Supporting Online Material for

Transient Regenerative Potential of the Neonatal Mouse Heart

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Materials and Methods

Apical resection

Apical resection surgeries were performed on neonatal mice (ICR/CD-1 strain, Charles River Laboratories, MA) at post natal day 1 (P1) and day 7 (P7). Neonates were anesthetized by cooling on an ice bed for 4 minutes. Neonatal rodents (up to 1 week old) can withstand long periods of hypothermia (*1*), which also causes reversible apnea and asystole (*2*), thus preventing excessive blood loss during surgery. Lateral thoracotomy at the fourth intercostal space was performed by blunt dissection of the intercostal muscles following skin incision. Iridectomy scissors were used to resect the apex of P1 hearts until the left ventricular chamber was exposed (removal of greater portions of the ventricle markedly increased surgical mortality). Following apical resection, neonates were removed from the ice bed, thoracic wall incisions were sutured with 7-0 non absorbable silk suture, and the skin wound closed using skin adhesive. Sham-operated mice underwent the same procedure without apical resection. Neonates were then placed under a heat lamp and warmed for several minutes until recovery. The entire procedure lasted approximately 10 minutes. Ninety percent of P1 neonates survived the surgical procedure, with all deaths occurring during or on the day of surgery. However, maternal cannibalization reduced survival rates the following day to ~70%. Surgical lethality was much higher in P7 pups, compared with P1 pups. Therefore, a smaller proportion of the apex was resected in P7 pups, with minimal chamber exposition. The day after surgery, mice that underwent apical resection were indistinguishable from sham-operated mice.

Our surgical protocol using chamber exposure as a landmark for the limit of resection ensured reproducibility of the procedure. To further demonstrate surgical reproducibility, we weighed 12 resected and 11 intact ventricles immediately after surgery. The hearts were excised after surgery and briefly rinsed in phosphate-buffered saline solution to remove residual blood from the ventricles. After dissecting non-cardiac tissue and atria, the ventricles were blotted and weighed.

Histology (Hematoxylin/eosin, Trichrome and Endomucin)

Hearts were fixed in 4% paraformaldehyde overnight at room temperature. The following day, hearts were moved to 50% ethanol and stored for up to one week before paraffin embedding. Paraffin sections (5 μm) were cut through the entire ventricle. Hematoxylin/eosin and Masson's trichrome staining were performed according to standard procedures. Hematoxylin/eosin stained sections were used to quantify muscle replacement throughout the first 21 days of cardiac regeneration. The largest two ventricular sections from each animal were quantified using Image J software (National Institutes of Health, MA). The mean area of resected ventricles was expressed as a percentage difference relative to sham-operated ventricles (set to 100%) at 1 and 21 days post-surgery. We performed an exhaustive analysis of the resected hearts at multiple time points. This analysis included inspection of serial sections throughout the entire myocardium antero-posteriorly. We only found 2 out of 140 sections that showed incomplete regeneration 21 days post resection.

For endomucin staining, which labels endothelial cells and marks blood vessels (*3*), slides were deparaffinized, blocked in 5% goat serum (Invitrogen, CA)

and incubated with a rat anti-mouse endomucin antibody (1:250 dilution in PBS clone# eBioV.7C7 (V.7C7), eBioscience, CA, USA) overnight at 4ºC. The following day, slides were washed 3 times in PBS and then incubated for 30 min at room temperature with a biotinylated rabbit anti-rat secondary antibody (1:500 dlution, Vector Labs, CA). Slides were next washed 3 times in PBS and then incubated for 30 minutes at room temperature with horseradish peroxidase streptavidin (1:500 dilution, Vector Labs, CA). Slides were then washed 4 times in PBS, incubated in the dark for 10 min at room temperature with diaminobenzodine (Dako, CA) and counterstained with hematoxylin. Sections were then dehydrated in ethanol twice for 3 min each, before being cleared with xylene (2 x 3 min) and mounted in Permount (Fisher Scientific, IL).

Echocardiography

Apical LV systolic function was calculated two months following apical resection echocardiographically on conscious mice using the Visual Sonics Vevo 2100, equipped with a 40 MHz mouse ultrasound probe. Fractional shortening was calculated based on end diastolic and end systolic dimensions obtained from M-mode ultrasound.

BrdU pulse-chase

For 5-bromo-2-deoxyuridine (BrdU) labeling experiments, neonates were injected subcutaneously on days 1, 7 and 14 after surgery with 0.035 ml of a 20 mg/ml solution of BrdU (Roche Applied Sciences, IN) dissolved in sterile 0.9% saline solution (Sigma-Aldrich, MO). In order to ensure detection of dividing cells and avoid false-positive labeling of binucleating cardiomyocytes, it was important to administer

BrdU prior to the onset of cardiomyocyte binucleation, which occurs before P7 in mice (*4*). The hearts were harvested 21 days after surgery.

Genetic Fate Mapping Study

We crossed cardiomyocyte-specific MerCreMer mice (Tg(Myh6-cre/Esr*)1Jmk/J, The Jackson Laboratory) with *Rosa26*-lacZ-flox-targeted mice (*Gtrosa26*^{tm1Sor}, The Jackson Laboratory) to generate a tamoxifen-inducible cardiomyocyte reporter strain for genetic fate mapping (*5*). MerCreMer is under the control of the cardiomyocyte-specific promoter alpha myosin heavy chain (αMHC). Induction of Cre recombinase activity and lacZ reporter expression was achieved by administering a single 2 mg dose of tamoxifen (Sigma), dissolved in sesame oil (Sigma), subcutaneously to neonatal mice at birth. The following day, neonates underwent apical resection. At 21 dpr, hearts were harvested, embedded in tissue freezing medium and flash frozen in 2-methylbutane cooled on liquid nitrogen (Sigma). Cryosections (8 μm) were stained with X-gal staining solution for 48 hours at 37ºC to detect lacZ activity throughout the heart (*5*). Sections were counterstained with nuclear fast red and the percentage of myocardial tissue stained positive for lacZ was quantified at 40x magnification using Image J software.

Immunofluorescence

All immunofluorescence was performed on paraffin sections. For phospho-histone H3/troponin T and Wt1/Troponin T co-staining, slides were rinsed 3 times in PBS, blocked in 10% goat serum for 20 minutes followed by 3 rinses in PBS. Sections underwent antigen retrieval by boiling in sodium citrate solution for 20

minutes. This was followed by overnight incubation with primary antibodies against phospho-histone H3 (Ser10) (1:100, rabbit polyclonal, Millipore, MA) or Wt1 (1:50, rabbit polyclonal, Santa Cruz Biotechnology, CA) and cardiac troponin T (1:100, mouse monoclonal, Thermo Scientific, IL). The following day, slides were washed 3 times in PBS and incubated with anti-mouse and anti-rabbit secondary antibodies conjugated to Alexa Fluor 488 or 555 (1:400 dilution; Invitrogen, CA) for 1 hour at room temperature. Slides were washed 3 times in PBS, stained with Hoechst 33342 (Invitrogen, CA) for 3 minutes to label nuclei and mounted in Vectashield (Vector Labs, CA).

For Aurora B and troponin T co-staining, a similar protocol was used excluding the antigen retrieval step. Overnight incubation was performed with primary antibodies against Aurora B (1:25, rabbit polyclonal, Sigma, MO) and cardiac troponin T (as above).

For BrdU and troponin T co-staining, slides were deparaffinized and DNA was then denatured by incubating slides in 2N HCl for 60 minutes at 37ºC. HCl was neutralized by immersing slides in 0.1M Borate buffer, pH 8.5, twice. Slides were rinsed with PBS 3 times, permeabilized with 0.3% Triton X-100 twice for 5 minutes, rinsed with PBS 3 times and then blocked with 10% goat serum for 20 minutes. This was followed by overnight incubation at 4 ºC with primary antibodies against BrdU (1:25, rat monoclonal, Abcam, Cambridge, MA) and cardiac troponin T (1:100, mouse monoclonal, Thermo Scientific, IL). The following day slides were incubated with an anti-rat biotinylated secondary antibody conjugated to FITC (1:200, Vector

Labs, CA) and an anti-mouse secondary antibody conjugated to Alexa Fluor 555 (1:400 dilution; Invitrogen, CA) for 1 hour at room temperature.

For BrdU and Nkx-2.5 co-staining, sections underwent antigen retrieval (see above), incubation with 0.1% Triton X-100 for 5 minutes, followed by blocking with universal blocking solution. Slides were then incubated overnight with primary antibodies (anti-BrdU, 1:50 dilution, mouse monoclonal, Roche Applied Sciences; anti-Nkx-2.5, 1:100 dilution, goat polyclonal, R&D Systems, MN) followed by secondary antibody staining with anti-mouse and anti-goat secondary antibodies conjugated to Alexa Fluor 488 or 555 (1:400 dilution; Invitrogen, CA), as described above. The slides were examined by fluorescence microscopy (Leica, IL) or laser confocal (510 META, Carl Zeiss MicroImaging, NY) microscopy, as indicated.

For wheat germ agglutinin (WGA) staining, slides were deparaffinized, rinsed 3 times in PBS and then incubated for 1 hour at room temperature with primary antibody against WGA conjugated to Alexa Fluor 594 (50 μg/ml, Invitrogen, CA) in PBS. Slides were then rinsed 3 times in PBS, stained with Hoechst 33342, mounted in Vectashield (Vector Labs, CA) and imaged by fluorescence microscopy, as described above.

RNA extraction and real-time PCR

To enrich for transcripts in the regenerating myocardium, we roughly dissected one third of the ventricle containing the regeneration plane, as previously described (*6*). Total RNA was extracted from resected and sham-operated ventricles at 1, 7, 14 and 21 days post-surgery using TRIzol (Invitrogen). Quantitative real-

time PCR was performed using an ABI 7000 cycler. cDNA was obtained by reverse transcription using random hexamers as primer (Invitrogen). Real-time PCR assays utilized either TaqMan or SYBR green chemistries depending on primer/probe design. Commercially available TaqMan mRNA expression assays for *Myh6*, *Myh7*, *Nppa* and *Nppb* were performed according to the manufacturer's recommended protocol (Applied Biosystems, CA). SYBR green chemistry was employed for analysis of *Col1a1*, *Col3a1*, *Il6*, *Il1b* and *Ccl3* mRNA expression levels. Real-time PCR reactions were amplified at 95°C for 30 sec and 60°C for 30 sec for 40 cycles, following an initial denaturation step at 95°C for 3 min. Amplification products were routinely checked using dissociation curve software (Applied Biosystems), and transcript quantities were compared using the $\Delta\Delta$Ct method (*7*). 18S ribosomal RNA was used as a housekeeping control. All primers for SYBR green reactions are listed in Supplementary Table 1.

Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). Student's unpaired t-test was used for comparisons between sham and resected groups. A value of $p<0.05$ was considered significant.

All protocols were approved by the Institutional Animal Care and Use Committee at UT Southwestern Medical Center.

Supplementary Figure Legends:

Supplementary Figure 1: An acute inflammatory response accompanies the early stages of cardiac regeneration in neonatal mice. **(A)** H&E stained heart section at 2 dpr showing infiltrating monocytes (arrowhead) surrounding a large fibrin clot (arrow) filled with erythrocytes (asterisks) adjacent to the wound. **(B)** Real-time PCR analysis of mRNA expression levels for several inflammatory markers (*Il6*, *Il1b* and *Ccl3*) at 1, 7, 14 and 21 days post injury. Gene expression changes are presented as a fold change relative to sham-operated controls, which are shown as a dashed line at 1.0. Values are presented as mean±SEM; n = 3-5 per group; *P<0.05.

Supplementary Figure 2: Formation of coronary vessels in regenerated myocardium. **(A** and **B)** H&E stained sections of regenerated myocardium 21 days after resection shown at low (5x) and high (20x) magnification. Note numerous coronary vessels lining the epicardial surface of the regenerated apex. **(C** and **D)** Endomucin staining of endothelial cells marks blood vessels in the newly formed apex. Images are shown at low (5x) and high (20x) magnification.

Supplementary Figure 3: Global de-differentiation and proliferation of cardiomyocytes in regenerating myocardium. **(A-D)** Cardiomyocyte mitoses were identified by staining for phosphorylated-histone H3 (green), troponin T (red) and nuclei (blue) in sham (A and C) and resected (B and D) hearts. Note the many de-differentiated cardiomyocytes with disassembled sarcomeres in the border zone and remote myocardium of regenerating hearts (B and D), compared to sham hearts (A and C). **(E-J)** Quantification of sarcomere disassembly and cardiomyocyte

proliferation in the apex, border zone and remote zone of sham and resected hearts at 1, 7, 14 and 21 dpr. Quantitative analysis represents counting of multiple fields from 3-4 independent samples per group at each time point. Quantification was performed at 20X magnification for pH3 staining, and at 10X magnification for sarcomere disassembly. These results indicate that apical resection induces a global myocyte proliferative response.

Supplementary Figure 4: Quantification of cardiomyocyte cytokinesis at 7 dpr. **(A)** Image showing a cytokinetic cardiomyocyte with aurora B kinase staining (green; arrow) localized to the cleavage furrow of a cardiomyocyte (red). **(B)** Quantitative analysis of cardiomyocyte cytokinesis in the apex, border zone and remote regions of sham-operated and resected hearts at 7 dpr. Quantification represents counts per field at 40x mag from 3 independent samples per group (~60 fields of cells per group). Values presented as mean±SEM; *P<0.05.

Supplementary Figure 5: The majority of newly formed cardiomyocytes within the regenerated apex are derived from pre-existing cardiomyocytes. **(A)** Schematic of the BrdU pulse-chase experiment designed to label proliferating cardiomyocytes. **(B)** BrdU-positive cardiomyocytes in the left ventricular apex following pulse-chase experiment demonstrating co-localization of BrdU (green) and Nkx2.5 (red) to cardiomyocyte nuclei (blue). Upper panel scale bar = 200 μm, lower 4 panels scale bar = 20 μm. **(C-F)** Co-localization of BrdU (green) and cardiac Troponin T (red) at the apex following resection. Images are shown for sham (C and D) and resected (E and F) hearts at low and high magnification. Note high number of BrdU positive cardiomyocytes within the resected apex at 21 dpr. Scale bar, 20 μm.

Supplementary Figure 6: Schematic of genetic fate mapping study design. αMHC-MerCreMer transgenic mice were crossed with *Rosa26*-lacZ reporter mice. Upon administration of a single dose of tamoxifen, the lacZ reporter gene switches on in the majority of cardiomyocytes. The presence of lacZ-positive cardiomyocytes within the regenerated apex following apical resection suggests that these cells are derived from an αMHC lineage.

Supplementary Figure 7: Cardiac regeneration in neonatal mice is not associated with significant cardiomyocyte hypertrophy or robust fibrosis. **(A)** Wheat germ agglutinin staining of apical myocardium in sham and resected hearts at 7, 14 and 21 dpr. **(B)** Quantification of cell size in apical myocardium of sham and resected hearts at 7, 14 and 21 days post injury. Values represent mean±SEM from 3 hearts per group (~200 cells assessed per heart). **(C)** Real-time PCR analysis of mRNA expression levels for several hypertrophic markers (*Myh6*, *Myh7*, *Nppa* and *Nppb*) at 21 days post injury. Gene expression changes are presented as a fold change relative to sham-operated controls. **(D)** Quantification of mRNA expression levels for *Col1a1* and *Col3a1* in regenerating hearts. Gene expression changes are presented as a fold change relative to sham-operated controls (dashed line at 1.0). Values presented as mean±SEM; n = 3-5 per group; *P<0.05.

Supplementary Figure 8: Evidence of epicardial activation following apical resection. **(A)** Real-time PCR for the epicardial genes *Wt1* and *Aldh1a2* demonstrating a modest up-regulation following apical resection. Gene expression changes are presented as a fold change relative to sham-operated controls (dashed

line at 1.0). Values presented as mean±SEM; *n* = 5 per group; **P*<0.05. **(B-D)** Immunostaining demonstrating Wt1 positive staining (green) in the epicardium at the injury site following resection.

Supplementary Figure 9: Cardiomyocyte mitoses were assessed 7 dpr at post-natal day 7 (P7) by staining for phosphorylated histone H3 (green), troponin T (red) and nuclei (blue). No de-differentiating or proliferating cardiomyocytes were noted around the regeneration plane in resected hearts **(A)**. A number of proliferating non-myocytes (arrows) were identified 7 days after resection within the fibrous scar **(B)**. Scale bar = 40 μm.

Supplementary Table 1: Primer sequences for real-time PCR reactions. S = sense; AS = antisense.

Gene	NCBI Reference Sequence	Primer Sequences
Il6	NM_031168.1	S 5'-TGATGCACTTGCAGAAAACA-3' AS 5'-ACCAGAGGAAATTTTCAATAGGC-3'
Il1b	NM_008361.3	S 5'-TGTGAAATGCCACCTTTTGA-3' AS 5'-GGTCAAAGGTTTGGAAGCAG-3'
Ccl3	NM_011337.2	S 5'-ACCATGACACTCTGCAACCA-3' AS 5'-GTGGAATCTTCCGGCTGTAG-3'
Col1a1	NM_007742.3	S 5'- TAGGCCATTGTGTATGCAGC-3' AS 5'- ACATGTTCAGCTTTGTGGACC-3'
Col3a1	NM_009930.2	S 5'- TAGGACTGACCAAGGTGGCT-3' AS 5'- GGAACCTGGTTTCTTCTCACC-3'

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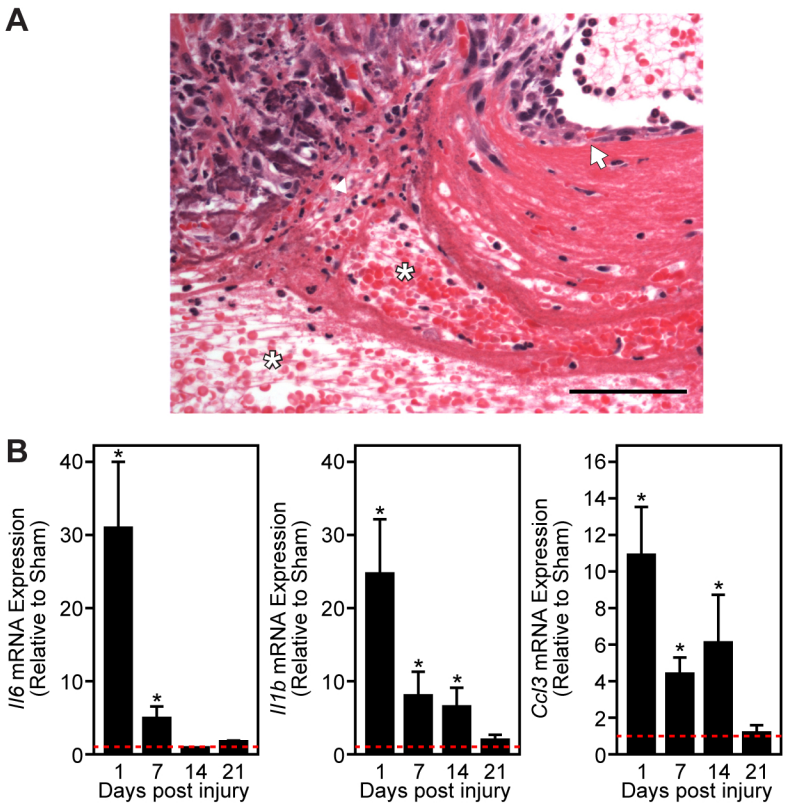
A
B
Il6 mRNA Expression (Relative to Sham)
Il1b mRNA Expression (Relative to Sham)
Ccl3 mRNA Expression (Relative to Sham)
Days post injury
1 7 14 21
0 10 20 30 40
0 2 4 6 8 10 12 14 16

Fig. S1

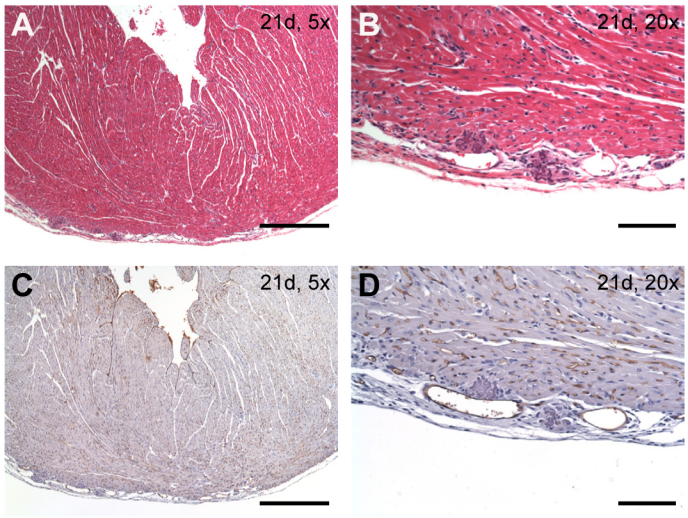

Fig. S2

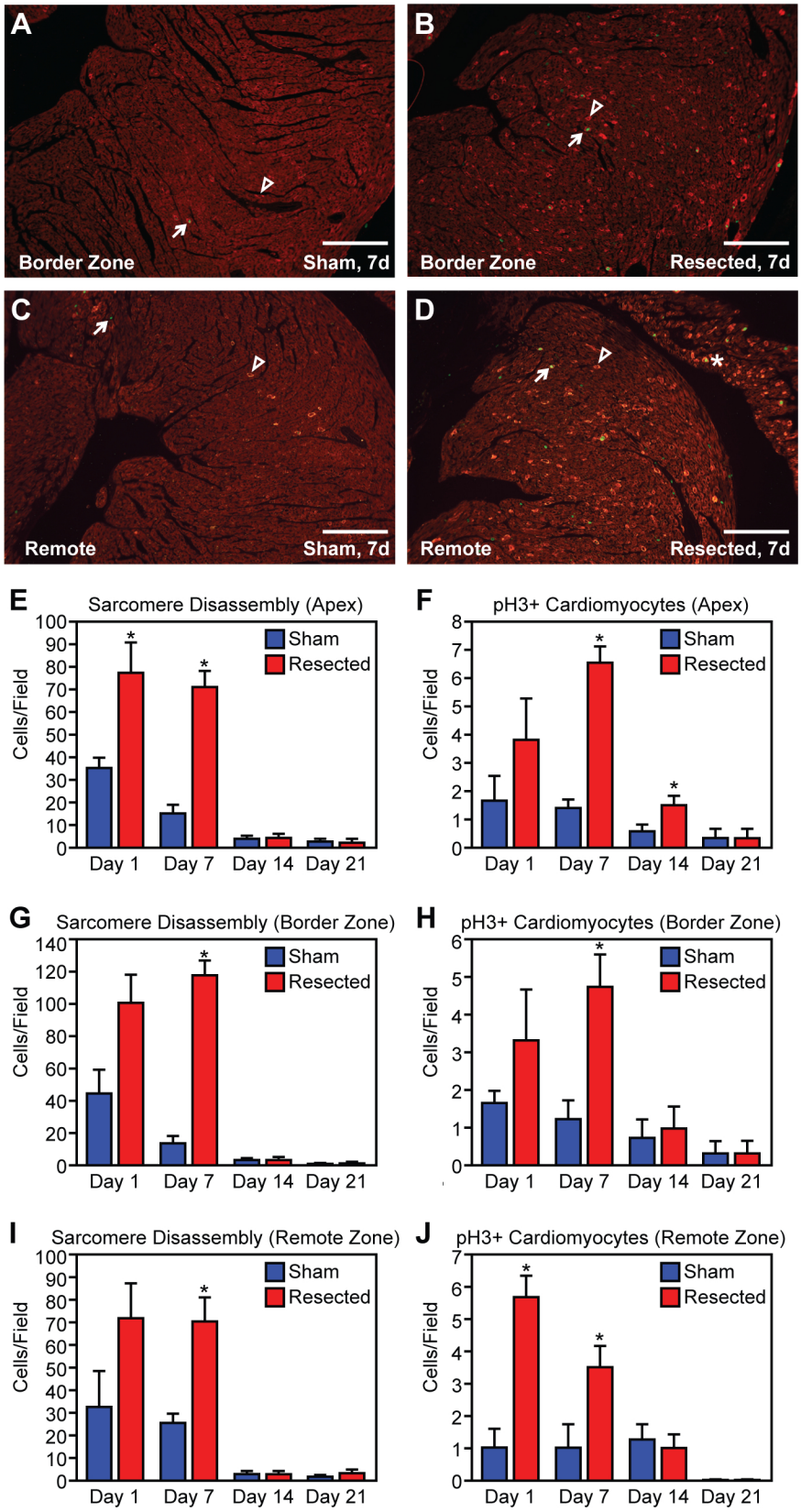
A
Border Zone
Sham, 7d
B
Border Zone
Resected, 7d
C
Remote
Sham, 7d
D
Remote
Resected, 7d
E
Sarcomere Disassembly (Apex)
Cells/Field
Sham
Resected
Day 1
Day 7
Day 14
Day 21
F
pH3+ Cardiomyocytes (Apex)
G
Sarcomere Disassembly (Border Zone)
H
pH3+ Cardiomyocytes (Border Zone)
I
Sarcomere Disassembly (Remote Zone)
J
pH3+ Cardiomyocytes (Remote Zone)

Fig. S3

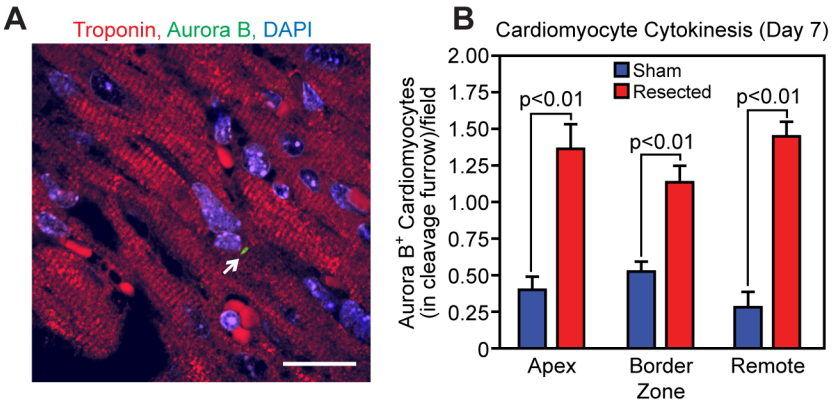
A
Troponin, Aurora B, DAPI
B
Cardiomyocyte Cytokinesis (Day 7)
Aurora B+ Cardiomyocytes (in cleavage furrow)/field
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0
Sham
Resected
p<0.01
p<0.01
p<0.01
Apex
Border Zone
Remote

Fig. S4

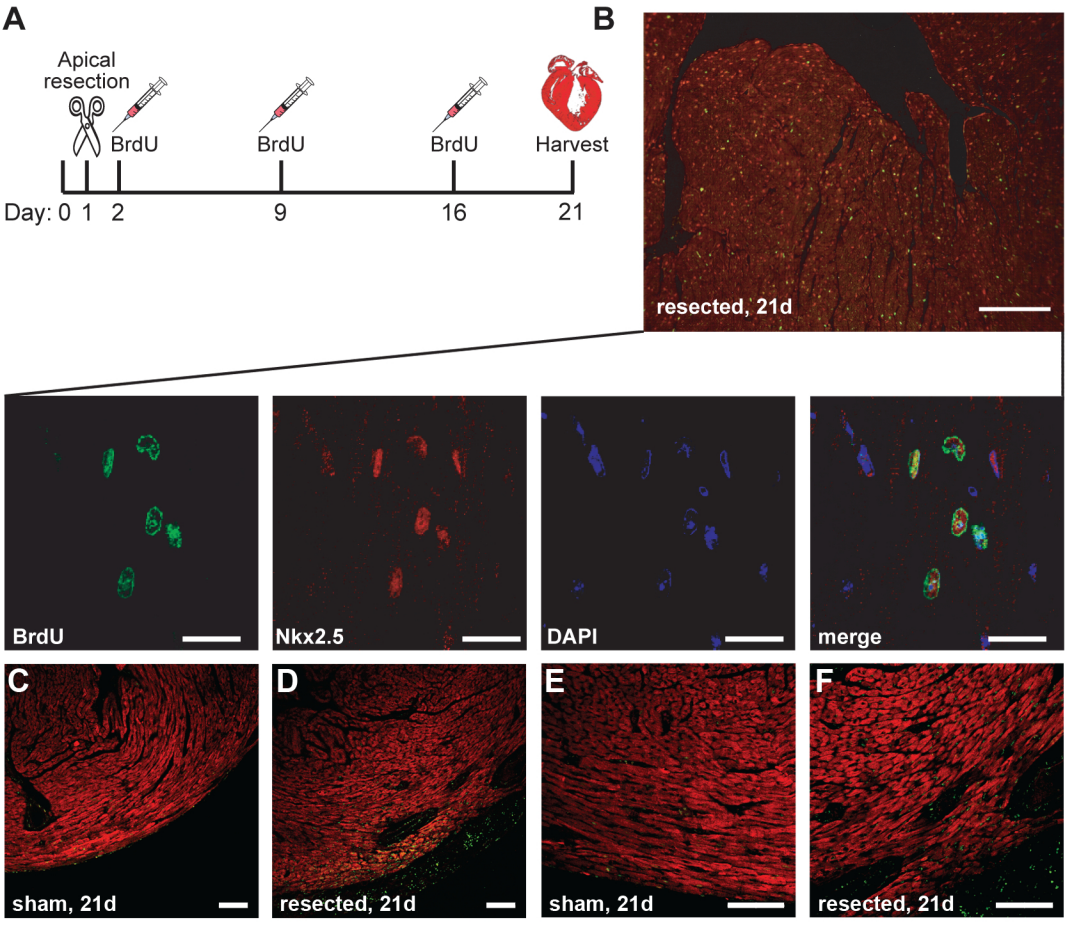
A
Apical resection
BrdU
BrdU
BrdU
Harvest
Day: 0 1 2 9 16 21
B
resected, 21d
BrdU
Nkx2.5
DAPI
merge
C
sham, 21d
D
resected, 21d
E
sham, 21d
F
resected, 21d

Fig. S5

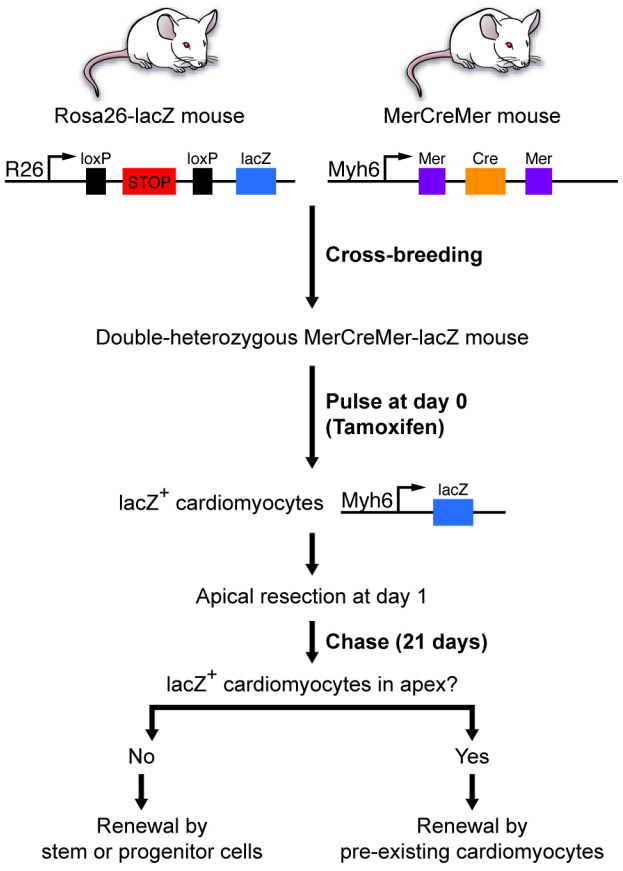
Rosa26-lacZ mouse
MerCreMer mouse
R26
loxP
STOP
loxP
lacZ
Myh6
Mer
Cre
Mer
Cross-breeding
Double-heterozygous MerCreMer-lacZ mouse
Pulse at day 0
(Tamoxifen)
lacZ+ cardiomyocytes
Myh6
lacZ
Apical resection at day 1
Chase (21 days)
lacZ+ cardiomyocytes in apex?
No
Yes
Renewal by
stem or progenitor cells
Renewal by
pre-existing cardiomyocytes

Fig. S6

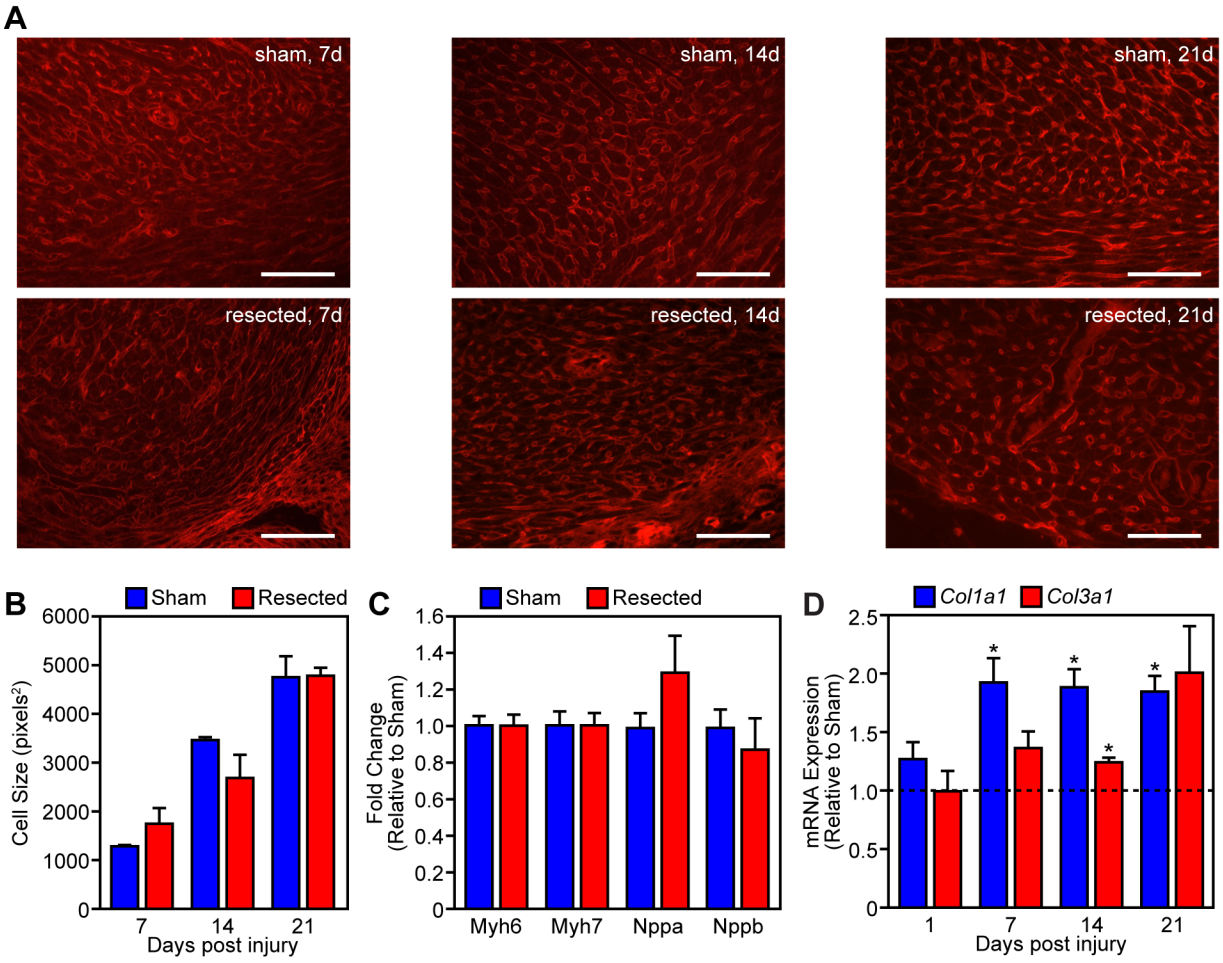
A
sham, 7d
sham, 14d
sham, 21d
resected, 7d
resected, 14d
resected, 21d
B
Sham
Resected
Cell Size (pixels²)
6000
5000
4000
3000
2000
1000
0
7
14
21
Days post injury
C
Sham
Resected
Fold Change (Relative to Sham)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Myh6
Myh7
Nppa
Nppb
D
Col1a1
Col3a1
mRNA Expression (Relative to Sham)
2.5
2.0
1.5
1.0
0.5
0
*
1
7
14
21
Days post injury

Fig. S7

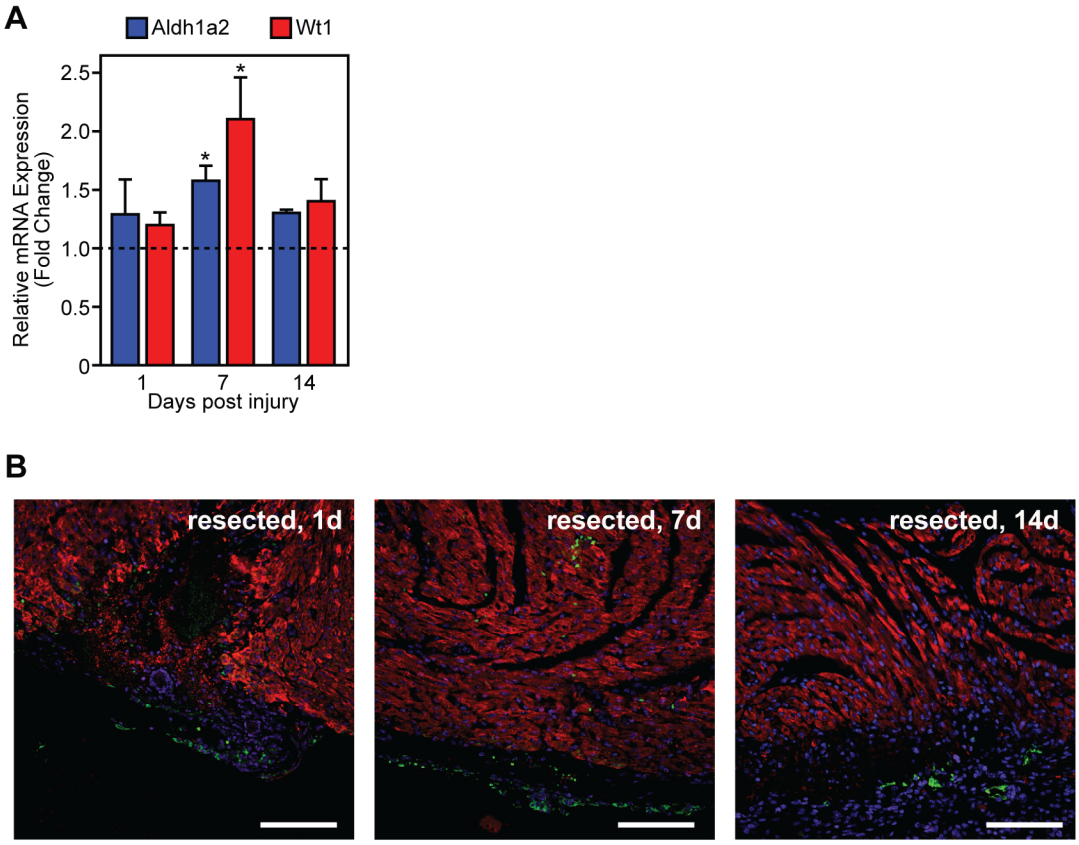
A
Aldh1a2
Wt1
Relative mRNA Expression
(Fold Change)
2.5
2.0
1.5
1.0
0.5
0
*
*
1
7
14
Days post injury
B
resected, 1d
resected, 7d
resected, 14d

Fig. S8

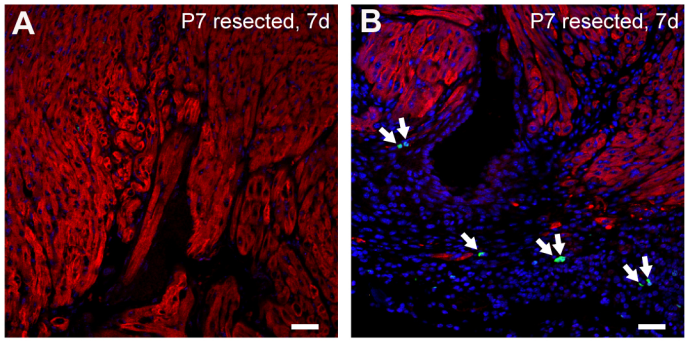

Fig. S9