derived craniofacial and skeletal elements, our work complements early grafting experiments that demonstrated the influence of the mesoderm on epidermal differentiation[9]. □

Methods

Generation of *CK14–Ikka* transgenic mice

A *Hind*III–*Not*I fragment encoding human *Ikka* was subcloned into the *SnaB*I site of pBS-R3, which contains the human *CK14* promoter. The *Sal*I–*Not*I fragment containing a *CK14–Ikka* poly(A) cassette was isolated, purified and microinjected into male pro-nuclei of fertilized C57BL/6 oocytes. The *CK14–Ikka(KM)* construct was similarly generated except that the expression cassette was injected into oocytes isolated from an $Ikka^{+/-}$ intercross. Transgenic founders were identified by PCR analysis and confirmed by immunoblotting. Subsequently, two different lines of transgenic founders were crossed with $Ikka^{+/-}$ mice. $Ikka^{+/-}$ *CK14–Ikka* mice were crossbred to generate rescued knockout mice. $Ikka^{-/-}$ *CK14–Ikka(KM)* mice were identified by genotyping of tissues derived from neonate mice.

Immunoblotting and immunohistochemistry

Protein lysates were prepared from different tissues of $Ikka^{-/-}$ *CK14–Ikka* newborn mice and immunoblotted with an anti-IKK-α antibody (Imgenex) and anti-IKK-β antibody (USB) (Fig. 1b). Protein extracts prepared from primary keratinocyte cultures (5–7 days old) were analysed by immunoblotting with an anti-IKK-α antibody (Imgenex) that recognizes both human and mouse IKK-α (Supplementary Fig. 1) or with an anti-filaggrin antibody (Babco) (Fig. 2b).

Back skin and oesophagus from newborn mice were fixed in formalin and Bouin's solution, respectively. Fixed tissues were paraffin-embedded, serially sectioned at 5 μM and stained with haematoxylin and eosin. Immunohistochemical staining for filaggrin was performed on paraffin sections with anti-filaggrin antibody (Babco). Immunohistochemical staining for IKK-α was performed on 4% paraformaldehyde-fixed cryosections using anti-IKK-α antibody (Cell Signalling) as per the manufacturer's recommendation. Bone and cartilage staining of newborn mice was performed using alcian blue and alizarin red as described previously[28].

In situ hybridization

Whole-mount *in situ* hybridization was performed on 4% paraformaldehyde-fixed E12.5 embryos as described[29]. A digoxigenin-labelled anti-sense mouse FGF8 probe, corresponding to the coding region, was used.

In vitro culture of mouse limb buds

Isolated embryonic limb buds were cultured as described[24] using serum-free, defined BGJB medium supplemented with $0.2\,\mathrm{mg\,ml^{-1}}$ ascorbic acid and penicillin–streptomycin.

Real-time PCR analysis

Real-time PCR was performed as described[2,30]. Total cellular RNA used for this analysis was isolated from limbs of E13 embryos. Cyclophilin mRNA was used to normalize the amount and quality of the RNA.

Received 29 December 2003; accepted 12 February 2004; doi:10.1038/nature02421.

1. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E. & Karin, M. A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. *Nature* **388,** 548–554 (1997).
2. Senftleben, U. *et al.* Activation by IKKα of a second, evolutionary conserved, NF-κB signaling pathway. *Science* **293,** 1495–1499 (2001).
3. Cao, Y. *et al.* IKKα provides an essential link between RANK signaling and cyclin D1 expression during mammary gland development. *Cell* **107,** 763–775 (2001).
4. Hu, Y. *et al.* IKKα controls formation of the epidermis independently of NF-κB via a differentiation inducing factor. *Nature* **410,** 710–714 (2001).
5. Takeda, K. *et al.* Limb and skin abnormalities in mice lacking IKKα. *Science* **284,** 313–316 (1999).
6. Li, Q. *et al.* IKK1-deficient mice exhibit abnormal development of skin and skeleton. *Genes Dev.* **13,** 1322–1328 (1999).
7. Schmidt-Ullrich, R. *et al.* Requirement of NF-κB/Rel for the development of hair follicles and other epidermal appendices. *Development* **128,** 3843–3853 (2001).
8. Hu, Y. *et al.* Abnormal morphogenesis but intact IKK activation in mice lacking the IKKα subunit of the IκB kinase. *Science* **284,** 316–320 (1999).
9. Wessells, N. K. *Tissue Interactions and Development* (Benjamin-Cummings, Menlo Park, California, 1997).
10. Vassar, R., Rosenberg, M., Ross, S., Tyner, A. & Fuchs, E. Tissue-specific and differentiation-specific expression of a human K14 keratin gene in transgenic mice. *Proc. Natl Acad. Sci. USA* **86,** 1563–1567 (1989).
11. Torrey, T. W. *Morphogenesis of the Vertebrates* 275–308 (Wiley, New York, 1970).
12. Takahashi, H., Shikata, N., Senzaki, H., Shintaku, M. & Tsubura, A. Immunohistochemical staining patterns of keratins in normal oesophageal epithelium and carcinoma of the oesophagus. *Histopathology* **26,** 45–50 (1995).
13. Delhase, M., Hayakawa, M., Chen, Y. & Karin, M. Positive and negative regulation of IκB kinase activity through IKKβ subunit phosphorylation. *Science* **284,** 309–313 (1999).
14. Zandi, E., Chen, Y. & Karin, M. Direct phosphorylation of IκB by IKKα and IKKβ: Discrimination between free and NF-κB-bound substrate. *Science* **281,** 1360–1363 (1998).
15. Dudley, A. T. & Tabin, C. J. Constructive antagonism in limb development. *Curr. Opin. Genet. Dev.* **10,** 387–392 (2000).
16. Solloway, M. J. *et al.* Mice lacking Bmp6 function. *Dev. Genet.* **22,** 321–339 (1998).
17. Yi, S. E., Daluiski, A., Pederson, R., Rosen, V. & Lyons, K. M. The type I BMP receptor BMPRIB is required for chondrogenesis in the mouse limb. *Development* **127,** 621–630 (2000).
18. Zou, H. & Niswander, L. Requirements for BMP signaling in interdigital apoptosis and scale formation. *Science* **272,** 738–741 (1996).
19. Winnier, G., Blessing, M., Labosky, P. A. & Hogan, B. L. Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. *Genes Dev.* **9,** 2105–2116 (1995).
20. Webster, M. K. & Donoghue, D. J. FGFR activation in skeletal disorders: too much of a good thing. *Trends Genet.* **13,** 178–182 (1997).
21. Martin, G. R. The roles of FGFs in the early development of vertebrate limbs. *Genes Dev.* **12,** 1571–1586 (1998).
22. Kawakami, Y. *et al.* MKP3 mediates the cellular response to FGF8 signalling in the vertebrate limb. *Nature Cell Biol.* **5,** 513–519 (2003).
23. Minowada, G. *et al.* Vertebrate Sprouty genes are induced by FGF signaling and can cause chondrodysplasia when overexpressed. *Development* **126,** 4465–4475 (1999).
24. Zuniga, A., Haramis, A. P., McMahon, A. P. & Zeller, R. Signal relay by BMP antagonism controls the SHH/FGF4 feedback loop in vertebrate limb buds. *Nature* **401,** 598–602 (1999).
25. Mohammadi, M. *et al.* Structures of the tyrosine kinase domain of fibroblast growth factor receptor in complex with inhibitors. *Science* **276,** 955–960 (1997).
26. Yamamoto, Y. *et al.* A nucleosomal role of IKKα is critical for cytokine-induced activation of NF-κB regulated genes. *Nature* **423,** 655–659 (2003).
27. Welscher, P. *et al.* Progression of vertebrate limb development through SHH-mediated counteraction of GLI3. *Science* **298,** 827–830 (2002).
28. Martin, J. F., Bradley, A. & Olson, E. N. The *paired*-like homeo box gene *MHox* is required for early events of skeletogenesis in multiple lineages. *Genes Dev.* **9,** 1237–1249 (1995).
29. Wilkinson, D. G. *In Situ Hybridization* (Oxford Univ. Press, Oxford, 1993).
30. Chen, L.-W. *et al.* The two faces of IKK and NF-κB inhibition: Prevention of systemic inflammation but increased local injury following intestinal ischemia-reperfusion. *Nature Med.* **9,** 575–581 (2003).

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

Acknowledgements We thank A. Ullrich for the FGF receptor inhibitor (SU5402), T. Kato, M. Delhase and S. Roy for technical assistance and discussions, and M. Ellisman and M. Mackey for the election microscopy performed at the National Center for Microscopy and Imaging Research. Work was supported by grants from the National Institutes of Health, and Superfund Basic Research Program and CERIES research awards.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to M.K. (karinoffice@ucsd.edu).

Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts

Charles E. Murry[1], Mark H. Soonpaa[2], Hans Reinecke[1], Hidehiro Nakajima[2], Hisako O. Nakajima[2], Michael Rubart[2], Kishore B. S. Pasumarthi[2*], Jitka Ismail Virag[1], Stephen H. Bartelmez[3], Veronica Poppa[1], Gillian Bradford[2], Joshua D. Dowell[2], David A. Williams[2*] & Loren J. Field[2]

[1] *Department of Pathology, Box 357470, Room D-514 HSB, University of Washington, Seattle, Washington 98195, USA*
[2] *Wells Center for Pediatric Research, Indiana University, 1044 West Walnut Street, R4 Bldg, Room W376, Indianapolis 46202-5225, USA*
[3] *Department of Pathobiology, University of Washington, Seattle, Washington 98195, USA*

* Present addresses: Department of Pharmacology Dalhousie University, Sir Charles Tupper Medical Bldg, Room 6-F1, 5850 College Street, Halifax B3H 1X5, Canada (K.B.S.P.); Division of Experimental Hematology, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine, 3333 Burnet Avenue, Cincinnati, Ohio 45229-3039, USA (D.A.W.)

The mammalian heart has a very limited regenerative capacity and, hence, heals by scar formation[1]. Recent reports suggest that haematopoietic stem cells can transdifferentiate into unexpected phenotypes such as skeletal muscle[2,3], hepatocytes[4], epithelial cells[5], neurons[6,7], endothelial cells[8] and cardiomyocytes[8,9], in response to tissue injury or placement in a new environment. Furthermore, transplanted human hearts contain myocytes derived from extra-cardiac progenitor cells[10–12], which may have originated from bone marrow[8,13–15]. Although most studies suggest that transdifferentiation is extremely rare under physiological conditions, extensive regeneration of myocardial infarcts

©2004 Nature Publishing Group

was reported recently after direct stem cell injection[9], prompting several clinical trials[16,17]. Here, we used both cardiomyocyte-restricted and ubiquitously expressed reporter transgenes to track the fate of haematopoietic stem cells after 145 transplants into normal and injured adult mouse hearts. No transdifferentiation into cardiomyocytes was detectable when using these genetic techniques to follow cell fate, and stem-cell-engrafted hearts showed no overt increase in cardiomyocytes compared to sham-engrafted hearts. These results indicate that haematopoietic stem cells do not readily acquire a cardiac phenotype, and raise a cautionary note for clinical studies of infarct repair.

A transgenic mouse line in which the cardiac-specific α-myosin heavy chain promoter drives expression of a nuclear-localized β-galactosidase reporter was used to monitor cardiomyogenic transdifferentiation events. These mice, designated as MHC–nLAC, have been used previously as donors for fetal cardiomyocyte transplantation[18], where the robust nuclear β-galactosidase signal readily permitted detection of grafted cardiomyocytes in a wild-type heart after 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) staining (Fig. 1a, b). Bone-marrow-derived haematopoietic stem cells (HSCs) were obtained from the MHC–nLAC mice by first isolating unfractionated marrow cells, and then removing cells expressing differentiated haematopoietic lineage cell-surface markers followed by fluorescence-activated cell sorting (FACS) to isolate cells expressing c-kit. The resulting population of primitive 'Lin$^-$ c-kit$^+$' cells, which are enriched in HSCs, was transplanted into the peri-infarct zone of adult congenic, non-transgenic recipients 5 h after coronary artery occlusion ($n = 42$). Our occlusion protocol typically resulted in infarct sizes of 38 ± 5% of the left ventricle[19]. The Lin$^-$ c-kit$^+$ stem cells were also transplanted into hearts injured by cauterization ($n = 26$), where the volume of injured myocardium is considerably less[20]. Mice were killed (1–4 weeks after infarction; Table 1), and the hearts were fixed and vibratome-sectioned at 300 μm from apex to base. The sections were then stained with X-gal and examined under a stereomicroscope. Despite the ability of this assay to detect a single transplanted fetal cardiomyocyte[21], no blue nuclei were detected in any of the hearts that received stem cell transplants (Fig. 1c and Table 1). Furthermore, immunostaining of these hearts revealed no ectopic expression of sarcomeric myosin heavy chain in the infarcts (Fig. 1d). These observations suggest that the transplanted Lin$^-$-c-kit$^+$ HSCs had not transdifferentiated into cardiomyocytes.

In vitro co-culture experiments were performed to explore further the cardiogenic potential of the Lin$^-$ c-kit$^+$ cells prepared from MHC–nLAC mice. Hanging-drop cultures were used to generate chimaeric embryoid bodies derived in part from non-transgenic mouse embryonic stem (ES) cells and in part from Lin$^-$ c-kit$^+$ HSCs prepared from MHC–nLAC mice ($n = 840$ embryoid bodies). Chimaeric embryoid bodies were also generated by bulk mixing of the ES and HSCs ($n =$ approximately 400 embryoid bodies). A similar approach was used previously to monitor transdifferentiation of adult-derived neuronal stem cells[22]. After 3 days of suspension culture, the embryoid bodies were transferred to adherent surfaces and allowed to grow for an additional 10 days, at which time widespread spontaneous contractile activity was present. The cultures were then fixed and stained with X-gal. Out of the more than 1,000 attached chimaeric embryoid bodies screened, no blue nuclei were identified (Fig. 1e). For each experiment, regions of the dish with contractile activity were microdissected with a Pasteur pipette, and DNA was prepared and subjected to polymerase chain reaction (PCR) amplification (Fig. 1e). The presence of the MHC–nLAC reporter gene was readily detected, indicating that MHC–nLAC HSCs were present but failed to give rise to overt cardiac transdifferentiation events after place-

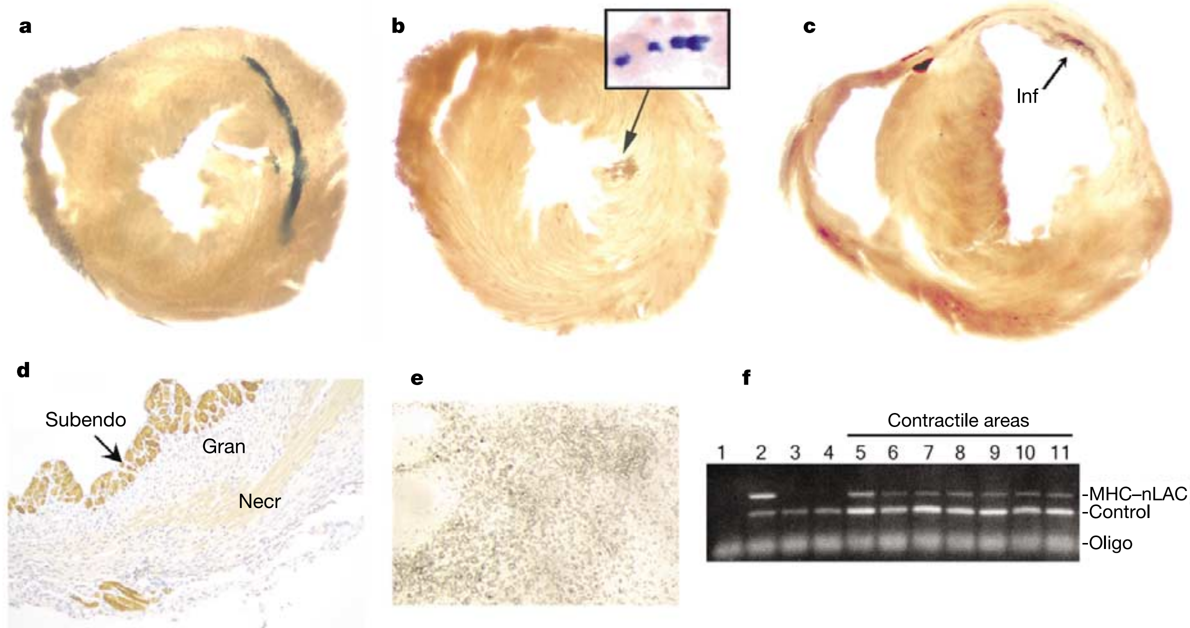

Figure 1 Failure of HSCs from MHC–nLAC mice to activate cardiac reporter genes or express endogenous myosin heavy chain. **a**, **b**, Positive control vibratome sections of hearts engrafted with fetal MHC–nLAC cardiomyocytes showing a large (**a**) and small (**b**) graft. Insert in **b** shows that a small cluster of donor cardiomyocytes is easily detectable despite fibrosis. **c**, X-gal-stained vibratome section from an infarcted heart receiving 100,000 MHC–nLAC Lin$^-$ c-kit$^+$ stem cells. No reporter gene activation is present. Inf, infarct. **d**, Histological section from an infarcted heart receiving 100,000 MHC–nLAC Lin$^-$ c-kit$^+$ HSCs and immunostained for sarcomeric myosin heavy chain. No myosin heavy chain is detected in the infarcted zone. Subendo, spared subendocardial myocardium; Gran, granulation tissue; Necr, unresorbed necrotic myocardium. **e**, Contractile region from a chimaeric embryoid body containing non-transgenic ES cells and MHC–nLAC Lin$^-$ c-kit$^+$ HSCs. No evidence for cardiomyogenic induction is apparent after X-gal staining. **f**, PCR from beating foci in chimaeric embryoid bodies demonstrating the presence of MHC–nLAC Lin$^-$ c-kit$^+$ cells. Lane 1, negative control without DNA; lane 2, positive control from a MHC–nLAC transgenic mouse; lane 3, negative control from a non-transgenic mouse; lane 4, negative control from an embryoid body with non-transgenic ES cells; lanes 5–11, PCR from microdissected contractile regions of chimaeric embryoid bodies containing MHC–nLAC Lin$^-$ c-kit$^+$ HSCs.

©2004 Nature Publishing Group

Table 1 **Summary of intracardiac HSC transplantation data**

Donor cell genotype	Haematopoietic stem cell used	Heart injury	Number of cells transplanted	Graft age at death (days)	Cardiomyogenic events per graft
MHC–nLAC	Lin^- c-kit^+	MI	100,000	14–28	0/42
	Lin^- c-kit^+	Cautery	100,000	7–26	0/26
	Lin^- c-kit^+ Sca-1^+	Cautery	31,000–75,000	7–36	0/11
	Lin^- c-kit^+ Sca-1^+	None	40,000–65,000	1–119	0/9
	Lin^- c-kit^- Sca-1^+	Cautery	17,000–25,000	7–36	0/11
	Lin^- c-kit^- Sca-1^+	None	7,000–37,000	1–119	0/9
MHC–EGFP	Lin^- c-kit^+	MI	100,000	14	0/10
β-Act–EGFP	Lin^- c-kit^+	MI	50,000	7–14	0/27

MI, myocardial infarct.

ment into a surrogate cardiomyogenic developmental field. In other studies, co-cultures were performed using variable inputs of Lin^- c-kit^+ cells from MHC–nLAC mice and embryonic day (E)15 fetal cardiomyocytes from non-transgenic mice. A similar approach was used to monitor transdifferentiation of adult endothelial progenitor cells[23]. The cultures were fixed after 7 days and stained with X-gal. In five independently established co-cultures, no blue nuclei were observed.

Modification of the sorting protocol was used to assess the cardiomyogenic potential of other populations of bone-marrow-derived cells. Lin^- c-kit^+ Sca-1^+ stem cells (a population further enriched in multipotential primitive HSCs) prepared from MHC–nLAC transgenic mice were transplanted into normal ($n = 9$) or cautery injured ($n = 11$) congenic, non-transgenic recipients. No blue nuclei were observed after whole-mount X-gal staining of vibratome sections prepared from these hearts (Table 1). Additionally, Lin^- c-kit^- Sca-1^+ cells prepared from MHC–nLAC transgenic mice were transplanted into normal ($n = 9$) or cautery injured ($n = 11$) congenic, non-transgenic recipients. Once again, no blue nuclei were detected in the recipient hearts (Table 1). Collectively these data suggest that HSCs do not undergo cardiomyogenic differentiation after transplantation into normal or injured hearts.

To rule out the possibility that the bacterial-derived β-galactosidase reporter gene might be subjected to excessive methylation when passed through non-cardiac cell lineages (that is, through the bone marrow), thus resulting in its silencing, additional experiments were performed using HSCs from mice with cardiac-restricted expression of the *Aequorea victoria* enhanced green fluorescent protein (MHC–EGFP mice). In isolated cell preparations, 100% of cardiac myocytes exhibit green fluorescence[24], and similar to the MHC–nLAC mice, the presence of even a single transplanted fetal cardiomyocyte was readily detected in control experiments. Using the same methodology as with MHC–nLAC donor cells, Lin^- c-kit^+ HSCs from MHC–EGFP mice were transplanted at the peri-infarct zone of adult congenic, non-transgenic recipients. Of the ten recipient hearts assayed, none exhibited EGFP

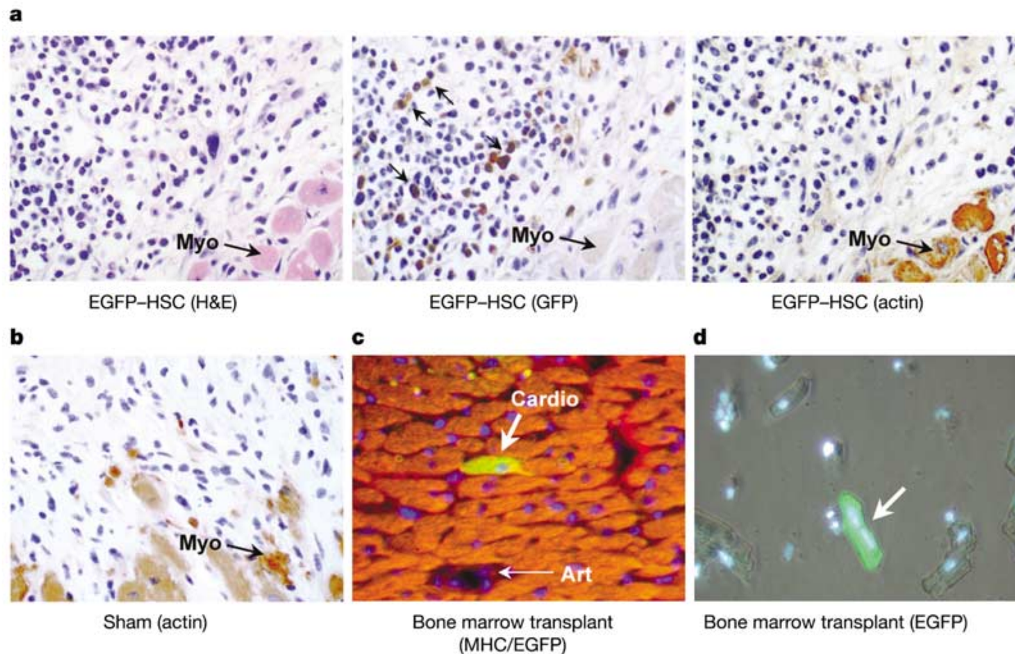

Figure 2 Absence of cardiac differentiation of HSCs after direct injection into infarcts, contrasted with rare transdifferentiation after bone marrow transplantation. β-Act–EGFP mice were cell donors. **a**, Left panel: haematoxylin- and eosin-stained section showing junction of host myocardium (Myo) with granulation tissue of 1-week-old, HSC-injected (EGFP–HSC) infarct. Granulation tissue contains numerous granulocytes and mononuclear inflammatory cells. Middle panel: serial section from the same heart immunostained for EGFP (brown), showing numerous $EGFP^+$ cells dispersed throughout granulation tissue (arrows). Host myocardium (Myo) is unstained. Right panel: serial section from the same heart immunostained for sarcomeric actin (brown). Host myocardium (Myo) is strongly stained, but no sarcomeric actin is present in the region containing EGFP-expressing cells. **b**, Sham-injected heart 1 week after infarction stained for sarcomeric actin (brown). Myocardium (Myo) at infarct border stains strongly, but infarct granulation tissue is negative. **c**, Histological detection of a rare $EGFP^+$ cardiomyocyte (Cardio; yellow) in the peri-infarct region after bone marrow transplantation, shown by immunostaining for α-myosin heavy chain (red) and EGFP (green). Approximately 2–4 such cells were identified per heart. A small arteriole (Art) is unstained. **d**, Rare transdifferentiation event after bone marrow transplantation detected in enzymatically dispersed cardiomyocytes. A single rod-shaped cardiomyocyte contains EGFP (arrow), while multiple other cardiomyocytes are negative.

©2004 Nature Publishing Group

positivity (Table 1). Thus, two independent, cardiac-restricted reporter transgenes failed to demonstrate that Lin$^-$ c-kit$^+$ stem cells undergo cardiomyogenic differentiation after transplantation into infarcted myocardium.

To follow more readily the fate of the transplanted Lin$^-$ c-kit$^+$ stem cells, as well as to test a reporter transgene that uses a constitutively active promoter, additional experiments were performed using transgenic mice in which EGFP was ubiquitously expressed from the chicken β-actin promoter (β-Act–EGFP mice). Lin$^-$ c-kit$^+$ HSCs derived from the β-Act–EGFP mice were transplanted into the peri-infarct zone of 15 adult congenic, non-transgenic recipients. EGFP-expressing cells, identified either by intrinsic fluorescence or by anti-GFP immunostaining, were abundant at 1 week after infarction (Fig. 2a, middle panel) but were much fewer in number at 2 weeks. The EGFP cells were small, round and did not co-localize with regions of sarcomeric actin staining in serial sections (Fig. 2a, right panel). To test whether an immune reaction to EGFP[25] might have prevented detection of transdifferentiation, these experiments were repeated using non-transgenic nude mice as recipients of transgenic Lin$^-$ c-kit$^+$ cells ($n = 12$). Once again, the EGFP-expressing cells were small, round and did not co-localize with sarcomeric actin or myosin staining. Thus, even when a ubiquitously expressed transgene was used to follow cell lineage, no evidence for cardiomyogenic differentiation of HSCs was detected.

Independent of transgenic analysis, the extensive regeneration of the infarct region reported previously by Orlic and colleagues[9] should be detectable in our studies by straightforward histological evaluation. Sections from mice receiving Lin$^-$ c-kit$^+$ cells (with either the MHC–nLAC or β-Act–EGFP transgenes) were compared to sham-injected mice at 1–2 weeks after infarction. All sections revealed changes typical for 1–2-week-old infarcts (Figs 1d and 2a, right panel, b): there was a rim of surviving subendocardial myocardium, patchy amounts of surviving subepicardial myocardium and unresorbed necrotic myocardium that decreased in size from 1–2 weeks. The region surrounding the necrotic zone consisted of typical granulation tissue evolving towards immature scar tissue, and this granulation tissue had only background levels of myosin staining. The actin- or myosin-stained hearts with HSC transplants were indistinguishable from hearts with sham transplants, with no evidence of regenerating myocardium (Table 1). In addition to documenting a failure of the Lin$^-$ c-kit$^+$ cells to undergo cardiomyogenic differentiation, these data also indicate that the presence of the transplanted HSCs does not result in the overt recruitment of endogenous cardiomyogenic stem cells.

Finally, the ability of circulating, bone-marrow-derived cells to give rise to cardiomyocytes after myocardial infarction was tested. Unfractionated bone marrow from β-Act–EGFP transgenic mice was transplanted into lethally irradiated, non-transgenic recipients. Mice were subjected to coronary artery ligation 2 months after engraftment, at which time their peripheral blood leukocytes consisted of >90% EGFP-expressing and thus donor-derived cells. Their hearts were studied from 1 week to 2 months after myocardial infarction, using double-label immunohistochemistry for EGFP and α-myosin heavy chain. Cells with anti-EGFP and anti-cardiac α-myosin heavy chain immune reactivity were detected in the peri-infarct zone (Fig. 2c), albeit only very infrequently (on average only 2–4 cells per heart were detected). As an independent method to ensure that the EGFP signal truly resided in cardiomyocytes, hearts were enzymatically dissociated and studied by fluorescence microscopy. Once again, fluorescent cardiomyocytes were observed at a similarly rare frequency in the dissociated cell preparations (1–3 cells per 100,000 cardiomyocytes; Fig. 2d).

The data presented here collectively suggest that direct injection of HSCs into the mouse heart does not result in *de novo* cardiomyogenic events or tissue regeneration. No lineage-restricted reporter gene activity was observed after injection of MHC–nLAC and MHC–EGFP HSCs into normal or injured hearts, indicating that the cardiac α-myosin heavy chain promoter is not activated in the transplanted cells. This view is supported by the absence of co-localization of EGFP and myosin or actin by immunostaining. Indeed, not a single cardiomyogenic event was detected in the 145 HSC transplants that were analysed. Rather, the implanted cells remained morphologically consistent with haematopoietic cells and decreased in number from 1–2 weeks. Importantly, the presence of bone-marrow-derived cardiomyocytes after bone marrow transplantation and infarction (Fig. 2c, d) suggests that transdifferentiation and/or fusion events can be detected using the methods employed here. The very low level of cardiomyocyte repopulation after bone marrow transplantation in the current study is consistent with the work of Jackson *et al.*[8], who estimated that 0.02% of cardiomyocytes in infarcted hearts arose from bone marrow sources. These data are also in agreement with most of the analyses of chimaerism in transplanted human hearts[12], including our own, where 0.04% of cardiomyocytes originated from extra-cardiac sources[10].

These results contrast with the work of Orlic *et al.*, who reported extensive cardiac regeneration after direct injection of Lin$^-$ c-kit$^+$ cells into infarcts[9]. The basis for this discrepancy is not clear, as considerable care was exercised to reproduce the methods for stem cell isolation and transplantation used by Orlic and colleagues (see the Supplementary Information for a more detailed comparison of the methods used). Nonetheless there still could be subtle differences in the protocols; moreover, differences in trace components in the stem cell preparation might contribute to the differential outcomes. An alternative and perhaps more likely explanation for the discrepant results lies in the different assays used to detect cardiomyogenic differentiation. The study by Orlic and colleagues[9] relied exclusively on immune fluorescence staining to track cell fate and to monitor cell differentiation after HSC transplantation. This approach requires the establishment of signal thresholds, above which cells are designated as positive for a given marker (as, for example, GFP and myosin immune fluorescence). Establishing thresholds in tissues with high levels of nonspecific autofluorescence, as is typically encountered in the infarcted heart, is by nature a subjective process. For this reason, the bulk of the experiments in the current study used transgenic markers of both lineage and phenotype, which have low background and hence are intrinsically less subjective than immunostaining.

The absence of cardiomyocytes with activated reporter transgenes after intracardiac injection of transgenic Lin$^-$ c-kit$^+$ stem cells suggests that cell fusion does not occur after this form of HSC delivery. Fusion of bone marrow cells with host cardiomyocytes in uninjured hearts has been reported recently[14]. Fusion between cardiomyocytes and donor cells was also observed after systemic delivery of genetically labelled adult heart-derived stem cells[26]. It is possible that cell fusion might underlie the apparent transdifferentiation events attributed to circulating bone-marrow-derived stem cells[8,10,12,13,15,27], as well as those observed after intracardiac injection of adult marrow-derived progenitors[28]. The absence of overt fusion events after intracardiac transplantation of HSCs in the current study may reflect differences in the mode of injury, the mode of delivery, and/or intrinsic properties of the stem cells used.

The data presented here did not address the potential beneficial effects of HSC injection on ventricular function after myocardial injury. Rather, the data indicate that Lin$^-$ c-kit$^+$ progenitor cells isolated according to our methodologies fail to undergo overt cardiomyogenic differentiation when transplanted into normal or injured hearts. In this regard, it is possible that the functional benefits observed by Orlic *et al.*[9] resulted from a beneficial impact on left ventricular remodelling and/or angiogenesis, rather than myocardial regeneration. Indeed, it is apparent that cell transplantation can result in improved cardiac function in the absence of donor cell participation in a functional syncytium with the host heart (reviewed in ref. 21). Finally, it is worth noting that several

©2004 Nature Publishing Group

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^- $c\text{-}kit^+$ $Sca\text{-}1^+$, Lin^- $c\text{-}kit^-$ $Sca\text{-}1^+$ or Lin^- $c\text{-}kit^+$ 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^- $c\text{-}kit^+$) 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.

1. Mallory, G. K., White, P. D. & Salcedo-Salgar, J. The speed of healing of myocardial infarction: A study of the pathologic anatomy in 72 cases. *Am. Heart J.* **18,** 647–671 (1939).
2. Ferrari, G. *et al.* Muscle regeneration by bone marrow-derived myogenic progenitors. *Science* **279,** 1528–1530 (1998); erratum *Science* **281**, 923 (1998).
3. Gussoni, E. *et al.* Dystrophin expression in the mdx mouse restored by stem cell transplantation. *Nature* **401,** 390–394 (1999).
4. Lagasse, E. *et al.* Purified hematopoietic stem cells can differentiate into hepatocytes *in vivo*. *Nature Med.* **6,** 1229–1234 (2000).
5. Krause, D. S. *et al.* Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell* **105,** 369–377 (2001).
6. Mezey, E., Chandross, K. J., Harta, G., Maki, R. A. & McKercher, S. R. Turning blood into brain: cells bearing neuronal antigens generated *in vivo* from bone marrow. *Science* **290,** 1779–1782 (2000).
7. Brazelton, T. R., Rossi, F. M., Keshet, G. I. & Blau, H. M. From marrow to brain: expression of neuronal phenotypes in adult mice. *Science* **290,** 1775–1779 (2000).
8. Jackson, K. A. *et al.* Regeneration of ischemic cardiac muscle and vascular endothelium by adult stem cells. *J. Clin. Invest.* **107,** 1395–1402 (2001).
9. Orlic, D. *et al.* Bone marrow cells regenerate infarcted myocardium. *Nature* **410,** 701–705 (2001).
10. Laflamme, M. A., Myerson, D., Saffitz, J. E. & Murry, C. E. Evidence for cardiomyocyte repopulation by extracardiac progenitors in transplanted human hearts. *Circ. Res.* **90,** 634–640 (2002).
11. Quaini, F. *et al.* Chimerism of the transplanted heart. *N. Engl. J. Med.* **346,** 5–15 (2002).
12. Muller, P. *et al.* Cardiomyocytes of noncardiac origin in myocardial biopsies of human transplanted hearts. *Circulation* **106,** 31–35 (2002).
13. Bittner, R. E. *et al.* Recruitment of bone-marrow-derived cells by skeletal and cardiac muscle in adult dystrophic mdx mice. *Anat. Embryol. (Berl.)* **199,** 391–396 (1999).
14. Alvarez-Dolado, M. *et al.* Fusion of bone-marrow-derived cells with Purkinje neurons, cardiomyocytes and hepatocytes. *Nature* **425,** 968–973 (2003).
15. Deb, A. *et al.* Bone marrow-derived cardiomyocytes are present in adult human heart: A study of gender-mismatched bone marrow transplantation patients. *Circulation* **107,** 1247–1249 (2003).
16. Strauer, B. E. *et al.* Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in humans. *Circulation* **106,** 1913–1918 (2002).
17. Assmus, B. *et al.* Transplantation of progenitor cells and regeneration enhancement in acute myocardial infarction (TOPCARE-AMI). *Circulation* **106,** 3009–3017 (2002).
18. Soonpaa, M. H., Koh, G. Y., Klug, M. G. & Field, L. J. Formation of nascent intercalated disks between grafted fetal cardiomyocytes and host myocardium. *Science* **264,** 98–101 (1994).
19. Virag, J. I. & Murry, C. E. Myofibroblast and endothelial cell proliferation during murine myocardial infarct repair. *Am. J. Pathol.* **163,** 2433–2440 (2003).
20. Soonpaa, M. H. & Field, L. J. Assessment of cardiomyocyte DNA synthesis in normal and injured adult mouse hearts. *Am. J. Physiol.* **272,** H220–H226 (1997).
21. Dowell, J. D., Rubart, M., Pasumarthi, K. B., Soonpaa, M. H. & Field, L. J. Myocyte and myogenic stem cell transplantation in the heart. *Cardiovasc. Res.* **58,** 336–350 (2003).
22. Clarke, D. L. *et al.* Generalized potential of adult neural stem cells. *Science* **288,** 1660–1663 (2000).
23. Badorff, C. *et al.* Transdifferentiation of blood-derived human adult endothelial progenitor cells into functionally active cardiomyocytes. *Circulation* **107,** 1024–1032 (2003).
24. Rubart, M. *et al.* Physiological coupling of donor and host cardiomyocytes after cellular transplantation. *Circ. Res.* **92,** 1217–1224 (2003).
25. Stripecke, R. *et al.* Immune response to green fluorescent protein: implications for gene therapy. *Gene Ther.* **6,** 1305–1312 (1999).
26. Oh, H. *et al.* Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proc. Natl Acad. Sci. USA* **100,** 12313–12318 (2003).
27. Kuramochi, Y. *et al.* Cardiomyocyte regeneration from circulating bone marrow cells in mice. *Pediatr. Res.* **54,** 319–325 (2003).
28. Kudo, M. *et al.* Implantation of bone marrow stem cells reduces the infarction and fibrosis in ischemic mouse heart. *J. Mol. Cell. Cardiol.* **35,** 1113–1119 (2003).
29. Reinecke, H. & Murry, C. E. Cell grafting for cardiac repair. *Methods Mol. Biol.* **219,** 97–112 (2003).
30. Reinecke, H., Poppa, V. & Murry, C. E. Skeletal muscle stem cells do not transdifferentiate into cardiomyocytes after cardiac grafting. *J. Mol. Cell. Cardiol.* **34,** 241–249 (2002).

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,

©2004 Nature Publishing Group