Inhibition of DNA Methyltransferases and Histone Deacetylases Induces Bone Marrow-derived Multipotent Adult Progenitor Cells to Differentiate into Endothelial Cells

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Introduction: Endothelial dysfunction plays a critical role in the pathogenesis of cardiovascular diseases and cancer. Bone marrow-derived multipotent adult progenitor cells (MAPC) have the potential to differentiate, at the single cell level, toward the three embryonic germ layers and may be the progenitors of the other tissue-specific stem cells. However, molecular mechanisms of endothelial differentiation from MAPC have not been defined. The importance of epigenetic changes such as DNA methylation and histone acetylation in gene regulatory networks during embryonic stem cell (ESC) differentiation has been documented. We postulated that endothelial cell (EC) differentiation from MAPC could be enhanced by inhibiting DNA methylation and histone deacetylation, reversing the repression of genes that specify EC fate.

Methods: MAPCs were derived from rat bone marrow and differentiated into EC by vascular endothelial growth factor (VEGF) treatment in the presence or absence of the specific DNA methyltransferase (DNMT) inhibitor 5′-aza-2′-deoxycytidine (aza-dC) and the histone deacetylase (HDAC) inhibitor trichostatin A (TSA). Expression of the endothelial marker genes was assessed by real time quantitative PCR and angiogenic potential of the differentiated EC was assessed by analysis of vascular network formation on fibronectin.

Results: Both aza-dC and TSA induced at least a three-fold increase in the expression of the EC marker genes VE-cadherin, vWF, and Flk1. This increase was also observed in the presence of the EC differentiation inducer VEGF, suggesting that factors other than VEGF mediate the response to the epigenetic agents. Both DNMT and HDAC inhibition stimulated vascular network formation.

Conclusion: Epigenetic therapy holds a potential in inducing self-repair, vascular tissue regeneration, controlling angiogenesis and endothelial dysfunction. (*Ethn Dis*. 2010;20:[Suppl 1]:S1-60–S1-64)

Key Words: Bone Marrow Stem Cells, DNA Methylation, Histone Acetylation, Endothelial Cells, Differentiation

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Introduction

Abuses such as atherosclerosis, hypertension, diabetes, and other cardiovascular risk factors affect the integrity and functional activity of the endothelial monolayer. Endothelial dysfunction plays a critical role in the pathogenesis of these diseases and has been shown to be an independent predictor of cardiovascular diseases.[1] Bone marrow transplantation therapy, with the goal of regenerating endothelial cells (EC), has been tested in preclinical experimental models and in clinical endothelial injury or dysfunction, and for ischemic vascular diseases, such as myocardial infarction or stroke. Indeed, bone marrow-derived endothelial cells have been shown to incorporate into sites of neovascularization and sites of endothelial injury, and replace dysfunctional endothelium.[2] However, using more defined, differentiated EC has obvious advantages to using crude bone marrow or partially purified bone marrow cells. For example, in the allogenic setting, this eliminates immunoreactive T cells and potentially tumorigenic cancer stem cells. Therefore, understanding the mechanisms of endothelial differentiation from bone marrow stem cells is essential for possible therapeutic applications.

Until recently, it was believed that adult bone marrow stem cells comprised mesenchymal stem cells (MSCs), which can differentiate into multiple cell lineages such as osteoblasts, chondrocytes, myoblasts, adipocytes, endothelial cells, neuron-like cells, cardiomyocytes and hepatocytes, and hematopoietic stem cells (HSCs), which give rise to all mature lineages of blood.[3] However, another bone marrow-derived stem cell, known as multipotentent adult progenitor cells (MAPC), has been found to have more plasticity and to be more pluripotent than the other adult stem cells.[4] MAPCs have the potential to differentiate, at the single cell level, toward cells derived from the three embryonic germ layers.[5,6] Like rodent embryonic stem cells, MAPC requires leukemia inhibitory factor (LIF) for culture and maintenance in the pluripotent state.[7] They express the primitive pluripotency markers characteristic of the embryonic stem cells such as oct4 and rex1, though expression of oct4 in MAPC is 1000-fold lower than that of embryonic stem cells.[5] Recent studies analyzing expression levels of oct4 in somatic stem cells, including freshly isolated bone marrow and cultured mesenchymal stem cells, have raised doubts about the expression of oct4 in adult stem cells[8–10] and its role in regulating the pluripotency of these stem cells.[8,9] However, a comparative transcriptome analysis of embryonic and the adult stem cells, such as MSC and MAPC, has suggested that MAPC are intermediate between embryonic stem cells and MSC and predicted that MAPCs may be the progenitors of the other tissue-specific stem cells such as mesenchymal, neural, gastrointestinal,

epidermal, hematopoietic, hepatic, and cardiac stem cells.[4] Therefore, they are an excellent tool to study the molecular mechanisms of self-repair during the recovery phase of cardiovascular diseases.

Recent studies have shown that Bmp4 is critical for differentiation of embryonic stem cells and bone marrow stem cells into the endothelial lineage.[11–13] They suggest a common molecular mechanism of differentiation of endothelial cells from both embryonic and adult stem cells. Previous studies have documented the importance of epigenetic changes such as DNA methylation and histone acetylation in gene regulatory networks during ESC differentiation.[14–16] We postulated that EC differentiation from MAPC could be enhanced by inhibiting DNA methylation and histone deacetylation, reversing the repression of genes that specify endothelial cell fate determination.

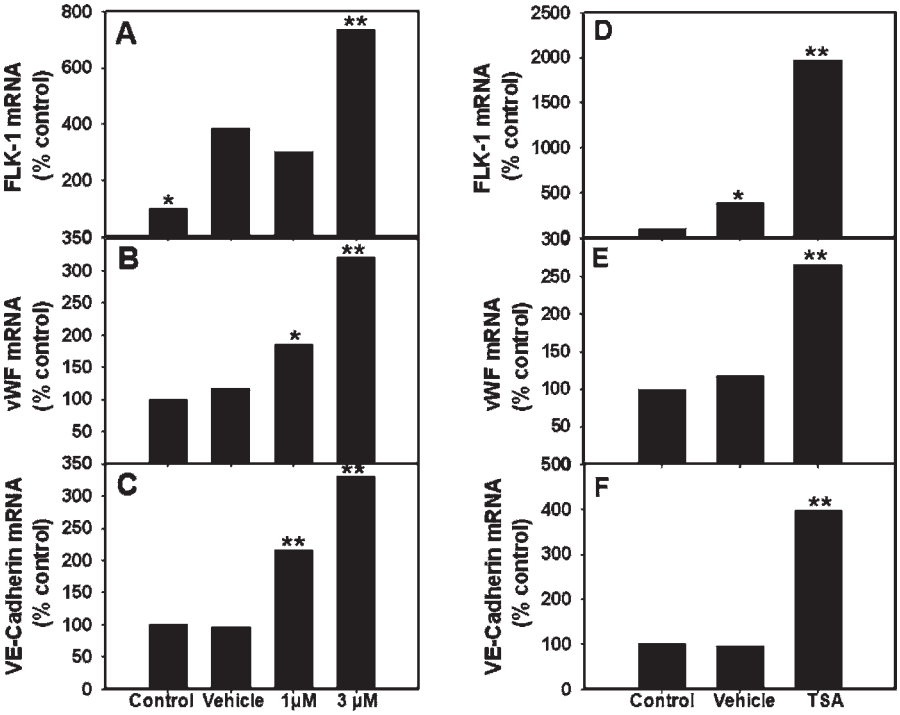

Fig 1. The DNMT and HDAC inhibitors increased expression of the endothelial marker genes on basal differentiation medium. Values for each gene are normalized by those of GAPDH and are presented in % of control (untreated). (A, B, C) Expression of flk1, vWF, and VE-cadherin in response to aza-dC treatment. (D, E, F) Expression of flk1, vWF, and VE-cadherin in response to TSA treatment. *$P \leq .05$ vs vehicle; ** $P \leq .01$ vs vehicle

METHODS

Rat MAPC Isolation and Culture

Rat MAPCs were isolated as described by Breyer et al.[7] Briefly, bone marrow cells were flushed out of the femur and tibia of a 4-week old Sprague Dawley rat. Six million bone marrow cells were plated per well of a fibronectin-coated 6-well plate (Corning) on rat MAPC maintenance medium. The medium was composed of 60% low-glucose DMEM (Gibco BRL), 40% MCDB-201, 1 × insulin-transferrin-selenium (ITS, Sigma), 1 × linoleic acid bovine serum albumin (LA-BSA, Sigma), 0.05×10^{-6} M dexamethasone (Sigma), 10^{-4} M ascorbic acid 3-phosphate (Sigma), 100 units penicillin, 1,000 units streptomycin (Gibco BRL), 55 μM 2-mercaptoethanol, 2% FBS (HyClone), 10 ng/mL hPDGF-BB (R&D Systems), 10 ng/mL mEGF (Sigma), and 1,000 units/mL mLIF (Chemicon). The cells were grown in a humidified, 5% O_2 and 6% CO_2, 37°C incubator. After 4 weeks, the cells were depleted of CD45 and TER119 positive cells using micromagnetic beads and a MACS separation CS column (Miltenyi Biotec, Sunnyvale, CA). $CD45^-TER119^-$ cells were subcultured at 10 cells/well of fibronectin-coated 96-well plates. The low density seeding increases the probability of clones growing from a single cell in each well. After 2 weeks, each colony with MAPC morphology was transferred into progressively larger plates. When enough cells were obtained, each clone that maintained the MAPC morphology was further characterized by QRT-PCR analysis of expression of specific markers, karyotyping to ascertain cytogenetic normalcy, and the ability to differentiate into the three embryonic layers. MAPCs were maintained on the MAPC maintenance medium and expanded by trypsinizing (0.05% trypsin) the cells and replating them every 2 days at a density of 200 cells/cm^2.

Karyotyping

Rat MAPCs were seeded on fibronectin-coated 10-cm plates in the maintenance medium at a density of 30,000 cells/plate. Forty eight hours after cell seeding, they were treated with 8 drops of Demecolcine (Sigma-D1925) for 3 h to stop cells in the metaphase cell cycle phase, harvested, washed, and exposed to 75 mM KCl hypotonic solution for 5 min. Hypotonic solution was washed out by centrifugation and cells were fixed in 10 mL methanol/acetic acid (3:1) fixative for 5 min. Fixed cells were collected by centrifugation and resuspended in 0.2 mL methanol/acetic acid (3:1) fixative. Cells were dropped onto a hot glass slide. Chromosomes were stained using Wright's stain. Chromosomal spreads were viewed under a microscope and 5 of them were counted per clone.

MAPC DIFFERENTIATION

Rat MAPCs were seeded on fibronectin-coated 24-well plates in the maintenance medium at a density of 45,000 cells/cm2 (81,000 cells/well). The next day, maintenance medium was switched completely to basal differentiation medium (maintenance medium without PDGF, EGF, and LIF) with or without 10 ng/mL hVEGF-A (R&D Systems). During the first 48 hours of differentiation, MAPCs were treated with vehicle, 1 or 3μM aza-dC, or 100 nM TSA in the differentiation medium. Thereafter, media were changed every other day using basal differentiation medium with or without VEGF (vascular endothelial growth factor). Differentiation was monitored for up to 18d.

RNA isolation and Q-RT-PCR

Total RNA was extracted using the RNeasy microkit (Qiagen, Valencia, CA). mRNA was reverse transcribed using Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) and random hexamer primers. cDNA was quantified using SYBR Green PCR Master Mix (Applied Biosystems) and an ABI PRISM 7700 sequence detector (Perkin Elmer/Applied Biosystems, Foster City, CA). Primers used for amplification and PCR conditions are available upon request. The data were processed with the ABI PRISM 7700 sequence detection software (V1.7α). Count values were exported into Excel software and used to compute normalized values. mRNA levels were normalized using the housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase).

Statistical Analysis

For the effect of treatment on mRNA expression, there were 3 replications per experimental unit and the data were analyzed by analysis of variance (one way ANOVA). Calculations were performed with the Statistical Analysis System (SAS, version 8.01, SAS Inc., Cary, NC). ANOVA was performed by the general linear model procedure and post-hoc multiple mean comparisons were performed by the Bonferoni test. P values $\leq .05$ were considered to be statistically significant. Unless otherwise stated, results are presented as percent of the untreated control.

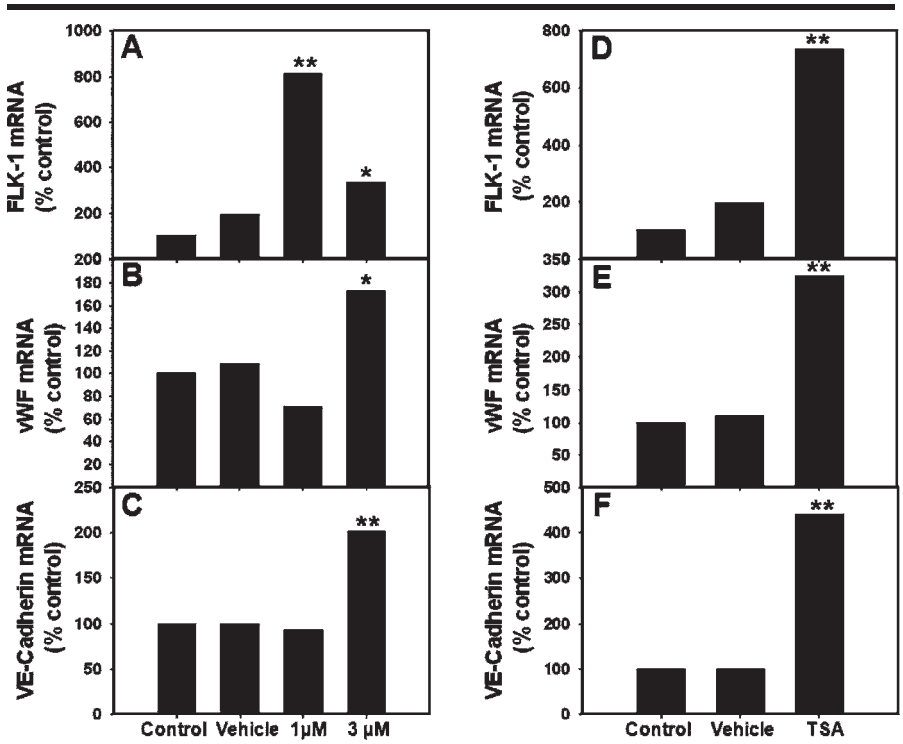

Fig 2. The DNMT and HDAC inhibitors increased expression of the endothelial marker genes on the EC differentiation medium. Values for each gene are normalized by those of GAPDH and are presented in % of control (untreated). (A, B, C) Expression of flk1, vWF, and VE-cadherin in response to aza-dC treatment. (D, E, F) Expression of flk1, vWF, and VE-cadherin in response to TSA treatment. *$P \leq .05$ vs vehicle; ** $P \leq .01$ vs vehicle

RESULTS

The DNMT and HDAC inhibitors increased expression of the endothelial marker genes in MAPC on basal differentiation medium. To begin defining the role of epigenetics in the differentiation of MAPC into EC, rMAPC were differentiated on basal differentiation medium in the presence of vehicle, 1 or 3 μM aza-dC, and 100 nM TSA for the initial 48h. Expression of the EC marker genes was determined 14 days after the initiation of differentiation. Figure 1 shows that expression of the endothelial marker genes was stimulated by both aza-dC and TSA treatment. Relative to the untreated control, expression of flk1, vWF, and VE-cadherin increased by 7.4-, 3.2-, and 3.3-fold, respectively, following DNMT inhibition (Fig. 1A–C). Expression of the same genes following HDAC inhibition by TSA increased by 19.7-, 2.7-, and 4.0-folds, respectively, relative to the untreated control (Figs. 1D–F). Vehicle treatment had no measurable effects (Fig. 1A–F).

The DNMT and HDAC inhibitors increased expression of the endothelial marker genes in MAPC on the EC differentiation medium. Previous studies had established that when MAPC are differentiated on basal differentiation medium, they differentiate into cells of

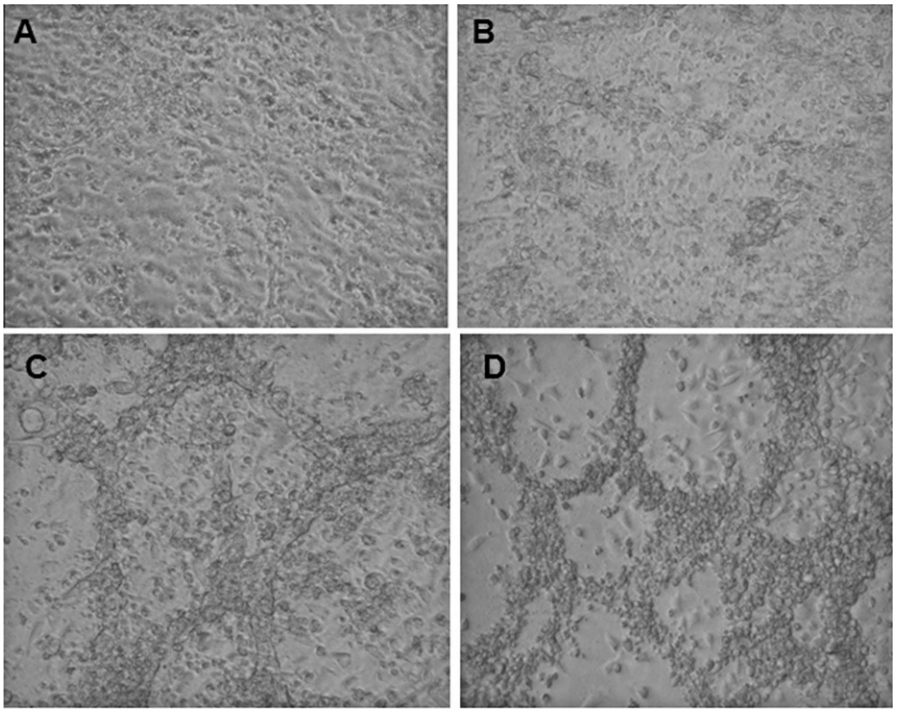

Fig 3. The DNMT and HDAC inhibitors induces MAPC to form vascular-like networks. The differentiation was performed on basal differentiation medium (A) in the presence of Vehicle (B), 1 μM aza-dC (C), or 100 nM TSA (D) for 48h. Vascular network formation was visualized by microscopy 18 d after initiation of differentiation

the three germ layers. However, when VEGF is added to the differentiation medium, they preferentially differentiate into EC.[5] To determine whether VEGF mediates signaling networks induced by epigenetic changes leading to MAPC differentiation into EC following DNMT and HDAC inhibition, we measured the expression of endothelial markers genes when VEGF was added to the differentiation medium. If VEGF is the sole mediator, DNMT and HDAC inhibition should fail to increase the expression of EC markers. On the other hand, if VEGF is not a mediator, there should be the same levels of expression increases as in its absence. Figure 2 indicates that both aza-dC and TSA treatment stimulated expression of the endothelial marker genes. Relative to the untreated control, expression of flk1, vWF, and VE-cadherin increased by 8.1-, 1.7-, and 2.0-fold, respectively, following DNMT inhibition (Fig. 2A–C). Expression of the same genes following HDAC inhibition by TSA increased by 7.4-, 3.3-, and 4.4-fold, respectively, relative to the untreated control (Figs. 2D–F). Vehicle treatment had no measurable effects (Fig. 2A–F).

The DNMT and HDAC inhibitors induce MAPC to form vascular-like networks on basal differentiation medium. Angiogenesis is the process where new blood vessels sprout out of preexisting vessels and this process involves the recruitment and proliferation of EC. *In vitro* angiogenesis assay has shown that mature ECs form a vascular-like network on matrix proteins. Therefore, angiogenesis assay is routinely used to assess the maturity and functionality of EC. We assessed vascular-like network formation by MAPCs on fibronectin following DNMT and HDAC inhibition. Figure 3 shows that both aza-dC (Fig. 3C) and TSA (Fig. 3D) treatments stimulated vascular-like network formation relative to the untreated or vehicle-treated control when MAPCs were grown on basal differentiation media.

DISCUSSION

Endothelial dysfunction is an independent predictor of cardiovascular diseases (CVD).[1] Bone marrow-derived stem cells can hone to sites of injured endothelium and MAPCs can induce angiogenesis.[17] MAPCs have been shown to have more plasticity than any other adult stem cell[4] and therefore represent an excellent tool to study the epigenetic regulation of adult stem cell differentiation into EC. However the molecular mechanisms mediating the differentiation of MAPCs into endothelial cells are not well understood. Previous studies had established the role of epigenetics, such as DNA methylation and histone acetylation reprogramming in the differentiation of embryonic stem cells into the mesodermal lineage. Indeed, the specific DNMT inhibitor aza-dC has been shown to induce the differentiation of ESC into cardiomyocytes and endothelial cells.[18,19] This effect could not be achieved by the other differentiation agents such as DMSO or retinoic acid, suggesting a role of epigenetics in the process. However, little is known about epigenetic regulation of adult stem cell differentiation into mesodermal lineages such as the EC.

Our data show that DNMT and HDAC inhibition induce MAPC to differentiate into the endothelial lineage. This is based on 1) the more than 3-fold increase in expression of the endothelial marker genes, including the marker of mature endothelial cells VE-cadherin; and 2) the increased formation of vascular-like structures when MAPC were treated with the

DNMT and HDAC inhibitors. Previous studies had established that VEGF stimulates MAPC differentiation into EC.[5] In our studies, the DNMT and HDAC inhibitors were able to stimulate expression of the endothelial marker genes in the presence of VEGF, even though the stimulation was sometimes less robust than in its absence. This suggests that besides VEGF, other factors mediate the response to the epigenetic agents. Futures studies will uncover the EC defining genes that are epigenetically repressed in MAPC and other endothelial progenitor cells.

Racial and ethnic disparities exist in both CVD prevalence and mortality[20] and EC dysfunction is the hallmark of these diseases.[1] This study, by beginning to define the molecular basis of EC dysfunction, will contribute to reducing this health disparity.

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