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Hypermethylation of *CXCR4* Promoter in CD34⁺ Cells from Patients with Primary Myelofibrosis

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Key Words. CXCR4 • Methylation • Myelofibrosis • CD34⁺ cell • Epigenetic

ABSTRACT

Constitutive mobilization of CD34⁺ cells in patients with primary myelofibrosis (PMF) has been attributed to proteolytic disruption of the CXCR4/SDF-1 axis and reduced CXCR4 expression. We document here that the number of circulating CD34⁺/CXCR4⁺ cells in PMF patients, as well as the cellular CXCR4 expression, was directly related to CXCR4 mRNA level and that reduced CXCR4 mRNA level was not due to SDF-1-induced downregulation. To address whether epigenetic regulation contributes to defective CXCR4 expression, we studied the methylation status of the CXCR4 promoter using methylation-specific polymerase chain reaction and methylation-specific sequencing in the JAK2V617F-positive HEL cell line and in CD34⁺ cells. We

found that CD34⁺ cells from PMF patients, unlike those from normal subjects, presented hypermethylation of CXCR4 promoter CpG island 1. Following incubation with the demethylating agent 5-Aza-2'-deoxycytidine (5-AzaD), the percentage of PMF CD34⁺ cells expressing CXCR4 increased 3–10 times, whereas CXCR4 mRNA level increased approximately 4 times. 5-AzaD-treated PMF CD34⁺ cells displayed almost complete reversal of CpG1 island 1 hypermethylation and showed enhanced migration in vitro in response to SDF-1. These data point to abnormal methylation of the CXCR4 promoter as a mechanism contributing to constitutive migration of CD34⁺ cells in PMF. STEM CELLS 2008;26:1920–1930

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Stromal cell-derived factor-1/CXC chemokine ligand 12 (SDF-1/CXCL12) is a major chemoattractant for normal hematopoietic stem and progenitor cells (HSCs/HPCs). SDF-1 is produced by endothelial cells, immature osteoblasts, and stromal cells within the bone marrow (BM) microenvironment, as well as by other cell types, including CD34⁺ cells themselves [1]. The canonical receptor for SDF-1 is chemokine receptor 4 (CXCR4), a member of a family of seven trans-membrane domain receptors coupled to heterotrimeric G1 proteins. Experimental deficiency in *SDF-1* or *CXCR4* in the embryo resulted in a lethal phenotype characterized by defective development of cardiac, gastrointestinal, blood vessel, and central nervous system and in the lack of BM seeding by HSCs/HPCs and maturing myeloid or lymphoid cells [2–5]. A role for the SDF-1/CXCR4 axis in human cancer cell metastatic spread has also been inferred [6–9].

Firm adhesion of HSCs/HPCs to BM endothelium in a murine transplantation model was mediated by locally produced SDF-1 [10], whereas, conversely, SDF-1 administration induced recruitment of HSCs/HPCs and maturing leukocytes to the circulation and the spleen [11]. Thus, a positive SDF-1 gradient is the mechanism (or one of the mechanisms) by which HSCs/HPCs are normally retained in the BM. The increased mobilization of HSCs/HPCs that occurs following administration of granulocyte colony-stimulating factor (G-CSF) or chemotherapeutic agents [12, 13] has been associated with the release of proteolytic enzymes, such as cathepsin G, neutrophil elastase, and matrix metalloproteinase-9 (MMP-9), which cause SDF-1 degradation and cleavage of the CXCR4 N terminus [12]. However, a negative correlation was found between the degree of HSCs/HPCs mobilized to peripheral blood (PB) and CXCR4 expression level in patients receiving chemotherapy plus G-CSF [14] or G-CSF alone [15]; furthermore, CD34⁺ cells in the BM of patients on G-CSF displayed lower CXCR4 membrane density than naïve cells, and the yield of mobilized CD34⁺ cells

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correlated with the proportion of BM CD34⁺/CXCR4⁻ cells [15]. These data support the contention that mobilization of CD34⁺ cells is also dependent on the density of CXCR4 on the cell membrane; accordingly, antagonist-induced desensitization of CXCR4 caused the egress of HSCs/HPCs from BM to PB [16]. The mechanisms by which G-CSF affects the expression of CXCR4 have not been completely elucidated, but at least in part they depend on proteolytic modifications with functional inactivation of the receptor [13]. However, it has been shown that G-CSF downregulates *CXCR4* in myeloid cells [17] through the transcriptional repressor growth factor independence-1 (Gfi-1) [18], whereas modulation of *CXCR4* expression at the post-translational level has been described in both hematopoietic [19] and nonhematopoietic [7, 20] cells. CXCR4 on the cell surface of migrating cells is physiologically downregulated upon SDF-1 binding through rapid phosphorylation and internalization of the receptor [21]. Therefore, mobilization of HSCs/HPCs may be the consequence of changes in the expression of either SDF-1 or CXCR4 or both.

Primary myelofibrosis (PMF) is a chronic myeloproliferative disorder (MPD) characterized by variable changes in blood cell count, leukoerythroblastosis, splenomegaly, extramedullary hematopoiesis, and bone marrow fibrosis with extensive angiogenesis [22]. Constitutive mobilization of CD34⁺ cells in the PB is one striking characteristic of PMF; the number of these cells is more than 300 times greater than in healthy subjects and 20–30 times greater than in other MPDs, such as polycythemia vera and essential thrombocythemia [23]. Abnormal cell mobilization in PMF is not limited to HSCs/HPCs, since more cells with a CD34⁺/CD133⁺/vascular endothelial growth factor receptor 2⁺ phenotype have been reported to circulate, preferentially at early phases of the disease [24]; although these cells do not function as true endothelial progenitor cells and their function in vessel formation and/or homeostasis is still unknown [25], they might be involved in the same mechanisms leading to enhanced mobilization as for the HSCs/HPCs. Mechanisms underlying the abnormal mobilization of CD34⁺ cells in PMF probably involve the establishment of a proteolytic environment causing SDF-1 degradation within the BM [26, 27]. However, it is likely that abnormalities of CXCR4 also play some role, as suggested by findings of reduced CXCR4 expression and an abnormally low *CXCR4* mRNA level in PMF CD34⁺ cells [28, 29]. The aim of this study was to address potential mechanisms for the reduced CXCR4 expression in PMF CD34⁺ cells and its contribution to the phenomenon of constitutive migration in the circulation.

SUBJECTS, MATERIALS, AND METHODS

Subjects

This study included a total of 40 PMF patients, 11 normal blood donors, and 31 volunteer donors of hematopoietic stem cells, of whom 20 donated BM cells and 11 were mobilized with G-CSF. Diagnosis of PMF was made according to the criteria identified by the Italian Consensus Conference [30]; all patients were primary forms in a typical fibrotic stage of the disease [31], who were studied at diagnosis or during follow-up, but were cytotoxic agent-free from at least 1 month. Twenty-four patients were *JAK2V617F* mutant (60%) [32], whereas none harbored *MPL* mutation [33]. Seventy-five percent of the patients were in the low-risk category, 20% in the intermediate category, and 5% in the high-risk category, according to Lille score [34]. The CD34⁺ cell count in the PB of PMF patients was $186 \pm 179 \times 10^6$ cells per liter (range, 10–379 $\times 10^6$ cells per liter). Informed consent from patients and controls was obtained under an IRB approval study protocol.

www.StemCells.com

Flow Cytometry Analysis of Circulating CD34⁺ Cells

CD34⁺ cells in the PB of PMF patients were enumerated using 50 μ l of EDTA-anticoagulated blood, after staining with CD45-fluorescein isothiocyanate (FITC)-/CD34-phycoerythrin (PE)-conjugated monoclonal antibodies (both from Becton, Dickinson and Company, San Jose, CA, <http://www.bd.com>) and 7-aminoactinomycin D (BD Pharmingen, San Diego, http://www.bdbiosciences.com/index_us.shtml) for excluding dead cells in a FACScan flow cytometer (Becton Dickinson), according to guidelines from the International Society of Hematotherapy and Graft Engineering [35]. Cell surface expression of CXCR4 (CD184) was determined using anti-human CD184(CXCR4)-PE antibody (BD Pharmingen) and the appropriate isotype control. Results were expressed both as the percentage of CD45⁺/CD34⁺ cells coexpressing CXCR4 and as the ratio of geometric mean fluorescence intensity (MFI) by dividing the value of specific antibodies with the corresponding isotype control antibody. Intracellular CXCR4 staining in cells migrating in Transwell cells (Corning Costar, Boston, <http://www.corning.com/lifesciences>) (described below) was performed after blocking cell surface CXCR4 with nonconjugated antihuman CXCR4 antibody (clone 12G5, 10 μ g/ml, for 1 hour at 4°C). Cells were fixed with paraformaldehyde and permeabilized with 1% Triton X-100 before fluorescence-activated cell sorting (FACS) analysis. Data analysis was performed using WinMDI software (version 2.9; <http://facs.scripps.edu/software.html>).

CD34⁺ Cell Purification

BM or PB mononuclear cells were separated over a Ficoll-Hypaque gradient (Lymphoprep; Nycomed Pharma, Oslo, Norway, <http://www.nycomed.at>), and CD34⁺ cells were immunomagnetically selected (Miltenyi Biotec, Bergisch Gladbach, Germany, <http://www.miltenyibiotec.com>) [36]. Purity of the isolated CD34⁺ cell population was evaluated by flow cytometry after labeling with PE-HPCA2 anti-CD34 monoclonal antibody (Becton Dickinson). Aliquots of CD34⁺ cells were immediately resuspended in lysis buffer for RNA and DNA purification or used in culture.

Ex Vivo Short-Term Incubation of CD34⁺ Cells

Purified CD34⁺ cells were cultured at 10⁵ cells per milliliter in Iscove's modified Dulbecco's medium (IMDM; Bio Whittaker) containing 10% fetal calf serum (FCS; HyClone, Logan, UT, <http://www.hyclone.com>) and incubated at 37°C and 5% CO₂ in a humidified atmosphere for 4 hours. Cells were then exposed for variable times up to 48 hours to different agents, including cytokines (SDF-1, transforming growth factor- β [TGF- β], and interferon- γ [IFN- γ]; all from Peprotech, Rocky Hill, NJ, <http://www.peprotech.com>) and the hypomethylating agent 5-Aza-2'-deoxycytidine (5-AzaD; Sigma-Aldrich, Milano, Italy, <http://www.sigmaaldrich.com>). Parallel cultures without added cytokines or 5-AzaD were prepared in each experiment as control. At the end of incubation, cells were collected and either processed directly for FACS analysis or nucleic acid extraction, or washed in fresh medium for use in migration experiments.

Cell Lines

The human erythroleukemia HEL and K562 cell lines and the myeloid HL60 cell line were purchased from the American Type Culture Collection (Manassas, VA, <http://www.atcc.org>). The cells were maintained in IMDM supplemented with 10% FCS.

Real-Time Quantitative Polymerase Chain Reaction Determination of *CXCR4* mRNA Level

Total RNA was extracted using Trizol (Invitrogen, Paisley, U.K., <http://www.invitrogen.com>); RNA concentration and purity/integrity were determined using NanoDrop technology (ND-1000 spectrophotometer; NanoDrop, Wilmington, DE, <http://www.nanodrop.com>). cDNA was reverse-transcribed from 100 ng of total RNA obtained from purified CD34⁺ cells using the High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, <http://www.appliedbiosystems.com>). TaqMan polymerase chain reactions

(PCRs) were carried out with the TaqMan Universal PCR Master Mix, using TaqMan Gene Expression Assays (HS-00607978 for *CXCR4*; Applied Biosystems), by means of the ABI Prism 7300 HT Sequence Detection System (Applied Biosystems). Assays were performed in quadruplicate. Gene expression profiling was achieved using the comparative cycle threshold (C_T) method of relative quantitation using VIC-labeled *RNaseP* probe as the housekeeping gene (Applied Biosystems) (ΔC_T).

Methylation-Specific PCR and Methylation-Specific Sequencing Analysis of *CXCR4* Promoter Methylation Status

To determine the location of CpG islands in the promoter region of *CXCR4*, we used the MethPrimer software (<http://www.urogene.org/methprimer/index1.html>); five CpG islands were identified, and primers for each CpG island were designed to be used in a methylation-specific polymerase chain reaction (MSP) assay (reported in supplemental online Fig. 1). We also designed primers for use in a methylation-specific sequencing (MSS) assay (described below), which allowed the amplification of a 230-nucleotide region within CpG island 1 containing 22 individual CpG dinucleotides. In brief, 1 μ g of DNA obtained from CD34⁺ cells or cell lines was treated with sodium bisulfite to induce conversion of unmethylated cytosine to uracil (Epitect Bisulfite kit; Qiagen, Hilden, Germany, <http://www1.qiagen.com>). The CpG methylase SssI from *Spiroplasma* sp., strain MQ1 (New England Biolabs, Beverly, MA, <http://www.neb.com>), was used to produce fully methylated methylated DNA from HL60 cells (positive control). MSP was performed using primers specific for either the methylated or unmethylated DNA under the following conditions for TaqGold polymerase (Applied Biosystems): 94°C for 13 minutes followed by 40 cycles at 94°C for 30 seconds, 59°C or 60°C (for methylated and unmethylated *CXCR4*, respectively) for 30 seconds, and 72°C for 30 seconds. Amplicons were resolved in 3% agarose gel and visualized by ethidium bromide staining. Bisulfite-modified DNA was also used as direct template for MSS. PCR conditions were as follows: 94°C for 13 minutes; 30 cycles consisting of 30 seconds at 94°C, 30 seconds at 56°C, and 30 seconds at 72°C; and 10 minutes at 72°C. The primer sequences were as follows: forward primer, 5'-gaggg-ggaaagtattgtt-3'; reverse primer, 5'-aaacttacacaaatcactcattca-3'. Amplicons were subjected to bidirectional sequencing on an ABI Prism 3730 DNA Analyzer (Applied Biosystems).

In Vitro Migration Assay for CD34⁺ Cells

For these experiments, 2×10^5 purified CD34⁺ cells were seeded in 500 μ l of IMDM supplemented with 10% FCS in the absence (control) or in the presence of 1 μ M 5-AzaD for 12 hours. At the end of incubation, cell aliquots were labeled with anti-CD34⁺ FITC and anti-human CD184(CXCR4) PE to evaluate changes in CXCR4 content upon 5-AzaD exposure. Immediately before migration assay, CD34⁺ cells were washed in serum-free media, resuspended in 100 μ l of IMDM, and loaded into Transwell filters (5 μ m pore; Transwell, 24-well cell clusters), which were then carefully transferred to wells containing 500 μ l of serum-free medium supplemented or not (for calculation of passive diffusion) with 125 ng/ml recombinant hSDF-1 α . Plates were incubated at 37°C in 5% CO₂ for 3 hours, and then the upper chamber was carefully removed and cells in top and bottom chambers were counted; the percentage of CD34⁺ cells undergoing migration in samples treated or not with 5-AzaD was calculated after subtracting passive diffusion.

Statistical Analysis

Comparison between groups was performed by the Mann-Whitney *U* test or Fisher test as appropriate, using the SPSS software (StatSoft, Inc., Tulsa, OK, <http://www.statsoft.com>), GraphPad In-Stat software (GraphPad Software, Inc., San Diego, <http://www.graphpad.com>), or Origin software (version 7.5; OriginLab, Northampton, MA, <http://www.originlab.com>) for computation. The chosen level of significance from two-sided tests was $p < .05$.

RESULTS

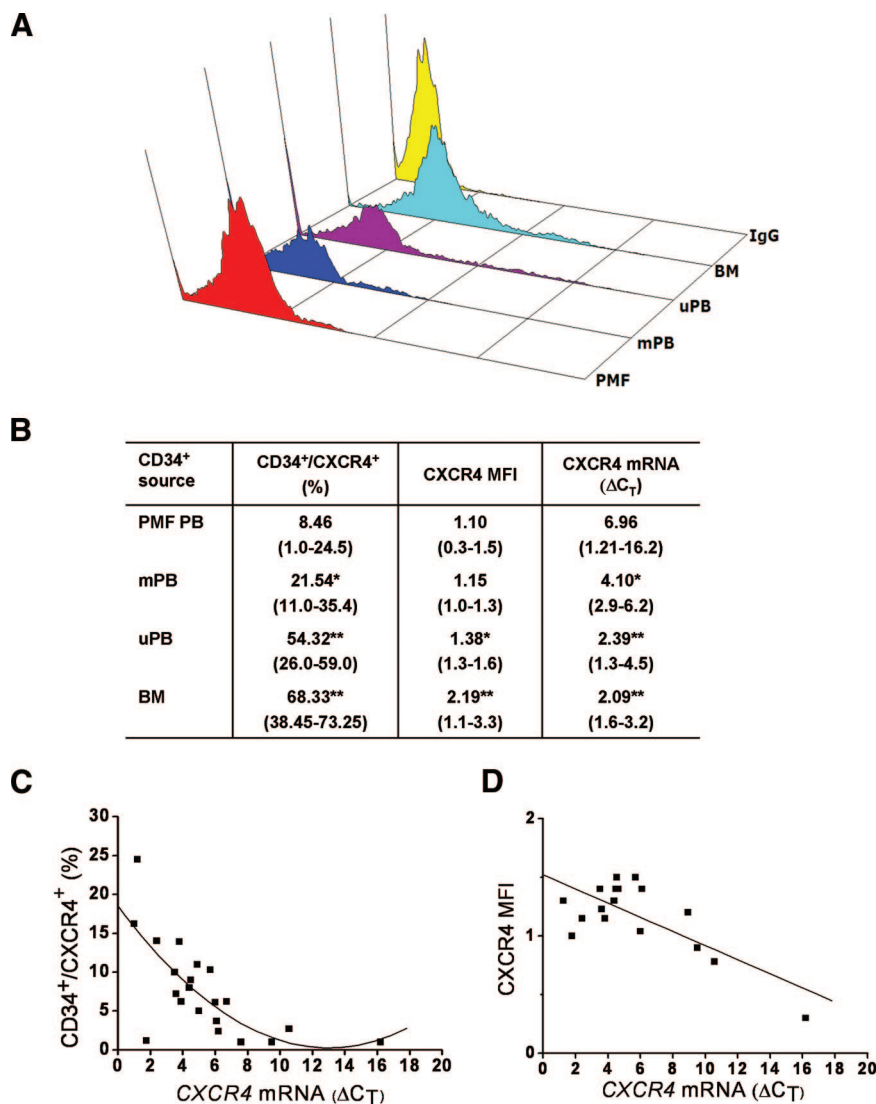
CXCR4 Expression in PMF CD34⁺ Cells Correlates with *CXCR4* mRNA Level

The expression of membrane CXCR4 in CD34⁺ cells from PB of PMF patients was determined by flow cytometry (FACS) and compared with normal different sources, which included unstimulated peripheral blood (uPB) and G-CSF-mobilized peripheral blood (mPB) and BM aspirates. As shown in Figure 1A and 1B, the frequency of CD34⁺ cells coexpressing CXCR4 in the PB of PMF patients was significantly lower than in normal samples ($p < .01$ compared with uPB or BM cells; $p < .05$ compared with mPB). In normal samples, CD34⁺CXCR4⁺ cells progressively decreased from a median of 68.3% of all CD34⁺ cells in the BM to 54.3% in uPB and to 21.5% in mPB. Also, the density of CXCR4 molecules, expressed as the MFI, was significantly lower in PMF CD34⁺ cells compared with normal uPB or BM CD34⁺ cells but not compared with mPB CD34⁺ cells (Fig. 1B). Purified PMF CD34⁺ cells expressed significantly lower *CXCR4* mRNA level than normal cells from all other sources; the highest and lowest mRNA levels were measured in CD34⁺ cells from control BM and PMF PB, respectively, differing on the order of 4.9 ΔC_T (Fig. 1B). On the basis of the assumption that in optimized real-time quantitative (RTQ) PCR experiments a ΔC_T $n = 3.3$ corresponds to a 10-fold difference in mRNA level [37], it was calculated that *CXCR4* mRNA expression in PMF CD34⁺ cells was ~40-fold lower than in normal BM CD34⁺ cells. Both the frequency of circulating CD34⁺/CXCR4⁺ cells and the CXCR4 MFI in PMF patients were correlated with *CXCR4* mRNA level, as shown in Figure 1C and 1D, respectively. The percentage of CD34⁺ cells coexpressing CXCR4, the CXCR4 MFI, and the level of *CXCR4* mRNA were all similar in patients harboring the *JAK2V617F* mutation and those not harboring it (not shown in detail).

Reduced *CXCR4* mRNA Level Is Not Due to SDF-1-Induced Downregulation

SDF-1 level has been found to be significantly increased in the BM microenvironment and in plasma of PMF patients [28, 38]; although downregulation of CXCR4 expression on the cell membrane would be expected because of its physiologic internalization upon SDF-1 cross-linking, we also asked whether the reduced *CXCR4* mRNA level observed in PMF CD34⁺ cells could follow ligand binding. To this end, we first determined the expression pattern of CXCR4 and *CXCR4* mRNA in three different hematopoietic cell lines: the HEL cell line, which harbors *JAK2 V617F* mutation; the BCR/ABL-positive K562 cell line; and the PML-RAR α -positive HL-60 cell line. We found that K562 cells were virtually negative for CXCR4 expression, whereas almost all HL60 cells expressed CXCR4 at a high level (MFI = 1.9); on the other hand, approximately 25% (range, 9.2%–49.3%) of HEL cells were CXCR4⁺ dim (MFI = 1.2) (Fig. 2). The level of *CXCR4* mRNA mirrored the results of FACS analysis (Fig. 2B); *CXCR4* mRNA was virtually undetectable in K562 cells, highly expressed in HL60 cells, and expressed at intermediate level, approximately 15-fold lower, in HEL cells.

HEL cells were exposed to increasing concentration of SDF-1 for 3–24 hours. Starting as soon as after 3 hours of incubation, we observed a dose-dependent decline of membrane CXCR4 that became undetectable in the presence of 500 ng/ml SDF-1 (Fig. 3A, 3B); on the contrary, *CXCR4* mRNA level remained stable (Fig. 3B). *CXCR4* was similarly downregulated in the presence of 500 ng/ml SDF-1 in CD34⁺ cells from normal uPB or PMF PB; again, no concurrent modification of mRNA



level was observed (Fig. 3C–3E). We concluded that SDF-1-induced decrease of CXCR4 expression in HEL cells and in CD34⁺ cells was due to ligand-induced internalization of the receptor but did not involve CXCR4 transcription. We also evaluated the effect of TGF- β , whose level is increased in PMF [39] and supposedly involved in disease pathogenesis [40, 41], and of IFN- γ , which was shown to downregulate CXCR4 mRNA in lymphocytes and neutrophils [42, 43]; we observed that neither cytokine induced modification of CXCR4 expression or of CXCR4 mRNA level in normal uPB- and PMF PB-derived CD34⁺ cells (supplemental online Fig. 2).

The CXCR4 Promoter Methylation Status Correlates with CXCR4 mRNA Level

Since epigenetic mechanisms have been shown to contribute to regulated expression of CXCR4 in solid cancer cell lines [44–46], we asked whether this mechanism might also be involved in the abnormally low CXCR4 expression in PMF CD34⁺ cells. The promoter region of CXCR4 contains five CpG islands (supplemental online Fig. 1); an MSP assay on bisulfite-treated DNA was designed to evaluate methylation status of each of these CpG islands, whereas MSS was used for sequencing a CpG dinucleotide-rich region within CpG island 1. In the first set of experiments, we used cell lines. We found that CpG 1 and

5 were partially methylated in HEL cells, whereas K562 cells had preferential representation of the methylated allele corresponding to CpG island 1, 3, and 5; on the other hand, there was no evidence for methylated alleles in HL60 cells (Fig. 4A). By using MSS, we confirmed that HL60 cells had 100% unmethylated cytosine compared with 40% \pm 8% and 74% \pm 16% in HEL and K562 cells, respectively (mean \pm SD of three determinations; $p < .01$ for both compared with HL60 cells) (Fig. 4B).

The methylation status of CXCR4 promoter was evaluated in CD34⁺ cells purified from the BM ($n = 4$) or mPB ($n = 4$) of healthy controls but not from uPB because of the insufficient number of purified cells that could be obtained. According to MSP, we found that all CpG islands in the CXCR4 promoter of BM-derived CD34⁺ cells were in an unmethylated status, whereas in CD34⁺ purified from mPB, a faint band corresponding to the methylated allele of CpG island 1 could be observed (Fig. 5A). On the other hand, all 18 PMF patients evaluated displayed comparable representation of methylated and unmethylated alleles corresponding to CpG island 1, with faint evidence of a methylated allele for CpG island 5 also (patients 3, 4, and 7) (Fig. 5A). By using MSS analysis, we calculated that 25% \pm 13% of cytosine residues in PMF CD34⁺ cells were methylated, compared with none in normal BM CD34⁺ cells and 9% \pm 4%

Figure 1. Frequency of CD34⁺CXCR4⁺ cells in the PB of PMF patients and CXCR4 cellular expression in CD34⁺ cells are correlated to CXCR4 mRNA level. The frequency of CD34⁺ cells coexpressing CXCR4 was determined by fluorescence-activated cell sorting analysis in the PB of PMF patients ($n = 40$), in the PB of healthy subjects in the steady state (uPB; $n = 11$) or after granulocyte colony-stimulating factor priming (mPB; $n = 11$), and in BM aspirate from volunteer donors ($n = 20$). A typical histogram for these different cell sources is presented in (A), and median value (range) of CD34⁺CXCR4⁺ cells and the CXCR4 expression expressed as the MFI are reported in (B). CXCR4 mRNA level was measured in purified CD34⁺ cells by real-time quantitative polymerase chain reaction and expressed as ΔC_T after being normalized to RNaseP as the housekeeping gene. Please note that higher ΔC_T values indicate lower mRNA content. *, $p < .05$; **, $p < .01$. The relationship between percentage of circulating CD34⁺CXCR4⁺ or the CXCR4 MFI and the level CXCR4 mRNA concurrently measured in purified CD34⁺ cells is presented in (C) and (D), respectively. In both instances, the correlation between the two variables was statistically significant at $p < .01$. Abbreviations: BM, bone marrow; MFI, mean fluorescence intensity; mPB, mobilized peripheral blood; PB, peripheral blood; PMF, primary myelofibrosis; uPB, unstimulated peripheral blood.

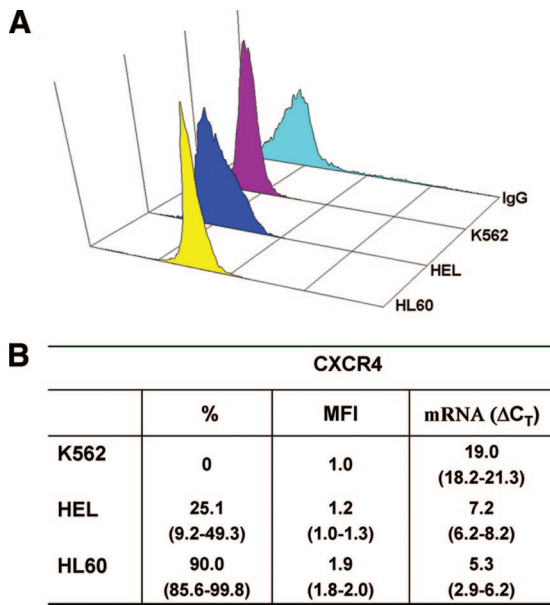


Figure 2. Expression of CXCR4 and level of *CXCR4* mRNA in three hematopoietic cell lines. Expression of CXCR4 on the membrane of HL60, K562, and HEL cells was measured by fluorescence-activated cell sorting and expressed as the percentage of CXCR4⁺ cells and as the CXCR4 MFI. A typical histogram is presented in (A), and median value (range) from at least three individual determinations is reported in (B). The *CXCR4* mRNA level was measured by real-time quantitative polymerase chain reaction and expressed as ΔC_T after being normalized to *RNaseP* as the housekeeping gene. Please note that higher ΔC_T values indicate lower mRNA content. Abbreviation: MFI, mean fluorescence intensity.

in mPB ($p < .01$ for both). These data, together with those presented in Figure 1, suggested that in CD34⁺ cells from healthy subjects and PMF patients, as was observed in cell lines, the level of *CXCR4* mRNA is correlated with the methylation status of CpG island 1 of the *CXCR4* promoter.

Hypomethylating Agent 5-AzaD Reverts Abnormal Methylation of *CXCR4* Promoter and Increases CXCR4 Expression

The above results prompted us to evaluate the effect of demethylating agent 5-AzaD on the transcriptional activity of *CXCR4*. First, we evaluated different concentrations of the agent, incubation times, and cell densities in cultures of HEL cells (not shown in detail); the best conditions were represented by a 1.0 μ M concentration of 5-AzaD for 6–48 hours of incubation, using cells that had been synchronized in serum-free medium for 12–16 hours. The number of HEL cells expressing CXCR4 in synchronized cultures was lower (median, 15%; range, 9%–19%) than in passage cultures (comparison shown in Fig. 2), allowing better appreciation of any changes induced by drug treatment. In synchronized HEL cells that had been exposed to 5-AzaD for 24 hours, the percentage of CXCR4⁺ cells increased up to a median of 47% (range, 30%–58%; $p < .01$ vs. untreated cultures (Fig. 4C, 4D). There was no further increase in the percentage of CXCR4⁺ cells at 48 hours of culture, but cell toxicity in the presence of 5-AzaD became significant (25% \pm 10% of the cells were trypan blue-positive at that time point).

The level of *CXCR4* mRNA was measured by RTQ-PCR at different times after drug exposure, from 2 to 48 hours. We observed a statistically significant increase of *CXCR4* mRNA in a very narrow time interval, peaking at 8 hours of incubation and returning to pretreatment level at 24 hours; the mean *CXCR4*

mRNA increase at 8 hours was 6.1 (\pm 0.9)-fold compared with control cultures ($p < .01$; Fig. 4E). We evaluated whether enhanced transcription of *CXCR4* after 5-AzaD exposure was associated with changes of promoter methylation status; to this end, cells were incubated with 5-AzaD for 24 hours, and bisulfite-treated DNA was analyzed using both MSP and MSS. As shown in Figure 4F and 4G, we observed a significant reduction in the proportion of methylated allele corresponding to CpG island 1, with no appreciable modification of CpG island 5, whereas the percentage of methylated cytosine using MSS analysis decreased from 40% \pm 12% to 14% \pm 8% ($n = 3$ experiments; $p = .003$). Overall, these results suggested that the *CXCR4* mRNA level in HEL cells was correlated mainly with the methylation status of CpG island 1, whereas the role of CpG island 5, if any, could not be clearly ascertained.

When purified CD34⁺ cells from PMF patients were incubated for 8–24 hours with 1 μ M 5-AzaD, a 3.9-fold median increase (range, 3.5–10) in the number of CD34⁺ cells coexpressing CXCR4 was observed at 12 hours of incubation ($p < .001$ vs. untreated cells) (Fig. 6A, 6B); concurrently, levels of *CXCR4* mRNA were already increased 3.7-fold (range, 3.1–4.1) at 8 hours (Fig. 6B). Changes in methylation status of *CXCR4* promoter were analyzed using MSP and MSS and are presented in Figure 6C. In all patients evaluated, MSP revealed the disappearance of the amplicon corresponding to the methylated allele of CpG island 1, whereas in cases where a methylated allele of CpG island 5 was present (i.e., patients 2, 3, and 5), no significant modification after 5-AzaD incubation could be demonstrated. These data were corroborated by the results of MSS analysis that showed a complete (as was the case for patients 1 and 3) or more than 70% (i.e., patients 4 and 5) reduction in methylated CpGs within the 230-base pair fragment corresponding to CpG island 1.

5-AzaD Treated PMF CD34⁺ Cells Showed Enhanced Migrating Properties

To assess the functional consequence of increased CXCR4 expression in PMF CD34⁺ exposed to 5-AzaD, we performed an in vitro migration assay in response to SDF-1. Purified CD34⁺ cells from eight PMF patients were incubated with or without 5-AzaD, and their migration in response to a gradient of SDF-1 added to the lower chamber was assessed at 3 hours. After subtracting passive diffusion (i.e., the number of CD34⁺ cells that migrated to the lower chamber in the absence of SDF-1), the percentage of CD34⁺ cells migrating to the lower chamber in response to SDF-1 increased from a mean (\pm SD) of 6.3% \pm 1.1% in controls to 23.2% \pm 8.9% in CD34⁺ cells preincubated with 5-AzaD ($p = .007$). We then measured the expression of membrane and intracellular CXCR4 in nonmigrating (upper chamber) and migrating (lower chamber) cells after 5-AzaD treatment in four experiments. Although we observed no change in the ratio of membrane versus intracellular CXCR4-associated fluorescence in the upper-chamber cells, those that had migrated presented a median fourfold increase in the relative amount of intracellular versus membrane CXCR4 (data not shown in detail), indicating receptor internalization upon SDF-1 binding.

DISCUSSION

The peripheral blood of PMF patients contains more HSCs/HPCs, including more immature progenitors capable of repopulating the BM of irradiated mice [27]. Stem cell mobilization is a normal phenomenon in healthy subjects that can be magnified after administration of cytokines, particularly G-CSF, and/or

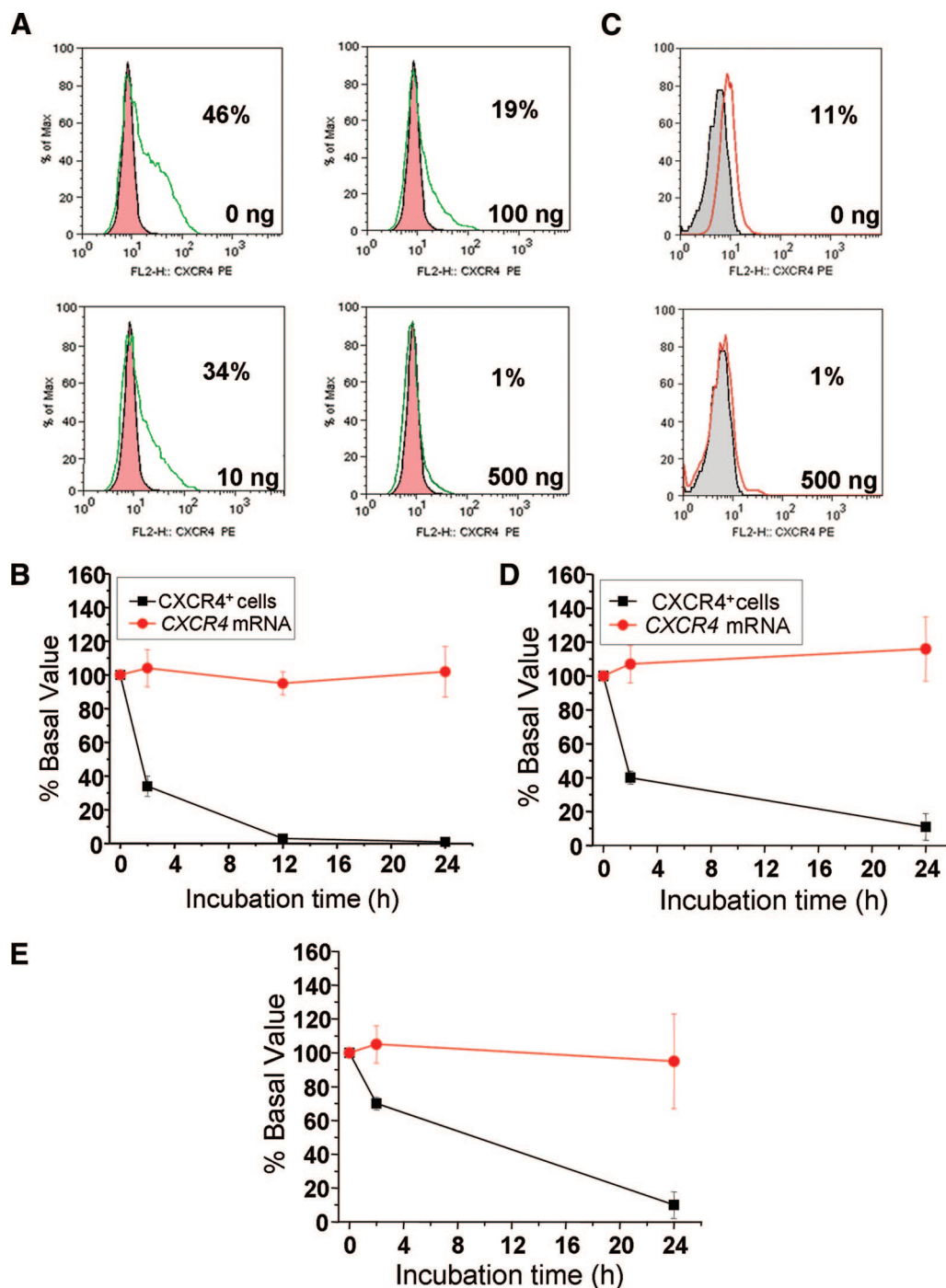


Figure 3. Downregulation of CXCR4 expression induced by SDF-1 in HEL or CD34⁺ cells does not involve changes in *CXCR4* mRNA level. HEL cells (A) or purified CD34⁺ cells from the peripheral blood from primary myelofibrosis (PMF) patients (C) were incubated with SDF-1 up to 500 ng/ml, as indicated in each plot. Variation of CXCR4 membrane expression by fluorescence-activated cell sorting analysis at 12 h in a typical experiment is depicted in (A) and (C) for HEL cells and PMF CD34⁺ cells, respectively; the percentage of CXCR4⁺ cells is reported inside each plot. The mean (\pm SD) percentage variation in the number of CXCR4⁺ cells, calculated from pooled experiments with HEL cells ($n = 3$) or CD34⁺ cells purified from PMF patients ($n = 5$) or normal donors ($n = 5$), after being exposed to 500 ng/ml SDF-1 for different times, is shown (square symbols) in (B), (D), and (E), respectively. Concurrent modification of *CXCR4* mRNA level measured by real-time quantitative polymerase chain reaction (circle symbols) is presented in (B) for HEL cells, (D) for PMF patients, and (E) for CD34⁺ cells obtained from unstimulated peripheral blood. Abbreviations: h, hours; Max, maximum; PE, phycoerythrin.

chemotherapeutics and is mediated by neutrophil-released proteases disrupting the adhesive interaction of HSCs/HPCs with the microenvironment. As a matter of fact, elevated levels of neutrophil elastase, MMP-9, and soluble vascular cell adhesion molecule 1 were found in plasma of PMF patients and correlated

with the number of circulating CD34⁺ cells [26]. Furthermore, it has been observed that both the number of CD34⁺ cells coexpressing CXCR4 and the CXCR4 cellular expression were significantly reduced in PMF, and such abnormalities have been attributed to reduced transcriptional activity of *CXCR4* [26, 28,

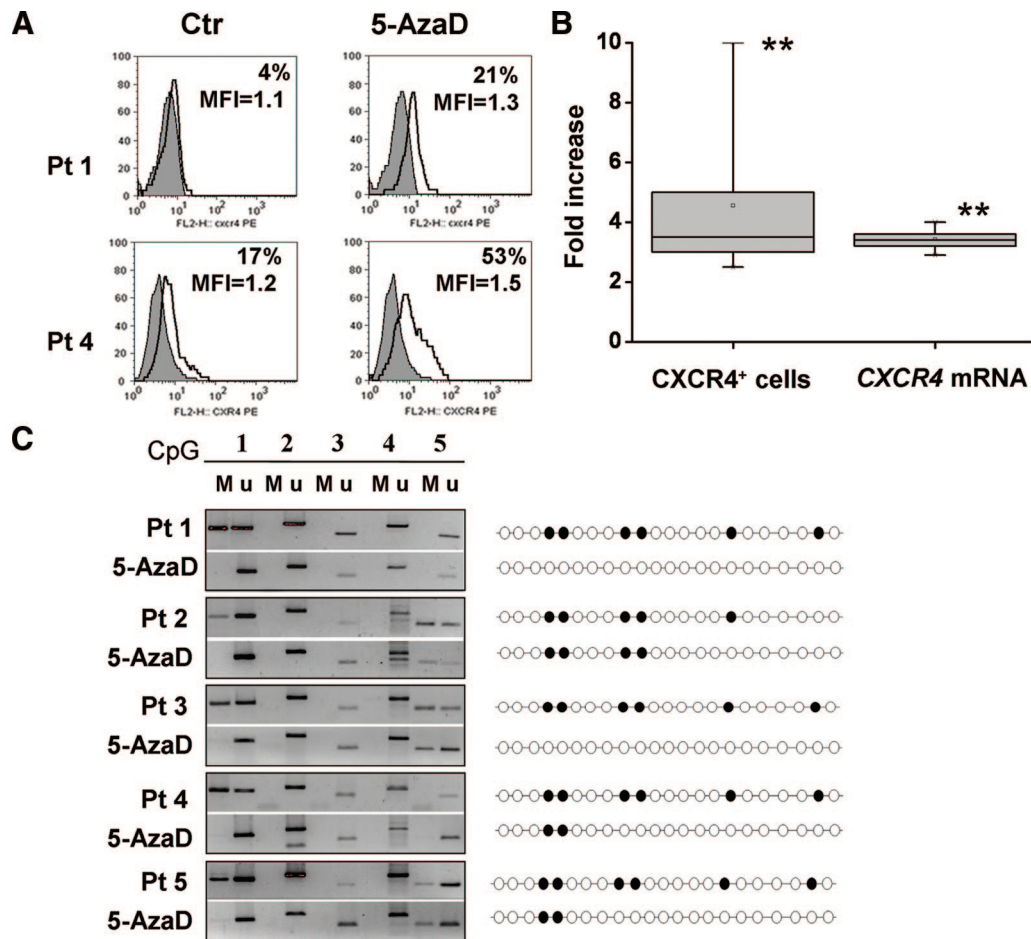


Figure 6. The hypermethylated status of *CXCR4* promoter in CD34⁺ cells from primary myelofibrosis (PMF) Pt is sensitive to the hypomethylating activity of 5-AzaD. CD34⁺ cells purified from peripheral blood of PMF Pts were incubated for 8–24 hours with 1 μ M 5-AzaD. In (A), a typical fluorescence-activated cell sorting analysis of the expression of CXCR4 by CD34⁺ cells is presented; the percentage of CD34⁺CXCR4⁺ cells, and the CXCR4 MFI, after exposure to 5-AzaD for 24 hours is reported inside each plot. Two representative Pts, corresponding to those also presented in (C), are shown. Cumulative results obtained in 10 Pts are presented in (B) and expressed as fold increase of CD34⁺ cells coexpressing CXCR4 versus untreated cells. The value measured in cultures containing 5-AzaD was significantly different from Ctr cultures at $p < .01$. Fold change in the level of *CXCR4* mRNA measured by real-time quantitative polymerase chain reaction at 8 hours of incubation with 5-AzaD of PMF CD34⁺ cells is presented in (B) (from a total of nine individual determinations). Boxes represent the interquartile range that contains 50% of the subjects, the horizontal line in the box marks the median, the small square inside indicates mean value, and bars show the range of values. The effect of 5-AzaD treatment on the methylation status of *CXCR4* promoter in PMF CD34⁺ cells was evaluated using MSP (C) and MSS (filled circles indicate M cytosine, and empty circles indicate u cytosine); representative results in five of nine PMF Pts evaluated are shown. Abbreviations: 5-AzaD, 5-Aza-2'-deoxycytidine; Ctr, control; M, methylated; MFI, mean fluorescence intensity; Pt, patient; u, unmethylated.

expansion of residual normal cells expressing physiologic level of CXCR4. On the other hand, the effects we observed in the short-term time in our experimental scheme are in support of a direct effect of 5-AzaD on the abnormal methylation status of *CXCR4* in progenitors. Overall, this would suggest that using demethylating agents in PMF, eventually in association with HDAC, might potentially have a double action mechanism against clonal progenitors, both inhibiting their growth and facilitating the re-emergence of normal progenitors, and reactivating abnormally repressed genes.

Some open questions still remain that might be addressed in future studies. One concerns possible relationships between downregulation of CXCR4 and abnormal tyrosine kinase activity due to *JAK2V617F* mutation or other functionally equivalent molecular abnormalities. In this work, we did not find differences in *CXCR4* expression in PMF CD34⁺ cells depending on the *JAK2* mutational status, confirming previous results in CD34⁺ cells [28]. On the other hand, the fact that *CXCR4* mRNA level was significantly reduced in the granulocytes of

PMF patients harboring a high mutant allele burden [29] would suggest additional regulatory levels dependent on the cell hierarchy or lineage belonging. In this regard, it is of interest that expression of p210/BCR-ABL has been shown to impair CXCR4 signaling through two different mechanisms depending upon its level of expression; at a low level of p210 expression, a signaling defect predominated without obvious modification of CXCR4 expression, whereas a higher level of p210 induced a marked downregulation of CXCR4 due to decreased *CXCR4* transcription [75]. In addition, it might be worthwhile to investigate possible effects of defective CXCR4 expression on the HSC/HPC proliferation and differentiation in PMF. In fact, in addition to regulating migration and homing of HSCs/HPCs, SDF-1 has an autocrine/paracrine effect by promoting survival of normal CD34⁺ cells through suppression of apoptosis and recruitment of quiescent G0 cells in G1 phase of the cell cycle [1]. Furthermore, SDF-1 intervenes in the regulation of proliferation and maturation of progenitors committed to megakaryocytopoi-

esis [76–79]; these progenitors are the most obviously involved hematopoietic cell lineage in PMF.

CONCLUSION

Overall, our data indicate that an abnormal degree of methylation of the *CXCR4* promoter might contribute to the constitutive migration of CD34⁺ cells in PMF patients. However, it might also be anticipated that defective expression of CXCR4 in PMF CD34⁺ cells might also influence other functional aspects of the cells in addition to their unique migration and homing properties.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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Hypermethylation of CXCR4 Promoter in CD34+ Cells from Patients with Primary Myelofibrosis

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