clinical trials of bone marrow progenitor cells for cardiac repair have been initiated over the last 2 yr[16,17]. The failure of HSCs to contribute significantly to formation of new cardiomyocytes in the present study may call into question the mechanistic underpinnings of such trials. □

Methods

Isolation of bone-marrow-derived HSCs

Tibias, femurs and iliac crests were collected from MHC–nLAC, MHC–EGFP or β-Act–EGFP mice, crushed in PBS containing 0.1% BSA and filtered through a 40-μm nylon mesh to obtain crude bone marrow. Crude marrow was then fractionated on Histopaque (1.083 g ml^{-1}, Sigma) at 740*g* for 25 min to collect low-density marrow cells from the interface. Both mature and immature haematopoietic cells were depleted from low-density marrow cells by pre-incubation with lineage-specific rat antibodies to murine CD4, CD8, Gr-1, B220 and Mac-1 (Pharmingen) and subsequently labelling with anti-rat IgG microbeads followed by magnetic cell sorting (MACS, Miltenyi Biotech). Briefly, unlabelled progenitor cells were separated from magnetically labelled low-density marrow cells on a column, which was placed in the magnetic field of a MACS separator. The magnetically labelled cells were retained in the column. Cells (Lin-depleted) present in the flow-through were pelleted by centrifugation at 435*g* for 5 min and incubated with c-kit (conjugated with fluorescein isothiocyanate or phycoerythrin, Pharmingen) and/or Sca-1 (conjugated with phycoerythrin, Pharmingen) antibodies and sorted by FACS for Lin$^-$ c-kit$^+$ Sca-1$^+$, Lin$^-$ c-kit$^-$ Sca-1$^+$ or Lin$^-$ c-kit$^+$ cell types.

Coronary artery ligation and intracardiac grafting

This model was performed as detailed previously[19,29]. Briefly, for studies involving stem cells from cardiac-restricted transgenic mice, recipient male and female mice were anaesthetized, supported on a ventilator, their left anterior descending coronary arteries ligated, and their chests closed aseptically. Five hours after ligation, mice were again anaesthetized, intubated, ventilated, and had their hearts exposed as above. The cell suspension was injected directly into the peri-infarcted area of the left ventricular free wall, as indicated in Table 1, using a 27 or 30 gauge needle. Sham-engrafted animals received comparable injections of serum-free medium. Closure and recovery were as above. All grafting experiments were done into histocompatible recipient mice such that no immune suppression was needed. Studies involving stem cells from β-Act–EGFP mice were performed as above, except that the HSCs were injected immediately after coronary ligation.

Histology

Histological methods are detailed in the Supplementary Information. For detection of LacZ reporter activity, hearts were fixed, vibratome sectioned at 300 μm from apex to base, and whole-mount stained with X-gal substrate as described[18]. The sections were then carefully examined under a stereomicroscope for the presence of blue nuclei, a procedure capable of detecting a single positively stained nucleus in a heart[21]. The whole-mount sections were subsequently paraffin-embedded. Immunostaining for sarcomeric myosin heavy chain and sarcomeric actin were performed as previously described[10,30].

Chimaeric embryoid bodies

HSCs (Lin$^-$ c-kit$^+$) were isolated from MHC–nLAC mouse bone marrow and mixed with undifferentiated mouse embryonic stem cells at 1:1, 1:2 and 1:8 ratios. Chimaeric embryoid bodies were formed as detailed in the Supplementary Information. Embryoid bodies were studied by X-gal staining and PCR analysis after 7–10 d of differentiation, when areas of spontaneous beating activity were present.

Bone marrow transplantation studies

Our bone marrow transplant protocol is detailed in the Supplementary Information. Wild-type C57Bl6/J mice were lethally irradiated and rescued by administration of ten million unfractionated bone marrow mononuclear cells obtained from β-Act–EGFP transgenic mice ($n = 13$). Myocardial infarction was performed 8–10 weeks post-transplant, when all animals showed >90% EGFP$^+$ cells in peripheral blood. Mice were killed from 2–10 weeks after infarction and studied by immunostaining of tissue sections or microscopic analysis of enzymatically dispersed cells.

Received 20 October 2003; accepted 1 March 2004; doi:10.1038/nature02446.
Published online 21 March 2004.

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements C.E.M. and L.J.F. thank L. Reinlib for his longstanding support of this collaboration. We thank C. Storey for assistance in sorting HSCs and in bone marrow transplantation, and L. Fernando Santana for assistance with enzymatic dissociation of mouse hearts. These studies were supported in part by NIH grants to C.E.M. and L.J.F., and by the HHMI (G.B., D.A.W.).

Competing interests statement The authors declare competing financial interests: details accompany the paper on **www.nature.com/nature**.

Correspondence and requests for materials should be addressed to C.E.M. (murry@u.washington.edu) or L.J.F. (ljfield@iupui.edu).

Haematopoietic stem cells adopt mature haematopoietic fates in ischaemic myocardium

Leora B. Balsam[1], Amy J. Wagers[2,3], Julie L. Christensen[2,3], Theo Kofidis[1], Irving L. Weissman[2,3] & Robert C. Robbins[1]

[1]*Departments of Cardiothoracic Surgery,* [2]*Pathology, and* [3]*Developmental Biology, Stanford University School of Medicine, Stanford, California 94305, USA*

Under conditions of tissue injury, myocardial replication and regeneration have been reported[1]. A growing number of investigators have implicated adult bone marrow (BM) in this process, suggesting that marrow serves as a reservoir for cardiac precursor cells[2–6]. It remains unclear which BM cell(s) can contribute to myocardium, and whether they do so by transdifferentiation or cell fusion. Here, we studied the ability of c-kit-enriched BM cells,

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Table 1 **Estimated number of GFP$^+$ cells present in injured myocardium**

Animal number	Cell type	Original no. of GFP$^+$cells implanted	Time of death (days)	Estimated number of GFP$^+$ cells at death	Original GFP$^+$ cells present at death (%)
1	c-kit^{Enr}	1×10^6	10	190,430	19.0
2	c-kit^{Enr}	1×10^6	10	471,930	47.2
3	c-kit^{Enr}	1×10^6	30	3,600	0.4
4	c-kit^{Enr}	1×10^6	30	3,000	0.3
5	c-kit^{Enr}	1×10^6	30	0	0
6	c-kit^{Enr}	1×10^6	30	0	0
7	KTLS LT-HSC	4×10^3	10	25,980	649.5
8	KTLS LT-HSC	4×10^3	10	11,820	295.5
9	KTLS LT-HSC	4×10^3	10	16,440	411.0
10	KTLS LT-HSC	4×10^3	30	0	0
11	KTLS LT-HSC	4×10^3	30	0	0
12	KTLS LT-HSC	4×10^3	30	720	18.0
13	Lin$^-$ c-kit$^+$	6×10^5	10	61,800	10.3
14	Lin$^-$ c-kit$^+$	6×10^5	10	64,890	10.8

Lin$^-$ c-kit$^+$ BM cells and c-kit$^+$ Thy1.1^{lo} Lin$^-$ Sca-1$^+$ long-term reconstituting haematopoietic stem cells to regenerate myocardium in an infarct model. Cells were isolated from transgenic mice expressing green fluorescent protein (GFP) and injected directly into ischaemic myocardium of wild-type mice. Abundant GFP$^+$ cells were detected in the myocardium after 10 days, but by 30 days, few cells were detectable. These GFP$^+$ cells did not express cardiac tissue-specific markers, but rather, most of them expressed the haematopoietic marker CD45 and myeloid marker Gr-1. We also studied the role of circulating cells in the repair of ischaemic myocardium using GFP$^+$–GFP$^-$ parabiotic mice. Again, we found no evidence of myocardial regeneration from blood-borne partner-derived cells. Our data suggest that even in the microenvironment of the injured heart, c-kit-enriched BM cells, Lin$^-$ c-kit$^+$ BM cells and c-kit$^+$ Thy1.1^{lo} Lin$^-$ Sca-1$^+$ long-term reconstituting haematopoietic stem cells adopt only traditional haematopoietic fates.

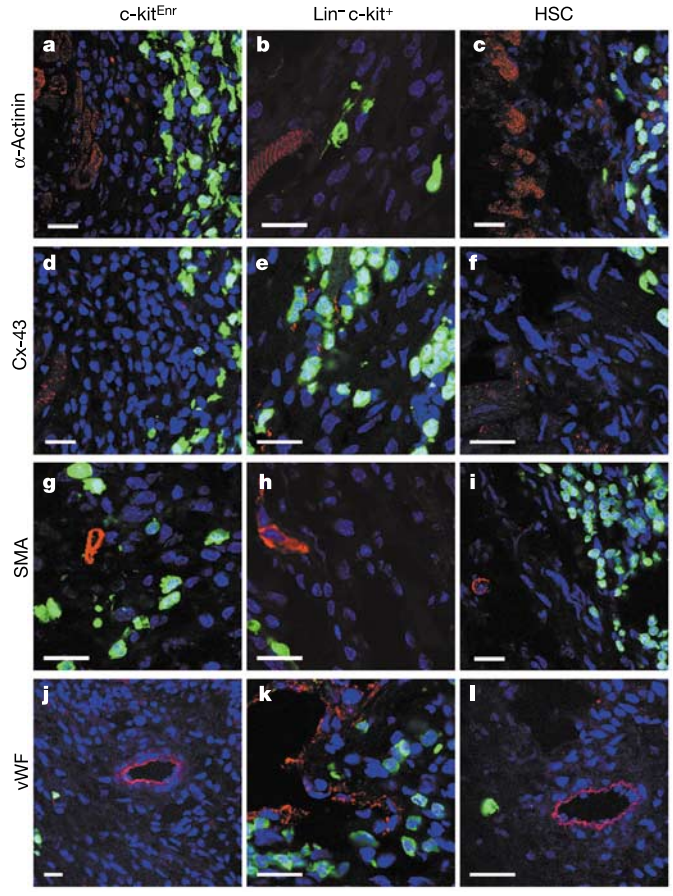

Figure 1 BM cells injected into ischaemic myocardium do not adopt cardiac, smooth muscle, or endothelial phenotypes. Representative confocal micrographs of infarct border zone 10 days after BM cell injection. **a**–**l**, DAPI nuclear staining is shown in blue, GFP expression in green, and tissue-specific markers in red (**a**–**c**, α-actinin; **d**–**f**, connexin-43; **g**–**i**, smooth muscle actin (SMA); **j**–**l**, von Willebrand's factor (vWF)). For panels **a**, **d**, **g**, **j** the donor BM cells were c-kit^{Enr}; for **b**, **e**, **h**, **k** they were Lin$^-$ c-kit$^+$; and for **c**, **f**, **i**, **l** they were purified KTLS LT-HSCs. Scale bar: 20 μm.

A study published by Orlic *et al.*[7] reported that an adult BM population enriched for haematopoietic stem cells (HSCs) (Lin$^-$ c-kit$^+$) transforms into myocardium and supporting vasculature within 9 days after direct injection into the ischaemic heart. To further characterize candidate cardiomyogenic populations within this BM subset, we isolated c-kit-enriched (c-kit^{Enr}), Lin$^-$ c-kit$^+$ and c-kit$^+$ Thy1.1^{lo} Lin$^-$ Sca-1$^+$ (KTLS) long-term reconstituting HSCs (LT-HSCs) from GFP-transgenic donor mice. Wild-type recipient mice underwent ligation of the left anterior descending coronary artery, and shortly thereafter, donor BM cells were injected into the border zone of the ischaemic myocardium. Animals were killed at 10 or 30 days after infarction (Supplementary Fig. 1) to evaluate the presence of GFP$^+$ donor-derived cells. At 10 days after infarction, large numbers of GFP$^+$ cells were present in c-kit^{Enr} ($n = 6$), Lin$^-$ c-kit$^+$ ($n = 2$) and KTLS LT-HSC ($n = 4$) injected hearts. These cells were located primarily in clusters throughout the infarcted tissue and in the bordering peri-infarct zone. At 30 days after infarction, few (if any) GFP$^+$ cells were detected in c-kit^{Enr} ($n = 5$) or KTLS LT-HSC ($n = 4$) injected hearts. The total number of GFP$^+$ cells per heart was estimated in a subset of animals (Table 1). For recipients of c-kit^{Enr} cells, 33.1 ± 19.9% of the original number of injected GFP$^+$ cells were detectable at 10 days, whereas only 0.2 ± 0.2% were noted at 30 days. Recipients of Lin$^-$ c-kit$^+$ cells contained 10.6 ± 0.4% of the original number of cells at 10 days. Recipients of KTLS LT-HSCs contained 452.0 ± 181% and 6.0 ± 10.4% of the original number of cells at 10 and 30 days, respectively. The data suggest that, at least in the case of injected KTLS LT-HSCs, cell division/proliferation may occur in the early time period after intramyocardial injection. However, over time, most of the cells in each group examined either die or migrate elsewhere.

We next performed two parallel experiments. In the first, donor cells (Lin$^-$ c-kit$^+$ or KTLS LT-HSCs) were injected into the myocardium 3–5 h after coronary ligation; in the second, donor cells (c-kit^{Enr} or KTLS LT-HSCs) were injected into the myocardium immediately after coronary ligation. We evaluated the phenotype of the intramyocardial GFP$^+$ cells in each treatment group by standard and laser-scanning confocal microscopy at 10 days, and in some cases, 30 days after infarction. Cardiac tissue was stained with myocyte-specific, smooth-muscle-specific and endothelial-specific antibodies and sections were analysed for potential transdifferentiating GFP$^+$ cells. As in our first study, there were many GFP$^+$ cells, but none of these co-expressed cardiac tissue-specific markers (Fig. 1a–l). GFP$^+$ cells also did not stain with antibodies against

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c-kit, suggesting that c-kit$^+$ LT-HSCs and haematopoietic progenitor cells within the originally injected cell populations had differentiated into alternate cell types. The pan-haematopoietic marker CD45 was co-expressed by many of the GFP$^+$ cells in the c-kit^{Enr} treatment group at both 10 (Fig. 2a–d) and 30 days. At 10 days, the majority of these cells also expressed the granulocyte marker Gr-1 (Fig. 3a–d). In the Lin$^-$ c-kit$^+$ and KTLS LT-HSC treatment groups, all GFP$^+$ cells co-expressed CD45 (Fig. 2e–l) and Gr-1 at 10 days (Fig. 3e–l). At 30 days, rare GFP$^+$ cells co-expressing the B-cell lymphoid marker B220 were also evident in the KTLS LT-HSC treatment group (Supplementary Fig. 2). No CD3-expressing (T-cell lineage) GFP$^+$ cells were detected in the injected myocardium. Taken together, these results demonstrate that after injection into ischaemic myocardium, HSC-enriched Lin$^-$ c-kit$^+$ cells and highly purified KTLS LT-HSCs differentiate exclusively into traditional haematopoietic cell fates, generating myeloid and lymphoid cell types. We did not observe a single case of transformation of these donor cells into cardiac myocytes, smooth muscle cells, or endothelial cells.

Although we found no evidence of myocardial transformation in cell-treated hearts, we hypothesized that cell treatment could still affect animal survival and preserve cardiac geometry and function after infarction. Peri-infarct treatment with c-kit^{Enr} BM cells ($n = 16$) or KTLS LT-HSCs ($n = 8$) did not improve 30-day survival after infarction when compared to control mice treated with saline only ($n = 12$). Survival was 87.5% in mice treated with c-kit^{Enr} cells, 87.5% in mice treated with KTLS LT-HSCs and 91.7% in control mice ($P =$ not significant). Infarct size and cardiac function were compared in a subset of mice treated with c-kit^{Enr} BM cells or saline only (control). Infarct size at 6 weeks after surgery was comparable between cell-treated and control mice (Supplementary Fig. 3). The percentage of left ventricle circumference below the left anterior descending artery ligature comprising scar tissue was 57.2 ± 6.5% in the cell-treated group ($n = 11$) and 59.6 ± 9.6% in the control group ($n = 7$) ($P =$ not significant). Parameters of cardiac geometry and function, including left ventricular chamber dimensions at end-diastole and end-systole, and per cent fractional shortening, were assessed *in vivo* by two-dimensional echocardiography at 2 and 6 weeks after infarction (Supplementary Table 1). At 2 weeks, a trend towards improved fractional shortening and decreased left ventricular chamber dimensions at end-diastole and end-systole was found in cell-treated versus control mice, yet these differences were not statistically significant; however, at 6 weeks, cell-treated mice showed a statistically significant, although modest, improvement in each of these parameters. Invasive haemodynamic parameters measured before death at 6 weeks after infarction did not differ significantly between cell-treated and control mice (Supplementary Table 2). Thus, we conclude that treatment with c-kit^{Enr} BM cells provides some long-term benefit in limiting ventricular dilatation and dysfunction after infarction, but it does not limit overall infarct size. A possible mechanism for this functional improvement could be potentiation of angiogenic activity of endogenous cells by injected c-kit^{Enr} cells.

Several studies have provided support for the hypothesis that BM cells are recruited to sites of cardiac injury through the blood and then transform into myocytes in the injured cardiac environment[3,6]. In the non-ischaemic heart, we (A.J.W., J.L.C. and I.L.W.) found no KTLS LT-HSC-derived myocytes after haematopoietic reconstitution with a single GFP$^+$ KTLS LT-HSC[8]. We also found no evidence of GFP$^+$ cardiomyocytes in wild-type mice that had been joined by parabiosis to GFP-transgenic partners for 6–7 months. Nonetheless, we hypothesized that circulating cells, including BM-derived

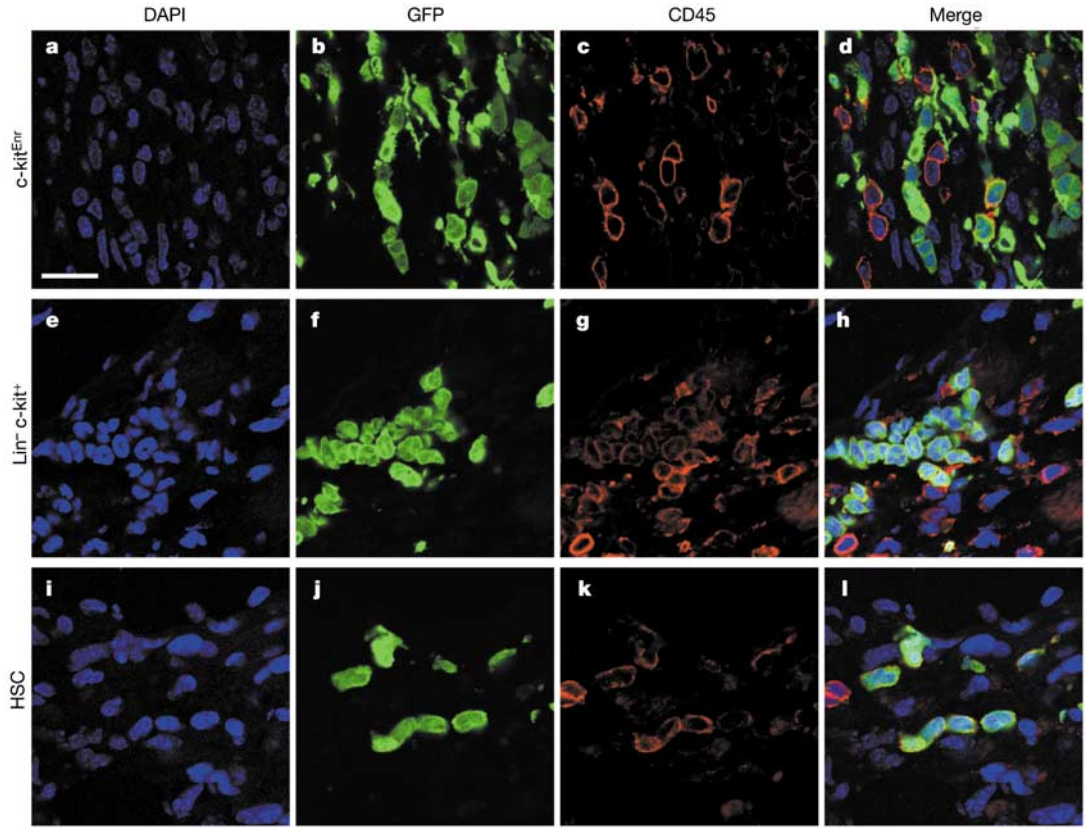

Figure 2 Haematopoietic phenotype of BM cells injected into ischaemic myocardium. **a**–**l**, Representative confocal micrographs of infarct 10 days after BM cell injection. DAPI nuclear staining (blue), GFP expression (green), CD45 expression (red), and merged images are shown. For panels **a**–**d** the donor BM cells were c-kit^{Enr}; for **e**–**h** they were Lin$^-$ c-kit$^+$; for **i**–**l** they were purified KTLS LT-HSCs. Scale bar: 20 μm.

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precursors, may transform into cardiomyocytes if significant tissue injury were present. To test this hypothesis, we used the classical parabiotic mouse model, in which surgically conjoined mice develop an anastomosed vasculature, leading to complete blood chimaerism within 7–10 days after surgery[8,9]. Wild-type mice underwent left anterior descending artery ligation followed by immediate parabiosis to a GFP-transgenic partner. After 8 weeks, parabiotic pairs were killed and both haematopoietic (Supplementary Table 3) and cardiac chimaerism were assessed. Robust cross-engraftment of the blood, spleen and BM occurred in the wild-type partners, with GFP^+ cells representing 43.5 ± 4.6%, 38.1 ± 3.2% and 6.7 ± 2.4%, respectively, of the total tissue cellularity. As described previously[9], cross-engraftment of KTLS LT-HSCs was also evident, with 5.6 ± 1.6% GFP^+ KTLS LT-HSCs present in the BM of non-transgenic parabiotic partners. Cardiac tissue of each wild-type parabiotic partner was assessed for the presence of GFP^+ cells. Scattered GFP^+ cells were present throughout the myocardium and scar; all of these cells expressed CD45 (Fig. 4a–h) and many co-expressed the B-cell marker B220 (Fig. 4i–p). Very rare cells co-expressed the T-cell marker CD3 (Fig. 4q–t). No co-expression of GFP with cardiac or smooth muscle markers was noted. These data suggest that even after cardiac injury, circulating cells do not detectably regenerate myocardium. Alternatively, it remains possible that regenerative cells circulate to the myocardium only early after myocardial injury, and such an event would be detected only if haematopoietic chimaerism were already established at the time of injury.

Recent years have seen tremendous excitement and controversy in the fields of stem cell biology and cardiac regeneration, with several studies suggesting that BM hosts precursors for cardiomyocytes. These studies were originally taken as evidence for trans-differentiation, although more recent studies suggest that cell fusion, rather than transdifferentiation, probably underlies such observations[10–13]. Here we demonstrate that three different populations of BM cells do not differentiate into cardiac muscle or vasculature when injected directly into ischaemic myocardium. Instead, they differentiate into mature haematopoietic lineages. Numerous donor BM-derived cells, many of them myeloid cells, are present in the heart at early time points, but by 1 month after injection, most of these cells are no longer detectable. Given the short half-life of myeloid cells, it is likely that these cells die in the intervening period of time.

Our studies are a logical extension to previous studies suggesting that BM cells contribute to myocardium after cardiac injury. Several studies have reported myocardial regeneration after transplantation in humans[2,14]. Using the Y chromosome as a marker of recipient cells in male recipients of female donor hearts, studies report anywhere from 0.01% to 20% recipient-derived cardiomyocytes. Another study demonstrated cardiac chimaerism of 0.23% in the hearts of female patients that underwent BM transplantation from male donors[5]. Experimental models using BM chimaeric mice provide evidence for contributions of BM-derived cells to cardiomyocytes[3] and suggest that direct fusion of blood lineage cells with myocytes is probably the mechanism by which such contributions occur[12]. Here we extend these findings by injecting a variety of stem-cell-enriched populations directly into injured myocardium. Our results suggest that intramyocardial injection of these cells bypasses important events that occur during BM transplantation into lethally irradiated recipients that are critical for the contribution of these cells, by cell fusion or other mechanisms, to myocardial cell lineages.

It is difficult to reconcile our data with that of Orlic *et al.*[7]. There

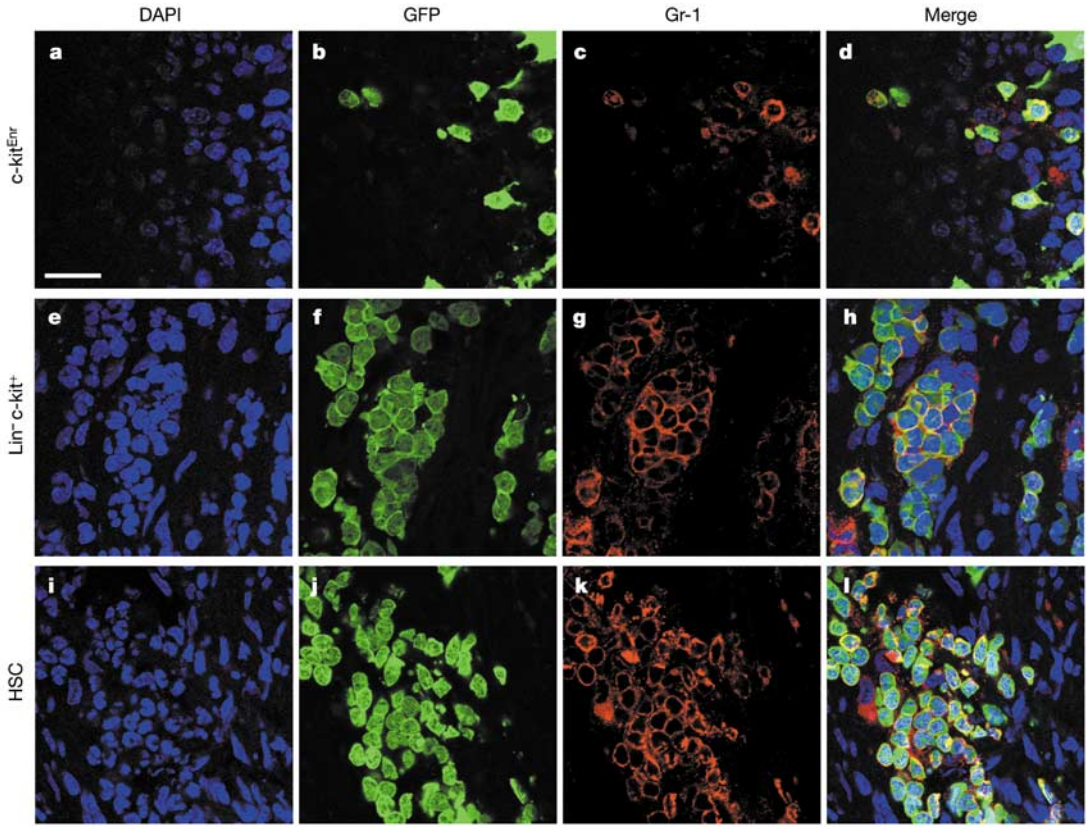

Figure 3 BM cells injected into ischaemic myocardium differentiate into granulocytes. **a**–**l**, Representative confocal micrographs of infarct 10 days after BM cell injection. DAPI nuclear staining (blue), GFP expression (green), myeloid Gr-1 expression (red), and merged images are shown. For panels **a**–**d** the donor BM cells were c-kit^{Enr}; for **e**–**h** they were Lin^- c-kit^+; for **i**–**l** they were purified KTLS LT-HSCs. Scale bar: 20 μm.

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may be differences in our anaesthetic and/or surgical technique, and one may postulate that these resulted in a different outcome. Notably, we were able to detect GFP^+ cells after 10 days in 14 out of 14 animals, whereas their group detected them in only 12 out of 30 animals, attributing this either to technical difficulty in implanting cells into the beating heart or to rejection of male donor cells implanted into female hearts. In our studies, we did not uniformly use gender-specific recipients or donors, with the exception of studies done with Lin^- $c\text{-}kit^+$ cells. In those experiments, females served as recipients of GFP^+ Lin^- $c\text{-}kit^+$ male donor cells. Regardless, in all 14 animals examined at the same 10-day post-infarct time point described by Orlic *et al.*[7], we detected abundant GFP^+ cells. This enhanced capacity for detection of GFP^+ cells may reflect better technique during the injection step or more rigorous analysis of the tissue after harvesting. Consistent with Orlic *et al.*[7], however, we do find that $c\text{-}kit^+$ GFP^+ input cells lose expression of the c-kit marker over time. The previous study noted only two $c\text{-}kit^+$ cells in the harvested tissue. Unfortunately, they do not report staining for additional haematopoietic markers, such as CD45 or lineage-specific blood cell markers.

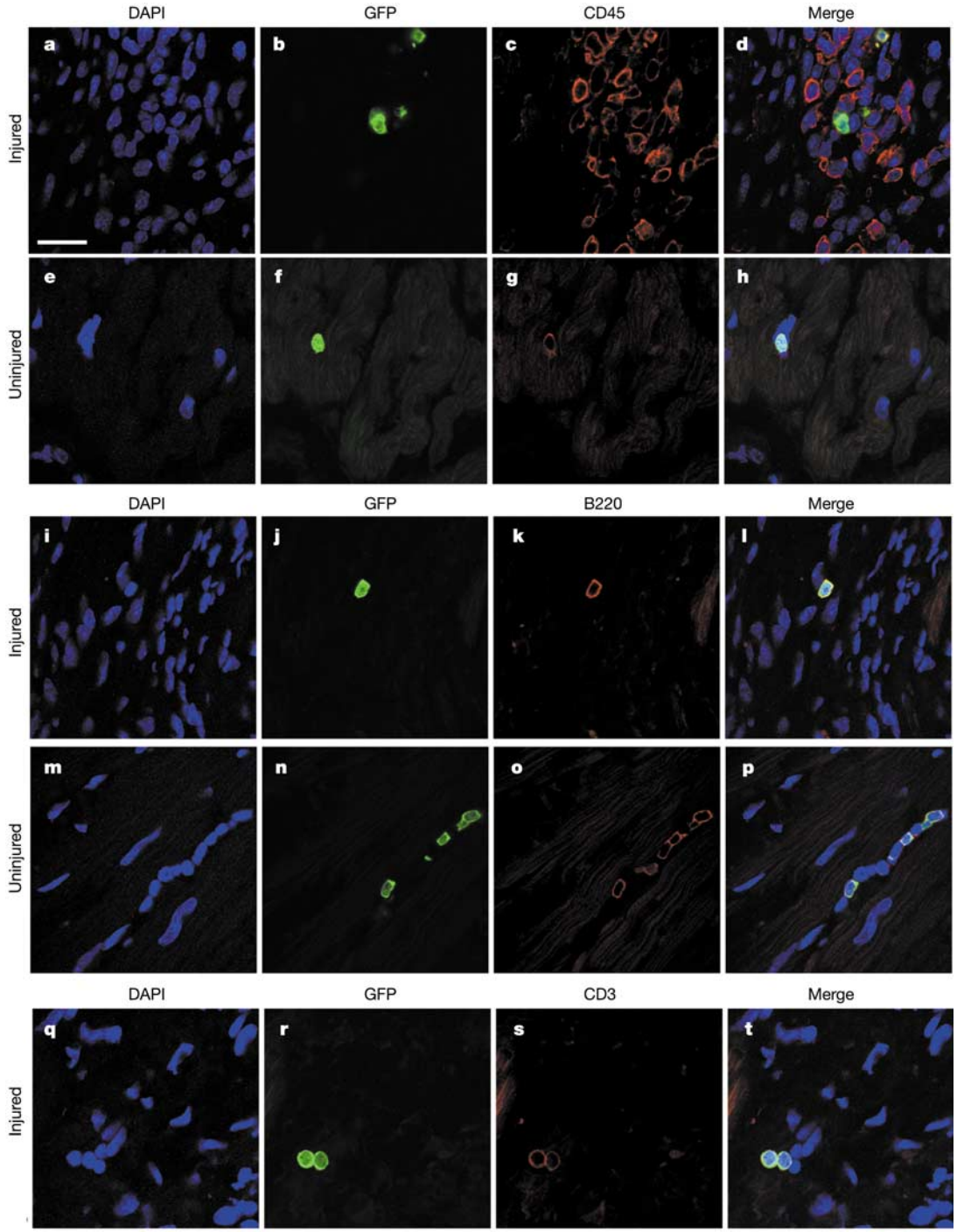

Figure 4 Chimaerism of cardiac tissue in non-transgenic parabiotic partners 8 weeks after cardiac injury and parabiosis. **a**–**t**, Representative confocal micrographs of left ventricular scar (injured area) and healthy left ventricular myocardium (uninjured area). DAPI nuclear staining (blue), GFP expression (green), haematopoietic marker (CD45, B220, or CD3) expression (red), and merged images are shown. Scale bar: 20 μm.

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Our data are of particular importance given the fact that a variety of groups worldwide have initiated clinical trials of BM transplantation into ischaemic myocardium[15,16]. Without additional preclinical experimental data, these studies are premature and may in fact place a group of sick patients at risk. At the very least, many more preclinical experimental data should be collected before such phenomena can be fully understood or clinically exploited. □

Methods

Animals

Eight- to twelve-week-old C57BL/Ka-Thy-1.1 and enhanced GFP-transgenic C57BL/Ka-Thy-1.1 mice were bred and maintained at the Stanford University Research Animal Facility. Transgenic mice were generated as previously described, with GFP expression driven constitutively by the chicken β-actin promoter[17]. All animal procedures were approved by an institutional review board.

Cell purification

BM cells were isolated from GFP-transgenic mice and enriched for c-kit by magnetic cell sorting as described[8]. KTLS LT-HSC or Lin^- c-kit^+ populations were further purified by double fluorescence-activated cell sorting. The antibodies used included 19XE5 (anti-Thy1.1, phycoerythrin or fluorescein isothiocyanate conjugate), 2B8 (anti-c-kit, APC conjugate) and E13-161.7 (anti-Sca-1, Ly6A/E, Texas red conjugate). The cocktail of lineage marker antibodies included KT31.1 (anti-CD3), GK1.5 (anti-CD4), 53-7.3 (anti-CD5), 53-6.7 (anti-CD8), Ter119 (anti-erythrocyte-specific antigen), 6B2 (anti-B220), 8C5 (anti-Gr-1) and M1/70 (anti-Mac-1). These antibodies were produced and purified in the I.L.W. laboratory.

Determination of haematopoietic chimaerism

Parabiotic partners were screened by flow cytometry for blood, spleen and BM chimaerism 8 weeks after surgery as described previously[8].

Myocardial injury model

C57BL/Ka-Thy-1.1 mice were endotracheally intubated and ventilated with a rodent ventilator (Harvard Apparatus). Anaesthesia was maintained with inhalational isoflurane. A thoracotomy was performed and an 8-0 ethilon ligature was placed around the left anterior descending artery just below the atrioventricular border. BM cells (1×10^6 c-kit^{Enr}, 6×10^5 Lin^- c-kit^+, or 4×10^3 KTLS LT-HSCs) or saline were injected into the anterior and posterior infarct border zones. The chest was then closed and the animals were weaned from the ventilator and extubated. Animals were killed at 10 days, 30 days, or 6 weeks after injury.

Parabiosis and myocardial injury model

In these experiments, C57BL/Ka-Thy-1.1 mice underwent left anterior descending artery ligation followed immediately by surgical parabiosis to a GFP-transgenic C57BL/Ka-Thy-1.1 partner. Parabiosis was performed as described previously[8,9]. Parabiotic pairs were housed individually with free access to food and water and were killed after 8 weeks.

Tissue collection and immunofluorescent histology

Hearts were perfused with saline and rapidly excised. They were fixed in 2% paraformaldehyde and cryoprotected in 30% sucrose. Tissue was frozen in optimum cutting temperature compound and stored at −70 °C until sectioning. Immunostaining was performed on 5-μm sections with a panel of cardiac, smooth muscle, endothelial and haematopoietic antibodies. Primary antibodies included rabbit anti-connexin-43 antibody, mouse monoclonal anti-α-actinin antibody, mouse monoclonal anti-smooth muscle actin antibody (all Sigma), sheep anti-von Willebrand's factor antibody (Serotec), rat anti-c-kit antibody (Research Diagnostics), rat anti-Gr-1 antibody, rat anti-CD45 antibody (both BD Pharmingen), rat anti-CD3 antibody, rat anti-B220 antibody, goat anti-GFP antibody (Rockland), and rabbit anti-GFP Alexa Fluor 488 conjugated antibody (Molecular Probes). Texas red conjugated secondary antibodies were used for cardiac (connexin-43, α-actinin), smooth muscle (smooth muscle actin), endothelial (vWF) and haematopoietic primaries (c-kit, Gr-1, CD45, CD3, B220), and fluorescein isothiocyanate conjugated secondary antibody was used for the goat anti-GFP primary. Sections were counterstained with 4,6-diamidino-2-phenylindole (DAPI) and analysed with a Zeiss LSM 510 two-photon confocal laser-scanning microscope.

Estimate of GFP^+ cells in post-infarcted hearts

The number of GFP^+ cells in the myocardium was estimated at 10 and 30 days after surgery in a subset of animals. Fixed hearts were sectioned at a thickness of 5 μm from apex to base on a cryostat. Every 150 μm (that is, every 30th tissue section), the number of GFP^+ cells was counted. Assuming a cell thickness of 5 μm, the total number of GFP^+ cells per heart was estimated as 30 times the total number of GFP cells on all sections sampled.

Calculation of infarct size

Infarct size was calculated in a subset of animals 6 weeks after surgery using computer-based planimetry. Fixed hearts were sectioned into six thick slices from base to apex, beginning at the level of the ligature on the left anterior descending artery. Thin sections from each level were stained with Masson's trichrome stain (Sigma). Infarct size was defined as the sum of the epicardial and endocardial infarct circumference divided by the sum of the total left ventricular epicardial and endocardial circumferences, and was averaged across the six slices.

Echocardiography

Two-dimensional echocardiography was performed at 2 and 6 weeks after surgery in cell-treated (1×10^6 c-kit^{Enr} cells) infarcted animals and saline-treated infarcted animals. Mice were anaesthetized with 2% inhalational isoflurane and imaged with a 15.8-MHz probe connected to a Sequoia C256 echocardiographic machine (Acuson).

Invasive haemodynamics

Closed-chest invasive haemodynamics were measured at 6 weeks after surgery in cell-treated (1×10^6 c-kit^{Enr} cells) infarcted ($n = 8$) and saline-treated infarcted animals ($n = 6$) with a Millar SPR-671 Mikro-tip pressure transducer (Millar Instruments). Mice were anaesthetized with 2% inhalational isoflurane. Recordings were analysed with Sonosoft 3.2.2 software (Sonometrics).

Statistics

Results are mean ± standard deviation. Survival was compared with the log-rank test. Comparisons for functional studies were made using the two-tailed Student's *t*-test. $P < 0.05$ was considered significant.

Received 21 September 2003; accepted 3 March 2004; doi:10.1038/nature02460.
Published online 21 March 2004.

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank G. Hoyt for technical assistance with animal surgery, and V. Mariano and L. Hildalgo for animal care. This work was supported by the Falk Cardiovascular research fund (R.C.R.) and an NIH grant (I.L.W.). L.B.B. was supported by Thoracic Surgery Foundation Nina Starr Braunwald Research Training Fellowship; A.J.W. was supported by an American Cancer Society grant and the Frederick Frank/Lehman Brothers, Inc. Irvington Institute Fellowship; J.L.C. was supported by a NIH Training Grant in Molecular and Cellular Immunobiology; and T.K. was supported by a German Research Society Training Grant.

Competing interests statement The authors declare competing financial interests: details accompany the paper on **www.nature.com/nature**.

Correspondence and requests for materials should be addressed to R.C.R. (robbins@stanford.edu).

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