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Expression of a transgene encoding mutant p193/CUL7 preserves cardiac function and limits infarct expansion after myocardial infarction

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Abstract

Objective—Transgenic mice expressing the dominant interfering p193 protein in cardiomyocytes (MHC-1152stop mice) exhibit an induction of cell cycle activity and altered remodeling following experimental myocardial infarction (MI). We hypothesized that the altered remodeling would result in improved cardiac function in the MHC-1152stop mice following MI, as compared to non-transgenic mice.

Methods—MHC-1152stop mice and non-transgenic littermates were subjected to experimental MI via permanent occlusion of the coronary artery. Infarct size was determined at 24 hrs and at 4 weeks post-MI, and left ventricular pressure-volume measurements were performed at 4 weeks post-MI in infarcted and sham-operated animals.

Results—Infarct size in MHC-1152stop mice and non-transgenic littermates was not statistically different at 24 hrs post-MI, as measured by tetrazolium staining. Morphometric analysis revealed that infarct scar expansion at 4 weeks post-MI was reduced by 10% in the MHC-1152stop mice ($p<0.05$). No differences in cardiac function were detected between sham-operated MHC-1152stop mice and their non-transgenic littermates. However, at 4 weeks post-MI ventricular isovolumic relaxation time constant (Tau) was decreased by 19% ($p<0.05$), and the slope of the dP/dt_{max}-EDV relationship was 99% increased ($p<0.05$), in infarcted MHC-1152stop mice as compared to infarcted non-transgenic littermates.

Conclusion—Expression of the dominant interfering p193 transgene resulted in a decrease in infarct scar expansion and preservation of myocardial function at 4 weeks post-MI. Antagonization of p193 activity may represent an important strategy for the treatment of MI.

Keywords

myocardial infarction; apoptosis; cardiac function; infarct size; heart regeneration

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There is a significant correlation between the severity of the loss of cardiac function and the level of cardiomyocyte death during the acute phase of myocardial infarction,[1] during the post-infarction period,[2] and/or during end-stage heart failure.[3] It follows that interventions aimed at antagonizing ischemia- and hypoxia-induced necrosis and/or apoptosis would have a positive effect on cardiac function following myocardial infarction (MI). Similarly, strategies aimed at increasing cardiomyocyte number, via re-initiation of cardiomyocyte cell cycle activity[4] or via delivery of cardiomyocytes or cardiomyogenic stem cells,[5,6] would also likely result in improved cardiac function.

Several studies have demonstrated that targeted expression of the SV40 large T Antigen DNA tumor virus oncoprotein induced atrial and/or ventricular cardiomyocyte cell cycle activity in transgenic mice.[7–9] Cellular and molecular studies identified several T Antigen binding proteins (namely, p107, p53 and p193/CUL7). [10–13] The function of several of these proteins has been well characterized. For example, p107 is a member of the retinoblastoma protein gene family[14] and is thought to regulate transit through a G1/S cell cycle check point. Similarly, p53 can regulate pro-apoptotic cell cycle checkpoints, and its activities as a tumor suppressor and transcription factor are well established.[15] The role of p193/CUL7 in cell cycle regulation is less clear. Recent studies showed that p193/CUL7 (also called p185) E3 ligase activity and is involved in ubiquitin-mediated protein degradation.[16,17] Structure-function studies demonstrated that introduction of a premature stop codon at p193/CUL7 amino acid residue #1153 resulted in a truncated protein with apparent dominant interfering activity (that is, expression of the truncated protein, designated 1152stop, mimicked the growth-promoting activites observed with a p193/CUL7 anti-sense expression construct in transfected NIH-3T3 cells).[12] Furthermore, expression of the 1152stop protein, in combination with a dominant interfering p53 transgene, rendered embryonic stem cell-derived cardiomyocytes responsive to the growth-promoting activity of the E1A DNA tumor virus oncoprotein. These studies indicated that p193/CUL7 might play a role in cardiomyocyte cell cycle regulation.

To determine if altering p193/CUL7 activity had an impact upon cardiomyocyte cell cycle regulation following cardiac injury, transgenic mice expressing a cardiomyocyte-restricted p193/CUL7 c-terminal truncation mutant were generated (MHC-1152stop mice).[18] A marked induction of cardiomyocyte cell cycle activity was observed at the infarct border zone and in the interventricular septum of MHC-1152stop transgenic mice at four weeks following coronary artery occlusion. Cell cycle activation was accompanied by a reduction in the level of injury-induced cardiomyocyte hypertrophy in the interventricular septum. These data confirmed that altering p193/CUL7 activity relaxes the otherwise stringent regulation of cardiomyocyte cell cycle re-entry in the injured adult heart. Additionally, cell cycle induction appeared to, at least partially, counteract adverse post-infarction ventricular remodeling.

The studies described above raise the possibility altering of p193/CUL7 activity might result in functional improvement following myocardial injury. To test this hypothesis, MHC-1152stop mice and their non-transgenic littermates were subjected to permanent coronary artery ligation. *In vivo* left ventricular pressure-volume analyses were performed at 4 weeks following injury to determine the effects of transgene expression on cardiac function. Hearts were then subjected to morphometric analyses to determine the effect of transgene expression on infarct size. These data revealed that altering p193/CUL7 activity was associated with a modest but significant reduction in post-infarction scar expansion, which was accompanied by enhanced left ventricular function. These data suggest that p193/CUL7 might be a therapeutic target for preserving cardiac structure and function after myocardial infarction.

METHODS

Transgenic mice

Generation and initial analysis of the MHC-1152stop transgenic mice was described previously.[18] Briefly, MHC-1152stop mice utilized the cardiac-restricted α-cardiac myosin heavy chain promoter[19] to target expression of a p193/CUL7 mutant with apparent dominant interfering activity.[12] The SV40 early region transcription terminator/polyadenylation site[20] was inserted downstream from the p193/CUL7 sequences. Mice were maintained in a DBA/2J background (Jackson Laboratories, Bar Harbor MA). The investigation conforms with the *Guide of the Care and Use of Laboratory Animals* published by the US Nationals Institutes of Health (NIH Publication NO. 85023, revised 1996). All studies used male mice from 12–16 weeks of age.

Western blot analyses

Hearts were homogenized directly in NP40 buffer and processed as described previously. [21,22] Anti-p193/CUL7 monoclonal antibody was generated using a recombinant protein. Signal was visualized by the ECL method according to the manufacturer's protocol (Amersham, Arlington Heights IL).

Myocardial infarction model

Animals were intubated and ventilated with 2% isoflurane/98% oxygen. Via left thoracotomy, the left coronary artery was tied off with an 8-0 Prolene ligature close to the inferior border of the left auricle. The intercostal space and the skin incision were then closed with interrupted sutures, the endotracheal tube was removed, and the animal placed on a 37°C heating pad (Cole Parmer, Vernon Hills IL) under an oxygen cover for 24 hours post surgery. Sham-operated animals underwent the same procedure without ligation of the coronary artery.

Left ventricular pressure-volume analysis

Mice were anesthetized with 2% isoflurane/98% oxygen and ventilated (Minivent 845; HugoSachs Elektronik, March-Hugstetten, Germany) at 125 cycles/min and a tidal volume of 6–7 μl/g. Mice were placed supine under a dissection microscope and connected to a feedback heating lamp (rectal temperature sensor, body temperature at 37°C). A polyethylene (PE)-50 catheter was inserted into the left external jugular vein for hypertonic saline infusion. A precalibrated four-electrode 1.4F pressure-volume (P-V) catheter (Model SPR-839; Millar Instruments, Houston, TX) was inserted into the right common carotid artery and advanced into the left ventricle (LV). The catheter was connected to a pressure-conductance unit (Sigma SA; CD Leycom, Zoetermeer, The Netherlands). The continuous pressure and volume signals were monitored in real time and digitized at a sample rate of 500/s, using Conduct NT software (CD Leycom, Zoetermeer, The Netherlands). Display of the on-line pressure-volume signals allowed for optimal positioning of the catheter in the left ventricle.

After a short stabilization period, LV pressure-volume loops were recorded at baseline (3 times for 5 seconds with the ventilator stopped). This yielded a total of 120–150 cardiac cycles from which the following parameters were determined using Circlab software (Leiden University Medical Center, Leiden, The Netherlands): heart rate, LV end-systolic pressure (Pes), LV end-diastolic pressure (Ped), LV peak positive and negative developed pressures (dP/dt_{max} and dP/dt_{min} respectively) and LV isovolumic relaxation time constant (Tau). After the steady state measurements, pressure-volume relations were measured 3 times by transiently occluding the inferior vena cava. From the acquired 10 to 20 successive cardiac cycles the following parameters were derived: the end-systolic pressure-volume

relation (ESPVR) slope, stroke work (SW) end-diastolic volume (EDV) relation (preload recruitable stroke work; PRSW) and the linear slope of the dP/dt_{max}-EDV relationship. Thereafter a 4-μl bolus of hypertonic saline was injected for determination of the parallel conductance volume coefficient (V_p) and absolute volumes. The saline bolus caused significant increase in LV volume, without affecting LV pressure; signals were acquired and used for parallel conductance (V_p) determination as described before.[23,24]

Infarct size analysis

For acute infarct size measurements, hearts were harvested at 24 hrs following MI. The hearts were then cannulated and perfused with phthalo blue to discriminate between perfused (blue) and ischemic (non-blue) tissue. The hearts were then sliced transversely at 1.2 mm intervals and stained en mass with triphenyltetrazolium chloride (TTC), which stains viable tissue red (dead tissue is white). Digital images were then analysed to determine the infarct size. For chronic infarct size measurements, hearts were harvested after the functional analyses were completed and perfusion-fixed in a solution of 1% paraformaldehyde, 1% cacodylic acid, pH 7.4, at room temperature. Hearts were sectioned on a vibratome from apex to base (1.2 mm), and representative cryo-sections[25] were prepared from each vibratome section and stained.[26] Coronal sections were sampled at 1.2 mm intervals from the apex to the base, and stained with Sirius red (stains collagen red) and Fast green (stains viable myocardium green, Figure 2*a*). Digital images were acquired and the percentage of the myocardium that was infarcted was determined at each 1.2 mm interval using the approach described by Pfeffer and colleagues.[27] These values were then averaged to calculate infarct size for each individual heart, and subsequently for each genotype (Figure 2*b*). For chronic infarct size determinations, digital images were captured and infarct sizes calculated according to the following formula[27]: (length of coronal infarct perimeter (epicardial + endocardial)/ total left ventricle transverse perimeter (epicardial + endocardial)) × 100. The very slight layers of surviving subendocardial and subepicardial myocardium were not taken into account since the amount of tissue is negligible.

Statistical analysis

All data are presented as mean ± SEM. Between-group comparisons were analysed by Student's unpaired t-test. Significance was assumed at $P<0.05$.

RESULTS

Generation of myocardial infarction in mice expressing the MHC-1152stop transgene

We have previously generated mice that express a mutant p193/CUL7 transgene in the myocardium (MHC-1152stop mice).[18] Western blot analyses were performed to confirm transgene expression (Figure 1*a*; expression of the endogenous p193/CUL7 is also apparent in the transgenic and non-transgenic hearts). Male MHC-1152stop mice and their non-transgenic siblings were subjected to thoracotomy at 12–16 weeks of age. In some animals the pericardium was opened and the mice underwent permanent coronary artery occlusion (MI group). In other animals, the pericardium was opened but the coronary artery was not ligated (sham-operated group). TTC staining was used to monitor viable myocardium at 24 hours after MI. As expected, virtually all of the unperfused myocardium was dead at 24 hrs after coronary artery occlusion (Figure 1*b*). Analyses of digital images revealed no statistical differences in the acute infarct size between the MHC-1152stop mice and their non-transgenic littermates (n=13 for both groups; Figure 1*c*).

Expression of the MHC-1152stop transgene results in a modest decrease in post-myocardial infarct scar expansion

MHC-1152stop and non-transgenic hearts were harvested at 4 weeks post-MI and sampled from apex to base as described above. Infarct size was slightly but significantly reduced in the MHC-1152stop mice compared with the non-transgenic animals (n=12 and 11 respectively).

In vivo cardiac function 4 weeks post MI

Cardiac function was determined in the sham-operated and MI groups using a Millar conductance catheter to monitor left ventricular pressure-volume relationships. The presence or absence of the MHC-1152stop transgene had no effect on hemodynamic measurements and P-V relations in the sham-operated groups (n=7 for both groups); these data did not significantly differ from each other (Table 1*a*). As expected, many parameters of myocardial function (i.e., Ped, dP/dt_{max}, dP/dt_{min}, Tau, ESPVR and PRSW) were depressed post-MI (Table 1*b*). Myocardial function was compromised to a lesser degree in the infarcted MHC-1152stop mice, with the average values for the left ventricular isovolumic relaxation time constant (Tau, which is a measure of diastolic function) and the slope of the dP/dt_{max}-end diastolic volume relation (dP/dt_{max}-EDV, which is a measure of systolic function) significantly improved ($p<0.05$) in the MHC-1152stop transgenic mice as compared to the non-transgenic animals (n=7 for both groups) (Table 1*b*). The improvement in dP/dt_{max} vs. EDV is further illustrated in Figure 3. Panel *a* shows a typical p-v loop obtained from an individual infarcted MHC-1152stop mouse at 4 weeks post-MI. Panel *b* shows the plot of dP/dt_{max} vs. EDV for an individual infarcted MHC-1152stop (circles) and an individual non-transgenic (triangles) mice. Collectively, these data indicate that cardiac function was enhanced in the transgenic mice at 4 weeks post-MI, as compared to the non-transgenic siblings.

DISCUSSION

The data presented here indicated that expression of the 1152stop transgene had a positive effect on cardiac structure and function at 4 weeks post-MI. Although infarct size was indistinguishable between transgenic and non-transgenic hearts at 24 hrs post-MI, a modest but significant decrease in infarct size was observed at 4 weeks post-MI. Functional analyses revealed that both Tau and dP/dt_{max}-EDV were significantly improved in infarcted MHC-1152stop hearts as compared to the non-transgenic siblings. While the absolute average number of tau in the sham groups was 20% lower in the non-transgenic mice compared with the transgenic animals at baseline (10,4 versus 12,5, non-significant), at 4 weeks post myocardial infarction tau was 16% higher in non-transgenic groups versus the transgenics (16,2 versus 13,6, significant), clearly showing the positive impact of antagonization of p193 activity on cardiac function. The pronounced enhancement of dP/dt_{max}-EDV in the MHC-1152stop hearts was of particular interest, as this measurement provided a load-independent index of cardiac function.[28] A similar albeit less pronounced trend was seen in several other functional parameters tested. Importantly, heart rate (which can influence global cardiac function) was not different in the infarcted transgenic and non-transgenic animals.

Previous studies demonstrated that cardiomyocyte cell cycle induction was observed in the MHC-1152stop mice, but not in the non-transgenic siblings.[18] A reduction of cardiomyocyte hypertrophy accompanied cell cycle induction in the transgenic mice. These observations led to the suggestion[18] that cell cycle activation in MHC-1152stop mice resulted in at least partial replacement of the cardiomyocytes lost at the infarct border zone and in the interventricular septum during the chronic phase of myocardial infarction, thereby

reducing the need for compensatory hypertrophic growth of the myocardium in the transgenic hearts. In contrast, the absence of cell cycle induction in infarcted non-transgenic hearts led to a comparably higher degree of compensatory hypertrophy. It is relatively easy to envision how these characteristics of the MHC-1152stop mice might have contributed to the improved cardiac structure observed in the present study. The similarity of infarct size in transgenic and non-transgenic mice at 24 hours post-MI indicated that transgene expression was not cardio-protective during the acute phase of infarction. Moreover, as shown in the previous study on these mice, no difference in cardiomyocyte-caspase activity was found.[18] Consequently, the presence of smaller infarcts in the MHC-1152stop hearts at 4 weeks post-MI must have resulted from events that occurred during the chronic phase of infarction. Infarct expansion, secondary to cardiomyocyte apoptosis, has frequently been observed during the chronic phase of myocardial infarction.[29] Cell cycle induction in MHC-1152stop hearts, particularly at the infarct border zone, might have resulted in the partial replacement of apoptotic border zone cardiomyocytes, thereby limiting the extent of infarct expansion.

It is likely that altered post-MI remodeling also had a direct impact on the improved cardiac function observed in the MHC-1152stop hearts. For example, Tau provides an index of left ventricular relaxation during isovolumic diastole (that is, the lusitropic state of the heart). Although it is well known that Tau is increased in failing hearts,[30] more recent studies have shown a direct relationship between increased values of Tau and hypertrophy in isolated cardiomyocytes harvested from mice with aortic stenosis[31] or from rabbits with myocardial infarction.[32] The marked reduction in injury-induced cardiomyocyte hypertrophy[18] could easily account for the preservation of Tau observed in the infracted MHC-1152stop mice.

Interpretation of the effect of transgene expression on the relationship between dP/dt_{max} and EDV in the MHC-1152stop mice is somewhat more complex. This functional parameter provides a sensitive index of cardiac contractility (i.e. the inotropic state of the heart). It is well established that, following MI, the decrease in cardiac contractility resulting from cardiomyocyte loss is initially compensated via hypertrophic growth of the surviving myocardium.[33] The enhanced cardiac contractility (i.e., dP/dt_{max}-EDV) seen in infracted MHC-1152stop mice would *a priori* seem somewhat paradoxical, given the attenuation of compensatory hypertrophy in these animals. However, a similar blunting of cardiac dysfunction following aortic banding has been reported previously for genetically altered mice with impaired capacity for hypertrophic growth,[34] leading these authors to speculate that under conditions of pressure overload, the development of cardiac hypertrophy and normalization of wall stress may not be necessary to preserve cardiac function. Although this study employed a very different model of cardiac injury, it is possible that a similar mechanism might be at play in the infarcted MHC-1152stop mice. It is however more likely that the increased cardiomyocyte cell cycle activity in the interventricular septum of these animals underlies the improved cardiac contractility following MI. That is, simply increasing cardiomyocyte number would almost certainly result in improved global cardiac contractility. Finally, the modest reduction of infarct size in the MHC-1152stop mice certainly contributed at least in part to the preservation of cardiac function following myocardial infarction.

In addition to p193/CUL7 1152stop, a number of other proteins have been shown to promote ventricular cardiomyocyte cell cycle activity when expressed in adult transgenic animals. These include SV40 Large T antigen,[9] the D-type cyclins,[35,36] an inducible form of c-myc[37] CDK-2[38], dominant interfering TSC2,[39] cyclin A2,[40] IGF-1,[41] bcl-2[42] and p38MAPK.[43] Of these, p193/CUL7 1152stop (this study), cyclin D2[44] and IGF-2[45] have been shown to improve cardiac function following myocardial injury. In the

case of p193/CUL7 1152stop and cyclin D2, a direct correlation between cardiomyocyte cell cycle activation and improved cardiac function was detected. In the case of IGF-1, it was not clear if the beneficial effect on cardiac function resulted from transgene-induced cardiomyocyte proliferation or alternatively from reduced levels of cardiomyocyte apoptosis.

Since p193/cul7 interacts with the F-box protein FBXW8, an E3 ligase which has published activity on D-type cyclins and stimulates or inhibits cell proliferation, we could argue that this, namely the relaxation of restriction point transit, is a possible mechanism of proliferation. [46] As shown in our previous publication on these mice, cardiomyocyte DNA synthesis at the infarct border zone could be detected. [18] Although we did not do a quantitative analysis of DNA synthesis in the present study, we did check for DNA synthesis activity in the peri-infarct zone at four weeks post myocardial infarction. Again, high levels of DNA synthesis were seen (Figure 4), hereby founding the idea that the reduction in infarct size and the improved cardiac function is due to cell cycle induction and cell proliferation.

In summary, the data presented here indicated altering p193/CUL7 activity had a positive impact upon post-MI cardiac structure and function. This improvement was likely due to injury-induced cardiomyocyte cell cycle activity in the interventricular septum and at the infarct border zone, resulting in a concomitant improvement in post-MI remodelling and infarct scar expansion during the chronic phase of infarction. Blockade of the p193/CUL7 pathway may thus represent an important strategy for treatment of myocardial infarction and cardiac remodelling.

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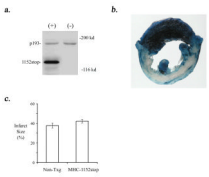

Figure 1.
Transgene expression and acute infarct size in non-transgenic and MHC-1152stop transgenic mice. (*a*) Western blot analysis demonstrating endogenous p193/CUL7 and/or p193-1152stop transgene expression in an MHC-1152stop mouse (+) and its non-transgenic littermate (−). Expression of the endogenous p193/CUL7 protein is apparent. Positions of molecular weight standards are indicated. (*b*) Phthalo blue-TTC staining demonstrating acute infarct size at 24 hrs following infarction. White area identifies dead tissue. (*c*) Infarct size in non-transgenic (n=13) and MHC-1152stop (n=13) mice at 24 hrs post-coronary artery ligation. There was no statistical difference in infarct size between the two groups.

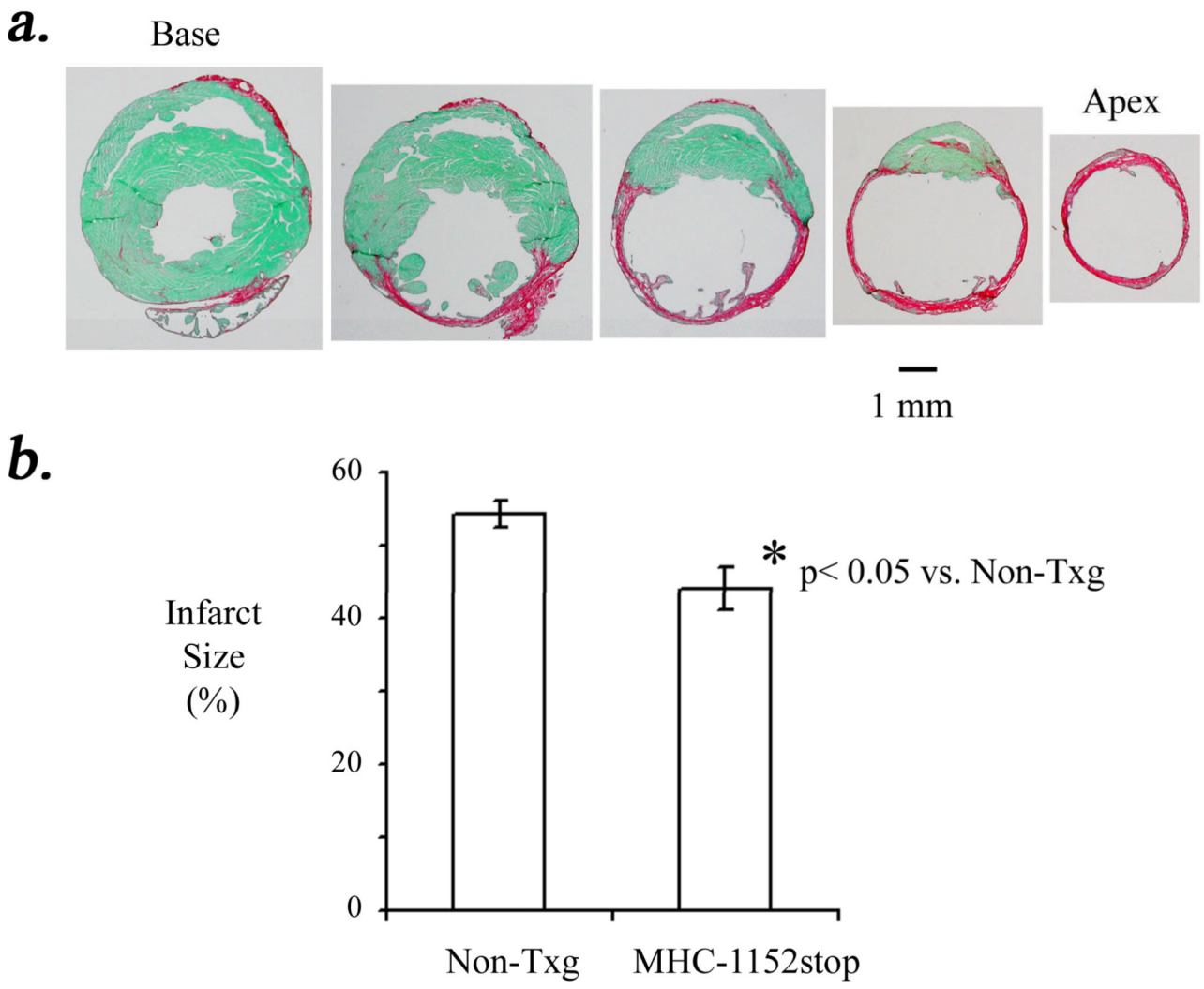

Figure 2.

Infarct size in non-transgenic and MHC-1152stop transgenic mice at 4 weeks following coronary artery ligation. (*a*) Sirius red/fast green staining of transverse sections demonstrating chronic infarct size at 4 weeks after MI in a MHC-1152stop mouse. Sections shown were collected at 1.2 mm intervals from apex to base. Red indicates collagen (scar) and green indicates viable myocardium. (*b*) Infarct size in non-transgenic (n=11) and MHC-1152stop (n=12) mice at 4 weeks following coronary artery ligation. Infarct size in the MHC-1152stop hearts was significantly smaller than in non-transgenic hearts.

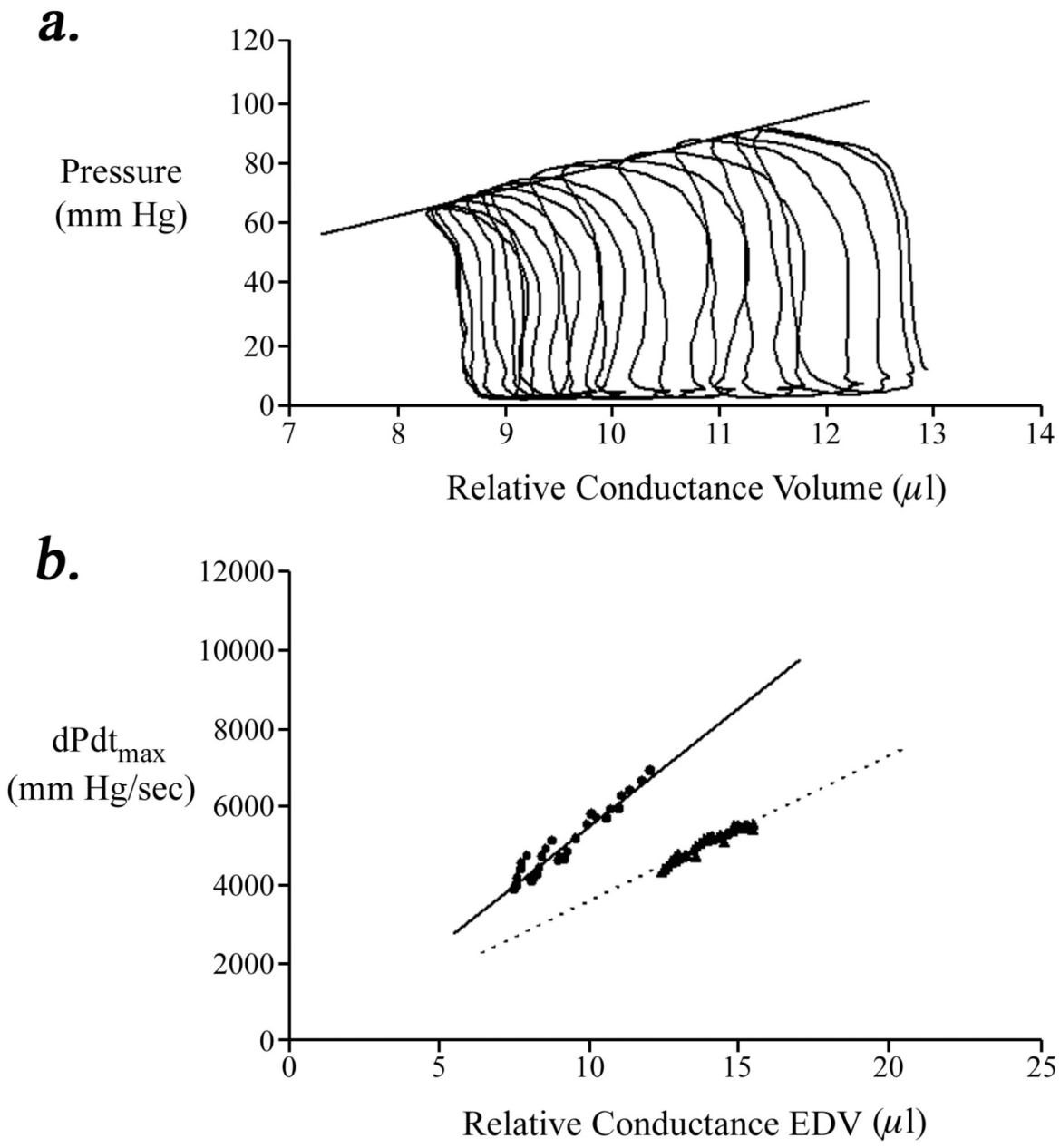

Figure 3.

(*a*) Representative pressure-volume loops from an individual MHC-1152stop mouse during transient inferior vena cava occlusion 4 weeks following myocardial infarction. The slope of the end-systolic p-v relation indicates ESPVR. (*b*) Relations between beat-to-beat dP/dt_{max} and end-diastolic volume 4 weeks following myocardial infarction. The slope of the dP/dt_{max}-EDV relation shows a significant shift upward to left in an MHC-1152stop mouse (circles, solid line; slope = 652.6 mmHg/s/ul) as compared to a non-transgenic mouse (triangles, dotted line; slope = 387.7 mmHg/s/ul).

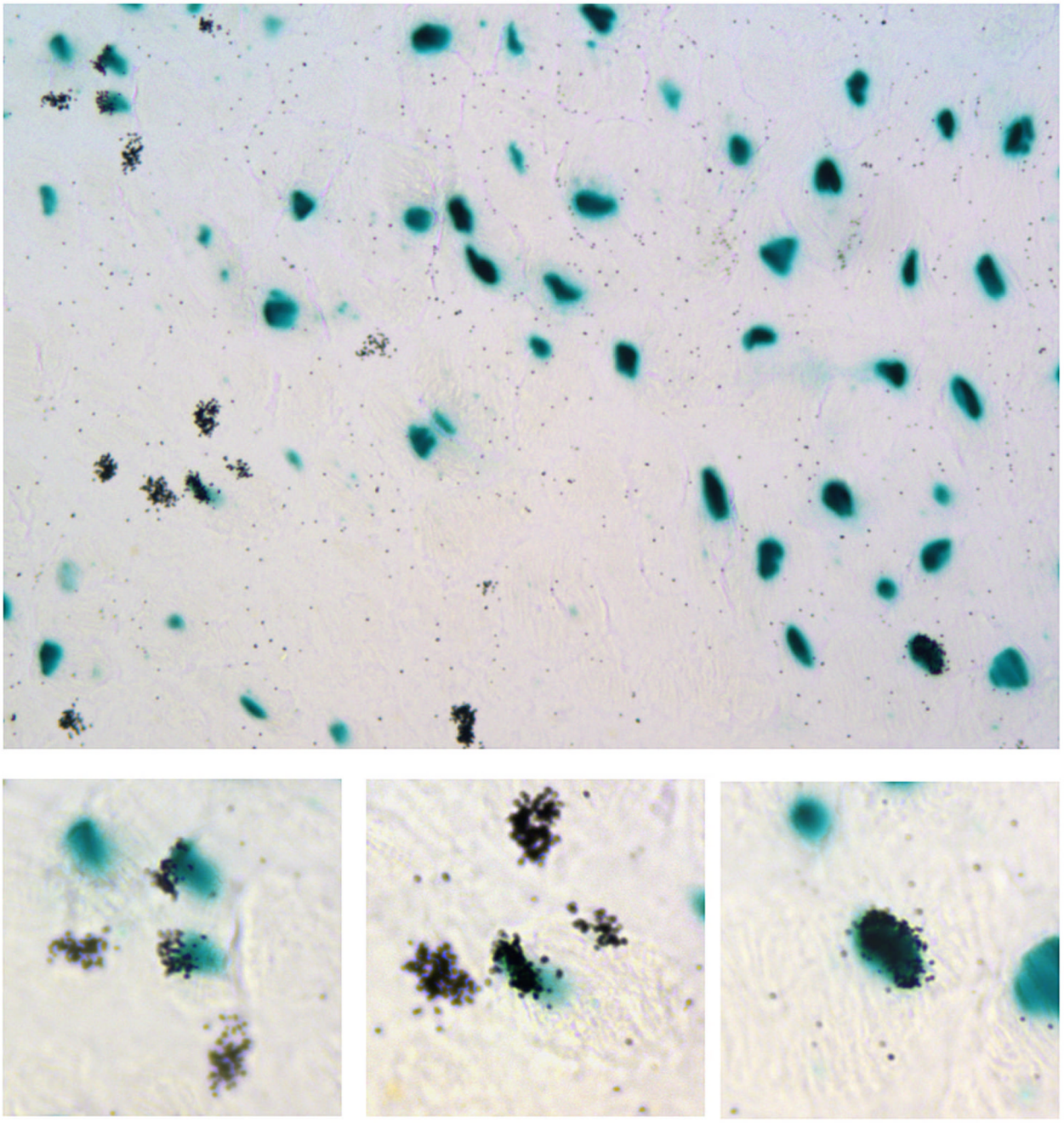

Figure 4.
Cardiomyocyte DNA synthesis in the septum/peri-infarct area of an MHC-nLAC/MHC-1152stop transgenic heart at four weeks after permanent coronary artery occlusion. Presence of silver grains (due to injection of tritiated thymidine four hours before tissue harvesting) over blue nuclei is indicative of cardiomyocyte DNA synthesis.

Table 1

Hemodynamic parameters in sham-operated and MI mice (4 weeks post operation).

	Non-transgenic	MHC-1152stop
a. Sham groups:	(n=7)	(n=7)
HR (beats/min)	528±15	474±34
Pes (mmHg)	91.1±8.7	86.4±7.6
Ped (mmHg)	2.4±0.5	3.9±1.1
dP/dt_{max} (mmHg/s)	7622.5±480.3	7051.7±1238.8
dP/dt_{min} (mmHg/s)	−6366.7±377.7	−5368.2±677.7
Tau (ms)	10.4±0.5	12.5±1.9
ESPVR (mmHg/μl)	24.6±6.4	17.3±4.5
PRSW (mmHg)	46.8±4.5	37.8±3.6
dP/dt_{max}-EDV (mmHg.s-1.μl-1)	797.7±213.5	813.6±205.3
b. MI groups:	(n=7)	(n=7)
HR (beats/min)	520±30	528±13
Pes (mmHg)	72.4±4.4	77.3±3.7
Ped (mmHg)	13.5±1.1	9.3±2.1
dP/dt_{max} (mmHg/s)	4430.0±465.2	5361.0±430.5
dP/dt_{min} (mmHg/s)	−3207.8±282.9	−3705.8±312.2
Tau (ms)	16.2±0.9	13.6±0.5*
ESPVR (mmHg/μl)	8.9±0.7	13.6±2.8
PRSW (mmHg)	22.6±5.2	24.9±2.2
dP/dt_{max}-EDV (mmHg.s-1.μl-1)	379.2±68.1	756.5±121.1*

Abbreviations: HR, heart rate; Pes, left ventricular end-systolic pressure; Ped, left ventricular end-diastolic pressure; dP/dt_{max}, left ventricular peak positive developed pressure; dP/dt_{min}, left ventricular peak negative developed pressure; Tau, left ventricular isovolumic relaxation time constant; ESPVR, end-systolic pressure-volume relation; PRSW, stroke work/end-diastolic volume relation; dP/dt_{max}-EDV, linear slope of the dP/dt_{max}-EDV relationship.

*$p<0.05$, MHC-1152stop vs. non-transgenic.