

## Original Article

# Cardiomyogenic induced bone marrow c-kit<sup>+</sup> cells improve cardiac repair in mice myocardium infarction model

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Received December 31, 2017; Accepted October 9, 2018; Epub December 15, 2018; Published December 30, 2018

**Abstract:** Cardiomyocyte loss plays a crucial role in ventricular remodeling following myocardial infarction (MI). Cell-based therapy approaches using bone marrow derived c-kit<sup>+</sup> cells may attenuate ischemic injury. Nevertheless, no type of cell has been conclusively shown to be effective. We sorted c-kit<sup>+</sup> bone marrow cells by MACS, then cardiomyogenic differentiation induced by coculturing with infarcted myocardium tissue fragments. Cardiac function and MI area were measured 4 weeks after transplantation of cardiomyogenic induced Bone marrow c-kit<sup>+</sup> cells into mice MI model. Our data showed that BM c-kit<sup>+</sup> cells cocultured with infarcted myocardium tissue fragments promoted proliferation and cardiomyogenic differentiation of *in vitro*. While, injected the cardiomyogenic induced BM c-kit<sup>+</sup> cells through tail vein in a murine model of MI significantly preserved cardiac function and reduced myocardial injury. Thus, we have successfully developed a type of cell for cardiac repair. Our *in vitro* and *in vivo* observations provide the first clinically relevant evidence for translating cardiomyogenic induced BM c-kit<sup>+</sup> cells into clinical strategies to treat myocardial infarction.

**Keywords:** Acute myocardial infarction, bone marrow c-kit<sup>+</sup> cells, cell proliferation, differentiation

## Introduction

Cardiovascular disease remains the leading cause of death worldwide, current therapeutic regimens remain limited [1]. Myocardial infarction (MI) is associated with a rapid loss of cardiomyocytes, result in cardiac dysfunction, and even heart failure [1]. Adult mammalian cardiomyocytes retain some capacity for renewal, but this response is inenough for prevention of heart failure after MI [2, 3].

As limited capacity of cardiomyocyte regeneration, stem cell therapy has emerged as a promising treatment of ischemic heart disease [4-7]. Accumulating preclinical and clinical studies have been reported that various stem cell populations can improve cardiac function and attenuate adverse left ventricular (LV) remodel-

ing in ischemic cardiomyopathy [4, 5, 8, 9]. Hematopoietic stem cells expressing c-kit have the capacity to drive efficient cardiac repair via differentiation, neovascularisation, myogenesis and angiogenesis [10-12]. Nevertheless, some researchers argue that c-kit may promote inflammatory reaction and can not transdifferentiated into cardiomyocyte, thus limited the therapeutic use of c-kit<sup>+</sup> stem cells [12, 13]. However, the therapeutic use of cardiomyogenic induced BM c-kit<sup>+</sup> cells remain unreported.

In this study, we showed that coculture with infarcted myocardium tissue fragments can promote BM c-kit<sup>+</sup> cells proliferation and cardiomyogenic differentiation *in vitro*. Moreover, when injected through tail vein in a murine model of MI, the cardiomyogenic induced BM c-kit<sup>+</sup> cells significantly preserved cardiac func-

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tion and reduced myocardial injury. Thus, this study presents cardiomyogenic induced BM derived c-kit<sup>+</sup> cells can improve cardiac repair.

### Materials and methods

#### *Induction of acute myocardial infarction (mouse AMI model) in adult mice*

Male C57BL/6 mice and CAG-EGFP transgenic mice in MI group (Shanghai Biomedical Organism, China) were used to induce acute myocardial infarction (AMI) model by coronary artery ligation as described before [14]. Control group mice underwent the same surgical procedure without coronary ligation. All experiments have been approved by Tai'an City Central Hospital Ethics Committee and were performed in accordance with ethical standards.

#### *Isolation and coculture/culture of murine BM c-kit<sup>+</sup> cells*

The protocols to harvest bone marrow mononuclear cells (BMMNC) and c-kit<sup>+</sup> cells described before [15]. The infarcted hearts were harvested, removed blood vessels and atrial tissue, then sliced into about 1 mm<sup>3</sup> fragments, digested by collagenase. BM c-kit<sup>+</sup> cells were co-cultured with 3 pieces of infarcted heart fragments, respectively, utilizing a 24-well plate with 0.4 µm transwell (Corning, USA) in Iscove's Modified Dulbecco's Medium (Gibco, USA) supplemented with 15% (v/v) fetal bovine serum (Biowest, France) for indicated days.

#### *Cell transplantation*

60 (46 survived) mice after induction of acute myocardial infarction 3 day were randomized into 3 groups (listed below), the murine model of MI as the recipient. and the tail veins were cannulated for infusion of cardiomyogenic induced c-kit<sup>+</sup> cells (CO c-kit<sup>+</sup>), c-kit<sup>+</sup> cells or PBS. Mice in the sham Group (n=12) received intravenous infusion of 100 µL PBS alone. Mice in c-kit<sup>+</sup> Group (n=16) underwent intravenous infusion of 5×10<sup>6</sup> c-kit<sup>+</sup> cells/100 µL in PBS. Mice in Co c-kit<sup>+</sup> Group (n=18) underwent intravenous infusion of 5×10<sup>6</sup> cardiomyogenic induced c-kit<sup>+</sup> cells/100 µL in PBS (10 days after cocultured with infarcted heart fragments).

#### *Flow cytometry (FCM) analysis of the mouse BM-MNCs*

Mouse BM-MNCs were washed with PBS, then blocked with 1% BSA and incubated with phycoerythrin (PE)-conjugated c-kit anti-mouse antibodies (Miltenyi Biotec, Germany) for 30 minutes at 4°C in the dark. Cells were then washed, resuspended in PBS, and analysed via BD Accuri C6 flow cytometer (BD, USA).

#### *Cell viability assays*

Murine BM c-kit<sup>+</sup> cells (3×10<sup>5</sup> cells/well) were cultured in a 24-well plate coculture with myocardium fragments or without. CellTiter 96® Aqueous One Solution Reagent (Promega, USA) was added to each well and the cells were incubated at 37°C for 4 hours on days 1, 5, 7, 10, and 15. The absorbance (OD490 nm) was measured using Biotek Synergy™ HT Multi-Mode Microplate Reader (Biotek, USA).

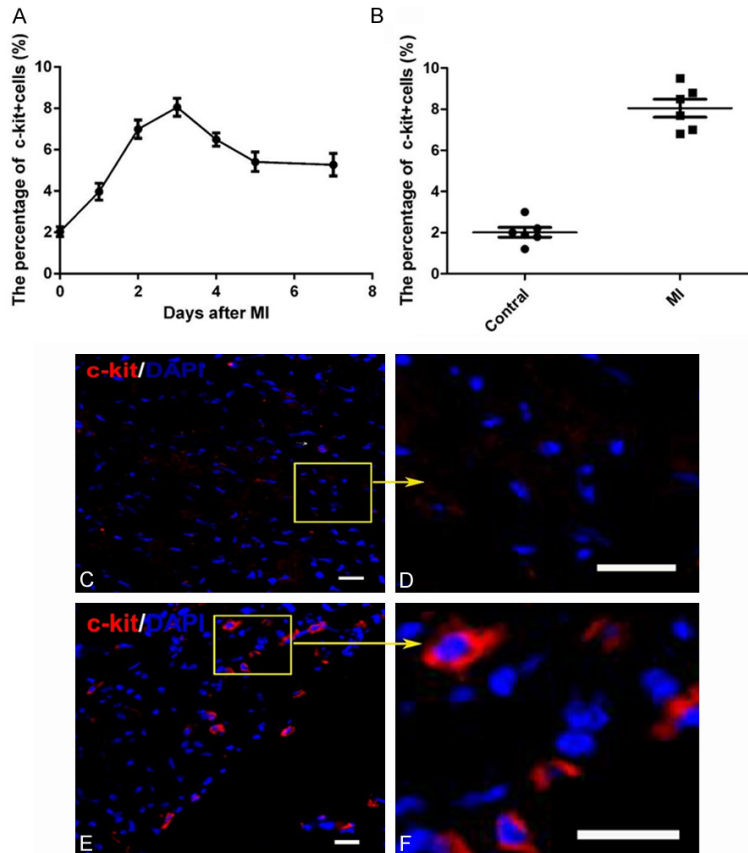
#### *Histological and immunofluorescent staining*

The BM c-kit<sup>+</sup> cells within or without coculture system were harvested in indicated time, fixed with the 4% paraformaldehyde, followed by a 1 hour incubation with 5% donkey serum (Jackson, USA). Cells were subsequently incubated with mouse anti-mouse ki67 (BD, USA), rabbit anti-mouse Phospho-Histone H3 (Cell signaling, USA) or goat anti-Gata4 (Santa Cruz, USA) overnight at 4°C, then incubated with secondary antibodies for 30 minutes. The cell nuclear was stained with DAPI. For the staining of myocardial tissues, they were fixed and embedded in Tissue-Tek OCT (Sakura, Japan), then cut into 5 µm sections, stained with chicken anti-GFP or goat anti-Gata4 (Santa Cruz, USA), followed by incubation with corresponding secondary antibodies. For visual inspection, Nikon Eclipse Ti-S Inverted Fluorescent Microscope (Nikon, Japan) was used.

#### *RNA extraction, reverse transcription-PCR and semi-quantitative PCR*

Total RNA was extracted using Quick-RNATM Micro Prep (Zymo, USA) following manufacturer's protocol. Reverse transcription was performed with 200 ng RNA in 10 µl of reaction reagent of GoScript™ Reverse Transcription System (Promega, USA). PCR was performed by ABI system, visualized and analyzed using an

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**Figure 1.** c-kit<sup>+</sup> cells ratio in adult BM-MNCs and the periphery of myocardial infarction after myocardial ischemia. (A) The relative ratio of c-kit<sup>+</sup> cells in adult BM-MNCs from day1 to 7 after MI. (B) The comparison of ratio of c-kit<sup>+</sup> cells in adult BM-MNCs between control group and MI group on day3 after MI. Increased c-kit<sup>+</sup> cells number in the periphery of myocardial infarction (E, F) than control group (C, D). Scale bar, 50  $\mu$ m. All data shown are representative of 3 independent experiments.

Image lab system (Bio-Rad, USA). Primer sequences are as follows:  $\beta$ -actin: forward primer 5'-GGCTGTATTCCCCTCCATCG-3' and reverse primer 5'-CCAGTTGGTAACAATGCCATGT-3', Ki67: forward primer 5'-CCTGCCGACCC-TACAAAAT-3' and reverse primer 5'-TCCGCCG-TCTTAAGGTAGGA-3', Nkx2.5: forward primer 5'-CAATGCCTATGGCTACAACGC-3' and reverse primer 5'-GAGTCATCGCCCTTCTCCTAAA-3', Gata4: forward primer 5'-CCCTACCCAGCCTACATGG-3' and reverse primer 5'-ACATATCGAGATTGGGGTGTCT-3', c-Tnt: forward primer 5'-CAGAGGAGGCCAACGTAGAAG-3' and reverse primer 5'-CTCCATCGGGGATCTTGGGT-3'.

### Evaluation of cardiac function

Four weeks after MI/implantation, recipient mice were subjected to transthoracic echocar-

diography. In brief, hearts were imaged 2-dimensionally in long-axis views at the level of the greatest LV diameter. The systolic and diastolic LV areas were measured at the same time. This view was used to position the M-mode cursor perpendicular to the LV anterior and posterior walls.

### Measurement of infarct size

Recipient mice were sacrificed 4 weeks after MI/implantation. The hearts were harvested and weighted, and then sliced into four transverse 3-mm sections from apex to base. Slices were stained with 2% triphenyltetrazolium chloride (Sigma-Aldrich, USA) and fixed with 4% paraformaldehyde. The caudal faces of each section were scanned using a flatbed color scanner, and the infarct volume was calculated.

### Statistical analysis

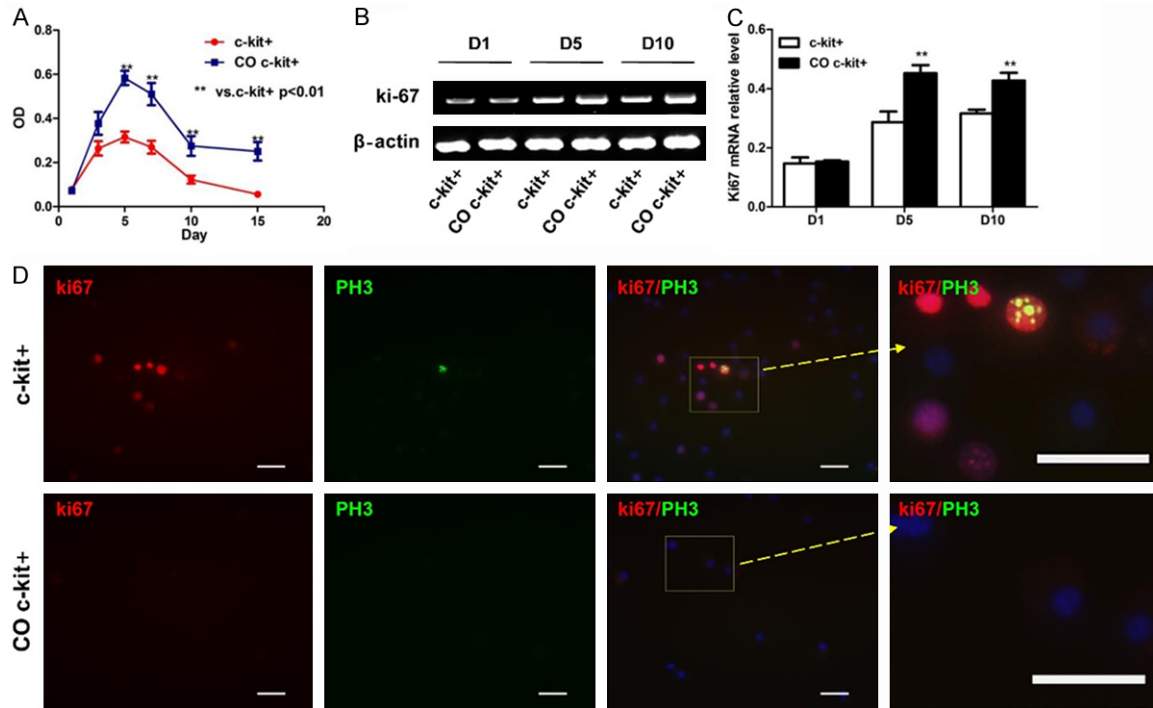
All data are expressed as mean  $\pm$  SEM. The comparison of cell proliferation ration and cardiomyogenic differentiation indexes (Gata4, Nkx2.5 and cTnt) between c-kit<sup>+</sup> group and CO c-kit<sup>+</sup> group was analyzed by unpaired *t* test with the software SPSS16.0 and Graphpad Prism 5. While the comparison of infarcted area and cardiac function indexes (LVEF, FS etal) between every two groups in three group (sham group , c-kit<sup>+</sup> group and CO c-kit<sup>+</sup> group) were performed by unpaired *t*-test with SPSS16.0 and Graphpad Prism 5. *P*<0.05 was considered to be significant difference.

## Results

### Increased c-kit<sup>+</sup> cells ratio in adult BM-MNCs in response to myocardial ischemia

After myocardium infarction operation, we checked the expression of c-kit in BM-MNCs from day 1-7. As shown in **Figure 1A**, the rela-

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**Figure 2.** Coculture with infarcted myocardium fragments promotes c-kit<sup>+</sup> cells viability. (A) Proliferation of c-kit<sup>+</sup> cells cocultured without infarcted myocardium fragments or not (n=8). RT-PCR (B) and semi-qPCR analysis (C) for mRNA levels of ki67 in c-kit<sup>+</sup> cells cocultured with infarcted myocardium or not on days 1, 5 and 10 (n=3). (D) Merged immunohistochemical staining of proliferating markers Ki67 (Red), PH3 (Green) and nuclei (DAPI). Scale bar, 50  $\mu$ m. CO c-kit<sup>+</sup>=cocultured with infarcted myocardium fragments.

tive ratio of c-kit<sup>+</sup> cells increased gradually from day 1 to day 3, then ratio decreased. The ratio is significantly higher in the MI group compared with control group (without coronary artery ligation) ( $8.02 \pm 0.43$  vs  $2.02 \pm 0.24$ ) on day 3 (**Figure 1B**).

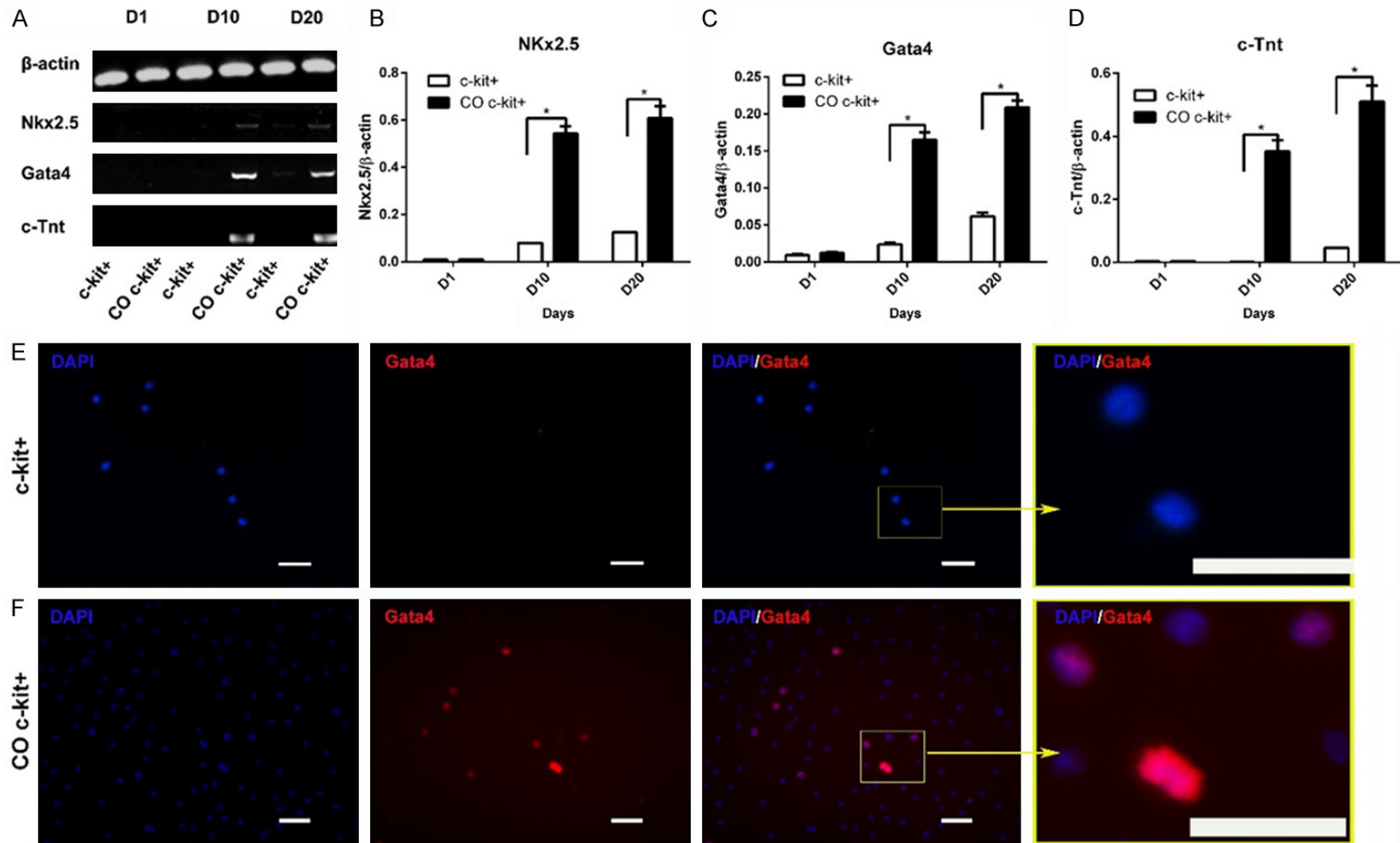
### *Increased c-kit<sup>+</sup> cells number in the periphery of myocardial infarction*

According to Shafie Fazel [10], the c-kit<sup>+</sup> cells number in the periphery of myocardial infarction was highest on day 3 after MI operation. We harvested the infarcted heart on day 3 and stained with c-kit antibody. Compared to control group, c-kit<sup>+</sup> cells number significantly increased in the periphery of myocardial infarction (**Figure 1C, 1D**). Though the sources of c-kit<sup>+</sup> cells still remain uncertain, there are many reports that bone marrow cells can home to the infarcted cardium [11, 12]. Thus to study the biological characteristics of bone marrow derived c-kit<sup>+</sup> cells is critical for studies on the role of c-kit<sup>+</sup> cells in cardiac repair mechanism in MI.

### *Coculture with MI myocardium fragments enhanced c-kit<sup>+</sup> cells viability*

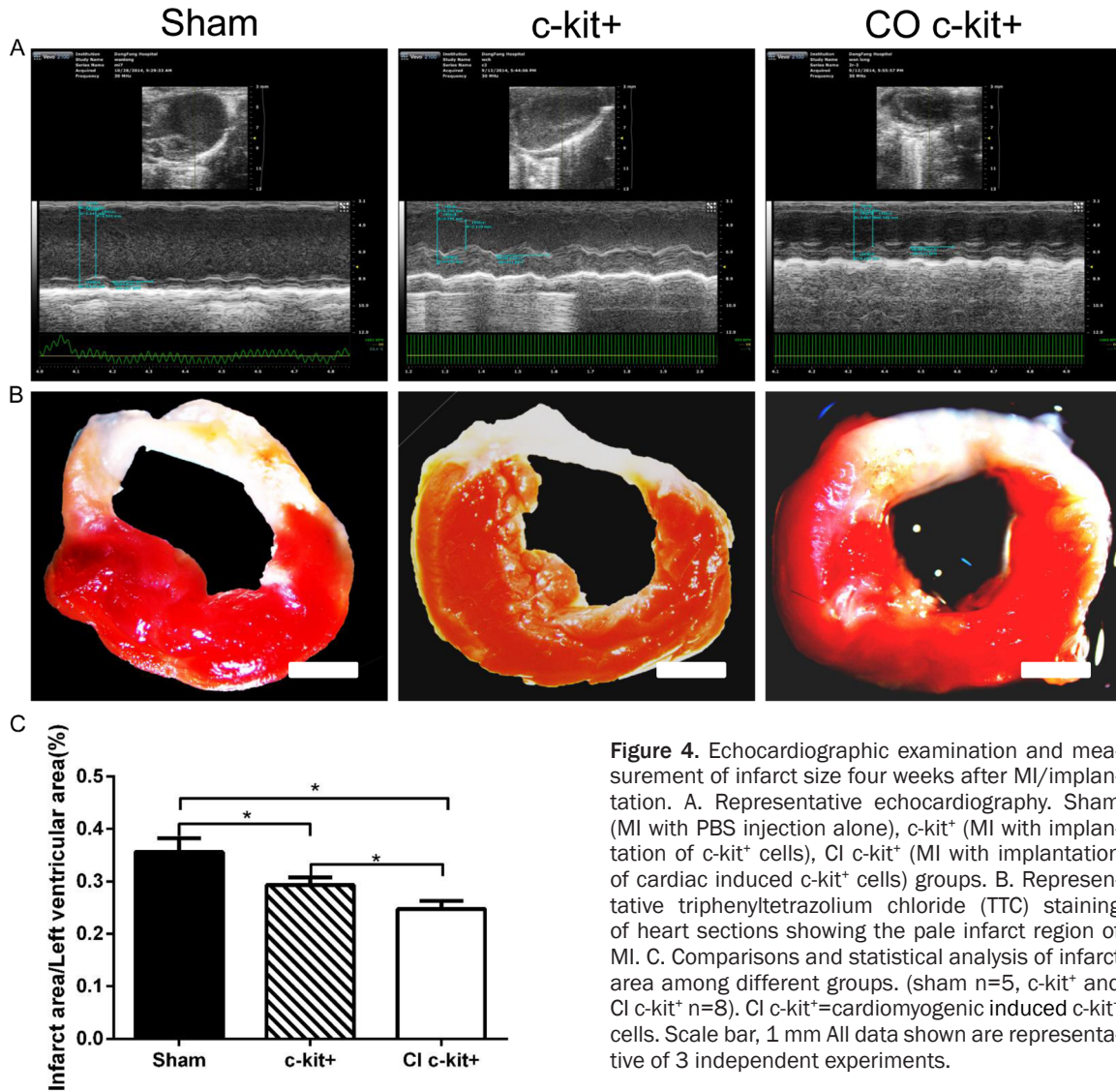
To evaluate the biological functions of myocardium fragments, we compared the proliferative behaviors of murine BM c-kit<sup>+</sup> cells coculture with infarcted heart fragments to that of the cells cultured without coculture. An increasing proliferation ration of the cells in coculture with infarcted heart fragments was observed from day 1 to day 15, reaching the maximal level on day 5 (**Figure 2A**). At all the measured time points (i.e. days 3, 5, 7, 10, and 15), the proliferation ration of cells cocultured with myocardium fragments was much higher than cells without coculture (**Figure 2A**). Reverse-transcription PCR analyses further confirmed that Ki67 mRNA in cells cocultured with myocardium fragments expressed higher than cells without coculture (**Figure 2B, 2C**). Cell proliferation was also shown by increased expression of proliferation markers Ki67/PH3 in cells cocultured with myocardium fragments by immunohistochemical staining (**Figure 2D**). Thus our results indicate that coculture with MI myocardium

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**Figure 3.** Cocultured with infarcted myocardium promotes cardiomyogenic differentiation of c-kit<sup>+</sup> cells. (A) RT-PCR and semi-qPCR (B-D) analysis showing the expression of cardiomyogenic differentiation markers Nkx2.5, Gata4, and c-Tnt in c-kit<sup>+</sup> cells cocultured with infarcted myocardium fragments or not at indicated time points of cell cultures. (E, F) Immunofluorescence staining of cardiomyogenic differentiation marker Gata4. Scale bar, 50  $\mu$ m. All data shown are representative of 3 independent experiments. CO c-kit<sup>+</sup>=BM c-kit<sup>+</sup> cells cocultured with infarcted myocardium fragments.





**Figure 4.** Echocardiographic examination and measurement of infarct size four weeks after MI/implantation. **A.** Representative echocardiography. Sham (MI with PBS injection alone), c-kit<sup>+</sup> (MI with implantation of c-kit<sup>+</sup> cells), CI c-kit<sup>+</sup> (MI with implantation of cardiac induced c-kit<sup>+</sup> cells) groups. **B.** Representative triphenyltetrazolium chloride (TTC) staining of heart sections showing the pale infarct region of MI. **C.** Comparisons and statistical analysis of infarct area among different groups. (sham n=5, c-kit<sup>+</sup> and CI c-kit<sup>+</sup> n=8). CI c-kit<sup>+</sup>=cardiomyogenic induced c-kit<sup>+</sup> cells. Scale bar, 1 mm All data shown are representative of 3 independent experiments.

fragments can promote BM c-kit<sup>+</sup> cells proliferation.

*Coculture with MI myocardium fragments induced cardiomyogenic differentiation of c-kit<sup>+</sup> cells*

To investigate the effects of MI myocardium fragments on cardiomyogenic differentiation of murine BM c-kit<sup>+</sup> cells, we analyzed the gene expressions of cardiomyogenic differentiation-related markers, such as Nkx2.5, Gata4, and c-Tnt in cells growing with MI myocardium fragments and cells without coculture. On day 10 and 20, of the cell cultures, reverse-transcription PCR (**Figures 3A-D**) and revealed that the Nkx2.5, Gata4, and c-Tnt mRNA expressions were almost exclusively upregulated in cells

growing cocultured with myocardium fragments. The immunohistochemical staining of Gata4 was also confirmed at protein level in a fraction of BM c-kit<sup>+</sup> cells growing with myocardium fragments (day 20) but not in the cells cultured without coculture (**Figure 3E**). These results demonstrate the capacity of the coculture with MI myocardium fragment to promote cardiomyogenic differentiation of BM stem cells.

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Our results have clearly shown the enhanced regenerative potential of BM stem cells while cocultured with MI myocardium fragments. This enlightened us to investigate the effect of car-

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**Table 1.** Echocardiography of Sham (MI with PBS injection alone), c-kit<sup>+</sup> (MI with implantation of c-kit<sup>+</sup> cells), Co c-kit<sup>+</sup> (MI with implantation of cardiac induced c-kit<sup>+</sup> cells)

	Sham	c-kit <sup>+</sup>	COc-kit <sup>+</sup>
EF (%)	23.27±2.94	31.27±3.57*	35.51±3.93**,&&
FS (%)	11.87±2.64	16.91±2.54	21.47±3.81**,&&
LVIDd (mm)	5.22±0.40	4.11±0.540	3.74±0.29*,&&
LVIDa (mm)	4.29±0.46	3.53±0.43	3.08±0.59*,&&
LV mass (mg)	65.93±12.02	70.90±14.58	45.35±2.25*,&
LVEDV (μl)	104.54±21.04	103.40±12.38	78.47±8.67**,&
LVESV (μl)	81.67±20.97	75.27±24.10	50.98±5.65**,&

EF=ejection fraction; FS=fractional shortening; LVEDV=left ventricular end-diastolic volume; LVEDP=left ventricular end-diastolic pressure; LVESV=left ventricular end-systolic pressure; LV mass=left ventricular mass; LVESV=left ventricular end-systolic volume; LVIDd=end-diastolic left ventricular dimension, LVIDs=end-systolic left ventricular dimension. Sham, n=10; c-kit<sup>+</sup>, n=10; cardiac induced c-kit<sup>+</sup>, n=10; \*P<0.05, \*\*P<0.01 compared with Sham group; &P<0.05, &&P<0.01 compared with c-kit<sup>+</sup> group. CO c-kit<sup>+</sup>=cardiomyogenic induced BM c-kit<sup>+</sup> cells.

cardiomyogenic induced c-kit<sup>+</sup> cell for cardiac repair in vivo. To this end, we applied the cardiomyogenic induced c-kit<sup>+</sup> cell (CO c-kit<sup>+</sup> group) or c-kit<sup>+</sup> cell (c-kit<sup>+</sup> group) or PBS (sham group) in a murine model of MI. Of note, transthoracic Doppler echocardiography (**Figure 4A**) showed that implantation of either the c-kit<sup>+</sup> cells or cocultured c-kit<sup>+</sup> cells significantly increased ejection fraction (EF; from 23% to 31% or to 35%) (**Table 1**) and fractional shortening (FS; from 12% to 17% or to 21%) (**Table 1**), respectively, 4 weeks after MI/implantation. Interestingly, the cocultured c-kit<sup>+</sup> cells were even more efficient in improving cardiac function than the c-kit<sup>+</sup> alone, as further evidenced by additional significant reductions in systolic and diastolic indices, such as end-systolic LV inner dimension (LVIDs) and end-diastolic LV inner dimension (LVIDd), LV end-systolic volume (LVESV) and LV end-diastolic volume (LVEDV) (**Table 1**). To explore whether transplanted cells could reduce cardiac injury, we evaluated the infarct size. The infarct size in MI mice was much smaller after transplanted of the cocultured c-kit<sup>+</sup> cell or c-kit<sup>+</sup> cells than that without transplantation (25% or 29% vs. 36%, respectively) (**Figure 4B, 4C**). In line with the improvement of cardiac function, the infarct size in MI mice treated with cardiomyogenic induced c-kit<sup>+</sup> cells was also significantly reduced, when compared with that treated with c-kit<sup>+</sup> cells (**Figure 4C**).

Moreover, Immunofluorescent staining of heart sections showed that the donor GFP<sup>+</sup> cells could be detected in peri-infarct myocardium of

MI mice 10 days after implantation (**Figure 5**) and the transplanted GFP<sup>+</sup> cells coexpressed Gata4 (**Figure 5**). Taken together, these results indicate that both the cardiomyogenic induced c-kit<sup>+</sup> cells or c-kit<sup>+</sup> cells improve cardiac performance and reduce cardiac injury while the cardiomyogenic induced c-kit<sup>+</sup> cells is superior to c-kit<sup>+</sup> cell in mediating cardiac repair.

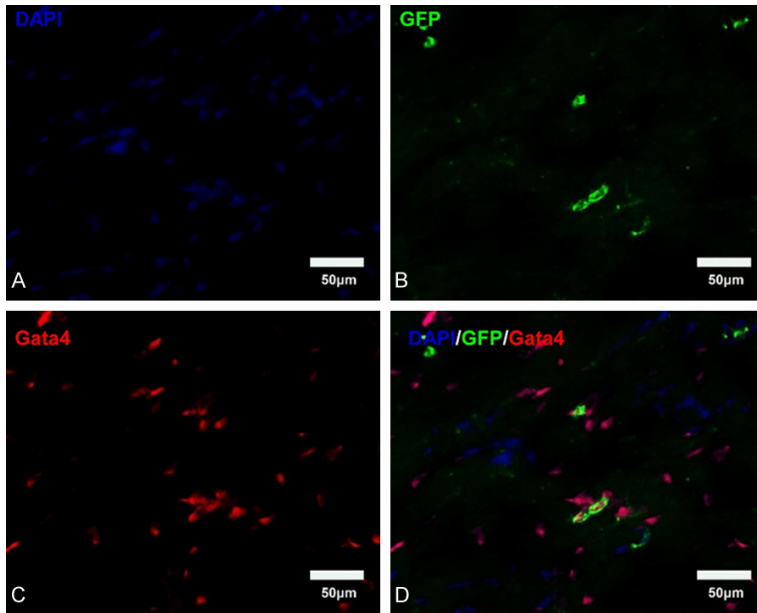
### Discussion

The present study was designed to augment the cardiogenic potential of cardiomyogenic induced bone marrow

c-Kit<sup>+</sup> cells and the potential therapeutic benefits. In this study, we have developed coculture system to harvest cells for cardiac repair. In the coculture system, post-infarct murine BM c-kit<sup>+</sup> cells exhibited an increased capacity for cell proliferation and cardiomyogenic differentiation in vitro. When injected in a murine model of MI, the cocultured BM c-kit<sup>+</sup> cells significantly improved cardiac performance and reduced infarct size. These in vitro and in vivo observations provide the first evidence for translating cardiomyogenic induced BM c-kit<sup>+</sup> stem cells into clinical strategies to treat MI.

Though current therapeutic improvements to MI (myocardium infarction)/HF (heart failure) can improve symptoms and prolong life, they do not settle the fundamental problem—the loss of cardiomyocytes [4, 7, 16]. Stem cell therapies have the potential to regenerate myocardium—what would have been unthinkable only a few years ago [5, 7, 11, 17-20]. Both clinical and experimental trials have showed that bone marrow derived stem/progenitor cells was the only possibility up to now to regenerate ischemic myocardium [8, 16, 20, 21]. Cell therapy to treat injured hearts has proven safe in humans, but the functional and survival benefits of these therapies have been modest and the mechanisms underlying putative benefits is still largely unknown [16, 21-24]. Hence, to identify new types of cell that can home to the ischemic heart and differentiate into new cardiomyocyte and elicit endogenous cardiac repair is still urgently needed. In this study, we went one step further and for the first time suc-

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**Figure 5.** Tracing of implantation cells. Immunofluorescence staining of GFP cardiomyogenic differentiation marker Gata4 periphery of infarction, A: DAPI, B: GFP, C: Gata4, D: Merge of DAPI, GFP and Gata4.

cessfully developed the cardiomyogenic induced BM c-kit<sup>+</sup> stem cells, which improved cardiac performance and reduced infarct size when transplantation into MI mice through intravenous injection, providing a clinically relevant novel cell type for treating MI.

Transplantation of BM-derived stem cells into infarcted heart holds promise as a novel option for the treatment of MI [9, 15, 19, 21, 22]. In this study, we tested the well characterized BM stem cell population, c-kit<sup>+</sup> cells, cocultured with infarcted myocardium fragments and analyzed whether cocultured c-kit<sup>+</sup> cells had additional advantage. Coculturing with infarcted myocardium fragments was shown to promote proliferation and cardiomyogenic differentiation of anchoring post-infarct BM c-kit<sup>+</sup> stem cells in vitro. Of note, when used to treat infarcted heart in a murine model of MI, cardiomyogenic induced c-kit<sup>+</sup> cells were superior to c-kit<sup>+</sup> cells in improving cardiac performance as well as in limiting infarct size. The mechanisms of protective capacities shown by cardiomyogenic induced c-kit<sup>+</sup> cells may associate with cardiomyogenic differentiation, but the exact mechanisms still merit further investigations.

Limitations of this research should also be signified. The assessment of myogenic differentia-

tion potential in transplanted c-kit<sup>+</sup> cells was limited to the overall cell content in the whole heart. Calculating the exact percentage of cells, which became cardiomyocytes, and distinguishing other cell types were hampered by an unknown rate of cell proliferation and loss in the cellular composition after 10 days in culture. The mechanisms of myocardium fragments in promoting proliferation and cardiomyogenic differentiation was not investigated.

In summary, we have identified bone marrow c-kit<sup>+</sup> cell population that increased in response to ischemic cardiac injury and c-kit<sup>+</sup> cells number increased in the periphery of myocardial infarction. In vitro,

we showed that coculture with MI myocardium fragments can promote BM c-kit<sup>+</sup> cells proliferation and cardiomyogenic differentiation. Furthermore, we provide evidence that intravenous implanted cardiomyogenic induced c-kit<sup>+</sup> cells, are superior to c-kit<sup>+</sup> cells, are able to improve heart function and reducing infarct size in the mice MI model. The possible mechanism of this protective phenomenon may rely on cardiomyogenic differentiation. In conclusion, our data raise the possibility that expansion and myocardial transplantation of autologous cardiomyogenic induced BM c-kit<sup>+</sup> cells may be a promising therapeutic approach in post-MI treatment.

### Acknowledgements

We are appreciating to every author for their assistance with the experiments and constructive comments on the manuscript.

### Disclosure of conflict of interest

None.

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