Mechanism of Improved Cardiac Function After Bone Marrow Mononuclear Cell Therapy
Role of Cardiovascular Lineage Commitment

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Background—Cell therapy is a promising option to improve functional recovery after ischemia. Several subsets of bone marrow–derived cells were shown to reduce infarct size and increase ejection fraction in experimental models of ischemia. The mechanisms underlying the functional improvement are diverse and have been shown to include paracrine effects of the injected cells, as well as a variable degree of differentiation to endothelial cells, pericytes, smooth muscle, and cardiac muscle.

Methods and Results—To elucidate the true nature of such plasticity and contribution to recovery, we engineered vectors that encoded inducible suicide genes under the control of endothelium (endothelial nitric oxide synthase)-, smooth muscle (SM22α)-, and cardiomyocyte (α-MHC)-specific promoters, thereby allowing selective depletion of the individual cell lineage acquired by the transplanted undifferentiated bone marrow–derived cells. Lenti-virally delivered thymidine kinase, which converts the prodrug ganciclovir into a cytotoxic agent, was used to selectively eliminate cells 2 weeks after transplantation of bone marrow mononuclear cells in an acute myocardial infarction model. We demonstrate that elimination of transplanted endothelium-committed or SM22α-expressing cells, but not cardiac-committed cells, induced a significant deterioration of ejection fraction. Moreover, elimination of endothelial nitric oxide synthase–expressing cells 2 weeks after injection reduced capillary and arteriole density.

Conclusions—This study demonstrates that elimination of bone marrow mononuclear cells reexpressing endothelial nitric oxide synthase particularly induced a deterioration of cardiac function, which indicates a functional contribution of the vascular cell fate decision of human bone marrow–derived mononuclear cells in vivo. (Circulation. 2010;121:2001-2011.)

Key Words: cell therapy ■ endothelial nitric oxide synthase ■ vasculature ■ angiogenesis-inducing agents
vessels, some of which contributed to the inner layer of endothelial cells, whereas others (eg, Tie2-positive bone marrow–derived cells) were more profoundly detected in the perivascular space, which suggests a pericyte function.5–9 Bone marrow–derived progenitor cells also formed robust and long-lasting functional vascular networks in vivo when tissue engineering approaches were used.10 However, transplant models raised some doubts about whether endogenously mobilized bone marrow–derived cells can indeed form endothelial cells in tumor or arteriogenesis models.11 Even more intensely debated is the question of whether bone marrow–derived cells can acquire a cardiac fate. Although selected murine c-kit–positive or human CD34+ bone marrow–derived cells showed robust engraftment and differentiation of the injected cells in all cardiovascular lineages in some studies,12,13 others failed to detect cardiac-committed bone marrow–derived cells.14

**Clinical Perspective on p 2011**

The different rates of cell retention, survival, and differentiation in the studies may reflect multiple ways to deliver cells,15,16 the use of different progenitor cell subsets,17 and the specific techniques used to detect the cells. In particular, immunofluorescence staining has been accused of yielding false-positive results. However, using bioluminescence imaging with firefly luciferase to detect living engrafted cells, van der Bogt et al17 demonstrated that injected BMCs were still detected up to 6 weeks after injection. The engraftment of BMCs up to 6 weeks after injection was further confirmed by polymerase chain reaction.17 In line with these findings, selective killing of injected cultured endothelial progenitor cells by thymidine kinase (TK) activation 2 weeks after injection was shown to be associated with deterioration of cardiac function,18 which indicates that at least in some of the studies, longer-term engraftment could be detected by different methods. Although these studies do not address the extent and role of cell differentiation, they do support the observation that bone marrow–derived cells are detectable and might exhibit longer-term functions.

To address the extent and functional relevance of cardiovascular cell fate acquisition, we engineered vectors encoding inducible suicide genes under the control of lineage-specific promoters, thereby allowing selective depletion of the individual cell lineage acquired by the transplanted undifferentiated human BMCs. Specifically, we used the endothelial nitric oxide synthase (eNOS) promoter (eNOSp), the transgelin (SM22-α) promoter (SM22p), and the α-myosin heavy chain (α-MHC) promoter (MHCp) to target endothelial cells, smooth muscle cells/fibroblasts, and cardiomyocytes, respectively.

**Methods**

**Cell Culture**

BMCs from healthy volunteers were isolated from bone marrow aspirates by density gradient centrifugation as described previously.19 The majority (>90%) of the isolated BMCs were CD45+, and ~70% expressed the monocyte and endothelial marker CD31. Approximately 50% expressed the lymphocyte marker CD3, and 10% were CD14+. Only a small fraction of cells (<5%) were CD34+, and mesenchymal cells (which express CD73 and give rise to colony-forming-unit fibroblasts) were <2%. Pooled human umbilical vein endothelial cells (HUVECs) and human aortic smooth muscle cells were purchased from Lonza (Verviers, Belgium). Cardiomyocytes were isolated from hearts of neonatal rats by collagenase digestion.

**Lentiviral Vectors and Transduction**

Lentiviral vectors that contained the enhanced green fluorescent protein (eGFP) gene or the viral TK gene of the herpes simplex type II virus and a woodchuck posttranscriptional regulatory element were generated as described previously.18 For lineage tracing, the constitutively active promoter (spleen focus-forming virus promoter) was replaced by the human eNOS promoter (accession number AF387340), the human SM22-α promoter (GenBank accession number FJ858739), and murine α-MHC promoter (GenBank accession number U71441) to control expression of either green fluorescent protein (GFP) or TK. HUVECs, smooth muscle cells, and cardiomyocytes were transduced by the addition of viral supernatant to the specific medium. After 24 hours, medium was changed and a second transduction was performed. To facilitate BMC transduction, dishes were coated with RetroNectin (Takara, Shiga, Japan), and viral supernatant was added for 2 hours at 37°C. After the supernatant was aspirated, the culture dishes were washed with PBS and then plated with freshly isolated BMCs in endothelial growth medium with 20% fetal calf serum. After 2 days, lentiviral transduction was repeated by adding viral supernatant to the plated cells.

Acute myocardial infarction was induced in male nu/nu mice (8 to 10 weeks; Harlan, Indianapolis, Ind) by permanent ligation of the apical branch of the left anterior coronary artery. A total of 1 million BMCs were resolved in 50 μL of PBS intramuscularly at 4 points in the border zone distal to the ligation of the coronary artery immediately after induction of the infarction. Echocardiography was performed with high-resolution echocardiography (Vevo 770 small-animal micro-ultrasound system, VisualSonics, Toronto, Canada).

**Statistical Analysis**

SPSS 11.0.1 (SPSS, Chicago, Ill) was used for statistical analysis of the data. Mortality rates after ganciclovir administration between TK-BMC and GFP-BMC were compared by Kaplan-Meier curves and log-rank analysis. Repeated-measures ANOVA was used for comparison of end-diastolic volume, end-systolic volume, and ejection fraction between GFP-BMC and PBS groups. Continuous data were analyzed by Mann-Whitney test between 2 groups or by 1-way ANOVA test and then Bonferroni post hoc analysis for more than 2 groups. For further details, including protocols for immunostaining, see the online-only Data Supplement.

**Results**

**Establishment of Transduction Protocols**

Given that the composition and specific isolation protocols of the injected cells may influence cell engraftment, BMCs were isolated according to the protocol used in the placebo-controlled, randomized, double-blind, multicenter REPAIR-AMI (Reinfluencing of Enriched Progenitor cells And Infarct Remodeling in Acute Myocardial Infarction) trial, which demonstrated significantly increased left ventricular contractile recovery and improved clinical outcome in the BMC group.19 Lentivirally delivered GFP was used to determine transduction efficiency. Approximately one third of the BMCs expressed GFP after viral transduction (Figure 1a and 1b). To determine which cell types of the heterogeneous BMC population were preferentially targeted by lentiviral transduction, we selected hematopoietic CD34+ progenitor cells, CD14+ myeloid cells, and CD3+ lymphocytes by means of magnetic beads. Although ~40% to 50% of CD34+ and CD14+ cells and >90% of cultured mesenchymal cells expressed GFP, CD3+ lympho-
cytes were not transduced by the vector (Figure 1b). Next, we delivered the suicide gene TK, which activates the prodrug ganciclovir to induce cell death. Ganciclovir induced toxicity in ≈50% of the TK-transduced BMCs and eliminated all outgrowing mesenchymal colonies (Figure 1c and 1d).

**Engraftment and Functional Activity of GFP-Transduced BMCs**

To determine clonal expansion and functional incorporation of lentivirus-transduced BMCs into vasculogenic lineages in vitro, we used a recently described coculture system in which endothelial cells were shown to form vessel-like structures on fibroblast or mesenchymal cells. After GFP-transduced endothelial cells were shown to form vessel-like structures on fibroblast monolayers, GFP-positive vessel-like structures were observed (Figure 2a). We confirmed that these cells expressed an endothelial marker (von Willebrand factor) and were connected with HUVECs (Figure 2b). Likewise, implantation of GFP-transduced BMCs into vasculogenic lineages in vivo revealed that GFP-expressing cells contributed to newly formed pericyte/smooth muscle fused vessels (Figure 2c) and expressed either the endothelial marker von Willebrand factor or the pericyte/smooth muscle marker factor receptor-β (Figure 2d), which demonstrates the vasculogenic characteristics, and only a few (<1%) coexpressed the cardiac marker troponin T.

**Depletion of Total BMCs Reduces Cardiac Function and Vascularization**

To determine the effect of cell ablation on heart function, we injected TK-transduced BMCs (TK-BMCs) or GFP-BMCs in mice immediately after myocardial infarction and induced cell depletion by ganciclovir at 2 weeks after acute myocardial infarction. Administration of ganciclovir selectively increased mortality in mice that had received TK-BMCs, whereas no effect was observed in mice that received GFP-BMCs lacking the suicide gene (P < 0.05 between groups; Figure 4e). Consistent with the induction of mortality in the TK-BMC group, ganciclovir treatment significantly reduced left ventricular ejection fraction within 3 days after ganciclovir treatment in the TK-BMC group, whereas no effect was seen when GFP-BMCs were implanted before treatment with ganciclovir (Figure 4f). Moreover, ganciclovir treatment abrogated the increase in capillary density and smooth muscle cells in the TK-BMC group but not in the GFP-BMC group (Figure...
4g and 4h). As a second control group, we injected TK-transduced HUVECs, which did not improve cardiac function (a finding that is consistent with previous studies\textsuperscript{22,23}). Depletion of TK-HUVECs did not induce a deterioration of cardiac function (Figure 4f).

**Generation of Lentiviral Vectors Allowing Lineage-Restricted Expression of GFP or TK**
To selectively eliminate cells that acquired a cardiovascular cell fate after injection, as indicated by reexpression of endothelial, smooth muscle, or cardiac marker genes, we constructed vectors

**Figure 2. Vasculogenic activity of BMCs.** a and b, GFP-transduced BMCs were cocultured with HUVECs and fibroblasts for 7 days. Representative images of GFP-positive vessel-like structures are shown. b, Higher magnification of the white square shown in panel a. HUVECs were labeled with UEA-1 lectin (Ulex1; red) before coculture. Immunostaining of von Willebrand factor (vWF; white) was performed to detect endothelial cells. c and d, GFP-transduced BMCs and yellow fluorescent protein–transduced HUVECs were transplanted in subcutaneously injected Matrigel plugs in vivo. c, Overview of the explanted plug in bright field (upper panel) and epifluorescence (lower panel). d, Counterstaining of 50-μm sections with GFP (green) and endothelial/smooth muscle/pericyte markers (red) as indicated. αSMA indicates α-smooth muscle actin; PDGFRβ, platelet-derived growth factor receptor-β.

**Figure 3. GFP-transduced BMCs augment functional recovery after acute myocardial infarction.** BMCs were virally transduced with GFP (n=9) and intraoperatively injected into the infarct border zone of nude mice after induction of acute myocardial infarction. Cardiac function was determined in comparison with PBS-injected mice (n=12) with acute myocardial infarction postoperatively and 2 and 4 weeks after BMC injection by high-resolution echocardiography. a, Representative examples of how volume index and ejection fraction were calculated (see the online-only Data Supplement for detail). b, Quantification of end-diastolic volume (EDV), end-systolic volume (ESV), and ejection fraction (EF) postoperatively (postop), at 2 and 4 weeks.
that encoded either GFP or TK under the control of the eNOS (eNOSp), SM22-α (SM22p), and α-MHC (MHCP) promoter (Figure 5a). GFP expression was documented in endothelial cells (HUVECs), smooth muscle cells, and cardiomyocytes when eNOSp-GFP, SM22p-GFP, and MHCp-GFP constructs were transduced, respectively (Figure 5b and 5c). The specificity of the constructs was confirmed by the absence of expression of eNOSp-GFP in smooth muscle cells and of MHCp-GFP in endothelial cells (Figure 5b). Moreover, consistent with the undifferentiated state, only a very few GFP-positive cells were detected in human BMCs transduced with the eNOSp-GFP or MHCp-GFP constructs (data not shown). SM22p-GFP, however, was detected in adherent mesenchymal cells (Figure 5b). When virally delivered TK under the control of the identical promoters was used, ganciclovir induced toxicity in SM22p-TK–expressing BMCs but not in eNOSp-TK– or MHCp-TK–expressing undifferentiated BMCs (Figure 5d). After transplantation of eNOSp-GFP–, SM22p-GFP–, and MHCp-GFP–transduced BMCs in nude mice after acute myocardial infarction, GFP-positive cells were preferentially detectable in the border and
infarct zones, which indicates that environmental cues induced the onset of expression of lineage-committed genes in the transplanted BMCs in vivo (Figure 6a through 6e). Consistent with the endothelial expression pattern of eNOS detected in the developing and adult heart,24 eNOSp-GFP was preferentially observed in capillaries, which were not stained with mouse-specific BS-1 lectin (Figure 6a and 6b). Human-specific von Willebrand factor staining confirmed the incorporation of these endothelial cell–committed GFP-positive cells (Figure 6a and 6b). SM22p-GFP was detected in smooth muscle cells but predominantly in cells that resembled fibroblasts, as confirmed by demonstration of expression of fibroblast-specific protein 1 (Figure 6c through 6e). In the MHCp-GFP group, troponin T–coexpressing cardiomyocytes were detected occasionally (Figure 6f). Overall, SM22-GFP–expressing cells were detectable with the highest frequency in the border zone of infarcted hearts (Figure 6g). Although at a lower incidence, eNOS-expressing BMCs contributed to approximately 40 cells/mm² (which reflects 1.3% of the total number of cells), whereas the absolute number of detectable α-MHC-GFP–positive cells was very low (<0.1% of the total number of cells; Figure 6g).

**Effect of Selective Elimination of Lineage-Committed BMCs In Vivo**

To determine the contribution of the subset of BMCs that reexpressed lineage-specific genes to the maintenance of cardiac function after cell therapy, lineage-specific TK-expressing BMCs were injected in nude mice immediately after myocardial infarction, and TK-expressing cells were eliminated by administration of ganciclovir after 2 weeks. Interestingly, after administration of ganciclovir, the survival of mice transplanted with lineage-committed TK-BMCs was superior to that of the TK-BMC group in which all lineages were traced by the vector (Figure 7a), which indicates that global depletion of all cells had more functional consequences than elimination of 1 of the 3 lineage-committed cell subsets. However, when we measured cardiac function by echocardiography, the eNOSP-TK group showed a profound and highly significant deterioration of left ventricular ejection fraction and a trend toward end-systolic volume expansion from 2 to 4 weeks when ganciclovir was injected at 2 weeks to deplete the cells (Figure 7b through 7d). Depletion of SM22p-TK BMCs also significantly reduced left ventricular ejection fraction, whereas selective killing of MHC-expressing cells did not significantly impair cardiac function, although a trend toward worsening of left ventricular ejection fraction was detectable (Figure 7b and 7c). Analysis of individual regions within the ventricle illustrates that the global reduction of ejection fraction in the eNOSp-TK group was predominantly caused by a deterioration of contractility in the apex and mid region, where myocardial infarction had been induced initially (Figure 7b). Paralleling the profound reduction in contractile function was a significant reduction of capillary and smooth muscle actin–positive vessel density after depletion of eNOS-expressing BMCs, whereas depletion of cardiac-committed MHC-expressing cells did not influence vascularization (Figure 7e and 7f). Surprisingly, elimination of SM22-expressing cells also affected capillary den-
sity, but smooth muscle actin–positive vessels were not significantly reduced either when the total number of smooth muscle actin–positive cells was analyzed or when the small and large arteries were counted selectively (Figure 7f and data not shown). The functional importance of capillary density was further substantiated by the close correlation of left ventricular ejection fraction with capillary density at 4 weeks (Figure 7g).

**Analysis of Bystander Effects**

It is well established that the activated prodrug ganciclovir can elicit an unspecific bystander effect on neighboring cells that may enhance the cytotoxicity of ganciclovir. Although we demonstrated in previous studies that the supernatant of ganciclovir-treated endothelial progenitor cells does not induce a cytotoxic effect on cultured cardiomyocytes or endothelial cells, we did not address the effect of ganciclovir-
induced cell death on physically associated cocultured cells. Therefore, we cocultured TK-expressing cells with nontransduced cells and induced cell death in the TK-expressing cells by adding ganciclovir. When 10% of TK-expressing HUVECs were cultured with 90% of GFP-transduced HUVECs (1:9 ratio), cell viability dropped to 30%, which indicates that roughly 60% of the HUVECs were killed by bystander effects (online-only Data Supplement Figure Ia and Ib). However, when TK-transduced progenitor cells were cocultured with HUVECs at a ratio of 1:9, viability of HUVECs was not affected after addition of ganciclovir (online-only Data Supplement Figure Ic and Id). Likewise, we performed an additional coculture experiment using a modified in vitro vascular tube formation assay as described above by adding TK-transduced CD14+ cell-derived macrophages to yellow fluorescent protein–transduced HUVECs (1:9 ratio) on fibroblast monolayers. Yellow fluorescent protein–transduced HUVECs produced well-organized vessel-like structures, as shown in Figure Ie and If of the online-only Data Supplement. When we depleted macrophages by addition of

**Figure 7.** Lineage-specific depletion of transplanted BMCs. a, Mice with acute myocardial infarction were treated with BMCs that had been transduced with the respective constructs. At 2 weeks, all mice were treated with ganciclovir. The graph illustrates the percentage of surviving mice at 4 weeks (2 weeks after ganciclovir treatment was begun; data are shown as delta 4 weeks to 2 weeks). *P < 0.05 vs GFP-BMC (χ² test). b, Ejection fraction (EF) at 4 weeks was determined in the apex and midventricular region as indicated in Figure 3. GFP-BMC: n = 8, eNOSp-TK: n = 9, SM22ap-TK: n = 9, aMHCP-TK: n = 6. c and d, Global EF (c) and end-systolic volume (ESV; d) at 2 weeks (immediately before onset of ganciclovir treatment) and 4 weeks. P values were calculated with paired t test. e and f, Automatic quantification of lectin-perfused vessel area (e) and the number of smooth muscle actin (SMA)–positive vessels (f) in the border zone at 4 weeks. Mice were treated with lineage-restricted TK-transduced BMCs and ganciclovir treatment after 2 weeks. n = 5 each. g, Correlation of EF and capillary area detected in the border zone at 4 weeks.
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with either acute or chronic myocardial ischemia\(^2\) and \(^3\) BMCs specifically increased coronary blood flow reserve and depletion of eNOS-expressing cells abrogates the enhanced previous findings; however, the demonstration that specific ejection fraction (Figure 7g). Thus, the finding that BMCs correlationship between capillary density and left ventricular support these early observations by demonstrating a strong correlation. First evidence that capillary density may influence cardiac function in acute myocardial infarction after BMC transplantation in an acute myocardial infarction model. Specifically, depletion of eNOS-expressing cells was associated with a reduction of capillary and arteriole density and induced a deterioration of regional and global left ventricular ejection fraction. The depletion of cells that expressed SM22-\(\alpha\), a marker expressed in smooth muscle cells but also in fibroblasts,\(^2\) induced a deterioration in contractile function, whereas the elimination of cells that expressed the cardiac myocyte marker \(\alpha\)-MHC did not significantly affect cardiac function.

Genetic studies using transgenic eNOS\(p\) reporter mice have demonstrated that eNOS expression is preferentially detected in the endothelial lining of capillaries and arteries.\(^2\) Consistently, BMCs transduced with eNOS\(p\)-driven GFP constructs have been shown to be incorporated in capillaries. The demonstration that depletion of eNOS-expressing cells induced the most severe cardiac dysfunction suggests that the mechanism underlying the therapeutic improvement of cardiac function in acute myocardial infarction after BMC administration preferentially involves effects on vascular function and neovascularization. Various studies demonstrated that therapeutic application of total BMCs (or selected isolated hematopoietic or mesenchymal progenitor cell subsets thereof) improves blood flow recovery and capillary density after ischemia.\(^5\) \(^7\) \(^26\) In particular, injected CD34+ and endothelial progenitor cells increased neovascularization and cardiac function in experimental models.\(^1\) In clinical studies, BMCs specifically increased coronary blood flow reserve and reduced vascular resistance in the target vessel in patients with either acute or chronic myocardial ischemia\(^27\) \(^28\) and ameliorated the symptoms of intractable angina,\(^29\) which suggests that bone marrow–derived cells target the vasculature. First evidence that capillary density may influence infarct size was provided in 1986 by Olivetti et al.,\(^30\) who showed that infarct size 30 days after ligation of the coronary artery is directly correlated with the number of capillaries in the infarct border zone. The results of the present study support these early observations by demonstrating a strong correlation between capillary density and left ventricular ejection fraction (Figure 7g). Thus, the finding that BMCs enhance capillary density and that this contributes at least in part to the functional benefit observed is consistent with previous findings; however, the demonstration that specific depletion of eNOS-expressing cells abrogates the enhanced capillary density induced by BMCs comes as a surprise, because this would imply that the majority of the effects of BMCs are mediated by the formation of capillaries via differentiation into eNOS-expressing endothelial cells in vivo. Although we could clearly demonstrate a vasculogenic activity of BMCs in vitro, in Matrigel plug assays in vivo, and in the infarct border zone, bone marrow–derived cells (particularly endothelial progenitor cells) also promote angiogenesis by releasing paracrine factors.\(^31\) \(^34\)

One explanation for the profound deterioration in left ventricular function might be that the activated cytotoxic form of ganciclovir might have induced cell death in physically connected neighboring cells, thereby enhancing the effects. Indeed, such bystander effects are well established and are even used to enhance the efficiency of tumor cell depletion.\(^33\) To address the relevance of bystander effects, we performed 2 control experiments in vivo. In the first experiment, we investigated the effect of depleting BMCs after injection in noninjured hearts; however, induction of suicide in TK-BMCs after injection in noninjured hearts did not affect cardiac function (online-only Data Supplement Figure II). Second, we evaluated the effect of depleting transplanted mature vascular cells; however, depletion of TK-transduced HUVECs did not alter cardiac function (Figure 4f), which indicates that the putative toxic effects induced by simply killing any transplanted cell are likely not the cause of the deterioration of function after depletion of BMCs.

In addition, we performed in vitro studies to more precisely determine the influence of suicide induction on neighboring cells. In these coculture assays, profound bystander effects were only detected when we cocultured suicide gene–loaded HUVECs with unmodified HUVECs, whereas coculture in the same 1:9 ratio of TK-loaded progenitor cells or macrophages with HUVECs did not profoundly affect cell death or network formation in our experience. This might be explained by the fact that HUVECs are well connected with each other in cell culture by gap junctions, which allows for the very efficient transfer of the cytotoxic drug. Progenitor cells or macrophages, however, appear less well connected with HUVECs in this in vitro assay. Furthermore, the number of terminal dUTP nick end-labeling–positive apoptotic cells in ganciclovir-treated TK-BMC–injected mouse hearts 3 days after induction of cell death by ganciclovir infusion did not exceed the number of GFP-positive cells detected in the control mice. Although this time point only represents a snapshot and does not allow us to calculate the exact sum of cells killed over the 3 days of ganciclovir injection, these data exclude a massive induction of unspecific cell death in the border zone. Moreover, the deterioration of cardiac function was more severe in the eNOS-TK group than in the SM22-TK group after ganciclovir treatment, although the number of SM22-expressing cells was 3 times as high as the number of eNOS-expressing cells. If induction of cell death, either by providing a “kiss of death” to neighboring cells or by inducing an inflammatory response, had caused a deterioration in function independent of cell fate and function, one would have expected to see a more profound response after depletion of SM22-expressing cells compared with eNOS-expressing cells; however, the present results showed a rather...
selective effect on regional functional recovery after depletion of eNOS-expressing cells in the apex and border zone of the infarcts that was more profound than the depletion of SM22-expressing cells.

Taken together, these data do not exclude bystander effects; however, they suggest that putative bystander effects are unlikely to account for the difference detected between the groups. Actually, it was surprising that the number of smooth muscle–positive vessels was reduced significantly after eNOS-expressing cells were eliminated and was only partially reduced after depletion of SM22-expressing cells. This might be related to the fact that in the present experiments, cells that expressed GFP under the control of the SM22 promoter did not contribute to the formation of entire new arterioles and were rarely detected in the smooth muscle layer of arteries but were incorporated preferentially around smaller capillaries in a perivascular location, or they phenotypically resembled fibroblasts. Fibroblasts are emerging as key components of normal cardiac function but also can contribute to pathological processes.36 Interestingly, recent data demonstrate that embryonic fibroblasts can induce cycling of cardiac myocytes.37 Therefore, the phenotypic and functional characterization of the bone marrow–derived newly generated fibroblasts deserves further detailed investigation.

Finally, the present data demonstrate that the depletion of BMCs that reexpress α-MHC does not induce a significant change in the global and regional function of the heart. This is consistent with the rather small number of cardiomyocyte–committed cells seen after injection of GFP-transduced total BMCs or by detection of GFP in BMCs that had been transduced with a vector to express GFP under the control of the α-MHC promoter. The number of cardiac-committed cells that may have derived from differentiation or fusion events in the present study (<1%) is in the middle of the large spectrum of published data showing no cardiac commitment at all versus a high level of cardiac commitment after injection of bone marrow–derived progenitor cells. The extent of cardiac commitment in the present study was not sufficient to induce a significant response by depleting these cells.

In summary, the present data provide further mechanistic insights into the role of lineage commitment of injected human BMCs for functional regeneration after acute myocardial infarction. These data do not preclude the contribution of paracrine factors, which surely contribute to the early and probably also late beneficial effects of injected cells. However, the specific deterioration of cardiac function and reduced vascular density caused by elimination of eNOS-expressing cells suggests that this population of cells particularly contributes to the effects observed at later time points. The present study intentionally used a clinically applied preparation of BMCs that has been shown to provide a beneficial effect in patients treated with this cell preparation.19 Although the present study is thus designed to provide insights into the mechanisms of a clinically used cell population, it has several limitations. First, we cannot entirely exclude indirect off-target effects caused by the induction of cell death, although we believe that our data showing a distinct response in cardiac function and vascularization after depletion of either eNOS- or SM22-expressing cells suggest that putative bystander effects are unlikely to fully account for the results of the present study. Second, the use of human cells requires the use of either immunodeficient or immuno-compromised animals, which may have impacts on cell integration, survival, and differentiation activity. Despite these limitations, the present study is the first attempting to elucidate the functional contribution of the cardiovascular cell fate decisions of clinically applied human cells in vivo.

Acknowledgments
We thank M. Muhly-Reinholz for expert technical assistance and Stefan Janssens for providing the α-MHC plasmid.

Sources of Funding
The project was supported by the Foundation Leducq, by the Deutsche Forschungsgemeinschaft (Exc 147/1), by the FP6 program contract HeartRepair (LSHM-CT-2005-018630) of the European Community, and by Angiosciff.

Disclosures
Drs Dimmeler and Zeiher are founders and advisors of Tueure GmbH.

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CLINICAL PERSPECTIVE

Cell therapy with bone marrow–derived mononuclear cells is a promising option to improve functional recovery after acute myocardial infarction; however, the mechanism underlying the functional improvement of cell therapy is unclear. Here, we demonstrate that selective depletion of committed-vascular cells that express endothelial nitric oxide synthase results in deterioration of cardiac function and a reduction of capillary density, which indicates that vascular-committed cells contribute to the beneficial effects of injected bone marrow–derived mononuclear cells.
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_Circulation_. 2010;121:2001-2011; originally published online April 26, 2010; doi: 10.1161/CIRCULATIONAHA.109.909291
_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/121/18/2001

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Supplemental Material

1. Supplemental Methods

Cells
Bone marrow mononuclear cells (BMC) from healthy volunteers were isolated from bone marrow aspirates by density gradient centrifugation with Biocoll (Biochrom AG, Germany) as previously described (Schachinger et al., 2006). The protocol has been approved by the Ethical committee and written informed consent was provided by each donor. Pooled HUVECs and human aortic smooth muscle cells were purchased from Lonza and were cultured in specific media provided by the company. HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% FCS. Cardiomyocytes were isolated from hearts of neonatal rat using collagenase and preplating to exclude fibroblasts. Cell culture was maintained in DMEM (GIBCO) with glutamax + 20% M199 medium + 8% inactivated FBS.

Preparation of lentiviral stocks
Self-inactivating lentiviral vectors containing the enhanced green fluorescent protein (eGFP) gene or the viral thymidine kinase gene of the herpes simplex type II virus and a woodchuck posttranscriptional regulatory element (WPRE) were generated as previously described (Ziebart et al., 2008) by transient transfection in 293T cells with packaging plasmid, pCMV8.91 and pMD.G for vesicular stomatitis virus–G protein (VSV-G) pseudotyping (Scherr et al., 2002). After 4 hours, the medium was replaced by EBM medium supplemented with EGM SingleQuots and 20% FBS for 24 hours. Lentiviral particles were collected every 24 hours for 3 days, and filtered through 0.22 μm filters. The constitutively active promoter (spleen focus forming virus promoter: SFFVp) of the lentivectors were replaced by promoters of the human eNOS promoter (accession number: AF387340), the human SM22α promoter (accession number: submitted to genebank) and murine α myosin heavy chain promoter (accession number: U71441) to control either GFP or TK expression. The myosin heavy chain promoter construct was kindly provided by Stefan Janssens, Belgium. Restriction enzymes for each cloning and full sequence of the plasmids are available upon request.

Transduction
HUVEC, smooth muscle cells, cardiomyocytes were transduced by adding viral supernatant to the specific medium. After 24 hours, medium was changed and a second transduction was performed.
To facilitate BMC transduction, culture dishes were coated with retronectin (Takara, Japan) and viral supernatant was added for 2 h at 37 °C. After aspirating the supernatant, the culture plates were washed with PBS and freshly isolated BMC were plated in EGM medium with 20% FBS for 2 days. To increase transduction efficiency, BMC were transduced again. In detail, floating cells were harvested, washed with PBS and then resuspended with fresh EGM medium with 20% FBS. Fresh medium was also added to the attached cells and combined with the floating cells and new viral supernatant for additional 2 days.

**Co-culture tube formation assay**

Fibroblast and HUVEC were co-cultured in the presence of either BMC or macrophages in EGM medium with 10% FCS on a fibronectin-coated plate. We covered the cells with 200 μl basement membrane matrix (Matrigel; BD Biosciences) per well of 12-well plates. Pictures were taken after 7 day-culture.

**Acute myocardial infarction (AMI) model**

AMI was induced in male nu/nu mice (8–10 weeks; Harlan) under mechanical ventilation and anesthesia with isoflurane and analgesie with carprofen (5mg/kg s.c.). AMI was induced by permanent ligation of the apical branch of the left anterior coronary artery. A total of 1 million BMC were injected as outlined in the material and method section.

**Echocardiographic assessment of left ventricular (LV) Function**

Echocardiography (Vevo 770 small animal microultrasound system, VisualSonics) was performed to evaluate LV function postoperatively, 2 weeks and 4 weeks after myocardial ischemia. Mice were restrained on a movable x-y-stage under isoflurane anesthesia. First, a parasternal long axis view including aortic valve and apex was taken to align the long axis of the left ventricle parallel with the horizontal line of the image by adjusting the position of the stage. Next, we rotated the echo probe by 90 degrees and then adjusted the position of the stage to scan the heart from base to apex. The apex level was defined as the level, where the papillary muscles disappear, when moving the y-axis from base to apex. The mid level was defined at a distance of 1 mm from the apex towards the base.

End-diastolic and end-systolic left ventricular dimensions were measured at each level. End-systolic volume (ESV in arbitrary unit) and end-diastolic volume (EDV) was calculated from the diameters (D) of the three levels (Volume = Dapex² + Dmid² +
Dbase²). Ejection fraction was calculated as (EDV-ESV)x100/EDV.

**Immunostainings**

Thirty minutes before sacrificing the mice, 200 μg BS-1 lectin were administered intravenously to stain perfused vessels. Before explantation, hearts were perfused with 1% paraformaldehyde by left ventricular canulation. Hearts were immersed in 5 % agarose for vibratome sectioning and then cut at 50-100 μm thickness. For frozen sections, hearts were immersed in OCT and then frozen at - 80°C. Frozen sections were cut with a cryotome with 10 to 50 μm thickness. The following antibodies were incubated overnight: FITC- or Alexafluoro633-labeled antibody against α-smooth muscle actin (Sigma), rabbit anti-human vWF antibody (DAKO) and alexa633-labelled anti-troponin T antibody. Tissues sections were imaged on a confocal laser scanning microscope 510 META (Zeiss, Germany). Samples were excited with a 405, 488, 543 and 633-nm laser lines with BP420-480, LP505, BP560-615, LP650 chroma filter sets using a Plan-Neofluar objectives (x20/0.5 or x40/1.3 Oil). To demonstrate that the GFP signal indeed derives from human cells, the spectra of the human cells expressing GFP were confirmed by Lambda Stack acquisition. The spectral distributions of fluorescence images were simultaneously recorded in a CHS-1 from 508–668 nm. Three-dimensional stacks of the heart were taken at 2-4 μm intervals spanning the whole GFP-positive cells (20-40 μm). The pinhole settings were at 2.0 airy units. Z-stack images were reconstructed as a 3D image or reformatted by projection view using Zeiss LSM image software.
**Supplemental figures: Figure 1**

**By-stander effects in vitro** (a) TK-expressing HUVEC were stained with vibrant DiI and were co-cultured with GFP-expressing HUVEC at a ratio of 1:9. Cell viability was detected 24 hours after addition of GCV (500 µM). (b) Quantification of cell viability with the MTT assay. (c) TK-expressing progenitors were stained with vibrant DiI and were co-cultured with GFP-expressing HUVEC at a ratio of 1:9. (d) Quantification of cell viability with the MTT assays. (e) Co-culture experiment using in vitro pseudovascular tube formation assay adding TK-transduced CD14+ cells-derived macrophages to YFP-transduced HUVEC on fibroblasts. YFP-HUVEC produced well-organized vessel-like structures. (f) Quantification of the area of YFP-positive HUVEC tubes with or without GCV.
**Effects of depletion of TK-BMC in uninjured hearts.** TK-BMC were injected in non-infarcted hearts of healthy control animals and EF was measured by high resolution echocardiography as indicated. At day14 mice were treated with GCV to induce the depletion of the injected cells.
3. Supplemental References

