Targeted Expression of Cyclin D2 Results in Cardiomyocyte DNA Synthesis and Infarct Regression in Transgenic Mice

Kishore B.S. Pasumarthi, Hidehiro Nakajima, Hisako O. Nakajima, Mark H. Soonpaa, Loren J. Field

Abstract—Restriction point transit and commitment to a new round of cell division is regulated by the activity of cyclin-dependent kinase 4 and its obligate activating partners, the D-type cyclins. In this study, we examined the ability of D-type cyclins to promote cardiomyocyte cell cycle activity. Adult transgenic mice expressing cyclin D1, D2, or D3 under the regulation of the α cardiac myosin heavy chain promoter exhibited high rates of cardiomyocyte DNA synthesis under baseline conditions. Cardiac injury in mice expressing cyclin D1 or D3 resulted in cytoplasmic cyclin D accumulation, with a concomitant reduction in the level of cardiomyocyte DNA synthesis. In contrast, cardiac injury in mice expressing cyclin D2 did not alter subcellular cyclin localization. Consequently, cardiomyocyte cell cycle activity persisted in injured hearts expressing cyclin D2, ultimately resulting in infarct regression. These data suggested that modulation of D-type cyclins could be exploited to promote regenerative growth in injured hearts. (Circ Res. 2005;96:110-118.)

Key Words: cardiomyocyte proliferation ■ DNA synthesis ■ heart regeneration

Cardiomyocyte death with an ensuing loss of myocardial function is observed in many forms of cardiovascular disease. This progressive decline in cardiac function might be partially abated if the surviving myocardium retained even a limited ability to proliferate. Although it is generally accepted that adult cardiomyocytes retain some capacity to synthesize DNA, there is considerable debate regarding the frequency at which this occurs, and if reinitiation of DNA synthesis necessarily leads to cell division. It is nonetheless clear that the intrinsic regenerative capacity of the adult mammalian heart is insufficient to restore cardiac function after significant injury. Consequently, considerable effort has been invested to study cardiomyocyte cell cycle regulation.

Cell cycle progression is regulated at multiple checkpoints to ensure that all requisite activities (ie, genome reduplication, DNA repair, and chromosome segregation) are completed before initiation of the next phase of the cell cycle. Checkpoint transit is regulated in part by a family of protein kinases (the cyclin-dependent kinases or CDKs) and their activating partners (the cyclins). For example, the initial commitment to a new round of cell division requires transit through the restriction point. Restriction point transit is regulated by CDK4 and the D-type cyclins. CDK4/cyclin D-mediated phosphorylation of members of the RB protein family disrupts RB-E2F binding, thereby permitting E2F-mediated transcription of genes involved in regulating DNA synthesis. Given the fundamental importance of restriction point transit in the activation of DNA synthesis and cell cycle progression, we have previously used the mouse α cardiac myosin heavy chain (MHC) promoter to target expression of cyclin D1. Cyclin D1 expression resulted in high rates of cardiomyocyte DNA synthesis in adult transgenic hearts, and was thus sufficient to drive cell cycle activity in the adult myocardium.

In this study, the capacity of the three mammalian D-type cyclins to drive cardiomyocyte cell cycle activity was directly compared. The MHC promoter was used to target expression of cyclin D1, D2, or D3 to the myocardium in transgenic mice (MHC-cycD1, MHC-cycD2, and MHC-cycD3 mice, respectively). In each case, transgene expression resulted in nuclear accumulation of cyclin D and CDK4 immune reactivity. Similar rates of cardiomyocyte DNA synthesis were detected in uninjured adult hearts in all three transgenic models. Myocardial injury resulted in cytoplasmic accumulation of transgene-encoded cyclin protein in MHC-cycD1 and MHC-cycD3 mice, with a concomitant reduction in cardiomyocyte DNA synthesis levels. In contrast, myocardial injury in MHC-cycD2 mice had no impact on the subcellular localization of transgene encoded cyclin protein, and in some cases, injury enhanced cardiomyocyte DNA synthesis levels. Cardiomyocyte cell cycle activity in MHC-cycD2 hearts was associated with a marked regression of infarct size after permanent coronary artery occlusion. These data indicated that the D-type cyclins are not functionally redundant, and furthermore suggested that modulation of D-type cyclins could be exploited to promote myocardial repair in injured hearts.
Materials and Methods

Transgenic Mice
The MHC promoter\(^8\) was used to target expression of cyclin D1\(^7\), cyclin D2\(^10\), and cyclin D3\(^11\) (Figure 1a). The SV40 transcription terminator/polyadenylation site\(^13\) was inserted after the cyclin cDNA sequences. Transgenic mice were generated as described\(^14\) and maintained in a DBA/2J background. Animal protocols were approved by the Institutional Animal Care and Use Committee. Studies were initiated in 11- to 13-week-old mice.

Western Blot Analyses
Antibodies used for the Western blot analyses\(^15\) were as follows: anti-cyclin D1 (sc-450), anti-cyclin D2 (sc-593), anti-cyclin D3 (sc-182), anti-CDK4 (sc-260), anti-CDK6 (sc-177), anti-CDK2 (sc-163), anti-cdc2 (sc-954), and anti-PCNA (sc-56); and anti-cyclin D2 antibody (CC07). Secondary antibodies were conjugated with horse-radish peroxidase. Signal was visualized by the ECL method.

Northern Blot Analysis
Northern blots\(^16\) used an SV40-specific oligonucleotide probe (5’-CCATGGGCGCAAGCTATCGCATGCTGAGCTCTAG-AGTCGAGGGCCCCGCTTACC-3’) to monitor transgene expression.

Immune Histology
Ten micron cryosections\(^17\) were reacted with anti-cyclin D1 (sc-450), anti-cyclin D2 (sc-593), anti-cyclin D3 (sc-182), anti-CDK4 (sc-260), or anti-phosphorylated histone H3 (07–081) antibodies. Signal were visualized with Vector M.O.M. or Vectastain ABC kits.

Myocardial Injury Models
Myocardial hypertrophy was induced by isoproterenol\(^18\). Cautery injury was performed as described previously.\(^19\) For myocardial infarction (MI), an 8-0 Prolene ligature was secured around the left coronary artery as described previously.\(^20\) In mice, the interventricular septum is spared after ligation of the left coronary artery.\(^21\)

Cardiomyocyte DNA Synthesis Assay
The MHC-nLAC reporter transgene was used to monitor cardiomyocyte DNA synthesis\(^19,22\) by scoring colocalization of blue nuclear β-galactosidase activity and silver grains. The border zone was defined as myocardial tissue within 0.5 mm of the fibrous scar tissue.

Infarct Size Measurements
Hearts were perfusion-fixed (1% paraformaldehyde, 1% cacodylic acid, 1 × PBS), sectioned coronally at 1.2-mm intervals,\(^20\) and stained with Sirius Red-Fast Green. Digital images were captured, and infarct sizes were calculated formulas follows: [coronal infarct perimeter (epicardial-endocardial)/total coronal perimeter (epicardial-endocardial)] × 100.\(^23\)

Statistical Analysis
Data are presented as mean±SEM. Between-group comparisons were analyzed by the nonparametric Mann-Whitney test (MI DNA synthesis), ANOVA, and Bonferroni multiple comparison test (baseline HW/HW, baseline, isoproterenol, and cautery DNA synthesis) or Student \(t\) test (isoproterenol HW/BW and MI size analysis). Significance was assumed at \(P<0.05\).

Results

Generation and Initial Analysis of the MHC-cycD1, MHC-cycD2, and MHC-cycD3 Transgenic Mice
The α-cardiac MHC promoter has been used previously to target expression of a cyclin D1 cDNA to the heart.\(^8\) A similar strategy was analyzed here to target expression of cyclin D2 and cyclin D3 cDNAs (Figure 1a). The MHC promoter comprised 4.5 kb of 5’ flanking sequence and 1 kb of the MHC 5’ untranslated region encompassing exons 1 to 3 up to (but not including) the MHC initiation codon. Thus, the first ATG encountered in transgene-derived transcripts resided in the cyclin D cDNA. To ensure appropriate processing of transgene transcripts, the SV40 early region transcription terminator\(^13\) was inserted after the cyclin cDNA sequences. A total of 9 MHC-cycD1, 8 MHC-cycD2, and 12 MHC-cycD3 lineages were established. Northern blot analyses were used to compare the relative level of transgene expression between MHC-cycD transgenic lines (Figure 1b). Hybridization with an oligonucleotide probe corresponding to the common SV40 terminator sequences revealed roughly similar levels of transgene mRNA expression in the MHC-cycD1, MHC-cycD2, and MHC-cycD3 lines used in this study.
Western blot analyses of nontransgenic hearts demonstrated that all three endogenous D-type cyclins were expressed at high levels in embryonic stage (day 15) and were barely detectable in adult nontransgenic hearts (Figure 1c). The level of cyclin D1 in adult MHC-cycD1 hearts was somewhat higher than that seen in nontransgenic hearts at embryonic day 15. Similarly, the level of cyclin D2 in adult MHC-cycD2 hearts and the level of cyclin D3 in adult MHC-cycD3 hearts were higher than that for the corresponding endogenous cyclin in nontransgenic hearts at embryonic day 15 (Figure 1c). High levels of transgene expression in adult MHC-cycD1 hearts had no detectable effect on expression of the endogenous cyclin D2 and cyclin D3. Similarly, transgene expression did not affect the levels of the endogenous D-type cyclins in the MHC-cycD2 or MHC-cycD3 adult hearts.

The expression of several additional cell cycle regulatory proteins was also monitored (Figure 1c). CDK4 levels (the obligate D-type cyclin binding partner) were elevated in the MHC-cycD transgenic animals, approaching those seen during cardiac development in nontransgenic hearts. PCNA levels were also observed to be increased, but to a much lesser degree. In contrast, transgene expression had no obvious effects on the levels of CDK6, CDK2, and cdc2. In all cases, equivalent sample loading was confirmed via Napthal Blue staining of the membranes before Western blot analyses (see online Figure 1 in the online data supplement available at http://circres.ahajournals.org). Transgene expression was accompanied by a moderate increase in cardiac mass. A 37%, 20%, and 31% increase in the heart weight/body weight ratio was observed for the MHC-cycD1, MHC-cycD2, and MHC-cycD3 mice, respectively, as compared with nontransgenic littermates for each group; see also online Table 1 in the online data supplement).

**D-Type Cyclins Are Nuclear Localized and Promote Ventricular Cardiomyocyte DNA Synthesis in Uninjured Adult Transgenic Hearts**

It has recently been reported that cell cycle withdrawal in neonatal cardiomyocytes was due in part to a relocalization of cyclin D1 from the nucleus to the cytoplasm.\(^{24}\) Immune histological analyses were therefore performed to ascertain the subcellular localization of transgene-encoded cyclin D. No overt D-type cyclin expression was noted in nontransgenic adult hearts reacted with anti-cyclin D1 (Figure 2a), anti-cyclin D2, or anti-cyclin D3 antibodies (not shown). In contrast, robust nuclear cyclin D1 immune reactivity was detected in hearts from adult MHC-cycD1 mice (Figure 2a). Similarly, nuclear cyclin D2 and cyclin D3 immune reactivity was detected in hearts from adult MHC-cycD2 and MHC-cycD3 mice, respectively. CDK4 immune reactivity was also monitored. No overt CDK4 immune reactivity was detected in adult nontransgenic hearts (Figure 2b). In contrast, robust nuclear CDK4 immune reactivity was present in the ventricles of each of the MHC-cycD transgenic hearts.

Given the subcellular localization of D-type cyclin and CDK4, and given the induction of PCNA expression, experiments were initiated to monitor cardiomyocyte DNA synthesis in the MHC-cycD mice. Accordingly, each transgenic lineage was crossed with MHC-nLAC mice, and the resulting MHC-nLAC/MHC-cycD and MHC-nLAC/(-) animals were sequestered. Cardiomyocyte nuclei in mice carrying the MHC-nLAC reporter transgene were readily identified in X-GAL–stained histological sections.\(^{19,22}\) To monitor cardiomyocyte DNA synthesis, the mice were given a single injection of tritiated thymidine and euthanized 4 hours later. The hearts were harvested and sectioned, and the sections were stained with X-GAL and processed for autoradiography. The presence of silver grains over blue nuclei was indicative of cardiomyocyte DNA synthesis.

No cardiomyocyte DNA synthesis was detected in mice that inherited the MHC-nLAC reporter gene only, although cardiac fibroblast DNA synthesis was readily detected (as evidenced by the presence of silver grains over nuclei lacking X-GAL staining; Figure 2c). In contrast, cardiomyocyte DNA synthesis was readily detected in the hearts of the adult D-type transgenic mice (as evidenced by the presence of silver grains over nuclei with blue X-GAL staining, Figure 2c). Elevated levels of cardiomyocyte DNA synthesis was detected in all three transgenic lineages, suggesting that the
activities of the D-types cyclins were functionally redundant under baseline conditions (although levels were somewhat greater in MHC-cycD2 and -cycD3 hearts; Figure 3a; see also online Table 1).

**Myocardial Injury Attenuates Ventricular Cardiomyocyte DNA Synthesis in MHC-cycD1 and MHC-cycD3 Mice, but not MHC-cycD2 Mice**

To determine whether cardiomyocyte DNA synthesis persisted during myocardial hypertrophy, MHC-nLAC(-) and MHC-nLAC/MHC-cycD double transgenic mice were subjected to 7 days of isoproterenol infusion. This treatment resulted in a marked increase in heart weight/body weight ratio (see online Table 1). The isoproterenol-treated mice were given an injection of tritiated thymidine 4 hours before euthanasia, and the hearts were processed as described. Surprisingly, isoproterenol-induced hypertrophy markedly reduced the levels of ventricular cardiomyocyte DNA synthesis in mice inheriting the MHC-cycD1 or the MHC-cycD3 transgene (Figure 3b; see also online Table 1). In contrast, isoproterenol infusion had no impact on cardiomyocyte DNA synthesis in the hearts of adult MHC-cycD2 mice; the level of DNA synthesis in the treated animals was not significantly different from that seen under baseline conditions. In agreement with previous studies, no cardiomyocyte DNA synthesis was detected in the ventricles of isoproterenol-treated mice inheriting only the MHC-nLAC transgene (Figure 3b, see also online data supplement). These data suggested that myocardial injury antagonized cell cycle activity in MHC-cycD1 and MHC-cycD3 mice, but not in MHC-cycD2 mice.

**Injury Alters D-Type Cyclin Subcellular Localization in MHC-cycD1 and MHC-cycD3 Mice, but not MHC-cycD2 Mice**

Western blot analyses of samples prepared from isoproterenol-treated transgenic hearts revealed a slight reduction in steady state cyclin levels in the MHC-cycD1 and MHC-cycD2 hearts, as compared with nontreated transgenic littermates (Figure 4a). Isoproterenol did not affect steady state cyclin levels in MHC-cycD3 mice. Moreover, isoproterenol infusion had no effect on CDK4 levels in any of the transgenic lineages. Thus, reduced cardiomyocyte DNA synthesis after isoproterenol treatment in MHC-cycD1 and MHC-cycD3 hearts was not attributable to decreased accumulation of cyclin and/or CDK4 proteins.

Immune histological analyses were performed to monitor cyclin subcellular localization. Robust nuclear cyclin expression was observed in the ventricles of hearts from both untreated and isoproterenol-treated MHC-cycD2 mice (Figure 4b). In contrast, isoproterenol treatment resulted in a marked redistribution of cyclin immune reactivity in the MHC-cycD1 and MHC-cycD3 mice, with the preponderance of the signal present in the cardiomyocyte cytoplasm. Cyclin D subcellular localization was also examined after cauterity injury. No nuclear cyclin D immune reactivity was detected in the injury border zone of MHC-cycD1 mice at 7 days after cauterization (Figure 4c). Similar results were observed with MHC-cycD3 mice (not shown). In contrast, cyclin D nuclear immune reactivity persisted in the injury border zone in MHC-cycD2 transgenic mice at 7 days after cauterization (Figure 4c). Thus the reduced cardiomyocyte DNA synthesis in isoproterenol-treated or cauterity-injured MHC-cycD1 and MHC-cycD3 hearts correlated with the loss of nuclear cyclin immune reactivity, and cyclin D2 was largely resistant to the signals that underlie this subcellular trafficking.
Cardiomyocyte DNA Synthesis and Infarct Regression in MHC-cycD2 Mice With Permanent Coronary Artery Occlusion

Given the sustained cardiomyocyte cell cycle activity in MHC-cycD2 mice after isoproterenol infusion and cautery damage, we reasoned that these animals might exhibit regenerative growth after a more clinically relevant form of myocardial injury. Accordingly, MHC-nLAC/(-) and MHC-nLAC/MHC-cycD2 transgenic mice were anesthetized, intubated, and subjected to permanent coronary artery occlusion. The mice received tritiated thymidine injections at 7 or 150 days after injury, and hearts were harvested 4 hours later and were processed for X-GAL staining and autoradiography. Cardiomyocyte DNA synthesis was detected at the infarct border zone and in the interventricular septum of hearts from MHC-nLAC/MHC-cycD2 mice at 7 days after injury (Figure 5a). These high levels of cardiomyocyte DNA synthesis persisted for as long as 150 days after injury, the latest date analyzed thus far (Figure 5b; see also online Table 1). In contrast, only very low levels of cardiomyocyte DNA synthesis were detected at the infarct border zone in MHC-nLAC/(-) hearts at 7 days after injury, and no cardiomyocyte DNA synthesis was detected in the interventricular septum. Moreover, no cardiomyocyte DNA synthesis was detected at either site in the MHC-nLAC/(-) hearts at 150 days after injury (Figure 5b; see also online Table 1).

Histological analyses were performed to monitor infarct size in the MHC-nLAC/(-) and MHC-nLAC/MHC-cycD2 hearts (n=10 mice/group per time point). Hearts were harvested at 7 days or 150 days after injury, fixed under physiological pressure, and coronal sections sampled at 1.2-mm intervals were stained with Sirius red (which stained collagen red) and fast green (which stained viable myocardium green). Digital images were acquired and the percentage...
of the myocardium that was infarcted was determined at each 1.2-mm interval using the quantitation approach described by Pfeffer and colleagues. The value from each 1.2-mm interval was then averaged to calculate infarct size for each individual heart, and subsequently for each genotype (Figure 6a). No significant difference in infarct size was apparent between the MHC-nLAC/MHC-cycD2 hearts at 7 days after injury, indicating that expression of cyclin D2 was not cardioprotective during the acute stages of myocardial infarction (MI). At 150 days after injury, a slight trend toward increased infarct size was observed in the MHC-nLAC/ hearts as compared with the earlier time point (Figure 6a), consistent with scar expansion. In contrast, infarct size in the MHC-nLAC/MHC-cycD2 hearts was significantly reduced at 150 days after injury, as compared with hearts at 7 days after injury. Infarct size reduction was observed in both male and female transgenic mice (Figure 6a) and was readily apparent when comparing representative sections from infarcted nontransgenic versus MHC-cycD2 hearts at 150 days after injury (Figure 6b).

The observed cardiomyocyte DNA synthesis at the infarct border zone, as well as the regression in infarct size, suggested that cell cycle activation resulted in regeneration of myocardial tissue after permanent coronary artery occlusion. To further explore this possibility, infarcted MHC-nLAC/MHC-cycD2 hearts were screened for the presence of cardiomyocyte phosphorylated histone H3 immune reactivity (a marker of mitotic cells). Colocalization of X-GAL activity (expressed by the cardiomyocyte-restricted MHC-nLAC reporter transgene) and histone H3 immune reactivity was readily detected (Figure 7a), suggesting that the MHC-cycD2 cardiomyocytes are able to undergo cytokinesis. To further document the presence of regenerative growth, cardiomyocyte number was determined in coronal sections harvested at 1.2-mm intervals from the apex to the base of the heart in nontransgenic and MHC-cycD2 hearts at 7 days and at 150 days after permanent coronary artery ligation. A similar number of cardiomyocytes were seen in sections sampled from nontransgenic hearts at 7 days and 150 days after injury, as well as from MHC-cycD2 hearts at 7 days after injury. In contrast, a marked and significant increase in cardiomyocyte number was observed in sections from the MHC-cycD2 hearts at 150 days after injury (Figure 7b). Cardiomyocyte minimal fiber diameter was similar in nontransgenic mice at 7 days after MI (19.31±0.89 μm), nontransgenic mice at 150 days after MI (17.94±0.79), MHC-cycD2 mice at 7 days after MI (19.95±1.16 μm), and MHC-cycD2 mice at 150 days after MI (18.4±0.98 μm; P>0.05 for all comparisons). Thus, differences in cardiomyocyte morphology did not confound the cardiomyocyte number assay. Interestingly, at 150 days after MI the increased relative area of viable cardiac tissue in apical sections of MHC-cycD2 as compared with nontransgenic mice as measured by Fast Green staining (187±5.3%) was very similar to that obtained by multiplying the total cardiomyocyte number times their average cross sectional area (192±6.5%). Thus, the increased cell number readily accounted for the decreases in infarct size in the MHC-cycD2 mice. Collectively, these data strongly support the notion that expression of cyclin D2 can promote cardiac regeneration after injury.
Several lines of evidence suggested that the activities encoded by the D-type cyclins were very similar. For example, genetic ablation of individual D-type cyclin genes was not lethal, and specific cellular defects were rescued by upregulation or by ectopic expression of other family members. The observation here that cyclin D1, D2, or D3 expression each resulted in a similar effect on heart weight and cardiomyocyte DNA synthesis was consistent with the notion of D-type cyclin functional redundancy (at least under baseline conditions). Other studies, however, suggested that some activities of the D-type cyclins were unique. For example, ectopic expression of cyclins D1, D2, and D3 resulted in distinct proliferative responses in the thymic epithelia of transgenic mice and in cultured cells. The sustained cardiomyocyte cell cycle activity, and the retention of nuclear cyclin D immune reactivity, observed after multiple forms of cardiac injury in MHC-cycD2, but not in MHC-cycD1 and -cycD3 mice, further underscored the notion that at least some aspects of D-type cyclin activity were not functionally redundant.

The strict correlation between nuclear cyclin immune reactivity and cardiomyocyte DNA synthesis suggested that differential subcellular trafficking underlies the phenotypic differences in the MHC-cycD transgenic mice after myocardial injury. This could have occurred as a consequence of differences in the rates of nuclear import and/or nuclear export between cyclins D1, D2, and D3. Altered cyclin stability is not likely to have been a factor, as no overt decrease in steady state protein levels were detected in isoproterenol-treated transgenic hearts (Figure 4a). Regulation of the subcellular localization of the D-type cyclins is complex. It has been shown that D-type cyclins must first associate with CDK4 before nuclear translocation. It has been suggested that p21 and p27 antagonized cyclin D nuclear export, which in turn resulted in enhanced nuclear accumulation. Other studies demonstrated that nuclear export of cyclin D was regulated by glycogen synthase kinase 3β (GSK-3β)-mediated phosphorylation. Phosphorylation of cyclin D1 at threonine residue 286 promoted cyclin D1 association with CRM1 (a nuclear exportin molecule), which in turn resulted in nuclear export of cyclin D1.

The potential impact of cytoplasmic D-type accumulation on the propensity for cell cycle progression in adult cardiomyocytes might also have contributed to the observed phenotypes. In other systems, failure to degrade cell cycle regulatory proteins resulted in cell cycle block. If cytoplasmic accumulation of cyclin D1 and/or D3 imparted a cell cycle block, this process could contribute to the exceedingly low rates of cardiomyocyte DNA synthesis observed in genetically naive, injured adult hearts.

The nuclear cyclin D localization and cell cycle activity observed in uninjured hearts were at odds with recent results from Ikeda and colleagues, who used adenoviral vectors to deliver cyclin D1 and CDK4 to cultured neonatal cardiomyocytes and adult rat hearts. Those investigators demonstrated that cardiomyocyte terminal differentiation was accompanied by cytoplasmic accumulation of cyclin D1. Furthermore, in the absence of a heterologous nuclear localization signal, ectopic cyclin D1 expression in postmitotic cardiomyocytes resulted in cytoplasmic accumulation and no cell cycle activation. Differences in the methods used for gene delivery could explain the discrepancies. The adenoviral delivery
studies used a multiplicity of infection of 100; other studies demonstrated that infection at this MOI resulted in hypertrophic growth of neonatal cardiomyocytes, which was thought to have been triggered by integrin clustering during viral attachment to the cell surface (Allen Samarel, unpublished data, 2004). Thus, adenoviral-induced cardiomyocyte hypertrophy could have resulted in the cytoplasmic accumulation of ectopic cyclin D1, similar to what was observed after injury in the MHC-cycD1 and MHC-cycD3 mice (Figure 4b).

Sustained cardiomyocyte DNA synthesis in injured MHC-cycD2 hearts was associated with infarct size regression, suggesting cyclin D2 expression led to regenerative myocardial growth after infarction. This view was supported by the presence of cardiomyocyte phosphorylated histone H3 immune reactivity and the increased cardiomyocyte cell number in the infarcted MHC-cycD2 hearts at 150 days after injury. Moreover, the absence of detectable levels of cardiomyocyte apoptosis in MHC-cycD2 hearts (0 TUNEL positive cardiomyocyte nuclei per 25,000 screened) in the presence of high rates of cardiomyocyte DNA synthesis indicates that cardiomyocyte cell cycle re-entry does not result in abortive mitosis and apoptosis in these animals. The importance of cell cycle activity in this process was underscored by the absence of overt infarct regression in MHC-cycD1 transgenic hearts after permanent coronary artery occlusion (Kishore B.S. Pasumarthi, unpublished data, 2004). Although the data presented here is mostly consistent with transgene-mediated cell cycle activation in fully differentiated cardiomyocytes, the experiments do not rule out the possibility that the reparative capacity of putative cardiomyogenic progenitor cells was also enhanced by transgene expression. If so, the differentiated phenotype and morphological appearance of the cardiomyocytes at the infarct border zone were most similar to the progenitor-derived cardiomyocytes reported by Schneider and colleagues.

In summary, the data presented in this study indicated that targeted expression of D-type cyclins was sufficient to promote cardiomyocyte cell cycle activation in adult mouse hearts. Cardiomyocyte DNA synthesis persisted in the MHC-cycD2 mice after injury, but not in the MHC-cycD1 or MHC-cycD3 mice. Cell cycle activation in injured MHC-cycD2 hearts correlated with the retention of nuclear cyclin D1 localization. Expression of the MHC-cycD2 transgene resulted in a progressive reduction of myocardial infarct size that was consistent with regenerative cardiac growth. These data suggested that modulation of D-type cyclin activity (as for example via pharmacological interventions aimed at altering subcellular localization or enhancing expression of specific D-type cyclin subtypes) could be exploited to promote myocardial repair in injured hearts.

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On Line Materials and Methods:

**Generation of transgenic mice**

cDNAs encoding cyclin D1 (nucleotide residues 158-1075), cyclin D2 (nucleotide residues 267-1152) and cyclin D3 (nucleotide residues 163-1047) were generated using RT-PCR. The mouse alpha-cardiac myosin heavy chain (MHC) promoter was used to target expression of each cyclin cDNA (Fig. 1a). The SV40 early region transcription terminator/poly-adenylation site (nucleotide residues 2586-2452) was inserted downstream from the cyclin D sequences. The three resulting transgenes (MHC-cycD1, MHC-cycD2, and MHC-cycD3, respectively) were each independently microinjected into inbred C3HeB/FeJ (The Jackson Laboratory, Bar Harbor, ME) zygotes, and transgenic animals were generated using standard methodologies. The resulting transgenic lineages were maintained in a DBA/2J background (The Jackson Laboratory). All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee. All analyses of transgenic mice were initiated at 11-13 weeks of age.

**Western blot analyses**

Hearts were homogenized in a NP-40 buffer, and samples were separated on acrylamide gels and electroblotted to nitrocellulose as described previously. The antibodies used were: anti-cyclin D1 (sc-450), anti-cyclin D2 (sc-593), anti-cyclin D3 (sc-182), anti-CDK4 (sc-260), anti-CDK6 (sc-177), anti-CDK2 (sc-163), anti-cdc2 (sc-954) and anti-PCNA (sc-56); all from Santa Cruz Biotechnology, Santa Cruz, CA) and anti-cyclin D2 antibody (CC07; Oncogene, Cambridge, MA). Secondary antibodies were conjugated with horseradish peroxidase, and signal was visualized by the ECL method according to the manufacturer’s protocol (Amersham, Arlington Heights, IL).
**Northern blot analysis**

Hearts were homogenized in RNA Stat-60 (Iso-Tex Diagnostics, Friendswood, TX). Total RNA (10 ug) was denatured with glyoxal, separated on a 1.2% agarose gel, transferred to a nitrocellulose membrane and hybridized with a radiolabeled oligonucleotide probe as previously described. The oligonucleotide probe (5’-CCATGGCGGCAAGCTCATCGCATGCCTGCAGAGCTCTAGAGTCGACGGGCCC-3’) was specific for the SV40 transcription terminator/poly-adenylation site.

**Immune histology**

Ten micron cryosections were reacted with anti-cyclin D1 (sc-450), anti-cyclin D2 (sc-593), anti-cyclin D3 (sc-182), anti-CDK4 (sc-260) or anti-phosphorylated histone H3 (07-081, Upstate, Lake Placid, NY) antibodies and signals were visualized with a Vector M.O.M. kit (for cyclin D1) or Vectastain ABC kit (for all others) from Vector Laboratories (Burlingame, CA).

**Myocardial injury models**

Myocardial hypertrophy was induced by isoproterenol. Cautery injury was performed as described previously. To induce myocardial infarction, an 8-0 Prolene ligature was placed around the left coronary artery close to the inferior border of the left auricle and tied off as described previously. In mice, the interventricular septum is spared following ligation of the left coronary artery.

**Cardiomyocyte DNA synthesis assay**

The MHC-nLAC reporter transgene was used to monitor cardiomyocyte DNA synthesis as described previously. Analyses were performed 4 hours following tritiated thymidine injection. Cardiomyocyte DNA synthesis was scored by the co-localization of
blue nuclear beta-galactosidase activity and silver grains. The border zone was defined as myocardial tissue within 0.5 mm of the fibrous scar tissue.

**Infarct size measurements**

Hearts were perfusion-fixed in 1% paraformaldehyde, 1% cacodylic acid, 1xPBS at room temperature as described previously\textsuperscript{13}. Hearts were then sectioned coronally on a vibratome at 1.2 mm intervals from apex to base, and representative thin cryo-sections (10 microns) were prepared from each vibratome section and stained with Sirius Red-Fast Green. Digital images were captured and infarct sizes were calculated according to the formula developed by Pfeffer and colleagues:\textsuperscript{16} \[\text{coronar infarct perimeter (epicardial + endocardial)/ total coronal perimeter (epicardial + endocardial)}\] \times 100. To quantitate cardiomyocyte number, coronal sections were stained with silver nitrate\textsuperscript{17} the total number of cardiomyocytes was determined.

**Statistical analysis**

All data are presented as mean ± SEM. Between-group comparisons were analysed by the non-parametric Mann-Whitney test (MI DNA synthesis), ANOVA and Bonferroni multiple comparison test (baseline HW/BW, baseline, isoproterenol and cautery DNA synthesis) or Student t test (isoproterenol HW/BW and MI size analysis). Significance was assumed at \(P<0.05\).

**References:**


## ON LINE SUPPLEMENTAL DATA

### Table 1: Cardiomyocyte attributes in normal and injured MHC-cycD hearts:

<table>
<thead>
<tr>
<th>MICE</th>
<th>BASE LINE HW/BW (mg/gm; % non-txg)</th>
<th>ISO HW/BW* (mg/gm; % base line)</th>
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<tr>
<td>MHC-nLAC / (-)</td>
<td>Parameter value: 100.0 + 7.52</td>
<td>142.6 + 8.78</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td></td>
<td>n: 15 mice</td>
<td>5 mice</td>
<td>100,000 nuc;</td>
<td>100,000 nuc;</td>
<td>25,000 nuc;</td>
</tr>
<tr>
<td>MHC-nLAC / MHC-cycD1</td>
<td>Parameter value: 136.9 + 10.58</td>
<td>134.2 + 6.54</td>
<td>0.09</td>
<td>0.005</td>
<td>0.023</td>
</tr>
<tr>
<td></td>
<td>n: 12 mice</td>
<td>6 mice</td>
<td>100,000 nuc;</td>
<td>100,000 nuc;</td>
<td>25,000 nuc;</td>
</tr>
<tr>
<td>MHC-nLAC / MHC-cycD2</td>
<td>Parameter value: 120.2 + 4.98</td>
<td>146.8 + 9.63</td>
<td>0.26</td>
<td>0.14</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>n: 8 mice</td>
<td>5 mice</td>
<td>100,000 nuc;</td>
<td>100,000 nuc;</td>
<td>25,000 nuc;</td>
</tr>
<tr>
<td>MHC-nLAC / MHC-cycD3</td>
<td>Parameter value: 130.9 + 7.52</td>
<td>148.3 + 8.35</td>
<td>0.22</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>n: 10 mice</td>
<td>5 mice</td>
<td>100,000 nuc;</td>
<td>100,000 nuc;</td>
<td>25,000 nuc;</td>
</tr>
</tbody>
</table>

*: In each case, the ISO HW/BW values were normalized to the non-isoproterenol treated animals with the same genotype.

#: Pooled data from all animals analyzed.

Abbreviations: HW/BW, Heart weight/body weight ratio; ISO, isoproterenol infusion; VENT CM, ventricular cardiomyocytes; SYN, synthesis; nuc, nuclei; N.D., not determined.
Figure 1. Loading control for Western blot analyses. After electrophoretic transfer, all Western blot membranes were stained with Napthol Blue to ensure that similar levels of proteins were analyzed. The image shows a Nathol Blue stained membrane prior to reaction with primary antibody.