Enhanced Angiogenic and Cardiomyocyte Differentiation Capacity of Epigenetically Reprogrammed Mouse and Human Endothelial Progenitor Cells Augments Their Efficacy for Ischemic Myocardial Repair

Melissa A. Thal, Prasanna Krishnamurthy, Alexander R. Mackie, Eneda Hoxha, Erin Lambers, Suresh Verma, Veronica Ramirez, Gangjian Qin, Douglas W. Losordo, Raj Kishore

Rationale: Although bone marrow endothelial progenitor cell (EPC)-based therapies improve the symptoms in patients with ischemic heart disease, their limited plasticity and decreased function in patients with existing heart disease limit the full benefit of EPC therapy for cardiac regenerative medicine.

Objective: We hypothesized that reprogramming mouse or human EPCs, or both, using small molecules targeting key epigenetic repressive marks would lead to a global increase in active gene transcription, induce their cardiomyogenic potential, and enhance their inherent angiogenic potential.

Method and Results: Mouse Lin-Sca1⁺CD31⁺ CD34⁺ EPCs and human CD34⁺ cells were treated with inhibitors of DNA methyltransferases (5-Azacytidine), histone deacetylases (valproic acid), and G9a histone dimethyltransferase. A 48-hour treatment led to global increase in active transcriptome, including the reactivation of pluripotency-associated and cardiomyocyte-specific mRNA expression, whereas endothelial cell–specific genes were significantly upregulated. When cultured under appropriate differentiation conditions, reprogrammed EPCs showed efficient differentiation into cardiomyocyte and vascular smooth muscle cells. Treatment with epigenetic-modifying agents show marked increase in histone acetylation on cardiomyocyte and pluripotent cell–specific gene promoters. Intramyocardial transplantation of reprogrammed mouse and human EPCs in an acute myocardial infarction mouse model showed significant improvement in ventricular functions, which was histologically supported by their de novo cardiomyocyte differentiation and increased capillary density and reduced fibrosis. Importantly, cell transplantation was safe and did not form teratomas.

Conclusions: Taken together, our results suggest that epigenetically reprogrammed EPCs display a safe, more plastic phenotype and improve postinfarct cardiac repair by both neocardiomogenesis and neovascularization. (Circ Res. 2012;111:00-00.)

Key Words: cardiomyogenesis • cell therapy • endothelial progenitor cell • epigenetic modification • histone acetylation • myocardial ischemia • transdifferentiation

Despite the pervasive belief that the heart has limited regenerative capacity, progenitor cell–based therapy has been shown to provide substantial clinical benefits for ischemic diseases such as chronic angina, acute myocardial infarction (AMI), and heart failure.1–6 Specifically, murine endothelial progenitor cells (EPCs)7 and the human equivalent CD34⁺ mononuclear cells are capable of homing to infarct and peri-infarct myocardium on ischemia, inducing angiogenesis/vasculogenesis, and augmenting cardiac function and survival through paracrine-mediated growth factor secretion.8–10 Although the revascularization appears to result in real improvements in quality of life, the ultimate goal of cardiovascular regenerative medicine is to regenerate lost myocytes in addition to neovascularization. There is no convincing evidence that EPCs have cardiomyocyte differentiation potential.11,12 Also, because the EPCs from aged patients with existing metabolic diseases and cardiovascular risk factors are known to have diminished functional properties, epige-
The epigenetic code, including posttranslational modifications in histones, is responsible for adding complexity to gene regulation beyond sequence specificity. It plays a critical role in regulating everything from gene expression, cell cycle, cell fate, differentiation, and, ultimately, the capacity for transdifferentiation in vivo.17,18 Endothelial cells, vascular smooth muscle cells, and cardiomyocytes all differentiate from a common progenitor in the mesoderm, suggesting that reprogramming endothelial cells back to an earlier state in mesodermal development could recapitulate their cardiomyogenic potential. Therefore, to remove the transcription-restrictive epigenetic marks and to harness all the differentiation potential the endothelial progenitor cell might possess, we treated the EPCs with epigenetic-modifying molecules 5′ Azacytidine, valproic acid, and BIX-01294. Based on clues from epigenetic-silencing mechanisms, we conceptualize that removal of epigenetic repressive marks by the aforementioned inhibitors will remodel chromatin surrounding cardiomyocyte-specific genes (as well as global genes), thus providing a window in which the cell epigenome is permissive to desired gene transcription and potentially transdifferentiation when cells are cultured under appropriate culture conditions or exposed to the proper microenvironment in vivo.

Our results suggest that small molecule–based epigenetic reprogramming of EPCs, both murine and human, results in an open epigenome conducive to enhanced global gene expression, including the induction of cardiomyocyte-specific gene expression. Further, intramyocardial transplantation of reprogrammed EPCs in the infarcted mouse hearts resulted in enhanced physiological and anatomic repair compared with nonreprogrammed EPCs, suggesting an improved paracrine capacity of reprogrammed cells. Most interestingly, drug-treated EPCs, both of murine and human origin, were able to transdifferentiate into cardiomyocytes in the border zone of the infarcted hearts. Collectively, our data suggest that epigenetic reprogramming of EPCs can enhance their therapeutic efficacy by enhancing their plasticity and functions.

Methods
Detailed Methods are provided in the Online Supplement.

Real-Time Polymerase Chain Reaction
RNA was isolated and reverse-transcribed into cDNA from sorted EPCs to assess gene mRNA expression of Oct4, Nanog, Sox2, Nkx2.5, connexin43, cardiac troponin T, eNOS, and VE cadherin using the Cells to Ct kit (Invitrogen) according to the suggested protocol. Relative mRNA expression of target genes was normalized to the endogenous 18S control gene.

Myocardial Infarction
Mice underwent surgery to ligate the left anterior descending coronary artery19 as reported previously:20 2.0 × 10⁵ mouse EPCs, 2.5 × 10⁵ or 5 × 10⁵ CD34⁺ cells resuspended in 20 µL phosphate-buffered saline were injected intramyocardially into the left ventricular wall (border zone) at two different locations immediately after left anterior descending ligation. The saline group underwent the same surgery but received phosphate-buffered saline without cells. Tissue was harvested at day 7, day 14, or day 28 after AMI for histological analysis.

Echocardiography
Transthoracic two-dimensional M-mode echocardiography was performed using the Vevo770 (VisualSonics, Toronto, ON, Canada) equipped with a 30-MHz transducer. Mice were anesthetized for analysis with a mixture of 1.5% isoflurane and oxygen (1 L/min) before AMI (baseline) and at days 7, 14, and 28 after AMI. M-mode tracings were used to measure left ventricular wall thickness and left ventricular inner diameter in systole and diastole. The mean value of three measurements was determined for each sample. Percentage fractional shortening and percentage ejection fraction were calculated as described previously.

Morphometric Studies
Infarcted hearts were perfused with phosphate-buffered saline followed by methanol fixation and paraffin embedding. Morphometric analysis including infarct size and percent fibrotic area was performed on Masson trichrome-stained tissue sections using ImageJ 1.43u software (US National Institutes of Health; http://rsb.info.nih.gov/ij/).

Chromatin Immunoprecipitation
The chromatin immunoprecipitation assay was performed as previously described.

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Non-standard Abbreviations and Acronyms

<table>
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<th>Abbreviation</th>
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<tr>
<td>5′Aza</td>
<td>5′ Azacytidine</td>
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<tr>
<td>AMI</td>
<td>acute myocardial infarction</td>
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<tr>
<td>BIX</td>
<td>BIX-01294</td>
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<tr>
<td>EPC</td>
<td>endothelial progenitor cell</td>
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<tr>
<td>FACS</td>
<td>fluorescence-activated cell sorting</td>
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<td>Lin</td>
<td>lineage</td>
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5′ Azacytidine, valproic acid, and BIX-01294. Based on clues from epigenetic-silencing mechanisms, we conceptualize that removal of epigenetic repressive marks by the aforementioned inhibitors will remodel chromatin surrounding cardiomyocyte-specific genes (as well as global genes), thus providing a window in which the cell epigenome is permissive to desired gene transcription and potentially transdifferentiation when cells are cultured under appropriate culture conditions or exposed to the proper microenvironment in vivo.
Methylation Analysis by Pyrosequencing
Methylation studies were performed as previously described.\textsuperscript{24}

Statistical Analyses
One-tailed, unpaired Student $t$ tests (Microsoft Excel) were used to measure statistical differences in which $P<0.05$ was considered statistically significant.

Results

Staggered Valproic Acid and 5’Azacytidine Treatment Results in Genome-Wide Enhanced Gene Expression in EPCs

Whole bone marrow was isolated from femurs, tibiae, and hip bones of C57BL/6 mice.\textsuperscript{25} Bone marrow mononuclear cells were FACS-sorted to $>95\%$ purity for the population of cells characterized as Lineage-negative (Lin; CD11b, Ly6G/C, B220, CD3e, Ter119) Sca-1$^{-}$CD31$^{+}$, which represents approximately $1.4\%$ of total mononuclear cells (Supplemental Figure IA). This sorting strategy allowed for the isolation of progenitor cell types (Lin-Sca-1$^{+}$) from the bone marrow with endothelial cell lineage (CD31$^{+}$).\textsuperscript{26} Lineage-negative Sca-1$^{-}$CD31$^{+}$ cells, which are referred to as EPCs henceforth, showed phenotypic characteristics consistent with their endothelial progenitor identity and incorporated into tubes formed by the mature murine endothelial cell line SVECs on Matrigel (BD Biosciences; Supplemental Figure IB). This suggests that this sorted population encompasses the functional effector cells found in the bone marrow–derived cultured EPCs without necessitating in vitro culture or differentiation.

In an attempt to increase their plasticity, $2.0\times10^{5}$ sorted EPCs were seeded on fibronectin-coated plates and then treated for 48 hours with individual or combinations of epigenetic modifying agents: $500$ nmol/L 5’Azacytidine (DNA methyltransferase inhibitor); 1 mmol/L valproic acid (VPA; histone deacetylase inhibitor); and 1 $\mu$mol/L BIX-01294 (histone methyltransferase inhibitor). Drug dosages comply with current literature suggestions\textsuperscript{27–29} and were verified as nontoxic by cell viability analysis (data not shown). As determined by real-time polymerase chain reaction, this resulted in a significant induction of pluripotency-associated gene expression ($Oct4$, Nanog, and Sox2), with the highest induction in the cells treated for 48 hours with 1 $\mu$mol/L BIX-01294 ($Oct4$ expression: $9.5\pm2.0$; $P=0.009$) or 24 hours with 1 $\mu$mol/L VPA followed by an additional 24 hours with $500$ nmol/L 5’Azacytidine ($Oct4$ expression: $5.5\pm1.6$; $P=0.007$; Figure 1A). All mRNA expression was normalized to 18S RNA and then presented as a fold difference compared with untreated EPCs. Interestingly, one of the most effective conditions included a delayed addition of the DNA methyltransferase inhibitor 5’Azacytidine, indicating that inhibition of histone deacetylase activity before DNA demethylation may be beneficial for enhanced transcriptional activity throughout the genome. Additionally, real-time polymerase chain reaction results show de novo induction of cardiomyocyte-specific transcripts (Figure 1B) in the two most effective drug treatment conditions immediately after treatment without necessitating cardiomyocyte differentiation conditions. Because the treated cells express cardiomyocyte genes at basal level, they may be more inclined to differentiate into mature cardiomyocytes under conditions conducive for cardiomyocyte differentiation. Similar changes in gene expression were found in human CD34$^{+}$ cells with the regimen of $2.5$ mmol/L VPA followed by $500$ nmol/L 5’Azacytidine
BIX-01294 treatment, even at reduced doses, was toxic to human CD34 cells and therefore was not used in human cell studies. Genome-wide expression profiling of drug-treated human CD34 cells confirmed that de novo induction of previously silent genes was not limited to any one cell type or cellular function (Supplemental Table) but was rather reflective of a global transcription-permissive chromatin and an open epigenome. Transcriptional profiling of control and treated cells identified 914 genes as significantly upregulated, whereas only 296 genes were significantly downregulated in the staggered VPA/5’Azacytidine–treated cells compared with untreated control CD34 cells (average of two sample sets performed in triplicate; Figure 1D, E). These findings indicate that epigenetic-modifying drug treatment results in a global increase in gene expression, which is not limited to induction of pluripotency or cardiomyocyte-specific gene expression. Interestingly, endothelial cell–specific gene expression (Figure 1C and Supplemental Figure II) in both mouse EPCs and human CD34+ cells treated with drugs was either maintained or marginally increased, suggesting that the cells do not acquire pluripotency and, therefore, a potentially tumorigenic phenotype. This was further supported by the inability of the treated cells to form teratomas when injected into immune-deficient mice and followed-up for up to 4 weeks (Supplemental Figure III).

Figure 2. Valproic acid (VPA)/5’Azacytidine or BIX-01294 treatment effects histone modifications in endothelial cells. A, Western blot analysis of acetylated H3K9 or pan acetylated H4 or (C) dimethyl H3K9 levels from 10 million SVEC cells after 24-hour treatment with 1 mmol/L VPA, followed by an additional 24 hours with 500 nmol/L 5’Azacytidine or 1 μmol/L BIX-01294. B and D, Quantitative assessment of modified histone levels relative to total histone levels. Values are fold change compared with untreated SVEC cells (n=3).

Figure 3. Epigenetically reprogrammed endothelial progenitor cells (EPCs) have increased acH3K9 associated with cardiac-specific promoters. A, Chromatin immunoprecipitation assay at cardiomyocyte-associated gene promoters and two regulatory regions of Oct4. CP, core promoter; RP, regulatory promoter. Values represent percent of total input. *P<0.05. B, Pyrosequencing of the Nkx2.5 promoter from isolated and bisulfite converted genomic DNA from SVECs with no drug, valproic acid (VPA)/5’Azacytidine, or BIX-01294. Converted NIH-3T3 gDNA was used as a positive control.

Chromatin-Remodeling Drugs Effectively Remove Targeted Transcription-Repressive Epigenetic Marks in Endothelial Cells

VPA, 5’Azacytidine, and BIX-01294 are effector molecules that remodel the chromatin to allow for less restricted gene expression and increased lineage differentiation potential. VPA and BIX-01294 are direct inhibitors of histone-modifying enzymes (histone deacetylase and histone methyltransferase G9a, respectively). Therefore, to confirm activity, murine endothelial cells (SVEC) were treated with either 1 μmol/L BIX-01294 or staggered addition of 1 mmol/L VPA and then 500 nmol/L 5’Azacytidine for 48 hours, and histone acetylation and methylation were assessed by Western blot. Acetylation of the lysine 9 position of 

[Image 82x309 to 545x725]
histone 3 (H3K9, a transcription-permissive epigenetic mark) was significantly higher in cells treated with VPA/5′Azacytidine compared with control cells (Figure 2A, B). Additionally, a pan acetylated histone 4 antibody detected a more than two-fold increase in total H4 acetylation in the drug-treated cells (Figure 2A, B). BIX-01294 directly inhibits G9a from forming dimethylated H3K9. H3K9 dimethylation was reduced by 64.3%±11% by BIX-01294 compared with untreated control cells (Figure 2C, D). This suggests both drug conditions, VPA/5′Azacytidine and BIX-01294, actively target the intended epigenetic enzymes.

Drug Treatment Enhances Histone Acetylation at Pluripotency and Cardiac-Specific Gene Promoters

To further characterize the epigenetic landscape within the regulatory region of cardiac and pluripotent genes, chromatin immunoprecipitation experiments were performed to evaluate the level of H3K9 acetylation. Chromatin from control and drug-treated SVEC cells was immunoprecipitated with anti-H3K9-acetyl antibodies followed by reverse-transcriptase polymerase chain reaction analysis to quantitate the amount of bound acetylated H3K9 to the activin 2 (Actn2), ryanodine receptor (Ryr2), and troponin T (TnnT2) cardiomyocyte gene promoters or two of the 5′ regulatory regions of Oct4. Cells treated with either BIX-01294 or VPA/5′Azacytidine showed significant increase in the amount of acetylated H3K9 bound to the core promoter and regulatory promoter regions of Oct4 compared with the untreated controls (P<0.05; Figure 3A). Further, all three cardiac-specific gene promoters analyzed in both drug conditions were more heavily bound by acetylated H3K9 than in the nontreated cells (P<0.05; Figure 3A). This increase in acetylated H3K9 associated with transcription regulatory regions is potentially responsible for the induction of Oct4 and cardiac-specific gene expression in treated EPCs. DNA methylation analysis of Nkx2.5, a cardiomyocyte-specific transcription factor, showed no significant difference in the CpG methylation patterns between treated and control SVEC cells (Figure 3B). These data suggest that increased H3K9 acetylation, rather than DNA methylation, largely accounts for de novo transcription of these previously silent transcripts.

Transplantation of Reprogrammed Mouse EPCs Improves Postinfarct Left Ventricular Function and Adverse Remodeling to a Greater Extent Than Untreated EPCs

In the next series of experiments, we determined the therapeutic efficacy and differentiation plasticity of drug-treated mouse EPCs in a mouse AMI model. AMI was induced by the permanent ligation of left anterior descending and EPCs, either untreated or treated with BIX-01294 alone or VPA/5′Azacytidine, were intramyocardially injected at the ischemic border zone. A subset of mice received saline as control. Left ventricular functions were evaluated by M-mode echocardiography on days 7, 14, and 28, after AMI. Left ventricular heart function analysis of left ventricular heart function and adverse remodeling was further corroborated by anatomic and histological evidence. An analysis of infarct size and percent left ventricular fibrosis of excised hearts at day 28 after AMI showed a significant reduction in the infarct size in mice transplanted with drug-treated EPCs, compared with controls (P<0.01; Figure 5A, B). Similarly mice receiving drug-treated EPCs showed significantly reduced left ventricular fibrosis on day 28 after AMI (P<0.01; Figure 5C).
Epigenetically Reprogrammed EPCs Augment Post-AMI Vascularity and Transdifferentiate Into Cardiomyocytes

Endothelial progenitor cells have been shown to enhance postinfarct left ventricular functions by augmenting neovascularization in the ischemic myocardium, largely by paracrine mechanisms. We assessed capillary density in the border zone of ischemic myocardium at 28 days post-AMI and cell injections. Capillaries were identified as vascular structures staining positive for CD31. EPCs reprogrammed with either VPA/5’Azacytidine or BIX-01294 showed significantly larger number of capillaries, especially in mice receiving EPCs treated with VPA/5’Azacytidine, when compared with untreated EPCs (Figure 5D, E), suggesting that epigenetic reprogramming of EPCs likely enhances their paracrine abilities, leading to increased vascularization and concomitant improved left ventricular function.

More interestingly, epigenetic reprogramming of the EPCs with VPA/5’Azacytidine or BIX-01294 rendered them a more plastic phenotype capable of transdifferentiating to cardiomyocyte lineage in vivo. The eGFP-positive Lineage-Sca-1+CD31+ cells were treated with the combination of drugs before transplantation into the ischemic myocardium after AMI. Although GFP+ cells were located in the EPC control group, none was found to costain with the cardiomyo-
cyte-specific protein marker, α-sarcomeric actin. However, some of the BIX-01294–treated EPCs and an even greater number of VPA/5′Azacytidine–treated EPCs sustained for both GFP and α-sarcomeric actin (Figure 5F). This suggests that, in addition to improving left ventricular function through enhancement of the inherent therapeutic properties of EPCs, treatment of EPCs with VPA/5′Azacytidine or BIX-01294 also enables acquisition of cardiomyocyte differentiation in vivo.

Epigenetically Reprogrammed Human CD34+ Cells Display Better Therapeutic Efficacy and Paracrine Activity, and Enhance Ischemic Myocardial Vascularity After AMI in Immune-Deficient Mice

We next determined whether the epigenetic reprogramming by these small molecules is limited to mouse EPCs or whether it can also be translated to human EPCs (bone marrow–mobilized CD34+ cells), currently used in clinical trials.6 CD34+ cells, obtained from healthy donors and provided by Baxter Healthcare, were treated or not with VPA (2.5 mmol/L and 5′Azacytidine 500 nmol/L) and mRNA expression of pluripotency and cardiomyocyte-specific transcripts was determined. Despite the promising results seen with BIX-01294 treatment of the mouse EPCs, similar drug doses and even two-fold reduced levels were toxic to the human CD34+ cells and, therefore, were omitted. Untreated CD34+ cells did not express significant level of either pluripotent gene or cardiomyocyte gene mRNA; however, VPA/5′Azacytidine treatment significantly induced the expression of these genes (Supplemental Figure II). To determine enhanced functional capacity of reprogrammed CD34+ cells in the repair of postinfarct myocardium, a previously determined subtherapeutic dose of 25,000 cells was used for post-AMI transplantation in an immune-deficient mouse AMI model. The dose of 25,000 CD34+ cells was chosen deliberately, because our unpublished studies have demonstrated that at this dose, these cells do not confer any therapeutic benefits. The rationale for using a subtherapeutic dose of cells was to test if the epigenetic reprogramming of cells will enhance the therapeutic efficacy of a cell dose that by itself is not therapeutic. At this dose, mice transplanted with untreated cells did not show any improvement in left ventricular functions, infarct size, or capillary density compared with saline group, whereas transplantation of CD34+ cells treated with VPA/5′Azacytidine resulted in a significant enhancement of left ventricular function based on percentage fractional shortening and percentage ejection fraction (percentage ejection fraction at day 28: CD34+ 12.96±1.49 and VPA/5′Azacytidine 28.82±3.94; P=0.01; Figure 6A). Additionally, transplantation of reprogrammed CD34+ cells resulted in significantly reduced infarct size and significantly increased capillary density in the border zone of infarcted myocardium (Figure 6B–D). Additionally, we observed significantly reduced apoptosis and increased proliferation in the infarcted myocardium in the hearts of mice receiving treated CD34+ cells compared with untreated cells (Supplemental Figure IV).

Because the cardioprotective effects (less apoptosis, increased proliferation, increased capillary density, improved overall left ventricular function) in mice that receive VPA/5′Azacytidine–treated CD34+ cells in the AMI model were considerable, we evaluated whether the paracrine secretory profile is enhanced in these treated cells. The Human Angiogenesis Array (R&D Systems) was used to quantitate changes in angiogenesis-promoting proteins in the conditioned medium of control and treated cells. CD34+ cells treated with VPA/5′Azacytidine showed an increase in a majority (15/19) of detected angiogenic proteins (Supplemental Figure V). These data suggest that the observed therapeutic effect with treated CD34+ cells in vivo was in part attributable to their enhanced angiogenic phenotype.

Treatment With VPA/5′Azacytidine Confers Cardiac Differentiation Potential to Human CD34+ Cells In Vivo

We also determined if increased repair capacity of treated CD34+ cells in vivo also reflect their increased plasticity toward cardiomyocyte differentiation in vivo. AMI was induced in immune-deficient mice and 4.0×105 DiI–labeled CD34+ cells with or without treatment with VPA/5′Azacytidine were injected into the heart at the time of coronary artery ligation. Heart tissue was harvested 7 days post-AMI and stained for α-sarcomeric actin, whereas DiI was used as an indicator of donor cells. Immunofluorescence staining identified donor DiI+ cells that co-stained with α-sarcomeric actin in hearts exclusively receiving treated CD34+ cells (Figure 6E). In hearts receiving untreated cells, no co-staining was observed. This suggests that in addition to enhancing their angiogenic activity, drug treatment also increased their plasticity toward de novo cardiomyocyte differentiation.

Discussion

Despite significant improvement in the prognosis of patients with myocardial ischemia by available therapies including thrombolysis and urgent revascularization, there remains significant mortality and a significant proportion of survivors are at risk for development of heart failure. Because the main underlying cause of this process represents the loss of cardiomyocytes and microvasculature of the infarcted wall, the development of treatment strategies aimed at preserving or regenerating myocardial tissue is currently acknowledged as a central therapeutic challenge. The available evidence demonstrating improvement in myocardial function after transplantation of autologous bone marrow–derived stem/progenitor cells including EPCs, both in preclinical as well as in available clinical trials, remains a potent force driving discovery and clinical development simultaneously and has provided new hope for patients with debilitating heart diseases. In the clinical setting, the functional improvement in patients receiving bone marrow EPCs has been reported and these findings have been replicated in recently completed, randomized,
double-blind, phase II clinical trial. In another randomized trial, authors reported an increase in ejection fraction and a reduction of end-systolic volumes in the group of patients receiving bone marrow cells. Recently, these authors published the long-term follow-up of these patients, revealing that the early benefit that had been observed was not preserved. The reasons for the apparent loss of benefit remain to be resolved; however, risk factors for coronary artery disease, including diabetes and age, are reported to be associated with a reduced number and impaired functional activity of EPCs in the peripheral blood of patients. Moreover, whereas it is generally accepted that EPCs participate in vascular repair of the ischemic myocardium, both via paracrine mechanisms and by physically integrating into neovasculature, there exists no convincing evidence that these cells are capable of transdifferentiating into functional cardiomyocytes. Because the ultimate goal of cardiac regenerative medicine is to replace both microvasculature and lost cardiomyocytes, it is therefore imperative to make efforts toward refining the strategies to improve this autologous source of cardiac cell therapy by augmenting both EPC function and plasticity.

Our studies reported in this article provide evidence that small molecule–mediated epigenetic reprogramming of EPCs, both mouse and human, significantly enhances their angiogenic and functional activity and leads to a cellular epigenome that is conducive to a more plastic phenotype capable of transdifferentiation into cardiomyocyte lineage. Several lines of evidence support this conclusion. Treatment of both mouse EPC and human CD34⁺ cells with small-molecule inhibitors of DNA methyltransferase, histone deacetylase, and G9a histone methyltransferase leads to globally upregulated gene transcription indicative of dynamic chromatin remodeling and transcription permissive epigenome. Cells treated with these small molecules show de novo induction of both cardiomyocyte-specific genes and yet retain their endothelial gene expression. Epigenetically reprogrammed EPCs show enhanced angiogenic activity in terms of paracrine factor secretion and increased ischemic angiogenesis in vivo, including the enhancement of both capillary density and the number of arterioles in the border zone of the infarct, suggesting both angiogenic and vasculogenic activity. Epigenetic reprogramming of both mouse and human CD34⁺ cells significantly enhances their myocardial repair capabilities, as evident from improved left ventricular functions and anatomic repair, including reduced fibrosis and apoptosis after acute myocardial infarction in mice. Finally, repro-
grammed cells differentiate into cardiomyocytes in vivo. We believe that these exciting data have a clear translational bearing on the bone marrow EPC-based cellular therapies for myocardial regenerative medicine.

There is growing recognition that epigenetic mechanisms largely govern the cell fate and function. Much of the information obtained from studies of nuclear cloning and iPSCs on the epigenetic changes in somatic nuclei points toward somatic cell chromatin remodeling mediated via the process of chromatin condensation, DNA methylation/demethylation, and posttranslational histone modifications (acetylation, phosphorylation, and methylation), two processes that are functionally linked. Although complex, the process of epigenetic gene silencing involves three critical steps: methylation of 5′ regulatory regions of target gene; deacetylation of histones 3/4 by histone deacetylases; and dimethylation of lysine 9 of histone 3 (H3K9). Chemical inhibitors of DNA methyltransferases (5-AzaCytidine) and histone deacetylases (trichostatin A, VPA) are capable of inducing multipotency, which is evidenced by their significant enhancement of reprogramming of somatic cells for somatic cell nuclear transfer or iPSC derivation. Both drugs can change the fate of a given cell by chromatin remodeling, leading to a heterochromatin status permissive for silenced gene transcription including reactivation of pluripotency-associated genes in somatic cells. Specifically in the cardiovascular system, studies have demonstrated that treatment with 5′Azacytidine leads to an increase in cardiomyocyte differentiation of mesenchymal stem cells with improved cardiac function after transplantation of 5′Azacytidine-treated mesenchymal stem cells compared with control mesenchymal stem cells. Although these chemical modifiers of key epigenetic repressive marks improve reprogramming efficiency, they are insufficient to induce pluripotency, eliminating the risk of teratoma formation associated with cell therapy. Our data corroborate these findings. We observed that treatment of EPCs with VPA/5′Azacytidine or BIX-01294 leads to robust increase in H3K9 acetylation and significantly reduced G9a-specific H3K9 dimethyltransferase activity. Importantly, a number of cardiomyocyte-specific gene promoters were enriched for H3K9 acetylation, indicating inhibition of histone deacetylases as primary epigenetic modification for de novo induction of cardiomyocyte genes. We did not see much change in the Nkx2.5 promoter methylation patterns in control and VPA/5′Azacytidine–treated EPCs. There could be several explanations to this unexpected finding. First, we only looked at the promoter region of Nkx2.5, which harbors only few CpG residues; it is possible that an analysis of methylation pattern 5′ to the Nkx2.5 promoter, including enhancer region and CpG islands, would have yielded different methylation patterns. Second, because EPCs are progenitor cells and phylogenetically related to the common mesodermal precursor from which both endothelial cells and cardiomyocytes are derived, the cardiomyocyte-specific gene promoters are still hypomethylated. When compared with fibroblasts, we did find significantly less methylated Nkx2.5 promoter region in EPCs.

Although the mouse EPC population used in our studies and characterized as lineage- Sca-1−CD31+ only recently has been characterized in the literature, our data support the claim that these cells function similarly to the human CD34+ cell population mobilized from patient bone marrow and are currently used clinically for cardiac cell therapy. The human population is heterogeneous and may contain cell types similar to outgrowth populations known as early or late EPCs found in mice. Although it is unclear whether the cell type we are using is also present in humans, the surface marker profile of these cells most closely resembles the early EPC population characterized by Asahara which arises after 7 to 10 days of culture in EBM-2 medium supplemented with IGF-1, vascular endothelial growth factor, FGF, and endothelial growth factor. These cells have been shown to acquire both Sca-1 and CD31 surface marker expression during culture. Potentially, the same well-characterized early EPC population created in culture already exists endogenously in the bone marrow.

In summary, the results described clearly suggest the current myocardial cellular therapy using EPC/CD34+ cells can be improved both in terms of functional outcome and potential regenerative capacity by pretreatment with epigenetic modifying small molecules. Our data indicate that epigenetic reprogramming of CD34+ cells boosted the functional cardiac repair capacity of an otherwise subtherapeutic dose of cells. Importantly, unlike pluripotent cells, transient reprogramming of EPC is safe because these cells did not show any evidence of teratoma formation in mice. Our approach to use small molecules to create a less condense and more accessible epigenome is a creative way to induce multipotency and cardiomyogenic differentiation potential in EPCs, without the undesired complications involved with induced pluripotent cells, and is novel. Additionally, our studies have a direct bearing on translation regenerative medicine and could potentially augment the efficacy of an existing clinical therapy for cardiovascular diseases.

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Disclosures

None.

References


Novelty and Significance

What Is Known?

- Transplantation of endothelial progenitor cells (EPCs) enhances neovascularization in the ischemic tissues.
- EPCs possess little to no cardiomyocyte transdifferentiation ability.
- Removal of inhibitory epigenetic marks can improve cellular plasticity.

What New Information Does This Article Contribute?

- Drugs targeting repressive epigenetic marks induce myogenic plasticity in EPCs.
- Epigenetic reprogramming upregulates genome-wide transcription, including cardiomyocyte-specific gene expression in EPCs.
- Reprogrammed EPCs are therapeutically superior to untreated cells, resulting in improved left ventricular function in an acute myocardial infarction model.
- Secretion of proangiogenic factors is enhanced in drug-treated EPCs.
- Drug-treated EPCs from both mouse and humans show cardiomyocyte differentiation potential in vivo.

The therapeutic benefits of bone marrow EPC therapy in preclinical and clinical trials have been attributed to paracrine factor-mediated vascular repair without myogenesis and/or myocardial regeneration. Although the revascularization appears to improve the quality of life, the ultimate goal is regeneration and repair of the afflicted myocardium. Therefore, it is of interest to improve the cardiomyogenic properties of existing autologous cell therapy that already has been approved for clinical use. This study demonstrates that removal of inhibitory epigenetic modifications in both mouse and human EPCs confers enhanced therapeutic potential in a mouse model of acute myocardial infarction. Not only is the inherent paracrine activity greater as evidenced by improved capillary density, cell survival, and proliferation within the border zone of the infarct but also the modified cells acquire cardiomyogenic potential. The suggested mechanism for the enhanced functionality and differentiation potential is the positive effect epigenetic modifying drugs have on global gene transcription, which primes the cell to respond to environmental stimuli. Clinically, this may be an effective way of modifying an existing cellular therapy with potentially significant improvements not only in revascularization of the ischemic tissue but also in regeneration of the damaged myocardium.
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Supplemental Material

Detailed Methods

Cell culture and drug treatment. Lineage - Sca-1+ CD31+ EPCs were cultured on 5µg/mL human fibronectin coated plates in DMEM supplemented with 10% FBS and penicillin/streptomycin at 37°C with 5% CO₂. Human CD34+ cells were cultured in X-VIVO (Lonza) media supplemented with 0.5% human serum albumin, 50ng/mL Flt3 ligand, 20ng/mL stem cell factor, 50ng/mL VEGF, and 10ng/mL thrombopoietin. Either cell type was treated with VPA for 24 hours then 500nM 5’Aza was added to the culture media and cells remained in culture for an additional 24 hours (48 hours total). Mouse EPCs were also cultured with 1µM BIX-01294 for 48 hours in the same culture conditions.

Mice. Eight - ten week old C57BL/6J (stock number 000664), C57BL/6-Tg eGFP (stock number 003291) or nude (stock number 00819) mice were purchased from Jackson Laboratory. All experiments conform to protocols approved by the Institutional Animal Care and Use Committee at Northwestern University (Chicago, IL) in compliance with all state and federal regulations governing the use of experimental animals.

Western blot analysis. Cell lysate from 1.5x10⁷ SVEC cells was prepared using whole cell lysate buffer (50mM Tris-HCl, 0.5% Igepal (NP-40), 150nM NaCl). Proteins (90 µg) were electrophoresed by SDS-PAGE and analyzed using antibodies against acetyl-histone H3K9 (C5B11), di-methyl-histone H3K9 (Cell Signaling), H3 (Cell Signaling), pan acetyl H4 (Active Motif) or H4 (Abcam). Equal protein loading in each lane was verified using antibodies against β-actin and changes in modified histone levels were quantified by first normalization to total H3 or H4 protein.
**Immunofluorescence.** Immunofluorescence was performed as previously described\(^1\). Deparaffinized tissue sections were stained for anti-CD31 antibody (BD Biosciences) for capillary density. Donor cells in host tissue were detected by anti-eGFP. Cardiomyocytes were detected by alpha sarcomeric actin antibody (Sigma). Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (1:5000, Sigma Aldrich), and sections were examined with a fluorescent microscope (Nikon ECLIPSE TE200).

**Pyrosequencing.** PCR reactions were carried out using the Hotstart Taq polymerase kit (Qiagen). For each PCR reaction, 50ng of the bisulfite converted DNA was used as a template with 50pm of forward and reverse primers. After 5 min of initial denaturation at 95°C, the cycling conditions of 44 cycles consisted of denaturation at 95°C for 15 s, annealing at 65°C for 30 s and elongation at 72°C for 45 s. Pyrosequencing was performed using the PyroMark MD Pyrosequencing System (Biotage) as described previously\(^2\). Pyrosequencing primer at a concentration of 0.3 \(\mu\) M was annealed to the purified single-stranded PCR product at 28°C. Methylation quantification was performed using the manufacturer-provided software.

**Microarray.** Genome-wide expression analysis was performed on RNA isolated from 1x10\(^6\) human CD34+ cells cultured 48 hours in X-VIVO (Lonza) media supplemented with 50ng/mL Flt3L, 20ng/mL stem cell factor, 50ng/mL VEGF, 10ng/mL TPO, 0.5% human serum albumin or 24 hours in the same media + 2.5mM VPA followed by an additional 24 hours with 500nM 5'Azacytidine. RNA was isolated and purified using RNA Stat-60 (Tel-test) as per the manufacturer’s suggested protocol. RNA quality and purity was assessed using NanoDrop ND-1000. Using the Human HOA5.1 OneArray (Phalanx Biotech Group), data was collected then analyzed on the Rosetta Resolved System (Rosetta Biosoftware). Differentially expressed gene list was produced by a
standard selection criteria as established by $|\text{Fold Change}| \geq 1$ and $P < 0.05$. Clustering analysis was performed to visualize the correlations among the replicates and different sample conditions. A subset of 398 genes was selected based on an intensity filter set such that the difference between the maximum and minimum intensity values exceeds 6000 among all microarrays.

**Angiogenesis ELISA array.** Two million human CD34+ cells were cultured as described for 48 hours at $1 \times 10^6$ cells/mL. Conditioned media was first collected by removal of cells by centrifugation ($400 \times g$ for 15 minutes) then applied to the human angiogenesis array (R&D Systems) as per the manufacturer’s suggested protocol. Data were analyzed by ImageJ 1.43u software (NIH). Trends were consistent with a second independent experiment.

**References**


Online Figure I: Isolation of Lineage-Sca-1+CD31+ EPCs from mouse bone marrow. (a) Sorting strategy for obtaining EPCs directly from mouse bone marrow. Percentages are of total bone marrow. (b) Tube formation assay shows incorporation of Dil labeled Lin-Sca-1+CD31+ cells into tubes formed by 5.0x10^4 SVEC cells. 10x magnification.
Online Figure II: Real-time PCR data represented as fold difference in mRNA expression in $5.0 \times 10^5$ VPA then 5'Aza treated CD34+ cells compared to untreated control cells. (n=3)
Online Figure III: No teratoma formation occurred when 1 million untreated, VPA/5’Aza or BIX-01294 treated bone marrow cells were injected into the flanks of nude mice. Positive teratoma formation with 1 million mouse embryonic stem cells (mES). (n=3)
Online Figure IV: VPA/5’Aza treated CD34+ cell therapy in mouse AMI results in less apoptosis and increased proliferation in the border zone. (a) Representative TUNEL stained sections with alpha-sarcomeric actin (red) and DAPI (blue). Scale bar is 20 μm. Quantification of TUNEL+ cells from 3 high power fields per heart of the border zone of myocardial infarcted mice, minimally 4 mice per condition, 14 days post-AMI. (b) Quantification of Ki67+ nuclei per high power field (HPF) at day 28 post-AMI. *p≤0.05, **p≤0.01
Online Figure V: Reprogrammed human CD34+ cells have increased angiogenic protein secretion. Human angiogenesis ELISA array shows a trend of increased angiogenesis protein levels in conditioned media from VPA/5’Aza treated CD34+ cells compared to the untreated cells. Representative results from one array, confirmed by an independent experiment.
Online Table I: Gene families of statistically significantly affected genes with VPA then 5′Aza treatment of CD34+ cells.

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