml in PBS) at room temperature for 30 minutes. Samples were then analyzed on a Becton & Dickinson FACScan to ascertain the nuclear DNA content. No overt evidence of endoreduplication was detected in MHC-cycD2 right atrial cardiomyocytes (i.e., the preponderance of nuclei retained a 2n content). Indeed, there appeared to be a slight reduction in the number of 4n nuclei in MHC-cycD2 right atria.

C. Assessment of right atrial multinucleation in MHC-cycD2 transgenic mice

To determine if cyclin D2 expression promoted right atrial cardiomyocyte multinucleation, dispersed cell preparations from MHC-nLAC control and from MHC-nLAC / MHC-cycD2 double transgenic right atria were prepared by digesting atrial appendages with collagenase. The resulting cell suspensions were fixed in several volumes of flow fix for one hour and smeared onto positively charged slides (Superfrost Plus). After drying the cells were stained with X-GAL and visualized by microscopy. Nuclear content was then scored. In MHC-nLAC right atria, 89.62 +/- 0.54% of the cardiomyocytes were mono-nucleated, and 10.38 +/- 0.54% were bi-nucleated. In MHC-nLAC / MHC-cycD2 double transgenic right atria, 88.54 +/- 0.7% of the cardiomyocytes were mono-nucleated and 11.46 +/- 0.7% were bi-nucleated. There was no statistical difference between the groups. Thus, expression of the MHC-cycD2 transgene does not promote right atrial multi-nucleation.