Cyclin A2 Induces Cardiac Regeneration After Myocardial Infarction and Prevents Heart Failure


Abstract—Mammalian myocardial infarction is typically followed by scar formation with eventual ventricular dilation and heart failure. Here we present a novel model system in which mice constitutively expressing cyclin A2 in the myocardium elicit a regenerative response after infarction and exhibit significantly limited ventricular dilation with sustained and remarkably enhanced cardiac function. New cardiomyocyte formation was noted in the infarcted zones as well as cell cycle reentry of perifocal myocardium with an increase in DNA synthesis and mitotic indices. The enhanced cardiac function was serially assessed over time by MRI. Furthermore, the constitutive expression of cyclin A2 appears to augment endogenous regenerative mechanisms via induction of side population cells with enhanced proliferative capacity. The ability of cultured transgenic cardiomyocytes to undergo cytokinesis provides mechanistic support for the regenerative capacity of cyclin A2. (Circ Res. 2007;100:1741-1748.)

Key Words: cyclin A2 ■ cardiac regeneration ■ side-population cells ■ heart failure ■ cell cycle

Normal adaptive mechanisms in response to myocardial infarction (MI) commence with scar formation in the damaged wall progressing to hypertrophy of the unaffected regions which ultimately succumbs to ventricular dilation and heart failure. Stem cell therapy to restore infarcted myocardium has been extensively studied, with numerous reports that hematopoietic2,3 and mesenchymal4 stem cells derived from bone marrow (BMCs) can give rise to new myocardium via transdifferentiation. This in turn has rapidly translated into a whirlwind of clinical activity aimed at duplicating these effects in the human heart. However, 3 recent studies9–11 have rigorously challenged the conclusions of these reports by independently demonstrating that BMCs transplanted into damaged hearts could not give rise to cardiomyocytes. Balsam et al9 have shown that not only do BMCs fail to give rise to cardiomyocytes, they actually develop into different cell types, despite being in the heart. The beneficial effects noted in earlier studies in terms of ventricular performance might be partially attributable to angioblast-mediated vasculogenesis12 which could prevent apoptosis of native cardiomyocytes rather than by direct myogenesis.

Given these limitations in BMCs, the search for naturally occurring authentic heart progenitor cells has begun in earnest, with several groups having reported on the existence of such cells.13–17 These native progenitors are, at their naturally occurring prevalence and levels of activation, clearly inadequate in reversing the downward spiral of events culminating in heart failure. Their differentiation in response to environmental cues might be expected to generate cardiomyocytes of a postmitotic nature, hence limiting the ability of such endogenous processes to counter the massive myocyte death in MI.

We have previously developed a model system in which constitutive expression of cyclin A2 in the myocardium led to an increase in cardiomyocyte mitoses, particularly during postnatal development.18 The increase in postnatal mitoses was also accompanied by the presence of mononuclear cardiomyocytes, indicative of less differentiation and perhaps greater plasticity. We then hypothesized that regeneration of infarcted myocardium may be potentiated in this model. We have now shown that the cyclin A2 transgenic mice exhibit enhanced cardiac function and a significant reduction in myocardial damage as assessed by MRI. This response appears to involve reentry of the perifocal myocardial cells into the cell cycle as demonstrated by an increase in mitotic index and DNA synthesis. Furthermore, mitosis is noted in small (≈5 μm) cells found in the infarct zone that express markers of cardiomyocytes as well as ABCG2, a known marker of side-population (SP) cells19 and a predominantly nuclear localization of cyclin A2 protein. These small cells appear to represent immature cardiomyocytes, and the enhancement of cell cycle activity in these cells is reminiscent of the enhanced cycling of postnatal cardiomyocytes noted in the transgenic cyclin A2 mouse model. In support of this
mechanism, we have illustrated that the mitotic index of early postnatal transgenic cardiomyocytes is significantly higher when placed in culture. Furthermore, the cultured transgenic cardiomyocytes retain the ability to undergo cytokinesis.

Materials and Methods

Surgical Procedures

Cyclin A2 transgenic mice (Jackson Laboratory, Bar Harbor, Maine) were maintained in a B6CBA background. Nontransgenic littermates were used as our controls. At 8 weeks of age, mice underwent left anterior artery (LAD) ligation to induce anterolateral MI. This was performed in a blinded manner. Each mouse was anesthetized, intubated, and subsequently underwent thoracotomy under 1.5% isoflurane (1.5% volume in 2 L/min air flow). The hearts were perfused with 1X phosphate buffered saline (PBS) and fat tissue was removed. The hearts were fixed in 4% paraformaldehyde overnight. The atria were removed under a dissecting microscope, and the ventricles were sectioned into serial 1-mm-thick slices with the first slice at the level of ligation of the LAD, dehydrated through ethanol series, and embedded in paraffin.

Sequential transverse sections (5 μm) were cut. Immunofluorescence staining was performed using anti–sarcomeric actin (FITC-tagged anti-mouse IgM) to identify cardiomyocytes. Antiphosphorylated histone H3 (anti–BrdU, anti–cyclin A2) were used to localize indices of cellular proliferation to cardiomyocyte nuclei. Rhodamine-conjugated anti-rabbit IgG was used as the secondary antibody against anti–H3P and anti–cyclin A2. Phospho-histone H3 and cyclin A2 staining were maintained for 4 weeks postdispersion.

Assessment of Infarction Size

To determine the extent of infarction, 5-μm serial paraffin-embedded sections of the heart underwent Masson trichrome staining. ImageJ (NIH) was used to measure the circumference of infarcted ventricle in each section. Based on these measurements and the mass of each slice used to generate the section, the infarction percent was calculated for each heart.

Myocyte Dispersion and Assessment of Mitotic Index in Cultured Myocytes

Between 20 to 26 postnatal day (PN) 2 or PN7 pups were used to isolate cardiomyocytes. Hearts were minced, the tissue was placed in 2 mL of Hanks Buffer, 2 mL of Pronase (0.01g/mL) was added and incubated at 37°C for 30 minutes. The muscle cell suspension was pelleted, and triturature of the tissue was performed in Dulbecco Modified Eagle Medium (DMEM) containing 2% fetal bovine serum, 1% penicillin, 1% glutamine, 1% hepes, and 20 μg/mL gentamycin. It was filtered, pelleted, and resuspended in 3 mL Hanks plus serum. It was pelleted again and resuspended in warm DMEM. Preplating with fibronectin solution (1 mg/mL DMEM) was used to minimize fibroblasts. After preplating, cells were counted. Approximately 4×10^6 cells per 2 mL of DMEM medium were transferred into Laboratory-Tek II slide wells (Nalge Nunc International) and incubated overnight. Transgenic cardiomyocytes were plated separately from nontransgenic cardiomyocytes. The Petri dishes were exposed to 30 minutes of gamma irradiation (Gamma Cell 40 using Cs-137 isotope) the next day to minimize fibroblasts. DMEM was aspirated from the slide wells and fresh 2 mL DMEM added. Cells were cultured for 4 to 7 days. One set of Petri dishes of transgenic cardiomyocytes and 1 set for nontransgenic cardiomyocytes was maintained for 4 weeks postdispersion.

The cells were fixed with 4% PFA, and double immunofluorescence staining was performed as described above to identify mitotic nuclei and cardiomyocyte cytoplasm. Cells were analyzed using confocal microscopy (Zeiss LSM 510 NLO Multiphoton Confocal Microscope). A mitotic index was computed for transgenic and nontransgenic cells by computing the ratio of mitotic cardiomyocytes to total number of cardiomyocytes.

Data are expressed as mean ± SEM. Student t test was used for data comparison, using a significance level of P<0.05.

Results

We have previously determined that cyclin A2 functions as a critical regulator of cardiomyocyte mitosis. To test our hypothesis that the continued expression of cyclin A2 in cardiomyocytes could mediate cardiac repair, we induced MI in transgenic and nontransgenic mice via permanent ligation of the LAD. Surgical survival rate was 79% at 1 week post-MI and did not differ significantly between groups. The percentage of infarcted LV volume was consistent between groups (transgenic: 46.8±3.6, nontransgenic: 49.5±4.3, P=0.64) indicating that the transgenic and nontransgenic groups were comparable at 1 week. Cardiac function was analyzed in a serial manner using MRI to measure volumetric ejection fraction (EF). Volumetric EF was markedly enhanced in transgenic mice at 3 weeks post-MI (Figure 1B) and at 3 months post-MI. Left ventricular end-diastolic and end-systolic volume (EDV and ESV, respectively) were markedly decreased in transgenic mice at both time points, implying that the presence of cyclin A2 expression prevents the normal ventricular dilation process after MI (Figure 1C). To define a time course for the enhancement of cardiac function noted in the transgenic mice, we induced infarctions in a second set of mice (6 transgenic and 5 nontransgenic) and
demonstrated that volumetric EF did not significantly differ between the 2 groups at 1 week post-MI (Figure 1D).

To begin to elucidate putative cellular and molecular mechanisms that could provide a basis for the marked enhancement of cardiac function in transgenic mice, DNA synthesis was analyzed by labeling with BrdU at 3 weeks post-MI and by using a sequential labeling protocol with BrdU for 3 months post-MI to assess cell cycle activity (Table 1). Five sections from each heart were analyzed at 1, 2, 3 weeks and 3 months post-MI (n = 3 to 5 per group at each time point). At 3 weeks, $2.65 \times 10^6$ cardiomyocytes/mouse (n = 6) were scored for nontransgenics and $2.52 \times 10^6$ cardiomyo-
TABLE 1. %BrdU-Positive Cardiomyocytes in the Left Ventricle and Distant Regions

<table>
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<th></th>
<th>LV</th>
<th>SEM</th>
<th>Distal</th>
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<tr>
<td>3 Weeks</td>
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<tr>
<td>Tg (n=7)</td>
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<td>0.064</td>
<td>0.000</td>
<td>0.000</td>
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<tr>
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<td>0.000</td>
<td>0.000</td>
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<tr>
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<tr>
<td>3 Months</td>
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<tr>
<td>P value</td>
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Cardiomyocyte DNA synthesis is significantly increased in infarcted transgenic mice. Percent BrdU-positive cardiomyocytes noted in the periinfarct (LV) and distal regions (RV) at 3 weeks postinfarct and after sequential labeling for 3 months postinfarct.

TABLE 2. Mitotic Indices (% H3P-Positive Cardiomyocytes) in the Left Ventricle and Distant Regions

<table>
<thead>
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<th>LV</th>
<th>SEM</th>
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<td>3 Weeks</td>
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<td>Tg (n=4)</td>
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<tr>
<td>P value</td>
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<td>0.36</td>
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<td>3 Months</td>
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<tr>
<td>Tg (n=5)</td>
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<td>0.009</td>
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</tr>
<tr>
<td>NTg (n=5)</td>
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<td>0.000</td>
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<td>0.000</td>
</tr>
<tr>
<td>P value</td>
<td>0.05</td>
<td>0.10</td>
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Cardiomyocyte mitotic indices are significantly enhanced in infarcted transgenic hearts. Percent of cardiomyocytes that are positive for phosphorylated histone H3 (H3P), a mitosis-specific marker, at 3 weeks and 3 months in the periinfarct/left ventricle (LV) and distal infarct/right ventricle (RV) regions. Measurements were confirmed under confocal microscopy.

At both time points, a greater number of mitoses were noted in the periinfarct zone compared with the distal zone of transgenic hearts. Conversely, no mitotic cardiomyocytes were noted in nontransgenic hearts at all time points. Confocal images of mitotic cardiomyocytes in the periinfarct zone of transgenic hearts are shown in Figure 2A.

Mitoses were also noted in the infarct zone in small, αSA-positive cells (~5 μm), which had a high nuclear to cytoplasmic ratio. Three mid-ventricular transverse sections from each infarcted zone of the nontransgenic hearts (n=5) and cyclin A2 transgenic hearts (n=5) were thoroughly examined under confocal microscopy at 3 weeks and 3 months. There were small cells coexpressing αSA and H3P identified in the infarct zone. At 3 weeks, there was an average of 0.4 cells/mouse coexpressing αSA and H3P in the nontransgensics, and an average of 3.4 cells/mouse coexpressing αSA and H3P in the transgensics. At 3 months, there was an average of 0.6 cells/mouse coexpressing αSA and H3P in the nontransgensics, and an average of 6.5 cells/mouse coexpressing αSA and H3P in the transgensics. They were not noted at 1 and 2 weeks post-MI in either group. Confocal images of these small cells that coexpress H3P and αSA noted in the infarct zone of transgenic hearts are illustrated in Figure 2B.

We sought to further characterize the small cells (potentially representing cardiomyocytes in early stages of differentiation) that were noted in the infarct zone. ABCG2, a member of the ATP-binding cassette transporter family of proteins, is well-established as a marker of side-population cells. These have been found in a variety of adult tissues and are thought to represent a class of pluripotent stem cells in which expression of ABCG2 diminishes as differentiation proceeds. ABCG2 has recently been shown to be expressed in cardiac progenitor cells, with the highest levels of expression in mice noted at embryonic day 8.5. Confocal analysis was used to detect αSA and ABCG2 commounfluorescence. Small cells that coexpressed both markers were noted in both transgenic and nontransgenic infarct zones at 2 weeks post-MI but not at 1 week post-MI (Figure 3A through 3F). They were noted to occur with equal frequency in transgenic...
and nontransgenic hearts, however mitoses in these small cells were predominantly limited to transgenic hearts as described above. In some sections, the typical membrane-expression pattern of ABCG2 was noted (Figure 3A, 3C, 3D, and 3E) whereas other sections exhibited a cytoplasmic localization (Figure 3B). Bright field microscopy was used to identify ABCG2 expression as further confirmation of specific signal and to exclude nonspecific autofluorescent signal (Figure 3F, a serial section adjacent to that pictured in 3D). An analysis of cKit-expressing stem cells was performed in transgenic and nontransgenic hearts at 2 weeks post-MI (n=3 for each group). Clusters of cKit-positive cells with high nuclear to cytoplasmatic ratio were seen occurring at equal frequency in both transgenic and nontransgenic infarct zones (data not shown).

The observation of small cells coexpressing aSA and ABCG2, representative of cardiac progenitors, in the infarct zones of both transgenic and nontransgenic hearts, but the significantly enhanced mitotic indices and parameters of cardiac function noted in transgenic hearts was indicative of increased cycling of cardiac progenitors in transgenic mice. As we previously determined that nuclear localization of cyclin A2 is associated with cardiomyocyte mitosis,18 cyclin A2 expression was assessed in both transgenic and nontransgenic hearts at 2 weeks post-MI. Nuclear expression of the cyclin A2 transgene protein product is only detected in early postnatal development, and by 2 weeks of age it is localized mainly in the cytoplasm of transgenic hearts (cyclin A2 is not detectable after postnatal day 2 in nontransgenic in either location). Cyclin A2 protein was detected in transgenic infarct zone cardiomyocyte nuclei, but not in nontransgenic infarct zones (Figure 4A and 4B). As the mice underwent infarction at 8 weeks of age, they were 10 weeks old at the time of this analysis. This indicates that the presence of cyclin A2 in the nuclei of cardiac progenitors of transgenic mice is associated with the enhanced cycling of cardiomyocytes in this model.

To examine the enhanced proliferative potential of the transgenic cardiomyocytes, cells were isolated from PN2 and PN7 transgenic and nontransgenic mice and cultured for 4 to 7 days, and examined for mitotic activity by staining with anti-aSA and anti-H3P. Cells at this stage of development were chosen for this analysis as they should closely mimic the immature cardiomyocytes noted in the infarct zones of the adult mouse hearts. A significantly enhanced mitotic index was noted in the PN2 transgenic cardiomyocytes compared with nontransgenic (0.089±0.010 versus 0.014±0.008, P<0.001; Figure 5A and 5B). Several cells from transgenic hearts were undergoing cytokinesis (Figure 5C, 5D, 5E, and 5F), whereas this was not noted in the cells from nontransgenic hearts. At PN7, occasional mitoses were noted among the cultured transgenic cells (Figure 5G, 5H, 5I, and 5J), but no mitoses were noted in the nontransgenic cells. When cardiomyocytes were cultured for 4 weeks with equal numbers plated for both transgenic and nontransgenic cells, a beating syncytium was observed in the transgenic culture (supplemental data) but no beating was observed in the nontransgenic cardiomyocytes.

**Discussion**

The overwhelming worldwide specter of heart failure has prompted a flurry of studies to identify mechanisms to promote cardiac repair, in particular focusing on the applicability of stem cells in this process. We postulated that harnessing the cell cycle machinery would promote cardiac repair. We have recently characterized cyclin A2 as a critical regulator of the cardiomyocyte cell cycle.18 Constitutive cardiac expression of cyclin A2, normally silenced in the heart after birth, induced cardiac hyperplasia that persisted through adulthood with increased mitoses noted in postnatal transgenic mice. Mitoses were associated with the nuclear localization of cyclin A2, which undergoes a shift in subcellular distribution to the cytoplasm as development proceeds. In nontransgenic mice, cyclin A2 was never detected after birth in either nuclei or cytoplasm.18

In this transgenic mouse model, we now demonstrate that cyclin A2 is able to mediate cardiac repair by inducing mitoses in the infarct zone, periinfarct zone, and distal myocardium after MI. The transgenic mice had markedly improved EF at 3 weeks and 3 months post-MI compared with nontransgenic mice, with significantly diminished ventricular remodeling. The lack of significant differences in EF between the groups at 1 week.
post-MI helps define a time-course for recovery. Mitoses were not noted before 2 weeks post-MI.

Mitoses noted in small cells of the infarct zone which also expressed αSA were predominantly noted in the transgenic hearts and raised questions regarding the source of such cells. Given their small size and high nuclear to cytoplasmic ratio, they appeared to represent cardiac progenitor cells. To further characterize these cells, ABCG2 expression was analyzed as it is a known marker of side-population cells, and has recently been shown to identify early cardiac progenitors. Interestingly, cells that coexpressed ABCG2 and αSA were noted with equal frequency in both transgenic and nontransgenic at 2 and 3 weeks post-MI. However, mitoses in small cells expressing αSA were not noted in nontransgenic infarct zones. Further studies examining colocalization of ABCG2 and H3P are needed in these immature cardiomyocytes.

Figure 3. ABCG2 Expression as a marker of SP cells. ABCG2 was found on putative cardiomyocyte progenitor cells in the infarcted hearts. ABCG2, a member of the ATP-cassette transporter family of proteins, has been shown to be a marker of SP cells that can be found in the myocardium. In A, C, D, and E, we have noted the presence of membrane ABCG2 localization (depicted by red signal) in what appear to be de novo cardiomyocytes (depicted by the green fluorescence signal for αSA). This analysis was performed using confocal microscopy. In B we note what appears to be cytoplasmic ABCG2 localization. To ensure we are not identifying nonspecific autofluorescent signals, ABCG2 expression was confirmed by DAB immunohistochemistry and identified using bright field microscopy (F).

Figure 4. Cyclin A2 expression localizes to nuclei of de novo myocytes of infarcted transgenic hearts. A, Coimmunofluorescence of cyclin A2 and αSA. B, The same section with coimmunofluorescence of DAPI and αSA. Red = cyclin A2, Green = αSA, Blue = DAPI staining of nuclei.
Nuclear expression of cyclin A2 was noted in transgenic infarct-zone cardiomyocytes but not in the nontransgenics. As the mice were 10 weeks of age for this analysis, and transgenic mice do not exhibit nuclear expression of cyclin A2 beyond 2 weeks of age, these cardiomyocytes appeared to represent immature cardiomyocytes. These results indicate that cardiomyocytes derived from ABCG2-expressing progenitors in the infarcted myocardium recapitulate the developmental paradigm noted in the early postnatal cyclin A2 transgenic hearts, that is mitosis is potentiated in postnatal cardiomyocytes expressing cyclin A2. Thus it appears that cyclin A2 is directing increased rounds of mitosis of the “immature” cardiomyocytes in the infarct zones of the transgenic mouse hearts. Additionally, the cell cycle reentry of periinfarct myocardium indicates a retention of perhaps a more “plastic” phenotype in the transgenic heart, possibly mimicking that observed in urodele amphibians such as the newt. Furthermore, postnatal (immature) transgenic cardiomyocytes in culture exhibit a significantly higher mitotic index than nontransgenic cells and even undergo cytokinesis, thus reinforcing the role of cyclin A2 in cardiac repair. Even after 4 weeks in culture, transgenic cardiomyocytes had formed a beating syncytium, a phenomenon that suggests greater contractility and survival may be associated with cyclin A2 independent of its effects on proliferation.

A previous report by Pasumarthi et al has demonstrated infarct regression in transgenic mice expressing cyclin D2 in the myocardium. Their data suggested that modulation of the D-type cyclins could be exploited to promote regeneration in injured hearts. We chose to focus our efforts on cyclin A2 as it is the sole cyclin regulating the two major transitions of the cell cycle, both G1/S and G2/M, and plays an essential role in development. It is the only cyclin to undergo postnatal silencing in rats, humans, and mice, and constitutive expression of

![Figure 5. Cyclin A2 expression drives proliferation of postnatal cardiomyocytes in culture. A and B depict cardiomyocytes dispersed from PN2 transgenic and nontransgenic hearts, respectively. Blue=DAPI staining of nuclei, Green=αSA, Red=H3P. Both panels depict merged images of all 3 signals. Note absence of H3P staining in B. In C through E, blue, green, and red signals are depicted individually and the merged image is depicted in F representing a PN2 transgenic cardiomyocyte undergoing cytokinesis with visualization of the contractile ring. G through J represent a PN7 transgenic cardiomyocyte undergoing mitosis, with blue, green and red signals depicted individually in G through I and the merged image in J.](image-url)
cyclin A2 in the myocardium elicits an upregulation in the D-type cyclin expression^1^ level as well as inducing a shift in the subcellular localization of cyclin B1 from cytoplasm to nucleus. Cyclin A2 thus appears to represent a master regulatory gene in cardiomyocyte cell cycle control.

Therefore, we have shown that targeted expression of this single gene, cyclin A2, a critical mediator of cardiomyocyte mitosis, can lead to repopulation in the infarct zone by new cardiomyocytes, cell cycle reentry of periinfarct myocardium and augment endogenous regenerative processes by potentially inducing side-population cell-derived cardiac progenitors with enhanced proliferative capacity. We have also demonstrated that cyclin A2 elicits significant recovery of myocardial function in a serial manner.

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Disclosures

Hina W. Chaudhry and Debra J. Wolgemuth are inventors on two patents pending from Columbia University on the use of cyclin A2 (to H.W.C.- K08 HL067048-03 and to D.J.W.).

References


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