The Fetal Mammalian Heart Generates a Robust Compensatory Response to Cell Loss

Running title: Sturzu et al.: Cardiac cell loss and recovery in embryonic mice

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Abstract

Background—Heart development is tightly regulated by signaling events acting upon a defined number of progenitor and differentiated cardiac cells. While loss-of-function of these signaling pathways leads to congenital malformation, the consequences of cardiac progenitor cell (CPC) or embryonic cardiomyocyte loss are less clear. In this study, we tested the hypothesis that embryonic mouse hearts exhibit a robust mechanism for regeneration following 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 CPC or cardiomyocyte ablation. Remarkably, ablation of up to 60% of CPCs at embryonic day 7.5 was well-tolerated and permitted embryo survival. Ablation of embryonic cardiomyocytes to a similar degree (50-60%) 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.

Conclusions—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.

Key words: cardiac development, regeneration, cardiac progenitor cells, cardiomyocyte, stem cell plasticity, cardiomyocyte proliferation, developmental plasticity, ablation, cardiogenesis, growth regulation
Introduction

The development of the mammalian four-chambered heart requires a close interplay between extracellular morphogens and signals (e.g. Wnt, TGF-β, FGF, Notch, and Hedgehog) and cell-intrinsic effectors such as transcription factors and epigenetic modifiers. Some of these factors regulate early lineage specification, while 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. Disruption in these formative events during embryonic development also has a profound impact on human life, as congenital heart disease remains the most common cause of birth defects world-wide.

While cardiogenesis is in many ways a delicate process, the heart is nonetheless able to withstand injury and regenerate throughout the lifespan of some animal species as demonstrated by studies in amphibians and lower vertebrates such as the newt and zebrafish. 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. Collectively, these non-mammalian 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. Furthermore, a prior study from Drenckhahn and colleagues found that healthy embryonic cardiomyocytes can augment their rate of proliferation to compensate for the reduced proliferation rate of diseased cardiomyocytes that are deficient in holocytochrome-c synthase, an enzyme that is essential for mitochondrial respiration. While this
approach and others examined the role of cell competition during cardiac development\textsuperscript{13}, they did not directly address the consequences of cardiomyocyte loss. Accordingly, the ability of an embryonic mammalian heart to regenerate following 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 by residual non-ablated cells if a robust regenerative mechanism exists at the cardiac progenitor cell (CPC) or immature cardiomyocyte (CM) stage of development and found that ablation of up to 50-60\% of CPCs or immature CMs 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 CMs in the embryonic heart. In the context of adult mammalian cardiac injury, advances in this area may lead to therapies that direct the post-injury response towards cellular regeneration rather than fibrosis.

**Methods**

An expanded Methods section is available in the Supplemental Materials.

**Experimental Animals**

An \textit{Nkx2.5\textsuperscript{Cre/+}} knock-in mouse line was kindly provided by Dr. Robert Schwartz\textsuperscript{14}. An \textit{\alpha \textbf{MHC}\textsuperscript{Cre/+}} transgenic mouse line was kindly provided by Dr. E. Dale Abel\textsuperscript{15}. \textit{ROSA26\textsuperscript{GFP-DTA}} and \textit{ROSA26\textsuperscript{LacZ}} mouse lines were purchased from the Jackson Laboratory\textsuperscript{16,17}. 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 ESC Lines

Derivation of the V6.5\textsuperscript{18} and R1\textsuperscript{19} ESC lines has been described previously. For generation of
\( Nkx2.5^{Cre/+};ROSA26^{eGFP-DTA} \) and \( \alpha MHC^{Cre/+};ROSA26^{eGFP-DTA} \) compound transgenic ESC lines, timed matings were performed between male \( Nkx2.5^{Cre/+} \) mice or \( \alpha MHC^{Cre/+} \) mice with female
\( ROSA26^{eGFP-DTA} \) mice. At 3.5 days post-coitum (dpc), females were sacrificed and blastocysts
were flushed from the uterine horns with M2 medium (Sigma-Aldrich, M7167) and washed
several times. Using a mouth pipette with a pulled glass capillary, blastocysts were plated
individually onto 24-well gelatin-coated plates containing mitomycin-C (Sigma-Aldrich, M4287)
inactivated mouse embryonic fibroblast (MEF) feeder layers in ESC Derivation Media and
cultured, undisturbed, at 37°C in 5% CO\textsubscript{2} in humidified air for 5–7 days without media changes.
As blastocysts hatched from their zona pellucidae, the inner cell mass (ICM) outgrowth was
identified and transferred into 200 \( \mu\)L of 0.25% trypsin-EDTA solution (Life Technologies,
25200) for 5 min at 37°C and gently dissociated by pipetting. Trypsin was inactivated with fetal
bovine serum (FBS, Atlanta Biologicals, S11550), and the ICM cells were centrifuged and
reseeded onto fresh MEFs in ESC Maintenance Media supplemented with 2\textsuperscript{i}\textsuperscript{20,21}.

Undifferentiated ES colonies were then gradually expanded to establish ESC lines. Lines were
selected for further use based on undifferentiated morphology, the presence of the \textit{Cre} transgene
and Y chromosome by PCR, and expression of eGFP. Primer sequences used for genotyping are
listed in Supplementary Table 1. ESC Derivation and Maintenance Media compositions are
reported in Supplementary Methods.
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, approximately 10-20 low passage (P5-P10) Nkx2.5\(^{Cre/+}\);ROSA26\(^{eGFP-DTA}\) or aMHC\(^{Cre/+}\);ROSA26\(^{eGFP-DTA}\) ESCs were microinjected into E3.5 blastocysts from superovulated CD-1 females (Charles River Laboratories). For the reverse complementation studies, P15-P25 V6.5 or R1 ESCs were microinjected into E3.5 blastocysts from superovulated ROSA26\(^{eGFP-DTA}\) females which had been mated to Nkx2.5\(^{Cre/+}\) males. For both approaches, the injected blastocysts were subsequently transferred into the uterus of 2.5 dpc pseudopregnant 6-8-week-old CD-1 foster mothers previously mated with vasectomized males\(^{22}\). Genotype was identified based upon expression of eGFP and the presence of the Cre transgene by PCR. Chimeric contribution was determined by flow-cytometric analysis as described in Supplementary Methods.

Ex vivo single-cell clonal analysis

Details regarding single-cell culture studies can be found in the Supplementary Methods.

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 OCT compound on dry ice. E9.5-E10.5 embryos and live-born mouse tissues were fixed overnight in 4% paraformaldehyde and embedded in paraffin. Tissue blocks were cut at 5 \(\mu\)m thickness. Paraffin-embedded sections underwent antigen retrieval in a pressure cooker for 3 minutes in citrate-based unmasking solution, pH 6.0 (Vector Labs, H-3300). 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\(^\circ\) C with
the primary antibody. Primary antibodies utilized are listed in Supplementary Table 2. 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 (Invitrogen, 1:400 dilution). Samples were subsequently rinsed in PBS and stained with NucBlue Fixed Cell ReadyProbes DAPI Reagent (Life Technologies, R37606). Slides were mounted in ProLong Gold Antifade Reagent (Life Technologies, P-36930). For tissue sections, epifluorescence images were acquired on a Zeiss AxioImager M1 upright widefield fluorescence/DIC microscope with dual CCD cameras. Immunostaining was performed on 3-5 embryos/group. For cell culture experiments, each well of a 96-well plate was individually imaged using 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 Immunohistochemistry using antibodies to cTnT, CD31, and pH3 or Ki-67. 1 mm z-stack immunofluorescence images of each section were acquired on a Zeiss LSM710 confocal microscope at 20x magnification to confirm that each DAPI+ cell nucleus counted was associated with a cardiomyocyte (if surrounded in three dimensions by cTnT stain) or an endocardial cell (if surrounded in three dimensions by a CD31 stain).

Echocardiography

Echocardiography was performed on 4-5 week-old mice using a 13-MHz linear-array transducer with a digital ultrasound system (Vivid 7; GE Medical Systems, Milwaukee, WI). The mice were lightly sedated using an intraperitoneal injection of ketamine (50 mg/kg). Heart rate, left ventricular internal diameter at end-diastole (LVIDED), and left ventricular internal diameter at end-systole (LVIDES) were measured, and the LV fractional shortening was calculated by using

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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 using GraphPad Prism version 6.02 for Windows (GraphPad Software, San Diego, California, USA) and RStudio version 0.98.1102 for Windows (Boston, Massachusetts, USA). For comparisons between groups where multiple measurements per mouse were recorded (embryonic cell proliferation, embryonic CM size, and adult CM size), a likelihood ratio test via ANOVA was computed on a linear mixed-effects model using RStudio. For comparisons between three groups (cardiomyocyte binucleation, adult heart weight ratios, left ventricular dimensions, and left ventricular fractional shortening), a one-way ANOVA was used with adjustment for multiplicity using Dunnett’s 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 Dunn’s correction for multiple comparisons. A p-value of <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 S1*)²³. We performed targeted genetic ablation of *Nkx2.5* cardiac progenitor cells during embryonic development in order to examine the innate recovery response by the remaining non-ablated cells. By crossing a previously described *Nkx2.5*-Cre knock-in mouse line (*Nkx2.5Cre/+*)¹⁴ with a mouse line that
expressed diphtheria toxin A (DTA) following Cre-dependent excision of a constitutively active eGFP transgene (ROSA26<sup>eGFP-DTA</sup>)<sup>16</sup>, we show that cardiac cells were abolished entirely in double transgenic embryos (Figure 1A).

While 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 prior experience with either doxycycline or tamoxifen-inducible mice suggests too much inter-embryo variability in drug metabolism <i>in utero</i> for that strategy to be reliable for our purposes<sup>24</sup>. Hence, we employed a novel strategy that involves injection of double transgenic Nkx2.5<sup>Cre/+</sup>;ROSA26<sup>eGFP-DTA</sup> embryonic stem cells (ESCs) into wild-type eGFP<sup>+</sup> blastocysts to ablate CPCs in quantifiable fractions (Figure 1B). Since ESC injection into mouse blastocysts is well-known to generate chimeric embryos and mice with a wide spectrum of ESC contribution<sup>25</sup>, this assay enabled us to determine the expected percentage of CPC ablation by assessing the overall contribution of the eGFP<sup>+</sup> Nkx2.5<sup>Cre/+</sup>;ROSA26<sup>eGFP-DTA</sup> ESCs to the embryo.

We first generated several independent ESC lines carrying both the Nkx2.5<sup>Cre/+</sup> and ROSA26<sup>eGFP-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 <i>in vitro</i> phenotype were subsequently injected into wild-type eGFP<sup>+</sup> blastocysts and fostered in pseudopregnant female mice. Following 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 due to Cre-mediated expression of DTA so that directly assessing the percentage of cardiac contribution by eGFP+ 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 (e.g. head, limbs, tail, etc.) by injecting wild-type eGFP+ ESCs into eGFP+ mouse blastocysts or red fluorescent tdTomato+ ESCs into wild-type mouse blastocysts. By flow cytometric (FACS) analysis, we found that 97% (29/30) of embryos showed a difference of ≤12% in ESC contribution to the heart compared to their mean contribution to the rest of the embryo (Figure S2A-D). Remarkably, our analysis of the embryonic day 10.5 (E10.5) chimeric embryos from Nkx2.5Cre+/;ROSA26eGFP-DTA ESC injections showed that the ablation of up to ~60% of CPCs, as determined by FACS, appears compatible with normal development, while degrees of ablation >60% lead to overall developmental arrest (Figure 1F).

Immunohistological analysis of chimeric embryos showed that eGFP+ cell contribution was homogenously distributed throughout the embryo, except for the heart region where eGFP+ cells were completely absent within the cardiac troponin T+ (cTnT) cell 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 non-ablated eGFP- cardiac cells (Figure 1F). Of note, sporadic eGFP+ cells could be found in the endocardium, consistent with the previously described incomplete expression of Nkx2.5 in these cells26 (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 (i.e. Gata4, Mef2c, Islet-1) and signaling mediators (i.e. β-catenin and Yap) that have been reported to regulate CPC number or myocardial growth \cite{27-29} (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 could be observed in the ablated embryo heart by immunofluorescence staining. We also examined whether the expression of sarcomeric proteins (i.e. cTnT, α-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 un-ablated CPCs and/or their myocardial descendants to rapidly recover full myocardial cellularity in the ablated heart.

Since 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 \textit{Nkx2.5\textasciicircum{2}}^{cre/+};ROSA26\textasciitilde{eGFP-DTA} 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 \textit{Nkx2.5\textasciicircum{2}}^{cre/+} and ROSA26\textasciitilde{eGFP-DTA} mice (Figure S3A). In particular, we assessed chimeric embryos or mice that are eGFP\textasciitilde{+} [i.e. derived from \textit{Nkx2.5\textasciicircum{2}}^{cre/+};ROSA26\textasciitilde{eGFP-DTA} (ablated) or \textit{Nkx2.5\textasciicircum{2}}^{+/+};ROSA26\textasciitilde{eGFP-DTA} (control) blastocysts]. Following injection of 639 blastocysts using two different wild-type ESC lines (V6.5 and R1), we obtained 394 embryos or mice, with roughly half (226) being eGFP\textasciitilde{+}. Similar to our \textit{Nkx2.5\textasciicircum{2}}^{cre/+};ROSA26\textasciitilde{eGFP-DTA} ESC injection results above, we found that wild-type V6.5 and R1 ESCs exhibit a broad range of contribution (Figure S3B). The total number of recovered control ROSA26\textasciitilde{eGFP-DTA} embryos and mice (144) was greater than the number of ablated \textit{Nkx2.5\textasciicircum{2}}^{cre/+};ROSA26\textasciitilde{eGFP-DTA} animals (82),
presumably due to 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 S3C). 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 (Figure S3D-S3E, Figure S4A-S4C). As we expected, no eGFP+ cells were present in the cTnT+ myocardium in the ablated mice (Figure S3F). Taken together, these findings support a robust compensatory mechanism in embryonic mouse hearts following CPC ablation.

**Fractional ablation of embryonic CMs by chimeric complementation**

While 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 (αMHCCre/+ ) transgenic mouse line (Figure S5).15 Embryos with compound αMHCCre/+ and ROSA26gfp-dta 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 αMHCCre/+;ROSA26gfp-dta ESC lines and selected three (MHC-DTA1, MHC-DTA2, and MHC-DTA3) for further study based upon their capacity to contribute to chimeric embryos and mice with a broad range of efficiency (Figure 3B-3C). Interestingly, we found that these chimeric embryos were also able to tolerate up to 50-60% ablation of their immature myocytes (Figure 3D and Figure S4D-S4E). Immunohistological staining for eGFP and cTnT expression showed a progressive decrease in myocardial cellularity in E9.5 chimeric embryos...
with higher than 50% contribution by $\alpha$MHC$^{\text{Cre}+/+}:\text{ROSA26}^{\text{eGFP-DTA}}$ ESCs (Figure 3E). A rare number of eGFP$^+$ CMs could be observed which may represent residual non-ablated CMs or CMs that were dying but had not been completely eliminated. Additional immunostaining for apoptotic cells confirmed that at least a fraction of these eGFP$^+$ CMs underwent cleavage of caspase-3 (cCsp3) or were positive for TdT-mediated dUTP nick end labeling (TUNEL) (Figure S6A-S6B). 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 (i.e. 50-60%) compared to 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**

Since the overall cardiac morphology in both ablation models was largely indistinguishable between surviving <50-60% ablated and control embryos, we examined whether there is a proliferative response to cell ablation by surveying the behavior of residual un-ablated cardiac cells ex vivo. We first dissected the heart tubes from E9.5 un-ablated control embryos, CPC-ablated embryos, and CM-ablated embryos. Next, we FACS-purified residual eGFP$^+$ cardiac cells, which are presumably responsible for recovering myocardial cellularity after ablation (Figure S7A), deposited one 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. 36% of single-cell clones from control hearts and 20% and 44% of CPC or CM-ablated clones, respectively, were classified as cardiomyocytes (Figure 4B), exhibiting spontaneous beating and well-organized Z-lines marked by sarcomeric $\alpha$-actinin expression (Figure 4C). Cardiomyocyte
binucleation was also observed, but did not significantly differ among groups (Figure S7B). A small minority of clones (2% of control, 1% of CPC-ablated, and 1% CM-ablated clones) expressed CD31 and were highly proliferative, possessing a polygonal morphology, consistent with an endocardial/endothelial cell phenotype (Figure 4D). The remaining majority of clones (62% of control, 79% of CPC-ablated, and 54% CM-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 CM ablation on clonal cell growth potential, we counted the cardiomyocyte colony sizes resulting from single-cell plating on day 7 or 14. A normality plot indicated the colony size distribution deviated significantly from a Gaussian distribution, necessitating a nonparametric analysis of the data (Figure S7C). 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 CM-ablated embryos compared to un-ablated 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 was most likely because CPC-ablated embryos had largely recovered their myocardial cellularity by E9.5, while the CM-ablated embryos were still actively recovering. These results indicate that CM ablation triggers a modest increase in cellular...
proliferation at E9.5 to increase cardiomyocyte number *ex vivo*.

**Embryonic CM 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 following cell ablation is due merely to a hypertrophic response in residual un-ablated CMs\(^{30}\). 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 CM ablation models using the mitosis marker phosphorylated histone H3 (pH3), and the cell proliferation marker Ki-67 (*Figure 4G*-4J). We found that embryos from both CPC and CM ablation models exhibited increases in expression of both proliferation markers within their cTnT\(^+\) CMs. To see whether this correlated with changes in cardiomyocyte size, we quantified the ratio of myocardial area to the number of nuclei within cTnT\(^+\) CMs. We found, however, the CM area per nucleus was not statistically different between ablated embryos and controls despite a trend towards smaller cell size (*Figure S8A*-S8B). 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\(^{31}\), we also examined whether the development of the endocardium is affected by ablation of CPCs or CMs. At E8.5 in the CPC ablation model and at E9.5 in the CM ablation model, the endocardium in ablated embryos exhibited similar CD31 expression relative to controls consistent with normal endocardial differentiation (*Figure 4G*-
In addition, we quantified the degree of endocardial cell proliferation in CPC or CM-ablated embryos by assessing the percentage of Ki-67\(^+\) or phospho-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).

Since 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\(^32\), we examined whether CM maturation was delayed in ablated embryos at the ultrastructural level by transmission electron microscopy (Figure S8C-S8E). 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 due to their need for an increased rate of cell proliferation.

CPC and CM-ablated embryos can survive into adulthood with no overt cardiac defect

The above results demonstrate that a majority of embryonic CPCs or CMs can be ablated without triggering gross embryonic defects. To rule out more subtle congenital phenotypes in CPC- or CM-ablated embryos that would not manifest until late gestation or after birth, we collected chimeric live-born mice that had undergone an average of approximately 40% CPC or CM ablation and assessed their cardiac structure by histology and their cardiac function by echocardiography at 4-5 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 to control un-ablated mice, their hearts showed similar gross overall size at the whole organ level (Figure 5C-5D) and had similar heart weights (Figure 5E), LV 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-5K). These findings further support our conclusion that acute CPC or CM loss during early embryogenesis is well-tolerated in mice due to a rapid and efficient compensatory proliferative response that supports the development of normal organ size, CM 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 CM 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 following CPC ablation at E7.5 was rapid and complete with little to no 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 CM ablation at E9.0 and found, with remarkable consistency, that the loss of 50-60% of immature CMs 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 CM proliferation was evident in both CPC and CM 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 employed 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 70-80% loss of cardiomyocytes. While 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 our knowledge, no study has demonstrated complete myocardial regeneration in the embryonic mouse heart following 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, as well as their regenerative potential given the increase in cardiomyocyte and/or cardiomyocyte precursor proliferation in the setting of cardiac cell ablation. Regardless, our data supports 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 50-60% of developing CPCs or immature CMs 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 embryonic day 8.5-9.5 cardiomyocytes of the proliferation marker Ki-67, which is detectable during the active stages of the cell-cycle, including late G1, S, G2 and M phases, but which is absent in resting cells (G0). While this supports that cardiomyocytes are highly proliferative during this stage of development, we found that both CPC and CM ablation triggered a further increase in myocyte proliferative capacity.
Likewise, histone H3 phosphorylation was also found to be increased upon CPC or CM ablation. However, its expression was only observed in a small fraction of developing myocytes, consistent with the fact that its phosphorylation is limited primarily to mitosis (M phase)\(^36\) (Figure 4G-4J). Due to the rapid differentiation of CPCs into immature cardiomyocytes at embryonic day 7.5 to 8.5 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 will be necessary to specifically address the role of first or second heart field CPCs in the regenerative process.

Our findings also raise questions regarding the potential mechanisms involved in replacing the number of CPCs and CMs following ablation. While we believe that CPC and CM 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 non-ablated cardiac cells may trigger the observed proliferative response. In addition, cell-cell contact has been well-described to regulate the rate of cell division\(^37\). The acute loss of CPCs or CMs may result in de-repression of this intrinsic negative regulator of cell proliferation. Furthermore, since the developing heart at this stage is continually receiving a new influx of cells from the second heart field (SHF), it is possible that an increase in migration and proliferation of SHF-derived cells may be involved in myocardial regeneration in the right ventricle and outflow tract. Further work will be 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 non-myocardial cells (e.g. endocardial and/or 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, as well as endocardial and epicardial cells. Consequently, this model may underestimate the degree of regeneration possible since it may ablate both the CPCs involved in myocardial formation as well as the endocardial/epicardial cells that regulate the compensatory process.

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

**Acknowledgments:** We thank Dr. Robert J. Schwartz at the University of Houston for providing us with the Nkx2.5\textsuperscript{Cre/+} knock-in mice, Dr. E. Dale Abel at the University of Iowa for providing us with the \(\alpha\text{MHC}\textsuperscript{Cre/+} \) 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. Author contributions: A.C.S. and S.M.W.
conceived the design of the project. A.C.S performed the experiments, analyzed the data, and prepared the figures. K.R. provided assistance with ESC derivation and ESC culture. D.P. assisted with tissue sectioning. K.P. and A.F.X. assisted with immunohistochemistry, blinded measurements, mouse colony management, and figure preparation. A.R. performed blastocyst microinjection and transfer surgery. A.C.S. and T.C.T. acquired echocardiographic images and T.C.T. performed related measurements. A.S. assisted with figure preparation and plate reader assays. M.C.E. assisted with ESC culture. R.F. assisted with mouse colony management and ESC culture. G.L. assisted with FACS analysis. A.C.S., M.K.S., and R.G. prepared sections for electron microscopy and acquired images. K.D.R. assisted with immunohistochemistry. A.C.S. and S.M.W. wrote the manuscript, and it was edited by M.S.C. and I.J.D.

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**Conflict of Interest Disclosures:** None.

**References:**


Figure Legends:

**Figure 1.** Fractional ablation of embryonic CPCs by chimeric complementation. (A) Schematic diagram of the eGFP/DTA construct before and after Nkx2.5-Cre-mediated excision of the floxed eGFP transgene (upper panel). Brightfield and GFP fluorescence images of E9.5 Nkx2.5<sup>Cre<sup>+/</sup></sup>, ROSA26<sup>GFP-DTA</sup>, and Nkx2.5<sup>Cre<sup>+/</sup></sup>;ROSA26<sup>GFP-DTA</sup> transgenic embryos (lower panel).
Note the absence of a heart in the compound transgenic embryo (arrow). (B) Schematic diagram of fractional ablation of CPCs by injection of Nkx2.5\textsuperscript{Cre\slash+};ROSA26\textsuperscript{eGFP-DTA} ESCs into wild-type CD-1 blastocysts. (C) Generation of Nkx2.5\textsuperscript{Cre\slash+};ROSA26\textsuperscript{eGFP-DTA} ESC lines. Brightfield and GFP fluorescence microscopy images are shown for the three ESC lines used. (D) Summary table demonstrating the complementation efficiency for each ESC line based upon the total number of animals collected. (E) Anticipated progression of CPC loss and regeneration in rescued embryos at approximately 50% ESC chimerism. (F) Brightfield and GFP images of E10.5 chimeric Nkx2.5\textsuperscript{Cre\slash+};ROSA26\textsuperscript{eGFP-DTA} embryos with varying degrees of CPC ablation. The embryo with \(~68\%\) CPC ablation shows a pericardial effusion and developmental arrest. Arrow: heart. Scale bars: 500 μm for (A); 200 μm for (C); 1 mm for (F).

Figure 2. Histological analysis of CPC-ablated embryos shows a rapid recovery in the heart. (A) eGFP, troponin T (cTnT), and DAPI staining of E8.5 and E10.5 chimeric embryos with up to ~50% CPC ablation. By E8.5, all eGFP\textsuperscript{+} myocardial cells have been ablated in the heart. Rare, scattered eGFP\textsuperscript{+} 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: eGFP\textsuperscript{+} staining in the atria of the E10.5 control embryo represents auto-fluorescent blood cells. (B-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). Expression of genes regulating cardiomyocyte proliferation is shown at E8.5 for Yap (E), nuclear β-catenin (F), and total β-catenin (G). Sarcomeric protein expression is shown at E8.5 for α-actinin (H) and cTnT (I), and at E9.5 for Mle2v (J). Nuclei are stained with DAPI and cardiomyocytes are identified by cTnT.
(A, B, C, E, G) or Mlc2v (D and F). For (B-J): degree of ablation in CPC-ablated sections = 41 ± 9% (mean ± SD). ca, common atrium; cv, common ventricle; oft, outflow tract. Scale bars: 100 μm for (A-G); 20 μm for (H-J).

**Figure 3.** Fractional ablation of embryonic CMs by chimeric complementation. (A) Schematic diagram of the eGFP/DTA construct before and after αMHC-Cre-mediated recombination (left panel). Brightfield and GFP fluorescence images of ROSA26αGFP-DTA control and αMHC

Cre/+;ROSA26αGFP-DTA transgenic embryos (right panel) at E8.5, E9.5, and E10.5 of development. Note 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/+;ROSA26αGFP-DTA ESC lines. Brightfield and GFP fluorescence microscopy images are shown for the three ESC lines used. (C) Fractional CM ablation by injection of αMHC

Cre/+;ROSA26αGFP-DTA ESCs into wild-type CD-1 blastocysts (upper panel). The summary table demonstrates the complementation efficiency for each ESC line tested based upon the total number of animals collected (lower panel). (D) Brightfield and GFP fluorescence images of E10.5 chimeric αMHC

Cre/+;ROSA26αGFP-DTA embryos. Note that embryos with >50% ablation are developmentally arrested and exhibit impaired cardiac morphogenesis. A pericardial effusion is occasionally observed. (E) eGFP, troponin T (cTnT), and DAPI staining of E9.5 chimeric embryos with progressively increasing amounts of cardiomyocyte ablation. Some residual un-ablated eGFP+ cardiomyocytes remain in the hearts at this stage. Scale bars: 500 μm for (A); 200 μm for (B and E); 1 mm for (D).

**Figure 4.** Embryonic CM proliferation is involved in myocardial recovery. (A) Schematic
diagram of single-cell isolation and culture of E9.5 cardiac cells. For (B-J): experimental
variables are represented as follows: [group: # of mice examined, mean percent ablation ± SD].
For (B-F): [control: n=9; CPC ablation: n=6, 45 ± 11%; CM ablation: n=6, 50 ± 12%]. (B)
Percentage of single-cell derived colonies classified as cardiomyocytes, endocardial/endothelial
cells, or other cells from dissociated E9.5 embryo hearts. (C-E) Histological analysis of single-
cell derived colonies from E9.5 hearts. (C) Sarcomeric α-actinin⁺ cardiomyocytes showing well-
organized Z-lines and sarcomeric structure. (D) CD31⁺ endocardial/endothelial cells. (E) Non-
cardiomyocyte/non-endothelial 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 cardiomyocyte colonies. Whiskers span the 10-90% range of
values. Statistical comparisons were performed using the Kruskal-Wallis test with Dunn’s
correction for multiple comparisons. For (G-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 cardiomyocytes and endocardial 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) cardiomyocytes in control and ablated hearts (filled arrows) was
defined by co-localization with a DAPI⁺ nucleus that is circumscribed in all dimensions by a
troponin T⁺ (cTnT) cell. Similarly, the percentage of phospho-histone H3⁺ or Ki-67⁺ endocardial
cells (thin arrows) was defined by co-localization 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. 100-600 cTnT⁺ cells and 50-300 CD31⁺ cells were counted per section. Statistical
comparisons were performed using a likelihood ratio test on a linear mixed-effects model. NS, non-significant. Scale bars: 100 μm for (C-E); 50 μm for (G-J).

**Figure 5.** CPC and CM-ablated embryos can survive into adulthood with no overt cardiac defect. (A) Strategy used for generation and analysis of 4-5 week-old mice undergoing CPC or CM ablation. (B) Representative images of control and ablated chimeric adult mice. (C) Brightfield and GFP fluorescence images of the dissected whole hearts. GFP signal is nearly absent in the hearts that have undergone ablation. (D) H&E staining of representative heart sections from control and ablated hearts. For (E-K): experimental variables are represented as follows: [group: # of mice examined, mean percent ablation ± SD]. For (E-I): results are displayed using 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-I) Representative M-mode echocardiography images (F) and left ventricular dimensions (G and H) and function (I) quantified by fractional shortening [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 32X fields per section were counted; each symbol shape represents an independent mouse; a horizontal bar indicates the mean value for each group. [control: n=3; CPC ablation: n=3, 51 ± 6%; CM ablation: n=3, 51 ± 7%]. FS, fractional shortening; LVID_{Ed}, left ventricular internal diameter at end-diastole; LVID_{Es}, left ventricular internal diameter at end-systole; NS, non-significant. Scale bars: 2 mm for (C and D); 50 μm for (J).
Figure 1

A

ROSA26

SA\(\rightarrow\)eGFP PGK-Neo\(\rightarrow\)tpA\(\rightarrow\)DTA\(\rightarrow\)bpA

\(\times\) Nkx2.5

CRE PGK-Neo

ROSA26

SA\(\rightarrow\)DTA\(\rightarrow\)bpA

B

Wild-type CD-1

Wild-type Blastocyst

Chimeric Embryo

C

Nkx2.5\(^{\text{Cre/+}}\); R26\(^{\text{eGFP-DTA}}\) ES cells

D

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E

Percentage Ablation

0% 25% 50% 75% 100%

Pre-Ablation During Ablation Post-Ablation

F

% CPC Ablation

0% 12% 19% 26% 48% 56% 68% 94%

Brightfield

E10.5

GFP

ES-Derived eGFP+ Cell

Blastocyst-Derived eGFP- Cell

0% 25% 50% 75% 100%

ES-Derived eGFP+ Cell

Blastocyst-Derived eGFP- Cell
Figure 2

A

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Figure 3

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<th>Total Blastocysts Transferred</th>
<th>Total Animals Recovered</th>
<th>Degree of CM Ablation: # Animals (% of Total Recovered)</th>
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% CM Ablation

E10.5

0% CM Ablation

38% CM Ablation

48% CM Ablation

60% CM Ablation

93% CM Ablation
Figure 4

A

Control Embryo
CPC or CM Ablated Embryo

Trypsin
Heart
FACS
Single-Cell Isolation
96-Well Plate
Cell Counting / Immunostaining

Merge
7 or 14 Days

B

Cell Lineage (% of Total Cells)

Control
CPC Ablation
CM Ablation

CPC or CM
Ablated Embryo

FACS

C

Cardiomyocyte
D
Endothelial
E
Other

α-Actinin
CD31
α-Actinin

DAPI
Merge
DAPI
Merge
DAPI
Merge

F

Cell Lineage (% of Total Cells)

Control
CPC Ablation
CM Ablation

G

Control
CPC Ablation

CD31
pH3

pH3+ CM's (%)
pH3+ Endocardial Cells (%)

H

Control
CPC Ablation

CD31
Ki-67

Ki-67+ CM's (%)
Ki-67+ Endocardial Cells (%)

I

Control
CM Ablation

CD31
pH3

pH3+ CM's (%)
pH3+ Endocardial Cells (%)

J

Control
CM Ablation

CD31
Ki-67

Ki-67+ CM's (%)
Ki-67+ Endocardial Cells (%)

Cell Counting / Immunostaining

7 or 14 Days
Wild-type CD-1
αMHCCre/+;R26eGFP-DTA

Wild-type Blastocyst
Nkx2.5Cre/+;R26eGFP-DTA

Cardiac Progenitor Cell Ablation
Cardiomyocyte Ablation
Nkx2.5Cre/+;R26eGFP-DTA

Chimeric Mouse
αMHCCre/+;R26eGFP-DTA

Chimeric Mouse

Transthoracic Echocardiography
Heart Weight Ratios
Cardiac Histology

Figure 5

Mouse Genotype
Brightfield
GFP
H&E

Heart Weight / Body Weight (mg/g)
Heart Weight / Tibia Length (mg/mm)
CM size (pixels²)
FS (%)

CM Ablation
CPC Ablation
Control

WGA

LVIDd (mm)
LVIDs (mm)
FS (%)
CM size (pixels²)

Control
CPC Ablation
CM Ablation

Control
CPC Ablation
CM Ablation
The 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

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SUPPLEMENTAL MATERIAL

Fetal Mammalian Heart Generates a Robust Compensatory Response to Cell Loss

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3Division of Cardiology, Department of Medicine and 4Department of Pathology, Massachusetts General Hospital, Boston, MA, 02114, USA
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*Corresponding author
SUPPLEMENTAL METHODS

Nkx2.5-Cre and αMHC-Cre Genetic Fate Mapping

Nkx2.5<sup>Cre<sup>+/</sup>;</sup>Rosa26<sup>LacZ</sup> or αMHC<sup>Cre<sup>+/</sup>;</sup>Rosa26<sup>LacZ</sup> whole mouse E9.5 embryos were assessed for β-Galactosidase (lacZ) activity according to previously published protocol¹.

FACS Analysis of ESC Chimerism

For embryonic tissues, the heart, limbs, tail, head, or body were minced with a scalpel blade into 1-4 mm³ 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 FACSArria 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⁺/eGFP⁻ 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⁺ 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\textsuperscript{Cre+};ROSA26\textsuperscript{eGFP-DTA} or αMHC\textsuperscript{Cre+};ROSA26\textsuperscript{eGFP-DTA} ESCs and individually transferred to microcentrifuge tubes containing 50 µL Hank's Balanced Salt Solution (HBSS) with Ca\textsuperscript{++}/Mg\textsuperscript{++} (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,
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% CO$_2$ 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$^\circ$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 4º C 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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<th>Primer</th>
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<td>Cre</td>
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<td>CGTATAGCCGAAATTGCCAG</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>CAAAACAGGTAGTTATTCGG</td>
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<tr>
<td>Y chromosome</td>
<td>Forward</td>
<td>TTGTCTAGAGAGCATGGAGGGCCATGTCAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
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**Supplemental Table 2: Antibodies Utilized for Immunohistochemistry**

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<th>Dilution</th>
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<td>DSHB</td>
<td>PY489</td>
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<td>1:100</td>
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<td>PECAM-1 (CD31)</td>
<td>rat</td>
<td>Dianova</td>
<td>DIA-310 / SZ31</td>
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<td>cleaved caspase-3 (Asp175)</td>
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<td>rabbit</td>
<td>Santa Cruz</td>
<td>sc-9053 / H-112</td>
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<td>Abcam</td>
<td>ab13970</td>
<td>1:400</td>
</tr>
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<td>DSHB</td>
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<td>Abcam</td>
<td>ab92721 / EPR3741</td>
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<td>#9701</td>
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SUPPLEMENTAL REFERENCES

Figure S-I

A  Nkx2.5^{Cre/+}, R26^{LacZ}

B  Nkx2.5^{Cre/+}, R26^{LacZ}

C  Nkx2.5^{Cre/+}, R26^{LacZ}

D  Nkx2.5^{Cre/+}, R26^{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

- **R26eGFP-DTA**
- **Nkx2.5Cre/+**

- **Ablated Chimeric Embryo**
- **Control Chimeric Embryo**

### B

<table>
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<tr>
<th>ES Line</th>
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<th>eGFP+ Animals Recovered</th>
<th>eGFP+ Genotype</th>
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<td></td>
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<td>72</td>
</tr>
<tr>
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<td>127</td>
<td>71</td>
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<td>Total</td>
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<td>Nkx-Cre +</td>
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</table>

### C

**% CPC Ablation to Nkx2.5Cre/+;R26eGFP-DTA Embryos**

- Brightfield
- GFP

### D

- **% Wild-Type ES Chimerism (Surviving Embryos)**

### E

- **Surviving Embryos (% of Expected)**

### F

- **Control R26eGFP-DTA**
- **50-60% CPC Ablation Nkx2.5Cre/+;R26eGFP-DTA**

**Scenario**

- **R26eGFP-DTA Nkx2.5Cre/+**
- **Nkx2.5Cre/+, R26**
**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<sup>Cre/+</sup> mice are mated to ROSA26<sup>eGFP-DTA</sup> mice to generate eGFP<sup>+</sup> embryos carrying either ROSA26<sup>eGFP-DTA</sup> only (control) or Nkx2.5<sup>Cre/+;Nkx2.5<sup>Cre/+;ROSA26<sup>eGFP-DTA</sup> (ablated) alleles. Unlabeled wild-type ESCs are injected into eGFP<sup>+</sup> 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<sup>Cre/+;ROSA26<sup>eGFP-DTA</sup> embryos. Embryos with >60% ablation show developmental arrest.

**D**, Degree of wild-type ES chimerism found in recovered surviving embryos or mice with ROSA26<sup>eGFP-DTA</sup> or Nkx2.5<sup>Cre/+;ROSA26<sup>eGFP-DTA</sup> alleles.

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

**F**, eGFP, troponin T (cTnT), and DAPI staining of a control ROSA26<sup>eGFP-DTA</sup> and a chimeric Nkx2.5<sup>Cre/+;ROSA26<sup>eGFP-DTA</sup> embryo. Only rare eGFP<sup>+</sup> endocardial cells are apparent in the ablated heart. Scale bars: 1 mm for C; 200 µm for F.
Figure S-IV

A

B

C

D

E

Ablation Tolerance at Various Developmental Stages

Age of Surviving Animal

% CPC Ablation

% CM Ablation
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.
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<th>Figure S-V</th>
<th>Description</th>
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<tr>
<td><strong>A</strong></td>
<td>( \alpha \text{MHC}^{\text{Cre}^+/+} \times R26^{\text{LacZ}} ) E9.5</td>
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<tr>
<td><strong>B</strong></td>
<td>( \alpha \text{MHC}^{\text{Cre}^+/+} \times R26^{\text{LacZ}} ) CV E9.5</td>
</tr>
<tr>
<td><strong>C</strong></td>
<td>( \alpha \text{MHC}^{\text{Cre}^+/+} \times R26^{\text{LacZ}} ) CV ott E9.5</td>
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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

A

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<th>% CM Ablation</th>
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<th>DAPI</th>
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E9.5

B

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**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

B

NS

C

Actual # Cardiomyocytes / Well

Predicted # Cardiomyocytes / Well
(Based on Gaussian Distribution)
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 Nkx2.5<sup>Cre/+;ROSA26<sup>eGFP-DTA</sup></sup> ESCs (CPC ablation), and an embryo chimerized with αMHC<sup>Cre/+;ROSA26<sup>eGFP-DTA</sup></sup> 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  

Average

Median
**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

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초록

배경
심장 발생에 있어서 심장전구세포(cardiac progenitor cell)와 분화한 심장 세포에서의 특정 신호전달 체계들이 중요하며, 이러한 신호전달 체계에 이상이 있으면 심장 분화에 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.

방법 및 결과
본 연구에서는 생쥐의 배아에서 심장 전구세포 혹은 초기 분화 심장 세포를 소말시키기 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 전여 심근세포에 의해 거의 회복되었고, 성체 생쥐의 심장 기능에는 특별한 이상이 발견되지 않았다. 두 개의 결론 모델 모두에서 심장 세포의 증식이 증가함을 관찰하였고, 이는 일부분 심근 세포질 및 심장 크기의 회복에 의한 것으로 설명할 수 있을 것이다.

결론
본 연구는 포유류에서 배아기의 심장세포 소실의 한계점을 확인해주었으며, 심장세포가 소실되지라도 심근세포의 재생 능력을 통해 심장이 정상적으로 발달할 수 있음을 보여준다.