Cell cycle withdrawal limits proliferation of adult mammalian cardiomyocytes. Therefore, the concept of stimulating myocyte mitotic divisions has dramatic implications for cardiomyocyte regeneration and hence, cardiovascular disease. Previous reports describing manipulation of cell cycle proteins have not shown induction of cardiomyocyte mitosis after birth. We now report that cyclin A2, normally silenced in the postnatal heart, induces cardiac enlargement because of cardiomyocyte hyperplasia when constitutively expressed from embryonic day 8 into adulthood. Cardiomyocyte hyperplasia during adulthood was coupled with an increase in cardiomyocyte mitosis, noted in transgenic hearts at all time points examined, particularly during postnatal development. Several stages of mitosis were observed within cardiomyocytes and correlated with the nuclear localization of cyclin A2. Magnetic resonance analysis confirmed cardiac enlargement. These results reveal a previously unrecognized critical role for cyclin A2 in mediating cardiomyocyte mitosis, a role that may significantly impact upon clinical treatment of damaged myocardium.

It is well established that the proliferative and hence, regenerative potential of adult mammalian cardiomyocytes is quite limited (1). This view has recently been challenged by a few reports, which contend that adult ventricular myocytes, under certain circumstances, appear to re-enter the cell cycle and proliferate (2–4). The potential to reactivate cardiomyocyte proliferation through manipulation of putative cellular regulators or conversion of pluripotent stem cells to cardiac myocytes (5–7) offers an exciting impetus for the design of novel therapeutic interventions to enhance cardiac function during disease conditions. The bulk of evidence maintains, however, that mammalian cardiomyocytes cease to proliferate in the early neonatal period (8–12), which is followed by an increase in cell size. In the murine heart, cardiomyocyte division is reportedly completed by birth, with DNA synthesis through postnatal day 3 contributing only to binucleation (13). The cessation of myocyte proliferation is attributed to an arrest of the cell cycle (14). In accordance with this hypothesis, adult rat cardiomyocytes have been shown to display a dual cell cycle blockade, with ≈80% of cells arresting in G0/G1 and 15–20% of cells arresting in G/M (14–16).

Progression through the cell cycle is tightly regulated and involves cyclins complexed with their catalytic partners, the cyclin-dependent kinases (cdks). A Among the cyclins, cyclin A2 is unique in that it regulates progression through two critical transitions: cyclin A2 complexed with cdk2 is essential for the G1/S transition and cyclin A2/cdk1 promotes entry into mitosis (17, 18). It is also the only cyclin to be down-regulated at both the message and protein level in rat, human (19), and mouse (the present study) cardiomyocytes, coincident with withdrawal of cardiomyocytes from the cell cycle (19). We hypothesized that down-regulation of cyclin A2 may thus play a crucial role in cardiomyocyte cell cycle exit, and conversely, that continued expression of cyclin A2 in the heart would result in altered cell division and importantly, cardiomyocyte hyperplasia.

EXPERIMENTAL PROCEDURES

Generation of Transgenic Mice—Mouse cyclin A2 cDNA was subcloned into a vector (clone 26 from Dr. Jeffrey Robins, University of Cincinnati, Cincinnati, OH) containing α-myosin heavy chain (MHC) promoter and the human growth hormone polyadenylation site (20). Transgenic mice were then generated according to previous protocols (21). All manipulations were performed according to Institutional Animal Care and Use Guidelines. Pups were screened for the presence of the transgene by genomic DNA blot hybridization (21) using cyclin A2 cDNA as a probe. Six lines of transgenic mice were established on a B6CBA background. Phenotypic characterization in this study was carried out using the F1 generation. All analyses in this study were performed on transgenic mice using nontransgenic littermates as controls.

Assessment of Heart Size/Body Weight Ratios—Each mouse was weighed and then anesthetized with avertin. 3 M KCl was injected into the beating heart to induce diastolic arrest. Hearts were gently perfused with 1× phosphate-buffered saline, and fat tissue was removed before weighing the hearts. Heart to body weight ratios were measured for postnatal day (PN) 7 and 14 and adult (3–18 months).

Assessment of Cell Sizes—Whole ventricular sections from adult (6 months) mice were fixed in 4% paraformaldehyde and embedded in paraffin. Sequential transverse sections (4 μm) were cut and stained with hematoxylin and eosin. Digital pictures were captured at ×40 magnification, and cell-analysis software (UTHSCSA Image Tool) was employed to measure cross-sectional areas of myocytes. At least 200

[1] The abbreviations used are: cdk, cyclin-dependent kinase; DAPI, 4′,6-diamidino-2-phenylindole; MHC, α-myosin heavy chain; PN, postnatal day; E, embryonic day; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; H3P, phosphorylated histone-3; N, non-transgenic; Tg, transgenic; HW/BW, heart weight to body weight ratio.
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cells/heart were measured for each of two lines (lines 1 and 58). Only cells with clearly delineated borders were measured. Cell lengths were measured utilizing the same program after immunostaining for pan-cadherin (Sigma), a structural protein found in the intercalated discs (22), again at ×40 magnification. Only those myocytes (>200 cells/heart) that could be visualized end-to-end were assessed.

Assessment of Myocyte Number—Ventricles were dissected from 10 transgenic and 10 control hearts and weighed. The average ventricular weight/total heart weight ratio was computed for both transgenic and control hearts and used to compute ventricular weight for the hearts used in measurements of myocyte cross-sectional area and length. Ventricular weight was multiplied by the known value for specific gravity of muscle tissue, 1.08 gm/ml to obtain ventricular volume (23). The ratio of myocyte ventricular volume was multiplied by 0.82 to determine the fraction occupied by myocytes (24). The average volume of each myocyte was calculated by multiplying myocyte cross-sectional area by length. The number of myocytes per ventricle was computed by dividing the myocyte fraction of ventricular volume by the average myocyte volume.

Northern Blot Analyses—RNA was isolated from total hearts using TriReagent (Sigma). RNA samples (10 μg/lane) were electrophoresed on denaturing 0.8% agarose/2.2% formaldehyde gels and transferred to nitrocellulose membranes according to standard protocols (25). The 1.7 kb cyclin A2 cDNA (25) was radiolabeled with [γ-32P]UTP and hybridized overnight at 65 °C in 4× SSC, 0.1% SDS, and 1 mg/ml salmon sperm DNA. Blots were washed at 65 °C in 2× SSC, 0.1% SDS, and signal was visualized by autoradiography at −70 °C. The integrity of RNA samples was established by staining blots with methylene blue (25).

Immunoblot Analyses—Heart tissue was homogenized in tissue lysis buffer, protein lysates were prepared, and immunoblots performed according to standard methods described by Soonpaa et al. (26). Blots were probed with antibodies to cyclin A2 (1:200), cdk2 (1:300), cyclin B1 (1:400), cyclins D2 (1:200), and D3 (1:200), p21 (1:200), p27 (1:100) (Santa Cruz Biotechnology), and cdk1 (1:300, Upstate Biotechnology, Lake Placid, NY). Detection with GAPDH antibody (1:200, Roche Applied Science) was used as a loading control. Subcellular fractionation was performed using the Nuclear Extraction Kit (Active Motif, Carlsbad, CA). Lamin A/C antibody (1:500, Santa Cruz Biotechnology) was the loading control for nuclear fractions. Signal was visualized by autoradiography using enhanced chemiluminescence according to the manufacturer's protocol (Amersham Biosciences). Autoradiograms were scanned and analyzed by Kodak One-Dimensional Image Analysis software (Eastman Kodak, Rochester, NY). Net intensity values were calculated by subtracting the background within the area measured for each band from the total intensity within this same measured area.

Immune Precipitations—Hearts were homogenized in tissue lysis buffer and processed as above. The following is a modification of Basu and Draetta (27). Homogenate from each sample (250 μg) was preadsorbed with protein A-Sepharose beads (50 μl of a 1 mg/ml suspension, Amersham Biosciences) for 1 h at 4 °C. The beads were removed by centrifugation, and the preadsorbed homogenate was reacted with 1 μg of antibody to cyclin A2 in a final volume of 750 μl in tissue lysis buffer. The mixture was incubated with rocking for 1 h at 4 °C. 50 μl of protein A beads were added, and the incubation was continued for an additional 1 h. Immune complexes were collected by centrifugation, washed three times with PBS buffer, and eluted in SDS-PAGE loading buffer containing 1 mM N-ethylmaleimide (to limit light chain migration) for 10 min at 95 °C. Samples were resolved on 12% SDS-PAGE gels, electroblotted to nitrocellulose, and subjected to immunoblot analysis as described above with antibodies to cdk1 and cdk2.

Histone H1 Kinase Assays—Preadсорbed tissue homogenate was prepared as above using cdk lysis buffer (16) and reacted with 1 μg of antibody to cyclin A2 with rocking for 1 h at 4 °C. 50 μl of protein A beads were added, and the incubation was continued for an additional 1 h at 4 °C. The immunocomplex beads were washed three times with 1 ml of cdk lysis buffer, then centrifuged, and the pellet was resuspended in 1 ml of 1× kinase buffer (50 mM Tris-Cl, pH 7.5, 1 mM MgCl2, 1 mM MnCl2, 10 mM NaF, 1 mM Na3VO4,containing 125 μM/ml histone H1 substrate protein and incubated for 5 min at 30 °C. To each reaction tube, 5 μl of ATP buffer was added and incubated for 10 min at 30 °C. Samples were resolved on a 12.5% SDS-PAGE gel, the gel was dried, and exposed to x-ray film overnight at −70 °C. Net intensity quantification was performed as described above.

Assessment of Cardiomyocyte DNA Synthesis—Whole ventricular sections from embryonic (E18), postnatal (PN2, PN7, PN14) and adult (6 month) hearts were fixed and embedded in paraffin as described above. Sequential transverse (4 μm) sections were cut and analyzed by immunohistochemistry as described previously (21), using antibody (1:100) to PCNA (BD Pharmingen, San Diego, CA) as an indicator of DNA synthesis (28). To localize PCNA-positive nuclei in cardiomyocytes, co-immunofluorescence (29) staining was performed utilizing α-sarcomeric actin (1:200, Sigma). Similar fields were compared at ×40 magnification, and the number of cardiomyocyte nuclei staining positively for PCNA were counted per field. The total number of cardiomyocyte nuclei per field were counted, and the ratio of PCNA-positive nuclei to the total number of nuclei was computed. This ratio was averaged over at least ten fields to compute the index of PCNA-positive nuclei.

Assessment of Cardiomyocyte Nuclei per Unit Area—Whole ventricular sections from 6-month-old mice from lines 1 and 58 were prepared for immunostaining (29) with α-sarcomeric actin to delineate cardiomyocytes as was performed on ventricular sections at various developmental stages. Cardiomyocytes were identified with antibody to α-sarcomeric actin as above. Anti-rabbit rhodamine ( Molecular Probes, Eugene, OR) was used as the secondary antibody for the H3P antibody, and anti-mouse IgM fluorescein isothiocyanate (Sigma) was used as the secondary against α-sarcomeric actin. The number of cardiomyocyte nuclei staining positively for H3P were counted per field at ×40 magnification under fluorescent field optics. The total number of cardiomyocyte nuclei per field were counted, and a mitotic index was computed as the ratio of H3P-positive nuclei to total nuclei. The mitotic indices for at least ten fields were averaged at each developmental stage analyzed. Rotational analysis for localization to cardiomyocytes was performed using confocal microscopy through 10-micron thick sections.

Isolated Cardiomyocyte Preparations—Isolated ventricular cells were dissociated from hearts obtained from PN7 transgenic and PN7 strain-matched wild-type mice using 0.07% trypsin/1 mM EDTA in Hank's solution, a modification of previously described methods (31). Whole hearts were dissected, minced into 1 mm cubes, and placed onto microtitre plates (Active Motif, Carlsbad, CA) to minimize fibroblast concentration, fixed with 4% paraformaldehyde, and examined by immunofluorescence staining with antibody (1:200) to α-sarcomeric actin and DAPI.

Assessment of Cardiac Function—MRI image acquisition and analyses were performed on a 9.4 Tesla Bruker WB400 microimaging system with 30 mm of quadrature RF coil (Bruker NMR Inc., Bellerica, MA). The mice were anesthetized with isoflurane (1.5% vol. in 2 liters/min air flow). The heart rate was ~450 bpm. Quantitation of ventricles was based on bright blood two-dimensional image stacks that were acquired using ECG-gated fast gradient echo cine sequence. The acquisition parameters were 250 ms repetition time, 1.8 ms echo time, 30° flip angle, 0.7 mm slice thickness, and 45° phase-encoding step size with a 3 sec scan time. Eight cardiac points were sampled over the cardiac cycle. The short axis images were acquired from which the left ventricle and myocardium was semi-automatically segmented using region-growing algorithm and histogram-based thresholding (32). Data are expressed as mean ± S.E. Student's t test was used for data comparison, using a significance level of p < 0.05.

RESULTS

To demonstrate that cyclin A2 is silenced in the mouse heart shortly after birth concomitant with withdrawal from mitosis, the relative levels of cyclin A2 mRNA and protein expression were assayed at selected times during murine cardiac development. Northern blot analysis revealed that cyclin A2 transcripts of 3.0 and 1.7 kb were observed at E12, E18, and PN2, but not at 6 weeks of age (Fig. 1A). Immunoblot analysis could not detect cyclin A2 protein at PN2 and later time points (Fig. 1B). The number of ventricular cardiomyocyte nuclei expressing cyclin A2 protein as detected by immunohistochemical analysis was high at E14 with a noticeable decline at E18, a further diminution at PN2, and complete absence by PN14 (Fig. 1C). This temporal pattern of decreased expression of cyclin A2 mRNA and protein levels is consistent with the previously described silencing of cyclin A2 in the hearts of rats and humans shortly after birth (19), and also coincides with cardiomyocyte cell cycle withdrawal (8–12).
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To test the hypothesis that sustained cyclin A2 expression would modulate cardiomyocyte proliferation, transgenic mice that constitutively express cyclin A2 in the cardiomyocyte lineage were generated (21). The MHC promoter was chosen because it is expressed from E7.5, throughout embryogenesis, and continues to be expressed through adulthood (20) (Fig. 2A).

Eight founders were identified after screening 60 pups derived from microinjected embryos, and six gave rise to transgenic lineages. Cyclin A2 transgenic mice were fertile, appeared healthy, and were not prone to alteration in morbidity and mortality over 1.5 years of observation. Cyclin A2 expression was assessed in at least three hearts from each line at 5–7 months of age by Northern analysis, with a representative blot shown in Fig. 2B. Lines 1, 2, 44, and 58 consistently express the expected cyclin A2 transgenic 2.3 kb mRNA, although there was some variation in the levels of expression from animal to animal within the same line. Other adult organs, such as the kidney (Fig. 2B and data not shown), failed to show cyclin A2 expression in transgenic mice, consistent with the previously described myocardial specificity of the MHC promoter (20).

Interestingly, a 3.0-kb band is also visualized in transgenic heart mRNA. This size is consistent with the larger endogenous cyclin A2 transcript and is not seen in control heart mRNA. Cyclin A2 protein expression, assayed by immunoblot analysis, was confirmed in all 4 lines above (data not shown), but line 58 was selected for more detailed analysis. Cyclin A2 protein expression is shown in transgenic hearts from line 58 at ages PN14 and 2 months and is absent in control hearts at both time points (Fig. 2C).

To determine if the continued expression of cyclin A2 altered the expression of cdk1 and cdk2, lysates were analyzed by immunoblot analysis (Fig. 2C). An average 1.6-fold and 1.2-fold increase in the levels of cdk1 protein at PN14 and 2 months, respectively, were observed in total transgenic hearts, notably different from the low levels of constitutive expression of cdk1 in controls. Immunoblot analysis demonstrated low levels of expression of cdk2 in control hearts, consistent with studies by others (33). Interestingly, an average 2.5- and 2.1-fold increase in the expression of cdk2 in transgenic hearts was observed at PN14 and 2 months of age, respectively.

To determine the subcellular distribution of cyclin A2 protein, lysates were prepared from nuclear and cytoplasmic fractions at PN7 and PN14. In transgenic hearts, cyclin A2 was noted to be predominantly localized to the nucleus at PN7, but largely in cytoplasm by PN14 (Fig. 2D). No protein was detected in either the nucleus or cytoplasm in control hearts at both ages.

To determine whether this temporally ectopically expressed cyclin A2 actually complexed with its normal cdk1 or cdk2 partner, immunoprecipitation followed by immunoblot analysis was performed using total heart lysates (Fig. 2E). Both cyclin A2-ckd1 and cyclin A2-ckd2 complexes were clearly detected at PN14 in transgenic hearts but never in controls. Cyclin A2-ckd2 complex was still detected at 2 months in transgenic hearts but not in controls. To assess the activity of these complexes, α-cyclin A2 immunoprecipitates were also assayed for histone H1 kinase activity. Histone H1 kinase activity was higher in transgenic hearts at PN7, PN14, and 2 months. At PN14, it was 2.0-fold higher as averaged over three independent assays performed at this stage (Fig. 2F).

Potential alterations in the expression profiles of other cell cycle regulators in response to deregulated cyclin A2 were explored. Interestingly, subcellular fractionation revealed that cyclin B1, the only other mitotic cyclin, was expressed in nuclear extracts of PN7 transgenic hearts in a more robust manner than in cytoplasmic extracts, whereas in the controls, cyclin B1 was detectable only in cytoplasmic extracts (Fig. 2G). Comparable amounts of cdk1-cyclin B1 complexes were noted in both transgenic and control heart protein extracts at PN7 and PN14 (Fig. 2H). There was no change in the levels of the cdk

Fig. 1. Cyclin A2 mRNA and protein expression are developmentally regulated in the normal mouse heart. A, cyclin A2 mRNA expression in normal mouse hearts detected by Northern blot: 6 week heart (6wk-H), 6 week kidney (6wk-K), E12 heart (E12-H), E18 heart (E18-H), PN2 heart (PN2-H). Ethidium bromide-stained ribosomal bands are shown as loading controls. B, immunoblot analysis of cyclin A2 protein expression in normal mouse heart: E17.5, PN2, PN7, and 2 months of age were generated (21). The brown staining indicates positively stained cardiomyocyte nuclei.

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FIG. 2. Transgenic mouse model constitutively expressing cyclin A2. A, diagram of MHC-CYCA2 transgenic construct. B, representative Northern blot analysis of transgenic mice (t23 and t27) and control mice (n1 and n2). H and K indicate heart and kidney, respectively. Blot was re-exposed for a shorter period of time to delineate detail in the t23-H lane. Ethidium bromide-stained ribosomal bands are shown as loading controls. C, immunoblot analysis of cyclin A2, cdkl and cdk2 with GAPDH as loading control in transgenic (Tg) and non-transgenic (N) hearts at PN14 and 2 months of age. HeLa lysate was the positive control. D, subcellular localization of cyclin A2 protein in Tg and N hearts at PN7 and PN14 utilizing nuclear and cytoplasmic protein extracts. GAPDH is the loading control for cytoplasm, whereas Lamin A/C is the loading control for nuclear extracts. E, immunoprecipitation of cyclin A2 complexes in Tg and N hearts at PN14, and 2-month a-cyclin A2-immunoprecipitated complexes were analyzed by immunoblot with a-cdk1 or a-cdk2. HeLa cell lysate was the positive control. F, H1 histone kinase assay of Tg and N heart protein lysates from PN7, PN14, and 2-month NC represents negative control (lysis buffer with no sample), and thymus is the positive control. G, subcellular localization of cyclin B1 protein in Tg and N hearts at PN7 utilizing nuclear and cytoplasmic protein extracts with GAPDH and Lamin A/C controls as described in two dimensions. H, immunoprecipitation of cdkl/cyclin B1 complexes in Tg and N hearts at PN7. α-Cdk1-immunoprecipitated complexes were analyzed by immunoblot with α-cyclin B1 with HeLa lysate as positive control. I and J, immunoblot analysis of p21 and p27 expression in Tg and N heart protein lysates from PN7, PN14, and 2 months of age. K, immunoblot analyses of cyclin D2 and D3 expression in Tg and N hearts.
inhibitor p21 in transgenic versus control hearts at all stages examined (Fig. 2I). However, p27 was significantly up-regulated (Fig. 2J, average for three immunoblots: 2.97-fold at PN7, 1.55-fold at PN14, and 1.48-fold at 2 months). Cyclins D2 and D3 were also found to be up-regulated in transgenic hearts at PN14 and 2 months of age as compared with controls (Fig. 2K).

No gross morphological abnormalities were noted in the transgenic hearts. However, the heart weight to body weight ratio (HW/BW) of adult transgenic mice was significantly increased when compared with controls across lines (1, 2, 44, and 58) that expressed cyclin A2 mRNA. A closer analysis of this phenotype was undertaken in lines 1 and 58. HW/BW ratios were determined at selected ages from post-natal development through adulthood, beginning at PN7 up to 1.5 years of age. There was no significant cardiac enlargement noted at PN7 and PN14 (Fig. 3A). However, the difference between the HW/BW ratios of transgenic versus controls increased with age, with statistical significance noted ≥6 months of age.

Microarray magnetic resonance imaging (MRI) analysis is the most technologically advanced modality for assessing cardiac mass and function, and is the most accurate and reliable method for noninvasively quantifying left ventricular mass and function in mice (34, 35). MRI images at end-diastole at mid-ventricular level were therefore utilized for the assessment of cardiac size in transgenic and gender-matched controls in line 58 at 8 months of age. The transgenic (n = 3) mouse hearts occupied an average of 41.0 ± 0.01% of the chest area compared with 30.5 ± 0.01% by the controls (n = 3, p = 0.0083). The MRI analysis thus confirmed in living hearts that the area of the chest cavity occupied by the heart is larger in the transgenic mice. Staining with Masson trichrome revealed no evidence of increased fibrosis in the transgenic hearts at 6 months of age.

Fig. 3. **Cyclin A2 transgenic mice exhibit cardiac hyperplasia due to an increase in postnatal mitosis.** A, heart enlargement in cyclin A2 transgenic mice. HW/BW ratios (mg/g) of Normal and Transgenic mice were plotted from PN7 through 1.5 years of age. The numbers of Tg and N mice examined at each age are detailed as follows: PN7 and PN14, 16 Tg, 15 N; 3–4mo., 5 Tg, 5 N; 6mo., 10 Tg, 10 N; 8–12mo., 5 Tg, 5 N; 1.5 yr., 1 Tg, 1 N. Asterisks indicate ages at which the HW/BW difference between Tg and N is statistically significant. B, myocyte cross-sectional areas were measured in N versus Tg ventricles (lines 1 and 58, age 6 months) using hematoxylin and eosin-stained sections. C, myocyte lengths measured in N versus Tg (Line 1 and 58, age 6 months) using Pan-cadherin staining. D, PCNA expression in N and Tg hearts at selected time points through development. E, isolated ventricular myocytes at PN7 demonstrate a predominantly mononuclear phenotype in the transgenic hearts. F, detection of H3P in N and Tg myocardium at selected time points through development.
(data not shown), suggesting that an increase in connective tissue did not contribute to increased cardiac size.

To determine if the continued expression of cyclin A2 resulted in hyperplasia, which would contribute to the enlarged heart phenotype, we calculated the total number of ventricular cardiomyocytes present in hearts from lines 1 and 58 at 6 months of age. The first step was to measure myocyte cross-sectional areas and length (Fig. 3, B and C). Myocyte cross-sectional areas were in fact slightly reduced in transgenic hearts (average of 209.7 ± 2.32 μm² versus 228.2 ± 2.29 μm² in line 1, p < 0.0001). In line 58, there was an average cross-sectional area of 193.2 ± 2.04 μm² in transgenic versus 231.0 ± 2.26 μm², p < 0.0001. Myocyte length was found to be shorter in transgenic hearts with an average length of 48.4 ± 0.599 μm in line transgenic versus 51.9 ± 0.583 μm, p < 0.0001. In line 58, there was an average length of 46.2 ± 0.522 μm in transgenic versus 50.2 ± 0.567 μm, p < 0.0001.

The measurements of cardiomyocyte cross-sectional area and length were utilized to calculate the average myocyte volume in representative hearts from line 1 and 58 in which the original measurements were made (Table 1). The quotient of the total ventricular volume and average myocyte volume gave the total ventricular volume and average myocyte volume.

| Heart weight | Ventricular weight | Ventricular volume | Myocyte fraction of ventricular volume | Myocyte volume | Number of myocytes | Average percent increase |
|--------------|-------------------|-------------------|----------------------------------------|----------------|--------------------|------------------------|
| Line 1       | N                  | 0.113             | 0.988                                  | 0.092          | 0.075              | 1.18 ± 0.025 × 10⁻⁷   | 6.36 ± 0.136 × 10⁶     | 43.4                   |
|              | Tg                 | 0.143             | 0.120                                  | 0.113          | 0.090              | 1.02 ± 0.022 × 10⁻⁷   | 9.12 ± 0.195 × 10⁶     | 67.3                   |
| Line 58      | N                  | 0.131             | 0.113                                  | 0.107          | 0.088              | 1.16 ± 0.025 × 10⁻⁷   | 7.59 ± 0.165 × 10⁶     | 67.3                   |
|              | Tg                 | 0.174             | 0.146                                  | 0.138          | 0.113              | 0.893 ± 0.019 × 10⁻⁷  | 12.7 ± 0.310 × 10⁶     | 67.3                   |

As cyclin A2 regulates progression through the G2/M transition in addition to the G/S checkpoint of the cell cycle, we sought to determine whether there was an increase in cardiomyocyte mitoses in our transgenic model utilizing an anti-phosphohistone-3 antibody. H3P on Ser10 is an established marker for chromosome condensation during mitotic prophase in animal cells (30). Histologic sections of hearts at various developmental stages were co-stained with antibodies to α-sarcomeric actin (to identify cardiomyocytes) and H3P. There was a significant increase in the mitotic index (ratio of H3P-positive cardiomyocyte nuclei to total cardiomyocyte nuclei) noted throughout all developmental time points examined (E18, PN2, PN7, PN14, and 6 months) in transgenic hearts (line 58, Fig. 3F). This analysis was repeated in line 1 for E18, PN7, and PN14 with similar results (data not shown). The data from the 6-month time point are not shown because there were only a few scattered mitoses noted in transgenic hearts, and none were noted in controls. The most dramatic elevation in the mitotic index in transgenic hearts was noted at PN7 at which time there was a 5-fold increase in the mitotic index.

Images obtained using confocal microscopy (similar ventricular sections of PN7 N in Fig. 4A and Tg in Fig. 4B) illustrate several stages of mitosis-prophase, prometaphase, and likely anaphase in transgenic cardiomyocytes (Fig. 4, C–E). Rotalational analysis of a 10 micron-thick histologic section further illustrated the presence of a mitotic prometaphase nucleus within an α-sarcomeric actin-stained cardiomyocyte (see Supplemental Data).

Cardiomyocyte number in transgenic versus normal hearts

Cardiomyocytes present in hearts from lines 1 and 58 at 6 months of age. The first step was to measure myocyte cross-sectional areas and length (Fig. 3, B and C). Myocyte cross-sectional areas were in fact slightly reduced in transgenic hearts (average of 209.7 ± 2.32 μm² versus 228.2 ± 2.29 μm² in line 1, p < 0.0001). In line 58, there was an average cross-sectional area of 193.2 ± 2.04 μm² in transgenic versus 231.0 ± 2.26 μm², p < 0.0001. Myocyte length was found to be shorter in transgenic hearts with an average length of 48.4 ± 0.599 μm in line transgenic versus 51.9 ± 0.583 μm, p < 0.0001. In line 58, there was an average length of 46.2 ± 0.522 μm in transgenic versus 50.2 ± 0.567 μm, p < 0.0001.

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DISCUSSION

One possible approach to cardiac regeneration involves manipulation of cellular proteins to promote cell cycle re-entry and proliferation of cardiomyocytes. This approach has received considerable interest in recent years because of the identification of key cell cycle regulatory proteins and several reports suggesting that manipulation of these factors can reactivate DNA synthesis \textit{in vivo} and \textit{in vitro} in the postmitotic ventricular myocardium (36–38). However, previous reports have not directly demonstrated that regulation of these factors can induce cardiomyocyte mitosis once the time line for cell cycle exit and thus terminal differentiation has been surpassed.

A significant gap in our understanding of the cardiomyocyte cell cycle is due to the limited number of studies that explore the effects of putative cellular regulators of the G2/M checkpoint. Cyclin A2 is unique among all cyclins in that it has been
shown to regulate transition through both G1/S and G2/M in cultured cell lines (17). Cyclin A2 is normally silenced in the heart shortly after birth when cardiomyocyte division ceases as the cells withdraw from the cell cycle. This was previously established in rat and human hearts (19), and we have confirmed this in the mouse. The temporal pattern of cyclin A2 mRNA and protein levels implicates a crucial role for cyclin A2 as a regulator of cardiomyocyte cell cycle exit. A recent report describing the effect of inhibiting the Rho family GTPases lends further support to the association of cyclin A2 and cardiomyocyte proliferation (39). That is expression of Rho GDIα, an inhibitor of Rho family proteins, in the mouse myocardium resulted in a decrease in cellular proliferation in the embryonic heart that was associated with down-regulation of cyclin A2.

To test the impact of its deregulated expression on cardiomyocyte proliferation and terminal differentiation, we have generated a mouse model of constitutive cyclin A2 expression in the myocardium. Phenotypic analysis reveals cardiac enlargement in the adult heart due to hyperplasia. Importantly, cardiomyocyte mitoses were significantly enhanced during postnatal development in the transgenic hearts, with the most dramatic difference occurring at PN7. The hyperplasia induced by constitutive cyclin A2 expression arises largely during postnatal development and not primarily during embryogenesis, as is likely the case in several other mouse models of altered or absent cell cycle proteins. Liao et al. (40) noted that cardiac overexpression of cdk2 elicited heart enlargement at PN2 but this did not persist in adults. c-Myc-overexpressing mice exhibited an enlargement in cardiac size that was most profound at 1 and 15 days of age (44 and 46%, respectively), but by 60 days of age, only a 34% increase was noted (24). The investigators concluded that this hyperplasia occurred during fetal development without acceleration of postnatal growth. The p27KIP1 knockout mice exhibited a significant increase in heart weight when compared with wild type as analyzed between 2 and 35 days of age (41). Interestingly, we note elevated levels of p27KIP1 in our transgenic mouse hearts, alluding to a feedback mechanism whereby p27KIP1 regulates cyclin A2 levels in the cardiomyocyte, but p21TIP1 does not.

Cyclin D1 overexpression in the mouse heart has been shown to promote increased DNA synthesis in adult transgenic hearts that resulted in multinucleation, but mitosis was not examined (26). Approximately 40% enlargement was noted when the HW/BW ratios of adult transgenic mice were compared with those of nontransgenic mice (n = 4, age not specified). Although >60% of the adult cyclin D1 transgenic cardiomyocytes exhibited a multinucleated phenotype, the authors concluded that it was unclear whether these cardiomyocytes retained the ability to undergo karyokinesis. This same group of investigators had previously demonstrated that cardiomyocyte division in the normal mouse heart does not occur after birth, with DNA synthesis through PN2 and PN3 contributing only to binucleation (13).

As cyclin A2 has been shown to regulate both G1/S and G2/M in cultured mammalian cell lines (17), we presume it is playing a role in the regulation of both gap phases in vivo. In Drosophila, when regulators of both gap phases are overproduced (i.e. cyclin E and string), cells are unable to compensate for the shortening of both gap phases and the cell cycle as a whole is abbreviated, resulting in small cells with a faster generation time (42). Previous investigators have demonstrated that overexpression of G1- to S-phase cell cycle regulatory proteins decreased cell size in vitro and in vivo (40, 43). This mechanism may account in part for the cardiomyocyte hyperplasia, with smaller cells noted in our model. Although an overall increase in heart size is not observed until the increased numbers of cardiomyocytes found in the postnatal hearts begin to grow, the most dramatic effect of “de-silencing” cyclin A2 occurs after birth, when cardiogenesis is normally complete.

Functional analysis indicated preservation of cardiac function at 3 months of age with a decline in ejection fraction and fractional shortening noted at 8 months of age in the transgenic hearts. This is also consistent with the observation of greater HW/BW differences between transgenic and normal after 5–6 months of age as compared with younger animals. Hyperplastic hearts would be expected to display hypercontractility that would ultimately progress to hypocontractility with age.

In conclusion, constitutive cardiac expression of normally silent cyclin A2 invokes an increase in cardiomyocyte mitosis and hyperplasia in the postnatal heart with resultant cardiac enlargement noted in the adult heart. This model differs from previous mouse models examining altered or absent cell cycle regulators in that it specifically addresses control of the G1/S checkpoint in addition to the G2/M checkpoint. Furthermore, karyokinesis in postnatal cardiomyocytes is specifically demonstrated. The enhanced HW/BW increase in the transgenic heart compared with normal during adulthood suggests that cytokinesis is indeed coupled with mitosis in the transgenic heart. The decline of cardiomyocyte mitoses noted between PN7 and PN14 with only scattered mitoses noted in the adult heart also correlated with a change in the subcellular localization of cyclin A2. The nuclear localization of cyclin A2 appeared to be critical for cardiomyocyte mitosis. Nuclear expression of cyclin A2 further appears to induce nuclear localization of cyclin B1, which normally is detected only in the cytoplasm after birth. Constitutive expression of cyclin A2 also increased expression of the δ-type cyclins, further highlighting a master regulatory role for cyclin A2 in the cardiomyocyte cell cycle. The manipulation of cyclin A2 expression to induce cardiac mitosis, and thus hyperplasia, should serve as a nidus for future studies examining therapeutic application by gene delivery of cyclin A2 to the infarcted, postmitotic myocardium.

Acknowledgments—We thank Drs. Laura Johnston and Jeffrey Holmes for discussions and suggestions related to phenotypic characterization, Dr. Jeffrey Robbins for the α-MHC promoter construct, Dr. Richard Valle for his insights into the stages of mitosis, and Theresa Swayne and the Confocal Microscopy Facility at Columbia Health Sciences for their assistance with confocal analyses.

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