Editorial

Fusion heals the broken-hearted

Daniel A. Zuppo, Michael Tsang

Department of Developmental Biology, School of Medicine, University of Pittsburgh, Pittsburgh, PA, USA

Correspondence to: Michael Tsang. Department of Developmental Biology, School of Medicine, University of Pittsburgh, Pittsburgh, PA, USA. Email: tsang@pitt.edu.

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Cardiovascular diseases (CVDs) remain one of the most severe conditions facing patients with reported mortality rates of 17.92 million in 2015 (1). Myocardial infractions (MIs), a prevalent form of CVD, trigger localized cardiomyocyte loss after ischemic injury, and the following inflammatory response and deposition of extracellular matrix causes scar formation (2). While significant advances have been made in diagnosing and treating CVDs, there currently are no treatments that can reverse the fibrotic scarring. Fibrosis induces ventricular remodeling, changing the pressure system, and the stressed heart is at greater risk for recurrent MIs and heart failure (2). Whether the scar tissue could be reversed with healthy myocardium has been at the forefront of cardiac research for the past couple decades because the adult mammalian heart is predominantly a quiescent organ, with limited regenerative potential. One particular focus has been directed at whether cardiomyocytes can proliferate in the adult heart after injury to replace lost tissue. Studies in zebrafish (3,4), newts (5), and in neonatal mice (6,7) have revealed that cardiomyocyte proliferation after injury can occur. Another focus was driven by the identification of endogenous, cardiac progenitor cells (CPCs) that sparked intense study and debate over the last decade about the possibility of resident stem cells restoring cardiac function. The possibility for resident progenitors to repopulate lost cardiac tissue could hold the key to restoring cardiac function after injury.

One major breakthrough came in 2001 when it was shown that bone marrow cells (BMCs) could be transplanted into post-MI mice and differentiate into cardiac cell types that reduced the severity of the injury and improve cardiac physiology (8-10). In these experiments, Orlic *et al.* demonstrated that injecting Lin$^-$ c-Kit$^+$ BMCs (Lin, endothelial cell and Kit, stem cell marker) from enhanced green fluorescent protein (EGFP$^+$) mice into the infarcted left ventricle increased the EGFP$^+$ cardiac muscle cells suggesting that c-Kit$^+$ BMCs differentiated into cardiomyocytes (9). They also demonstrated that Lin$^-$ c-Kit$^+$ BMCs differentiated into endothelial cells and smooth muscles cells which indicated that these progenitor cells behaved as stem cells since they could give rise to multiple lineages (9). In 2003 Beltrami *et al.* isolated Lin$^-$ c-Kit$^+$ CPCs from adult rat myocardium, cultured under *in vitro* conditions, and were able to differentiate them into cardiomyocytes, smooth muscle cells, and endothelial cells, however, they were morphologically and functionally immature (11). Moreover, transplanted cardiac-derived Lin$^-$ c-Kit$^+$ CPCs into adult rat myocardium post-MI resulted in the appearance of a band of differentiated cardiomyocytes, smooth muscle and endothelial cells within the infarct (11). These initial findings excited the cardiac regeneration field because it showed that resident cells within the myocardium behaved as stem cells and could differentiate into functional cardiac cells lineages to improve recovery after MI.

More recently, Ellison *et al.* expanded on Beltrami's findings to show that c-Kit$^+$ CPCs were both necessary and sufficient for cardiac regeneration. They administered 5-fluorouracil (an agent that disrupts mitosis) after cardiac injury and found new cardiomyocyte formation and c-Kit$^+$ CPC expansion was absent. In addition, heart

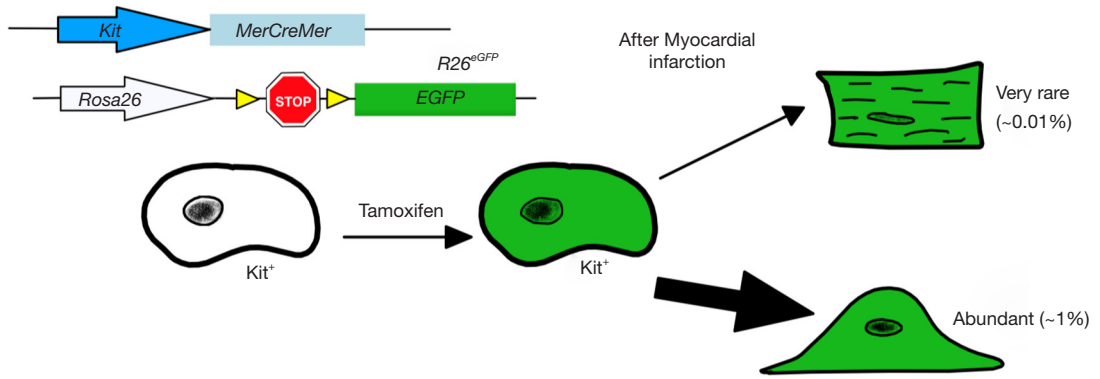

Figure 1 Schematic showing lineage tracing of c-Kit+ cells after myocardial infarction labels mostly endothelial cells and in very rare occasions in cardiomyocytes.

failure symptoms in these rats were prevalent (12). Ellison *et al.* then transplanted GFP– expressing c-Kit$^+$ CPCs into 5-fluorouracil treated rats with MI and found GFP$^+$ cardiomyocytes and vascular cells within the damaged myocardium: suggesting that c-Kit$^+$ CPCs were critical for endogenous regeneration of the heart (12). While these findings increased the interest in manipulating this cell population, other groups attempted to directly test the resident c-Kit$^+$ CPCs contribution to cardiomyocyte differentiation and heart physiology after MI (13-16). van Berlo *et al.* performed c-Kit$^+$ CPC lineage tracing by developing a mouse line that expressed Cre recombinase and endogenous GFP at the *Kit* locus and bred these mice into a *Rosa26-eGFP* line so that all *Kit*$^+$ cells would express EGFP (*Figure 1*) (16). They observed minimal expression of EGFP$^+$ cardiomyocytes in uninjured and post-MI hearts (less than 0.03%), but they did observe colocalization of the CD31$^+$ cells and EGFP signal which suggested endothelial cell expansion (16). Interestingly, using the *Rosa26*-membrane-Tomato-loxP-stop-loxP-membrane-eGFP line (*R26*$^{mT/mG}$), van Berlo *et al.* observed cell-fusion events, and not *de novo* cardiomyocyte formation as a mechanism of c-Kit$^+$ derived myocyte cells (16). They concluded that this low abundance of observed cardiomyocytes derived from c-Kit$^+$ CPCs suggested that this population was not sufficient to contribute to improving heart function after MI. Similar findings were reported by three other studies (13-15). Sultana *et al.* further answered these questions by developing multiple knock-in lineages that would express different fluorescent proteins fused with histone *H2B* protein (15). They crossed mice expressing the knock-in allele *c-kit*$^{H2B\text{-}tdTomato}$ with those containing *Nkx2.5*$^{H2B\text{-}GFP}$ (marking the early cardiogenic transcription factor *Nkx2.5*) or *cTnT*$^{H2B\text{-}GFP}$ (which labels the mature sarcomeric structures), and found minimal colocalization of these markers within c-Kit$^+$ cells (15). Sultana *et al.* also observed increased colocalization of c-kit with endothelial protein PECAM and noted c-Kit$^+$ CPCs demonstrated limited differentiation into cardiomyocytes (15). Liu *et al.* sought to determine the fates of the c-Kit$^+$ CPC-derived cells by reassessing the findings from Ellison and van Berlo, and they employed an instant pulse-chase model where they collected *Kit-Cre*^{ER}; *Rosa26-RFP* hearts at 24 and 48 hours after tamoxifen treatments (14). Using RFP$^+$ cardiac troponin I (Tnni3)$^+$ as markers of c-Kit$^+$ cardiomyocytes, they observed that 58-71% of all cardiomyocytes were labeled by both markers (14). They postulated that these RFP$^+$Tnni3$^+$ cardiomyocytes did not differentiate from Kit$^+$ CPCs, because was unlikely that such a large percentage of myocytes would be generated within 48 hours, and that a population of pre-existing cardiomyocytes expressed c-Kit which could create artifactual findings with basic lineage tracing (14). A more definitive set of experiments were conducted by He *et al.* (13). They generated a dual-recombinase reporter system to determine if c-Kit$^+$ CPCs differentiated into *de novo* cardiomyocytes because the traditional, single-recombinase lineage-tracing system often had off-target expression of the fluorescent tracer protein (13). This was achieved by the development of a mouse (*Tnni3-Dre* × *Kit-Cre*^{ER} × *IR1*) that expressed *Dre* recombinase under the control of *Tnni3*, tamoxifen-inducible Cre recombinase controlled by *Kit* promoter, and an interleaved reporter (IR1) labeling Dre-*rox* recombination as tdTomato, while ZsGreen is expressed if Cre-*loxP* recombination occurred (13). He *et al.* observed a majority of ZsGreen$^+$ endothelial cells but

no ZsGreen$^+$ cardiomyocytes in *Tnni3-Dre* × *Kit-Cre^{ER}* × IR1 hearts, while *Kit-Cre^{ER}* × IR1 control hearts showed ZsGreen in both cardiomyocytes and non-myocytes (13). These findings confirmed the traditional Cre-recombinase system was not as specific as previously believed, as some cardiomyocytes express Kit, and that a significant portion of the *de novo* cardiomyocytes previously reported are artifacts of the system, i.e., Kit expression in mature cardiomyocytes. The question still unresolved is whether the observed c-Kit$^+$ derived cardiomyocytes is actually the result of cell-fusion.

In their recent publication, Maliken *et al.* determined if endogenous CPCs can give rise to *de novo* cardiomyocytes or fused with resident cardiomyocytes (17). This was addressed by using *Kit* lineage-specific deletion of the cardiogenic transcription factors *Gata4* and *Gata6*. *Gata4* is required for heart tube formation during embryogenesis (18,19) and *Gata4/Gata6* double mutants failed to develop a functional heart entirely (20), indicating that *Gata4* and *Gata6* are necessary genes for cardiac development and cardiomyocyte differentiation. Maliken *et al.* hypothesized that if CPCs from the *Kit* lineage were differentiating into *de novo* cardiomyocytes, then the loss of *Gata4* and *Gata6* would inhibit this differentiation of CPCs into cardiomyocytes (17). To test this, Maliken *et al.* generated mouse lines containing tamoxifen-inducible MerCreMer (MCM) under the control of the *Kit* allele and floxed alleles of *Gata4* and *Gata6* (*Figure 2*). In the presence of tamoxifen, *Kit*$^+$ cells would selectively recombine and disrupt the *Gata* genes. These lines were also bred to have either the *Rosa26*-loxP-stop-loxP-eGFP (*R26^{eGFP}*) to lineage trace all *Kit*-allele derived cells (17). First, mice were fed tamoxifen after weaning, and were sacrificed after 1-, 2-, or 4-month of treatment to determine if loss of *Gata4*, *Gata6*, or both reduced the number of cardiomyocytes derived from the *Kit*$^+$ *R26e^{GFP}* CPCs. Unexpectedly, loss of *Gata4* (*Gata4$^{fl/fl}$*) and the double knockout of *Gata4/Gata6* (*Gata4$^{fl/fl}$/Gata6$^{fl/fl}$*) increased the number of eGFP$^+$ cardiomyocytes compared to the control mice. These results were surprising because it was previously reported that the deletion of *Gata4* and *Gata6* blocked cardiomyocyte differentiation and caused acardia (20). To determine if these myocytes were the result of *de novo* differentiation from the *Kit*$^+$ CPCs or fusion events, Maliken *et al.* used *Kit^{MCM}R26$^{mT/mG}$* mice containing either the floxed allele for *Gata4* or *Gata4/6* and counted mTomato$^+$/m-eGFP$^+$ cells. This dual reporter method showed an increase in mTomato$^+$/m-eGFP$^+$ cardiomyocytes in *Gata4$^{fl/fl}$* and *Gata4$^{fl/fl}$/Gata6$^{fl/fl}$* when compared to controls and indicated the labeled myocytes were from fusion events instead of *de novo* origins. In the original experiments that showed *Kit*$^+$ could give rise to multiple lineages in the myocardium, transplanted Lin-*Kit*$^+$ BMCs were used (8,9,11). In this study, Maliken *et al.* used BMCs from *Kit^{MCM}*: *Gata4$^{fl/fl}$/Gata6$^{fl/fl}$* mice to determine if other cell lineages were affected. They observed an expansion in CD133$^+$ endothelial progenitors from *Kit^{MCM}*: *Gata4$^{fl/fl}$/Gata6$^{fl/fl}$* BMCs compared to controls which suggests that Gata genes functions in endothelial cells. Previous lineage tracing studies have shown cells derived from *Kit^{Cre}* progenitors to be either endothelial cells (CD31$^+$) or leukocytes (CD45$^+$) (16), and Maliken *et al.* found that CPCs from either *Kit^{MCM}*: *Gata4$^{fl/fl}$* or *Kit^{MCM}*: *Gata4$^{fl/fl}$/Gata6$^{fl/fl}$* lineages contained increased levels of CD31$^+$ and CD45$^+$ expression compared to controls (17). These findings further confirmed that *Gata4* plays a role in suppressing endothelial cell and leukocyte differentiation since loss of *Gata4* allowed for an expansion of these cell populations.

Since the Kit$^+$ CPCs deficient in *Gata4* or *Gata4/Gata6* displayed increased eGFP$^+$ cardiomyocytes and increased differentiation into endothelial and leukocyte lineages, Maliken *et al.* next wanted to determine whether the increase in eGFP-expressing myocytes was the result of leukocyte fusion or altered endothelial cell differentiation (17). To understand this phenomenon, they performed two different bone marrow transplantation experiments to determine how loss of *Gata4/6* increased eGFP$^+$ myocytes. In the first experiment, they used donor bone marrow from *Kit^{MCM}*:*Gata4$^{fl/fl}$Gata6$^{fl/fl}$*: *R26e^{GFP}* (and *Kit^{MCM}R26e^{GFP}* control) mice that underwent 6 weeks of tamoxifen treatment and transplanted this marrow into irradiated recipient *R26^{mT}* mice (*Figure 2*) (17). Two months later eGFP$^+$ cardiomyocytes were found in host hearts, and Maliken *et al.* concluded that these cardiomyocytes were formed from fusion events because they expressed both eGFP and m-Tomato. Maliken *et al.* also performed flow cytometry on isolated cardiac interstitial cells and found no difference in CD31$^+$ endothelial cell population size which indicated that *Gata4$^{fl/fl}$/6$^{fl/fl}$* bone marrow did not contribute to the expansion in endothelial cells that they originally detected in the *Kit^{MCM}*:*Gata4$^{fl/fl}$Gata6$^{fl/fl}$* lineage (17). To see if it was the endogenous *Kit*$^+$ CPCs that were responsible for increased eGFP$^+$ cardiomyocyte presence and CD31$^+$ endothelial cell expansion, they performed a reverse bone marrow experiment. *R26^{mT}* bone marrow from donor mice were

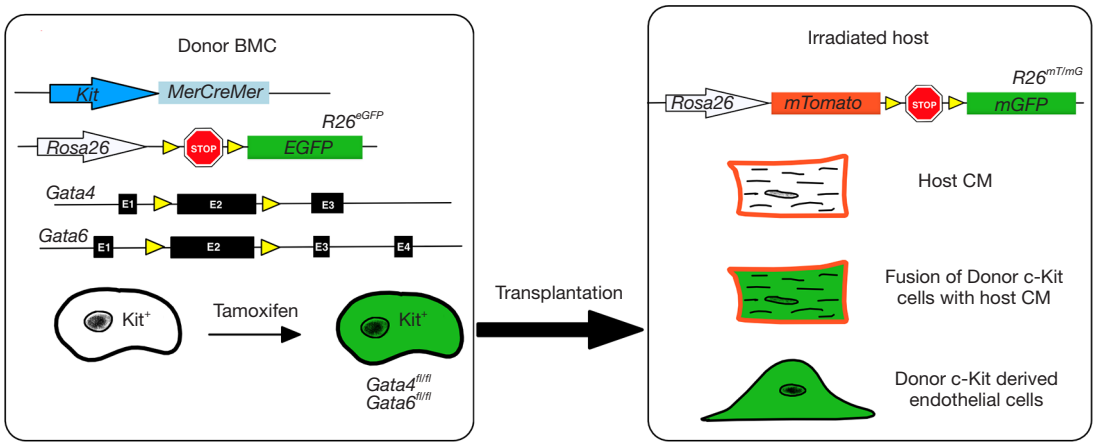

Figure 2 Schematic showing transplantation of c-Kit labelled (green) donor bone marrow cells (BMCs) into irradiated host mice expressing membrane tagged tomato in cardiomyocytes. Transplantation of Gata4 and Gata6 deficient c-Kit cells resulted in increased green cardiomyocytes that were also expressing membrane tagged tomato protein, indicative of cell fusion.

transplanted into irradiated Kit^{MCM}:$R26^{eGFP}$ control and Kit^{MCM}:$Gata4^{fl/fl}Gata6^{fl/fl}$: $R26^{eGFP}$ mice. Mice received 2 months of tamoxifen treatment to inactivate the Gata factors, and analysis of cardiac sections showed increased cardiomyocytes expressing m-Tomato from the donor marrow in Kit^{MCM}:$Gata4^{fl/fl}Gata6^{fl/fl}$: $R26^{eGFP}$ hearts compared to controls. These findings further support that deletion of *Gata4/6* within endogenous Kit^{+} CPCs is the source of the increase in fusion between native cardiomyocytes and bone marrow-derived leukocytes observed in these hearts.

Since deletion of *Gata4/6* within Kit^{+} CPCs increased progenitor differentiation toward an endothelial lineage, Maliken *et al.* hypothesized that if they deleted these genes within adult endothelial cells that they would observe weakened vascular permeability since they found greater leukocyte infiltration and fusion to cardiomyocytes. First, they overexpressed vascular endothelial growth factor-A (VEGF-A), a known inducer of endothelial cell permeability, in Kit^{MCM}:$R26^{eGFP}$ mice and observed increased eGFP^{+} cardiomyocytes and leukocyte infiltration. This recapitulated their previous data from the Kit^{MCM}:$Gata4^{fl/fl}$/$Gata6^{fl/fl}$:$R26^{mT/mG}$ experiment where they lineage traced cardiomyocyte fusion events, suggesting that modulating endothelial leakiness was sufficient to increase the appearance of c-Kit^{+} derived cardiomyocytes (17). Next, a tamoxifen-inducible Tie2 Cre mouse line ($Tie2^{CreERT2}$:$R26^{eGFP}$) was used to specifically disrupt *Gata4* in all adult endothelial cells. $Tie2^{CreERT2}$:$R26^{eGFP}$ controls and $Tie2^{CreERT2}$:$R26^{eGFP}$:$Gata4^{fl/fl}$ mice were fed tamoxifen for 2 months and endothelial specific loss of *Gata4* displayed a 2-fold increase in eGFP^{+} adult endothelial cells from increased endothelial cell proliferation. In addition, a shift from CD31^{+}-High expression to CD31^{+}-Low expression was observed, suggesting that Gata4– endothelial cells are less differentiated that negatively altered microvasculature integrity. They also detected increased CD45^{+} leukocyte presence in $Tie2^{CreERT2}$:$R26^{eGFP}$:$Gata4^{fl/fl}$ hearts, which further confirms that vascular permeability has been compromised due to increased leukocyte infiltration (17). This was further confirmed when they bred the Kit^{MCM} allele into $Tie2^{CreERT2}$:$R26^{eGFP}$:$Gata4^{fl/fl}$ mice to determine if increased cardiomyocyte fusion events would occur when compared to Kit^{MCM} or $Tie2^{CreERT2}$ alone. As predicted, Kit^{MCM}: $Tie2^{CreERT2}$:$R26^{eGFP}$:$Gata4^{fl/fl}$ hearts showed significantly increased eGFP^{+} cardiomyocytes and approximately double the number of eGFP^{+} cells from their Kit^{MCM} lineage tracing fusion experiments with loss of *Gata4* in both the adult endothelial cells and Kit^{+} CPCs (17). Finally, $R26^{mT}$ bone marrow transplantation into irradiated $Tie2^{CreERT2}$:$R26^{eGFP}$:$Gata4^{fl/fl}$ recipient mice resulted in increased leukocyte-driven cardiomyocyte fusion. These studies demonstrated that the loss of *Gata4* in adult endothelial cells allows leukocyte infiltration as vascular permeability is increased in this less differentiated endothelium and that this amplified leukocyte-cardiomyocyte fusion events.

Understanding how endogenous progenitors within the heart respond after injury and whether they can be

manipulated into specific cell lineages are critical questions in regenerative medicine. So determining Kit$^+$ CPCs behavior is necessary to advance our current knowledge. Maliken *et al.* used multiple Cre recombinase lines, under the control of *Kit* and *Tie2* promoters, and show that *de novo* cardiomyocytes previously thought to differentiate from *Kit*$^+$ CPCs are the result of leukocyte-fusion and the deletion of *Gata4* enhanced the frequency of these events. Cell fusion, specifically bone marrow cell-derived fusion, is a known phenomenon as BMCs were reported to fuse with neurons, Purkinje cells, and cardiomyocytes (21), so the findings here advances our understanding that Gata factors are important in this process. Future studies could explore whether increasing leukocyte to cardiomyocyte fusion can be beneficial to the native cardiomyocytes, especially after myocardial infarction. Orlic, Beltrami, and Ellison saw that endogenous *Kit*$^+$ CPCs and transplanted *Kit*$^+$ BMCs improved cardiac physiology (8-12). Thus, leukocyte-fusion may impart a cardioprotective phenotype onto the native cardiomyocytes that allows them to survive within the ischemic environment of the infarct. Elucidating if the leukocyte-fused cardiomyocytes plays an important role post-MI would advance our understanding of how cardiomyocytes can be protected after injury and mend broken hearts.

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Footnote

Conflicts of Interest: The authors have no conflicts of interest to declare.

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