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The cardiomyocyte cell cycle

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Abstract

Many forms of cardiac disease are characterized by cardiomyocyte death due to necrosis, apoptosis and/or oncosis. Recently, the notion of promoting cardiac regeneration as a means to replace damaged heart tissue has engendered considerable interest. One approach to accomplish heart muscle regeneration entails promoting cardiomyocyte cell cycle activity in the surviving myocardium. Genetically modified mice have provided useful model systems to test the efficacy of specific pathways to promote cardiomyocyte proliferation in normal and diseased hearts. For example, expression of a heart-restricted dominant interfering version of p193 (an E3 ubiquitin ligase also known as Cul7) resulted in an induction of cardiomyocyte cell cycle activity at the infarct border zone and ventricular septum 4 weeks after permanent coronary artery occlusion. A concomitant reduction in hypertrophic cardiomyocyte growth was also observed in this model, suggesting that cell cycle activation partially counteracted the adverse ventricular remodeling that occurs post-infarction. In other studies, targeted expression of cyclin D2 promoted cardiomyocyte cell cycle activity in adult hearts. The level of cardiomyocyte cell cycle activity increased after myocardial infarction, ultimately resulting in a marked increase in cardiomyocyte number and a concomitant regression of infarct size. Collectively, these data suggest that modulation of cardiomyocyte cell cycle activity can be exploited to promote regenerative growth in injured hearts.

Keywords

cardiac myocyte proliferation; heart regeneration; p193; cul7; cyclin D2

Introduction

Cardiovascular diseases that lead to cardiomyocyte death are frequently associated with a progressive and irreversible reduction in cardiac function, which can ultimately culminate in heart failure (Swynghedauw, 1999). Although it is clear that the adult myocardium retains at least a limited capacity for cardiomyocyte cell cycle re-entry, the fate of these cells with respect to polyploidization, kariokinesis and/or cytokinesis is not entirely clear (Soonpaa and Field, 1998). Recent data suggest that adult-derived stem cells undergoing cardiomyogenic differentiation might contribute to myocardial renewal following injury, however the level at which this occurs is currently highly debated (Dowell et al., 2003). Indeed it has been suggested that previously reported cardiomyogenic differentiation events were due to fusion between stem cells and host cardiomyocytes (Rodic et al., 2004), or alternatively, that cardiomyogenic differentiation failed to occur at all (Chien, 2004). Regardless of the absolute level of regenerative activity intrinsic to the adult heart, or enabled by remote organs, heart failure frequently develops following significant cardiac injury. This underscores the fact that, in the

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absence of intervention, intrinsic regenerative activities are insufficient to stem the tide of cardiomyocyte loss in diseased hearts.

Given that cardiomyocyte loss is the causal etiology in many forms of heart disease, a number of therapeutic strategies have been developed to counteract the initial cell loss following injury, and to repopulate the damaged heart with “replacement” myocytes. To date a number of myocyte replacement strategies have been tested, including direct transplantation of myocytes or myogenic stem cells (Dowell et al., 2003), mobilization of endogenous cardiomyogenic stem cells (Orlic et al., 2001), and the induction of cardiomyocyte cell cycle activity in the surviving myocardium (Pasumarthi and Field, 2002). These approaches are based on the premise that the replacement of cardiomyocytes in diseased hearts would be accompanied by a concomitant restoration of cardiac structure and, more importantly, a rescue of cardiac function.

This report is focused on the induction of cardiomyocyte cell cycle activity as a potential mechanism for cardiac repair. Initially, issues relevant to monitoring cardiomyocyte cell cycle activity in normal and diseased hearts are discussed. In addition, two recently described transgenic mouse models which exhibit enhanced cardiomyocyte cell cycle activity following myocardial infarction are reviewed. In the first model, expression of a dominant interfering version of the E3 ubiquitin ligase p193 (also known as Cul7) was targeted to the myocardium (Nakajima et al., 2004). In the second model, expression of the D-type cyclins was targeted to the myocardium (Pasumarthi et al., 2005). In both cases, induction of cardiomyocyte cell cycle activity was observed after myocardial injury, with a concomitant amelioration of post-injury ventricular remodeling. The potential therapeutic impact of cardiomyocyte cell cycle activation is discussed.

Monitoring cardiomyocyte DNA synthesis in vivo

A numbers of assays have been employed to monitor cardiomyocyte cell cycle activity in adult hearts. In the absence of actual quantitation of cell numbers, these analyses have typically relied upon measuring some aspect of cardiomyocyte DNA synthesis directly (i.e., nuclear cardiomyocyte DNA content in tissue sections, incorporation and subsequent detection of labeled deoxyribonucleotides, etc.) or indirectly (i.e., expression of proteins involved in DNA replication or mitosis). A key issue in such analyses is the criteria used to distinguish cardiomyocyte nuclei from non-myocyte nuclei. Most studies have relied on the simultaneous assessment of a cell cycle marker and a myocyte-restricted gene product, such as components of the myofiber (i.e., myosin, cardiac actin, troponin, etc). The presence of a nuclear cell cycle marker over a cardiac cell body (as identified, for example, by the presence of cytoplasmic myosin immune reactivity) is taken to be indicative of cardiomyocyte cell cycle activity.

While such assays are in principle sound, there are a number of factors that can contribute to the mis-identification of cardiomyocyte nuclei. A major concern comes from the fact that cardiomyocytes comprise less than 20% of the cells present in the adult heart. Consequently, non-cardiomyocyte nuclei can frequently appear on top of cardiomyocyte cell bodies in histologic sections. This potential problem is illustrated by examining sections prepared from MHC-nLAC transgenic mice, which express a nuclear localized beta-galactosidase reporter gene under the regulation of the cardiomyocyte-restricted alpha-cardiac myosin heavy chain promoter (Soonpaa and Field, 1997; Soonpaa et al., 1994). Staining heart sections prepared from these mice with X-GAL (a chromogenic beta-galactosidase substrate) resulted in the accumulation of dark blue signal within cardiomyocyte nuclei, whereas non-myocycte nuclei lacking the beta-galactosidase gene product did not stain. Staining of the same section with Hoechst identified non-cardiomyocyte nuclei, which appeared cyan under fluorescence illumination (cardiomyocyte nuclei did not appear cyan due to quenching of Hoechst

fluorescence by the X-GAL reaction product). Examination of the co-stained sections clearly revealed the presence of non-cardiomyocyte nuclei over cardiomyocyte cell bodies (Figure 1). In the absence of such reporter gene activity, cell cycle activity in noncardiomyocytes which overlie cardiomyocyte cell bodies could easily be mis-identified as originating in the cardiomyocyte. The potential for nuclear mis-identification increases dramatically in injured hearts, where non-cardiomyocytes can exhibit high rates of cell cycle activity. Several reviews have focused on caveats and issues to consider when attempting to quantitate cardiomyocyte cell cycle activity in normal and injured intact hearts (Field, 2004; Soonpaa and Field, 1998).

Characterization of cardiomyocyte cell cycle activity in mice expressing a dominant interfering p193 transgene in the myocardium

Previous studies identified a 193 kD SV40 Large T Antigen binding protein in cardiomyocyte tumor cell lines generated from transgenic mice (Daud et al., 1993). Structure/function experiments revealed that p193 induced apoptosis when expressed in NIH-3T3 cells, and that apoptosis was dependent upon the presence of a sequence containing consensus BH3 and nedd-8 motifs (Pan et al., 2004; Tsai et al., 2000). Other studies indicated that p193 has E3 ligase activity (Dias et al., 2002), and thus is likely to be involved in ubiquitin-mediated protein degradation. Truncation of p193 at amino acid residue 1152 resulted in a molecule, designated 1152stop, with apparent dominant interfering activity. Expression of 1152stop rendered NIH-3T3 cells resistant to apoptosis; moreover, when co-expressed with a dominant interfering p53 molecule, 1152stop rendered embryonic stem cell-derived cardiomyocytes resistant to E1A-induced apoptosis (Pasumarthi et al., 2001).

To determine the impact of the dominant interfering p193 activity in the adult myocardium, transgenic mice were produced which expressed the 1152stop protein under the regulation of the alpha-cardiac myosin heavy chain promoter (Nakajima et al., 2004). Ventricular transgene expression in these mice (designated MHC-1152stop) was observed to initiate around neonatal day 5, and no overt cardiac abnormalities were noted during neonatal development or in adult transgenic hearts. To monitor the level of cardiomyocyte DNA synthesis in un-injured adult transgenic hearts, MHC-1152stop mice were intercrossed with MHC-nLAC mice, and offspring inheriting both transgenes were sequestered. Adult double transgenic mice received a single injection of tritiated thymidine, and hearts were harvested 4 hours later. The hearts were sectioned, stained with X-GAL, and then processed for autoradiography. The presence of silver grains in the autoradiogram (due to the incorporation of tritiated thymidine) over dark blue nuclei (due to reporter transgene activity) was taken to be indicative of cardiomyocyte DNA synthesis, and consequently, cardiomyocyte cell cycle activity. No cardiomyocyte DNA synthesis was seen in uninjured adult MHC-1152stop hearts when a total of 43,236 nuclei were screened (<0.002%); similarly, no cardiomyocyte DNA synthesis was detected in mice carrying the MHC-nLAC transgene alone when 30,048 nuclei were screened (<0.003%). Thus, under base line conditions, expression of the MHC-1152stop transgene had no overt effect on cardiomyocyte cell cycle activity (Nakajima et al., 2004).

Cardiomyocyte DNA synthesis was also monitored in the MHC-1152stop/MHC-nLAC double transgenic mice following myocardial infarction (MI) induced by permanent occlusion of the coronary artery (Nakajima et al., 2004). At 4 weeks post-MI, 0.4% of the cardiomyocytes at the infarct border zone and 0.05% of the cardiomyocytes in the ventricular septum exhibited DNA synthesis (Figure 2A). Cardiomyocytes with phosphorylated histone H3 immune reactivity (Figure 2B), a marker for mitosis (Wei et al., 1998) were also detected. In contrast, mice inheriting the MHC-nLAC transgene alone exhibited DNA synthesis in only 0.008% of the cardiomyocytes at the infarct border zone, and no cardiomyocyte DNA synthesis was observed in the septum when 44,233 nuclei were screened (<0.002%). Thus, expression of the MHC-1152stop transgene permitted cardiomyocyte cell cycle entry following myocardial

injury. These data also underscore the exceedingly low rates of cardiomyocyte cell cycle activity in the absence of growth-promoting transgenes. Interestingly, cardiomyocyte cell cycle activity in the injured MHC-1152stop hearts was accompanied by a marked reduction of hypertrophic cardiomyocyte growth in the ventricular septum (as evidenced by the higher cardiomyocyte nuclear content per unit area tissue, as well as by a reduction in the two-dimensional area of the cardiomyocyte cell bodies, see Figure 2C).

Characterization of cardiomyocyte cell cycle activity in mice expressing a cyclin D2 transgene in the myocardium

Commitment to a new round of cell division requires transit through the restriction point of the cell cycle. Restriction point transit is regulated largely by the activity of the cyclin dependent kinases and their obligate cofactors, the D-type cyclins (Schang, 2003). A series of experiments were initiated to determine if expression of the D-type cyclins would impact upon cardiomyocyte cell cycle regulation in adult hearts. Accordingly, transgenic mice were generated wherein the alpha-cardiac myosin heavy chain promoter was used to drive expression of each of the D-type cyclins (cyclin D1, D2 or D3) in the heart; the individual transgenic lines were designated MHC-cycD1, MHC-cycD2 and MHC-cycD3, respectively. Preliminary analyses revealed that the transgenic hearts were approximately 20-30% larger as compared to their non-transgenic littermates, which was attributable to increased cardiomyocyte numbers (Pasumarthi et al., 2005; Soonpaa et al., 1997).

To determine if cyclin D expression resulted in sustained cardiomyocyte cell cycle activity, adult mice from each MHC-cycD line were intercrossed with MHC-nLAC mice. The resulting double transgenic animals received an injection of tritiated thymidine and were sacrificed 4 hours later. The hearts were then sectioned, and cardiomyocyte DNA synthesis was assessed as described above. Under base line conditions, 0.09%, 0.26% and 0.22% of the cardiomyocytes exhibited DNA synthesis in the MHC-cycD1, MHC-cycD2 and MHC-cycD3 lines, respectively. In contrast, no cardiomyocyte DNA synthesis was observed in mice inheriting the MHC-nLAC reporter transgene alone when 100,000 nuclei were screened (<0.001%).

To determine if DNA synthesis in the cyclin D transgenic mice persisted in response to injury, animals were subjected to 7 days of isoproterenol infusion, a treatment that typically results in a ca. 40% increase in heart weight and development of interstitial fibrosis (Soonpaa and Field, 1994). Isoproterenol-induced injury resulted in a marked decrease in the level of cardiomyocyte DNA synthesis in the MHC-cycD1 and MHC-cycD3 transgenic hearts. In contrast, cardiomyocyte DNA synthesis in the MHC-cycD2 transgenic hearts was largely unaffected by isoproterenol infusion (Pasumarthi et al., 2005). Interestingly, reduction of cardiomyocyte DNA synthesis rates in the isoproterenol-treated MHC-cycD1 and MHC-cycD3 hearts was associated with cytoplasmic localization of the cyclin proteins. In contrast cyclin protein in the isoproterenol-treated MHC-cycD2 hearts was predominantly localized in the nucleus.

To determine if cardiomyocyte DNA synthesis persisted in the MHC-cycD2 transgenic hearts following a more physiologically relevant form of injury, MHC-cycD2/MHC-nLAC double transgenic mice were subjected to permanent coronary artery occlusion. Mice inheriting the MHC-nLAC transgene alone were also subjected to coronary occlusion for comparison. A marked induction in cardiomyocyte DNA synthesis, approaching 1% of the total number of cardiomyocytes after a single injection of tritiated thymidine, was seen at the infarct border zone in the MHC-cycD2/MHC-nLAC double transgenic mice at 7 days post-MI (Figure 3). In contrast, only 0.008% of the border zone cardiomyocyte synthesized DNA in infracted mice carrying only the MHC-nLAC transgene. Relatively high levels of cardiomyocyte DNA synthesis persisted in the MHC-cycD2 hearts for as long as 150 days post-MI, the longest time

point examined. A concomitant increase in the levels of phosphorylated histone H3 immune reactivity was also observed (Pasumarthi et al., 2005). In contrast, no cardiomyocyte cell cycle activity was apparent in the mice inheriting the MHC-nLAC reporter transgene alone at 150 days post-MI.

To determine the impact of sustained cardiomyocyte cell cycle activity on cardiac architecture following MI, MHC-cycD2 mice and their non-transgenic siblings were subjected to permanent coronary artery occlusion and their hearts were harvested at 7 or 150 days post-MI. The hearts were perfusion fixed, and coronal sections were prepared. Sections were sampled at 1.2 mm intervals from the apex to the base, and infarct size was determined using the approach described by Pfeffer and colleagues (Pfeffer et al., 1991). Coronary artery ligation resulted in similar infarct size in MHC-cycD2 and non-transgenic mice at 7 days post-MI, indicating that transgene expression was not acutely cardioprotective. At 150 days post-MI, no reduction in infarct size was noted in the non-transgenic animals; in contrast a marked and statistically significant reduction in infarct size was seen in the MHC-cycD2 hearts (Figure 4A). The reduction in infarct size was accompanied by an increase in cardiomyocyte number in the MHC-cycD2 hearts at 150 days post-MI as compared to 7 days post-MI; in contrast no increase in cardiomyocyte number was detected in the infarcted non-transgenic animals (Figure 4B). Thus, expression of the MHC-cycD2 transgene resulted in apparent regenerative growth of the myocardium following permanent coronary artery occlusion.

Summary and Conclusions

The studies described here demonstrated that cardiomyocyte cell cycle activation can antagonize the adverse ventricular remodeling typically observed following MI. In the case of mice expressing a dominant interfering form of p193, elevated levels of cardiomyocyte DNA synthesis and phosphorylated histone H3 immune reactivity were detected at the infarct border zone and in the surviving septum at 4 weeks post-MI as compared to infarcted non-transgenic littermates. Moreover the marked hypertrophic response seen in cardiomyocytes present in the septum of the infarcted non-transgenic hearts was largely attenuated in the infarcted MHC-1152stop mice. Interestingly, the level of cardiomyocyte cell cycle activity in the septum of the transgenic mice was roughly equal to the level of cardiomyocyte apoptosis, as evidenced by activated caspase 3 immune reactivity (Nakajima et al., 2004). This correlation suggested that cell cycle-induced replacement of cardiomyocytes lost to remodeling-induced apoptosis might have abrogated the necessity for hypertrophic growth in the surviving cardiomyocytes. The improvement in cardiac architecture at 4 weeks post-MI was associated with a modest improvement in cardiac function, as determined by intra-ventricular pressure-volume measurements in intact animals (Hassink et al., 2002).

In contrast to the relatively low levels of cardiomyocyte DNA synthesis seen in mice expressing dominant interfering p193, high rates of cardiomyocyte cell cycle activity were seen in cyclin D2-expressing hearts following MI. Cell cycle induction was associated with an improvement in cardiac architecture by 150 days post-MI, as evidenced by a reduction in infarct size as well as by an increase of cardiomyocyte number. The improvement in cardiac architecture following MI was accompanied by an equally impressive improvement in function; infarcted MHC-cycD2 hearts recovered as much as 80% of the cardiac function present in their age-matched, sham-operated littermates (Hassink et al., Submitted).

These studies support the notion that the restoration of cardiomyocyte numbers in diseased hearts, via induced cardiomyocyte cell cycle activity, can rescue cardiac structure and function. Perhaps not surprising, the level of cell cycle activation appeared to dictate the extent of the therapeutic effect. In the case of MHC-1152stop mice, low rates of cardiomyocyte cell cycle activity appeared to be sufficient to alter post-MI remodeling, but was insufficient to impact

upon gross scar content. In the case of the MHC-cycD2 mice, high rates of cell cycle activity appeared to result in bona fide regenerative growth and the emergence of newly formed myocardium. Moreover, scar tissue near the base of the MHC-cycD2 transgenic hearts was largely resolved by 150 days post-MI (Pasumarthi et al., 2005). These results were consistent with previous observations wherein cell cycle activation resulted in regeneration of myocardial injury in newts (Bader and Oberpriller, 1978) and zebra fish (Poss et al., 2002). Thus, these data suggest that induction of sufficiently high rates of cardiomyocyte cell cycle activity could have a positive impact on cardiac structure and function, even after scar formation.

Finally, it is important to note that quiescent adult cardiomyocytes retained the capacity to respond to the p193 and cyclin D pathways (Nakajima et al., 2004; Tamamori-Adachi et al., 2003). Thus, pathway modulation in genetically naïve adult cardiomyocytes should result in similar reparative effects following myocardial injury. While the data presented above indicated that the pathways could be modulated via gene transfer, a more rationale approach would entail the development of small molecules which are able to mimic the effects of transgene expression. If sufficient cell type specificity could be attained (either via local drug delivery or by taking advantage of unique properties of the p193 and/or cyclin D pathways in cardiomyocytes), then pharmacologic modulation of these pathways might be sufficient to promote therapeutic growth of the myocardium following injury.

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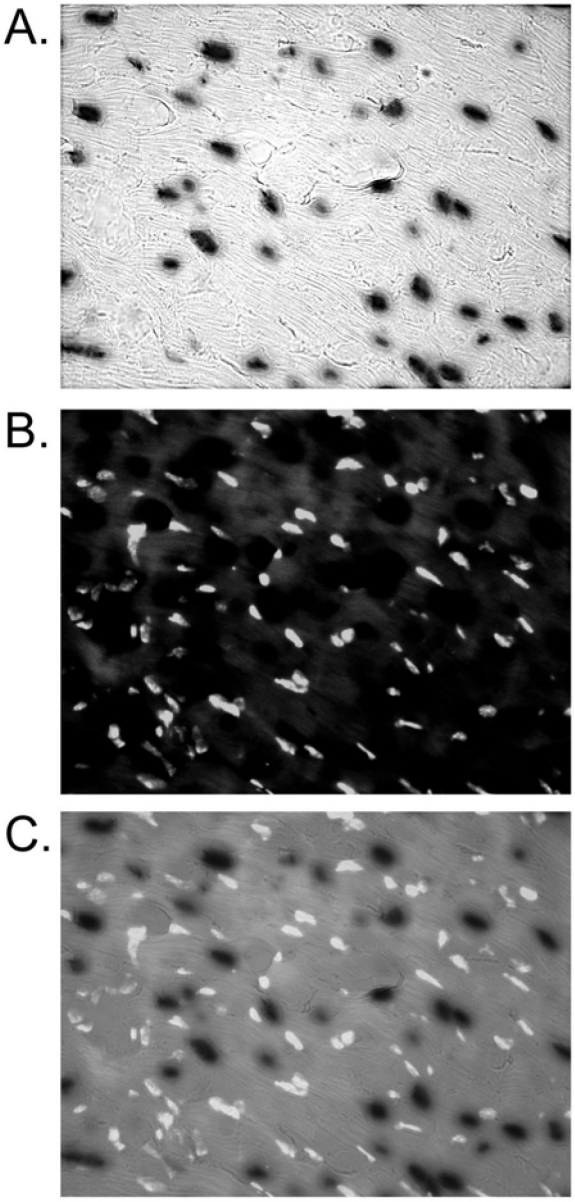

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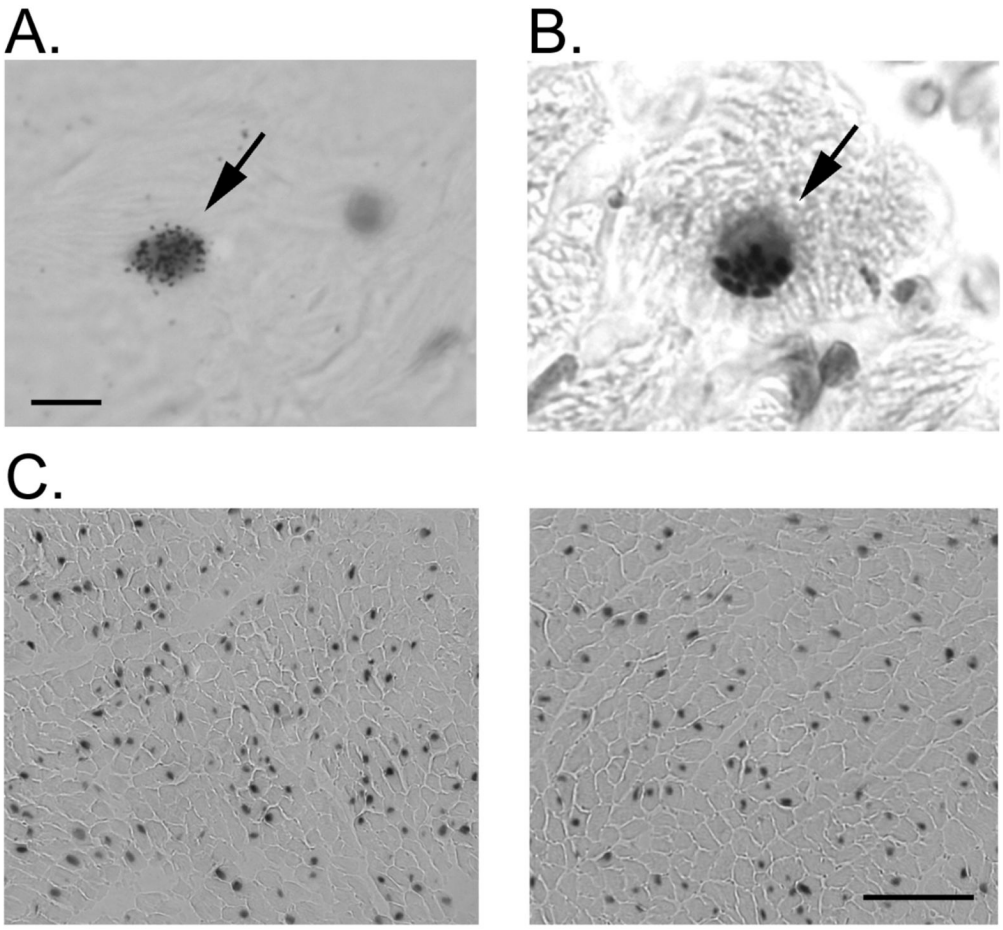
Figure 1.

Heart section (10 microns) prepared from a MHC-nLAC transgenic mouse was stained with X-GAL (Panel A; viewed under bright field illumination) and Hoechst (Panel B, viewed under fluorescent illumination). Cardiomyocyte nuclei stain dark blue (and appear black in the reproduction in Panel A), and non-myocyte nuclei stain cyan (and appear white in the reproduction in Panel B). Panel C shows a merged image; non-cardiomyocyte nuclei can be seen over the cytoplasm of cardiomyocytes.



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Figue 2.

Cardiomyocyte characterization in MHC-1152stop/MHC-nLAC double transgenic mice at 4 weeks post-MI. A) Example of cardiomyocyte DNA synthesis in the interventricular septum of a double transgenic mouse as evidenced by the presence of silver grains over an X-GAL-stained nucleus (arrow; bar = 10 microns). B) Example of cardiomyocyte phosphorylated histone H3 immune reactivity in the interventricular septum of a double transgenic mouse as evidenced by the dark signal over an X-GAL-stained nucleus (arrow; bar = 10 microns). C) Expression of the MHC-1152stop transgene reduces post-MI cardiomyocyte hypertrophy, as evidenced by the increased cardiomyocyte nuclear content per unit area tissue as well as by the reduced cardiomyocyte cell body area in MHC-1152stop/MHC-nLAC double transgenic hearts (left panel) vs. MHC-nLAC singe transgenic hearts (right panel). Bar = 100 microns. These data have been described previously (Nakajima et al., 2004).

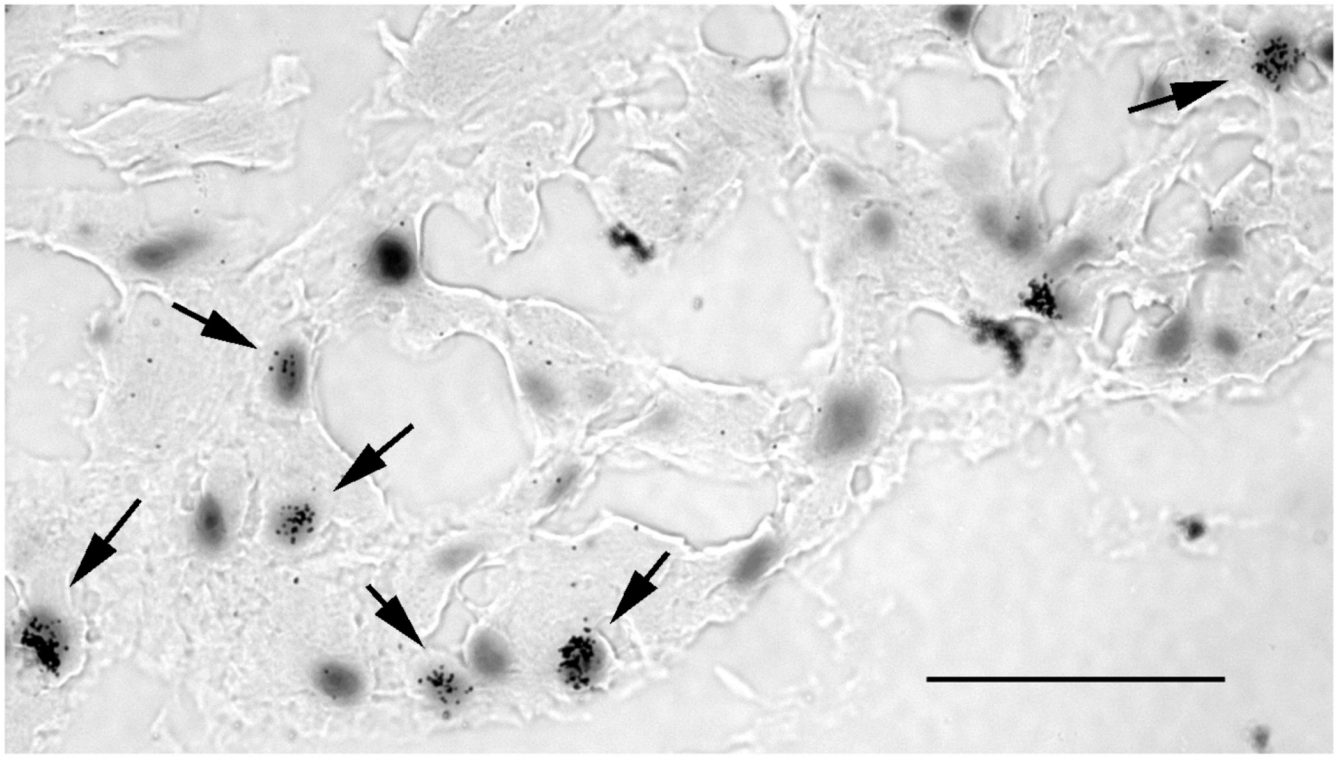

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Figure 3.
Cardiomyocyte DNA synthesis at the infarct border zone in MHC-cycD2/MHC-nLAC double transgenic mice at 7 days post-MI, as evidenced by the presence of silver grains over X-GAL-stained nuclei (arrows; bar = 50 microns). These data have been described previously (Pasumarthi et al., 2005).

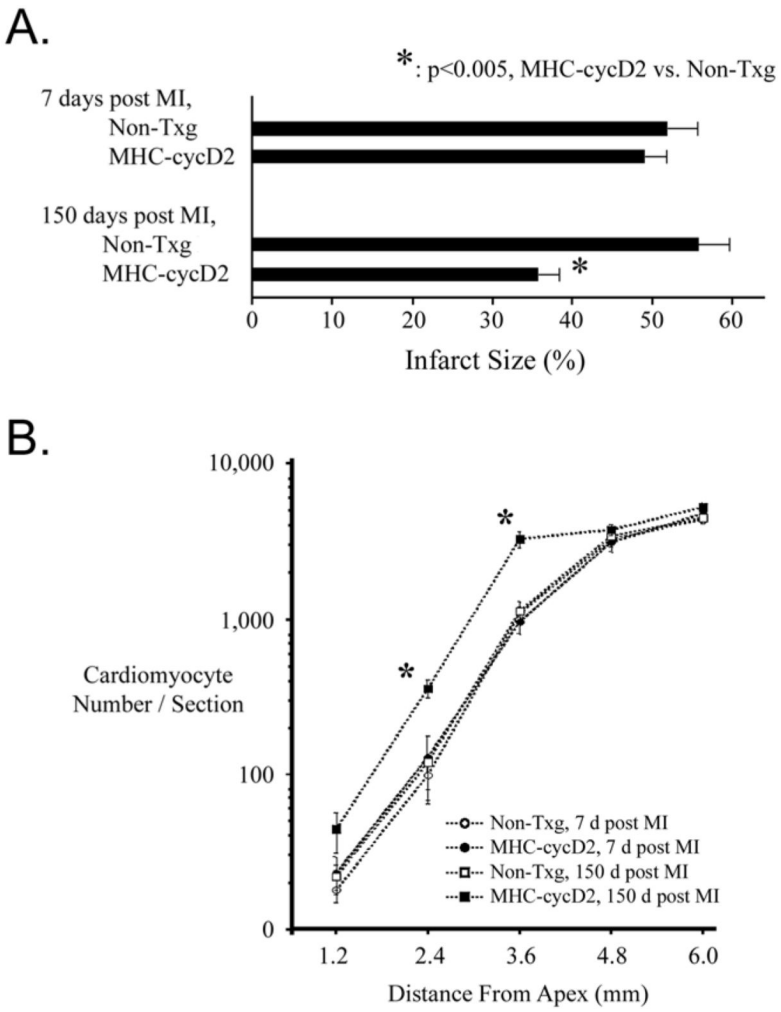

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Figure 4.

Characterization of myocardial infarcts in MHC-cycD2 transgenic mice. A) Infarct size in non-transgenic and MHC-cycD2 transgenic mice at 7 days and 150 days following permanent coronary artery occlusion. B) Cardiomyocyte content in sections sampled at 1.2 mm intervals from the apex to the base of MHC-cycD2 transgenic mice and their non-transgenic siblings at 7 and 150 days post-MI. These data have been described previously (Pasumarthi et al., 2005).