Molecular Cardiology

Fetal Mammalian Heart Generates a Robust Compensatory Response to Cell Loss

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Background—Heart development is tightly regulated by signaling events acting on a defined number of progenitor and differentiated cardiac cells. Although loss of function of these signaling pathways leads to congenital malformation, the consequences of cardiac progenitor cell or embryonic cardiomyocyte loss are less clear. In this study, we tested the hypothesis that embryonic mouse hearts exhibit a robust mechanism for regeneration after extensive cell loss.

Methods and Results—By combining a conditional cell ablation approach with a novel blastocyst complementation strategy, we generated murine embryos that exhibit a full spectrum of cardiac progenitor cell or cardiomyocyte ablation. Remarkably, ablation of up to 60% of cardiac progenitor cells at embryonic day 7.5 was well tolerated and permitted embryo survival. Ablation of embryonic cardiomyocytes to a similar degree (50% to 60%) at embryonic day 9.0 could be fully rescued by residual myocytes with no obvious adult cardiac functional deficit. In both ablation models, an increase in cardiomyocyte proliferation rate was detected and accounted for at least some of the rapid recovery of myocardial cellularity and heart size.

Conclusion—Our study defines the threshold for cell loss in the embryonic mammalian heart and reveals a robust cardiomyocyte compensatory response that sustains normal fetal development. (Circulation. 2015;132:109-121. DOI: 10.1161/CIRCULATIONAHA.114.011490.)

Key Words: heart ■ mice ■ myocytes, cardiac ■ regeneration ■ stem cells

The development of the mammalian 4-chambered heart requires a close interplay between extracellular morphogens and signals (eg, Wnt, transforming growth factor-β, fibroblast growth factor, Notch, and Hedgehog) and cell-intrinsic effectors such as transcription factors and epigenetic modifiers.1,2 Some of these factors regulate early lineage specification; others mediate cellular expansion and maturation. Pioneering work on model organisms such as Drosophila, Xenopus, chick, zebrafish, and mouse has revealed the exquisite sensitivity of the early embryo to perturbations in these pathways that result in morphological defects and subsequent early embryonic lethality.3 Disruption in these formative events during embryonic development also has a profound impact on human life; congenital heart disease remains the commonest cause of birth defects worldwide.4

Although cardiogenesis is in many ways a delicate process, the heart is nonetheless able to withstand injury and to regenerate throughout the life span of some animal species, as demonstrated by studies in amphibians and lower vertebrates such as the newt and zebrafish.3,4 In these organisms, it has been proposed that an increased rate of proliferation by preexisting cardiomyocytes is the predominant endogenous mechanism responsible for cardiac injury repair.5-6 Collectively, these nonmammalian models have motivated efforts to define the extent of heart regeneration present in mice and humans and to identify underlying repair mechanisms that could represent potential targets for therapeutic intervention. Indeed, recent reports suggest that the neonatal murine heart can regenerate after experimental injury in a process that depends on Meis1-regulated cardiomyocyte proliferation.7-11 Furthermore, a prior study from Drenckhahn and colleagues12 found that healthy embryonic cardiomyocytes can augment their rate of proliferation to compensate for the reduced proliferation

Received June 17, 2014; accepted April 17, 2015.
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Presented at the AHA Scientific Sessions, November 16-20, 2013, Dallas Convention Center, Dallas, TX.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.114.011490/-/DC1.

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Circulation is available at http://circ.ahajournals.org DOI: 10.1161/CIRCULATIONAHA.114.011490

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rate of diseased cardiomyocytes that are deficient in holocytochrome-c synthase, an enzyme that is essential for mitochondrial respiration. Although this approach and others examined the role of cell competition during cardiac development, they did not directly address the consequences of cardiomyocyte loss. Accordingly, the ability of an embryonic mammalian heart to regenerate after rapid and extensive cell loss has yet to be addressed.

In this study, we used a novel blastocyst complementation strategy to test the ability of a mammalian embryonic heart to compensate for rapid cardiac cell loss and to quantitatively define the extent of cardiac lineage cell ablation that is compatible with normal development. We hypothesized that genetically mediated cell loss in the mouse heart can be fully compensated for by residual nonablated cells if a robust regenerative mechanism exists at the cardiac progenitor cell (CPC) or immature cardiomyocyte stage of development and found that ablation of up to 50% to 60% of CPCs or immature cardiomyocytes is well tolerated and supports live birth to adulthood. Furthermore, we found that proliferation of residual cardiomyocytes is involved in the cell replacement process. Our findings encourage further investigation of the endogenous mechanisms responsible for replacing lost CPCs and cardiomyocytes in the embryonic heart. In the context of adult mammalian cardiac injury, advances in this area may lead to therapies that direct the postinjury response toward cellular regeneration rather than fibrosis.

Methods
An expanded Methods section is available in the online-only Data Supplement.

Experimental Animals
An Nkx2.5<sup>Cre/+</sup> knock-in mouse line was kindly provided by Dr Robert Schwartz. An αMHC<sup>Cre/+</sup> transgenic mouse line was kindly provided by Dr E. Dale Abel. ROSA26<sup>GFP-DTA</sup> and ROSA26<sup>Cre</sup> mouse lines were purchased from The Jackson Laboratory. Experimental animal protocols were approved by the Institutional Animal Care and Use committees of Massachusetts General Hospital and Stanford University. All experiments were performed on somite-matched embryos or sex-matched adult mice.

Establishment of Embryonic Stem Cell Lines
Derivation of the V6.5<sup>α</sup> and R1<sup>α</sup> embryonic stem cell (ESC) lines has been described previously. For generation of Nkx2.5<sup>Cre/+</sup>, ROSA26<sup>GFP-DTA</sup> and αMHC<sup>Cre</sup>;ROSA26<sup>GFP-DTA</sup> compound transgenic ESC lines, timed matings were performed between male Nkx2.5<sup>Cre/+</sup> mice or αMHC<sup>Cre/+</sup> mice with female ROSA26<sup>GFP-DTA</sup> mice. At 3.5 days postcoitum, females were euthanized and blastocysts were flushed from the uterine horns with M2 medium (M1767, Sigma-Aldrich) and washed several times. With the use of a mouth pipette with a pulled glass capillary, blastocysts were plated individually onto 24-well gelatin-coated plates containing mitomycin-C (M4287, Sigma-Aldrich) inactivated mouse embryonic fibroblast feeder layers in ESC derivation media and cultured, undisturbed, at 37°C in 5% CO2 in air. Embryos were then gradually expanded to establish ESC lines. Lines were selected for further use on the basis of undifferentiated morphology, the presence of the Cre transgene and Y chromosome by polymerase chain reaction, and expression of enhanced green fluorescent protein (eGFP). Primer sequences used for genotyping are listed in Table I in the online-only Data Supplement. ESC derivation and maintenance media compositions are reported in the online-only Data Supplement.

Chimera Production
Embryos were staged by vaginal plugging of the mother, with noon on the day of appearance of the plug designated as embryonic day (E) 0.5. For the initial studies, 10 to 20 low-passage (P5–P10) Nkx2.5<sup>Cre/+</sup>;ROSA26<sup>GFP-DTA</sup> or αMHC<sup>Cre</sup>;ROSA26<sup>GFP-DTA</sup> ESCs were microinjected into E3.5 blastocysts from superovulated CD-1 females (Charles River Laboratories). For the reverse complementation studies, P15 through P25 V6.5 or R1 ESCs were microinjected into E3.5 blastocysts from superovulated ROSA26<sup>GFP-DTA</sup> females that had been mated to Nkx2.5<sup>Cre/+</sup> males. For both approaches, the injected blastocysts were subsequently transferred into the uterus of 2.5 days postcoitum pseudopregnant 6- to 8-week-old CD-1 foster mothers previously mated with vasectomized males. Genotype was identified on the basis of the expression of eGFP and the presence of the Cre transgene by polymerase chain reaction. Chimeric contribution was determined by flow cytometric analysis, as described in the online-only Data Supplement.

Ex Vivo Single-Cell Clonal Analysis
Details of the single-cell culture studies can be found in the online-only Data Supplement.

Immunohistochemistry
For cell culture experiments in 96-well plates, cells were fixed for 5 minutes in 4% paraformaldehyde and washed with PBS. E8.5 embryos were fixed for 10 minutes in 4% paraformaldehyde and embedded in optimal cutting temperature compound on dry ice. E9.5 to E10.5 embryos and live-born mouse tissues were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Tissue blocks were cut at 5-μm thickness. Paraffin-embedded sections underwent antigen retrieval in a pressure cooker for 3 minutes in a citrate-based unmasking solution, pH 6.0 (H-3300, Vector Laboratories). Plated cells and tissue sections were blocked and permeabilized with 10% goat serum, 1% BSA, and 0.1% saponin in PBS for 1 hour and then incubated overnight at 4°C with the primary antibody. Primary antibodies used are listed in Table II in the online-only Data Supplement. Samples were then washed with PBS and incubated at room temperature for 1 hour with corresponding secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 546, or Alexa Fluor 647 (1:400 dilution, Invitrogen). Samples were subsequently rinsed in PBS and stained with NucBlue Fixed Cell Staining (Invitrogen). Samples were then washed with PBS and incubated at room temperature for 1 hour with corresponding secondary antibodies conjugated to Alexa Fluor 488, Alexa Fluor 546, or Alexa Fluor 647. For cell culture experiments, each well of a 96-well plate was individually imaged with a BioTek Cytation 3 Cell Imaging Multi-Mode Reader.

Quantification of Cardiomyocyte Proliferation
For assessment of cardiomyocyte proliferation, embryo tissue sections were prepared as described in the Immunohistochemistry section with antibodies to cardiac troponin T (cTnT), CD31, and phosphorylated histone H3 or Ki-67. To confirm that each DAPI<sup>+</sup> cell nucleus counted was associated with a cardiomyocyte (if surrounded in 3 dimensions by cTnT stain) or an endocardial cell (if surrounded in 3 dimensions by a CD31 stain), 1-μm z-stack immunofluorescence...
images of each section were acquired on a Zeiss LSM710 confocal microscope at ×20 magnification.

**Echocardiography**
Echocardiography was performed on 4- to 5-week-old mice with a 13-MHz linear-array transducer with a digital ultrasound system (Vivid 7; GE Medical Systems, Milwaukee, WI). The mice were lightly sedated with an intraperitonal injection of ketamine (50 mg/kg), heart rate, left ventricular internal diameter at end diastole, and left ventricular internal diameter at end systole were measured, and the left ventricular fractional shortening was calculated by use of an M-mode echocardiogram obtained at the midpapillary level. For each mouse, all values were averaged from measurements obtained from 9 cardiac cycles. All measurements and calculations were performed by an operator blinded to the mouse genotype.

**Statistical Analysis**
Statistical analysis was performed with GraphPad Prism version 6.02 for Windows (GraphPad Software, San Diego, CA) and RStudio version 0.98.1102 for Windows (Boston, MA). For comparisons between groups in which multiple measurements per mouse were recorded (embryonic cell proliferation, embryonic cardiomyocyte size, and adult cardiomyocyte size), a likelihood ratio test via ANOVA was computed on a linear mixed-effects model with RStudio. For comparisons between 3 groups (cardiomyocyte binucleation, adult heart weight ratios, left ventricular dimensions, and left ventricular fractional shortening), a 1-way ANOVA was used with adjustment for multiplicity with the Dunnett multiple-comparison test, or, when data did not conform to a normal distribution (ex vivo cardiomyocyte colony sizes), the Kruskal-Wallis test was used with the Dunn correction for multiple comparisons. A value of P<0.05 was considered significant.

**Results**

**Fractional Ablation of Embryonic CPCs by Chimeric Complementation**
The myocardial lineage of the heart arises from first and second heart field cells that express *Nkx2.5*, a key transcription factor in early heart formation (Figure I in the online-only Data Supplement). We performed targeted genetic ablation of *Nkx2.5* CPCs during embryonic development to examine the innate recovery response by the remaining nonablated cells. By crossing a previously described *Nkx2.5-Cre* knock-in mouse line (Nkx2.5<sup>Cre<sup>*</sup></sup>)<sub>Simes</sub> with a mouse line that expressed diphertheria toxin A (*DTA*) after Cre-dependent excision of a constitutively active eGFP transgene (*ROSA26<sup>eGFP</sup>*), we show that cardiac cells were abolished entirely in double-transgenic embryos (Figure 1A)

Although the complete ablation of CPCs demonstrates the essential requirement of these cells in normal embryogenesis, it does not lend itself to a strategy for examining the regenerative potential of the fetal heart. One solution may be to use a drug-inducible cardiac-specific Cre model; however, our previous experience with either doxycycline- or tamoxifen-inducible mice suggests too much interembryo variability in drug metabolism in utero for that strategy to be reliable for our purposes. Hence, we used a novel strategy that involves injecting double-transgenic *Nkx2.5<sup>Cre<sup>*</sup></sup>*;<*ROSA26<sup>eGFP</sup>*<sup>*DTA</sup>) ESCs into wild-type eGFP<sup>*-</sup> blastocysts to ablate CPCs in quantifiable fractions (Figure 1B). Because ESC injection into mouse blastocysts is well known to generate chimeric embryos and mice with a wide spectrum of ESC contribution, this assay enabled us to determine the expected percentage of CPC ablation by assessing the overall contribution of the eGFP<sup>*</sup> ESCs to the embryo.

We first generated several independent ESC lines carrying both the *Nkx2.5<sup>Cre<sup>*</sup></sup>* and *ROSA26<sup>eGFP</sup>*<sup>*DTA</sup>* alleles (Figure 1C). These ES clones were eGFP<sup>*</sup> under fluorescence microscopy, indicating the presence and expression of the floxed eGFP transgene. Three such clones (*Nkx-DTA1*, *Nkx-DTA2*, and *Nkx-DTA3*) that exhibited normal proliferation rates and an otherwise healthy in vitro phenotype were subsequently injected into wild-type eGFP- blastocysts and fostered in pseudopregnant female mice. After injection of 602 wild-type blastocysts, we obtained 414 embryos and mice with a broad range of eGFP<sup>*</sup> ESC contribution (Figure 1D). Within each chimeric embryo, CPCs derived from injected eGFP<sup>*</sup> ESCs were expected to die as a result of Cre-mediated expression of *DTA* so that directly assessing the percentage of cardiac contribution by eGFP<sup>*</sup> ESCs would not have been possible (Figure 1E). To overcome this limitation, we examined the expected variance in ESC contribution to the heart relative to other portions of the embryo body (head, limbs, tail, etc.) by injecting wild-type eGFP<sup>-</sup> ESCs into eGFP<sup>*</sup> mouse blastocysts or red fluorescent tdTomato<sup>*</sup> ESCs into wild-type mouse blastocysts. By flow cytometric (fluorescence-activated cell sorter [FACS]) analysis, we found that 97% of the embryos (29 of 30) showed a difference of ≤12% in ESC contribution to the heart compared with their mean contribution to the rest of the embryo (Figure II.A–II.D in the online-only Data Supplement). Remarkably, our analysis of the E10.5 chimeric embryos from *Nkx2.5<sup>Cre<sup>*</sup></sup>*;<*ROSA26<sup>eGFP</sup>*<sup>*DTA</sup>* ESC injections showed that the ablation of up to ≈60% of CPCs, as determined by FACS, appears compatible with normal development, whereas degrees of ablation >60% lead to overall developmental arrest (Figure 1F).

Immunohistochemical analysis of chimeric embryos showed that eGFP<sup>*</sup> cell contribution was homogenously distributed throughout the embryo except for the heart region, where eGFP<sup>*</sup> cells were completely absent within the eTnt<sup>*</sup> population (Figure 2A). Consistent with this, the hearts of chimeric embryos showed an absence of eGFP fluorescence (arrows), supporting the high efficiency of CPC ablation and compensation from adjacent nonablated eGFP<sup>*</sup> cardiac cells (Figure 1F). Of note, sporadic eGFP<sup>*</sup> cells could be found in the endocardium, consistent with the previously described incomplete expression of *Nkx2.5* in these cells (Figure 2A). Interestingly, the gross morphology and myocardial cellularity were similar between surviving embryos with up to 60% ablation and their stage-matched littermate controls without ESC chimerism, supporting the presence of a regenerative process to compensate for lost CPCs and their myocardial descendants (Figure 2A).

To elucidate potential compensatory processes that might account for the recovery of lost CPCs, we performed immunostaining for transcription factors (ie, Gata4, Mef2c, Islet-1) and signaling mediators (ie, β-catenin and Yap) that have been reported to regulate CPC number or myocardial growth (Figure 2B–2G). However, these proteins exhibited high baseline expression during this early stage of development in control embryos; thus, no qualitative difference in expression...
Figure 1. Fractional ablation of embryonic cardiac progenitor cells (CPCs) by chimeric complementation. A, Schematic diagram of the enhanced green fluorescent protein (eGFP)/diphtheria toxin A (DTA) construct before and after Nkx2.5-Cre-mediated excision of the floxed eGFP transgene (top). Bright-field and GFP fluorescence images of E9.5 Nkx2.5Cre/+;ROSA26eGFP-DTA, and Nkx2.5Cre/+;ROSA26eGFP-DTA transgenic embryos (bottom). Note the absence of a heart in the compound transgenic embryo (arrow). B, Schematic diagram of fractional ablation of CPCs by injection of Nkx2.5Cre/+;ROSA26eGFP-DTA embryonic stem (ES) cells (ESCs) into wild-type CD-1 blastocysts. C, Generation of Nkx2.5Cre/+;ROSA26eGFP-DTA ESC lines. Bright-field and GFP fluorescence microscopy images are shown for the 3 ESC lines used. D, Summary table demonstrating the complementation efficiency for each ESC line based on the total number of animals collected. E, Anticipated progression of CPC loss and regeneration in rescued embryos at ≈50% ESC chimerism. F, Bright-field and GFP images of embryonic day (E) 10.5 chimeric Nkx2.5Cre/+;ROSA26eGFP-DTA embryos with varying degrees of CPC ablation. The embryo with ≈68% CPC ablation shows a pericardial effusion and developmental arrest. Arrow indicates the heart. Scale bars, 500 µm for A, 200 µm for C, and 1 mm for F.
could be observed in the ablated embryo heart by immunofluorescence staining. We also examined whether the expression of sarcomeric proteins (\(\alpha\)-actinin, myosin light chain 2v) varied between ablated and control hearts and found no difference in these parameters (Figure 2H–2J). Together, these data support an efficient compensatory response by residual unablated CPCs or their myocardial descendants to rapidly recover full myocardial cellularity in the ablated heart.

Figure 2. Histological analysis of cardiac progenitor cell (CPC)–ablated embryos shows a rapid recovery in the heart. A, Enhanced green fluorescent protein (eGFP), cardiac troponin T (cTnT), and DAPI staining of embryonic day (E) 8.5 and E10.5 chimeric embryos with up to \(\approx 50\%\) CPC ablation. By E8.5, all eGFP+ myocardial cells have been ablated in the heart. Rare, scattered eGFP+ endocardial cells can be identified (arrow). Cardiac chamber morphology and cellularity in the compact and trabecular compartments are similar between control and ablated hearts, suggesting a rapid restoration of myocardial cell number after ablation. Note that eGFP+ staining in the atria of the E10.5 control embryo represents autofluorescent blood cells. B through J, Immunostaining of control and ablated hearts. Cardiac transcription factor expression is shown at E8.5 for Gata4 (B) and Mef2c (C) and E9.5 for Islet-1 (D). The expression of genes regulating cardiomyocyte proliferation is shown at E8.5 for Yap (E), nuclear \(\beta\)-catenin (F), and total \(\beta\)-catenin (G). Sarcomeric protein expression is shown at E8.5 for \(\alpha\)-actinin (H) and cTnT (I) and at E9.5 for Mlc2v (J). Nuclei are stained with DAPI, and cardiomyocytes are identified by cTnT (A–C, E, G) or Mlc2v (D and F). For B through J, the degree of ablation in CPC-ablated sections is \(41\pm9\%\) (mean\(\pm SD\). ca Indicates common atrium; cv, common ventricle; and oft, outflow tract. Scale bars, 100 \(\mu\)m for A through G and 20 \(\mu\)m for H through J.
Because the robustness of our experimental conclusions depends heavily on having an accurate assessment of the degree of ESC contribution to the hearts of chimeric embryos, we sought to exclude the possibility that Nkx2.5<sup>Cre+/−</sup>;ROSA26<sup>GFp-DTA</sup> ESCs harbor an unexpected defect in their ability to contribute to heart-forming cells. To address this, we developed a complementary strategy involving the injection of wild-type eGFP-ESCs into blastocysts derived from the mating of Nkx2.5<sup>Cre+/−</sup> and ROSA26<sup>GFp-DTA</sup> mice (Figure IIIA in the online-only Data Supplement). In particular, we assessed chimeric embryos or mice that are eGFP<sup>+</sup> (ie, derived from Nkx2.5<sup>Cre+/−</sup>;ROSA26<sup>GFp-DTA</sup> [ablated] or Nkx2.5<sup>Cre+/−</sup>;ROSA26<sup>GFp-DTA</sup> [control] blastocysts). After injection of 639 blastocysts using 2 different wild-type ESC lines (V6.5 and R1), we obtained 394 embryos or mice, with roughly half (226) being eGFP<sup>+</sup>. Similar to our Nkx2.5<sup>Cre+/−</sup>;ROSA26<sup>GFp-DTA</sup> ESC injection results above, we found that wild-type V6.5 and R1 ESCs exhibit a broad range of contribution (Figure IIIB in the online-only Data Supplement). The total number of recovered control ROSA26<sup>GFp-DTA</sup> embryos and mice (144) was greater than the number of ablated Nkx2.5<sup>Cre+/−</sup>;ROSA26<sup>GFp-DTA</sup> animals (82), presumably because of the lethality in ablated embryos with insufficient wild-type ESC contribution. Consistent with our earlier findings, embryos with up to 60% CPC loss were able to maintain normal embryonic development (Figure IIIC in the online-only Data Supplement). When all surviving embryos and mice were stratified by their degree of wild-type ESC chimerism, it was apparent that up to 60% ablation of CPCs could be tolerated (Figures IIID, IIIE, and IVA–IVC in the online-only Data Supplement). As we expected, no eGFP<sup>+</sup> cells were present in the cTnT<sup>+</sup> myocardium in the ablated mice (Figure IIIF in the online-only Data Supplement). Taken together, these findings support a robust compensatory mechanism in embryonic mouse hearts after CPC ablation.

**Fractional Ablation of Embryonic Cardiomyocytes by Chimeric Complementation**

Although our CPC ablation model demonstrates loss of CPCs with exquisite temporal resolution, it does not reveal whether the regenerative response to ablation requires CPCs or whether developing cardiomyocytes are capable of responding to the loss of cardiac cells during development. To address this, we performed fractional ablation of developing immature cardiomyocytes using a previously published α-myosin heavy chain-Cre (αMHC<sup>Cre+</sup>) transgenic mouse line (Figure V in the online-only Data Supplement).<sup>15</sup> Embryos with compound αMHC<sup>Cre+</sup> and ROSA26<sup>GFp-DTA</sup> transgenes have a well-formed heart tube at E8.0 but exhibit a hypocellular looped heart at E9.5 and the absence of a heart with pericardial edema and developmental arrest at E10.5 (Figure 3A). We generated several αMHC<sup>Cre+</sup>;ROSA26<sup>GFp-DTA</sup> ESC lines and selected 3 (MHC-DTA1, MHC-DTA2, and MHC-DTA3) for further study on the basis of their capacity to contribute to chimeric embryos and mice with a broad range of efficiency (Figure 3B and 3C). Interestingly, we found that these chimeric embryos were also able to tolerate up to 50% to 60% ablation of their immature myocytes (Figure 3D and Figure 1VD and 1VE in the online-only Data Supplement). Immunohistological staining for eGFP and cTnT expression showed a progressive decrease in myocardial cellularity in E9.5 chimeric embryos with >50% contribution by αMHC<sup>Cre+</sup>;ROSA26<sup>GFp-DTA</sup> ESCs (Figure 3E). A small number of eGFP<sup>+</sup> cardiomyocytes could be observed that may represent residual nonablated cardiomyocytes or cardiomyocytes that were dying but had not been completely eliminated. Additional immunostaining for apoptotic cells confirmed that at least a fraction of these eGFP<sup>+</sup> cardiomyocytes underwent cleavage of caspase-3 or were positive for TdT-mediated dUTP nick-end labeling (Figure VIIA and VIIB in the online-only Data Supplement). Similar to embryos that had undergone CPC ablation, there was no apparent phenotypic difference after E10.5 between surviving embryos with a high degree of ablation (ie, 50%–60%) compared with those with less fractional ablation. Taken together, these results demonstrate a robust myocardial compensatory response when cell ablation occurs during the immature cardiomyocyte stage of development.

**Cell Ablation Triggers Cardiomyocyte Expansion Ex Vivo**

Because the overall cardiac morphology in both ablation models was largely indistinguishable between surviving <50% to 60% ablated and control embryos, we examined whether there is a proliferative response to cell ablation by surveying the behavior of residual unablated cardiac cells ex vivo. We first dissected the heart tubes from E9.5 unablated control embryos, CPC-ablated embryos, and cardiomyocyte-ablated embryos. Next, we FACS-purified residual eGFP<sup>+</sup> cardiac cells, which are presumably responsible for recovering myocardial cellularity after ablation (Figure VIIA in the online-only Data Supplement), deposited 1 cell per well into a 96-well plate, and cultured the cells ex vivo for 7 or 14 days (Figure 4A). Approximately 9.5% of 5605 plated cells survived single-cell cloning.

Examination of cells within each well revealed several patterns of specification. Thirty-six percent of single-cell clones from control hearts and 20% and 44% of CPC- or cardiomyocyte-ablated clones were classified as cardiomyocytes (Figure 4B), exhibiting spontaneous beating and well-organized Z lines marked by sarcomeric α-actinin expression (Figure 4C). Cardiomyocyte binucleation was also observed but did not significantly differ among groups (Figure VIIIB in the online-only Data Supplement). A small minority of clones (2% of control, 1% of CPC-ablated, and 1% of cardiomyocyte-ablated clones) expressed CD31 and were highly proliferative, possessing a polygonal morphology, consistent with an endothelial/endothelial cell phenotype (Figure 4D). The remaining majority of clones (62% of control, 79% of CPC-ablated, and 54% cardiomyocyte-ablated clones) lacked significant sarcomeric α-actinin or CD31 expression (Figure 4E). These might have represented hematopoietic, epicardial, endocardial cushion mesenchyme, or neural crest-derived cells. No clones expressed smooth muscle myosin heavy chain, the definitive marker for the smooth muscle lineage.

To examine the effect of CPC or cardiomyocyte ablation on clonal cell growth potential, we counted the cardiomyocyte clone sizes resulting from single-cell plating on day 7 or 14. A normality plot indicated that the colony size distribution deviated significantly from a gaussian distribution, necessitating a
Figure 3. Fractional ablation of embryonic cardiomyocytes (CMs) by chimeric complementation. **A**, Schematic diagram of the enhanced green fluorescent protein (eGFP)/diphtheria toxin A (DTA) construct before and after αMHC-Cre-mediated recombination (left). Bright-field and GFP fluorescence images of ROSA26×eGFP-DTA control and αMHC-Cre×ROSA26×eGFP-DTA transgenic embryos (right) at embryonic day (E) 8.5, E9.5, and E10.5 of development. Note that the compound transgenic embryo shows a progressive defect in cardiac morphogenesis by E9.5 and is completely missing a heart structure at E10.5. **B**, Generation of αMHC-Cre/+;ROSA26×eGFP-DTA embryonic stem (ESC) lines. Bright-field and GFP fluorescence microscopy images are shown for the 3 ESC lines used. **C**, Fractional cardiomyocyte ablation by injection of αMHC-Cre/++;ROSA26×eGFP-DTA ESCs into wild-type CD-1 blastocysts (top). The summary table demonstrates the complementation efficiency for each ESC line tested based on the total number of animals collected (bottom). **D**, Bright-field and GFP fluorescence images of E10.5 chimeric αMHC-Cre/++;ROSA26×eGFP-DTA embryos. Note that embryos with >50% ablation are developmentally arrested and exhibit impaired cardiac morphogenesis. A pericardial effusion is occasionally observed. **E**, eGFP, cardiac troponin T (cTnT), and DAPI staining of E9.5 chimeric embryos with progressively increasing amounts of cardiomyocyte ablation. Some residual unablated eGFP+ cardiomyocytes remain in the hearts at this stage. Scale bars, 500 µm for **A**, 200 µm for **B** and **E**, and 1 mm for **D**.
Figure 4. Embryonic cardiomyocyte (CM) proliferation is involved in myocardial recovery. A, Schematic diagram of single-cell isolation and culture of embryonic day (E) 9.5 cardiac cells. For B through J, experimental variables are represented as follows: group, number of mice examined, mean percent ablation±SD. For B through F, control, n=9; cardiac progenitor cell (CPC) ablation, n=6, 45±11%; CM ablation, n=6, 50±12%; B, Percentage of single-cell derived colonies classified as CMs, endocardial/endothelial cells, or other cells from dissociated E9.5 embryo hearts. C through E, Histological analysis of single-cell derived colonies from E9.5 hearts. C, Sarcomeric α-actinin+ CMs showing well-organized Z lines and sarcomeric structure. D, CD31+ endocardial/endothelial cells. E, Non-CM/nonendothelial cell lacking significant sarcomeric α-actinin or CD31 expression. F, Box-and-whisker plot demonstrating median cell number and interquartile range among wells classified as containing CM colonies. Whiskers span the 10% to 90% range of values. Statistical comparisons were performed with the Kruskal-Wallis test with the Dunn correction for multiple comparisons. For G through J, control, n=4; CPC ablation, n=4, 41±9%; CM ablation, n=4, 53±12%. Immunofluorescence confocal microscopy with 1 mm z-stack imaging for proliferative CMs and endothelial cells in CPC-ablated (G and H) or CM-ablated (I and J) hearts at E8.5 or E9.5, respectively. The percentage of phospho-histone H3+ (pH3; G and I) or Ki-67+ (H and J) CMs in control and ablated hearts (solid arrows) was defined by colocalization with a DAPI+ nucleus that is circumscribed in all dimensions by a cardiac troponin T+ (cTnT+) cell. Similarly, the percentage of pH3+ or Ki-67+ endocardial cells (thin arrows) was defined by colocalization with a DAPI+ nucleus that is circumscribed in all dimensions with a CD31+ cell. Each symbol in the dot plot represents the fraction of cTnT+ or CD31+ cells staining positive for pH3 or Ki-67 in a single cardiac histology section; each symbol shape represents an independent mouse; a horizontal bar indicates the mean value for each group. One hundred to 600 cTnT+ cells and 50 to 300 CD31+ cells were counted per section. Statistical comparisons were performed by a likelihood ratio test on a linear mixed-effects model. FACS indicates fluorescence-activated cell sorter. Scale bars: 100 µm for C through E and 50 µm for G through J.
nonparametric analysis of the data (Figure VIIIC in the online-only Data Supplement). The presence of rare colonies with many cardiomyocytes suggests a hierarchical contribution to normal myocardial development and to the recovery of cardiomyocytes after ablation. On day 7, the median cardiomyocyte number per colony was significantly greater in colonies derived from cardiomyocyte-ablated embryos compared with unablated controls (5 versus 3; Figure 4F). In contrast, no statistically significant difference was observed in cardiomyocyte number per colony between control and CPC-ablated embryos, although there was a trend toward greater colony size in CPC-ablated embryos at day 14 (Figure 4F). The lack of a significant difference in cardiomyocyte colony size between CPC-ablated and control embryos most likely resulted because CPC-ablated embryos had largely recovered their myocardial cellularity by E9.5, whereas the cardiomyocyte-ablated embryos were still actively recovering. These results indicate that cardiomyocyte ablation triggers a modest increase in cellular proliferation at E9.5 to increase cardiomyocyte number ex vivo.

Embryonic Cardiomyocyte Proliferation Is Involved in Myocardial Regeneration After Ablation In Vivo

We next examined whether there is a proliferative response in vivo to compensate for the loss of cardiomyocytes, as suggested by our ex vivo experiments, or whether the recovery after cell ablation is due merely to a hypertrophic response in residual unablated cardiomyocytes. In the case of hypertrophy, the individual cardiomyocyte size should increase without a corresponding change in myocardial cell proliferation rate, whereas in the case of proliferation, cardiomyocyte size should remain constant or decrease, accompanied by an increase in the rate of proliferation. We first performed immunostaining for proliferation markers in embryo sections from both CPC or cardiomyocyte ablation models using the mitosis marker phosphorylated histone H3 and the cell proliferation marker Ki-67 (Figure 4G–4J). We found that embryos from both CPC and cardiomyocyte ablation models exhibited increases in expression of both proliferation markers within their cTnT+ cardiomyocytes. To see whether this correlated with changes in cardiomyocyte size, we quantified the ratio of myocardial area to the number of nuclei within cTnT+ cardiomyocytes. We found, however, that the cardiomyocyte area per nucleus was not statistically different between ablated embryos and controls despite a trend toward smaller cell size (Figure VIIIA and VIIIB in the online-only Data Supplement). This suggests that an increase in proliferation is the prevailing mechanism involved in the morphological recovery of the ablated heart.

In light of the interdependence between the myocardium and neighboring endocardium to maintain normal cardiac development, we also examined whether the development of the endocardium is affected by ablation of CPCs or cardiomyocytes. At E8.5 in the CPC ablation model and at E9.5 in the cardiomyocyte ablation model, the endocardium in ablated embryos exhibited CD31 expression similar to that of controls, consistent with normal endocardial differentiation (Figure 4G–4J). In addition, we quantified the degree of endocardial cell proliferation in CPC- or cardiomyocyte-ablated embryos by assessing the percentage of Ki-67+ or phosphorylated histone H3+ endocardial cell nuclei. However, we did not observe a consistent difference in endocardial cell proliferation between ablated embryos and controls (Figure 4G–4J).

Because cardiomyocyte maturation is accompanied by changes in structure of the contractile unit, including greater myofibril density, more organized alignment, and an increase in sarcomere length, we examined whether cardiomyocyte maturation was delayed in ablated embryos at the ultrastructural level by transmission electron microscopy (Figure VIIIC–VIIIE in the online-only Data Supplement). We found that at E10.5 hearts from CPC-ablated embryos exhibited a modest decrease in sarcomere thickness despite a lack of change in sarcomere length. This suggests that compensating cardiomyocytes may be phenotypically less mature because of their need for an increased rate of cell proliferation.

CPC- and Cardiomyocyte-Ablated Embryos Can Survive Into Adulthood With No Overt Cardiac Defect

The above results demonstrate that a majority of embryonic CPCs or cardiomyocytes can be ablated without triggering gross embryonic defects. To rule out more subtle congenital phenotypes in CPC- or cardiomyocyte-ablated embryos that would not manifest until late gestation or after birth, we collected chimeric live-born mice that had undergone an average of ~40% CPC or cardiomyocyte ablation and assessed their cardiac structure by histology and their cardiac function by echocardiography at 4 to 5 weeks of age (Figure 5A). We found that these chimeric mice displayed the expected coat color chimerism (Figure 5B) with few to no residual eGFP+ cells in the heart (Figure 5C). Compared with control unablated mice, their hearts showed similar gross overall size at the whole-organ level (Figure 5C and 5D) and had similar heart weights (Figure 5E), left ventricular chamber dimensions (Figure 5F–5H), and contractile function (Figure 5I) as measured by echocardiography. We also assessed whether a hypertrophic response occurred in ablated hearts, which could indicate whether there had been a transient history of heart failure with remodeling changes. We found, instead, no evidence of hypertrophic changes as measured by mean cardiomyocyte size in ablated versus control hearts (Figure 5J and 5K). These findings further support our conclusion that acute CPC or cardiomyocyte loss during early embryogenesis is well tolerated in mice owing to a rapid and efficient compensatory proliferative response that supports the development of normal organ size, cardiomyocyte number, and contractile function into early adulthood.

Discussion

In this study, we developed a novel strategy to quantitatively examine the ability of a mammalian embryonic heart to compensate for rapid cell loss. Specifically, we made use of a blastocyst complementation assay that gives rise to chimeric embryos with fractional CPC or immature cardiomyocyte ablation during embryonic development. We found that embryos with up to 60% CPC loss were fully viable and could survive to adulthood. The compensatory process after CPC ablation at E7.5 was rapid and complete with little to no
Figure 5. Cardiac progenitor cell (CPC)– and cardiomyocyte (CM)-ablated embryos can survive into adulthood with no overt cardiac defect. **A**, Strategy used for generation and analysis of 4- to 5-week-old mice undergoing CPC or CM ablation. **B**, Representative images of control and ablated chimeric adult mice. **C**, Bright-field and green fluorescent protein (GFP) fluorescence images of the dissected whole hearts. GFP signal is nearly absent in the hearts that have undergone ablation. **D**, Hematoxylin and eosin (H&E) staining of representative heart sections from control and ablated hearts. For **E** through **K**, experimental variables are represented as follows: group, number of mice examined, mean percent ablation±SD. For **E** through **I**, results are displayed with box-and-whisker plots demonstrating median value and interquartile range. Whiskers span the minimum and maximum range of values. **E**, Heart weight–to–body weight ratio and heart weight–to–tibia length ratio in wild-type and ablated hearts (control, n=28; CPC ablation, n=11, 39±9%; CM ablation, n=6, 42±11%). **F** through **I**, Representative M-mode echocardiography images (**F**) and left ventricular dimensions (**G** and **H**) and function (**I**) quantified by fractional shortening (FS; control, n=16; CPC ablation, n=9, 40±10%; CM ablation, n=7, 41±10%). **J** and **K**, Wheat germ agglutinin (WGA) staining of control and ablated hearts (**J**) and dot plot (**K**) displaying mean CM size. Each symbol in the dot plot represents the mean CM size in an individual heart section; a minimum of three ×32 fields per section were counted; each symbol shape represents an independent mouse; horizontal bar indicates the mean value for each group (control, n=3; CPC ablation, n=3, 51±6%; CM ablation, n=3, 51±7%). LVIDES indicates left ventricular internal diameter at end systole; and LVIDes, left ventricular internal diameter at end diastole. Scale bars: 2 mm for **C** and **D** and 50 µm for **J**.
evidence of molecular alteration or cardiac cellular deficiency by E10.5. To examine the capacity of immature cardiomyocytes to respond to cell loss, we induced cardiomyocyte ablation at E9.0 and found, with remarkable consistency, that the loss of 50% to 60% of immature cardiomyocytes was well tolerated during embryonic development and that adult heart size and function in these animals were completely normal. Consistent with findings from newt and zebrafish cardiac regeneration, increased cardiomyocyte proliferation was evident in both CPC and cardiomyocyte ablation models. These data illustrate the significant capacity for myocardial injury repair in the embryonic mouse heart and raise the prospect that postnatal cardiac regeneration may be achievable in the mammalian heart if key mechanisms for triggering the expansion of proliferation-competent cardiomyocytes can be identified for therapeutic gain.

Our study used a Cre-mediated conditional cell ablation strategy in mice to examine the response of an embryonic heart to fractional cell loss. Earlier studies have shown the remarkable ability of an adult zebrafish heart to replenish as much as a 70% to 80% loss of cardiomyocytes. Although the study by Drenckhahn and colleagues illustrated the ability of an embryonic mammalian heart to increase cardiomyocyte proliferation in healthy cells to compensate for impaired proliferation in cardiomyocytes with metabolic derangement, the approach used was associated with an absence of apoptosis in the diseased cells and their subsequent retention. To the best of our knowledge, no study has demonstrated complete myocardial regeneration in the embryonic mouse heart after extensive and temporally controlled cell ablation. However, it should be recognized that fetal cardiomyocytes are proliferative at baseline and the ablation studies described here are likely an assessment of both the normal generative potential of fetal cardiomyocytes during cardiac development and their regenerative potential, given the increase in cardiomyocyte and cardiomyocyte precursor proliferation in the setting of cardiac cell ablation. Regardless, our data support the remarkable capacity of the fetal heart to recover from experimental cell ablation during embryonic development.

We found that the embryonic mouse heart can replace as much as 50% to 60% of developing CPCs or immature cardiomyocytes after Cre-mediated ablation and that proliferation of immature cardiomyocytes is involved in the compensatory response. In the absence of cell ablation, we observed expression by the majority of E8.5 to E9.5 cardiomyocytes of the proliferation marker Ki-67, which is detectable during the active stages of the cell cycle, including the late G1, S, G2, and M phases, but which is absent in resting cells (G0). Although this finding supports that cardiomyocytes are highly proliferative during this stage of development, we found that both CPC and cardiomyocyte ablation triggered a further increase in myocyte proliferative capacity. Likewise, histone H3 phosphorylation was also found to be increased on CPC or cardiomyocyte ablation. However, its expression was observed in only a small fraction of developing myocytes, consistent with the fact that its phosphorylation is limited primarily to mitosis (M phase) (Figure 4G–4J). As a result of the rapid differentiation of CPCs into immature cardiomyocytes at E7.5 to E8.5 of development, we were unable to document an increase in the number of Isl-1+ CPCs in the residual heart after cell ablation (Figure 2D). Further studies using a combination of inducible cell ablation and blastocyst complementation assays are necessary to specifically address the role of first or second heart field CPCs in the regenerative process.

Our findings also raise questions about the potential mechanisms involved in replacing the number of CPCs and cardiomyocytes after ablation. Although we believe that CPC and cardiomyocyte ablation by intracellular DTA expression should be a relatively cell-autonomous process without an exogenous inflammatory response from circulating leukocytes, differences in the amount of locally secreted signals from nonablated cardiac cells may trigger the observed proliferative response. In addition, cell-cell contact has been well described to regulate the rate of cell division. The acute loss of CPCs or cardiomyocytes may result in derepression of this intrinsic negative regulator of cell proliferation. Furthermore, because the developing heart at this stage is continually receiving a new influx of cells from the second heart field, it is possible that an increase in migration and proliferation of second heart field–derived cells may be involved in myocardial regeneration in the right ventricle and outflow tract. Further work is necessary to clarify the precise mechanisms responsible for embryonic myocardial regeneration at distinct stages of development and within each heart field.

One important caveat of our findings that should be considered carefully is the potential role of other nonmyocardial cells (eg, endocardial and epicardial cells) in the compensatory process. It is well known that signaling factors released from endocardial and epicardial cells play important roles in myocardial development. Our CPC ablation model depends on the expression of Cre from the endogenous Nkx2.5 promoter, which is known to express in pharyngeal endoderm at later stages of development, and endocardial and epicardial cells. Consequently, this model may underestimate the degree of regeneration possible because it may ablate both the CPCs involved in myocardial formation and the endocardial/epicardial cells that regulate the compensatory process.

Conclusions

We found that quantitative and fractional ablation of developing mouse CPCs and immature cardiomyocytes lead to cardiac cell loss that can be compensated for by residual unablated cells. This process is efficient and can rescue normal fetal development in hearts that have undergone up to 50% to 60% CPC or cardiomyocyte ablation. The mechanism for this compensatory response involves, in part, increased proliferation from residual immature cardiomyocytes. Identification of the mechanisms involved in responding to the loss of CPC and cardiomyocyte number by triggering their increase in proliferation may enable us to devise improved therapies for heart failure caused by myocardial cell loss.

Acknowledgments

We thank Dr Robert J. Schwartz at the University of Houston for providing us with the Nkx2.5cre Cre knock-in mice; Dr E. Dale Abel...
at the University of Iowa for providing us with the αMHCCre/+ transgenic mice; Dorothy Hu and Kimberly Atkin McCurdy at the MGH Endocrine Histology Core for assistance with tissue sectioning; Andrew Olson at the Stanford Neuroscience Microscopy Service for assistance with microscopy; Laura Prickett-Rice at the Harvard Stem Cell Institute Flow Cytometry Core Facility and Patty Lovelace and Jennifer Ho at the Stanford Stem Cell FACS Core for assistance with FACS analysis; Anusha Kumar for assistance with cell culture and manuscript critique; R. Sharon Chinthrajah and Peter Sturzu for assistance with blinded cell counting; and David Knowles and Joseph Garner at Stanford University for assistance with statistical analysis.

Sources of Funding

This work was supported in part by a Harvard Stem Cell Institute Seed Grant, a National Institutes of Health Director’s New Innovator Award (DP2OD000401), the National Institutes of Health/National Heart, Lung, and Blood Institute Progenitor Cell Biology Consortium (U01HL099796), the California Institute for Regenerative Medicine (RB3-05129), an Endowed Faculty Scholar Award from the Lucile Packard Foundation for Children/Child Health Research Institute at Stanford (to Dr Wu), the National Institutes of Health T32 (HL007208 and HL94274), and a post-doctoral fellowship award from the American Heart Association Founders Affiliate and the Lawrence J. & Florence A. DeGeorge Charitable Trust (to Dr Sturzu).

Disclosures

None.

References


**CLINICAL PERSPECTIVE**

A wealth of studies support the importance of developmental signaling molecules in promoting proper heart formation, from lineage commitment to cellular expansion and maturation. Disruption in these formative events during embryonic development has a profound impact on human life; congenital heart disease remains the commonest cause of birth defects worldwide. However, whether there is a requirement for a specific number of progenitor cells to fashion a normal mammalian heart and the potential mechanisms that exist to compensate for unexpected cell loss during embryonic development have not been explored. To investigate whether a mechanism is present in the developing heart to compensate for the loss of cardiac cells during the earliest stages of heart formation, we used a novel cell ablation strategy that could temporally ablate cardiac progenitor cells or immature cardiomyocytes in quantifiable fractions. By doing so, we found that, remarkably, mouse embryos could compensate for up to a 60% loss of cardiac cells without obvious morphological or functional defect. Furthermore, we show that increased proliferation of residual immature cardiomyocytes is at least partly responsible for this regenerative activity. These data provide direct evidence for the existence of a robust regenerative mechanism in the embryonic heart to compensate for cardiac progenitor cell and immature cardiomyocyte loss during development. In addition, the strategy we developed for fractional lineage-specific cell ablation should be applicable for other organ systems to address the role of progenitor cell number in development and disease and their mechanism of regeneration.
Fetal Mammalian Heart Generates a Robust Compensatory Response to Cell Loss

Anthony C. Sturzu, Kuppusamy Rajarajan, Derek Passer, Karolina Plonowska, Alyssa Riley, Timothy C. Tan, Arun Sharma, Adele F. Xu, Marc C. Engels, Rebecca Feistritzer, Guang Li, Martin K. Selig, Richard Geissler, Keston D. Robertson, Marielle Scherrer-Crosbie, Ibrahim J. Domian and Sean M. Wu

_Circulation_. 2015;132:109-121; originally published online May 20, 2015;
doi: 10.1161/CIRCULATIONAHA.114.011490
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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SUPPLEMENTAL MATERIAL

Fetal Mammalian Heart Generates a Robust Compensatory Response to Cell Loss

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SUPPLEMENTAL METHODS

**Nkx2.5-Cre and aMHC-Cre Genetic Fate Mapping**

*Nkx2.5<sup>Cre<sup>+</sup>;ROSA26<sup>LacZ</sup></sup> or aMHC<sup>Cre<sup>+</sup>;ROSA26<sup>LacZ</sup></sup> whole mouse E9.5 embryos were assessed for β-Galactosidase (lacZ) activity according to previously published protocol<sup>1</sup>.

**FACS Analysis of ESC Chimerism**

For embryonic tissues, the heart, limbs, tail, head, or body were minced with a scalpel blade into 1-4 mm<sup>3</sup> pieces and individually digested in Hank's Balanced Salt Solution (HBSS) with Ca<sup>++</sup>/Mg<sup>++</sup> (Life Technologies, 14025-134) containing 10 mg/mL collagenase A&B (Roche; 11088785103, 11088823103) at 37º C for 30-60 minutes with periodic pipetting. Following centrifugation at 300xg for 30s, the supernatant was discarded and the digested cells were resuspended in 1X Red Blood Cell Lysis Solution (Miltenyi Biotec; 130-094-183) and incubated for 10 minutes at room temperature. Cells were again centrifuged and resuspended in HBSS with Ca<sup>++</sup>/Mg<sup>++</sup> containing 4% fetal bovine serum (FBS, Atlanta Biologicals, S11550) for flow cytometric analysis. For live-born mice, the same protocol was applied to tissue from the tail or ears at ~postnatal day 7. Following addition of propidium iodide to gate out dead cells, flow cytometric cell counting was performed on a FACSCalibur or FACSaria II flow cytometer (BD Biosciences) using CellQuest v3.3 software (BD Biosciences, San Jose, CA). Doublet discrimination and exclusion was performed by gating cells according to their FSC-H versus FSC-W and SSC-H versus SSC-W distributions. To determine the proportion of eGFP<sup>+</sup>/eGFP<sup>-</sup> cells in each tissue sample, the data was analyzed with FlowJo v7.6 software (Tree Star, Ashland, OR). Degree of CPC or CM ablation was computed using the average percentage of eGFP<sup>+</sup> cell chimerism to tail and limb samples for embryonic mice or tail and ear samples for live-born mice.
**Media Composition for Establishment of ESC Lines**

ESC Derivation Media: DMEM with High Glucose/4.0 mM L-Glutamine (Thermo Scientific HyClone, SH30022), 20% KnockOut Serum Replacement (KOSR, Life Technologies, 10828), 4 mM Glutamax (Life Technologies, 35050), 100 µM MEM Non-Essential Amino Acids (Life Technologies, 11140), 50 U·µg/mL Penicillin-Streptomycin (Life Technologies, 15070), 100 µM 2-Mercaptoethanol (Sigma, M6250), and 500 U/mL leukemia inhibitory factor (LIF, Millipore, ESG1107).

ESC Maintenance Media: DMEM with High Glucose/4.0 mM L-Glutamine, 10% KOSR, 7.5% FBS, 4 mM Glutamax, 100 µM MEM Non-Essential Amino Acids, 50 U·µg/mL Penicillin Streptomycin, 100 µM 2-Mercaptoethanol, 500 U/mL LIF, and the “2i” inhibitors: 3 µM glycogen synthase kinase-3 inhibitor CHIR99021 (StemGent, 04-0004), and 1 µM mitogen-activated protein kinase inhibitor PD0325901 (Stem-Gent, 04-0006).

**Media Composition for Ex Vivo Cardiac Cell Culture**

Cardiomyocyte Maintenance Media: IMDM (Life Technologies, 12440), 15% FBS (Hyclone, SH30071.HI), 4 mM Glutamax (Life Technologies, 35050), 25 U·µg/mL Penicillin-Streptomycin (Life Technologies, 15070), 50 µg/mL Ascorbic Acid (Sigma, A4544), 100 µM Monothioglycerol (Sigma, M6145).

**Ex vivo single-cell clonal analysis**

Heart tubes were dissected on ice at E9.5 from CD-1 control embryos, or CD-1 embryos which had been chimerized by Nkx2.5^{Cre+};ROSA26^{EGFP-DTA} or αMHC^{Cre+};ROSA26^{EGFP-DTA} ESCs and individually transferred to microcentrifuge tubes containing 50 µL Hank’s Balanced Salt Solution (HBSS) with Ca^{++}/Mg^{++} (Life Technologies, 14025-134). To each tube, 200 µL of 0.25% trypsin-EDTA solution (Life Technologies, 25200) was added and incubated for 5-10 minutes at 37°C with gentle dissociation by pipetting. Following inactivation of trypsin with FBS (Hyclone, Sh30022).
SH30071.HI), the cells were centrifuged and re-suspended in HBSS with Ca**+/Mg**+ containing 4% FBS and placed on ice. Flow cytometric sorting was performed on a FACSARia II flow cytometer (BD Biosciences) and analyzed using FACSDiva v6.1.3 software (BD Biosciences, San Jose, CA). Doublet discrimination was performed by gating cells according to their FSC-H versus FSC-W and SSC-H versus SSC-W distributions. The proportion of eGFP+/eGFP− cells in each tissue sample was determined as described in FACS Analysis of ESC Chimerism in Supplementary Methods. eGFP− single cardiac cells were FACS-purified and individually sorted into gelatinized 96-well tissue culture plates containing Cardiomyocyte Maintenance Media and cultured at 37°C in 5% CO2 in humidified air for 7 or 14 days without media changes.

**TUNEL Staining**
Following deparaffinization, TdT-mediated dUTP nick end labeling (TUNEL) was performed on heart sections using an ApopTag Red In Situ Apoptosis Detection Kit (Chemicon, S7165, antidigoxigenin rhodamine antibody) according to manufacturer’s recommendations. The heating method was used for the DNA exposure step by incubating the slides in a pressure cooker for 3 minutes using citrate-based unmasking solution, pH 6.0 (Vector Labs, H-3300). Slides were subsequently washed in PBS and incubated overnight at 4°C in PBS with 10% goat serum, 1% BSA, 0.1% saponin, and primary antibodies to eGFP (Abcam, ab13970, 1:400 dilution) and troponin T (Thermo Scientific, MS-295, 1:500 dilution). Sections were washed with PBS and incubated at room temperature for 1 hour with corresponding secondary antibodies conjugated to Alexa Fluor 488 or Alexa Fluor 647 (Invitrogen, 1:200 dilution). Slides were then mounted in ProLong Gold Antifade Reagent containing DAPI (Life Technologies, P-36931). All staining was performed on 3-5 hearts/group, with 4 distinct sections/heart.
**Wheat Germ Agglutinin (WGA) Staining**

Following removal of OCT (E8.5 sections) or paraffin (E9.5 & adult heart sections), slides were washed in PBS and then incubated for 1 hour at room temperature with a primary antibody against WGA conjugated to FITC (Sigma, L4895, 5 μg/ml stock, 1:200 dilution). The slides were subsequently blocked and permeabilized with 10% goat serum, 1% BSA, and 0.1% saponin in PBS for 1 hour and incubated overnight at 4ºC with a primary antibody to troponin T (Thermo Scientific, MS-295, 1:500 dilution). Sections were washed with PBS and incubated at room temperature for 1 hour with a secondary antibody conjugated to Alexa Fluor 546 (Invitrogen, 1:400 dilution). Slides were mounted in ProLong Gold Antifade Reagent containing DAPI (Life Technologies, P-36931). To quantify cardiomyocyte size, images at 32X magnification were captured and ImageJ was used to determine the average cell area by an investigator blinded to mouse genotype. Quantitative analyses involved counting a minimum of 3 fields from 3-4 independent hearts per group, and 2-4 distinct sections/heart (~30-50 cells per field assessed, total ~100-150 cells per sample).

**Electron Microscopy**

E10.5 embryos were dissected in 0.1M sodium cacodylate buffer, pH 7.4 and placed into electron microscopy fixative (2.5% glutaraldehyde, 2.0% paraformaldehyde, 0.025% calcium chloride in a 0.1M sodium cacodylate buffer, pH 7.4) and allowed to fix overnight at 4ºC. The fixative was replaced with cacodylate buffer and the embryos were stored at 4ºC until further processing in a Leica Lynx automatic tissue processor. The embryos were post fixed with osmium tetroxide, en bloc stained with 2.0% uranyl acetate dehydrated in a graded ethanol series, embedded in pure epoxy resin and polymerized overnight at 60ºC. 1 μm thick sections were cut using glass knives and a Sorvall MT-1 (Dupont) ultramicrotome and floated on water droplets on glass slides. The slides were dried in a humidity chamber on a warm hot plate. Toluidine blue stain (0.5% toluidine blue in aqueous 0.5% sodium borate) was pipetted over the
sections and placed onto the hot plate until a slight gold rim could be seen around the stain droplet. The sections were rinsed in a stream of distilled water, dried, cover slipped and examined by light microscopy. Tissues representing the common ventricle of the heart were chosen, and the blocks trimmed accordingly. Thin sections were cut using a diamond knife and an LKB 2088 ultramicrotome and placed on copper grids. Sections were stained with lead citrate and examined in a FEI Morgagni transmission electron microscope. Images were captured with an Advanced Microscopy Techniques 2K digital CCD camera. Quantitative analysis involved measuring the average sarcomeric length and width per field on images acquired at a direct magnification of 10,000-20,000X by an investigator blinded to mouse genotype. Multiple fields were examined from 3 independent hearts per group, and 2 distinct sections/heart.

**H&E Staining**

Hematoxylin-eosin staining on adult heart sections was performed according to standard protocol at the MGH Endocrine Core Histology Facility.
**SUPPLEMENTAL TABLES**

**Supplemental Table 1: Primers Utilized for PCR-based Genotyping**

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**Supplemental Table 2: Antibodies Utilized for Immunohistochemistry**

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SUPPLEMENTAL REFERENCES

Figure S-I

A  Nkx2.5\textsuperscript{Cre/+}, R26\textsuperscript{LacZ}  
B  Nkx2.5\textsuperscript{Cre/+}, R26\textsuperscript{LacZ}  
C  Nkx2.5\textsuperscript{Cre/+}, R26\textsuperscript{LacZ}  
D  Nkx2.5\textsuperscript{Cre/+}, R26\textsuperscript{LacZ}  

ca
CV
Figure S-I. Genetic fate mapping of Nkx2.5 lineage cells, related to Figure 1. A and B, Sagittal views of an E9.5 embryo. Robust β-galactosidase activity is evident throughout the common atrial and ventricular chambers, as well as pharyngeal arch ectoderm. C and D, Transverse section of an E9.5 embryo. β-galactosidase is detected in the myocardium, with minimal activity in the endocardium. ca, common atrium; cv, common ventricle. Scale bar: 500 μm.
Figure S-II. Determination of the variance of ESC chimerism in the heart versus other tissues, related to Methods. A, FACS contour plots showing distribution of eGFP\(^+\) versus eGFP\(^-\) cells from the tissues of three representative E10.5 eGFP\(^+\) embryos that have been complemented with unlabeled wild-type ESCs. Mean ESC chimerism percentage is shown within the embryo image. Scale bar: 1 mm. B, Graphical representation of flow cytometry-based cell counting in selected embryos demonstrating ESC chimerism in the heart correlates well with chimerism in other tissues. C, Difference between percent chimerism in the heart and the mean extra-cardiac tissue chimerism (i.e. head, body, limb, tail). Each data point represents one embryo. D, Likelihood of variation between extra-cardiac tissue chimerism and heart chimerism. 67% of embryos exhibited a variation ≤5%; 90% of embryos exhibited a variation ≤10%; and 97% of embryos exhibited a variation of ≤12%. For C and D, n=30 embryos.
Figure S-III

A B

![Diagram of Ablated Chimeric Embryo and Control Chimeric Embryo](image)

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<th>Animals Recovered</th>
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C

% CPC Ablation to Nkx2.5<sup>Cre+/+;R26eGFP-DTA</sup> Embryos

![Images of Embryos at Different CPC Ablation Levels](image)

D

% Wild-Type ES Chimerism (Surviving Embryos)

![Graph showing % Wild-Type ES Chimerism](image)

E

Surviving Embryos (% of Expected)

![Graph showing Surviving Embryos](image)

F

Control R26<sup>eGFP-DTA</sup> vs 50-60% CPC Ablation Nkx2.5<sup>Cre+/+;R26eGFP-DTA</sup>

![Images of Embryos with DAPI, GFP, and cTnT staining](image)
Figure S-III. Recapitulation of the CPC ablation phenotype using a reverse complementation approach, related to Figure 1. 

A, Strategy for rescue of CPC ablation phenotype by wild-type ESC chimerism. Nkx2.5\textsuperscript{Cre/+} mice are mated to ROSA26\textsuperscript{eGFP-DTA} mice to generate eGFP\textsuperscript{*} embryos carrying either ROSA26\textsuperscript{eGFP-DTA} only (control) or Nkx2.5\textsuperscript{Cre/+};ROSA26\textsuperscript{eGFP-DTA} (ablated) alleles. Unlabeled wild-type ESCs are injected into eGFP\textsuperscript{*} blastocyst-stage embryos from each group to generate chimeras. 

B, Summary table demonstrating the number of embryos and live-born mice recovered from wild-type ESC injection into control and CPC-ablating blastocysts. The number of animals derived from injection into the control genotype is expected to be higher due to early lethality of CPC-ablated embryos with low ESC chimerism. 

C, Brightfield and GFP images of E10.5 chimeric Nkx2.5\textsuperscript{Cre/+};ROSA26\textsuperscript{eGFP-DTA} embryos. Embryos with >60% ablation show developmental arrest. 

D, Degree of wild-type ES chimerism found in recovered surviving embryos or mice with ROSA26\textsuperscript{eGFP-DTA} or Nkx2.5\textsuperscript{Cre/+};ROSA26\textsuperscript{eGFP-DTA} alleles. 

E, Rate of recovery of surviving Nkx2.5\textsuperscript{Cre/+};ROSA26\textsuperscript{eGFP-DTA} chimeric animals relative to the number of un-ablated control (i.e. ROSA26\textsuperscript{eGFP-DTA}) chimeric embryos stratified by degree of wild-type ES chimerism. Note the drastic reduction in the percentage of recoverable Nkx2.5\textsuperscript{Cre/+};ROSA26\textsuperscript{eGFP-DTA} live animals when ES chimerism is <50%. 

F, eGFP, troponin T (cTnT), and DAPI staining of a control ROSA26\textsuperscript{eGFP-DTA} and a chimeric Nkx2.5\textsuperscript{Cre/+};ROSA26\textsuperscript{eGFP-DTA} embryo. Only rare eGFP\textsuperscript{*} endocardial cells are apparent in the ablated heart. Scale bars: 1 mm for C; 200 µm for F.
Ablation Tolerance at Various Developmental Stages

B

% CPC Ablation

Brightfield

0% 26% 51%
E10.5

0% 24% 41%
E12.5

0% 35% 45%
E15.5

C

% CPC Ablation

10-20% Ablation

30-40% Ablation

40-50% Ablation

Brightfield

E10.5

E12.5

E15.5

D

% CM Ablation

Brightfield

E10.5-E12.5

E15.5-E18.5

4 weeks

E10.5-E12.5

E15.5-E18.5

4 weeks

E10.5

E12.0

E15.5
Figure S-IV. Embryonic survival and additional embryo images, related to Figure 1 and 3. A, Degree of CPC ablation found in recovered surviving embryos or mice at mid (E10.5-E12.5) or late (E15.5-E18.5) gestation, and early life (4 weeks). B, Brightfield images demonstrate the tolerance of ablated embryos to survive throughout gestation. Note: embryos derived from both chimeric rescue approaches are shown. C, Brightfield images demonstrate consistency of normal embryo phenotype at various ranges of ablation. D, Degree of CM ablation found in recovered surviving embryos or mice at mid (E10.5-E12.5) or late (E15.5-E18.5) gestation, and early life (4 weeks). E, Brightfield images demonstrate stability of CM ablation tolerance throughout development. Scale bars: 1 mm.
Figure S-V

A αMHC^{Cre+}, R26^{LacZ}

B αMHC^{Cre+}, R26^{LacZ}

C αMHC^{Cre+}, R26^{LacZ}
Figure S-V. Genetic fate mapping of αMHC lineage cells, related to Figure 3. **A**, Sagittal view of an E9.5 embryo. Robust β-galactosidase activity is evident throughout the atrial and ventricular chambers. **B**, Transverse section through the mid-ventricle of an E9.5 embryo. β-galactosidase is detected throughout the myocardium. **C**, Transverse section through the outflow tract and common ventricle of an E9.5 embryo. cv, common ventricle; oft, outflow tract. Scale bar: 500 µm.
Figure S-VI. Cardiomyocyte apoptosis during CM ablation, related to Figure 3. A and B, Cleaved caspase-3 (cCsp3, A) or TdT-mediated dUTP nick end labeling (TUNEL, B), eGFP, troponin T (cTnT), and DAPI staining of E9.5 embryos undergoing CM ablation. In the absence of CM ablation, no cCsp3⁺ (A) or TUNEL⁺ (B) cardiomyocytes are observed (upper row). However, a growing number of cCsp3⁺ or TUNEL⁺ cardiomyocytes can be identified as the severity of ablation increases (middle, lower rows). ca, common atrium; cv, common ventricle.
Scale bar: 50 µm.
Figure S-VII

A

Control

CPC Ablation

CM Ablation

Propidium iodide

Tail

Heart

Propidium iodide

100% 0%

100% 0%

100% 0%

100% 0%

53% 47%

95% 5%

53% 47%

95% 5%

44% 56%

62% 38%

44% 56%

62% 38%

Propidium iodide

100% 0%

100% 0%

100% 0%

100% 0%

Control

CPC Ablation

CM Ablation

B

C

Actual

# Cardiomyocytes / Well

Predicted # Cardiomyocytes / Well (Based on Gaussian Distribution)

Binucleated Cardiomyocytes / Well (%)

Control | CPC Ablation | CM Ablation

NS
**Figure S-VII.** *Ex vivo* clonal analysis of cultured cardiomyocytes following cardiac ablation, related to Figure 4. **A,** Representative FACS contour plots of dissociated tail and heart samples from E9.5 embryos used for single-cell clonal analysis. The proportion of eGFP+/eGFP- cells in these tissues is shown from an embryo with no ESC chimerism (control), an embryo chimerized with *Nkx2.5Cre*/*;ROSA26*^{EFP-DTA} ESCs (CPC ablation), and an embryo chimerized with *αMHCCre*/*;ROSA26*^{EFP-DTA} ESCs (CM ablation). Few eGFP+ cardiac cells remain in the CPC-ablated embryo by this stage of development. In the CM-ablated embryo, the fraction of eGFP+ cells in the heart relative to the tail is reduced due to the loss of eGFP+ cardiomyocytes. **B,** Mean percentage of binucleated cardiomyocytes per well among wells containing cardiomyocyte colonies. Error bars represent mean ± s.e.m. **C,** Q-Q normality plot of actual versus predicted single-cell derived cardiomyocyte colony size demonstrates marked systematic deviation from linearity indicating lack of conformity to a Gaussian distribution.
Figure S-VIII

A  Control  CPC Ablation

B  Control  CM Ablation

C  Control  CPC Ablation

D  

E  

WGA

Sarcomere Length

Average

Median

Control  CPC Ablation

Control  CM Ablation

E9.5

E8.5

E10.5

Embryo #

Embryo #

Embryo #

536 nm

369 nm

1477 nm

1360 nm

p=0.30

p=0.28

Co
trol

CM Ablation

CM size (pixels²)

1 2 3 1 2 3

0 500 1000 1500 2000

2500

0 200 400 600 800

1000

Sarcomere Width (nm)

Sarcomere Length (nm)
**Figure S-VIII.** Embryonic cardiomyocyte size and ultrastructural features following ablation, related to Figure 4. For A through E, experimental variables are represented as follows: group, number of mice examined, mean percent ablation±SD. A and B, Wheat germ agglutinin (WGA) staining and dot plots displaying mean CM size for CPC-ablated hearts at E8.5 (A) and CM-ablated hearts at E9.5 (B). Each symbol in the dot plot represents the mean CM size in an individual heart section; a minimum of three 32X fields per section were counted; each symbol shape represents an independent mouse; a horizontal bar indicates the mean value for each group. Statistical comparisons were performed using a likelihood ratio test via ANOVA on a linear mixed-effects model. E8.5 embryos were embedded frozen in OCT; E9.5 embryos were embedded in paraffin. Control, n=4; CPC ablation, n=4, 41±9%; CM ablation, n=4, 53±12%. C, Transmission electron micrographs (EM) showing sarcomeric structure from a control and a CPC-ablated heart section at E10.5. D and E, Dot plots of sarcomere width (D) and length (E) based upon EM analysis. Control, n=3; CPC ablation, n=3, 40±7%. Each point represents the average sarcomere width/length from a single field; bars represent median ± interquartile range; dashed lines represent the average of the three median values for each group. Scale bars: 50 µm for A and B; 500 nm for C.
척추동물의 배아 심장 세포는 뛰어난 재생 능력을 가지고 있다

이상연 교수 서울대학교병원 순환기내과

초록

배경
심장 생장에 있어서 심장전구세포(cardiac progenitor cell)의 분화 능력이 심장 세포에서의 특별 신호전달 체계들이 중요하며, 이와 관련된 신호전달 체계에 이상이 있으면 심장 세포에 문제가 발생한다는 것은 잘 알려져 있다. 하지만 심장 발생시 심장전구세포나 분화한 심장 세포가 소실되면 심장의 발생에 어떠한 영향을 미치는지는 잘 알려져 있지 않다. 본 연구에서는 생쥐의 심장에서 세포가 소실되어도 남은 세포에 의해 재생될 수 있을 것이라는 가설을 평가하였다.

결론
본 연구는 포유류에서 배아의 심장세포 소실의 한계점을 확인하였으며, 심장세포가 소실되더라도 심근세포의 재생 능력을 통해 심장이 정상적으로 발달할 수 있음을 보여준다.

방법 및 결과
본 연구에서는 생쥐의 배아에서 심장 전구세포 혹은 초기 분화 심장 세포를 소멸시키기 위해 "blastocyst complementation"이라는 방법을 사용하였다. 특히, 배아기 7.5일째에서 심장전구세포의 60% 이상 소실은 특별한 이상 없이(well tolerated) 배아 생존이 가능하였으며, 배아기 9.0일째에서 초기 분화 심장 세포의 50~60%가 소실되어도 잔여 심근세포에 의해 거의 회복되었고, 성체 생쥐의 심장 기능에는 특별한 이상이 발견되지 않았다. 두 개의 결합 모델 모두에서 심장 세포의 증식이 증가함을 관찰하였고, 이는 일부분 심근 세포질 및 심장 크기의 회복에 의한 것으로 설명할 수 있을 것이다.