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RESEARCH PAPER

Epigenetic dysregulation of the erythropoietic transcription factor *KLF1* and the β-like globin locus in juvenile myelomonocytic leukemia

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ABSTRACT

Increased levels of fetal hemoglobin (HbF) are a hallmark of more than half of the children diagnosed with juvenile myelomonocytic leukemia (JMML). Elevated HbF levels in JMML are associated with DNA hypermethylation of distinct gene promoter regions in leukemic cells. Since the regulation of globin gene transcription is known to be under epigenetic control, we set out to study the relation of DNA methylation patterns at β-/γ-globin promoters, mRNA and protein expression of globins, and epigenetic modifications of genes encoding the globin-regulatory transcription factors *BCL11A* and *KLF1* in nucleated erythropoietic precursor cells of patients with JMML. We describe several altered epigenetic components resulting in disordered globin synthesis in JMML. We identify a cis-regulatory upstream *KLF1* enhancer sequence as highly sensitive to DNA methylation and frequently hypermethylated in JMML. The data indicate that the dysregulation of β-like globin genes is a genuine attribute of the leukemic cell clone in JMML and involves mechanisms not taking part in the normal fetal-to-adult hemoglobin switch.

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Introduction

Juvenile myelomonocytic leukemia (JMML) is an aggressive malignant haematopoietic disorder occurring in infants and young children.[1] The leukemic stem/progenitor cell clone differentiates not only toward granulocytes and monocytes, but also the red cell lineage.[2,3] A key characteristic observed in the majority of JMML patients is excessive production of fetal hemoglobin (HbF) even when corrected for patient age.[4] The presence of elevated levels of HbF at diagnosis is a strong adverse prognostic factor linked with higher risk of treatment failure and shorter survival.[4-6] Most studies on leukemic erythropoiesis in JMML date back several decades.[7-12] With the caveat in mind that the diagnosis of JMML was not well defined at the time, the data suggested that the malignant clone maintained (or reverted to) a fetal type of erythropoiesis, as concluded from reverted red cell isoenzyme patterns, reduced expression of the I antigen, decreased hemoglobin A2 production, and shifted ratio of $^{G}\gamma/^{A}\gamma$ globin chains.[7-12]

The erythropoietic transcription of human β-like globin genes, which are integrated in a broader locus on chromosome 11, switches from the ε-globin gene in embryonal and early fetal life to the γ-globin genes in most of fetal life and then on to β-globin late in fetal life and postnatally.[13] The regulation of globin switching involves a set of powerful upstream enhancer elements (the "locus control region"), which are indispensable for high expression of the β-like globin genes. The elements represent active transcription factor-binding sites and form a complex structure that loops the chromosome to bring the control region near the appropriate globin gene, thereby delivering powerful coactivators to the site of transcription and insulating the locus from surrounding inactive chromatin.[14,15] This process is supported by 2 major developmental-specific transcription factors, KLF1[16] and BCL11A.[17] KLF1 is an activator of β-globin and primarily relevant during postnatal erythropoiesis.[18] BCL11A interacts with the NuRD chromatin remodeling complex and other erythroid transcription factors and is a key repressor of γ-globin, probably through direct physical interaction.[17] Importantly, the physiologic fetal-to-adult hemoglobin switch in humans is also associated with differential DNA methylation at intergenic cis-elements and globin gene promoters.[19]

Leukemic DNA hypermethylation of distinct genetic regions is a cardinal feature of JMML[20-23] and defines a high-risk variety of JMML with poor prognosis.[20,24] Aberrant DNA methylation and elevated levels of HbF were consistently shown to be highly concordant in JMML, in line with the adverse prognostic impact of both features.[20-23] At least one previous case report demonstrated disordered globin synthesis in the absence of a β-globin gene mutation.[25] All this led us to investigate how the globin-regulatory network in JMML erythroid cells is altered at the epigenetic level. We identify erythroid-specific epigenetic changes

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associated with the leukemic clone. We emphasize that these changes are different from mere reactivation of fetal physiology.

Materials and methods

Human cells

Human cells were collected after obtaining informed consent from participants or guardians and approval from institutional review committees. Spleen cells from 14 patients diagnosed with JMML were collected at study centers of the European Working Group of Myelodysplastic Syndromes in Childhood (EWOG-MDS). Splenectomies were performed for clinical indication. Cord blood was obtained from 6 healthy newborns, and peripheral blood (PB) was collected from 17 healthy volunteers.

Isolation of primary erythroblasts, in vitro-differentiated erythroblasts, granulocytes, and monocytes

Biocoll density gradient centrifugation (Biochrom, Berlin, Germany) was used to separate and cryopreserve mononuclear cells (MNC) and granulocytes from leukocyte suspensions. Glycophorin A-positive erythroid precursor (GPEP) cells were isolated from MNC by magnetic-activated cell sorting using CD235a MicroBeads (Miltenyi, Bergisch Gladbach, Germany) or by fluorescence-activated cell sorting after double-staining with anti-CD235a-fluorescein isothiocyanate (Biolegend, San Diego, CA) and diamidinophenylindole (DAPI). For human erythroid massive amplification (HEMA), progenitor cells were expanded from peripheral blood MNC for 10 d and differentiated for 4 d in culture media as described elsewhere.[26] Monocytes ($CD14^+$) cells were isolated from MNC using CD14 MicroBeads (Miltenyi).

Reverse transcriptase quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated using Quick-RNA MicroPrep (Zymo Research, Freiburg, Germany) and transcribed into cDNA using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). RT-qPCR analysis was performed with ABsolute qPCR SYBR Green Mix (Thermo Fisher Scientific, Waltham, MA) on a CFX384 system (Biorad, München, Germany) using *GAPDH* for internal normalization and HEMA GPEP cells from a healthy adult as calibrator. Primer sequences are provided in **Table S1**.

High-pressure liquid chromatography (HPLC)

Hemoglobin variants from filtered lysates of GPEP cells were separated using a PolyCAT A cation exchange column (PolyLC, Columbia, MD) and analyzed on an Elite LaChrom HPLC system (Hitachi, San Jose, CA) using gradient elution with a bis-tris buffer. Hemoglobins were detected by absorbance at 415 nm.

Quantitative high-resolution DNA methylation analysis

Genomic DNA was isolated using Gentra Puregene (Qiagen), bisulfite-converted using EZ DNA Methylation Kit (Zymo Research), and analyzed for *HBG1/HGB2, BCL11A*, and *KLF1* methylation by EpiTYPER mass spectrometry (Agena, Hamburg, Germany) using protocols described previously,[27] or for *HBB* methylation by bisulfite sequencing of subcloned alleles (pCR2.1-TOPO, Life Technologies). Primer sequences are provided in **Table S1**.

Dual luciferase reporter gene assay

The pCpG-free-promoter-Lucia plasmid (Invivogen, San Diego, CA) was used. In vitro methylation was performed using the *M.Sss*I methyltransferase. Efficiency was monitored by methylation-sensitive cleavage with *Hpa*II and methylation-insensitive cleavage with *Msp*I (all enzymes from New England Biolabs, Ipswich, MA). The HEK293T human embryonal kidney cell line and the K562 erythroleukemia cell line were co-transfected with pCpG-free-promoter-Lucia or methylated/unmethylated pCpG-free-promoter-Lucia-KLF1 and a Firefly vector for normalization (pCpGL-CMV/EF1, courtesy of Michael Rehli, Regensburg, Germany). Twenty-four hours later, the cells were lysed and incubated with coelenterazine (Sigma-Aldrich, Steinheim, Germany) and D-luciferin (Biosynth, Staad, Switzerland). Reporter gene activity was measured on a luminometer at 480 nm (Lucia) and 560 nm (Firefly).

KLF1 target activation

Cells were double-stained with fluorescein isothiocyanate-labeled anti-human CD235a antibody and allophycocyanin-labeled anti-human CD44 antibody (both from Biolegend) and analyzed by flow cytometry.

Results

Globin expression analysis in JMML erythroid precursor cells

Epigenetic modifications of globin regulation cannot be studied in bulk red cells as erythrocytes do not contain DNA. We therefore isolated nucleated glycophorin A-positive erythroid precursor cells from MNC by immunomagnetic cell sorting with anti-CD235a beads (Fig. 1A). Cryopreserved mononuclear spleen cells of JMML patients (n = 14), peripheral blood of healthy adults (n = 17), and umbilical cord blood of healthy newborns (n = 6) were used. GPEP cell separation from cryopreserved samples containing excess dead cells was performed by fluorescence-activated cell sorting, thus permitting double selection for $CD235a^+/DAPI^-$ cells. To obtain sufficient amounts of GPEP cells from peripheral blood, erythroid progenitors were first expanded by *ex vivo* differentiation in a 2-phase cell culture system (HEMA culture) and then purified immunomagnetically. The median purity of GPEP cells as assessed by $CD235a^+$ flow cytometry was 92.2% (**Table S2**).

The expression level of β-like globins in GPEP cells was measured by RT-qPCR. As expected, GPEP cells from healthy adults predominantly expressed β-globin (*HBB*) mRNA [median $\gamma/(\gamma+\beta)$ quotient 5.1%] whereas GPEP cells from healthy newborns expressed large amounts of γ-globin (*HBG1/HBG2*) mRNA and low levels of *HBB* mRNA [median $\gamma/(\gamma+\beta)$

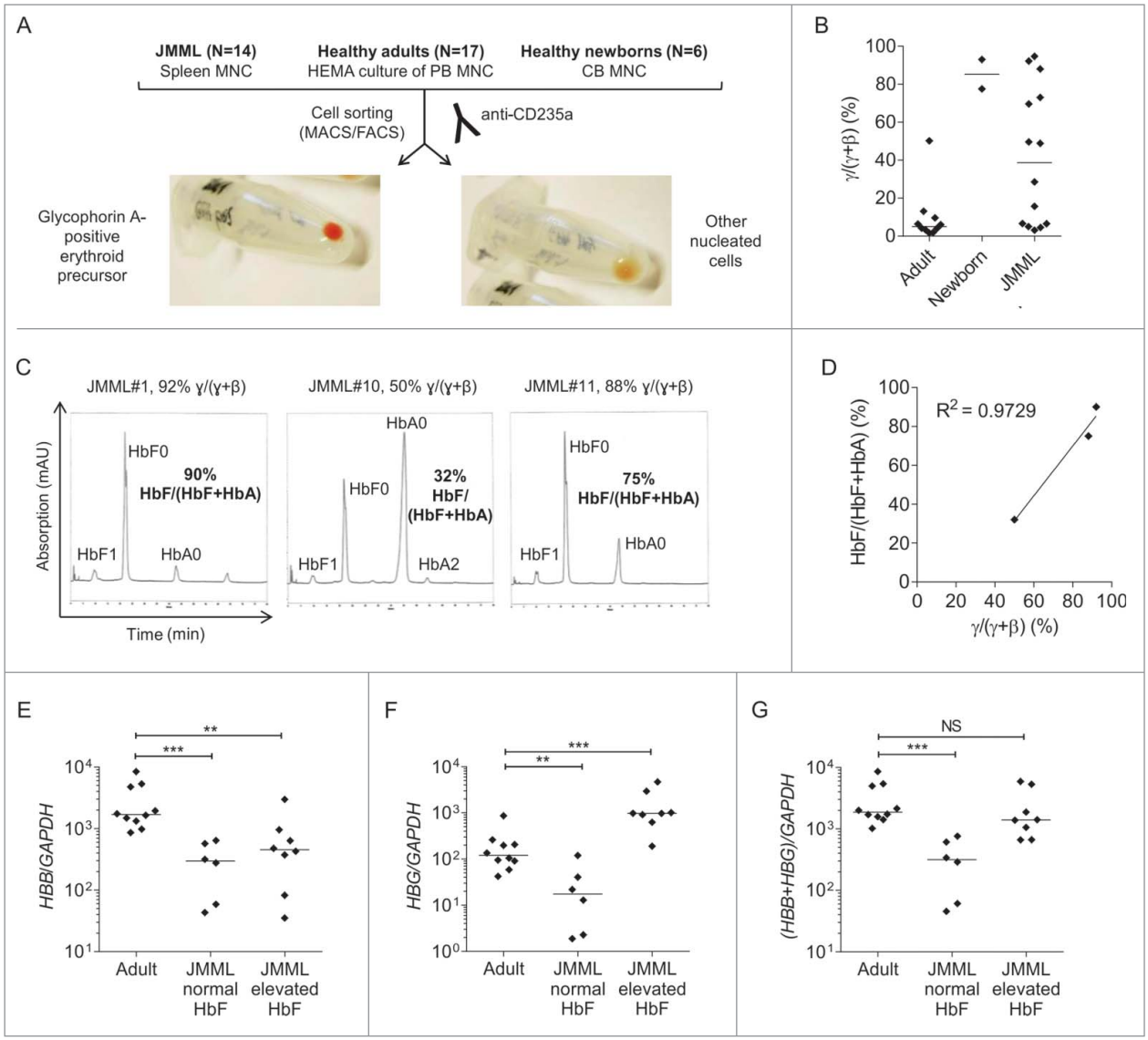

Figure 1. β-like globin expression in glycophorin A-positive erythroid precursor cells. (A) Isolation of GPEP cells and other nucleated cells from cryopreserved spleen cells of JMML patients, *in vitro*-differentiated blood cells of healthy adults and cord blood cells of healthy newborns. (B) β-like globin mRNA level [$\gamma/(\gamma+\beta)$ mRNA quotient] measured by RT-qPCR in GPEP cells of adults, newborns, and JMML patients. (C) High pressure liquid chromatography to determine hemoglobin variants in GPEP cells of 3 patients with JMML. HbF0/HbA0/HbA2: unmodified, HbF1: glycosylated; for the calculation of %HbF as HbF/(HbF+HbA) the amount of unmodified and glycosylated HbF was considered. For each patient, the corresponding globin mRNA quotient is indicated above the chromatogram. (D) Correlation between globin mRNA quotient and %HbF in GPEP cells of 3 patients with JMML. (E) *HBB* transcript levels relative to the *GAPDH* reference gene in GPEP cells of adults, JMML patients classified as normal HbF, and JMML patients classified as elevated HbF. (F) *HBG* transcript levels relative to *GAPDH* in GPEP cells of adults, JMML patients classified as normal HbF, and JMML patients classified as elevated HbF. Due to high sequence homology, the assay did not discriminate between *HBG1* and *HBG2*. (G) Total β-like globin transcription relative to *GAPDH* in GPEP cells of adults, JMML patients classified as normal HbF, and JMML patients classified as elevated HbF. Mann-Whitney test: NS, not significant; $^{**}P \leq 0.01$; $^{***}P \leq 0.001$. MNC, mononuclear cells; HEMA, human erythroid massive amplification; CB, cord blood.

quotient 85.3%] (Fig. 1B, Table S3). The expression of β-like globin mRNA in JMML GPEP cells was highly variable between patients, with $\gamma/(\gamma+\beta)$ quotients ranging from 3.1% to 95.0%. We next sought to determine how well the relative globin mRNA levels reflected the actual hemoglobin composition in red precursor cells. Although the limited number of purified GPEP cells posed a technical challenge, Hb-HPLC analysis succeeded in quantifying hemoglobin fractions of GPEP cells from 3 JMML patients (Fig. 1C). The percentage of HbF (%HbF) determined by Hb-HPLC was in good agreement with $\gamma/(\gamma+\beta)$ mRNA quotient (Fig. 1D). This indicates that the relative expression of β-like globin mRNA translates directly into hemoglobin composition in JMML GPEP cells and that RT-qPCR of globin mRNA can be used to estimate %HbF if a direct determination by Hb-HPLC is not possible. Based on γ-globin mRNA quotient, 6 of 14 JMML patients were classified as patients with normal HbF and 8 were classified as JMML with elevated HbF (Fig. S1, Table S3). The %HbF values measured in bulk erythrocytes at time of diagnosis before first red blood cell transfusion were available from clinical records in 8 cases. The classification as "HbF normal" or "HbF elevated for age" was invariably consistent with the analysis of β-like globin mRNA levels in JMML GPEP cells (**Table S3**).

We observed that *HBB* transcript levels relative to *GAPDH* were lower in GPEP cells from JMML patients than healthy

adults, whether HbF was elevated or not (Fig. 1E). *HBG* transcripts were increased in JMML patients with elevated HbF as expected (Fig. 1F). As a consequence, the overall transcription of β-like globins was reduced in GPEP cells from JMML patients with normal HbF compared with healthy adults (Fig. 1G). By contrast, increased *HBG* expression in JMML patients with elevated HbF resulted in total β-like globin transcription at a similar level as in healthy adults (Fig. 1G).

DNA methylation of globin gene promoters in JMML erythroid precursor cells

We next explored if altered β-like globin transcription was paralleled by aberrant DNA methylation of the β-like globin gene promoters in JMML patients. Quantitative mass spectrometry covering 6 of the 7 γ-globin promoter CpG dinucleotides (Fig. 2A) showed that the CpG methylation was dense in GPEP cells from healthy adults as well as JMML patients with normal HbF (Fig. 2B), in line with the low γ-globin expression observed in these groups. By contrast, the level of γ-globin promoter CpG methylation was low in GPEP cells from healthy newborns and JMML patients with increased HbF (Fig. 2B). Although a direct quantitative relationship between γ-globin promoter CpG methylation and γ-globin mRNA expression would not necessarily be expected because of the complex transcriptional regulation of hemoglobins, we observed a surprisingly robust correlation between γ-globin methylation and expression in JMML GPEP cells (Fig. 2C). We also measured the γ-globin promoter CpG methylation in non-GPEP cell populations (CD235a-negative mononuclear cells, $CD14^+$ monocytes, and granulocytes) of patients and controls (Fig. 2D). Methylation was dense in all groups, consistent with lineage-specific silencing of hemoglobin transcription in non-GPEP cells.

When attempting to analyze CpG methylation of the β-globin promoter we encountered technical limitations of mass spectrometry at this target region. We therefore resorted to the less quantitative method of bisulfite sequencing, with material

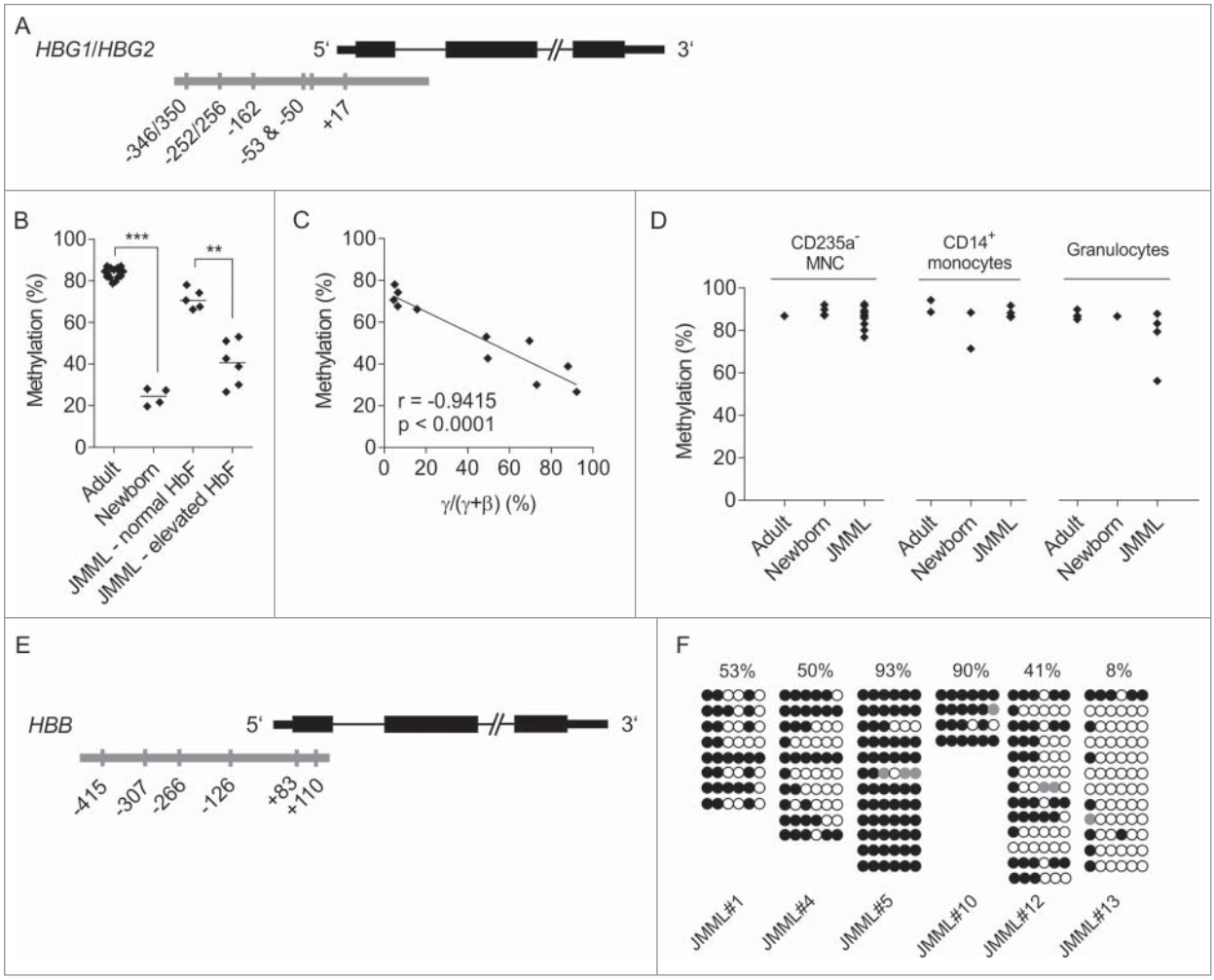

Figure 2. DNA methylation of the β-like globin gene promoters. (A) Target region (gray) in the γ-globin (*HBG*) promoter with CpG dinucleotide sites that were analyzed for methylation by mass spectrometry. Due to high sequence homology, the assay did not discriminate between *HBG1* and *HBG2*. (B) γ-globin promoter CpG methylation (mean of 6 CpG sites) in GPEP cells of adults, newborns, JMML patients classified as normal HbF, and JMML patients classified as elevated HbF. (C) Correlation between globin expression [$\gamma/(\gamma+\beta)$ mRNA quotient] and γ-globin promoter methylation in GPEP cells of patients with JMML. r, Pearson coefficient. (D) γ-globin promoter CpG methylation (mean of 6 CpG sites) in non-GPEP cell populations of adults, newborns, and patients with JMML. (E) Target region (gray) in the β-globin (*HBB*) promoter with CpG dinucleotide sites that were analyzed for methylation by bisulfite sequencing. (F) β-globin promoter CpG methylation in GPEP cells of 6 JMML patients assessed by bisulfite sequencing. Lines represent individual alleles; filled circles, methylated cytosines; open circles, unmethylated cytosines. The average level of methylation across CpG sites and alleles is indicated at the top for each patient. Mann-Whitney test: $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

availability allowing for a limited analysis of only 6 JMML cases (Fig. 2E). In contrast to healthy GPEP cells, which express β-globin and carry an unmethylated β-globin promoter,[19] GPEP cells from children with JMML exhibited variable β-globin promoter hypermethylation (Fig. 2F), a finding that generally fits with diminished *HBB* expression levels as described above. However, the variability of *HBB* transcript levels (Fig. 1E) did not correlate well with variable promoter DNA methylation (Fig. 2F) in JMML GPEP cells.

Together, the results illustrate that aberrant hemoglobin expression in JMML is associated with epigenetic modification of the β-like globin promoters in GPEP cells. In addition, this is the first description of epigenetic differences between JMML cell fractions differentiating along various lineages.

The β-globin activator KLF1 is subject to DNA methylation changes in JMML erythroid precursor cells

We then asked if regulatory networks of β-like globin expression were affected by leukemic DNA hypermethylation in JMML GPEP cells. We focused on the 2 major regulators governing the fetal-to-adult hemoglobin switch, BCL11A and KLF1. KLF1 directly induces β-globin transcription in erythroid cells and acts as an activator of BCL11A, which in turn represses γ-globin mRNA expression (Fig. 3A).

We assessed CpG methylation by mass spectrometry at 4 *BCL11A* gene regions, 2 located in a promoter CpG island and 2 within intragenic CpG islands of *BCL11A*. All 4 regions were unmethylated in GPEP cells from JMML patients or healthy controls, as well as in non-erythroid cells from JMML patients or healthy controls (data not shown). We conclude that the *BCL11A* gene locus is not subject to changes in CpG island methylation, neither as a regulatory mechanism under physiologic conditions nor as a consequence of leukemic transformation in JMML.

Three *KLF1* gene regions were investigated: the ~0.5 kb upstream enhancer element, the ~0.2 kb upstream promoter and the 5′ CpG island (Fig. 3B). The analysis showed that the *KLF1* enhancer, but not the promoter and canonical CpG island were affected by CpG hypermethylation in GPEP cells from patients with JMML (Fig. 3C, Fig. S2). By contrast, all 3 regions were unmethylated in GPEP cells from healthy adults, compatible with active KLF1 function in this cell type. Of note, all 3 *KLF1* regions were devoid of methylation in GPEP cells from healthy newborns although *KLF1* transcription is downregulated in neonatal erythroblasts to enable physiologic HbF expression.[18] By contrast, the *KLF1* enhancer exhibited dense methylation in non-erythroid cells from healthy individuals or patients with JMML (Fig. 3D), suggesting that DNA methylation is used to repress transcription of the erythroid-specific factor *KLF1* in cells not differentiating toward the red lineage. To corroborate the idea of lineage-specific epigenetic KLF1 control we compared the *KLF1* enhancer DNA methylation in 2 acute myeloid leukemia (AML) cell lines: K562, derived from an erythroleukemia, and HL60, derived from an AML with myeloid maturation. Methylation was nearly absent in K562 whereas the region was densely methylated in HL60 (Fig. 3E).

Taken together, the data suggest that *KLF1* enhancer DNA methylation is used under normal conditions to keep the gene silent in non-erythroid cells but does not participate in the fetal-to-adult hemoglobin switch in red cells. However, the

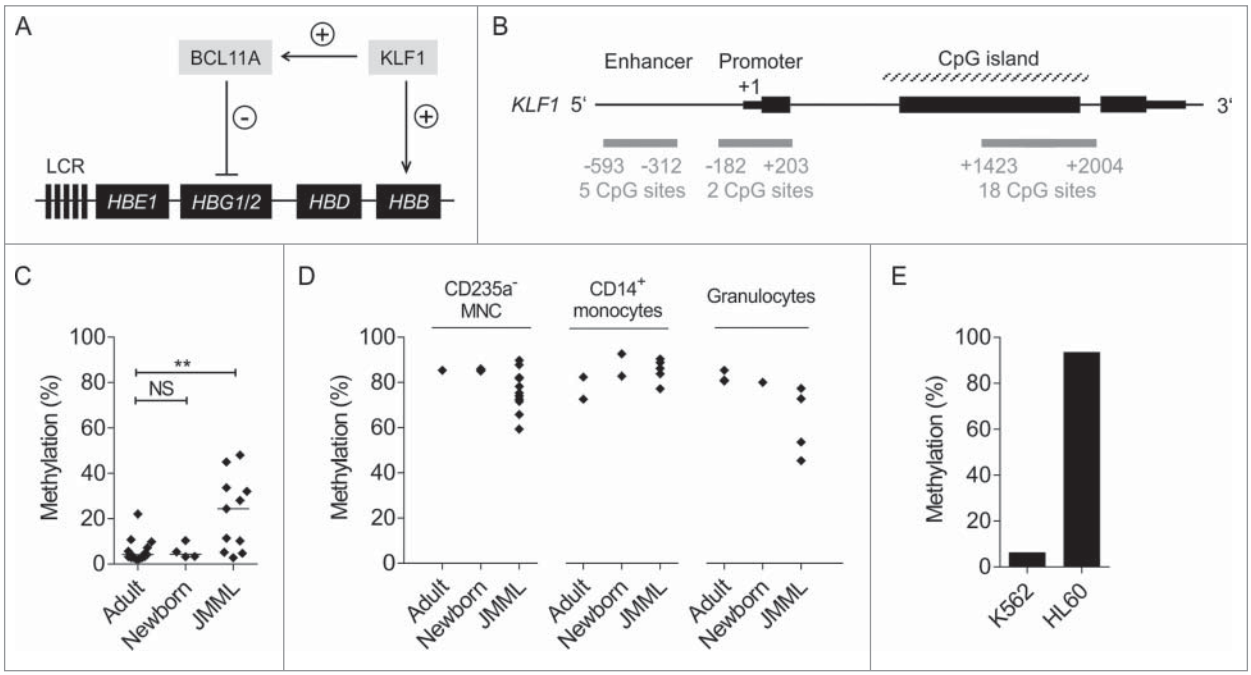

Figure 3. DNA methylation analysis of the *KLF1* gene. (A) Regulation of β-like globin expression in erythropoietic cells by transcription factors *BCL11A* and *KLF1*. LCR, locus control region. (B) Target regions (gray) in the *KLF1* gene locus that were analyzed for methylation by mass spectrometry. (C) CpG methylation of the *KLF1* enhancer region (mean of 5 CpG sites) in GPEP cells of adults, newborns, and patients with JMML. (D) *KLF1* enhancer methylation in non-GPEP cell populations of adults, newborns, and patients with JMML. (E) *KLF1* enhancer methylation in the erythroleukemia cell line K562 and the non-erythroid myeloid leukemia cell line HL60. Mann-Whitney test: NS, not significant; $^{**}P \leq 0.01$.

KLF1 enhancer region acquires leukemia-associated hypermethylation specifically in the erythroid lineage in some patients with JMML.

DNA methylation of the KLF1 enhancer leads to transcriptional repression

To determine if the epigenetic changes observed alter the expression of *KLF1* we used a dual luciferase reporter assay that allowed us to directly test how CpG methylation affects the function of the enhancer sequence. A 356-bp fragment of the *KLF1* enhancer region was ligated into a reporter plasmid completely devoid of CpG dinucleotides and enhancer CpG sites were methylated enzymatically. The transcriptional activity of the reporter gene was assessed after transfecting unmethylated and methylated construct into erythroid K562 cells and non-erythroid HEK293T cells. We found that the unmethylated *KLF1* enhancer construct was highly active in K562 cells, whereas methylation resulted in a dramatic loss of reporter gene transcription (Fig. 4A). By contrast, *KLF1* enhancer activity was barely detectable in HEK293T cells irrespective of methylation (Fig. 4B), consistent with its specificity to the erythroid lineage.[28] The experiment confirms the cis-regulatory nature of the *KLF1* enhancer sequence and that epigenetic modification modulates its activity.

Variable expression of KLF1 in JMML erythroid precursor cells is reflected by expression changes of regulatory KLF1 target molecules

We next studied *KLF1* mRNA expression in erythroid cells and its relation to downstream regulatory targets. RT-qPCR confirmed that the level of *KLF1* mRNA expression was significantly lower in GPEP cells from healthy newborns than healthy adults (Fig. 5A), consistent with the role of *KLF1* in hemoglobin switching. *KLF1* expression in JMML GPEP cells was highly variable, with the median level being similar to that in healthy newborns (Fig. 5A). Consistent with the cis-regulatory effect of *KLF1* enhancer methylation described above, there was a negative correlation between methylation and expression in JMML GPEP cells (Fig. 5B). As expected, the expression of *KLF1* correlated positively with that of *HBB* (Fig. 5C). Unexpectedly, however, we failed to demonstrate a relationship between *KLF1* and *HBG* expression in JMML GPEP cells (data not shown).

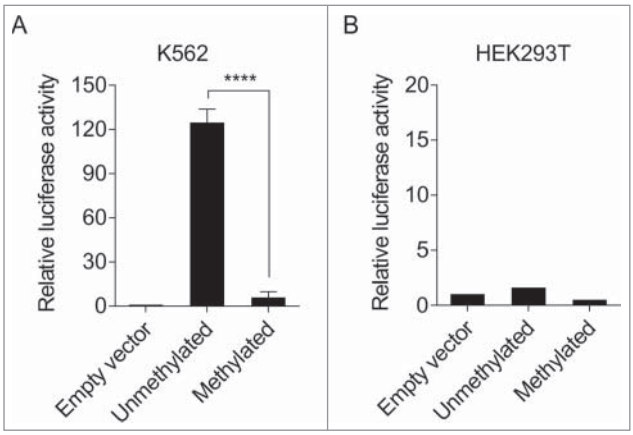

Figure 4. Hypermethylation inhibits the activity of the *KLF1* enhancer. (A) Analysis of *KLF1* enhancer activity by a luciferase reporter assay in K562 cells. Luciferase activity was normalized to the activity of the pCpGL-CMV/EF1 Firefly control vector. Normalized activity of the construct containing unmethylated *KLF1* enhancer CpG sites and the construct containing methylated *KLF1* enhancer CpG sites are shown relative to the normalized activity of the reporter construct not containing the *KLF1* enhancer sequence (empty vector). Error bars represent standard deviation of 3 transfection experiments. T test: $^{****}P \leq 0.0001$. (B) Same experiment in non-haematopoietic HEK293T cells, confirming specificity of *KLF1* enhancer function to erythropoietic cells.

Assessing KLF1 protein expression in JMML GPEP cells proved difficult because of material constraints that led to insufficient specificity of the immunoblots. As an alternative we used flow cytometry to measure the surface expression of the CD44 protein, a direct regulatory target of KLF1.[29] CD44 expression on non-erythroid cells was the same in JMML and control (Fig. 5D), in concordance with the lack of KLF1 function in non-erythroid cells.[16] However, CD44 expression was reduced on GPEP cells from JMML patients compared with the control (Fig. 5D). As predicted, CD44 expression was lower on JMML GPEP cells with higher *KLF1* enhancer methylation/lower *KLF1* mRNA expression, and vice versa (Fig. 5E). Together, the data support that epigenetic alterations affect the function of the erythroid-specific transcription factor KLF1 in JMML and contribute, at least in part, to disordered β-like globin expression.

Discussion

JMML is a unique myeloproliferative disorder of childhood that has long been recognized as a model disease for oncogenic dysregulation of Ras signal transduction.[30] In addition to genetic changes, the significance of leukemia-associated epigenetic modifications in JMML is now being increasingly appreciated. Non-random CpG island hypermethylation occurs in at least half of JMML cases and portends a poor prognosis.[20,23,24] The concordance between abnormal hemoglobin composition and aberrant DNA methylation in high-risk JMML, together with the paradigmatic contribution of epigenetic mechanisms to globin gene regulation,[19,31] prompted us to study the globin gene-regulatory network in JMML erythroid precursor cells with a focus on epigenetic alterations. We found that the canonical role of promoter CpG methylation for globin transcription was also reproducible in JMML cells. JMML GPEP cells with increased γ-globin transcripts exhibited decreased methylation of *HBG* promoter CpG dinucleotides as compared with those with low γ-globin expression. In JMML non-GPEP cell populations, the γ-globin promoter was silenced by dense methylation. We noted a surprisingly tight correlation of γ-globin transcript levels with HbF protein measured in leukemic red precursor cells or in full blood of JMML patients at time of diagnosis. The data indicate that increased HbF in JMML originates in the clonal leukemic erythropoiesis and is distinct from elevated levels of HbF observed under certain circumstances of stress erythropoiesis or in non-malignant haematopoietic disorders such as β-thalassemia or sickle cell disease.[32-34]

Increased levels of HbF are also noted in other malignant myeloid disorders, such as MDS or AML,[9,35-37] but it must be emphasized that the levels are not nearly as high as those encountered in JMML.[9,37] To our knowledge, it has not been determined experimentally in MDS or AML whether HbF

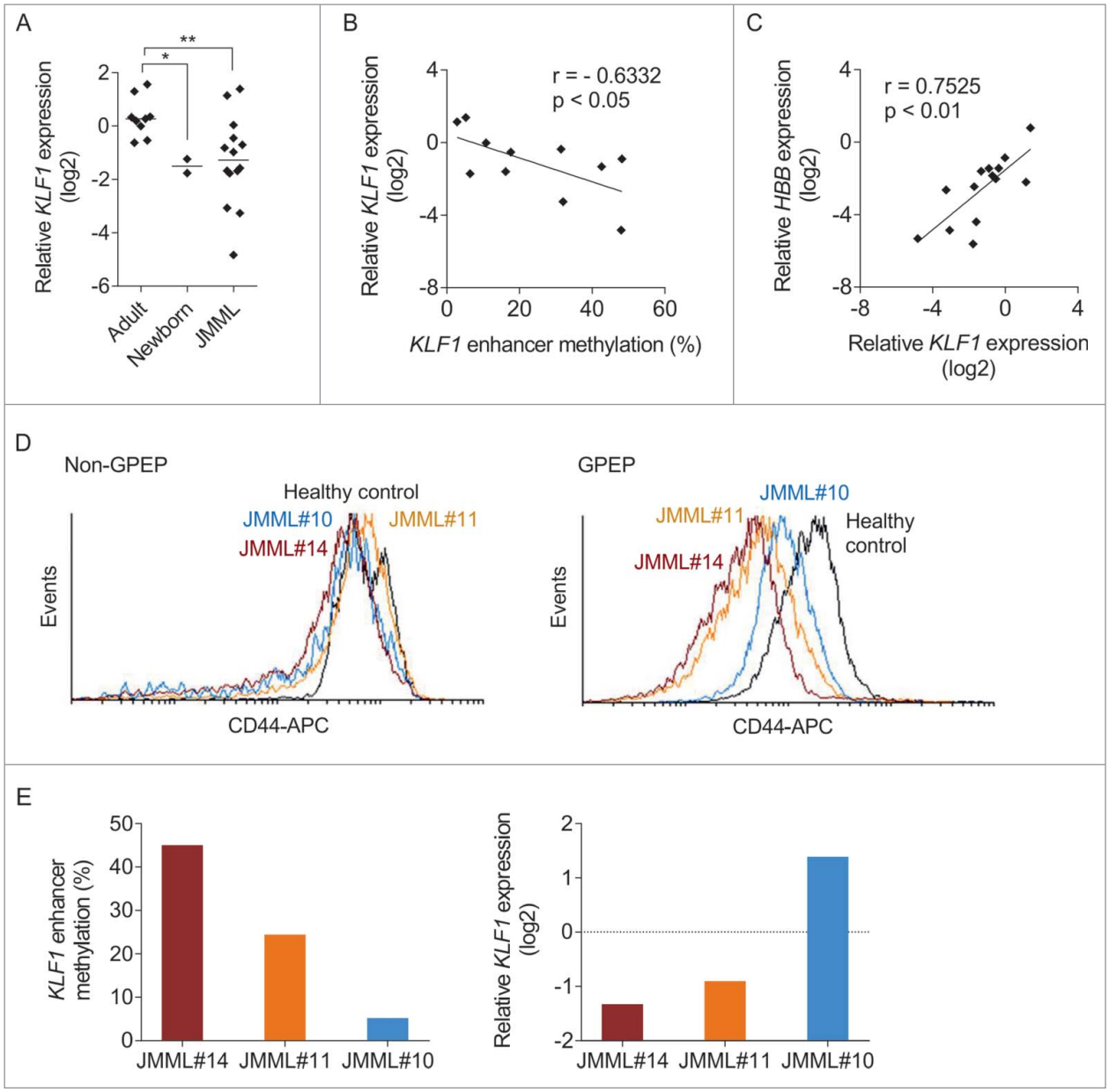

Figure 5. Expression analysis of KLF1 and its regulatory target CD44. (A) *KLF1* transcript levels relative to the *GAPDH* reference gene measured by reverse transcriptase quantitative polymerase chain reaction in GPEP cells of adults, newborns, and patients with JMML. Cells of a healthy adult were used as calibrator (relative log2 expression = 0). Mann-Whitney test: $^{*}P \leq 0.05$; $^{**}P \leq 0.01$. (B) Correlation between *KLF1* enhancer methylation and *KLF1* mRNA expression in GPEP cells of patients with JMML. r, Pearson coefficient. (C) Correlation between *KLF1* and *HBB* mRNA expression in GPEP cells of patients with JMML. r, Pearson coefficient. (D) Surface expression of CD44 on glycophorin A-negative mononuclear (non-GPEP) cells (left panel) or GPEP cells (right panel) of 3 JMML patients and a healthy control measured by flow cytometry. APC, allophycocyanin. (E) *KLF1* enhancer methylation (left panel) and *KLF1* mRNA expression relative to *GAPDH* (right panel) in GPEP cells of 3 JMML patients analyzed in.Fig. 2D Cells of a healthy control were used as calibrator (dashed line).

production occurs in the neoplastic clone, as we show for JMML, or results from low-abundant normal erythroid precursor cells that express the γ-globin genes during adult erythroid development[38] and expand under erythropoietic pressure. We prefer the latter possibility as it also helps explain the observation that, in contrast to JMML, the prognostic significance of increased HbF in MDS or AML is favorable.[37,39]

A remarkable finding is the specificity to the erythroid lineage of some leukemic epigenetic modifications, such as *KLF1* enhancer hypermethylation or γ-globin hypomethylation. The JMML-initiating clone, deriving from an early haematopoietic multipotent stem/progenitor cell,[40] differentiates not only to the myelomonocytic but also the erythroid lineage.[2,3,12] Accordingly, gene mutations acquired during leukemogenesis are usually present in nucleated red cells of JMML patients, whether isolated by clonogenic progenitor cell culture[3] or direct separation.[20] Similarly, we described previously that epigenetic alterations identified in myelomonocytic cells of JMML patients could be traced back to the early progenitor compartment and were also reflected in the erythroid lineage.[20] Hence, it appears that both global and lineage-specific epigenetic alterations occur in JMML. The observed variability of *de novo KLF1* enhancer methylation in erythroid JMML cells may call into question that this is an early event during leukemogenesis. However, it is possible that the epimutation occurs early but becomes unstable in later stages of the disease.

With the identification of the *KLF1* upstream enhancer element as highly sensitive to methylation and variably methylated in JMML cells, the present study is the first to describe the epigenetic control of a haematopoietic transcription factor in this leukemia. Importantly, erythroid-specific *KLF1* enhancer methylation in JMML demonstrates that the leukemic dysregulation in JMML GPEP cells goes beyond reversion to an earlier or fetal developmental stage, as methylation of the *KLF1* enhancer sequence does not occur during normal fetal-to-adult development of red cells. As expected, higher methylation of the *KLF1* enhancer in JMML cells was associated with lower transcription of *KLF1* and reduced expression of the KLF1 targets *HBB* and CD44. However, an unexpected limitation was that there was no obvious correlation of *KLF1* methylation with *HBG* transcription or level of HbF. A possible explanation could be the influence of other γ-globin-regulating factors, such as LIN28B[41] or LRF.[42]

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Authors contribution

S.F. devised the study, performed experiments, interpreted data, and wrote the manuscript. C.F.K., A.M., T.E. and O.M. performed experiments. C.P. interpreted data and contributed to manuscript writing. C. M.N. provided clinical samples, interpreted data and contributed to manuscript writing. C.F. devised the study, interpreted data and wrote the manuscript.

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