Circulating Cells Contribute to Cardiomyocyte Regeneration After Injury

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Running title: Circulating Cells Contribute to Myocardial Repair

Subject codes:
[4] Acute myocardial infarction
[137] Cell biology/structural biology
[151] Ischemic biology-basic studies

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In October, 2014, the average time from submission to first decision for all original research papers submitted to Circulation Research was 16 days.

DOI: 10.1161/CIRCRESAHA.116.304564
ABSTRACT

Rationale: The contribution of bone marrow-borne hematopoietic cells to the ischemic myocardium has been documented. However, a pivotal study reported no evidence of myocardial regeneration from hematopoietic-derived cells. The study did not take into account the possible effect of early injury-induced signaling as the test mice were parabiotically paired to partners immediately after surgery-induced myocardial injury when cross circulation has not yet developed.

Objective: To re-evaluate the role of circulating cells in the injured myocardium.

Methods and Results: By combining pulse-chase labeling and parabiosis model, we show that circulating cells derived from the parabiont expressed cardiac-specific markers in the injured myocardium. Genetic fate-mapping also revealed that circulating hematopoietic cells acquired cardiac cell fate by means of cell fusion and transdifferentiation.

Conclusions: These results suggest that circulating cells participate in cardiomyocyte regeneration in a mouse model of parabiosis when the circulatory system is fully developed before surgery-induced heart injury.

Keywords:
Parabiosis, circulating cells, fusion, transdifferentiation, circulation, stem cell, regeneration

Nonstandard Abbreviation and Acronyms:
WT Wild type
GFP green fluorescent protein
MI myocardial infarction
MCM MerCreMer
cTnT cardiac troponin T
SM22α smooth muscle 22α
β-Gal β-Galactosidase
TAM tamoxifen
INTRODUCTION

The mammalian heart is a terminally differentiated organ with very limited regenerative capacity to repopulate lost cardiac myocytes following injury. Over the past decade, numerous studies have provided evidence that mammalian heart contains different populations of resident cardiac progenitors or stem cells. These cells possess the ability to differentiate into cardiac myocytes and also give rise to other cardiac cell types such as vascular smooth muscle cells and endothelial cells. Furthermore, the heart may not be the only organ where cardiac progenitors or stem cells can be found. Several lines of evidence suggest that bone marrow serves as a reservoir for stem or progenitor cells which are responsive to myocardial injury and home to the site of damage via circulation. However, the ability of bone marrow- or circulation-borne cells to adopt cardiac cell fate remains controversial. Results from bone marrow transplantation studies have consistently shown that genetically labelled donor cells could engraft into the damaged myocardium of the recipient and express mature cardiomyocyte markers. Nevertheless, parabiosis animal models used to study the contribution of bone marrow-borne cells in the injured heart have yielded various results.

Bone marrow-borne stem cells can egress from the bone marrow and mobilize into circulation in response to external cues. Because of this unique property, parabiosis that allows a shared circulatory system to be established in two surgically conjoined animals is therefore suitable to assess the contribution of bone marrow-borne cells to tissue repair under normal physiological conditions. Indeed, parabiosis has been used to characterize blood or bone marrow-borne circulating cells during homeostasis and in response to tissue injuries, including myocardial injury. Results from parabiosis study have challenged the notion that bone marrow-derived circulating cells could adopt cardiac phenotype after injury. After left anterior descending artery ligation wild type (WT) mice were immediately joined by parabiosis to green fluorescent protein (GFP) transgenic mice to investigate the contribution of circulating bone marrow cells to myocardial regeneration. In this report, the circulating GFP+ cells did not express cardiac markers but only gave rise to the cells of hematopoietic lineage in the injured heart. Unlike bone marrow transplantation, parabiosis requires additional time for the cross circulatory system to be developed in the animals. As injury-induced signals are important modulators of stem cell activities and are only transiently elevated at the early stage of injury, we hypothesized that contribution of bone marrow-borne circulating cells to cardiac repair may only be observed when cross circulation has already been established at the time of injury. To test this hypothesis, in this study, the fate of circulating bone marrow cells in the injured myocardium was re-evaluated by subjecting parabiotic mice to surgery-induced myocardial infarction (MI) only after peripheral circulation had been stabilized.
METHODS
All experiments involving animals were conducted in accordance with the Guide for the Use and Care of Laboratory Animals, and all of the animal protocols have been approved by the Institutional Animal Care and Use Committee at National Cheng Kung University.

Animals.
The $\alpha$-MHC promoter driven-MerCreMer (MCM) and Z/EG transgenic mice in the C57BL/6 background were purchased from the Jackson Laboratory. For ease of tail vein injection, FVB mice were chosen for the microbubble experiments conducted in this study. FVB, C57BL/6J-Tg(Pgk1-EGFP) transgenic mice and WT mice were purchased from the National Laboratory Animal Center, Taiwan. Animals of 12-16 weeks of age were used for the experiments conducted in this study.

In vivo contrast-enhanced microbubble imaging.
MicroMarker Contrast Agent microbubbles (VisualSonics) were resuspended in normal saline according to the manufacturer’s protocol. At each time point, a 100 $\mu$l bolus containing $2 \times 10^8$ microbubbles was systemically delivered by intravenous injection into one parabiotic mouse, the donor. The heart of the parabiont was viewed in a contrast mode, and the contrast signal intensity reflecting the flow of microbubbles was analyzed by appropriate VisualSonics software validated for the Vevo 2100 system. Prior to microbubble injection in the donor mouse, the background noise was recorded. Soon after injection, the microbubble contrast signal in the donor parabiont was detected and recorded. The probe was then moved to the recipient parabiont and the background noise was obtained before the microbubble contrast signal was detected. Three minutes post-injection, contrast signal intensity in the recipient heart was recorded. Changes in microbubble contrast signal intensity in the heart of the recipient parabiont at each time point was calculated by dividing the contrast signal intensity value by its corresponding baseline value.

Parabiosis and myocardial injury model.
The mice were anesthetized by intraperitoneal injection with Zoletil (25 mg/Kg containing 0.2% Rompun). The mice were then shaved and unilateral flank skin incision from elbow to the knee joints was created. The skin edge of mice was sutured with 5.0 prolene (Ethicon). One month post-parabiotic surgery, experimental myocardial infarction (MI) was induced in WT or MCM parabionts. The mice were anesthetized by isoﬂurane inhalation (2-5% isoﬂurane in oxygen) and subjected to artificial ventilation through endotracheal cannulation. The left anterior descending coronary artery at 2-3 mm distal to the left atrial appendage was ligated to induce infarction. The MCM mice subjected to tamoxifen (TAM, Sigma-Aldrich) injection were separated from their partners at 2 weeks post-MI. An incision was created at the conjoined surgical site and the edge of skin wound was then sutured with 5.0 prolene. To induce Cre-mediated recombination, the mice were injected intraperitoneally with of TAM for 14 days at a dosage of
40 μg per 1 g body weight. For short-term treatment to induce Cre nuclear translocation, the mice were injected with the same dosage of TAM twice daily for 3 days.

**Adult cardiomyocyte isolation.**

Adult ventricular cardiac myocytes were isolated from MCM parabionts on a Langendorff apparatus. Ten minutes after heparinization, hearts were removed from the anaesthetized mice. The hearts were cannulated for retrograde perfusion with Ca²⁺-free Tyrode solution (NaCl 120.4 mM, KCl 14.7 mM, KH₂PO₄ 0.6 mM, Na₂HPO₄ 0.6 mM, MgSO₄ 1.2 mM, HEPES 1.2 mM, NaHCO₃ 4.6 mM, Taurine 30 mM, BDM 10 mM, Glucose 5.5 mM). Following perfusion for 3 minutes, the hearts were digested by the Ca²⁺-free Tyrode solution supplemented with collagenase B (0.4 mg/g body weight; Roche), collagenase D (0.3 mg/g body weight; Roche) and protease type XIV (0.05 mg/g body weight; Sigma-Aldrich). After digestion was completed, the ventricles were cut from the cannula and teased into small pieces in the digestion solution neutralized by the Ca²⁺-free Tyrode solution containing 10% FBS. Individual cardiac myocytes were dissociated from the digested tissues by gentle pipetting and undigested tissues were removed by filtering through a nylon mesh of 250 μm pore size.

**Immunohistochemical staining.**

For immunohistochemical analyses, only the ischemic region below the site of ligation was excised. Harvested heart tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Immunostaining was performed on 5-μm tissue sections with the following primary antibodies at 4°C overnight: rabbit anti-GFP (1:200; GeneTex), mouse anti-Cre (1:200; Covance), mouse anti-cTnT (1:200; DSHB), mouse anti-sarcomeric α actinin (1:100; Sigma-Aldrich), rat anti CD45 (1:50; BD Biosciences), rabbit anti-SM22α (1:200; abcam) and isolectin-488 (Invitrogen). The appropriate secondary antibodies (Invitrogen) were used for visualization under a fluorescence microscope. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, 1 μg/ml; Sigma-Aldrich). For DAB immunostaining of GFP (1:200; GeneTex) and β-Gal (1:500; Invitrogen), a DAB substrate kit (DAKO) was used.

**Immunocytochemistry and X-Gal staining.**

Following isolation of ventricular cardiac myocytes, the cells were fixed in 2% paraformaldehyde for 30 minutes at room temperature. The cells were then permeabilized with 0.2% Triton X-100 in PBS for 15 minutes followed by blocking in 5% goat serum in PBS for 1 hour. The cells were immunostained with the following primary antibodies: rabbit anti-cTnI (1:200; abcam), mouse anti-Cre (1:100; Covance) and rabbit anti-GFP (1:200; GeneTex). Appropriate secondary antibodies (Invitrogen) were used for visualization. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, 1 μg/ml; Sigma-Aldrich).
X-Gal staining of adult cardiac myocytes was carried out by incubating the fixed cells at 37°C overnight in a staining solution containing 0.01% sodium deoxycholate, 0.02% NP-40, 1 mM MgCl₂, 2 mM K₂Fe(CN)₆, 2 mM K₄Fe(CN)₆, 1 mg/ml X-Gal. All chemicals were purchased from Sigma-Aldrich.

**Cardiomyocyte quantification.**

To quantify cells from tissue sections, cell number was determined by quantifying 5-6 sections, spaced at 20-25 μm intervals, from each heart and 3 images were taken at a magnification of 200× for each section. To quantify dissociated cardiac myocytes, total number of cells was calculated with a hemocytometer. For analysis, 1 × 10⁵ cells were mounted onto to each slide for image acquisition. The quantification results are presented as the percentage of labelled cells calculated per total number of cardiac myocytes dissociated from the heart.

**Flow cytometric analyses.**

The peripheral blood of the WT parabiont was collected from the retro-orbital sinus and the red blood cells were lysed by ACK lysing buffer (Lonza). Cardiomyocyte-depleted cardiac small cells were isolated by digesting the minced heart with 0.1% collagenase B (Roche), 2.4 U/ml dispase II (Roche), and 2.5 mmol/L CaCl₂ at 37°C for 30 minutes. Undigested tissues were removed by filtering through a 40 μm filter. The cells were then stained with fluorochrome-conjugated antibodies against CD45, CD3, B220 and F4/80 (BD Biosciences) on ice for 30 minutes. Flow cytometry was performed using FACSCanto II (BD) and FACSDiva software (BD) and FlowJo was used for data analysis.

**RESULTS**

*Cross circulatory system is stabilized 7 to 10 days after parabiotic surgery.*

To examine when blood circulation was developed after parabiosis, the whole body of a GFP transgenic mouse was surgically joined to a WT mouse. Peripheral blood chimerism in the WT parabiont was then analyzed at different time points after surgery. Flow cytometric analysis revealed that the majority of GFP⁺ cells engrafted in the WT parabiont co-expressed the pan-hematopoietic cell marker CD45 (Figure 1A), suggesting they are of hematopoietic lineage. We observed that a cross circulation was established at day 3 and the blood chimerism was stabilized at 7-10 days after parabiosis (Figure 1A and Online Table I). To assess whether cross circulation between parabionts affected the flow of systemic factors in the vascular network of the heart, we also employed an in vivo imaging system using contrast-enhanced ultrasound agent microbubbles. The size of microbubbles used was 2.3-2.9 μm; therefore, they were able to travel freely through the circulation in the body, even the microvasculature. Following parabiotic surgery...
microbubbles were systemically injected into the donor parabiont at different time points (Figure 1B). Soon after the injection, strong microbubble contrast signals could be readily detected in the heart of the donor parabiont, suggesting the microbubbles had entered the circulatory system (Figure 1C). Upon establishment of vascular communication, microbubbles in the donor parabiont circulated to the recipient partner and their signal intensity could be examined. At day 3 post-parabiotic surgery, we detected a relatively weak contrast signal in the heart of the recipient mouse. However, the contrast signal of circulating microbubbles increased dramatically at day 5 and leveled off at day 7 after parabiotic joining (Figure 1C). Taken together, flow cytometric analysis and microbubble contrast intensity evaluation indicate that the cross circulatory system requires at least 7-10 days to stabilize in parabionts.

Early injury-induced signaling is critical for tissue repair; however, these signals are only transiently elevated upon injury. Consistent with a previous finding, we found that the shared circulatory system requires 7-10 days to be stabilized. The additional time required for a single shared circulation to be developed represents a major difference between a bone marrow transplantation model and a parabiosis model. Such a difference may contribute to the discrepancy in the potential of bone marrow/hematopoietic-derived cells to repair the myocardium observed in these two animal models. We speculated that the establishment of a common circulation at the time of heart injury is required for bone marrow-borne circulating cells to contribute to the injured myocardium.

Circulating cells integrated into microvasculature and acquired mature hematopoietic cell fates in injured heart.

Following stabilization of cross circulation, we examined the cell fates of circulating cells in the injured myocardium. To trace the fate of the circulating cells, a WT mouse was joined to a GFP transgenic mouse and MI was induced by surgery after stabilization of cross circulation at 1 month post-parabiotic surgery. Because cardiomyocyte replenishment is already saturated on day 10 post-MI, the injured heart was harvested 2 weeks after myocardial injury for further analyses (Figure 2A). The immunostaining result showed that the GFP− cells were positive for isolecitin and smooth muscle 22α (SM22α), which are endothelial and smooth muscle cell markers, respectively (Figure 2B), suggesting the circulating cells participate in micro-vasculature reconstitution. Although cells co-expressing both GFP and smooth muscle cell marker could be found, the number of these cells was relatively low. The majority of circulating cells remained negative for smooth muscle cell marker but positive for hematopoietic cell marker CD45 (Online Figure I). To examine what hematopoietic lineage the circulating GFP+ cells had acquired, cardiomyocyte-depleted small cells isolated from the injured heart of the WT mouse were stained with mature hematopoietic cell markers CD3, B220 and F4/80 for identification of T cells, B cells and macrophages, respectively (Figure 2A). Flow cytometric analyses indicated that the majority of circulation-derived
GFP+/CD45+ cells had adopted hematopoietic cell fates, of which 21.25±0.05% of cells were T cells while 39.25±2.35% and 29.9±1.90% of circulating cells had committed to B cell and macrophage lineages, respectively (Figure 2C and 2D).

Circulation-derived cardiac myocytes integrated into injured myocardium.

The observation that circulating cells participate in microvasculature reconstitution in the injured myocardium prompted us to test whether these cells also contribute to cardiomyocyte regeneration. To examine this possibility, the MerCreMer (MCM) transgenic mice expressing α-MHC promoter-driven MerCreMer fusion protein was surgically paired to GFP transgenic mice. Because the resident cardiac myocytes in MCM mice expressed MerCreMer fusion protein (GFP-/Cre+) (Online Figure IIA), cardiac myocytes arising from the GFP+ circulating cells via cell fusion (GFP+/Cre+) or transdifferentiation (GFP+/Cre−) could be distinguished. Following stabilization of cross circulation at 1 month post-parabiotic surgery, the MCM parabiont was subjected to surgery-induced MI and the ischemic region below the site of ligation was excised from the injured heart for immunohistochemical analyses (Figure 3A). We observed that GFP+ cells scattered throughout the ischemic myocardium (Online Figure III) and these cells expressed mature cardiomyocyte markers cardiac troponin T (cTnT) and sarcomeric α-actinin in the MCM parabiont (Figure 3B and Online Figure III). Furthermore, quantification of cells in the ischemic region of injured hearts revealed that 9.39% of GFP+ cells engrafted into the myocardium were positive for Cre expression (GFP+/Cre+), suggesting that the circulating GFP+ cells had fused with the resident cardiac myocytes (Figure 3C and Online Table II). We also detected GFP+/Cre− cardiac myocytes, which are representative of transdifferentiated cells, at a frequency of 0.17% (Figure 3C and Online Table II). In addition to histological analyses, individual adult cardiac myocytes were dissociated from the injured heart of the MCM parabiont for identification of GFP+/Cre+ and GFP+/Cre− cells. In the absence of TAM induction, subcellular localization of Cre proteins was cytosolic and peri-nuclear (Online Figure IIA). However, TAM injection led to nuclear translocation of Cre (Online Figure IIB and IIC). To identify Cre+ cardiac myocytes more easily, the MCM mouse paired with the GFP transgenic mouse was separated at day 14 post-MI and then injected with TAM for 3 days. From dissociated ventricular cardiac myocytes, we identified 0.17±0.034% GFP+ cardiac myocytes co-expressing Cre, suggesting they arose from cell fusion (Figure 3D). Furthermore, we also found 0.0016±0.00063% of cardiac myocytes expressed GFP only (Figure 3D), indicating they were the transdifferentiated cells. Our findings suggested that contribution of circulating cells to cardiomyocyte regeneration could be observed following stabilization of the cross circulatory system.
Circulation-derived cardiac myocytes arisen from cell fusion and transdifferentiation.

In the MCM and GFP mouse paired model, any fused or transdifferentiated cardiac myocytes could be discriminated by their expression of GFP with or without Cre. To provide more direct evidence to assess the contribution of bone marrow-borne circulating cells to myocardial repair, a genetic pulse-trace labeling system was employed, in which a MCM mouse was parabiotically joined to a Z/EG transgenic mouse (Figure 4A). Two weeks post-MI, the parabiotic mouse pair was separated and Cre-mediated recombination was induced in the MCM parabiont by 14-day TAM injection. In the injured heart of the MCM, the cells originating from the Z/EG parabiont could be identified by their expression of β-Gal. If the circulating β-Gal+ cells fused with Cre+ resident cardiac myocytes in the MCM, TAM treatment would lead to Cre-dependent excision of the loxP-flanked LacZ sequence and therefore the fused cells would become GFP+. However, Cre-dependent gene excision would not be induced in cardiac myocytes arising from a transdifferentiation event and the cells would remain β-Gal+ (Figure 4A). Immunohistochemical analyses showed both GFP+ and β-Gal+ cardiac myocytes could be identified in the ischemic heart region of MCM parabiont (Figure 4B). Similar degrees of fusion and transdifferentiation events, 10.1% and 0.1%, respectively (Online Table III), to those in the MCM and GFP parabionts were also detected in the MCM and Z/EG parabionts. Further examination revealed that a higher percentage of cell fusion events occurred in the MI border zone than in the remote areas (Online Figure IV). To provide more definitive evidence that the circulating cells could acquire cardiac fate via cell fusion and transdifferentiation, individual adult cardiac myocytes were isolated from the injured hearts of MCM mice for examination of their GFP expression and β-Gal activity. In dissociated cardiac myocytes, we identified 0.19±0.046% of GFP+ cardiac myocytes, suggesting these cells had fused with the resident cardiac myocytes (Figure 4C). Furthermore, we observed that 0.00064±0.00034% of cells were positive for X-Gal staining, indicating these cells arise from an event of transdifferentiation (Figure 4C).

Stabilized cross circulation is important for acquisition of cardiac fate by circulating cells.

Stabilization of a shared circulatory system between parabiotic mice required at least 7 days (Figure 1). To strengthen our findings that a stabilized circulatory system at the time of injury was the key for cardiac fate acquisition by bone marrow-borne circulating cells in the injured myocardium, MCM mice were subjected to MI surgery when the cross circulation had been partially developed or not yet developed. MI was induced in mice before parabiotic surgery (D0) or at day 3 (D3) or day 5 (D5) post-parabiotic surgery (Figure 5A). Following 14 days of TAM-induced recombination, the ischemic region of the injured myocardium was harvested for analyses. In mice with fully developed cross circulation, we could observe 10% of fused GFP+ cardiac myocytes in the ischemic cardiac tissue (Figure 4B and Online Table III). However, the frequency of cell fusion decreased to 7.93±0.9% when cross circulation had only been
established for 5 days at the time of MI surgery (Figure 5B and 5D). The number of fused cardiac myocytes dropped further to 6.05±1.01% in the MCM mice that had only been joined to Z/EG for 3 days before MI was induced (Figure 5B and 5D). Although at much lower frequency, we still observed 1.33±0.24%, of fused cardiac myocytes in the mouse that had been subjected to MI surgery before parabiosis (Figure 5C and 5D). Collectively, these observations indicate that a limited cross circulation has a profound impact on the acquisition of cardiac cell fate by circulating cells via fusion.

DISCUSSION

Here we employed a parabiosis model to explore the natural power of bone marrow-borne circulating cells to repair an injured heart. The results from both immunohistochemical staining and analyses of dissociated cardiac myocytes revealed that circulating cells mobilized to the injured heart could contribute to cardiomyocyte regeneration through both cell fusion and transdifferentiation. In the injured heart of a MCM mouse that had been parabiotically joined to a GFP transgenic mouse, we detected GFP+ cells expressing cardiomyocyte markers, suggesting that circulating bone marrow cells may participate in myocardial repair. Quantification of GFP+ and Cre+ cells revealed that cell fusion is likely to be the dominant cellular mechanism for such contribution, particularly in the infarct border zone of the ischemic heart. This is in agreement with previous bone marrow transplantation data.10, 11, 23 In support of this finding, TAM-induced Cre-lox recombination in the MCM mouse that was formerly conjoined to a Z/EG mouse also showed that the majority of circulating cell-derived cardiac myocytes expressed GFP+ at the ischemic region of injured heart, indicating the Z/EG-derived β-Gal+ cells fused with resident Cre+ cardiac myocytes. In addition to cardiac myocytes, the circulating cells from the parabiont partner were observed to be incorporated into the vasculature of the injured myocardium. This finding is in accordance with studies demonstrating that bone marrow-originated cells could mobilize to a distant site and contribute to vasculature reconstitution after injury.12, 24

A previous study reported that hematopoietic cells do not acquire cardiac cell fate in the ischemic myocardium.18 In that study, contribution of bone marrow circulating cells to myocardial repair induced by surgery-induced MI was examined immediately after parabiosis, which contrasts with our study that was performed at 7-10 days after parabiosis. Several lines of evidence have indicated that injury-induced factors, such as immune modulators and chemokines, that are up-regulated after injury are important in modulating tissue regeneration. These factors are particularly important for regulating stem cell activities such as mobilization, proliferation and differentiation.20, 21, 25-28 More importantly, some of these signals are only transiently activated at the early stage of injury and usually diminish by day 10 post-injury.7, 20, 21, 25, 29 In the present study, we have demonstrated that there was very limited blood chimerism 3 days after parabiotic
surgery, and that the cross circulatory system required 7-10 days to be fully established. Real-time microbubble contrast imaging showed that incomplete vascular connection impeded delivery of systemic factors to the heart. Since timely and efficient transmission of injury-induced factors is necessary for tissue regeneration, we suspect the timing of MI induction may underlie the apparent discrepancies between our current results and those of Balsam’s group.

In the present study we show that both cell fusion and transdifferentiation are involved in bone marrow cell-modulated cardiac repair. Bone marrow cells can fuse with different types of somatic cells, thereby contributing to repair of various tissue injuries. In addition to the anti-apoptotic effect, cell fusion facilitates tissue repair by helping phenotypic and functional reversion of the fused cells. In accordance with these studies, it has been reported that cell fusion promotes re-entry of cardiac myocytes into the cell cycle; cardiac myocytes are terminally differentiated cells with very limited proliferative ability, therefore, such results suggest a potential benefit of cell fusion in cardiomyocyte repair. In addition to cell fusion, transdifferentiation of bone marrow-derived circulating cells was also detected in the injured heart. The ability of bone marrow cells to transdifferentiate into cardiac cells is controversial and cell fusion has been postulated to be the underlying mechanism contributing to the plasticity of hematopoietic stem cells to generate non-hematopoietic somatic cells. However, observation of GFP+/Cre and X-Gal+ cardiac myocytes in the dissociated cells provides compelling evidence that the circulating cells have the ability to transdifferentiate into cardiac myocytes in response to tissue damage, although at a lower frequency than cell fusion. Contribution of cardiac stem/progenitor cells to repair the injured heart has been documented. Furthermore, it is suggested that the bone marrow serves as a reservoir to replenish stem/progenitor cells in the injured heart. However, further examination would be required to identify the bone marrow cell population involved in such a process. Taken together, bone marrow-borne circulating cells possess the potential to give rise to non-hematopoietic cells under pathological conditions and may serve as an alternative cell source to replenish cardiac myocytes.

A study employing an isotope-labeling system to assess the contribution of progenitor cells and pre-existing cardiac myocytes to cardiomyocyte regeneration revealed that pre-existing cardiac myocytes are the primary cell population to replenish lost cells. Here we demonstrate the ability of bone marrow-derived cells to repair the injured heart via cell fusion and direct transdifferentiation, and that cell fusion is the dominant mechanism for bone marrow cell-derived cardiac myocytes. Whether the fusion between cardiac myocytes and bone marrow-borne circulating cells, including progenitor and stem cells, is the major contributor to cardiomyocyte proliferation requires further examination.

In conclusion, our findings provide evidence that bone marrow-derived circulating cells have the ability to engraft into the injured myocardium and to repair heart by fusing with resident cardiac myocytes.
and transdifferentiation. How cell fusion and transdifferentiation of bone marrow-derived cells are regulated in the injured heart remains unknown. Uncovering the signaling pathway governing these processes may lead to a greater understanding of how endogenous bone marrow cells could be augmented to repair injured myocardium.

**SOURCES OF FUNDING**
This study was supported by grants from the National Science Council grants 100-2314-B-006-046 and 102-2321-B-006-027; the National Health Research Institutes grant EX10123SI; the National Research Program for Biopharmaceuticals grants NRPB100CV021 and 102TM1055; and the Academia Sinica Translational Medicine Program.

**DISCLOSURES**
Patrick Hsieh received research grants from Celgene Cellular Therapeutics and Meridigen Biotech Co.

**REFERENCES**


DOI: 10.1161/CIRCRESAHA.116.304564


FIGURE LEGENDS

**Figure 1.** Development of stabilized cross circulation requires 7-10 days. **A,** Left, Lineage of GFP+ cells observed in the WT parabiont was analyzed by co-staining with CD45. **Right,** Blood chimerism was determined by analyzing the percentage of partner-derived GFP+ cells circulating in the peripheral blood of a WT parabiont (n = 2). **B,** Schematic diagram explaining the in vivo imaging analysis of blood flow using contrast enhanced agent microbubbles. **C,** Left, Background noise and microbubble contrast signal intensity in the ventricles of both donor and recipient mice after parabiotic surgery. **Right,** Fold change in microbubble contrast signal intensity at different time points post-surgery (n ≥ 2). Results are presented as the mean ± SEM.

**Figure 2.** Circulating cells engraft into the injured myocardium and acquire mature hematopoietic cell fate. **A,** Diagram illustrating the experimental procedure designed to analyze the vascular and hematopoietic cell fates of GFP+ circulating cells in the injured heart of a WT parabiont. **B,** Immunostaining result demonstrating the GFP+ cells co-expressing vascular cell markers SM22α and isolectin in the ischemic myocardium of a WT mouse. Scale bar, 5 μm. **C,** Flow cytometric analyses of the hematopoietic phenotypes adopted by GFP+ circulating cells isolated from the injured heart of a WT parabiont. **D,** Percentages of different mature hematopoietic cell lineages acquired by CD45+/GFP+ cells in the injured heart 14 days post-MI (n=2). Results are presented as the mean ± SEM.

**Figure 3.** Circulating cells adopt cardiac cell fate after heart injury. **A,** Schematic illustration of the experimental procedure using MCM and GFP parabiotic mice to examine the fate of circulating cells in the injured myocardium. CM; cardiomyocyte. **B,** Representative images of circulation-derived GFP+ cells co-stained with cardiac specific markers cTnT (Left) and sarcomeric α-actinin (Right). Scale bars, 20 μm. **C,** Representative immunohistochemical images showing the cells co-expressing both GFP and Cre (Left) and a GFP+ cardiomyocyte with absence of Cre expression (Right). Scale bars, 20 μm. **D,** Following TAM injection for 3 days to induce Cre translocalization, individual cardiac myocytes were dissociated from injured hearts. Shown are representative images of isolated cardiac myocytes arisen from cell fusion (Left) and transdifferentiation (Right) of circulating cells originated from the GFP parabiont. Scale bars, 100 μm. Numbers of GFP+/Cre+ fused cardiac myocytes and GFP+/Cre- transdifferentiated cells were quantified as the percentage of labelled cells in cardiac myocytes dissociated from injured hearts (n=3). Results are presented as the mean ± SEM.

**Figure 4.** Circulating cells contribute to myocardial repair through both cell fusion and transdifferentiation. **A,** Scheme depicting the experiment using a pulse-chase labeling system to examine the contribution of circulating cells in the injured heart. The parabiotic mice were separated at 2 weeks post-
MI for TAM injection. CM; cardiomyocyte. **B,** Representative immunohistochemical images of circulating cell-derived GFP⁺ (**Left**) and β-Gal⁺ (**Right**) cardiac myocytes arisen from cell fusion and direct transdifferentiation, respectively. Scale bars, 20 μm. **C,** Representative images of fused GFP⁺ cardiac myocytes (**Left**) and transdifferentiated X-Gal⁺ cells (**Right**) dissociated from injured myocardium following 14 days of TAM-induced recombination. Scale bars, 100 μm. Quantification result shows percentages of fused and transdifferentiated cells in total cardiac myocytes isolated from injured hearts (n=2). Results are presented as the mean ± SEM.

**Figure 5.** Limited cross circulation affects the number of circulating cells adopting cardiac cell fate in the injured myocardium. **A,** Schematic diagram explaining the experiment designed to examine whether limited cross circulation could affect the circulating cells to acquire cardiac cell fate. Heart injury was induced in MCM parabionts before parabiotic surgery (D0) or at day 3 (D3) or day 5 (D5) of parabiotic surgery. CM; cardiomyocyte. **B,** Representative immunohistochemical images of GFP⁺ cardiac myocytes derived from fusion of circulating cells mobilized from the Z/EG parabiont. Images were taken from the ischemic region of MCM parabionts that had been parabiotically joined for 5 days (**Left**) or 3 days (**Right**) before MI was induced. Scale bars, 20 μm. **C,** Representative image of a rare GFP⁺ cardiomyocyte observed in the ischemic heart of the MCM that had undergone MI surgery before being surgically joined to the Z/EG parabiont. Scale bar, 20 μm. **D,** Quantification of fused GFP⁺ cells identified in the injured hearts of MCM parabionts shared limited cross circulation with their Z/EG partners. Results show percentages of fused GFP⁺ cells in total cardiac myocytes quantified (n≥1). Results are presented as the mean ± SEM.
Novelty and Significance

What Is Known?

- Stem/progenitor cells residing in the bone marrow are capable of responding to heart injury.

- Acquisition of cardiac cell fates by hematopoietic cells is observed in the injured myocardium of animals that have undergone hematopoietic reconstitution by bone marrow transplantation.

- The ability of bone marrow-borne cells to give rise to cardiac myocytes is still controversial because a pivotal study involving surgically conjoined animals reported that these cells only adapt to hematopoietic cell fates after heart injury.

What New Information Does this Article Contribute?

- Hematopoietic cells participate in micro-vasculature reconstitution and contribute to cardiac myocyte regeneration in injured myocardium.

- Bone marrow-borne circulating cells acquire cardiac myocyte fate by means of cell fusion and transdifferentiation.

- A shared circulation, well developed between two surgically conjoined animals, is the key for cardiac cell lineage acquisition of hematopoietic cells.

The contribution of bone marrow-borne cells to cardiac myocyte regeneration is well documented. Using an experimental animal model in which two animals are surgically joined to develop a shared circulation, we evaluated the contribution of circulating cells to injured myocardium after cross circulation was stabilized in the conjoined animals. This is to ensure the important early injury-related signals can be transmitted efficiently and effectively between both animals upon heart injury. We found that the circulating cells are involved in micro-vasculature reconstitution and contribute to cardiac myocyte regeneration via fusion and transdifferentiation. This contribution could be observed only when the cross circulation is well established in conjoined animals.
Figure 2

A

Parabiosis for 1M

MI in MCM for 2 weeks

IHC staining analysis of vascular cell fate

or

Cardia small cell isolation for flow cytometry

B

GFP Isolectin SM22α DAPI

C

T cells

B cells

Macrophages

CD45

CD3

B220

F4/80

D

% of CD45^+ / GFP^+ cells

T cells

B cells

Macrophages
Circulating Cells Contribute to Cardiomyocyte Regeneration After Injury
Jasmine M Wu, Ying-Chang Hsueh, Hui-Ju Ch'ang, Chwan-Yau Luo, Li-Wha Wu, Hiromitsu Nakauchi and Patrick C Hsieh

Circ Res. published online November 14, 2014;
Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/early/2014/11/14/CIRCRESAHA.116.304564

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2014/11/14/CIRCRESAHA.116.304564.DC1
SUPPLEMENTAL MATERIAL

Circulating Cells Contribute to Cardiomyocyte Regeneration after Injury

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Supplemental Methods

Animals
The α-MHC promoter driven-MerCreMer (MCM) and Z/EG transgenic mice in the C57BL/6 background were purchased from the Jackson Laboratory. For ease of tail vein injection, FVB mice were chosen for the microbubble experiments conducted in this study. FVB, C57BL/6J-Tg(Pgk1-EGFP) transgenic mice and WT mice were purchased from the National Laboratory Animal Center, Taiwan. Animals of 12-16 weeks of age were used for the experiments conducted in this study.

Genotyping
The following primers were used:
GFP: 5’-CACATGAAGCAGCACGACTT-3’ and 5’-TGCTCAGGTAGTGTTGTCG-3’
Cre: 5’-ATACCGGAGATCATGCAAGC-3’ and 5’-AGGTGGACCTGATCATGGAG-3’

In Vivo Contrast-Enhanced Microbubble Imaging
MicroMarker Contrast Agent microbubbles (VisualSonics) were resuspended in normal saline according to the manufacturer’s protocol. At each time point, a 100 μl bolus containing 2 × 10⁸ microbubbles was systemically delivered by intravenous injection into one parabiotic mouse, the donor. The heart of the parabiont was viewed in a contrast mode, and the contrast signal intensity reflecting the flow of microbubbles was analyzed by appropriate VisualSonics software validated for the Vevo 2100 system. Prior to microbubble injection in the donor mouse, the background noise was recorded. Soon after injection, the microbubble contrast signal in the donor parabiont was detected and recorded. The probe was then moved to the recipient parabiont and the background noise was obtained before the microbubble contrast signal was detected. Three minutes post-injection, contrast signal intensity in the recipient heart was recorded. Changes in microbubble contrast signal intensity in the heart of the recipient parabiont at each time point was calculated by dividing the contrast signal intensity value by its corresponding baseline value.

Parabiosis and Myocardial Injury Model
The mice were anesthetized by intraperitoneal injection with Zoletil (25 mg/Kg containing 0.2% Rompun). The mice were then shaved and unilateral flank skin incision from elbow to the knee joints was created. The skin edge of mice was sutured with 5.0 prolene (Ethicon). One month post-parabiotic surgery, experimental myocardial infarction was induced in WT or MCM parabionts. The MCM mice subjected to tamoxifen (TAM, Sigma-Aldrich) injection were separated from their partners at 2 weeks post-MI.
An incision was created at the conjoined surgical site and the edge of skin wound was then sutured with 5.0 prolene. To induce Cre-mediated recombination, the mice were injected intraperitoneally with of TAM for 14 days at a dosage of 40 µg per 1 g body weight. For short-term treatment to induce Cre nuclear translocation, the mice were injected with the same dosage of TAM twice daily for 3 days. The TAM solution was prepared by dissolving the powder in sunflower oil (Sigma-Aldrich) at a concentration of 5 mg/ml.

**Adult Cardiomyocyte Isolation**

Adult ventricular cardiomyocytes were isolated from MCM parabionts on a Langendorff apparatus. Ten minutes after heparinzation, hearts were removed from the anaesthetized mice. The hearts were cannulated for retrograde perfusion with Ca²⁺-free Tyrode solution (NaCl 120.4 mM, KCl 14.7 mM, KH₂PO₄ 0.6 mM, Na₂HPO₄ 0.6 mM, MgSO₄ 1.2 mM, HEPES 1.2 mM, NaHCO₃ 4.6 mM, Taurine 30 mM, BDM 10 mM, Glucose 5.5 mM). Following perfusion for 3 minutes, the hearts were digested by the Ca²⁺-free Tyrode solution supplemented with collagenase B (0.4 mg/g body weight; Roche), collagenase D (0.3 mg/g body weight; Roche) and protease type XIV (0.05 mg/g body weight; Sigma-Aldrich). After digestion was completed, the ventricles were cut from the cannula and teased into small pieces in the digestion solution neutralized by the Ca²⁺-free Tyrode solution containing 10% FBS. Individual cardiomyocytes were dissociated from the digested tissues by gentle pipetting and undigested tissues were removed by filtering through a nylon mesh of 250 µm pore size.

**Immunohistochemical Staining**

For immunohistochemical analyses, only the ischemic region below the site of ligation was excised. Harvested heart tissues were fixed with 4% paraformaldehyde and embedded in paraffin. Immunostaining was performed on 5-µm tissue sections with the following primary antibodies at 4°C overnight: rabbit anti-GFP (1:200; GeneTex), mouse anti-Cre (1:200; Covance), mouse anti-cTnT (1:200; DSHB), mouse anti-sarcomeric α actinin (1:100; Sigma-Aldrich), rat anti CD45 (1:50; BD Biosciences), rabbit anti-SM22α (1:200; abcam) and isolectin-488 (Invitrogen). The appropriate secondary antibodies (Invitrogen) were used for visualization under a fluorescence microscope. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, 1 µg/ml; Sigma-Aldrich). For DAB immunostaining of GFP (1:200; GeneTex) and β-Gal (1:500; Invitrogen), a DAB substrate kit (DAKO) was used.

**Immunocytochemistry and X-Gal Staining**

Following isolation of ventricular cardiomyocytes, the cells were fixed in 2% paraformaldehyde for 30 minutes at room temperature. The cells were then permeabilized with 0.2% Triton X-100 in PBS for 15 minutes followed by blocking in 5% goat serum in PBS for 1 hour. Both permeabilization and blocking steps were carried out at room temperature. The cells were immunostained with the following primary antibodies: rabbit anti-cTnI (1:200; abcam), mouse anti-Cre (1:100; Covance) and
rabbit anti-GFP (1:200; GeneTex). Appropriate secondary antibodies (Invitrogen) were used for visualization. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI, 1 μg/ml; Sigma-Aldrich).

X-Gal staining of adult cardiomyocytes was carried out by incubating the fixed cells at 37°C overnight in a staining solution containing 0.01% sodium deoxycholate, 0.02% NP-40, 1 mM MgCl₂, 2 mM K₃Fe(CN)₆, 2 mM K₄Fe(CN)₆, 1 mg/ml X-Gal. All chemicals were purchased from Sigma-Aldrich.

**Cardiomyocyte Quantification**

To quantify cells from tissue sections, cell number was determined by quantifying 5-6 sections, spaced at 20-25 μm intervals, from each heart and 3 images were taken at a magnification of 200× for each section. To quantify dissociated cardiomyocytes, total number of cells was calculated with a hemocytometer. For analysis, 1 × 10⁵ cells were mounted onto each slide for image acquisition. The quantification results are presented as the percentage of labelled cells calculated per total number of cardiomyocytes dissociated from the heart.

**Flow Cytometric Analyses**

The peripheral blood of the WT parabiont was collected from the retro-orbital sinus and the red blood cells were lysed by ACK lysing buffer (Lonza). Cardiomyocyte-depleted cardiac small cells were isolated by digesting the minced heart with 0.1% collagenase B (Roche), 2.4 U/ml dispase II (Roche), and 2.5 mmol/L CaCl₂ at 37°C for 30 minutes. Undigested tissues were removed by filtering through a 40 μm filter. The immunostaining was carried by incubating 1× 10⁶ cells with fluorochrome-conjugated antibodies against CD45, CD3, B220 and F4/80 (BD Biosciences) on ice for 30 minutes. Flow cytometry was performed using FACSCanto II (BD) and FACSDiva software (BD) and FlowJo was used for data analysis.
Supplemental Tables

Online Table I. Blood chimerism analysis in parabiotic mice

<table>
<thead>
<tr>
<th></th>
<th>D0</th>
<th>D1</th>
<th>D3</th>
<th>D5</th>
<th>D7</th>
<th>D10</th>
<th>D14</th>
<th>1M</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pair # 1</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.0</td>
<td>0.1</td>
<td>15.0</td>
<td>36.3</td>
<td>40.0</td>
<td>45.3</td>
<td>47.2</td>
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<tr>
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<td>0.2</td>
<td>12.3</td>
<td>23.1</td>
<td>31.1</td>
<td>39.8</td>
<td>44.1</td>
<td>42.6</td>
</tr>
<tr>
<td><strong>Average</strong></td>
<td>0.100</td>
<td>0.150</td>
<td>13.65</td>
<td>29.70</td>
<td>35.55</td>
<td>42.55</td>
<td>45.65</td>
<td>41.50</td>
</tr>
<tr>
<td><strong>±SEM</strong></td>
<td>±0.10</td>
<td>±0.05</td>
<td>±1.35</td>
<td>±6.59</td>
<td>±4.45</td>
<td>±2.75</td>
<td>±1.55</td>
<td>±1.09</td>
</tr>
</tbody>
</table>

A GFP transgenic mouse was parabiotically conjoined to a WT mouse. At different time points following parabiotic surgery, the peripheral blood was collected for flow cytometric analysis. The blood chimerism was determined by analyzing the percentage of GFP+ cells observed in the WT parabiont (n=2).
Online Table II. Quantification of GFP\(^+\) cardiomyocytes engrafted in the injured myocardium

<table>
<thead>
<tr>
<th></th>
<th>Cre(^-)/GFP(^-)</th>
<th>Cre(^-)/GFP(^+)</th>
<th>Cre(^+)/GFP(^+)</th>
<th>Total cardiomyocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total number counted</strong></td>
<td>5247</td>
<td>545</td>
<td>10</td>
<td>5802</td>
</tr>
<tr>
<td><strong>percentage</strong></td>
<td>90.43</td>
<td>9.39</td>
<td>0.17</td>
<td></td>
</tr>
</tbody>
</table>

The numbers of resident (Cre\(^-\)/GFP\(^-\)), fused (Cre\(^-\)/GFP\(^+\)) and transdifferentiated (Cre\(^+\)/GFP\(^+\)) cardiomyocytes in the injured myocardium of MCM parabionts were quantified (n=3).
Online Table III. Quantification of circulating cell-derived cardiomyocytes in the injured heart

<table>
<thead>
<tr>
<th>Labeled cardiomyocytes</th>
<th>Unlabeled cardiomyocytes</th>
<th>Total cardiomyocytes</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFP⁺</td>
<td>598</td>
<td>5270</td>
<td>5866</td>
</tr>
<tr>
<td>β-Gal⁺</td>
<td>8</td>
<td>7888</td>
<td>7898</td>
</tr>
</tbody>
</table>

The numbers of GFP⁺ and β-Gal⁺ cardiomyocytes, which represent fused and transdifferentiated cells, respectively, were calculated (n=3).
Online Figure I. The majority of circulating cells do not express smooth muscle cell marker. 

A, The cells originating from the GFP parabiotic partner expressed hematopoietic cell marker CD45 but not smooth muscle cell marker smooth muscle actin (SMA) in the injured heart of the MCM parabiont. Scale bar, 20 μm. B, Representative image showing the circulation-derived GFP^+^ cells did not co-express smooth muscle cell marker smooth muscle 22α (SM22α). Scale bars, 5 μm.
Online Figure II. **Immunostaining examination of Cre expression in MCM cardiomyocytes.**

A, Representative images demonstrating subcellular localization of Cre protein in MCM cardiomyocytes could be cytosolic ([Left](#)) or peri-nuclear, as indicated by the arrow, ([Right](#)) in the absence of TAM induction. Cells were stained with mature cardiomyocyte marker cardiac troponin I (cTnI). Scale bars, 20 μm. B, Following 3 days of TAM treatment, nuclear translocation of Cre was observed in dissociated MCM cardiomyocytes ([Left](#)). No Cre$^+$ signal could be observed in the isotype control ([Right](#)). Scale bars, 20 μm. C, Representative immunohistochemical image showing translocation of Cre to nuclei following TAM induction ([Left](#)). The isotype control is shown on the [Right](#). Scale bars, 20 μm.
Online Figure III. GFP⁺ circulating cells scatter throughout the injured myocardium.
Representative images showing the circulation-derived GFP⁺ cells co-expressing cardiac marker cardiac troponin T (cTnT) could be observed in both the peri-infarct and remote regions of the ischemic heart. Scale bars, 20 µm.
Online Figure IV. More GFP+ fused cells are observed in the border zone of the infarct myocardium.

A, Representative images showing circulation-derived cardiomyocytes arisen from cell fusion in the infarct/border zone or remote area of the ischemic heart. Scale bars, 20 μm. B, Degree of cell fusion was calculated as the percentage of fused GFP+ cardiomyocytes per total cells quantified in different regions of injured hearts (n=3). Results are presented as the mean ± SEM.