Functional Regulation of Pre-B-cell Leukemia Homebox Interacting Protein 1 (PBXIP1/HPIP) in Erythroid Differentiation*†§

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Background: HPIP is a pre-B-cell leukemia homebox 1 (PBX1) interacting protein with unknown function in hematopoiesis.

Results: The HPIP gene is a target of GATA1 and CTCF and regulates erythroid differentiation involving PI3K/AKT-dependent mechanisms.

Conclusion: HPIP is a novel downstream target of GATA1 and serves as an essential regulator of erythroid differentiation.

Significance: A new regulator of erythroid differentiation is discovered. This finding may help in better understanding erythropoiesis.

Pre-B-cell leukemia homebox interacting protein 1 or human PBX1 interacting protein (PBXIP1/HPIP) is a co-repressor of pre-B-cell leukemia homebox 1 (PBX1) and is also known to regulate estrogen receptor functions by associating with the microtubule network. Despite its initial discovery in the context of hematopoietic cells, little is yet known about the role of HPIP in hematopoiesis. Here, we show that lentivirus-mediated overexpression of HPIP in human CD34+ cells enhances hematopoietic colony formation in vitro, whereas HPIP knockdown leads to a reduction in the number of such colonies. Interestingly, erythroid colony number was significantly higher in HPIP-overexpressing cells. In addition, forced expression of HPIP in K562 cells, a multipotent erythro-megakaryoblastic leukemia cell line, led to an induction of erythroid differentiation. HPIP overexpression in both CD34+ and K562 cells was associated with increased activation of the PI3K/AKT pathway, and corresponding treatment with a PI3K-specific inhibitor, LY-294002, caused a reduction in clonogenic progenitor number in HPIP-expressing CD34+ cells and decreased K562 cell differentiation. Combined, these findings point to an important role of the PI3K/AKT pathway in mediating HPIP-induced effects on the growth and differentiation of hematopoietic cells. Interestingly, HPIP gene expression was found to be induced in K562 cells in response to erythroid differentiation signals such as DMSO and erythropoietin. The erythroid lineage-specific transcription factor GATA1 binds to the HPIP promoter and activates HPIP gene transcription in a CTCF-binding factor (CTCF)-dependent manner. Co-immunoprecipitation and colocalization experiments revealed the association of CTCF with GATA1 indicating the recruitment of CTCF/GATA1 transcription factor complex onto the HPIP promoter. Together, this study provides evidence that HPIP is a target of GATA1 and CTCF in erythroid cells and plays an important role in erythroid differentiation by modulating the PI3K/AKT pathway.

The human hematopoietic system is composed of a heterogeneous population of cells that range in function from mature cells with limited proliferative potential to pluripotent stem cells known as hematopoietic stem cells (HSC)4 with extensive proliferation, differentiation, and self-renewal capacities (1, 2). This process is governed by the interplay of a number of transcription factors and various signaling pathways, which altogether facilitate proper hematopoietic development (3, 4). Emerging evidence indicates that human leukemias, lymphomas, and possibly myelodysplastic syndromes are initiated at the level of HSCs and/or early multipotent progenitors that have been transformed due to genetic/chromosomal aberrations or deregulation of gene expression (5). Of several regulators of HSC, PBX transcription factors play an important role in the establishment and maintenance of definitive hematopoiesis, and PBX overexpression has been linked to leukemia development (6). PBX proteins mainly act as cofactors for HOX pro-

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4 The abbreviations used are: HSC, hematopoietic stem cell; PBX1, pre-B-cell leukemia homeobox 1; PBXIP1 or HPIP, pre-B-cell leukemia homebox interacting protein 1; Epo, erythropoietin; q, quantitative; CFC, colony-forming cell assay; E/Meg, erythroid and megakaryocyte; 4-OHT, 4-hydroxytamoxifen; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; CTCF, CTCF-binding factor.
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Cell Culture—The human leukemic cell lines K562 and HL60 were obtained from the American Type Culture Collection (Manassas, VA). Cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 10 units/ml penicillin, and 10 μg/ml streptomycin.

Plasmids—To study the regulation of HPIP gene expression, we amplified by PCR an ~2.3-kb 5'-flanking region of the HPIP gene using BAC clone ID RP11-307C12 (gift from J. D. Shaugnessy, Jr., University of Arkansas for Medical Sciences) as template using specific primers as follows: forward primer, 5' -CGCGGATCCACGAGAGGAAAGGATG-3' (Xhol site), and reverse primer, 5' -ATGGAAGCTTACAGGCGACATAGT TGCTG-3' (HindIII site). The PCR fragment was subsequently cloned into a promoter probe vector, pGTL. The cloned promoter region was sequence-verified and found correctly located between the −154,934- and −154,937-kb region on human chromosome 1q21.3 (Fig. 6A). In humans, the HPIP gene is located upstream of the PYGO2 gene and downstream of the PMVK gene on chromosome 1q21.3. Using web-based PROSCAN suite 7.1 software, we have identified the annotated (putative) transcription start site that is located at −740 bp from the start codon ATG (supplementary Fig. S5), and the TATA box is located at −769 bp. The cDNA that encodes HPIP was amplified by PCR with the following the primers using pMIG-HPIP plasmid as template: forward primer, 5’ -CTG-GGATCCATGCGACATGACAAAGC-3’ (underlined sequence denotes MfeI site; sequence in italics encodes partial sequence of FLAG tag), and reverse primer, 5’ -CTG-GATCCATGCGACATGACAAAGC-3’. HPIPshRNA vector in pGIPz vector was provided by Dr. Sam Aparicio, British Columbia Cancer Agency, University of British Columbia, Vancouver, Canada.

Induction of K562 and G1E-ER4 Differentiation—To induce K562 differentiation, cells at a density of 10^5 cells per ml were treated with DMSO (1.6%) or sodium butyrate (1.5 mM), as indicated. To examine the effects of Epo on K562 cells, cells were grown in the presence or absence of EPO (5–15 units/ml) in RPMI 1640 medium supplemented with heat-inactivated 10% FBS for various time points. For inhibitor studies, cells were treated with PI3K inhibitor LY-294002 (50 μM) as indicated. G1E-ER4 cells were cultured as described previously (21) and induced with 4-hydroxytamoxifen (4-OHT) (10^{-8} M) whenever required for GATA1 induction.

Isolation and Lentiviral Transduction of Cord Blood-derived CD34+ Cells—To isolate CD34+ cells, cord blood was obtained from the stem cell assay lab (Terry Fox Laboratory, British Columbia Cancer Agency). CD34+ cell-enriched populations (65–98% CD34+ cells) were obtained by positive selection using magnetic beads (Easy Sep Stem Cell Technologies Inc., Vancouver, Canada). Purified CD34+ cells were stimulated overnight for 48 h for in vitro experiments at densities less than or equal to 2 × 10^5 cells/ml in Iscove’s medium supplemented with 1% BSA, 10 μg/ml bovine pancreatic insulin, and 200 μg/ml human transferrin (BIT; Stem Cell Technologies Inc.), 10^{-4} mol 2-mercaptoethanol, 2 mM glutamine, 100 ng/ml FL-3 (Immunex Corp.), 100 ng/ml steel factor, 50 ng/ml thrombo-

teins (7). Particularly, PBX1 together with HOX genes are essential for normal HSC development, and its deregulation led to leukemogenesis (8, 9). Ablation of the Pbx1 gene in mice causes an embryonic lethal phenotype with severe homeotic malformations, hypoplasia (or absence) of many organs, but also lymphoid, myeloid, and erythroid deficiencies (10). Therefore, understanding the protein regulatory network linked to PBX1 is important for normal hematopoiesis as well as leukemia development.

In an attempt to map the interactome of PBX1, we have previously identified human PBX interacting protein (HPIP), also known as pro-B-cell leukemia homeobox interacting protein 1 (PBXIP1), as a PBX1 interacting protein through a yeast two-hybrid approach employing a human hematopoietic cDNA-based library (11). HPIP is a nucleo-cytoplasmic shuttling protein (12). HPIP also interacts with PBX2 and PBX3. HPIP inhibits the ability of PBX-HOX heterodimers to bind to target sequences. Moreover, HPIP strongly inhibits the transcriptional activation capacity of E2A-PBX suggesting HPIP is a newly recognized regulator of PBX function (11). The same study also reported that similar to many HOX family members, HPIP is expressed in the most primitive hematopoietic stem cell-enriched CD34+ population, whereas its expression is found very low in terminally differentiating CD34− hematopoietic populations (11).

Recent studies have also revealed the role of HPIP in cell migration and proliferation in breast cancer cells (13, 14). Mouse xenograft studies and anchorage-independent growth assays demonstrated the oncogenic nature of HPIP (13). HPIP regulates these functions by activation of PI3K/akt and Src/MAPK pathways (13). Accumulating evidence supports that the PI3K/AKT signaling pathway is also a key player in developmental hematopoiesis, hematopoietic stem cell survival, and self-renewal (15–17). For example, PI3K/AKT transduces Src-induced erythroid cell differentiation (18). In addition, AKT, which is one of the main downstream targets of PI3K, mediates erythropoiesis in response to erythropoietin signaling by controlling GATA1 transcriptional activity on the TIMP-1 gene (19). In particular, DMSO-induced erythroid differentiation is dependent on PI3K activity (20). Altogether, these reports support the central role for the PI3K/AKT pathway in erythropoiesis.

Based on HPIP being expressed in primitive human hematopoietic stem and progenitor cell-enriched CD34+ populations, a close relationship of HPIP with PBX functions, and a demonstrated role for HPIP in regulating the PI3K/AKT signaling pathway, we hypothesized that HPIP has important functional roles in hematopoiesis. To test this, we have employed overexpression and knockdown of HPIP in primary human CD34+ cells and in K562 cells and assessed the impact on colony formation and differentiation. These functional and molecular studies demonstrate that HPIP expression is induced in response to erythropoietin inducers such as DMSO and erythropoietin (Epo) and regulates erythroid differentiation by activating the PI3K/AKT pathway.
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pontamine sulfate and lentivirus with 0.5 \times 10^8 to 5 \times 10^8 infectious units/ml, placed in a 96-well plate coated with 5 \mu g/cm^2 fibronectin (Sigma), and then incubated at 37 °C for 6 h. Lentivirus was produced for pMNDUS vector, pMNDUS-HPIP, pGIPz control shRNA vector, and pGIPz-HPIPshRNA constructs using a standard four-plasmid packaging system by calcium phosphate transfection method in HEK293T cells. Harvested virus-containing supernatants were concentrated by two pulses using a 3000 sonicator (Misonix, Inc), with 2 min on ice.

Clonogenic Progenitor Assays—Appropriate aliquots of CD34+ cells transduced with various HPIP constructs were plated in 1 ml of methylcellulose-containing medium (Methocult H4230; Stem Cell Technologies Inc.) supplemented with 50 ng/ml human stem cell factor (Stem Cell Technologies Inc.), and 20 ng/ml each of human IL-3 (Novartis), IL-6 (Cangene), GM-CSF (Novartis), granulocyte-CSF (G-CSF; Novartis), and 3 units/ml Epo (Stem Cell Technologies Inc.) as described previously (23). The cultures were then incubated for 14 days at 37 °C, and colonies of terminally differentiating erythroid, myeloid, and mixed erythroid-myeloid cells (from BFU-E, CFU-GEMM) formed were scored based on their morphological characteristics.

Generation of HPIP Stable Clones in K562 Cells Using Lentivirus Transduction—For ectopic expression of HPIP in K562, cells were transduced with lentivirus carrying pMNDUS vector or pMNDUS-HPIP with 0.5 \times 10^8 infectious units/ml, placed in a 6-well coated plate, and then incubated at 37 °C for 24 h. GFP-positive cells were isolated using FACS and used for various assays.

Cell Proliferation Analysis—For analyzing proliferation activity, cells were cultured in growth medium at a starting density of 5000 cells in a 96-well plate, and growth of cells was quantified by MTT assay for indicated time points. Cell counts were performed in quadruplicate every 24 h using a plate reader.

Cell Differentiation by Benzidine Staining Assay—Erythroid differentiation was assayed by the method of Orkin by benzidine staining of hemoglobin accumulated in the cells. Stable clones of HPIP in K562 cell suspension (200 \mu l) was mixed with 20 \mu l of freshly prepared benzidine solution (10:1 mixture of 0.2% 3,3-dimethoxybenzidine in 0.50 mM acetic acid and 30% hydrogen peroxide), and stained cells were scored under a microscope. At least 400 cells were examined (in duplicate) at each assay.

Western Blot Analysis—For Western blot analysis, cells were harvested by centrifugation and washed once with phosphate-buffered saline (PBS). Cells were lysed in RIPA buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EGTA, 1% Nonidet P-40, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and complete mini protease inhibitor mixture (Roche Applied Science)) and were centrifuged to remove cell debris. To study phosphorylated proteins, cell lysates were prepared in RIPA buffer supplemented with phosphatase inhibitor mixture (Sigma). The protein concentration was determined by the RC-DC protein assay (Bio-Rad), and each lysate containing 50–70 \mu g of protein was loaded and resolved on an SDS-polyacrylamide gel and transferred to nitrocellulose membrane (Invitrogen) and then probed with specific antibodies. After incubation with HRP-conjugated secondary antibodies (GE Healthcare), the blots were visualized with chemiluminescence (ECL) detection reagents (Bio-Rad) followed by autoradiogram using Kodak developing system or by Versadoc imaging system (Bio-Rad). Western blotting was performed using antibodies against HPIP from Bethyl Laboratories; GAPDH, acetyl-H3K4, and CTCF from Millipore; GATA1, phospho-AKT Ser-473, phosphor-GSK3β, total AKT, and total GSK3β from Cell Signaling Technologies; C/EBPα from Santa Cruz Biotechnology and Alexis; and FLAG from Sigma.

Real Time Quantitative RT-PCR—Total RNA from cultured cells was purified using the TRIzol method (Invitrogen), following the manufacturer’s instructions. All RNA extracts were treated with RNase-free DNase to remove genomic DNA contamination. After purification, 1 \mu g of RNA was reverse-transcribed at 42 °C for 60 min with oligo(dT) primer followed by enzyme inactivation at 70 °C for 15 min. The cDNAs thus synthesized were amplified by PCR with SYBR Green, using the following primers in Applied Biosystems qPCR machine: HPIP forward, 5’-GTC CCC TCG AGG AGT TGT GT-3’, and reverse, 5’-ATC TTC CAT CAT CTG AGG GC-3’; hipp1 forward, 5’-GTC CCC TCG AGG AGT TGT GT-3’, and reverse, 5’-ATC TTC CAT CAT CTG AGG GC-3’; GAPDH forward, 5’-ATC TTC CAT CAT CTG AGG GC-3’, and reverse, 5’-ATC TTC CAT CAT CTG AGG GC-3’; GATA1 forward, 5’-GTC CCC TCG AGG AGT TGT GT-3’, and reverse, 5’-ATC TTC CAT CAT CTG AGG GC-3’; GATA1 forward, 5’-GTC CCC TCG AGG AGT TGT GT-3’, and reverse, 5’-ATC TTC CAT CAT CTG AGG GC-3’; GATA1 forward, 5’-GTC CCC TCG AGG AGT TGT GT-3’, and reverse, 5’-ATC TTC CAT CAT CTG AGG GC-3’; GAPDH forward, 5’-GTC CCC TCG AGG AGT TGT GT-3’, and reverse, 5’-ATC TTC CAT CAT CTG AGG GC-3’; GAPDH forward, 5’-GTC CCC TCG AGG AGT TGT GT-3’, and reverse, 5’-ATC TTC CAT CAT CTG AGG GC-3’; GAPDH forward, 5’-GTC CCC TCG AGG AGT TGT GT-3’, and reverse, 5’-ATC TTC CAT CAT CTG AGG GC-3’.
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FIGURE 1. Expression of HPIP in hematopoietic tissues and cell lines. A, Western analysis shows the expression of HPIP in various myeloid cell lines. GAPDH was used as loading control. Real time qPCR analysis shows the relative mRNA levels of hpip to gapdh in various hematopoietic tissues (B), and common myeloid progenitors (CMP) and granulocyte-megakaryocyte progenitors (G). The data presented are representative of one of two independent experiments.

RESULTS

HPIP Expression in Hematopoietic Tissues and Myeloid Cell Lines—We have previously documented HPIP expression at the transcript level in K562 and HL60 leukemic cell lines and also in hematopoietic stem/progenitor-enriched CD34+ cells (11). Consistent with our previous report, Western blot analysis (Fig. 1A) revealed HPIP expression in various myeloid cell lines, HL60, K562, THP-1, and U937 cells and in human cord blood CD34+ cells. In addition, real time qPCR analysis showed HPIP expression in mouse hematopoietic tissues such as spleen, bone marrow, and thymus (Fig. 1B). HPIP expression was also detected in purified mouse common myeloid progenitors and common granulocyte and macrophage progenitors (Fig. 1C). In support of these results, gene expression data deposited in the human protein atlas data bank also showed HPIP expression in several hematopoietic cell lines as well as in hematopoietic organs, which include bone marrow, spleen, tonsils, and lymph node (supplemental Fig. S1) (24). These initial findings of HPIP expression in hematopoietic organs and also in hematopoietic cells strongly suggest a possible role for HPIP in hematopoiesis.

HPIP Is a Positive Regulator of Colony-forming Cell (CFC) Activity of CD34+ Cells—To evaluate the effects of HPIP expression on hematopoietic differentiation and lineage commitment, we carried out both HPIP ectopic (over)expression and knockdown studies using human CD34+ cells. For overexpression, we utilized a lentiviral delivery system confirmed to yield readily detectable levels of HPIP expression in HeLa cells by Western analysis using HPIP antibody (Fig. 2A). To assess the effects of HPIP knockdown in CD34+ cells, we employed an HPIP shRNA lentiviral construct (pGIPz-HPIP-shRNA) or control shRNA construct (pGIPz-control shRNA) and confirmed strong suppression (~90%) in transduced HeLa cells (Fig. 2B). First, we examined whether engineered overexpression/ectopic HPIP modulated the number of clonogenic progenitors using the CFC. Transduced GFP+ CD34+ cells were isolated by FACS 48 h post-infection and assayed for clonogenic progenitor content in methylcellulose. HPIP-expressing progenitor cells formed 159 (S.E. 7.0) colonies/500 cells initially plated versus 98 (S.E. 19.7) colonies in the vector control or 104.5 colonies with more erythroid colonies (BFU-E plus CFU-E) in the vector control (S.E. 19.7) colonies in the vector control or 104.5 colonies (S.E. 4.9) in untransduced control (Fig. 2C). Interestingly, the lineage distribution of colonies was also significantly altered with more erythroid colonies (BFU-E plus CFU-E) in the HPIP-transduced versus the control-transduced cells (110/500 cells initially plated in HPIP+ versus 27.5/500 cells initially plated in

between pulses. After lyse clarification, extracts were pre-cleared with a 50-μl bed volume of protein A/G-Sepharose ChIP beads (Millipore) for 1 h at 4 °C on a rotator. Protein-DNA complexes were immunoprecipitated with respective antibodies at a final concentration of 1:500, incubated 4 °C for 3 h, and then incubated with a 50-μl bed volume protein A/G beads on a rotator at 4 °C for 1 h. The beads were washed twice with 1 ml of ml of washing buffer, once with 1 ml of washing buffers at 500 mM NaCl concentration, once with 1 ml of LiCl/detergent solution (10 mM Tris-Cl, pH 8, 500 mM NaCl, 0.025% sodium azide, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate), and once with 1 ml of 1× TBS (20 mM Tris-Cl, pH 7.5, 150 mM NaCl). Beads were then collected by centrifugation at 1500 rpm for 2 min at 4 °C after each wash. Immunocomplexes were eluted from the beads with 1 ml of 1% SDS, 1× TE (10 mM Tris-Cl, pH 7.6, 1 mM EDTA, pH 8); the SDS was diluted with 1.5 ml of 0.67% SDS, 1× TE. Cross-links were reversed by heating at 65 °C overnight. DNA was purified and subjected to real time qPCR analysis with the following primers: HPIP-PR1 forward, 5′-CTGCTATACTCTTACAGG-3′, and reverse, 5′-GGTCCAACCCCTGATACCTCT-3′; HPIP-PR2 forward, 5′-CAGGCTGCACACAAGCTAG-3′, and reverse, 5′-ACCCTGGCATGATCCTGTA-3′; HPIP-PR3 forward, 5′-GATGGGCAATTGGGAAAGAC-3′, and reverse, 5′-GACCAGGCATAAAGGTGGAA-3′; and HPIP-PR4 forward, 5′-GTGTCAGCTCAGGCTTTGGA-3′, and reverse, 5′-AACTTGGAAGGTGAAGTTGAAG-3′.

Co-localization Studies—HeLa cells grown on cover glass in DMEM were transfected with pcDNA-GATA1 plasmid (encodes T7-tagged GATA1) using Lipofectamine 2000 (Invitrogen). Thirty six hours post-transfection, cells were fixed in 4% paraformaldehyde (PFA) at room temperature for 20 min and processed as described previously (13) to study co-localization of transfected T7-tagged GATA-1 and endogenous CTCF. Anti-rabbit CTCF antibody (1:200) (Sigma), rabbit IgG-FITC, and mouse Anti-rabbit CTCF antibody (1:200) (Millipore) and anti-mouse CD34 expression was also detected in purified mouse common myeloid progenitors and common granulocyte-megakaryocyte progenitors (G). The data presented are representative of one of two independent experiments.

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FIGURE 2. HPIP promotes erythroid lineage decisions of human hematopoietic stem cells, CD34+ cells in vitro. Effect of HPIP expression on CFC activity of CD34+ cells. A, following lentivirus transduction, HPIP ectopic expression was verified by Western blotting using anti-HPIP antibody. B, similarly HPIP knockdown was analyzed using anti-HPIP antibody. C, methylcellulose-based CFC assay for HPIP overexpression in CD34+ cells. CFCs colonies were scored after 14 days, and the increase of clonogenic progenitors was determined in cells transduced with HPIP versus the empty control (pMNDUS) or untransduced control CD34+ cells (UC). The number of CFCs plotted is for 500 cells plated initially (C) and various lineages, i.e. burst forming units-erythroid (BFU-E), granulocyte-megakaryocyte (GM) and granulocyte-erythroid-megakaryocyte-monocyte (GEMM) colonies were counted and plotted (D). E, methylcellulose-based CFC assay for HPIP knockdown in CD34+ cells. Similar to HPIP overexpression in CD34+ cells, CFCs colonies were scored after 14 days, and the decrease of clonogenic progenitors was determined in cells transduced with HPIPshRNA versus the control shRNA or untransduced control CD34+ cells (UC). Vec, vector. The number of CFCs plotted is for 500 cells plated initially (E), and various lineages i.e. burst forming units-erythroid (BFU-E), granulocyte-megakaryocyte (GM), and granulocyte-erythroid-megakaryocyte-monocyte (GEMM) colonies were counted and plotted (F). Inset shows the expression of HPIP in CD34+ cells. The data presented are representative of one of two independent experiments.

the vector control arm or 33.5/500 initially plated in the untransduced control arm) (Fig. 2D). Similarly significant differences in the formation of CFU-GM and CFU-GEMM colonies were also observed (Fig. 2D). Furthermore, the cells derived from these colonies were assessed by FACS analysis for glycophorin A, erythroid-specific marker, and CD33, a myeloid cell marker, expression. HPIP-transduced CD34+ cells showed ~48% of glycophorin A-positive cells versus 28% vector control cells (supplemental Fig. S2). Next, the effect of HPIP knockdown on clonogenic capacity of CD34+ cells was evaluated. Consistent with the above results, HPIP knockdown resulted in decreased CFC number of 70.5 (S.E. 2.12) colonies/500 cells initially plated versus 130 (S.E. 0.7) colonies in the vector control or 123 (S.E. 5.6) colonies in untransduced control (Fig. 2, E and F). Together, these results point to an important functional role for HPIP in hematopoietic progenitor function and notably as a positive regulator at the level of erythroid progenitors.

HPIP Expression Alters Erythroid Differentiation Potential of K562 Cells—Because HPIP expression shows a distinct stimulatory effect on BFU-E, we sought to test whether HPIP expression alters erythroid differentiation in K562 cells. K562, a multipotent erythro-megakaryoblastic leukemia cell line, has been used to study in vitro erythroid differentiation as it can be differentiated into mature erythroid or myeloid cells (25). To further examine whether HPIP expression alters erythroid differentiation, we used lentiviral transduc-
tion with HPIP or shRNA to HPIP to generate K562 clones in which HPIP was stably overexpressed or knocked down. Stable transformants were sorted using GFP as a tracker by FACS, and HPIP overexpression or knockdown was confirmed by Western analysis (Fig. 3, A and B, respectively). Overexpression of HPIP did not alter K562 cell proliferation significantly as assessed by MTT assay up to day 3 of monitoring (Fig. 3C). HPIP knockdown increased the proliferation capacity of K562 starting from day 2, and this was sustained until day 4 (Fig. 3D). Using a benzidine staining assay to measure hemoglobin accumulation as a measure of differentiation, we detected a significant increase in erythroid differentiation in HPIP-overexpressing cells (Fig. 3E). Conversely, HPIP knockdown resulted in a significant decrease, some 2-fold, in DMSO-induced differentiation of K562 cells (Fig. 3F). These results reinforce a model in which HPIP plays an important positive regulatory role in erythroid differentiation.

**FIGURE 3.** HPIP expression in K562 alters differentiation. A, Western blot analysis of HPIP overexpression determined using anti-HPIP antibody. B, HPIP knockdown in K562 cells using anti-HPIP antibody. β-Actin was used as loading control. C and D, MTT cell proliferation assay. Differences in proliferation among K562 (untransduced control (UC)), K562 + vector, and K562 + HPIP (C), or K562 (untransduced control-UC) and K562 + control (ctrl) shRNA and K562 + HPIPshRNA (D) after 4 days were detected by measuring the absorbance (Abs) of MTT at 550 nm and subtracting the background absorbance at 690 nm. E and F, erythroid differentiation assay. K562 (UC), K562 + vector and K562 + HPIP (*, p < 0.001) (E) or K562 (UC), K562 + control (ctrl) shRNA, and K562 + HPIPshRNA (F) in the presence and absence of DMSO (1.6%) (*, p < 0.001) were monitored for hemoglobin content after 4 days using benzidine/hydrogen peroxide staining. The histogram shows the percentage of benzidine-positive cells that were scored by light microscopy. The data presented are representative of one of two independent experiments.
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HPIP-mediated Erythroid Differentiation Follows PI3K/AKT Pathway—The PI3K/AKT pathway has been well documented to play an important role in erythroid differentiation (16, 18). AKT, which is the downstream target of PI3K, requires PI3K-dependent phosphorylation at Ser-473 for its optimal activity (26). Upon growth factor signaling, AKT phosphorylation of GSK3β at Ser-9 leads to reduced GSK3β activity (27). Based on the fact that HPIP activates the PI3K/AKT pathway (13), we predicted that HPIP-mediated differentiation of K562 cells was linked to the PI3K/AKT pathway. To test this, we examined the activation of downstream targets of PI3K signaling such as AKT and GSK3β in K562-HPIP and K562-HPIP shRNA cells by Western analysis. Ectopic expression of HPIP substantially increased the AKT and GSK3β phosphorylation over control cells indicating the requirement of HPIP for the activation of PI3K/AKT pathway in K562 cells.

Next we have tested the effect of the PI3K inhibitor, LY-294002, on HPIP-mediated colony forming capacity of CD34+ cells. LY-294002 treatment reversed the stimulatory effect of HPIP on CFC formation by CD34+ cells (Fig. 4C and supplemental Fig. S3A). Similarly, LY-294002 treatment of HPIP-transduced K562 cells also significantly decreased erythroid differentiation as assessed by benzidine staining assay (Fig. 4D and supplemental Fig. S3B).

Chemical and Physiological Modulators of Erythroid Differentiation Induce HPIP Expression in Leukemic Cell Lines—Because HPIP expression influenced erythroid differentiation in K562 cells, we sought to study its regulation in these cells. Treatment with the differentiation inducer DMSO (1.6%) led to enhanced HPIP expression as revealed by immunoblotting (Fig. 5A). Further real time quantitative RT-PCR analysis confirmed that HPIP RNA levels increased by nearly 5-fold in response to

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**FIGURE 4.** Effect of HPIP expression on phosphorylation of AKT and GSK3β in K562 cells. HPIP overexpressing (A) or knockdown (B) K562 cell lysates were subjected to Western blot analysis using indicated phosphorylation-specific and protein-specific antibodies. Untransduced K562 cell lysate was used as untreated control. C, effect of PI3K inhibitor on CFC ability of CD34+ HPIP versus CD34+ vector (Vec) cells or CD34+ -untransduced cells. Cells were treated or untreated with LY-294002 (50 µM), and CFC colonies were scored after 14 days, and the number of clonogenic progenitors was determined in cells transduced with HPIP versus the empty control or untransduced control (*, p = 0.003). D, effect of PI3K inhibitor on differentiation ability of K562 + HPIP versus K562 + vector cells or K562-untransduced cells. Cells were treated or untreated with LY-294002 (50 µM) for 24 h, and benzidine-positive cells were determined (*, p < 0.001). The data presented are representative of one of two independent experiments. UC, untransduced control.
inducer treatment (Fig. 5A, lower panel). Similar results were also obtained in HL60, another leukemic cell line, indicating HPIP induction by DMSO is not restricted to K562 cells (Fig. 5B). However, treatment with sodium butyrate did not change the expression of HPIP significantly in K562 or in HL60 (supplemental Fig. S4).

Next, we tested whether Epo was also capable of inducing HPIP expression. K562 cells were treated with various concentrations of erythropoietin, ranging from 1 to 15 units/ml, and HPIP expression was verified by Western blot analysis. As shown in Fig. 5C, HPIP protein levels increased significantly by Epo at 5 units/ml concentration in parallel to induction of GATA1, which is a known inducer of Epo signaling. This result is further confirmed by real time quantitative PCR analysis (Fig. 5D). Next, we carried out time-dependent treatment with Epo in K562 cells. HPIP expression was induced after 4 h of treatment and then slowly decreased as shown in Fig. 5E. Together, these results suggest that chemical modulators of erythroid differentiation such as DMSO and physiological inducer of erythropoiesis induce HPIP expression in leukemic cell lines.

E/Meg Transcription Factors Activate HPIP Gene Transcription—Because erythropoietin induced HPIP expression in K562 leukemic cells, we sought to elucidate the mechanism of HPIP gene expression in hematopoietic cells. Sequence inspection of the human HPIP 2.3-kb promoter region using TF search tool revealed 16 GATA1–2 consensus binding sites (Fig. 6A and supplemental Fig. S6). In addition, we also found several other E/Meg-binding sites, which include C/EBPα and SCL (Tal-1). Next, to check whether these E/Meg transcription factors indeed activate HPIP gene transcription, we cloned the 2.3-kb 5′-flanking regions of the HPIP gene into the promoter probe vector, pGL3, as described under “Experimental Procedures,” and performed luciferase assays using co-transfection studies in K562 cells. As shown in Fig. 6B, all known E/Meg transcription factors, which include GATA1–2, C/EBPα, SCL, PU1, Gif-1, and Fli-1, activated HPIP gene transcription. GATA-1, GATA-2, and C/EBPα were notably effective in the activation of HPIP gene transcription by nearly 130-, 6-, and 28-fold over control vector, respectively. These results suggested E/Meg transcription factors activate HPIP gene transcription in K562 cells.

GATA1 Activates HPIP Transcription in G1E-ER4 Cells—Because GATA1 showed the highest transcriptional activity on the HPIP promoter, we further validated the above findings in G1E-ER4 cells. G1E-ER4 cell lines are derived from GATA1-deficient ES cells but stably express a conditional form of GATA1 upon exposure to β-estradiol or 4-OHT (21). As reported previously, treatment of G1E-ER4 cells with 4-OHT

FIGURE 5. Effect of erythroid differentiation inducers, DMSO and Epo, on HPIP expression in leukemic cell lines. Either K562 (A) or HL60 cells (B) were treated with DMSO (1.6%) for the indicated time points, and HPIP expression was analyzed by Western blot (upper panel) or RT-qPCR using gene-specific primers (lower panel). GAPDH serves as internal control. C and D, K562 cells were treated with various doses of Epo for the indicated time points, and then HPIP expression was analyzed by either Western blot (C) or RT-qPCR (D). GAPDH serves as internal loading control and GATA1 as positive control for Epo effect. E, Western analysis shows the effect of Epo at various time points on HPIP expression. The data presented are representative of one of two independent experiments.
induced GATA1 accumulation (Fig. 7A). As predicted, HPPIP protein levels were increased in parallel to GATA1 protein accumulation upon treatment with 4-hydroxytamoxifen in G1E-ER4 cells. Consistent with Western data, quantitative RT-PCR analysis also showed HPPIP mRNA synthesis along with /H9252-globin, which is a known target gene for GATA1 (Fig. 7, B and C), demonstrating GATA1-mediated induction of HPPIP gene expression in erythroid cells.

**GATA1 and C/EBPα Are Recruited to HPPIP Chromatin and Activate Its Gene Transcription**—Because GATA1 and C/EBPα showed strong transcriptional activity on the HPPIP promoter as shown in luciferase assays, we next examined whether they regulate HPPIP gene expression by recruitment to the HPPIP chromatin locus. Because the HPPIP promoter region contains 16 GATA1- and 4 C/EBPα-binding sites, we designed the primers in such a way that the amplified region covers at least one binding site for C/EBPα and 1–3 binding sites for GATA1 (Fig. 8A). K562 cells were either untreated or treated with DMSO for 24 h or with Epo for 4 h, and then cell lysates were analyzed by ChIP assay followed by qPCR. As shown in Fig. 8B, both GATA1 and C/EBPα readily recruited to all four regions of the HPPIP promoter (HPPIP-PR1, HPPIP-PR2, HPPIP-PR3, and HPPIP-PR4). However, upon treatment with DMSO, GATA1 appears to slightly dissociate from the HPPIP promoter region compared with untreated samples, but C/EBPα binding is enriched by 3–4-fold at promoter regions 2–4 and completely dissociates from region 1 (HPPIP-PR1). Interestingly, GATA1 binding is enriched by 2-fold in region 1 upon Epo treatment but slightly reduced at regions 3 and 4 compared with untreated samples (Fig. 8C). GATA1 occupancy at region 2 is more or less unchanged. In contrast, Epo treatment did not detectably influence C/EBPα binding onto HPPIP chromatin region 1, but binding at regions 2–4 is decreased (Fig. 8C). These results indicate the differential recruitment of GATA1 and C/EBPα onto the HPPIP promoter upon treatment with DMSO and Epo. Next, we checked the active chromatin status of the HPPIP promoter upon DMSO and Epo treatment. ChIP analysis of K562 cells treated with either DMSO or Epo showed enrichment of his-
tone acetylation at lysine 4 (H3K4Ac) (Fig. 8D). Together, these results indicate differential regulation of HPIP gene transcription by GATA1 and C/EBPα in response to cell differentiation signals such as DMSO or Epo in K562 cells.

**CTCF and GATA1 Coordinately Regulate HPIP Gene Expression**—Bioinformatic analysis of the 5′-flanking region of the HPIP gene further revealed the presence of CTCF, a chromatin insulator, binding sites on HPIP promoter region (supplemental Fig. S7). Because CTCF, a genomic insulator protein, is one of the critical regulators of β-globin gene expression and participates in erythroid differentiation (28–30), we sought to test whether CTCF also regulates HPIP gene transcription. To address this, we treated K562 cells with Epo (5 units/ml) for 4 h, and cell lysates were subjected to ChIP analysis using CTCF antibody. As shown in Fig. 9A, Epo treatment enhanced CTCF binding to the HPIP promoter regions 1 and 2 (HPIP-PR1 and HPIP-PR2) but not regions 3 and 4. Furthermore, CTCF knockdown in K562 cells by CTCF-specific siRNA also affected the HPIP expression in response to Epo treatment (Fig. 9B) indicating CTCF acts as a positive regulator of HPIP gene expression in K562 cells.

Next, we checked CTCF requirement for GATA1-mediated HPIP gene activation. CTCF siRNA or control siRNA along with a GATA1 expression plasmid and a HPIP promoter-Luc plasmid were transfected into K562 cells, and luciferase activity was measured. As shown in Fig. 9C, CTCF knockdown reduced the transcriptional activity of GATA1 by ∼50% over control siRNA-transfected cells. Moreover, co-immunoprecipitation analysis revealed a likely direct interaction of CTCF with GATA1 in K562 (Fig. 9D). Furthermore, to support these results, we carried out co-localization studies using fluorescence microscopy in HeLa cells. Transiently transfected T7-tagged GATA-1 localized to both cytoplasmic and nuclear compartments, whereas CTCF localized only to the nucleus (Fig. 9E). Nuclear co-localization of GATA1 with endogenous CTCF in HeLa cells suggests a coordinated role in HPIP gene regulation.

Because CTCF binding to DNA is methylation-sensitive, we treated K562 cells with the DNA methylation inhibitor, decitabine, to check if HPIP expression is altered. Indeed, treatment with decitabine increased HPIP protein levels as demonstrated by Western analysis (Fig. 9F). Furthermore, luciferase assay data showed increased promoter activity upon decitabine treatment (Fig. 9G). Together these results suggest that CTCF regulates HPIP gene transcription probably by promoting the formation of an active transcription complex with GATA1 in a DNA methylation-sensitive manner.

**DISCUSSION**

Although HPIP is reported to be expressed in primitive human hematopoietic stem/progenitor cell-enriched CD34+ populations and to act as a co-repressor for pre-B-cell leukemia transcription factor PBX1 (11), its role in hematopoiesis and cell differentiation is not explored. In this study, we attempted to characterize HPIP functions in hematopoiesis *in vitro*.
through lentiviral gene transfer into cord blood cells and also its potential role in cell differentiation using K562 cells as a model system. Constitutive expression of HPIP in hematopoietic stem cells significantly increased the frequency of clonogenic progenitors (CFC) (Fig. 2). In particular, HPIP ectopic expression in CD34<sup>+</sup>/H11001 cells increased the number of erythroid colonies. These data are consistent with our cell differentiation assays carried out in K562 as a model system where ectopic expression of HPIP in K562 cells also promoted erythroid differentiation, whereas K562 cell proliferation was unaffected, suggesting that HPIP promotes erythroid cell differentiation. The arrest of cell proliferation observed in K562 cells after HPIP transduction may be the consequence of entry of the cells into a differentiation program. Our previous studies demonstrated the inhibitory activity of HPIP on PBX-HOX heterodimers to bind to target sequences and the co-repressor activity on transcriptional activation capacity of E2A-PBX suggesting opposing roles of HPIP on PBX1 functions (11). Accumulating evidence suggests that HOX proteins, which require PBX1 as a cofactor, inhibit erythropoiesis (31–33). For example, mice transplanted with marrow cells overexpressing HOXA10 are anemic (33). Another HOXA gene, HOXA5, has also been shown to suppress erythroid differentiation when overexpressed in human CD34<sup>+</sup>/CD38<sup>+</sup> cells (34). Similar results were also seen with overexpression of HOXB6, which suppresses hemoglobinization in both cell lines and primary bone marrow cells (31). In

**FIGURE 8. GATA1 and C/EBPα bind to HPIP promoter.** A, schematic representation of HPIP promoter and regions 1–4 (HPIP-PR1–4) selected for ChIP assay. K562 cells were either treated or untreated with DMSO (1.6%) for 24 h or Epo (5 units/ml) for 4 h, and ChIP assay was performed using either anti-GATA1 antibody or anti-C/EBPα antibody. IgG used as control. GATA1 (B), or C/EBPα (C), or acetyl H3K4 (D) occupancies over human HPIP promoter regions (HPIP-PR 1–4) were determined by ChIP assay. The bar values indicate the relative values of qPCR-amplified products using HPIP promoter-specific primers for the specified regions (HPIP-PR 1–4). The data presented are representative of one of two independent experiments.
FIGURE 9. CTCF regulates HPIP expression in K562 cells. A, K562 cells were either treated or untreated with Epo (5 units/ml) for 4 h, and ChIP assay was performed using anti-CTCF antibody. IgG was used as control. CTCF occupancy over human HPIP promoter regions (HPIP-PR 1–4) was determined by ChIP assay. The bar values indicate the relative values of real time qPCR-amplified products using HPIP promoter-specific primers for the specified regions (HPIP-PR 1–4). B, K562 cells transfected with either control (ctrl) siRNA or CTCF siRNA were lysed, and the cell lysates were subjected to Western analysis using indicated antibodies. C, luciferase assay show the decrease in promoter activity upon CTCF knockdown in GATA1 and hHPIP-promoter luciferase (Luc) co-transfected K562 cells. D, co-immunoprecipitation shows the interaction of CTCF and GATA1 in K562 cells. E, nuclear co-localization of transiently transfected T7-tagged GATA1 with endogenous CTCF in HeLa cells. F, effect of DNA demethylation agent, decitabine (Dcbn), on HPIP expression. K562 cells were treated with DNA demethylation agent, decitabine, at indicated concentrations, and cell lysates were subjected to Western analysis using specified antibodies. GAPDH serves as internal loading control. G, effect of decitabine at various concentrations on HPIP promoter-Luc activity determined by luciferase assay. The data presented are representative of one of two independent experiments.
addition, recipients of HOXB6-transduced marrow are anemic and have lower numbers of splenic BFU-E. Conversely, HOXB6-deficient mice have higher numbers of adult and fetal BFU-E (32) suggesting that HOX genes oppose erythropoiesis. Given the repressive effect of HPIP on PBX-HOX activity and its stimulatory effect on BFU-E, a key role for HPIP appears to be its ability to oppose HOX-PBX1-mediated inhibition of erythroid differentiation.

Our data also implicate the PI3K/AKT pathway in HPIP-mediated erythroid differentiation. The PI3K pathway is well connected to erythropoiesis. For example, activation of phosphatidylinositol 3-kinase is important for erythropoietin-induced erythropoiesis from CD34+ hematopoietic progenitor cells (35). AKT1, which is one of the main downstream targets of PI3K, is reported to mediate erythropoiesis in response to Epo signaling by controlling GATA1 transcriptional activity on the TIMP-1 gene (19). Particularly, DMSO-induced erythroid differentiation is dependent on PI3K activity indicating the central role for the PI3K/AKT pathway in erythropoiesis (20). As shown in this study, LY294002, a selective inhibitor of PI3K, caused decreased numbers of CFC colonies in HPIP-overexpressing CD34+ cells and blocked differentiation of K562 cells. In agreement with our previous studies in MCF7, HPIP expression in K562 also activated PI3K/AKT signaling as demonstrated by Western analysis (Fig. 4). The mechanism of PI3K/AKT activation by HPIP may be based on the interaction of HPIP with PI3K as demonstrated previously in MCF7 breast cancer cell lines (13). However, it remains possible that HPIP could interact directly or indirectly with the EPO receptor through PI3K, which we have yet to analyze.

As HPIP expression is important for erythroid differentiation, we hypothesized that HPIP gene expression may be influenced by erythroid differentiation signals. Accordingly, in this report we also present evidence that HPIP gene expression is induced by Epo as well as by DMSO, a chemical inducer of erythroid differentiation, in K562 cells. Upon DMSO treatment for 5 days in K562 cells, we observed ~4-fold induction of HPIP mRNA (Fig. 5A), which correlates with increased HPIP protein levels. Similarly, DMSO also induces HPIP expression in HL60 (Fig. 5B). It is well documented that erythropoietin regulates the expression of many genes that are involved in erythropoiesis. For instance, c-MYB and NLM genes are induced in response to Epo treatment at day 8 in CD34+ cells (36). Indeed GATA1 itself is induced in response to Epo signaling (37). Similarly, tumor suppressor transcription factor p73 is also induced in response to Epo signaling and is involved in erythroid differentiation (38). It is likely that GATA1 activates various genes, including HPIP, whose activity is required for cellular differentiation program.

GATA transcription factors, particularly GATA-1, GATA-2, and GATA-3, are important in regulating gene expression during hematopoiesis and in determining hematopoietic cell lineages. For instance, GATA-1 is an essential transcription factor for the development of erythroid cells (39, 40). GATA-2 is critical in the development of hematopoietic stem cells (41). The HPIP promoter contains 16 predicted GATA-binding sites, and reporter assays revealed a strong stimulatory effect of GATA1 on HPIP gene transcription, ~130-fold activation. In G1E-ER4 cells, endogenous HPIP gene expression is induced upon GATA1 expression (Fig. 7). However, we also observed basal levels of HPIP expression in GATA1 knock-out cells (data not shown). This is similar to basal β-globin expression in GATA1 knock-out cells (42). Other than GATA1, there may be other transcription factors that could activate HPIP expression in GATA1 knock-out cells. Indeed, our luciferase data show that other known E/Meg signature transcription factors, such as GATA2, C/EBPα, PU1, SCL, Gfi-1 and Fli-1, could also activate the HPIP gene promoter suggesting HPIP expression is not only dependent on GATA factors. Furthermore, our ChIP analysis also shows the direct recruitment of GATA1 onto the HPIP promoter, and upon Epo signaling GATA1 binding to the HPIP promoter was enhanced (Fig. 8B) consistent with a direct role for GATA1 on HPIP gene expression. This is consistent with the result from genome-wide analysis of GATA1-binding sites in G1E-ER4 cells carried out by Hardison and co-workers (43). As shown in supplemental Fig. S8, their data reveal that GATA1 and GATA2 occupy the HPIP promoter region. Because DMSO and Epo induce HPIP expression in K562 cells, enrichment of acetyl histone H3 lysine 4 upon treatment with either agent also reflects the active chromatin status of the HPIP promoter during erythroid differentiation (Fig. 8D). GATA1 regulates a number of genes involved in erythropoiesis, including its cofactor Fog-1 (42). Because GATA1 regulates HPIP gene expression, which in turn regulates the PI3K pathway for erythroid differentiation, this suggests a “feedforward” mechanism in HPIP-mediated erythroid differentiation.

Erythroid lineage commitment from progenitor cells requires the precisely coordinated activation of erythroid genes. It is dependent on the coordinated regulation of various erythroid transcription factors (44). In accordance with this, during erythroid differentiation HPIP transcription may be dependent on various transcription factors. In this context, we have also investigated the role of CTCF on HPIP gene expression for two reasons. One, CTCF-binding sites are mapped to the HPIP gene locus (supplemental Fig. S8) (45). Second, CTCF is reported to regulate erythroid differentiation (29, 30). Our experimental results show that CTCF is required for Epo-induced HPIP gene expression (Fig. 9B). Furthermore, ChIP analysis shows the binding of CTCF to the HPIP promoter. Intriguingly, we also found that GATA1-mediated activation of the HPIP promoter requires CTCF (Fig. 9C) perhaps due to direct protein-protein interaction as evidenced by our co-immunoprecipitation results (Fig. 9D). CTCF binding to DNA is dependent on the methylation status of its target DNA (46). Intriguingly our studies show that decitabine, a DNA methylation inhibitor, treatment enhanced HPIP gene expression, consistent with increased recruitment of CTCF. CTCF-dependent HPIP gene expression, as shown here, partly explains how CTCF regulates erythroid differentiation in addition to its direct effect on β-globin synthesis.

In conclusion, we propose a model wherein erythroid differentiation factor, Epo signaling, regulates HPIP expression through coordination between erythroid lineage-specific transcription factor GATA1 and CTCF. HPIP thus expressed in turn mediates erythroid differentiation through the activation of the PI3K/AKT signaling pathway. Epo influences HPIP gene expression.
expression through the GATA1 transcription factor, and HPIP in turn activates GATA1 through the PI3K pathway indicating a feedforward mechanism operating in erythroid differentiation.

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