

# The Type of Injury Dictates the Mode of Repair in Neonatal and Adult Heart

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**Background**—The neonatal heart possesses the unique power to regenerate in response to resection of the left ventricular apex. We sought to determine whether the type of injury affects the mode of repair and regeneration.

**Methods and Results**—Apical resection, or permanent left anterior descending coronary artery ligation, was induced in neonatal 1-day-old mice. Echocardiography was used to confirm and monitor cardiac injury and remodeling. Histological and immunohistochemical examinations of the resected and infarcted neonatal hearts revealed inflammation and granulation tissue formation. From day 3, early regeneration was identified at the injured sites and was characterized by dedifferentiation and proliferation of cardiomyocytes around the injured areas. The young cardiomyocytes infiltrated the granulation tissue and replaced it with a new myocardium. The ability of neonatal cardiomyocytes to proliferate was confirmed in neonatal heart organ cultures. Notably, myocardial infarction in neonatal mouse produced incomplete regeneration with a residual small infarct and, sometimes, aneurysm at 28 days after myocardial infarction. We then repeated the same experiments in the adult heart. Remarkably, myocardial infarction in the adult mouse heart produced a typical thin scar, whereas apical resection revealed an abnormal, epicardial, hemorrhagic scar 21 days after injury.

**Conclusions**—Our findings suggest that the type of injury, resection, or infarction affects the mode of repair in both neonatal and adult mouse hearts. Identifying the differences in the mechanisms or repair of these 2 types of injuries could help to develop novel regenerative therapies relevant to human patients. (*J Am Heart Assoc.* 2015;4:e001320 doi: 10.1161/JAHA.114.001320)

**Key Words:** cardiomyocytes • fibrosis • inflammation • macrophages • myocardial infarction • myocardial regeneration

The injured human heart has limited ability to replace lost cardiomyocytes.<sup>1–3</sup> The healing and repair process after myocardial injury is characterized by inflammation, scar formation, fibrosis, and minimal regenerative response.<sup>4,5</sup> A clear unmet need exists for new effective regenerative therapies.

The finding that rat and mouse hearts can regenerate in the first 7 days after birth<sup>6,7</sup> has raised interest and hope that

it may be possible to reactivate this regenerative process in humans so as to renew injured myocardium. It has also provided an exclusive system in which to study the basic mechanisms of myocardial regeneration versus repair in mammals. This model could be used in transgenic mice to determine the molecular and cellular mechanisms that regulate mammalian heart regeneration.<sup>8–10</sup> Consequently, apical resection and myocardial infarction (MI) in neonatal mice provide an opportunity to understand the mechanisms responsible for the age-dependent decline in cardiac regenerative capacity.

The ability of the neonatal heart to regenerate completely has been questioned.<sup>11–13</sup> It is possible that the repair response to apical resection is different from the response to ischemic or cryoinjury. We sought to determine whether the type of injury dictates the mode of repair and regeneration after MI. The correlation between the type of injury and the succeeding regenerative response could provide additional mechanistic insight into myocardial regeneration versus repair. Understanding how neonatal mammalian hearts regenerate would be an important step toward regenerative therapy for patients with irreversible myocardial damage and end-stage heart failure.

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## Materials and Methods

### Animal Experiments

This study was approved by the Sheba Medical Center institutional review board committee and was performed in accordance with the guidelines of the animal care and use committee of the Sheba Medical Center, Tel Aviv University.

### Neonatal Mouse Model of Partial Apical Resection or MI

To determine the regeneration ability of neonatal mouse, we used a similar method to that used by Sadek's group.<sup>6,8</sup> One-day-old neonatal ICR mice (n=240; Harlan Laboratories, Jerusalem, Israel) were subjected to apical resection (n=150), MI (n=90), or sham operation (n=25). To avoid pain and stress, newborn mice were anesthetized by inhalation of 2% isoflurane and 100% oxygen. The mice were cooled down on an ice bed for 4 minutes, causing asystole and reversible apnea<sup>14</sup> and preventing excessive blood loss during surgery. The cooling-down period also provided additional anesthesia. The chest was opened by left thoracotomy, and iridectomy scissors were used to carefully and gradually resect thin segments from the left ventricle apex, as described previously.<sup>6,8</sup> The thoracic wall and skin were then sutured with an 8-0 prolene suture.

For MI induction, we repeated this anesthesia procedure and permanently occluded the mid-LAD coronary artery by 8-0 prolene suture (Ethicon). Finally, animals were placed on a heating pad (37°C), allowed to recover, and returned to their mothers. Sham-operated mice underwent the same procedure except for the resection or LAD ligation. Average survival rates after apical resection and MI were 70% and 40%, respectively. Survival after sham operation was 95%.

### Organ Culture from a Neonatal Mouse Heart

To confirm the proliferative and growth power of the neonatal myocardium, we used a model of organ culture. We harvested hearts from neonatal, 1-day-old mice and then sliced the hearts into 2 to 3 transverse sections, which were cultured on a 100-mm coated culture dish with cardiomyocyte growth media for 32 days.<sup>15</sup> Cardiac tissue growth was assessed by cell budding (outgrowth) from the cultured heart slices. The outgrowth was stained with antibodies against  $\alpha$ -cardiac actin (Santa Cruz Biotechnology) and c-kit (Santa Cruz Biotechnology), the marker most commonly associated with cardiac and cardiovascular precursors.<sup>16</sup>

### Myocardial Resection and Infarction in Adult Mouse

To determine whether the regenerative process in neonatal mouse hearts is specific to age or mode of injury, we repeated the myocardial resection procedure in adult mice. Female 12-week-old ICR mice (Harlan Laboratories) were anesthetized with 1% to 3% isoflurane. The mice were intubated and ventilated with 100% oxygen. The chest was shaved and opened by left thoracotomy, and iridectomy scissors were used to carefully and gradually resect small segments from the left ventricle apex (n=15). For LAD ligation, we used 8-0 prolene (Ethicon) to permanently occlude the LAD coronary artery (n=4). The chest was closed and the skin sutured with a 5-0 prolene suture (Ethicon). Mice were placed on a heating pad (37°C) until recovery. Average survival rates after apical resection and MI were 20% and 80%, respectively. Survival from sham operation was 95%.

### Histological Examination

For histological examination, we used 60 hearts from the apical resection group, 30 from the MI group, and 15 from the sham-operated group. Hearts were harvested at days 1, 3, 5, 7, 21, and 28 after the procedure, washed with PBS, and then fixed in 4% paraformaldehyde overnight. Adjacent blocks were embedded in paraffin and sectioned into 5- $\mu$ m slices. Hematoxylin and eosin and Masson's trichrome staining (to detect scarring and fibrosis) were performed according to standard procedure. To identify neonatal cardiomyocytes, cell proliferation, macrophages, and cardiac stem cell accumulation, we stained the heart sections with antibodies against  $\alpha$ -cardiac actin (Santa Cruz Biotechnology), phosphorylated histone H3 (PS10; Epitomics), Mac-3 (BD Bioscience), and c-kit (Santa Cruz Biotechnology). Cell proliferation was quantified by the average of 3 adjacent fields at magnification of  $\times 600$  (n=9).

### Echocardiography to Evaluate Cardiac Function

To confirm myocardial damage, transthoracic echocardiography was performed 3, 7, 21, and 28 days after the procedure. Mice were anesthetized by isoflurane 3%. The chest was shaved, and mice were placed in a supine position. Echocardiograms were performed with a special small animal echocardiography system (Vevo 2100 imaging system; VisualSonics) equipped with a 22- to 55-MHz linear-array transducer (MS250 MicroScan transducer; VisualSonics). All measurements, averaged for 3 consecutive cardiac cycles, were performed by an experienced technician.

## Statistical Analysis

All values are expressed as mean±SEM. Differences in left ventricular ejection fraction by echocardiography at day 28 were compared by 1-way ANOVA, with Bonferroni's multiple comparison post-test. Differences were considered significant at  $P<0.05$ . Differences between phosphorylated histone H3 stain for mitosis were compared by 2-way ANOVA, with Bonferroni's multiple comparison post-test. Differences were considered significant at  $P<0.05$ . Graph-Pad Prism version 5.00 (GraphPad Software) was used for analysis.

## Results

### Regeneration Ability of the Neonatal Heart After Apical Resection

To determine the regeneration ability of neonatal mice, we subjected 1-day-old newborn ICR mice to apical resection or sham operation, as described previously.<sup>6,8</sup> Heart regeneration was determined on days 1, 3, 5, and 28. In response to apical resection, an intensive healing and regenerative process occurred (Figure 1A through 1D). The healing process began with a blood clot that covered and sealed the injury site and prevented massive bleeding (Figure 1A). In some cases, these apical clots were formed against the chest wall and prevented fatal bleeding. During the next 3 days, inflammation and granulation tissue appeared (Figure 1B). Interestingly, we noticed robust accumulation of monocytes and macrophages at the site of the blood clots from day 1 after resection and then in the granulation tissue on day 3 (Figure 1E and 1F). Subsequently, the granulation tissue was replaced by new myocardial tissue (Figure 1C and 1D) that replaced the damaged myocardium. Significantly, we noticed massive dedifferentiation of cardiomyocytes at the border zone, which was characterized by a disorganized sarcomeric structure along with the appearance of intercellular spaces and the relocation of sarcomeric structures toward the cell wall (Figure 2A through 2C). This was followed by cardiomyocytes that invaded the granulation tissue and replaced it as early as day 3 after resection (Figure 2A).

In addition, we noticed robust cardiomyocyte nuclei division both at the border zone and at the remote myocardium. Nuclei division was assessed by phosphorylated histone H3 immunostaining (Figure 2D and 2E). Histone H3 is specifically phosphorylated during both mitosis and meiosis and is considered to be a marker of cell division.<sup>17</sup> At day 3 after resection, the amount of nuclei division (average of 3 fields) was nearly 2-fold higher in the injured neonatal heart compared with hearts subjected to sham operation (Figure 2F). Remarkably, at 21 days following resection, the

injured myocardium regenerated completely, with a minimal, superficial thin scar at the site of resection (Figure 1C and 1D).

### Outgrowth of Cardiac Cells From Neonatal Heart in Organ Culture

To explore the proliferative ability of neonatal cardiomyocytes in vitro, we used an organ culture method by which cells budding from the cultured heart tissue were extracted and examined. A beating outgrowth of cells was evident as early as 1 week and continued beating for 4 weeks (Figure 3A and 3B). Notably, after 4 to 5 weeks in culture, about 25% of outgrowth cells began to express c-kit (Figure 3C), the marker most commonly associated with cardiac and cardiovascular precursors.<sup>16</sup> Our findings suggest that the in vitro outgrowth was enriched with cardiovascular precursors,<sup>16</sup> dedifferentiating cardiomyocytes,<sup>18</sup> or both.

### Incomplete Regeneration After MI in Neonatal Mouse Heart

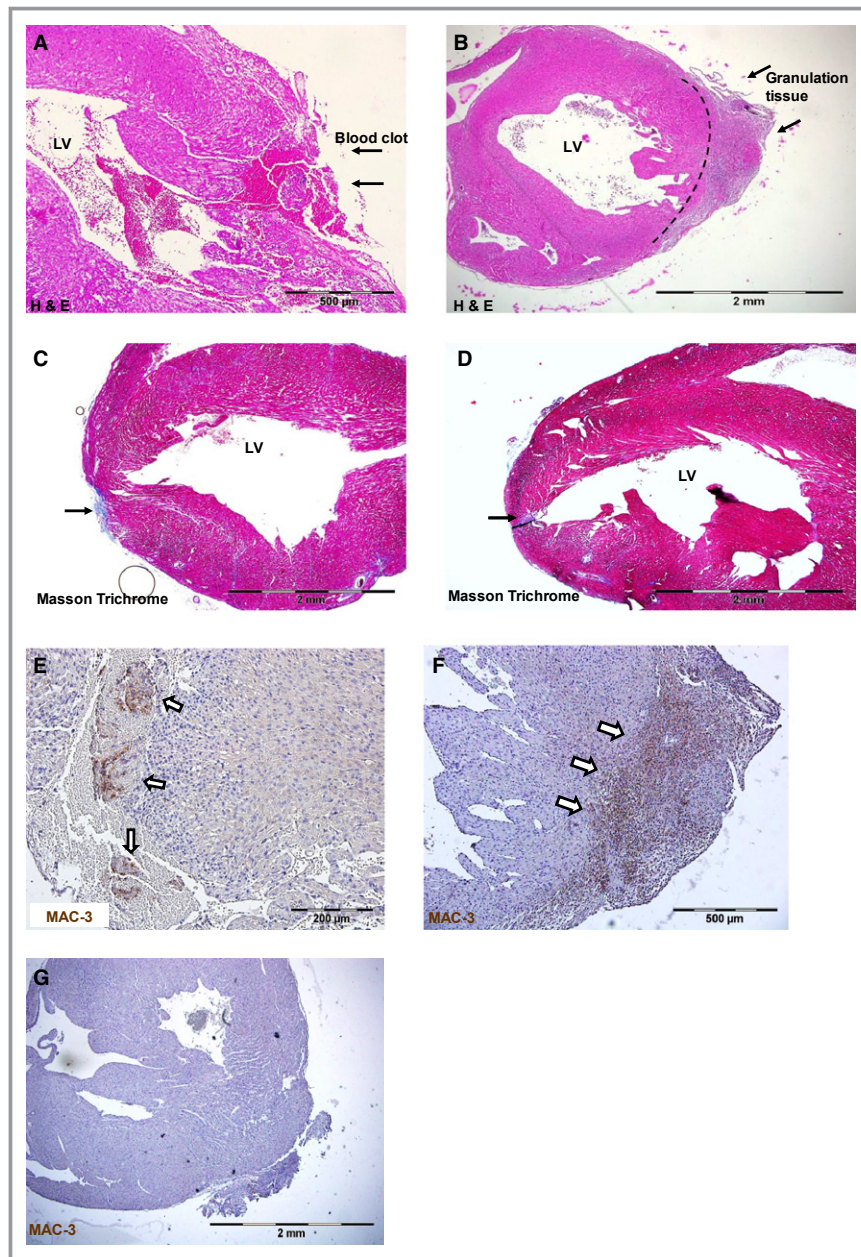
To determine whether myocardial regeneration is effective after MI, we subjected newborn mice to permanent occlusion of the LAD coronary artery or sham operation (Figure 4A and 4B). An intensive inflammatory response, particularly macrophages (Figure 4C), was evident by 3 days after MI. Seven of 40 mice were euthanized 21 to 28 days following MI. Surprisingly, unlike apical resection, regeneration after MI was incomplete, and a transmural, small infarct was evident at 21 and even 28 days after MI (Figure 4D). Furthermore, the number of nuclei divisions in the border zone and remote myocardium, after MI, was smaller than in the heart after apical resection (Figure 4G and 4H), with less evidence of proliferating and dedifferentiated cardiomyocytes (Figure 4E through 4H).

In addition, apical aneurysm formation and left ventricle remodeling were evident by echocardiography scan 28 days after MI (Figure 4I through 4K). Compared with the sham operation, left ventricle ejection fraction was slightly lower after apical resection and MI 28 days after injury (Figure 4K). Moreover, unlike neonates after apical resection, some neonates after MI experienced delay in growth and were distinctively smaller than sham-operated neonates at the same age (Figure 4L).

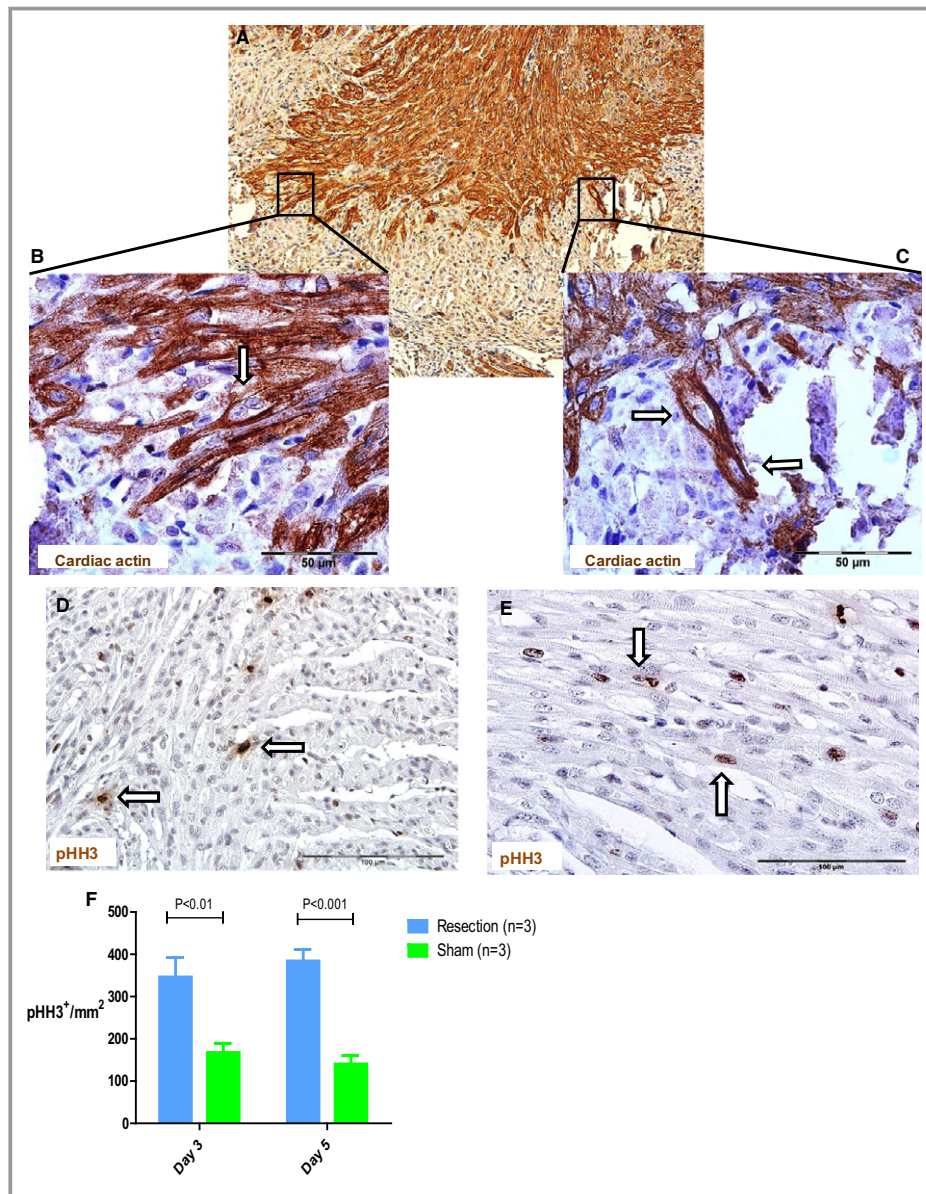
### Myocardial Injury in Adult Heart

To determine whether the regenerative process in neonatal mouse hearts is specific to age or mode of injury, we repeated the myocardial injury procedure in 12-week-old adult mice. Resection in the adult mouse needed to be



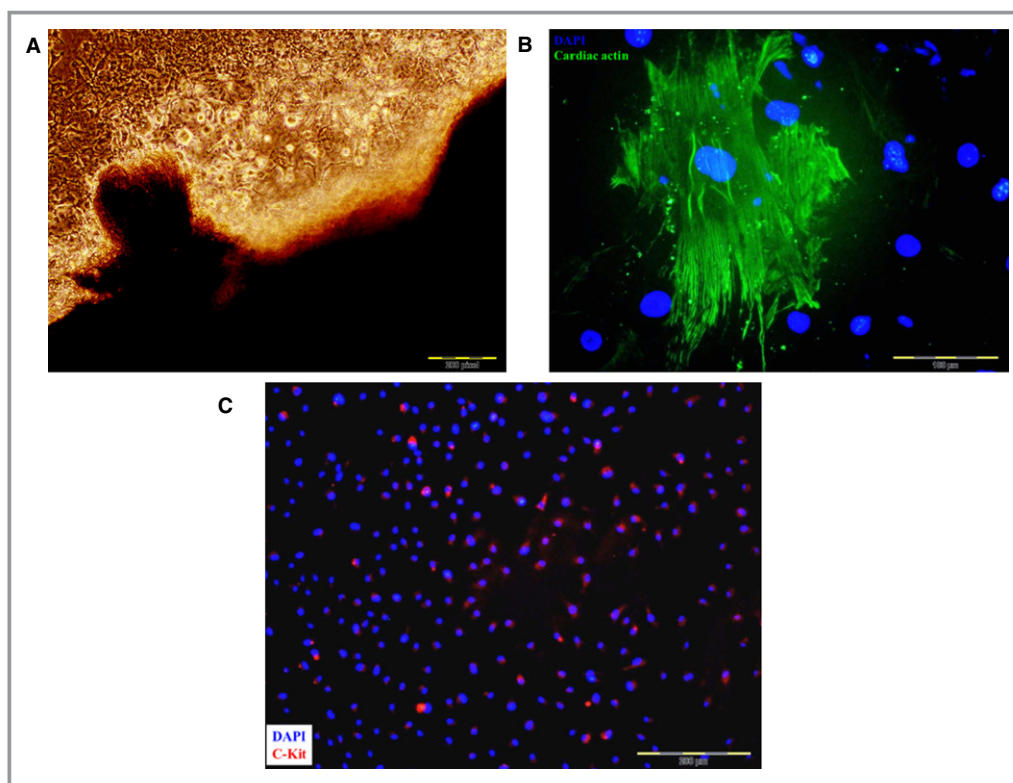


**Figure 1.** Myocardial regeneration after apical resection in neonatal mice. Newborn mice (1 day old) were anesthetized and cooled down on ice, and the chest was opened by left thoracotomy. Iridectomy scissors were used to resect the heart apex until the LV chamber was exposed. A, At 1 day after resection, H&E staining revealed blood clot formation at the resection area (arrow). Scale bar: 500 μm. B, At 3 days after resection, H&E staining showed inflammation and granulation tissue at the injured area (arrow). The dashed line marks the resection plane. Scale bar: 2 mm. C, At 21 days after resection, Masson's trichrome staining showed that robust myocardial regeneration replaced the inflammation and granulation tissue, with minimal epicardial fibrosis (arrow). Scale bar: 2 mm. D, At 21 days after resection, Masson's trichrome staining of another neonatal heart demonstrated minimal fibrosis (arrow). Scale bar: 2 mm. E, Monocytes and macrophages (dark brown) penetrated the blood clot that covered the injured area 1 day after resection (arrows). Scale bar: 200 μm. F, Robust infiltration of monocytes and macrophages (dark brown) into the apex 3 days after myocardial infarction. Scale bar: 500 μm. G, Sharp decrease in monocyte and macrophage number at the regenerating apex and in other parts at 5 days after resection. Scale bar: 2 mm. H&E indicates hematoxylin and eosin; LV, left ventricle.



**Figure 2.** Dedifferentiation and proliferation of cardiomyocytes at the injured area 3 days after resection. A, Cardiac actin staining reveals that cardiomyocytes (brown) infiltrated the injured tissue and replaced the granulation tissue. Nuclei are stained blue with hematoxylin. Scale bar: 200  $\mu\text{m}$ . B, Higher magnification of the penetrating cardiomyocytes at the border zone demonstrates cardiomyocyte dedifferentiation, characterized by sarcomeric disorganization, appearance of intercellular spaces, rearrangement of sarcomers toward the cell wall (arrow), and nuclei division (arrow). The manifestation of dedifferentiating, double-nuclei cardiomyocytes is clearly different from the adjacent, single-nuclei, sarcomere-full cardiomyocytes. Scale bar: 50  $\mu\text{m}$ . C, Higher magnification of different area at the border zone shows double-nuclei cardiomyocytes with disorganization of sarcomeres. Again, the look of these divided cardiomyocytes is different from adjacent single-cell cardiomyocytes. Scale bar: 50  $\mu\text{m}$ . D, pHH3 immunostaining for dividing nuclei showed intensive mitotic activity at day 3 after resection. Scale bar: 100  $\mu\text{m}$ . E, At day 5 after resection, pHH3 immunostaining (brown) revealed dividing cardiomyocytes. Note typical sarcomeric striation in cells with stained nuclei (arrows). Scale bar: 100  $\mu\text{m}$ . F, At days 3 and 5 after resection, the amount of nuclei division was 2-fold higher in the injured neonatal heart compared with sham-operated hearts. Differences between pHH3 stain for mitosis were compared by 2-way ANOVA, with Bonferroni's multiple comparison post-test. Differences were considered significant at  $P < 0.05$ . pHH3 indicates phosphorylated histone H3.





**Figure 3.** Beating outgrowth of cells from neonatal heart in organ culture expressed dedifferentiation markers. One-day-old mouse hearts were harvested, sliced, and placed on a coated tissue culture dish (100 mm) using cardiomyocyte growth media. Cells budding from the cultured heart were stained with specific antibodies. A, Beating outgrowth of neonatal heart at day 14 in culture. Scale bar: 200  $\mu$ m. B, Cardiomyocyte budding from cultured heart at day 35 in culture. Cardiomyocytes were stained green with cardiac actin, nuclei were stained blue with DAPI. Scale bar: 100  $\mu$ m. C, The percentage of outgrowth cells that expressed the stem cell marker c-Kit was near 25% after 35 days in culture. C-kit expressing cells in red, nuclei stained blue with DAPI. These findings suggest resident cardiac stem-cell proliferation or cardiomyocyte dedifferentiation. Scale bar: 200  $\mu$ m. DAPI indicates 4',6-diamidino-2-phenylindole.

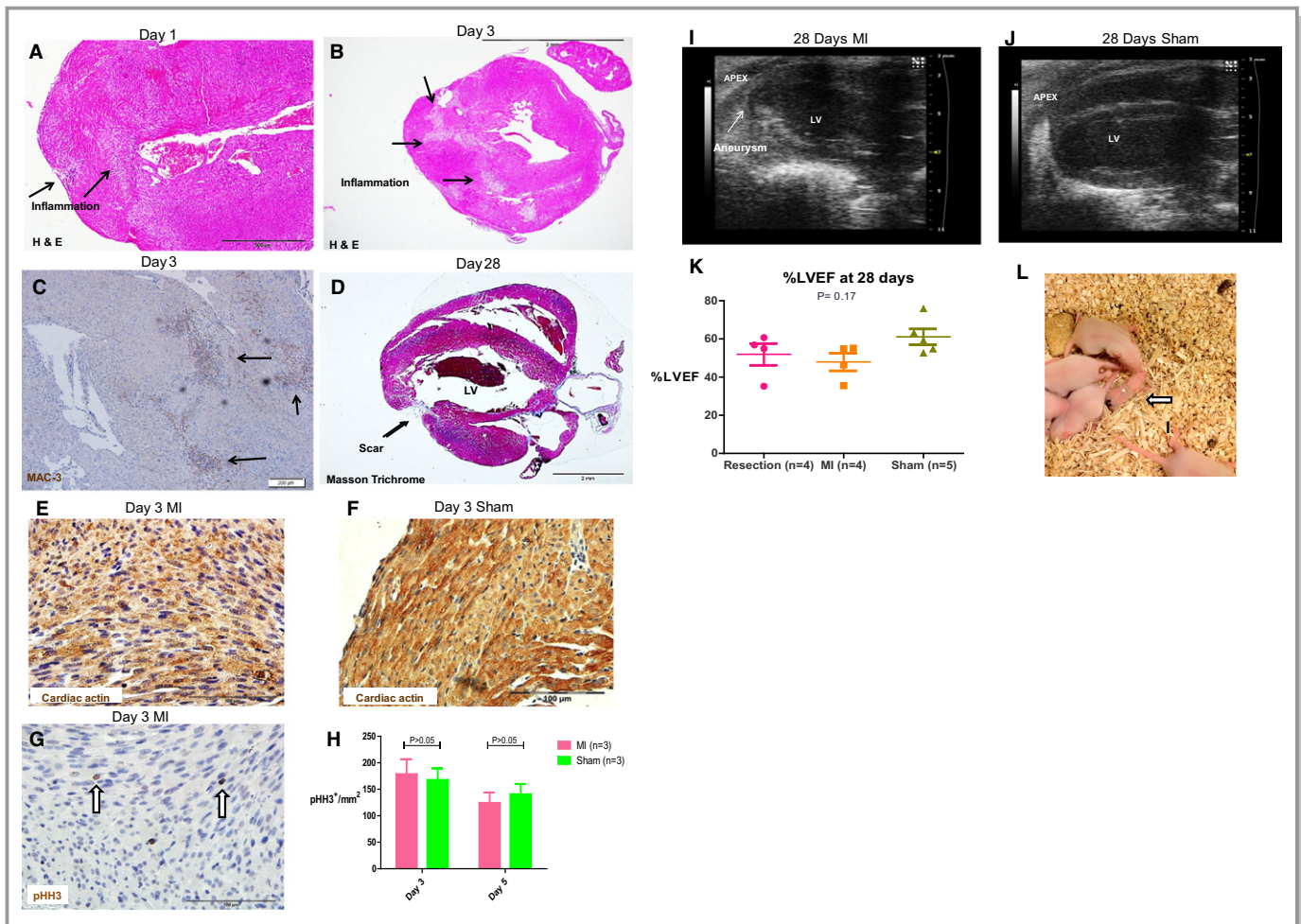
superficial and delicate because of raised blood pressure levels and to avoid massive fatal bleeding. The resection injury in the adult mouse produced massive bleeding, a blood clot that sealed the resected segment, inflammation, and an epicardial scar that covered a large hematoma 21 days after resection (Figure 5A). Interestingly, the blood clot, which appeared as part of the regenerative process in neonatal heart, was still evident in the adult heart scar, as were inflammation and granulation (Figure 5B). This extraordinary scar was clearly different from the typical transmural thin scar formed by MI 21 days after coronary artery occlusion in mouse (Figure 5C).

## Discussion

The major new finding of the present study suggests that the type of injury determines the mode of repair after MI. First, we confirmed previous reports<sup>6,8,19–21</sup> and showed that surgical resection of the left ventricle apex in 1-day-old mouse

stimulates a regenerative response that restores the damaged segment. New myocardium replaces the site of resection in neonatal hearts, whereas the new cardiomyocytes originate, at least to a large extent, from dedifferentiation and proliferation of cardiomyocytes at the border zone.<sup>6,8</sup> After MI, however, incomplete regeneration, small scar formation, aneurysm formation, and growth retardation were observed. Consequently, the type of tissue damage dictates the regenerative versus repair response. This difference in the mode of repair occurs in both neonatal and adult hearts. Our findings suggest that MI and subsequent inflammation might inhibit complete regeneration. Identification of the cells or molecules that stimulate or inhibit the regenerative response after myocardial injury could help us develop new therapies for myocardial regeneration in adult mice.

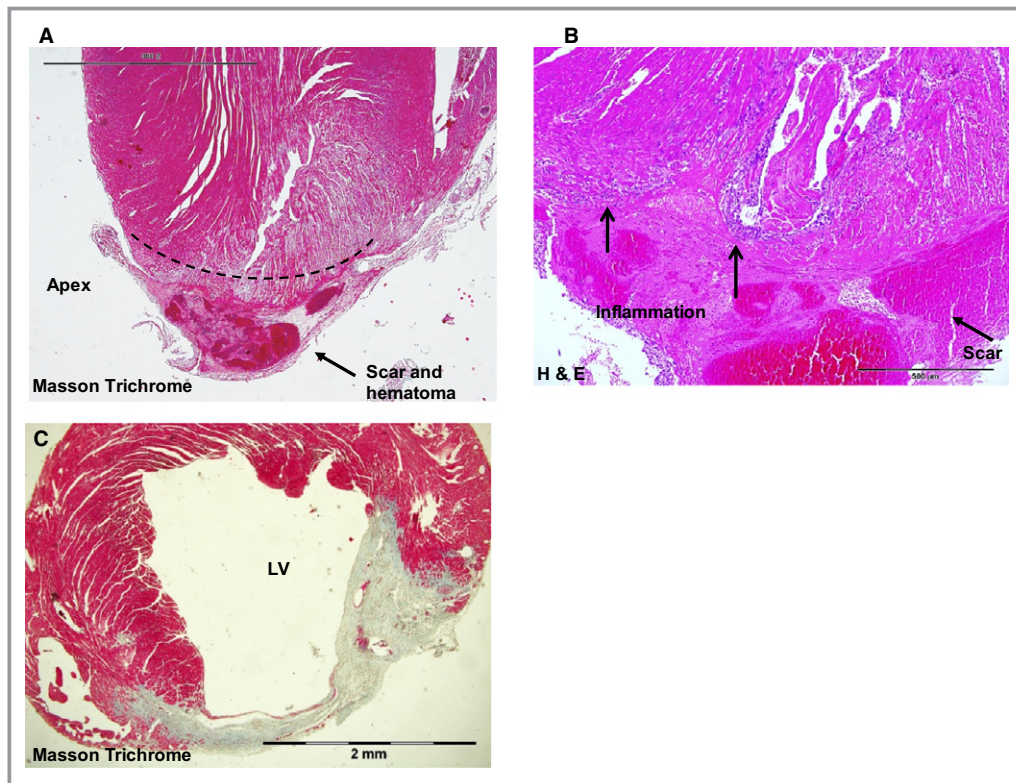
Some findings in our study differ from previous reports.<sup>9,10,22–25</sup> We found incomplete regeneration and scar formation after MI in neonatal mice, in contrast to the complete regeneration seen after MI in other studies.<sup>9,10,25</sup>



**Figure 4.** Incomplete myocardial regeneration after MI in neonatal heart. To determine the effect of ischemic injury on myocardial regeneration, newborn mice (1 day old) were anesthetized and cooled down on ice. The chest was opened by left thoracotomy. An 8-0 prolene suture was used to permanently occlude the left anterior descending artery. **A**, At 1 day after MI, H&E staining revealed cell necrosis and initial inflammatory response at the infarcted myocardium. Scale bar: 500  $\mu$ m. **B**, At 3 days after MI, H&E staining demonstrated extensive inflammation around the necrotic anteroapical areas (arrows). Scale bar: 2 mm. **C**, At day 3 after MI, mac-3 staining revealed robust accumulation of monocytes and macrophages (brown staining) at the necrotic myocardium. Scale bar: 200  $\mu$ m. **D**, Masson's trichrome staining showed a small thin scar at 28 days after MI (arrow). Scale bar: 2 mm. **E**, Cardiac actin staining of the border zone 3 days after MI revealed less evidence of proliferating cardiomyocytes or sarcomere disassembly. Cardiomyocytes were stained brown with cardiac actin, nuclei were stained blue with hematoxylin. Scale bar: 100  $\mu$ m. **F**, Cardiac actin staining of sham-operated heart 3 days after procedure revealed intact myocardium without inflammation or cardiomyocyte dedifferentiation. Scale bar: 100  $\mu$ m. **G**, pHH3 staining demonstrated mitotic nuclei at day 3 after MI (arrows). Scale bar: 100  $\mu$ m. **H**, At days 3 and 5 after MI, the number of mitotic events in the myocardium was similar to sham-operated hearts. Differences between pHH3 stain for mitosis were compared by 2-way ANOVA, with Bonferroni's multiple comparison post-test. Differences were considered significant at  $P < 0.05$ . **I**, Transthoracic echocardiography was performed after MI or sham MI using a special small animal echocardiography system equipped with a 22- to 55-MHz linear-array transducer. Images of 2D echocardiogram 28 days after MI in 1-day-old mouse demonstrated apical aneurysm (arrow) and LV dilatation. **J**, Images of 2D echocardiogram of a sham-operated mouse of the same age showed normal LV size and function. **K**, LVEF was slightly lower after apical resection and MI at 28 days after injury. Differences between echocardiography measurements were compared by 1-way ANOVA, with Bonferroni's multiple comparison post-test. Differences were considered significant at  $P < 0.05$ . **L**, Mouse at 6 days after MI (arrow) next to sham-operated mice at the same age. Note growth retardation of the mouse subjected to MI, suggesting the impact of significant myocardial injury on development. 2D indicates 2-dimensional; H&E, hematoxylin and eosin; LV, left ventricle; LVEF, left ventricle ejection fraction; MI, myocardial infarction; pHH3, phosphorylated histone H3.

Indeed, incomplete regeneration after neonatal heart cryoinjury has been described.<sup>12</sup> In the present study, we used sagittal rather than transverse sections to increase the sensitivity to detect small scars. We identified a marked increase in cardiomyocyte dedifferentiation and proliferation

after apical resection; however, after MI and subsequent incomplete regeneration, the percentage of proliferating cells was similar to that of sham MI. The reason for less proliferation and incomplete regeneration after MI is unclear. First, a small scar could be created by local, continuous injury



**Figure 5.** No regeneration and scar formation is noted in adult heart after apical resection or MI. ICR 12-week-old female mice were anesthetized, intubated, and ventilated. Chests were shaved and opened by left thoracotomy. Iridectomy scissors were used to carefully resect the heart apex to a smaller extent than neonatal resection because of severe bleeding and high mortality rate. A, At day 21 after resection in 12-week old mouse, Masson's trichrome revealed an apical, hemorrhagic scar at the resected area (arrow). Dashed line marks the resection plane. Scale bar: 2 mm. B, At day 21 after resection in adult mouse, H&E staining revealed active healing with inflammation and hematoma encapsulated within the apical scar. Scale bar: 500  $\mu$ m. C, In contrast, MI induced a typical thin, transmural scar at day 21 after MI. Scale bar: 2 mm. H&E indicates hematoxylin and eosin; LV, left ventricle; MI, myocardial infarction.

from the ligature, as previously suggested.<sup>10</sup> Second, we considered the possibility that the necrotic and apoptotic cells in the infarcted myocardium release factors that intensify reactive inflammation and compromise the survival of neighbor cardiomyocytes, destroy extracellular matrix integrity, and inhibit cardiomyocyte dedifferentiation and proliferation. Tissue excision, in contrast, minimizes the inflammatory response. In view of these plausible mechanisms, it is likely that larger infarct size compromises healing and regeneration compared with fine apical resection. Third, sometimes substantial regeneration after MI requires >28 days. Jesty et al,<sup>12</sup> for example, have described significantly more regeneration at day 94 than at day 21 after cryoinjury in a neonatal heart. It is possible that longer follow-up could disclose complete regenerative response similar to apical resection in neonatal heart. Together, these explanations could partially explain less cardiomyocyte proliferation and incomplete regeneration after MI and suggest that the mode of injury affects the extent of regeneration in neonatal heart. Finally, the discrepancy among different researchers may

arise from difficulties in standardizing the size of MI, such as small versus large infarcts.

Interestingly, we have shown that the mode of injury also affects myocardial repair in the adult heart. Although permanent coronary artery occlusion produced a transmural thin fibrotic scar, myocardial resection in the adult mouse induced an unusual superficial hemorrhagic scar without extensive scarring. These differences in mode of repair are similar to the differences observed in human patients. In patients with hypertrophic cardiomyopathy, for example, surgical septal resection produces a myocardial defect without necrosis or fibrosis, as indicated by the absence of delayed hyperenhancement, by cardiovascular magnetic resonance.<sup>26</sup> In contrast, alcohol septal ablation produces a morphologic appearance of MI: large areas of transmural necrosis and fibrosis, as indicated by hyperenhancement with cardiovascular magnetic resonance.<sup>26</sup> This observation in human patients supports our findings that the type of injury, mechanical versus ischemic, directs the healing and repair process in both neonatal and adult hearts.



The reason for the differences in repair response of the adult heart is unclear. We can speculate that the microenvironment at the injury site guides the mode of regeneration versus repair. Inflammation and scar tissue formation that replaces the necrotic myocardium after MI are essential to prevent cardiac wall rupture. Maintenance of extracellular matrix integrity has emerged as a critical factor in myocardial regeneration and repair.<sup>27–29</sup> It is possible that ischemic injury, with subsequent cell necrosis, apoptosis, and damage to the extracellular matrix, dictates the course of inflammation that inhibits complete regeneration. Mechanical resection, in contrast, induces mild inflammation without damage to the extracellular matrix at the border zone and supports regeneration. Finally, vascularization is essential for regeneration and repair, even in the neonatal heart.<sup>25</sup> It is possible that complete occlusion of the coronary artery interferes with complete vascularization and regeneration in the neonatal heart.

Surprisingly, a recent provocative report suggested that regeneration does not occur after apical resection in newborn mice.<sup>13</sup> These findings contradict several previous reports.<sup>6,9,10,12,19,20,23,24,30</sup> It is possible that the extent of the resection is critical. Extensive resection (>15%) used by the authors<sup>13</sup> could create intensive inflammation that impairs healing and regeneration. In addition, adhesions to the chest wall could either impair regeneration or cover the regenerated myocardium with fibrotic tissue. These adhesions are difficult to remove during pathology processing. Indeed, in 1 heart presented by Anderson et al (Figure 2C), the fibrotic tissue was spread over several discrete locations, including the right ventricle, at 37 days after resection. This diffuse scarring suggests that intensive inflammation, with or without adhesions to the injured myocardium, could impair healing and regeneration.

## Macrophages and Myocardial Regeneration

Within a day after resection we noticed robust accumulation of monocytes and macrophages in the clot that covered the resection segment and, subsequently at day 3, in the granulation tissue. These findings confirmed previous reports and suggest that circulating monocytes are the major source of macrophages in the heart after injury.<sup>25,31–34</sup> Interestingly, a most recent report suggested that a population of resident embryonic cardiac macrophages expands in response to diffuse toxic injury (mainly apoptosis) in the neonatal heart.<sup>35</sup> This distinct population of neonatal macrophages contributes to neonatal heart regeneration.<sup>35</sup> Nevertheless, this study supports the findings of Aurora et al, who have suggested that macrophages are key mediators of neonatal heart regeneration and that monocyte and macrophage depletion blocked myocardial regeneration.<sup>25</sup> The mecha-

nism by which macrophages mediate myocardial regeneration and repair is unclear. Aurora et al showed that the number of proliferating cardiomyocytes was not different from macrophage-depleted and control mice at day 7 after MI. They suggested that cardiomyocyte proliferation is not controlled by monocytes and macrophages but rather that a defect in macrophage-mediated neovascularization regulates cardiac regeneration.<sup>25</sup>

## Limitations

The model of apical resection and MI induction in neonatal mice is prone to a high death rate and mother cannibalism.<sup>8</sup> When testing various interventions, it is difficult to assess whether the mouse died from the intervention or from technical difficulties. In addition, most methods used to detect cell proliferation actually label DNA duplication and nuclear division rather than real cytokinesis. Some reagents for measuring proliferation, such as labeled ribonucleotide analogs, could lead to confusion between DNA repair and proliferation, labeling as “proliferative” cells that are in fact damaged and have activated their repair machinery. In the present study, we estimated cell proliferation by phosphorylated histone H3 staining, which marked both mitosis and meiosis and is considered to be a marker of cell division.<sup>17</sup> The source of dedifferentiated cardiomyocytes is unclear. Dedifferentiation is difficult to demonstrate *in vivo*, and the eligible markers are not definitive. Furthermore, it is difficult to prove that dedifferentiated cells are the source of proliferating cardiomyocytes. In the present study, we described histological characteristics of dedifferentiating cardiomyocytes. In some of them, we clearly demonstrated dividing nuclei (Figure 2A through 2C). Finally, it is possible that the less differentiated, proliferating cardiomyocytes are the progeny of activated resident cardiac stem cells in the phase of lineage specification. A lineage-mapping study is needed to determine the exact source of the proliferating cardiomyocytes.<sup>36,37</sup>

## Conclusions and Implications

Our study suggests that mechanical versus ischemic myocardial damage dictates the mode of healing and repair in both neonatal and adult mouse hearts. Taken together, differences in sequelae between these 2 types of injuries may have important implications for the development of new regenerative therapies relevant to human patients and underscore the use of a myocardial ischemic model. It is possible that, unlike myocardial resection, ischemic cell death by necrosis and apoptosis dictates the inflammatory response and inhibits complete regeneration. These findings are important because

the relationship between the type of injury and the subsequent regenerative response could provide mechanistic insight into myocardial regeneration. The role of macrophages and the differences between neonatal and adult macrophages need further evaluation. Understanding what causes the neonatal heart to regenerate completely after resection but to develop incomplete regeneration after ischemic injury may contribute toward the development of new tools to induce myocardial regeneration in the adult human heart.

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## Disclosures

None.

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