Bone Marrow Cells Differentiate in Cardiac Cell Lineages After Infarction Independently of Cell Fusion

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Abstract—Recent studies in mice have challenged the ability of bone marrow cells (BMCs) to differentiate into myocytes and coronary vessels. The claim has also been made that BMCs acquire a cell phenotype different from the blood lineages only by fusing with resident cells. Technical problems exist in the induction of myocardial infarction and the successful injection of BMCs in the mouse heart. Similarly, the accurate analysis of the cell populations implicated in the regeneration of the dead tissue is complex and these factors together may account for the negative findings. In this study, we have implemented a simple protocol that can easily be reproduced and have reevaluated whether injection of BMCs restores the infarcted myocardium in mice and whether cell fusion is involved in tissue reconstitution. For this purpose, c-kit–positive BMCs were obtained from male transgenic mice expressing enhanced green fluorescence protein (EGFP). EGFP and the Y-chromosome were used as markers of the progeny of the transplanted cells in the recipient heart. By this approach, we have demonstrated that BMCs, when properly administered in the infarcted heart, efficiently differentiate into myocytes and coronary vessels with no detectable differentiation into hemopoietic lineages. However, BMCs have no apparent paracrine effect on the growth behavior of the surviving myocardium. Within the infarct, in 10 days, nearly 4.5 million biochemically and morphologically differentiated myocytes together with coronary arterioles and capillary structures were generated independently of cell fusion. In conclusion, BMCs adopt the cardiac cell lineages and have an important therapeutic impact on ischemic heart failure. (Circ Res. 2005;96:127-137.)

Key Words: transdifferentiation • myocardial regeneration • cell fusion

Several studies have suggested that adult bone marrow cells (BMCs) can differentiate into cell lineages distinct from the organ in which they reside.1 The recognition that BMCs maintain some of the growth potential of younger cells has promoted a heated debate about stem cell plasticity and the utilization of BMCs in the treatment of ischemic heart failure.2 The efficacy of BMCs for myocardial regeneration after infarction was documented 3 years ago,3 and this protocol was rapidly applied clinically.4 Nine clinical trials have been completed and several are ongoing and, with the exception of one,5 all other show positive results.4,6–12 Because of the difficulty to demonstrate myocardial regeneration in humans in the absence of cardiac biopsies, three possibilities have been raised in the interpretation of the improvement of cardiac function in patients. They include the development of coronary vessels that rescue hibernating myocardium,11,12 de novo formation of myocytes8,10 and vascular structures4,8,9,12 or the activation and growth of resident progenitor cells via a paracrine effect12 mediated by BMCs. These are important biological and clinical questions that can be addressed experimentally to acquire a better understanding of the relevance of this form of therapy for the human disease. Similarly, the controversy on differentiation of BMCs into cardiac lineages13–16 can be resolved by an accurate and reproducible experimental design complemented by an adequate methodological analysis.

In spite of the therapeutic efficacy of BMCs in heart failure4,6–12 and models mimicking the human disease,3,10,17–19 two studies13,14 and two commentaries15,16 have presented and discussed negative results, criticizing the early experimental data and clinical trials. They question the ability of BMCs to regenerate dead myocardium and claim that the original findings were a collection of artifacts and all clinical trials were premature and “may have in fact place a group of sick patients at risk.”14 Because of the impact that these positive and negative findings have in the future treatment of the postinfarcted heart in humans, we have implemented a simple protocol that can easily be reproduced in laboratories with experience in models of myocardial infarction in small animals. Additionally, we have applied and emphasized the
type of analysis that has to be performed to obtain reliable information. By this approach, we have demonstrated that BMCs differentiate into myocytes and coronary vessels replacing the infarcted myocardium independently of cell fusion.

**Materials and Methods**

An expanded Materials and Methods can be found in an online data supplement available at http://circres.ahajournals.org.

**Results**

**BMCs for Myocardial Repair**

The bone marrow of male mice heterozygous for enhanced green fluorescence protein (EGFP) was collected and the cells were sorted with anti–c-kit coated immunobeads. Immunocytochemically (Figure 1A through 1C), sorted c-kit–positive cells were 63% negative for a cocktail of antibodies recognizing B and T lymphocytes (CD5, CD45R), monocytes and granulocytes (CD11b, Gr-1), neutrophils (7-4), and erythrocytes (TER-119). Similarly, FACS analysis (Figure 1D) showed that the c-kit–positive cells were 52% negative for markers of hematopoietic cell lineages (CD3e, CD11b, CD45R/B220, Gr-1, TER-119). Hematopoietic stem cell and endothelial progenitor cell markers (CD34, Sca-1, flk1) were present in 11% to 47% of the cells (Figure 1E through 1G). Unexpectedly, EGFP was detected in 27% of BMCs (Figure 1H and 1I). Thus, this enriched c-kit BMC population was used here.

**Myocardial Infarction and BMCs**

BMCs were combined with rhodamine-labeled microspheres for the identification of the injection sites. Because EGFP was present in a small fraction of cells, male EGFP-positive BMCs were injected in female infarcted mice so that EGFP and the Y-chromosome were detected in the progeny of the

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**Figure 1.** BMCs and myocardial regeneration. A through C, c-kit–positive cells (A, green) are largely negative for a cocktail of antibodies of hematopoietic cell lineages (B and C, white). D through G, FACS profiles of c-kit–positive BMCs showing markers of hematopoietic cell lineages (D) and antigens of endothelial progenitor cells (E through G). H and I, Detection of EGFP in c-kit–positive cells by immunocytochemistry (H, green) and FACS (I). Bars = 10 μm.
BMCs in vivo. Two injections of $5 \times 10^5$ BMCs each mixed with rhodamine particles were made in proximity of the border zone (Figure 2A through 2F). We knew that the procedure was difficult with an inherent variability in infarct size and a 50% probability of correct injection. The mouse heart beats $\approx 600$ times per minute and has a left ventricular (LV) wall that is less than 1 mm thick. These factors make the injection of cells within the LV wall highly problematic. Thus, the unsuccessfully injected mice (no rhodamine particles) were considered the most appropriate control animals for the successfully treated mice. 5-Bromodeoxyuridine (BrdUrd) was given daily for the recognition of newly formed cells with time.

Thirty-seven infarcted mice were obtained, and 18 of these mice were studied at 5 days and 19 at 10 days after surgery. Rhodamine particles were found in 9 and 11 mice at 5 and 10 days, respectively. Thus, a 54% rate of proper injection was accomplished. Infarct size varied from 15% to 60% in both groups of mice (see online data supplement). Therefore, without an appropriate protocol it is impossible to predict the successful or unsuccessful treatment of animals and the actual size of the infarct in each mouse.

### Myocardial Regeneration and BMCs

Myocardial regeneration was found in each of the 20 infused-treated mice showing rhodamine particles (Figure 2G through 2J). Conversely, myocardial regeneration was not found in the 17 infarcted mice with unsuccessful administration of BMCs as documented by the absence of rhodamine particles. In these cases, the lost myocardium was replaced by collagen (Figure 2K) and there were no EGFP or Y-chromosome labeled cells. This was in sharp contrast to treated infarcts in which the quantity of collagen was minimal and the cells contained in the regenerated myocardium expressed in $\approx 20\%$ to $25\%$ of the cases EGFP and in $\approx 60\%$ to $80\%$ of the cases carried the Y-chromosome (Figure 2L through 2O). EGFP-Y chromosome–positive cells within the infarct were all CD45 negative, indicating that there was no commitment to the hematopoietic lineages (see online data supplement). However, in nontreated infarcts, numerous CD45-positive cells were detected at 5 and significantly less at 10 days (see online data supplement). CD45-positive cells were EGFP and Y-chromosome negative. Thus, consistent with previous results, myocardial regeneration with BMCs attenuates inflammation and myocardial scarring.

In all cases, the regenerated myocardium contained new myocytes that expressed GATA-4, Nkx2.5, MEF2C, $\alpha$-sarcomeric actin, cardiac myosin heavy chain, troponin I, and desmin. Connexin 43 and N-cadherin were detected at 5 days but were more apparent at 10 days (Figure 3A through 3J). Fibroblasts were identified between developing myocytes (see online data supplement). Because BrdUrd was injected daily, most of the new myocytes were labeled by BrdUrd confirming their formation after the injection of BMCs (Figure 4A). Two markers of cell proliferation, Ki67 and MCM5, were used at euthanasia to evaluate cell growth at 5 and 10 days after BMC implantation. A significant fraction of cycling cells was found at both intervals with both markers (Figure 4B and 4C). The presence of EGFP and/or the Y-chromosome offered the unequivocal documentation of the origin of these myocytes from the BMCs. The new myocytes were predominantly mononucleated with a small fraction binucleated (Figure 4D). Conversely, differentiated mouse myocytes are $\approx 94\%$ binucleated and $\approx 5\%$ mononucleated. Thus, these results exclude the contribution of preexisting myocytes to the generation of new myocytes. The volume of new myocytes was $\approx 350$ and $\approx 600$ $\mu$m$^3$ at 5 and 10 days, respectively. Together, $\approx 2.5$ and $\approx 4$ million myocytes were formed within the infarct at 5 and 10 days, respectively (Figure 4E). New myocytes were $\approx 1/40$ of spared myocytes: 23 000 to 25 000 $\mu$m$^3$. In fact, surviving myocytes showed a 16% and 25% hypertrophy at 5 and 10 days after infarction, respectively (Figure 4F). As a result of myocyte formation, infarct size was reduced by 6% at 5 and by 17% at 10 days (Figure 4G and 4H).

The repair of the infarct involved the formation of arterioles and capillaries (Figure 4I through 4N). At 10 days, there were 13±9 mm of arterioles and $98\pm 31$ mm of capillaries per mm$^3$ of new myocardium. For comparison, there are $\approx 10$ arterioles and $\approx 3500$ capillaries per mm$^2$ of tissue in the adult heart. Thus, the size of myocytes and the characteristics of the coronary vasculature were consistent with a rather immature phenotype of the regenerated myocardium. In the spared myocardium of treated hearts, only two EGFP-positive vascular cells were found. At most, only a few vessels were formed by BMCs in the noninfarcted myocardium. Therefore, BMCs appear to acquire the cardiac cell phenotype repairing the infarcted heart.

### Cell Fusion and Myocardial Regeneration

Myocardial regeneration could be the result of fusion of the injected BMCs with existing cells and formation of hybrid cells, the consequence of BMC differentiation and cardiac lineage commitment, or both. Because male BMCs were injected in female infarcted mice, the X- and Y-chromosomes were measured to evaluate whether cell fusion was implicated in de novo myocardial growth. Additionally, DNA content/nucleus was determined. Newly formed myocytes and vascular cells had only one set of X- and Y-chromosomes, whereas two X-chromosomes were detected exclusively in the surviving myocytes (Figure 5A through 5L). There were no cells in the area of cardiac repair that showed Y-chromosome labeling in combination with more than one X-chromosome signal. Also newly formed binucleated myocytes carried only one Y- and one X-chromosome in each nucleus (Figure 5G and 5H). Thus, the regenerated myocytes were of male origin, whereas the spared myocytes retained their female phenotype. Finally, a 2C DNA content was found in each nucleus of noncycling myocytes (see online data supplement) further minimizing the role of cell fusion in myocardial repair.

### Myocyte and Vessel Growth in the Surviving Myocardium

To determine whether the injection of BMCs had a paracrine effect on the spared myocardium, BrdUrd-labeled myocytes were measured in the border and distant region of treated and untreated infarcted hearts. Additionally, the fraction of Ki67-positive myocytes was determined to evaluate the degree of cell replication at death. In both regions, the percentage of BrdUrd- and Ki67-positive myocytes was comparable be-
Figure 2. Myocardial regeneration. A through F, Anatomical images of the heart. Three infarcts are shown (A through C) together with the localization of rhodamine particles (RPs) in A and B (red, arrowheads) and their absence in C. D through F, Injection of RPs and by inference of BMCs in the border zone (D and E, arrowheads). Unsuccessful injection (F). G through J, Transverse section of a treated infarct (MI, arrows) at 10 days. RPs in the border zone (circles) are shown at higher magnification (H and I, white dots). New myocytes within the infarct are identified by α-sarcomeric actin (red); the area in the rectangle is illustrated at higher magnification (J). Regenerated myocytes express, at times, EGFP (yellow-green). K, Collagen type I and III (yellow) in an untreated-infarct. The area in the rectangle is also shown in the inset. Asterisks indicate spared myocytes. L through O, New myocytes in treated-infarcts at 5 (L and M) and 10 (N and O) days express cardiac myosin (red), EGFP (yellow-green), or Y-chromosome (N and O, white nuclear dots) and EGFP. Sarcomere striation in developing myocytes at 10 days (N and O, arrows). G, Bar=1 mm. H through K, Bars=100 μm. L through O, Bars=10 μm.
between treated and nontreated infarcted mice (Figure 6A and 6B). Also, capillary length density was similar in treated and untreated mice (Figure 6C). These data are not consistent with a paracrine effect of BMCs on myocytes and vessels of the noninfarcted portion of the heart.

**Ventricular Function**

Myocardial regeneration did not ameliorate LV end-diastolic pressure, developed pressure and + and −dP/dt at 5 days. However, at 10 days, these hemodynamic parameters were improved in treated-infarcted mice (Figure 6D). These positive effects on LV performance were not observed in untreated-infarcted mice. The improvement in cardiac function with BMCs can only be accounted for by the regeneration of myocardial mass and reduction of infarct size. Whether few or numerous vessels are formed within the infarct, the contractile behavior of this region does not change. Vessels do not contract or generate force; force is developed by myocytes. It is erroneous to assume that regeneration of vessels only can restore contractile activity in the infarcted myocardium.

Figure 3. BMCs adopt the cardiomyocyte fate. A through J, Regenerating myocytes at 5 (A and B) and 10 (C through J) days express in nuclei GATA-4 (A and B, white), Nkx2.5 (C and D, bright blue), and MEF2C (E and F, yellow) and in the cytoplasm α-sarcomeric actin (A, B, G through J, red), cardiac myosin (C and D, red) and troponin I (E and F, red). Connexin 43 between developing myocytes is visible in the insets (G and H; white, arrowheads). N-cadherin is also visible in the insets (I and J; white, arrowheads). Bars=10 μm.
The ability of BMCs to commit to the myocyte lineage has been challenged by negative results and observations suggesting that the identification of GFP in skeletal myofibers is the consequence of autofluorescence. The study performed in Goodell’s laboratory erroneously implies that native GFP fluorescence present in frozen sections of skeletal myofibers can be confused with autofluorescence. This has nothing to do with the detection of the overexpression of EGFP in cardiomyocytes by immunolabeling with specific antibodies.

**Figure 4.** Regeneration of myocytes and vessels. A through D, *Significant vs 5 days. E, Volume distribution of regenerated myocytes at 5 (n=562) and 10 (n=620) days. F, Hypertrophy of spared myocytes in untreated-infarcts (U-MI) and treated-infarcts (T-MI). *Significant vs SO. G and H, Aggregate volumes of tissue components in the new myocardium (G); reduction of infarct size by tissue regeneration (H). The size of the infarct, 31% in U-MI and 33% in T-MI, allowed us to compute the volume of myocardium remaining (R) and lost (L) in the two groups of infarcted mice. The volume of new myocardium (F, solid segments) increased the volume of remaining myocardium (R) and decreased the volume of lost myocardium (L minus F) by the same amount. In treated-mice, infarct size was reduced by 6% at 5 days and by 17% at 10 days. *Significant vs SO. I through N, EGFP-positive cells (I, K, L, and N, green) in the regenerated myocardium express in nuclei the endothelial cell transcription factor Ets-1 (J and K, yellow) and the smooth muscle cell transcription factor GATA-6 (M and N, white), and in the cytoplasm von Willebrand factor (J and K, red) and α-smooth muscle actin (M and N, red). Capillaries, arrowheads. Bars=10 μm.
Figure 5. Newly formed myocytes have one set of X- and Y-chromosomes. A through L, Regenerated myocytes (A through J) have in their nuclei one Y-chromosome (white) and one X-chromosome (magenta). Regenerated binucleated myocytes show in each nucleus only one set of X- and Y-chromosome (G and H). Spared myocytes (K and L) have nuclei each with two X-chromosomes (magenta). EGFP, yellow-green; α-sarcomeric actin, red; nuclei, blue (PI). Bars=10 μm.
anti-GFP antibody performed in this study and previously.\textsuperscript{3,21} It is difficult to understand why this unusual protocol was used\textsuperscript{24} because immunostaining is the standard procedure today. The advantage of the use of the antibody is apparent in the amplification of the signal associated with the expression of the transgene (Figure 7A through 7D). By this approach, the signal-to-background ratio increases dramatically, \textasciitilde300-fold. To confirm the presence of EGFP in the regenerated myocardium, thick sections of the paraffin-embedded tissue that corresponded to the areas of newly formed myocytes and coronary vessels were used for the detection of the EGFP transgene by PCR. A distinct band reflecting the amplified EGFP-DNA sequence was identified (Figure 7E).

It is common practice to stain samples in the presence of a well-established positive and negative control. Moreover, the level of autofluorescence of the formalin-fixed, paraffin-embedded and cut tissue section is always determined and compared with the intensity of the fluorescence signal of the labeled epitope. Also, the background fluorescence generated by staining sections with the secondary antibody only is determined. Our working policy is that the fluorescence signal generated by immunostaining of a structure has to be at least 30-fold higher than that accounted for by background autofluorescence together with secondary antibody unspecific staining (Figure 7F through 7I). Therefore, autofluorescence is not a relevant factor when an adequate protocol of tissue labeling and detection is used.

**Discussion**

**BMCs Mediate Myocardial Regeneration**

In the current study, we document that BMCs differentiate into cardiac cell lineages, reconstitute the dead myocardium after infarction, and improve ventricular function. These positive results appear to occur independently from a paracrine effect of BMCs on the surviving myocardium. Our findings argue strongly in favor of differentiation of the injected cells into the myogenic and vascular lineages as the mechanism of cardiac repair and against cell fusion as the cause of the new cardiac phenotype. The formed cardiac cells in the female infarcted hearts have a male phenotype as the injected BMCs. Cell fusion remains essentially an in vitro phenomenon with few implications in vivo.\textsuperscript{25} Cell fusion in vivo in different organs including the skin, the lung, the brain, and the heart is restricted at most to a few cells which, by inference, have no physiological consequences on baseline function or on tissue repair in pathological states.\textsuperscript{25} Studies of cell fusion in the liver and skeletal muscle are problematic because cell fusion is an inherent aspect of the growth pathway of hepatocytes and myofibers. Under normal physiological turnover, fusion of BMCs with parenchymal cells is an extremely rare event in these tissues.\textsuperscript{25}
There are several other factors that support BMC transdifferentiation rather than cell fusion in myocardial regeneration after infarction. After permanent coronary occlusion, all cells in the supplied myocardium die in less than 5 hours. Essentially, there are no partner cells left for fusion. Adult myocytes have a volume of $20,000 \mu m^3$ and if cell fusion occurred in our conditions, new myocytes should have a volume of at least $20,000 \mu m^3$ or larger. In contrast, these myocytes have a maximum size of $\approx 2000 \mu m^3$ and a minimum size of $\approx 100 \mu m^3$. Donor-derived cells divide rapidly and extensively, whereas tetraploid cells divide slowly and might not divide at all if one of the partners is a terminally differentiated myocyte. Fusion of a BMC to a myocyte that has reached irreversible growth arrest cannot stimulate its reentry into the cell cycle.19

Figure 7. EGFP immunolabeling and detection of the transgene. A through D, The same field of regenerated myocardium is shown without immunostaining (A). Fluorescence signal is the combination of the native EGFP fluorescence (green) and the autofluorescence associated with formalin fixation. Green signal enhanced by 30-fold is shown for the same field in B. In both cases, the EGFP-positive cells are barely detectable. C, When the anti-EGFP antibody is applied the fluorescence signal for the myocytes expressing EGFP is increased $\approx 400$-fold with respect to A. Labeling of myocytes for cardiac myosin is documented in D (red). E, EGFP band is evident in the DNA collected from samples of regenerated myocardium in treated hearts (T). Cells obtained from EGFP transgenic mice were used as a positive control (+) and myocardium obtained from untreated-mice (U) was used as negative control. F through I, Levels of fluorescence signal in the same field of regenerated myocardium under four different conditions. F illustrates the degree of autofluorescence of the section before immunostaining. G, Enhancement of the autofluorescence signal by 50-fold. Fluorescence of H reflects the nonspecific binding of the secondary antibody in the absence of labeling with the primary antibody. I, Intensity of fluorescence signals after labeling with antibodies specific for $\alpha$-sarcemeric actin (red) and the subsequent staining with the appropriate secondary antibody. The Y-chromosome was detected by FISH (white nuclear dots). The real signals are at least 100-fold stronger than the baseline autofluorescence. Bars=10 $\mu m$. 
Cell fusion should generate binucleated myocytes, with one tetraploid and one diploid nucleus, or myocytes with three diploid nuclei. This was not the case.

The cre-lox genetic system is frequently used to detect cell fusion. However, this system is not perfect. It is surprising that the possibility of metabolic cooperation was not considered because this phenomenon may account for some of these observations. By metabolic cooperation, a cell acquires the cytoplasm from a neighboring cell and undergoes excision of the flox-flanked DNA segment in the absence of cell fusion. The exchange of the cytoplasm between the donor cell and the recipient cell occurs through intercellular junctions. Metabolic cooperation is important in a tissue that is functionally a syncytium. Theoretically, Y-chromosome-EGFP-positive myocytes could have resulted from hybrid cells that underwent reductive cell division converting the hyperploid cell to a diploid karyotype, which concealed their fusion history. Reductive cell division of hybrid cells in vivo has only been documented in hepatocytes generated under a stringent selection pressure that conferred them survival and growth advantage. These "pseudodiploid" cells accumulate slowly and are found together with a large number of fused cells. Conversely, we found the 2.5 to 4 million donor-derived myocytes to be diploid with an XY-chromosome complement as early as 5 and 10 days after cell implantation. The short interval between the injection of BMCs and the generation of diploid male cells makes reductive mitosis an unlikely possibility.

Controversy on BMC Transdifferentiation

The current results are consistent with previous observations made in our laboratory in which an enriched population of c-kit-positive BMCs regenerated the infarcted myocardium. Similarly, BMCs and endothelial progenitor cells improve cardiac function in humans. So far, only one negative study has been reported. Moreover, a variety of bone marrow-derived cells capable of differentiating into the cardiac myogenic lineage have been described. It is difficult to reconcile our findings and the clinical and experimental studies with the claim made recently. The most likely possibility is a technical difference in the experimental protocol, identity of the therapeutic cell(s), tissue preparation, and immunocytochemical analysis of the myocardium.

The utilization of frozen tissue samples has severe limitations in terms of the quality of the sections, immunolabeling, and microscopic resolution. The infarct is rarely preserved in frozen sections. Similarly, the 100% degree of success in the injection of cells in the mouse heart has no precedent and will never be matched. Moreover, the lack of changes in LVEDP and an 8% mortality with infarcts of 60% is astonishing. This unusual result has been attributed to a better postoperative care of the animals. Infarct size is a critical determinant of survival in animals and humans. In spite of the perfect care that patients have in the most sophisticated medical centers, a 46% infarct results in intractable heart failure. Rodents are not different although they can survive slightly larger infarcts. The hearts analyzed immunocytochemically for the presence of cardiac regeneration were not the same studied functionally or with routine histology. Thus, whether coronary ligation was unsuccessful or a small or large infarct was obtained was not determined. This is critical because of the complexity of the model and the difficulty of producing an infarct and a correct injection of cells. Most importantly, as shown here, the size of new myocytes averages 500 μm² and cells of this volume would not be recognized by the approach and methodology used in these studies. This is apparent in the micrographs that illustrate clusters of EGFP-positive cells; the EGFP signal is diffuse to the cell cytoplasm and can easily obscure a thin rim of myocyte specific proteins. Double labeling for the EGFP-transgene and myocyte cytoplasmic proteins was never performed on the same section and negative claims were based on only two animals that were supposedly properly infarcted and injected with cells comparable to those used in our early study. The improvement in ventricular function claimed in chronically treated animals in the absence of myocardial regeneration is based on unusual echocardiographic data that are not supported by hemodynamic measurements.

The same limitations can be found in another study in which engraftment of BMCs was observed within the infarct but myocyte formation was considered modest and restricted to the surviving myocardium. In fact, myocyte regeneration in the infarcted region seems to be present in some of the illustrations although the colocalization of β-Gal and GFP indicative of cell fusion was never determined in the same sections. Histochemistry was used to detect β-Gal, and immunolabeling was used for the identification of GFP. Moreover, the utilization of frozen sections precludes the subsequent recognition of small cells of the size of newly formed myocytes. Also, the diffuse localization of CD45 to the cell cytoplasm further questions some of the technical aspects of this report.

Intrinsic genetic markers have been proposed as the gold standard for these types of studies. We believe that the detection of the Y chromosome in newly generated myocytes implemented in the early report and used in this study falls well within this category. Most importantly, any genetic marker requires its subsequent identification by histochemical or immunocytochemical procedures. If limitations exist in these protocols, the powerful genetic markers lead to false collection of data and erroneous interpretations and conclusions. The assumption made by Balsam et al and Murry et al that the technical approach that they have used in the identification and measurement of myoccardial structures is superior to that used in our laboratory does not reflect any scientific reality but the emotional disbelief that BMCs can adopt myocardial cell lineages and repair the injured heart. It is unfortunate that two editorials accompanying the publication of these studies further promoted this negative view.

The esoteric nonphysiological models of parabiosis with complete blood chimerism or with reconstituted EGFP-positive bone marrow were introduced to question the ability of BMCs to acquire a cardiomyocyte lineage. Surprisingly, the negative results were considered of great relevance for the understanding of BMC transdifferentiation. The limitation of the therapeutic potential of circulating BMCs is not new. Therefore, the paradigm offered in these studies defeats any clinical reality and the dramatic problem of ischemic heart failure. If circulating BMCs would have the ability to spontaneously repair damaged organs, infarcts of the heart, brain, skin, kidney, and intestine would be easily recon-
stituted and the majority of current human diseases would not exist. These models are of little value to resolve the controversy at hand and can only add confusion to the confusion. The issue in need of resolution is whether BMCs injected directly in the infarct or in the border zone differentiate into the cardiac cell lineages and contribute to myocardial regeneration.

Over the past 2 years, most of the results claiming hematopoietic stem cell (HSC) plasticity have been questioned because of imprecise identification of the administered cells, incomplete characterization of their differentiated progeny, and/or the formation of hybrid cells by fusion of the donor to the differentiated recipient cells. At the same time, there have been an increasing number of detailed reports documenting the existence of multipotent cells in the bone marrow and, among them, cells able to differentiate into the cardiac myocyte lineage. Why myocardial regeneration from BMCs has been caught in the HSC controversy remains unexplained.

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References

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**Materials and Methods**

**c-kit^POS cells:** Bone marrow was harvested from the femurs and tibias of male transgenic mice expressing EGFP under the control of the β-actin promoter. Cells were suspended in phosphate buffered saline (PBS) containing 5% fetal calf serum and enriched for c-kit by immunomagnetic cell sorting (Miltenyi, Auburn CA). c-kit^POS cells were suspended at a concentration of 1 x 10^5 cells in 5 µl of PBS containing polystyrene microspheres conjugated with rhodamine (Molecular Probes, Eugene, OR). The phenotype of the sorted cell population was defined by FACS analysis and immunocytochemistry.

**Myocardial infarction:** Myocardial infarction was induced in female C57BL/6 mice at 2 months of age as previously described. Three-five hours after infarction, the thorax was reopened and 2.5 µl PBS containing c-kit^POS cells were injected in the anterior and posterior aspects of the viable myocardium bordering the infarct or in two regions within the infarcted area. Infarcted mice in which rhodamine particles were not detected were used as untreated infarcted controls. Sham-operated mice (5 days, n=5; 10 days, n=12) were used as baseline. Animals were sacrificed at 5 and 10 days after surgery. Protocols were approved by Institutional review board.

**Ventricular function:** Mice were anesthetized with chloral hydrate (400 mg/kg body weight, i.p.), and the right carotid artery was cannulated with a microtip pressure transducer (model SPR-671, Millar) for the measurements of left ventricular (LV) pressures and LV + and - dP/dt in the closed-chest preparation. The abdominal aorta was cannulated, the heart was arrested in diastole and the myocardium was perfused retrogradely with 10% buffered formalin. Three tissue sections, from the base to the apex of the left ventricle, were stained with hematoxylin and eosin. Infarct size was measured by the fraction of myocytes lost from the left ventricle inclusive of the interventricular septum.
Cell proliferation and EGFP detection: Animals were injected with BrdU daily and BrdU localization was detected by anti-BrdU (Roche, Minneapolis, MN). Expression of Ki67 and MCM5 in nuclei was evaluated by treating samples with a rabbit polyclonal anti-Ki67 antibody (Novocastra, New Castle upon Tyne, United Kingdom) and mouse monoclonal anti-MCM5 (Serotec, Raleigh, NC).\textsuperscript{1,5,8,9} EGFP was detected with a rabbit polyclonal anti-GFP (Molecular Probes). Myocytes were recognized with rabbit polyclonal anti-Csx/Nkx2.5, goat polyclonal anti-GATA-4 (Santa Cruz, Santa Cruz, CA), goat polyclonal anti-MEF2C (Santa Cruz), mouse monoclonal anti-cardiac myosin heavy chain (MAB 1552; Chemicon, Temecula, CA), mouse monoclonal anti-α-sarcomeric actin (clone 5C5; Sigma, St. Louis, MS), rabbit polyclonal anti-troponin I (Santa Cruz), rabbit polyclonal anti-desmin (Sigma), rabbit polyclonal anti-connexin 43 (Sigma) and rabbit polyclonal anti-N-cadherin (Santa Cruz). Endothelial cells were identified with rabbit polyclonal anti-Ets1 (Santa Cruz) and rabbit polyclonal anti-von Willebrand factor (Sigma). Smooth muscle cells were labeled with rabbit polyclonal anti-GATA-6 (Santa Cruz) and mouse monoclonal anti-α-smooth muscle actin (clone 1A4; Sigma). Nuclei were stained with propidium iodide (PI). Sections were analyzed by confocal microscopy.\textsuperscript{1-9}

X and Y chromosomes: For the FISH assay, sections were exposed to a denaturating solution containing 70% formamide. After dehydration with ethanol, sections were hybridized with the DNA probe Whole Mouse Chromosome Paint (Cambio, Dry Drayton, Cambridge, United Kingdom) for 3 hours.\textsuperscript{1,11} Nuclei were stained with PI.

PCR and EGFP Detection: Sections, 10 µm in thickness, were cut from the heart of 5 mice in which myocardial regeneration was detected by immunohistochemistry. Peripheral blood cells obtained from an EGFP transgenic mouse were used as positive control while heart tissue sections from unsuccessfully injected animals were used as negative control. Tissue sections were de-paraffinized for 30 minutes at 70°C and subsequently immersed in xylene and ethanol at decreasing concentration. Slides were
then washed in distilled water. The regenerated myocardium in the infarcted area and the surviving myocardium were scraped off the slide separately. Genomic DNA was extracted with the QIAamp DNA micro kit according to the manufacturer’s protocol (Qiagen, Valencia, CA). DNA, 200 ng, was mixed with the primers 2R (5’-CACGAACTCCAGCAGGACCAT-3’) and 6F (5’-CAAGACCCCGCGCGAGGT-3’) and amplified for 31 cycles (96°C for 30 sec, 55°C for 45 sec, 72°C for 45 sec). Two µl of the amplified PCR products were subjected to a second nested PCR with the primers 2R and 5R (5’-GGAGGACGGCAACATCCT-3’) for additional 31 cycles at the same conditions. The final PCR products were run in agarose gel for the detection of the EGFP band.

**Fluorescence signals, morphometric measurements and statistical analysis:** It is common practice in our laboratory to establish the degree of autofluorescence for each probe used, each time immunolabeling of structures is evaluated. In the current study, the level of background autofluorescence was 0.58±0.33 and the intensity of the actual fluorescence was 87±31; these values are expressed in intensity units in an 8-bit scale. There was no overlapping between the signals due to background autofluorescence and the signals generated by immunolabeling.

The morphometric procedures utilized here for the determination of infarct size, volume of newly formed and spared myocytes, capillary and arteriolar density have been described previously. All results are presented as mean±SD. The statistical significance between two measurements was determined by the Student's t test, and in multiple comparisons by the Bonferroni method. P <0.05 was considered significant.

**References**


**Legends to Supplementary Figures**

**Figure 1.** Morphometric measurement of infarct size. Number of myocyte lost in each infarct. The percentage of myocyte lost in each case is indicated on the top of each bar. The average number of myocytes in the left ventricle and septum of sham-operated animals is shown. Average infarct size is also included.

**Figure 2.** BMCs do not adopt the hematopoietic fate in the myocardium. A-F, Nuclei (PI, blue) and CD45-positive blood cells (white) in the regenerating (A-D) and non-regenerating (E and F) infarcted myocardium. Most of CD45-negative cells in the regenerating myocardium correspond to small developing myocytes (B and D, cardiac myosin heavy chain; red). The developing myocytes have a volume of ~150-250 µm³ (arrowheads). Yellow-green = EGFP-positive cells. The CD45-positive blood cells...
(white) and the maturing myocytes included in the rectangles of panels A-D are illustrated at higher magnification in the insets. The CD45-positive blood cells (white) in panels E and F are EGFP-negative.

**Figure 3.** Regenerating myocytes are negative for vimentin. A-F, Fibroblasts, identified by the expression of procollagen (A and B, white, arrows) and vimentin (C-F, white, arrows), are distributed between forming myocytes (B, D and F, α-sarcomeric actin; red). Fibroblasts are EGFP-negative while some newly formed myocytes are EGFP-positive (A-F, green, yellow-green).

**Figure 4.** DNA content in newly formed myocytes. Frequency distribution of DNA content in non-cycling (red line) and cycling (green line; Ki67 positive) myocyte and lymphocyte nuclei. Each nucleus in mononucleated and binucleated newly formed myocytes has a 2C DNA content. Similarly, surviving myocytes and lymphocytes showed a 2C population. DNA content greater than 2C was restricted to cycling cells.
Supplementary Figure 1
Supplementary Figure 2A-2F
Supplementary Figure 4