Therapeutic Delivery of Cyclin A2 Induces Myocardial Regeneration and Enhances Cardiac Function in Ischemic Heart Failure

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Background—Heart failure is a global health concern. As a novel therapeutic strategy, the induction of endogenous myocardial regeneration was investigated by initiating cardiomyocyte mitosis by expressing the cell cycle regulator cyclin A2.

Methods and Results—Lewis rats underwent left anterior descending coronary artery ligation followed by peri-infarct intramyocardial delivery of adenoviral vector expressing cyclin A2 (n = 32) or empty adeno-null (n = 32). Cyclin A2 expression was characterized by Western Blot and immunohistochemistry. Six weeks after surgery, in vivo myocardial function was analyzed using an ascending aortic flow probe and pressure-volume catheter. DNA synthesis was analyzed by proliferating cell nuclear antigen (PCNA), Ki-67, and BrdU. Mitosis was analyzed by phosphohistone-H3 expression. Myofilament density and ventricular geometry were assessed. Cyclin A2 levels peaked at 2 weeks and tapered off by 4 weeks. Borderzone cardiomyocyte cell cycle activation was demonstrated by increased PCNA (40.1 ± 2.6 versus 9.3 ± 1.1; P < 0.0001), Ki-67 (46.3 ± 7.2 versus 20.4 ± 6.0; P < 0.0001), BrdU (44.2 ± 13.7 versus 5.2 ± 5.2; P < 0.05), and phosphohistone-H3 (12.7 ± 1.4 versus 0 ± 0; P < 0.0001) positive cells/hpf. Cyclin A2 hearts demonstrated increased borderzone myofilament density (39.8 ± 1.1 versus 31.8 ± 1.0 cells/hpf; P = 0.0011). Borderzone wall thickness was greater in cyclin A2 hearts (1.7 ± 0.4 versus 1.4 ± 0.4 mm; P < 0.0001). Cyclin A2 animals manifested improved hemodynamics: Pmax (70.6 ± 8.9 versus 60.4 ± 11.8 mm Hg; P = 0.017), max dP/dt (3000 ± 588 versus 2500 ± 643 mm Hg/sec; P < 0.05), preload adjusted maximal power (5.75 ± 4.40 versus 2.75 ± 0.98 mWatts/µL²; P < 0.05), and cardiac output (26.8 ± 3.7 versus 22.7 ± 2.6 mL/min; P = 0.004).

Conclusions—A therapeutic strategy of cyclin A2 expression via gene transfer induced cardiomyocyte cell cycle activation yielded increased borderzone myofilament density and improved myocardial function. This approach of inducing endogenous myocardial regeneration provides proof-of-concept evidence that cyclin A2 may ultimately serve as an efficient, alternative therapy for heart failure. (Circulation. 2006;114[suppl I]:I-206–I-213.)

Key Words: cyclin ■ heart failure ■ myocardial regeneration

Heart failure has become a major global health concern. Current therapies ranging from revascularization to remodeling to replacement are limited and variably effective. Cellular cardiomyoplasty, with its myriad forms of cell types and delivery routes, has shown great experimental promise and some benefit in early clinical application.

An effective endogenous repair strategy would be a theoretically ideal therapy. However, because of the post-mitotic state of adult cardiomyocytes, the predominant myocardial response to injury is cardiomyocyte hypertrophy. Although recent research has identified a putative population of resident cardiac progenitor stem cells, the native role of these cells during injury is clearly clinically insufficient for maintaining cardiac function and the potential role of the manipulation of these cells is still highly uncertain. A mechanically more attractive approach is to attempt to induce native cardiomyocytes to reenter the cell cycle and replicate. Multiple potential cell cycle regulators such as cyclins and cyclin-dependent kinases (cdk) serve as potential therapeutic targets. Manipulation of the restriction point control cyclin D has shown initial promise.

Cyclin A2 possesses a unique role in its 2-point control of the cell cycle, first by interacting with cdks 1 and 2 to control the G1/S transition into DNA synthesis and then by interacting with cdks 1 and 2 to control the G2/M entry into mitosis. We have previously demonstrated that constitutive expres-
sion of the cell cycle regulator cyclin A2 in a transgenic mouse yields robust postnatal cardiomyocyte mitosis and hyperplasia. To examine the potential role of this regenerative strategy as a therapy for heart failure, a cyclin A2-expressing adenoviral vector was constructed and delivered to rat hearts after left anterior descending coronary artery infarction and subsequent evidence of cardiomyocyte proliferation, peri-infarct geometric enhancement, and cardiac functional improvement were observed.

Methods

Animal Care and Biosafety

Adult, male Lewis rats weighing 250 to 300 grams were obtained from Charles River Laboratories (Boston, Mass). Food and water were provided ad libitum. This study was performed in accordance with the standard humane care guidelines of the Guide for the Care and Use of Laboratory Animals and the Institutional Animal Care and Use Committee of the University of Pennsylvania, which conform to federal guidelines.

Induction of Heart Failure

Male Lewis rats (n = 64) were anesthetized with intraperitoneal ketamine (75 mg/kg) and xylazine (7.5 mg/kg), endotracheally intubated with a 14-gauge angiocatheter, mechanically ventilated (Hallowell EMC, Pittsfield, Mass) and supplemented with 1.5% isoflurane as maintenance anesthesia. A left fourth intercostal space thoracotomy was performed and the pericardium was incised. The left anterior descending coronary artery (LAD) was identified and encircled with a 7-0 prolene suture at the level of the left atrial appendage. The suture was briefly snared to confirm adequate consistent ligation, as evidenced by blanching of the arterial region of distribution, and then permanently ligated. This highly reproducible method causes an infarction of 30% of the left ventricle. The animals were closed in 3 layers over a temporary thoracostomy tube and allowed to recover for six weeks. Over the course of 6 weeks, the animals predictably progressed into ischemic cardiomyopathy. A subset of animals received serial intraperitoneal injections of bromodeoxyuridine (BrdU; BD Pharmingen San Diego, Calif) at a concentration of 80 μg/kg at 4-day intervals to examine DNA synthesis.

Adenoviral Vector Delivery

Replication-deficient (E1, E3 deleted) adenoviral vectors containing murine cyclin A2 (Pubmed gene bank ID X75483) driven by the cytomegalovirus promoter were made by the University of Iowa Gene Transfer Vector Core (Iowa City, Iowa). Corresponding empty replication-deficient adenovirus with null content was used as a control. At the time of coronary ligation, animals were randomized to either therapy with adeno-cyclin A2 or adeno-null injection. Immediately after LAD ligation, a total of 3×10⁹ plaque forming units were injected into 5 predetermined regions in the peri-infarct borderzone as previously described.

Confirmation of Protein Expression

In vivo expression of the cyclin A2 protein was confirmed in a subset of normal noninfarct animals (n = 8/group) by direct intramyocardial injections of adeno-cyclin A2 or adeno-null vector into the cardiac apex. Myocardial tissue was harvested at 3 days, 1 week, 2 weeks, 3 weeks, and 4 weeks and frozen immediately in liquid nitrogen. Myocardium was subsequently dounced in homogenization buffer consisting of 50 mmol/L tris/HCl (pH 7.5), 100 mmol/L NaCl, 5 mmol/L EDTA, 1% v/v Triton X-100, 1 mmol/L NaF, 1 mmol/L Na₂VO₄, 0.2 mmol/L phenylmethylsulfonyl fluoride, 10 μg/mL leupeptin, and 10 μg/mL aprotinin. Lysates were cleared by centrifugation at 12,000 rpm for 10 minutes at 4°C and analyzed for protein content via the Bradford method (BioRad, Hercules, Calif). Then, 40 μg of each protein sample was then denatured at 70°C for 10 minutes and electrophoresed onto a 4% to 12% sodium dodecyl sulfate-polyacrylamide gel. Equal loading of protein was verified by Coomassie blue staining. Proteins were transferred to Immobilon-P PVDF membranes (Millipore, Bedford, Mass) and immunoblotting was performed using a mouse anti-cyclin A monoclonal antibody (Abcam, Cambridge, Mass). This antibody has been tested in mice and has demonstrated cross-reactivity with murine cyclin A. Proteins were visualized via horseradish peroxidase conjugated anti-mouse antibody (Amersham Biosciences, Piscataway, NJ) and chemiluminescence detection (Amersham Biosciences). Immunohistochemistry was used to confirm protein expression within the harvested hearts; 10-μm thin myocardial sections were fixed in 4% paraformaldehyde, permeabilized with Triton X-100, and blocked with 5% normal goat serum for 2 hours at room temperature, followed by incubation with rabbit anti-human cyclin A (1:600; Abcam, Cambridge, Mass) overnight at 4°C. Antibody cross-reactivity with mouse and rat cyclin A has been demonstrated by the manufacturer. The slides were washed 3 times in phosphate-buffered saline and incubated with FITC-conjugated goat anti-rabbit secondary antibody (1:1000; Abcam) for 45 minutes at room temperature. Cyclin A2 expression was quantitated in 4 fields/specimen of the region of interest of both cyclin A2 and null hearts by fluorescence microscopy (40× air magnification; Leica Microsystems, Wetzlar, Germany).

Tissue Section Preparation

Six weeks after infarction and before explantation, the right atrium was incised and the hearts were perfused and rinsed with 10 mL phosphate-buffered saline via the aortic root with resultant blanching of the myocardium. The hearts were arrested in diastole, distended with Optimum Cutting Temperature embedding compound at a fixed distending pressure, and snap-frozen in liquid nitrogen. Explanted hearts were analyzed in a blinded fashion. Sequential transverse 10-μm tissue sections were made at the level of the papillary muscles. Sections were immediately fixed in 4% paraformaldehyde, washed, and blocked in 5% normal goat serum/phosphate-buffered saline.

Immunohistochemical Assessment of Cardiomyocyte DNA Synthesis and Proliferation

Myocardial thin sections were fixed, permeabilized, and blocked as described. Immunohistochemical analysis of cyclin A2 DNA synthesis was performed by staining for PCNA (1:600 rabbit anti-human PCNA; Abcam) and Ki-67 (1:500 goat anti-mouse (Ki-67; Santa Cruz Biotechnology, Santa Cruz, Calif) as indicators of DNA synthesis and cellular proliferation. Positive nuclei were co-localized to α-sarcromeric actin (1:700 mouse anti-rabbit α-sarcomeric actin; Sigma Aldrich, St. Louis, Mo) to confine PCNA and Ki-67-positive staining specifically to cardiomyocytes. The α-isofor of sarcomeric actin will label only α-cardiac muscle actins and α-skeletal muscle actins, and will not stain smooth muscle actin. Dual-positive cells in 4 high-power peri-infarct fields were then quantitated in a blinded fashion for each specimen and averaged (40× air magnification; Leica Microsystems, Wetzlar, Germany).

From the subset of animals that had received serial injections of BrdU, tissue sections were processed as detailed and BrdU staining was analyzed. After fixation and antigen retrieval, tissue sections were incubated with a biotinylated mouse anti-BrdU antibody (1:600; BD Pharmingen, San Diego, Calif). Streptavidin-horseradish peroxidase was used to detect the presence of antibody via the Diaminobenzidine (DAB) substrate system. (BrdU detection assay; BD Pharmingen).

Immunohistochemical Assessment of Mitosis

Sections were analyzed for mitosis using the nuclear mitosis marker, phosphohistone-H3 (1:600 mouse anti-human phosphohistone-H3; Upstate Biotechnology, Lake Placid, NY). Dual-positive staining for both phosphohistone-H3 and cardiomyocytes localized to DAP-stained nuclei was performed as described. The number of dual-
positive stained cells was counted in a blinded fashion in 4 high-power fields per specimen.

**Measurement of Ventricular Geometry**

Tissue sections were obtained at the level of the papillary muscle. The sections were stained with hematoxylin and eosin to delineate morphology. Digitized photomicrographs were taken with a Nikon Coolpix 4300 camera using standardized imaging distances. Geometric measurements were then computed in a blinded fashion using Scion Image Beta Release 4 (Scion Corporation, Frederick, Mass) from 2 representative tissue sections for each animal. The left ventricular diameter was measured in 2 perpendicular axes and averaged for each animal. The wall thickness of the left ventricular borderzone was measured and analyzed from 2 separate areas of each tissue section and averaged for each animal. The borderzone was defined as 1 field lateral to myocardial scar. The wall thickness index was calculated for each specimen and was defined as the ratio of borderzone wall thickness to remote normal myocardial wall thickness × 100.

**Assessment of Myofilament Density**

Myocardial borderzone hematoxylin and eosin-stained tissue sections were imaged and myofilament density was analyzed per high-power field. Myofilament density was defined as the total myocytes per high-power field. Four high-power fields were quantitated and averaged per tissue section in both borderzone and remote noninfarcted areas. Data analysis was performed in a blinded fashion, recorded, and analyzed for statistical significance.

**Assessment of Hemodynamic Function**

Six weeks after initial LAD ligation, a median sternotomy was performed and hemodynamic measurements were obtained using an ascending aortic Doppler flow probe and an intraventricular pressure-volume catheter. A 2.5-mm peri-aortic flow probe (Model 2.5PSL492; Transonic Systems Ithaca, NY) was placed around the ascending aorta to measure the cardiac output. A 2-French pressure-volume conductance microcatheter (model SPR838; Millar Instruments Houston, Tex) was volume and pressure calibrated and placed via the left ventricular apex into the left ventricular cavity. Hemodynamic measurements were analyzed in a blinded fashion utilizing Chart v4.1.2 software (AD Instruments, Colorado Springs, Colo) and ARIA1 Pressure Volume Analysis software (Millar Instruments). In addition to steady-state hemodynamic measurements, contractility was measured from pressure-volume relationships obtained via preload reduction after occlusion of the inferior vena cava. Volume measurements were calibrated by 2-point linear interpolation with fixed-volume cuvettes of heparinized rat blood, and parallel conductance was excluded with the hypertonic saline injection technique.

**Statistical Analysis**

Statistical analysis was performed using JMP IN 5.1 software using 2-way ANOVA to test for differences among means. All results were expressed as means ± SEM. Statistical significance was determined as a P < 0.05.

**Statement of Responsibility**

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

**Results**

**Adenoviral Expression**

Western blot analysis of cyclin A2 protein levels demonstrated statistically significant cyclin A2 levels in the adenocyclin A2 samples as compared with adeno-null controls. As expected, adeno-null control samples did not contain measurable amounts of cyclin A2 protein. In the experimental animals, cyclin A2 protein reached peak levels at 14 days and tapered off by 28 days (Figure 1). Fluorescent immunocytochemical labeling confirmed cyclin A2 protein levels as early as 3 days after adenoviral injection. Immunocytochemical labeling for cyclin A2 was absent in the adeno-null animals at all time points, and absent in the adeno-cyclin A2 animals at 28 days.

**Cardiomyocyte Proliferation**

PCNA expression colocalized to α-sarcomeric actin stained cells was significantly upregulated in cyclin A2 animals compared with null controls, suggesting borderzone cardiomyocyte cell cycle activation. Ki-67 expression colocalized to α-sarcomeric actin-stained cells was likewise significantly upregulated in adeno-cyclin A2 animals compared with null controls, also suggesting borderzone cardiomyocyte cell cycle activation. Furthermore, BrdU uptake studies demonstrated a significant increase in the proportion of BrdU-positive cells per 1000 cardiomyocytes with adeno-cyclin A2 therapy, thereby confirming active DNA synthesis (Table 1). Colocalization of phosphohistone-H3 expression to α-sarcomeric actin stained cells in cyclin A2 animals compared with null controls was significantly elevated, confirming cardiomyocyte mitosis (Figure 2). These cellular proliferation studies provided multiple independent measures of cell cycle activation and cardiomyocyte mitosis with adeno-cyclin A2 therapy. These data demonstrate convincing evidence for cardiomyocyte proliferation with cyclin A2 activation.
Myofilament Density
Myofilament density analysis demonstrated a statistically significant increase in peri-infarct borderzone myofilaments in the adeno-cyclin A2 treatment animals as compared with null control animals. Quantitation of myofilament density in the remote, normal myocardium demonstrated no change in myofilament density or structure between the treatment and control groups. The similarity in remote myofilament composition and structure validated the assay and denoted similar baseline myocardial structure (Figure 3).

Ventricular Geometry
Computerized planimetric analysis of ventricular geometry demonstrated a statistically significant increase in borderzone wall thickness in the adeno-cyclin A2 treated animals as compared with the adeno-null treated animals (1.8±0.04 versus 1.4±0.04 mm; P<0.001). The calculated wall thickness index (defined as the percent ratio of borderzone wall thickness to remote normal myocardial wall) was significantly increased in cyclin A2 experimental tissue sections as compared with controls (73.9±6.7 versus 62.4±5.4; P<0.001). Enhanced preservation of left ventricular geometry and diameter (8.8±0.2 versus 9.9±0.1 mm; P<0.001) was noted in the adeno-cyclin A2 group compared with control animals (Figure 4).

This combination of enhanced borderzone wall thickness and preservation of ventricular geometry increases the potential for enhanced myocardial function.

Cardiac Function
Myocardial functional analysis 6 weeks after LAD ligation indicated a statistically significant increase in cardiac output as measured by Doppler analysis of ascending aortic blood flow in the adeno-cyclin A2 animals as compared with adeno-null animals. Dynamic pressure-volume analysis showed a statistically significant improvement in maximum generated ventricular pressure (Pmax), maximum dP/dT, elastance, and preload-adjusted maximal power with adeno-cyclin A2 therapy. Also, the ventricular volumes appear to be smaller in the cyclin hearts. There was no difference in the baseline heart rate between the treatment and control groups. Therefore, it appears that the adeno-cyclin A2-treated animals had statistically significant preservation of cardiac function compared with control animals (Table 2). Cyclin A2-treated animals also had significantly improved cardiac contractility, as indicated by an increased slope of the pressure–volume relationship during caval occlusion as compared with controls (0.75±0.19 versus 0.57±0.18; P=0.048). Representative pressure–volume loops from each group are shown in Figure 5.

Discussion
In infarcted hearts, targeted expression of the cell cycle regulator cyclin A2 induced cardiomyocyte mitotic activity, increased borderzone myofilament density, and improved ventricular function.

Controlling myocardial cell cycle regulation is a complex interaction between cyclins, cyclin-dependent kinases, cdk inhibitors such as p57, p21, p27, and other regulators including retinoblastoma protein, PCNA, and E2F.21–23 The cyclins, which play a central role in cell cycle control, form an
appealing therapeutic target for inducing cardiomyocyte replication. Subsequent endogenous myocardial regeneration would seem an intuitively well-matched therapy to address necrotic and apoptotic cardiomyocyte loss in heart failure.

Cyclin A2 is unique in its control of both major transitions of the cell cycle at G1/S and G2/M. Cyclin A2 is the only cyclin that is completely silenced after birth in mice,13 rats,24 and humans.24 This disappearance of cyclin A2 occurs at a rate consistent with the rate of withdrawal of cardiomyocytes from the cell cycle.24 A targeted deletion of cyclin A2 in the mouse exhibited embryonic lethality at embryonic day 5.5.25 A recent study has demonstrated that the development of the majority of mouse tissues can occur independently of all 3 D-type cyclins.26 It has also been shown with a double knockout that cdk4 and cdk6 are not essential for cellular proliferation.27 It has also been reported that E-type cyclins are largely dispensable for normal development in mice.28 Both E1 and E2 knockout mice (−/−) developed normally. The D-type cyclins control restriction point movement into DNA synthesis and have been studied as a potential therapeutic target; however, the potential for cell cycle arrest before mitosis may be a limitation.29 Thus, cyclin A2 expression offers enhanced potential for inducing cell cycle re-entry given its control of the G2/M transition in addition to G1/S and makes cyclin A2 a particularly attractive therapeutic target.

We have previously demonstrated biologic marker evidence of cardiomyocyte mitosis and histomorphologic evi-
dence of cardiomyocyte hyperplasia in a transgenic mouse expressing cyclin A2. Up to 70% increase in the calculated number of cardiomyocytes was noted, as well as a corresponding increase in heart weight-to-body weight ratio. These encouraging findings have prompted us to develop a therapeutic strategy based on inducing cardiomyocyte cyclin A2 expression.

In our model, cyclin A2 adenoviral expression peaked at 2 weeks and tapered off by 4 weeks. This limited duration of adenoviral expression may be of utility; providing cyclin A2 expression during an active period of adverse post-infarction ventricular remodeling when the presence of additional functional cardiomyocytes may be of particular benefit. Potential detrimental hypertrophy from excessive hyperplasia or perhaps neoplasia from prolonged cyclin A2 expression consequently may be avoided, because we did not observe any nests of aberrant appearing cells in any of the specimens. Furthermore, our examinations of transgenic animals constitutively expressing cyclin A2 for as long as 1.5 years did not reveal any neoplastic transformation.

We examined multiple biologic markers of cellular proliferation in this study. While PCNA and Ki-67 are well-accepted indicators of cellular proliferation, phosphohistone-H3 expression is a very precise assay of mitosis and thus serves as the ultimate marker of the desired product of cell cycle re-entry. The complete absence of phosphohistone-H3 in borderzone and remote myocardium in our control animals confirmed the post-mitotic state of the adult rat heart. The expression levels in the remote uninjured myocardium were equivalent between null and cyclin A2 groups, thereby excluding any systemic factors to which differences could potentially be attributed. Additionally, each

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N=18 for each subset.

Figure 5. Pressure–volume loops. Improved contractility was measured in cyclin A2-treated animals as compared with control animals (P-V slope 0.75±0.19 mm Hg/µL vs 0.57±0.18 mm Hg/µL). Representative pressure–volume loops are shown.
heart was matched to an internal control to confirm that the therapy was localizable and contained within the region of administration. BrdU labeling further verified that DNA synthesis actively occurred within cardiomyocytes.

This rationale was extended to the comparison of myofilament density. The remote myocardium revealed equivalent myofilament density among groups. Ischemic injury resulted in a notable decrement in myofilament density which was partially rescued with cyclin A2 therapy, yielding a 25% increase in density. Interestingly, the observed increase in borderzone wall thickness in cyclin A2 hearts equaled a 23% improvement. Although the absolute values may appear less than significant, on a relative scale, a 25% increase in myocytes is substantive. Additionally, in a heart which at baseline is only millimeters thick, any increase may be sufficient to yield considerable improvement in functional capacity. These histologic improvements were mirrored in multiple functional parameters.

Two theoretical limitations of this study warrant discussion. In using an adenoviral expression system to effect a nuclear process, a question arises as to whether an adenoviral gene product will undergo appropriate processing and targeting to attain appropriate intranuclear localization. One study reported that the fusion of a nuclear localization signal to an adenoviral expressed cyclin D1 was necessary to target the cyclin D1 to the nucleus and prevent cytoplasmic accumulation, which precluded cell cycle activation.31 A more recent study questioned the necessity of such a nuclear localization signal and postulated that the observation of cytoplasmic accumulation had more to do with the adenoviral delivery protocol and an overwhelming multiplicity of infection with the in vitro cardiomyocyte model used in the Tamamori-Adachi study.31 Furthermore, 3 other studies of adenoviral-mediated expression of indirect cell-cycle regulators E2F,21 E1A,32 and FGF-533 have all demonstrated cell cycle re-entry to some degree. These studies imply that adenovirally transcribed and translated proteins can be appropriately processed by intrinsic post-translational modification and subcellular localizing machinery and thus can target and impact nuclear events.

The second limitation of this study pertains to the proposed existence of a putative resident cardiac progenitor stem cell population. Although the data in this study are consistent with the induction of native cardiomyocyte cell cycle activity and replication, there exists the theoretical possibility that it is in fact this stem cell population that may have been activated, thus providing a second potential mechanism to explain the degree of cardiac repair noted as a result of cyclin A2 administration. This putative mechanism is being investigated further. If such a population of stem cells is in fact being stimulated with this cyclin expression strategy, this may provide an unexpected but added benefit.

In this report, the therapeutic strategy of myocardial gene transfection to express cyclin A2 induced cardiomyocyte cell cycle activation. This activation yielded increased borderzone myofilament density and improved myocardial function. This approach details proof-of-concept evidence that can be further used to design a novel therapeutic strategy to administer or activate cyclin A2 and, hence, endogenous myocardial regeneration may be an attainable goal in the near future to limit the morbidity and mortality of human heart failure.

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Disclosures
None.

References


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