Chamber Specification During Direct Cardiac Reprogramming

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Chamber specification during direct cardiac reprogramming

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ABSTRACT

Forced expression of core cardiogenic transcription factors can directly reprogram fibroblasts to induced cardiomyocyte-like cells (iCMs) *in vitro* and *in vivo*. This cardiac reprogramming approach provides a proof of concept for induced heart regeneration by converting a fibroblast fate to a cardiomyocyte fate. However, it remains elusive whether chamber-specific cardiomyocytes can be generated by cardiac reprogramming. Therefore, we assessed the ability of the cardiac reprogramming approach for chamber specification *in vitro* and *in vivo*. We found that *in vivo* cardiac reprogramming post-myocardial infarction exclusively generates ventricular-like iCMs, while a major fraction of iCMs generated *in vitro* fail to determine their chamber identities. Our results indicate that *in vivo* cardiac reprogramming may have an inherent advantage of generating chamber-matched new cardiomyocytes as a potential heart regenerative approach.

INTRODUCTION

Heart disease is the leading cause of morbidity and mortality worldwide. The central problem of heart disease remains irreversible loss of cardiomyocytes replaced by cardiac fibrosis. Thus, the cardiac reprogramming approach is particularly attractive in that it directly targets a major source of cardiac fibrosis, cardiac fibroblasts, to induce new cardiomyocytes. It has been shown that forced expression of core cardiogenic transcription factors (i.e. Gata4, Mef2c, and Tbx5 with or without Hand2) is able to reprogram fibroblasts into induced cardiomyocyte-like cells (iCMs) *in vitro* and *in vivo*[1-3]. These initial studies provided an irrefutable evidence that new cardiomyocytes can be generated by fibroblast to cardiomyocyte fate conversion, proposing an entirely new potential heart regenerative strategy.

There are two different types of working cardiomyocytes in the heart (i.e. atrial and ventricular), each of which are anatomically confined to respective chambers with distinctive functional properties. Highly orchestrated contraction of these two independent types of

cardiomyocytes is essential for effective blood circulation. Therefore, a viable heart regenerative strategy should be able to generate chamber-matched working cardiomyocytes in their respective anatomical locations. For example, after myocardial infarction (MI), which mainly affects the ventricles, ventricular cardiomyocytes need to be regenerated in the affected ventricle(s). However, the ability of the cardiac reprogramming approach to generate a specific type of working cardiomyocytes has not been carefully determined.

In this study, we sought to determine chamber identities of the iCMs generated by either *in vitro* or *in vivo* cardiac reprogramming. Using multi-channel high content imaging, we simultaneously analyzed induction of ventricular and atrial phenotypes following *in vitro* cardiac reprogramming. We found that a significant fraction of pan-cardiac marker expressing cells express both atrial and ventricular markers *in vitro*. In contrast, *in vivo* cardiac reprogramming post-MI exclusively induced ventricular-like iCMs in the ventricle. Our results showed successful chamber specification of iCMs by *in vivo* cardiac reprogramming, in contrast to stochastic and non-exclusive atrial and ventricular specification of iCMs by *in vitro* cardiac reprogramming. These findings point to an important advantage of *in vivo* cardiac reprogramming as a potential heart regenerative approach.

RESULTS

Chamber specification during *in vitro* cardiac reprogramming

We previously observed heterogeneous population of iCMs, which resemble diverse individual subtypes of cardiomyocytes, following *in vitro* cardiac reprogramming[4]. However, it remains elusive whether atrial and ventricular specifications during cardiac reprogramming are mutually exclusive processes as shown during heart development or whether they are progressed simultaneously. Thus, we sought to determine chamber specification during *in vitro* cardiac reprogramming using multi-channel high content imaging analysis. We transduced mouse

embryonic fibroblasts (MEFs) with the quad-cistronic retroviral vector encoding four core cardiogenic transcription factors with the splicing order of *Mef2c*, *Gata4*, *Tbx5*, and *Hand2* (referred to as MGTH) as described previously[5]. Three weeks after transduction, we analyzed induction of a pan-cardiac Troponin I, a ventricular specific MLC-2v, and an atrial specific MLC-2a proteins (Figure 1). About 40% of cells expressed Troponin I, indicating that these cells adopt a cardiomyocyte fate. While ~26% of whole population expressed both MLC-2a and Troponin I (~65% of Troponin I^+ cells), ~16% of cells induced both MLC-2v and Troponin I (~39% of Troponin I^+ cells). We noted that atrial specification is more permissive than ventricular specification during *in vitro* cardiac reprogramming. Unexpectedly, ~14% of whole cellular population, which represents most MLC-$2v^+$Troponin I^+ cells, exhibited both atrial and ventricular markers (Figure 1C). A major fraction of a pan-cardiac marker expressing cells, which are often defined as iCMs, fail to specify their chamber identities during *in vitro* cardiac reprogramming. These results demonstrated that atrial and ventricular specifications of iCMs can be simultaneously progressed in a non-exclusive manner during *in vitro* cardiac reprogramming.

Chamber specification during *in vivo* cardiac reprogramming post-MI

Next, we examined chamber specification during *in vivo* cardiac reprogramming post-MI. To identify iCMs derived from cardiac fibroblasts, we used Tcf21-MerCreMer (iCre):R26R-tdTomato cardiac fibroblast lineage reporter mouse line which was generated by cross breeding Tcf21-MerCreMer knock-in mice[2,6] with Rosa26-CAG-LoxP-stop-LoxP-tdTomato mice. In this mouse line, cardiac fibroblasts become labeled with tdTomato upon tamoxifen administration[2]. We directly introduced retroviruses expressing MGTH or control vector into the infarcted left ventricle. At 3 weeks post-MI, we processed heart sections and immunostained them for pan-cardiac Troponin I and ventricular specific MLC-2v or atrial specific MLC-2a (Figure 2A-2C). We did not observe any tdTomato$^+$Troponin I^+ iCMs in the control vector injected hearts. However,

we found that MGTH introduction induces a significant number of tdTomato^{+}Troponin I^{+} iCMs in the border zone of the infarcted hearts, consistent with the previous studies[2,3,7-9] (Figure 2A). Nearly all tdTomato^{+}Troponin I^{+} iCMs expressed MLC-2v, while nearly no iCMs expressed MLC-2a (Figure 2B). These results demonstrated that ventricular-like iCMs are exclusively generated in the ventricle by *in vivo* cardiac reprogramming. In addition, we isolated cardiomyocytes using Langendorff perfusion following *in vivo* cardiac reprogramming. By immunostaining these isolated iCMs for MLC-2v and MLC-2a, we confirmed a ventricular phenotype as well as organized myofibrillar structures of iCMs in a single cell level (Figure 2D). Our findings suggest that the native intra-ventricular environment, in which iCMs are surrounded by continuously contracting native ventricular cardiomyocytes and other types of cells in three dimensions, may provide necessary conditions for ventricular specification which cannot be recapitulated in a plastic dish.

DISCUSSION

Either atrial or ventricular chamber identity of a cardiomyocyte is acquired in an anatomically defined restrictive location through chamber-specific independent processes during heart development[10]. In contrast, the previous study showed that diverse subtypes of cardiomyocytes (i.e. atrial, ventricular, and pacemaker) are co-induced next to each other by *in vitro* cardiac reprogramming[4]. However, it remains unclear whether atrial or ventricular chamber specification is progressed independently or simultaneously in individual iCMs during *in vitro* cardiac reprogramming. Through this study, we found that both atrial and ventricular chamber specifications can be simultaneously progressed in individual cells during *in vitro* cardiac reprogramming. As a result, a significant fraction of iCMs exhibit both atrial and ventricular phenotypes. However, such a hybrid cell does not exist in the post-natal heart. A very small fraction of iCMs showed a pure ventricular-like phenotype without co-expressing an atrial marker, indicating that induction of a ventricular chamber specification is a less efficient process

as opposed to induction of an atrial phenotype in fibroblasts. This is consistent with our previous study demonstrating inability of adult fibroblasts to induce a ventricular phenotype, while diverse cardiac phenotypes including ventricular can be induced in MEFs[4]. This uncontrolled chamber specification would be an important obstacle to overcome for potential *in vitro* clinical application of directly reprogrammed iCMs in the future. We speculate that successful ventricular chamber specification may require suppression of simultaneously developing atrial specification processes and vice versa.

In contrast to undetermined chamber specification during *in vitro* cardiac reprogramming, ventricular-like iCMs were exclusively induced in the ventricle following *in vivo* cardiac reprogramming post-MI. Ventricular specification of iCMs during *in vivo* cardiac reprogramming may be analogous to that of native cardiomyocytes in the ventricle during heart development. Our results suggest that intra-ventricular environment containing native ventricular cardiomyocytes and other ventricular cells may enforce iCMs to adopt a ventricular phenotype. Continuous and vigorous contraction of native ventricular cardiomyocytes as well as secreted molecules from various ventricular cells may be necessary for ventricular specification of iCMs. Despite unsuccessful chamber specification by in vitro cardiac reprogramming, it is important to note that the *in vivo* cardiac reprogramming approach may have an inherent advantage of generating chamber-matched cardiomyocytes over other heart regenerative approaches using pluripotent stem cell-derived cardiomyocytes which may have to overcome incomplete chamber specification of newly generated cardiomyocytes. In addition, it would be important to identify ventricular specific signaling molecules which specify chamber identities of iCMs in intra-ventricular environment during *in vivo* cardiac reprogramming for generation of clinically useful chamber-specific iCMs *in vitro*.

METHODS

Animals

All animal procedures performed in this study were approved by the Institutional Animal Care and Use Committee at Vanderbilt University Medical Center. All experimental procedures were performed in compliance with NIH and ARRIVE guidelines.

In vitro cardiac reprogramming

Retroviruses were generated using the quad-cistronic retroviral vector encoding mouse *Mef2c*, *Gata4*, *Tbx5* and *Hand2* as described previously[5,11]. Briefly, Platinum E cells (Cell Biolabs) were transfected with pBabe-X-MGTH construct using Fugene 6 (Promega). The growth medium (DMEM with 10% FBS and 1% penicillin/streptomycin) on transfected Platinum E cells was changed with the fresh growth medium 16-20 hours after transfection. The medium containing viruses was filtered through a 0.45 µm polyethersulfone (PES) filter. Polybrene was added to the viral medium at a concentration of 6 µg/ml. MEFs were isolated, counted and then frozen as described previously[5,12]. After thawing, pre-counted MEFs (5 - 7 x 10^4 cells/ a well) were seeded into a 24 well black clear bottom plate (Greiner, cat# 662892) 16-20 hours prior to infection. Following removal of fibroblast growth medium, the viral medium was transferred into the cell culture plate containing MEFs. Another transfection was independently performed for generating the second viral medium 24 hours after the first transfection. The second viral medium replaced the first viral medium in the culture plate containing MEFs 24 hours after first infection. Twenty-four hours after the second infection, the viral medium was removed from the culture plate containing MEFs, and then replaced with the cardiac induction medium, composed of DMEM/199 (4:1), 10% FBS, 5% horse serum, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% essential amino acids, 1% B-27, 1% insulin-selenium-transferrin, 1% vitamin mixture, 1% sodium pyruvate (Invitrogen), 1 µM SB431542 (Sigma), and 0.5 µm A83-01 (Tocris) as described previously[5,12]. The induction medium was changed every 3 days until cells were fixed for immunocytochemistry.

Immunocytochemistry

Immunocytochemistry was directly performed on a 24 well plate as described previously[5,11,12]. After fixation with 2% paraformaldehyde for 15 minutes, cells were permeabilization with permeabilization buffer (0.05% Triton-X in PBS) for 5 minutes three times at room temperature. After incubating with blocking buffer (Universal blocking buffer, BiogeneX, cat# HK083-50K) for ~45 min at room temperature, cells were incubated with primary antibodies against Troponin I (goat polyclonal, Abcam, cat# 188877, 1:400 dilution), MLC-2v (rabbit polyclonal, proteintech, cat# 10906-1-AP, 1:200 dilution), and MLC-2a (mouse monoclonal, Synaptic Systems cat# 311 011, 1:400 dilution) overnight at 4 ºC. Following three times of 5-minute wash with permeabilization buffer, cells were incubated with respective Alexa fluorogenic secondary antibodies (Invitrogen) at 1:400 dilution at room temperature for 1 hour. Cells were washed again with permeabilization buffer for 5 minutes three times. During the third wash, DAPI solution at 300 nM (Invitrogen) was added at 1:100 concentration.

High content imaging and analysis

High content imaging analysis was performed as described previously[5,11,12]. Briefly, the Immunostained cells were analyzed with ImageXpress Micro XL Automated Cell Imaging system (Molecular Device). Cell images were acquired with a 10X objective at 36 fields per well. DAPI, FITC, Texas Red, Cy5 filter sets were used to detect DAPI, Troponin I, MLC-2v, and MLC-2a, respectively. Thirty-six captured images per each 24 well were analyzed with MetaXpress software (Molecular Device) to quantify the number of Texas Red, FITC and/or Cy5 fluorescent positive cells among DAPI positive cells.

***In vivo* cardiac reprogramming**

For *in vivo* reprogramming, retroviruses expressing MGTH were generated as described above for *in vitro* cardiac reprogramming without changing growth media 16-20 hours post- transfection.

The viral medium was collected and filtered through a 0.45 µm polyethersulfone (PES) filter 48 hours post-transfection. The viral medium was concentrated with retrovirus precipitation reagent (Alstem) as per manufacturer's protocol. The concentrated retroviruses were stored at 4 ºC and used within two days. Tamoxifen dissolved in corn oil (90%) and ethanol (10%) at concentration of 50mg/ml was administered to 10-14 week old Tcf21-iCre:R26R-tdTomato mice via intraperitoneal injection for 5 consecutive days. Five days after the last dose of tamoxifen, permanent ligation of the left anterior descending coronary artery (LAD) was performed in Tcf21-iCre: R26R-tdTomato mice to induce MI as described previously[13]. The mice were anesthetized with continuous inhalation of isoflurane without ventilation. A minimal size of left thoracotomy over the left chest was performed, and the heart was temporarily displaced. The ligation was made at ~3 mm below the origin of the LAD using 6.0 silk. Immediately after LAD ligation, 30 µL of concentrated virus solution was directly injected to the infarcted left ventricle using gastight syringe (Hamilton, cat# 7637-01) and 34-gauge needle (Hamilton, cat# 207434). After confirming the anterior wall of the left ventricle become pale by intramyocardial infiltration of virus solution, the heart was replaced immediately into the thoracic cavity. After manual evacuation of air, the chest was closed.

Immunohistochemistry

After euthanizing the mice by CO_2 inhalation and cervical dislocation 3 weeks post-reprogramming, the frozen heart sections were processed. After washing with ice-cold PBS, the dissected hearts were fixed with pre-chilled 4% paraformaldehyde in PBS for 30 minutes, and then incubated in 30% sucrose in PBS overnight at 4ºC. The fixed hearts were embedded in O.C.T compound and frozen in pre-chilled isopentane. The frozen hearts were sectioned at 8 µm thickness. After 5 minute air dry, the heart sections were washed with PBS three times, fixed again with pre-chilled 4% paraformaldehyde in PBS on ice for 20 minutes, followed by PBS washing three times. The fixed heart sections were permeabilized in 0.1% Triton X-100 in PBS

for 20 minutes. Following PBS washing three times, the heart sections were blocked with M.O.M mouse IgG blocking reagent (Vector Labs) for 1 hour and 5% goat serum in M.O.M protein diluent (Vector Labs) for 30 minutes at room temperature. The heart sections were incubated with primary antibodies against Troponin I (goat polyclonal, Abcam, cat# ab56357, 1:400 dilution) and MLC-2v (rabbit polyclonal, proteintech, cat# 10906-1-AP, 1:200 dilution), or MLC-2a (rabbit polyclonal, Proteintech cat# 17283-1-AP, 1:200 dilution) overnight at 4ºC. After washing with PBS three times, the heart sections were incubated with respective Alexa fluorogenic secondary antibodies (Invitrogen) at 1:400 dilution at room temperature for 1 hour. tdTomato was visualized without immunostaining. Images were captured using Olympus IX81 epifluorescent microscope.

Isolation of cardiomyocytes

Adult mouse cardiomyocytes were isolated from Tcf21-iCre:R26R-tdTomato mice 4 weeks post-reprogramming using Langendorff perfusion as described previously[14]. The mouse hearts were perfused retrogradely via aortic cannulation in a Langendorff apparatus with three types of buffer solutions: 1) perfusion buffer (NaCl 120.4 mM, KCl 14.7 mM, Na_2HPO_4 0.6 mM, Ka_2HPO_4 0.6 mM, MgSO4 1.2 mM, Na-HEPES 10 mM, $NaHCO_3$ 4.6 mM, Taurine 30 mM, BDM 10 mM, Glucose 5.5 mM, pH 7.0), 2) digestion buffer without $CaCl_2$ (Collagenase II 2.4 mg/mL in perfusion buffer), and 3) digestion buffer with $CaCl_2$ (Collagenase II 2.4 mg/mL, and $CaCl_2$ 40 µM in perfusion buffer)[14]. After removing the hearts from the Langendorff apparatus, ventricular cardiomyocytes were mechanically dissociated and triturated using a fine scalpel and scissors and resuspended in stopping buffer ($CaCl_2$ 11.7 µM in calf serum 2 ml plus perfusion buffer 18 ml). After centrifuging cells at low speed, the pellet was collected for cardiomyocytes. The isolated cardiomyocytes were fixed with fixation buffer (BD Bisoscience, cat# 554655) for 20 minutes on ice followed by washing with Perm/Wash buffer (BD Bisoscience, cat# 554723) once. The fixed cells were incubated with primary antibodies against MLC-2v (rabbit polyclonal, proteintech, cat# 10906-1-AP, 1:200 dilution) and MLC-2a (mouse monoclonal, Synaptic Systems cat# 311 011, 1:400

dilution) overnight at 4 ºC. Following washing with Perm/Wash buffer, cells were incubated with respective Alexa fluorogenic secondary antibodies (Invitrogen) at 1:400 dilution at room temperature for 1 hour. After Perm/Wash buffer wash, cells were re-suspended with stain buffer (BD Bisoscience, cat# 554656) containing DAPI solution. The immunostained cardiomyocytes were placed on a glass slide and covered by a glass coverslip. Cardiomyocyte images were captured with Zeiss LSM 500 confocal microscope

Statistical Analyses

Statistical significance was determined using unpaired two-tailed Student's t-test. P-values of < 0.05 were regarded as significant.

ACKNOWLEDGEMENT

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AUTHOR CONTRIBUTIONS

Z.Z. and Y.-J.N. designed research. Z.Z., J.V., and W.Z. performed the experiments and analyzed data. Z.Z. and Y.-J.N. wrote the manuscript. All authors reviewed the manuscript.

CONFLICT OF INTEREST

The authors declare no competing interests.

DATA AVAILABILITY

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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FIGURES AND FIGURE LEGENDS

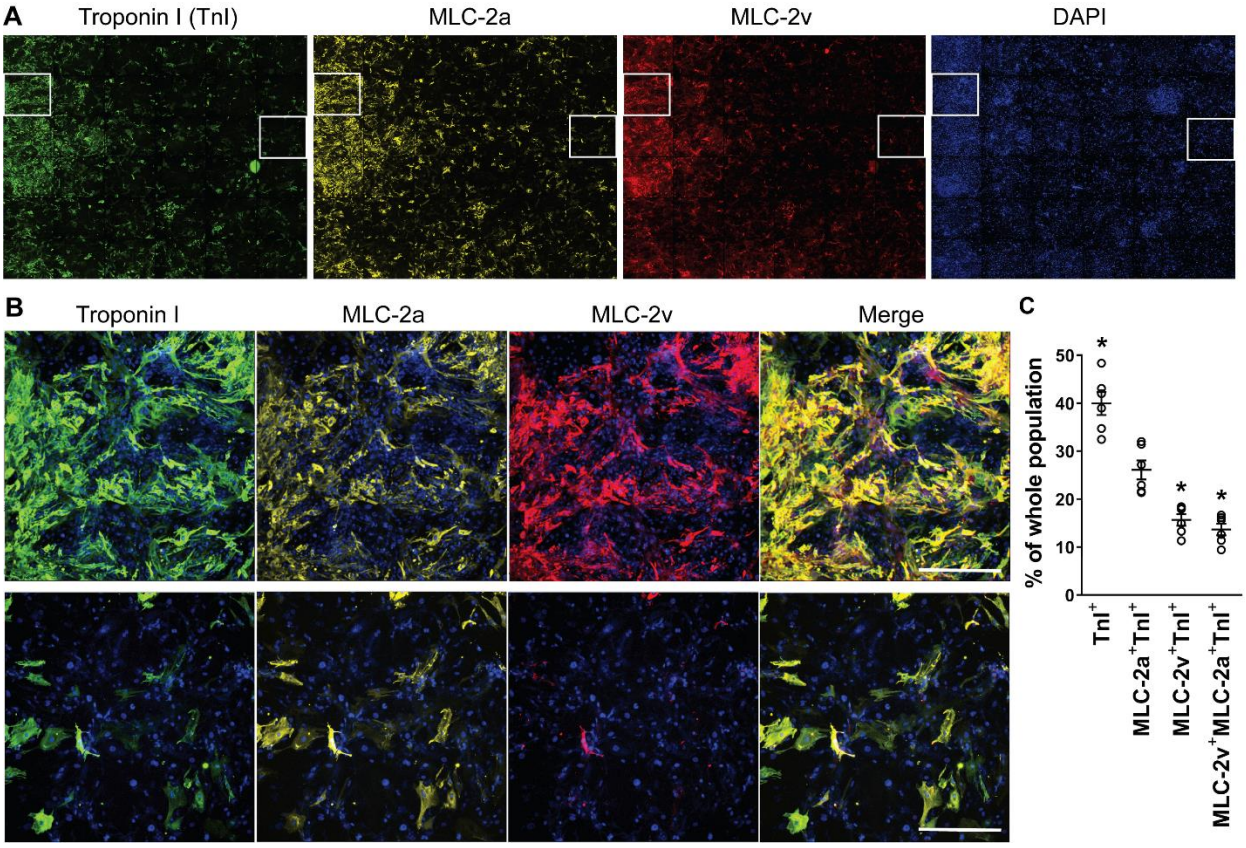
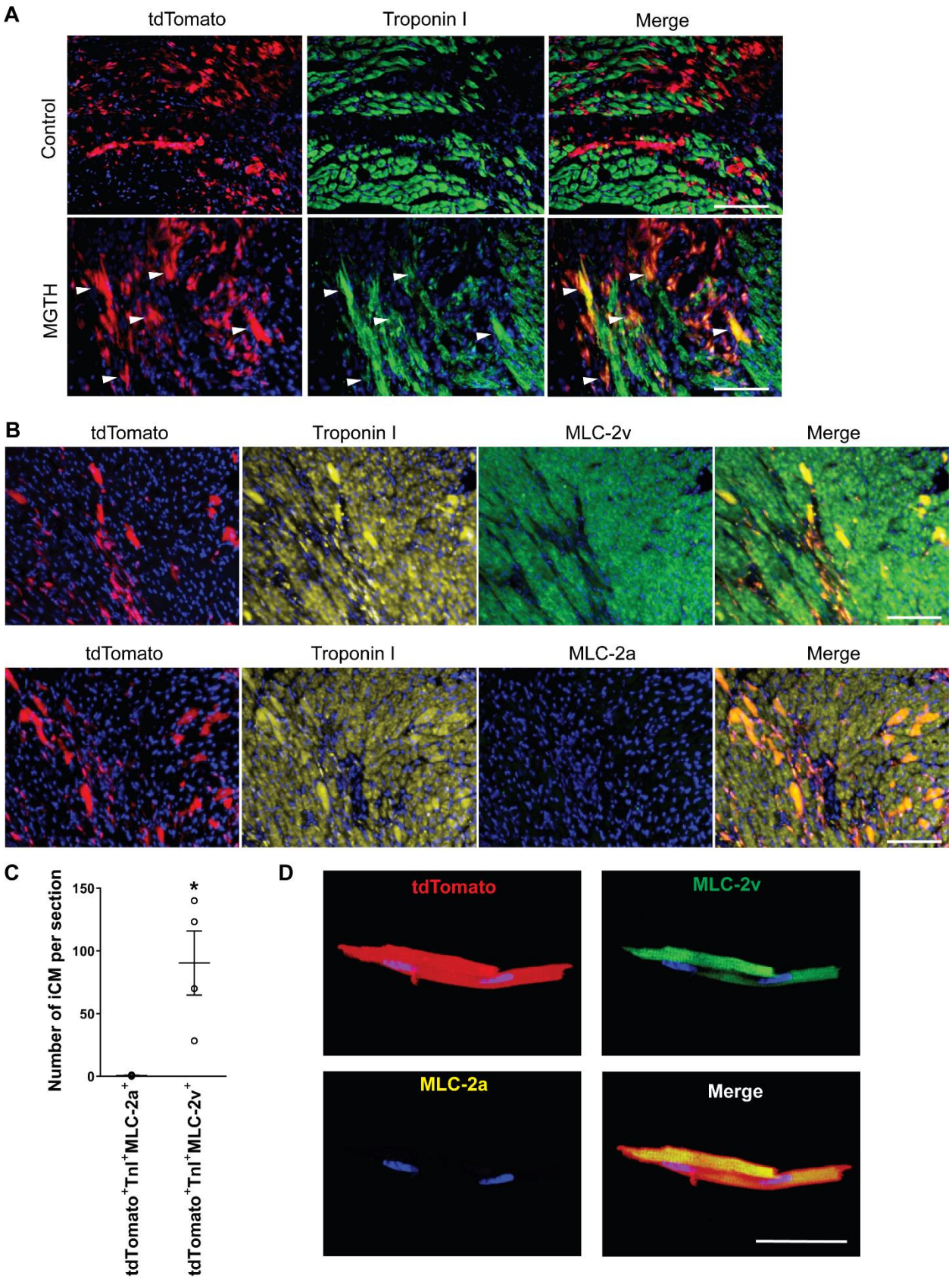


Figure 1. Incomplete chamber specification during *in vitro* cardiac reprogramming. (A) Composite immunofluorescent images used for high content imaging analyses to quantify Troponin I, MLC-2a, and MLC-2v induction 3 weeks after MGTH transduction into MEFs. Each panel shows a composition of 36 images taken by the high content imaging system using a 10X objective. (B) An enlarged single image of a white inlet in (A) (left inlet: top; right inlet: bottom). Scale bar, 400 μM. (C) Summary of high content imaging analyses. n=6. **P*<0.01 versus MLC-2a^{+}TnI^{+}.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Merge
C
Number of iCM per section
150
100
50
0
*
tdTomato+TnI+MLC-2a+
tdTomato+TnI+MLC-2v+
D
tdTomato
MLC-2v
MLC-2a
Merge

Figure 2. Exclusive induction of ventricular-like iCMs following *in vivo* cardiac reprogramming post-MI. (A) Lineage tracing of iCMs using Tcf21-iCre:R26R-tdTomato mice following MGTH or control vector injection post-MI. Heart sections were immunostained for Troponin I. White arrows indicate tdTomato$^+$Troponin I$^+$ iCMs. Scale bar, 100 μM. (B) Heart sections as described in (A) were immunostained for Troponin I and MLC-2v or MLC-2a. tdTomato$^+$Troponin I$^+$MLC-2v$^+$ cells indicate ventricular-like iCMs. Scale bar, 100 μM. (C) Quantification of ventricular-like iCMs generated by *in vivo* cardiac reprogramming. The average number of iCMs from 6 heart sections per heart is presented. TnI: Troponin I. n=4. *P<0.05. (D) Isolated iCMs exhibiting a ventricular phenotype. Scale bar, 50 μM.

Figures

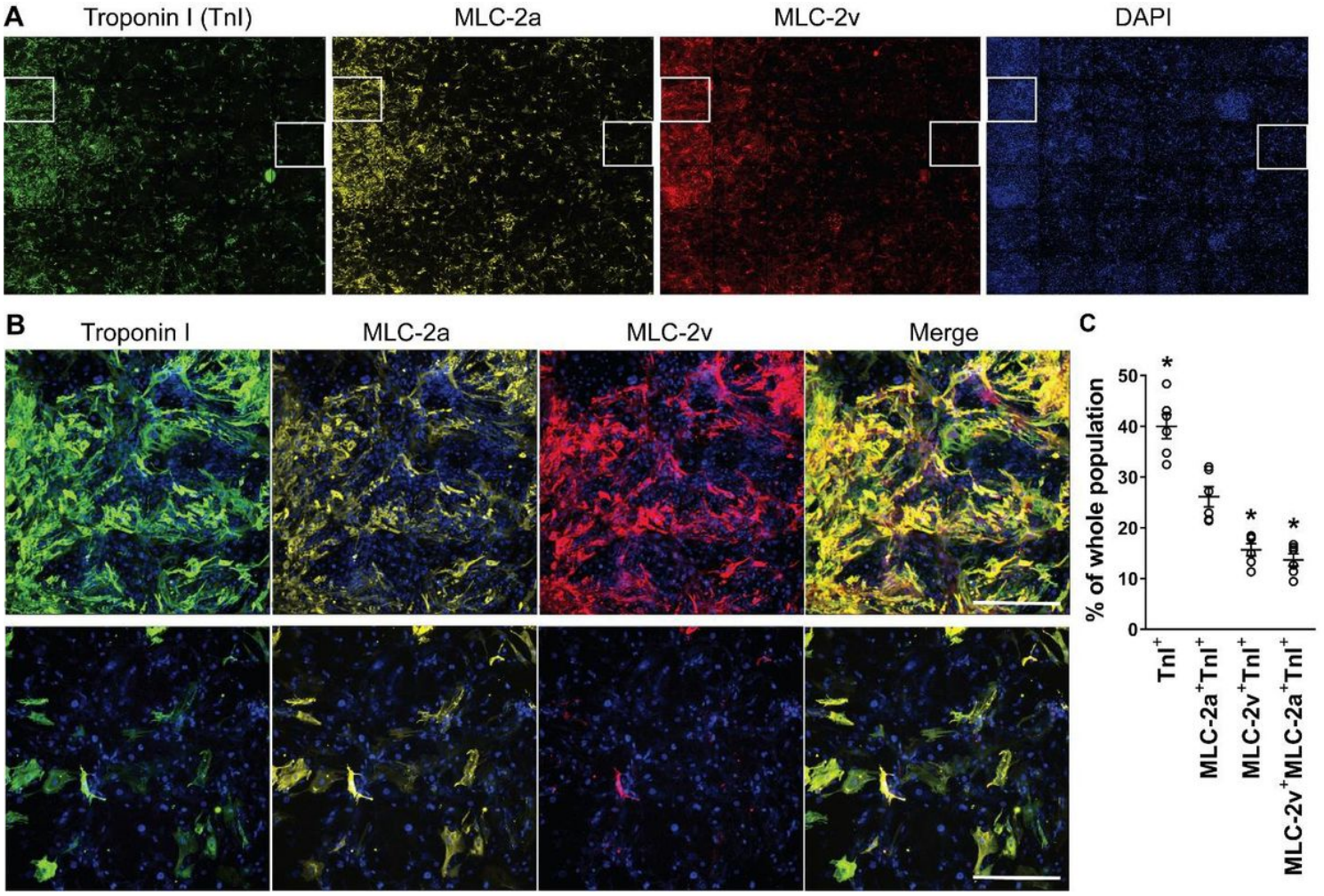

Figure 1

Incomplete chamber specification during in vitro cardiac reprogramming. (A) Composite immunofluorescent images used for high content imaging analyses to quantify Troponin I, MLC-2a, and MLC-2v induction 3 weeks after MGTH transduction into MEFs. Each panel shows a composition of 36 images taken by the high content imaging system using a 10X objective. (B) An enlarged single image of a white inlet in (A) (left inlet: top; right inlet: bottom). Scale bar, 400 μM. (C) Summary of high content imaging analyses. n=6. *P<0.01 versus MLC-2a+TnI+.

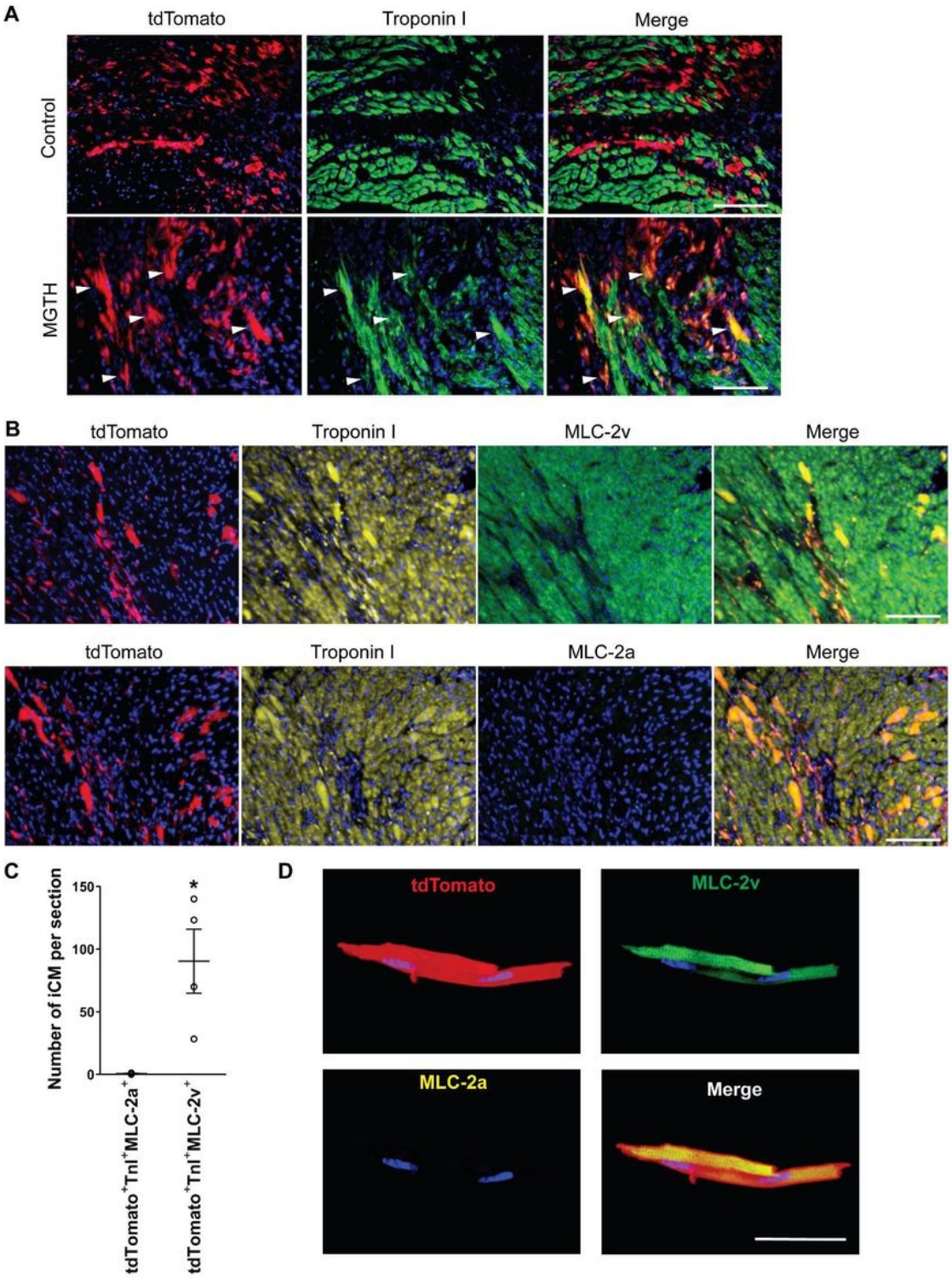

Figure 2

Exclusive induction of ventricular-like iCMs following in vivo cardiac reprogramming post-MI. (A) Lineage tracing of iCMs using Tcf21-iCre:R26R-tdTomato mice following MGTH or control vector injection post-MI. Heart sections were immunostained for Troponin I. White arrows indicate tdTomato+Troponin I+ iCMs. Scale bar, 100 μM. (B) Heart sections as described in (A) were immunostained for Troponin I and MLC-2v or MLC-2a. tdTomato+Troponin I+MLC-2v+ cells indicate ventricular-like iCMs. Scale bar, 100 μM.

(C) Quantification of ventricular-like iCMs generated by in vivo cardiac reprogramming. The average number of iCMs from 6 heart sections per heart is presented. TnI: Troponin I. n=4. *P<0.05. (D) Isolated iCMs exhibiting a ventricular phenotype. Scale bar, 50 μM.