ARTICLE

Fetal hemoglobin induction during decitabine treatment of elderly patients with high-risk myelodysplastic syndrome or acute myeloid leukemia: a potential dynamic biomarker of outcome

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ABSTRACT

Hematologic responses to hypomethylating agents are often delayed in patients with myelodysplastic syndrome or acute myeloid leukemia. Fetal hemoglobin is a potential novel biomarker of response: recently, we demonstrated that a high fetal hemoglobin level prior to decitabine treatment was associated with superior outcome. Here we investigated whether early fetal hemoglobin induction during decitabine treatment also had prognostic value, and studied the potential of decitabine to induce erythroid differentiation and fetal hemoglobin expression *in vitro*. Fetal hemoglobin levels were measured by high-performance liquid chromatography in patients with higher-risk myelodysplastic syndrome (n=16) and acute myeloid leukemia (n=37) before treatment and after each course of decitabine. Levels above 1.0% were considered induced. Patients achieving complete or partial remission as best response had attained a median fetal hemoglobin of 1.9% after two courses of treatment, whereas the median value in patients who did not reach complete or partial remission was 0.8% (P=0.015). Fetal hemoglobin induction after two courses of decitabine treatment was associated with early platelet doubling (P=0.006), and its subsequent decrease with hematologic relapse. In patients with myelodysplastic syndrome, induction of fetal hemoglobin after course 2 of treatment was associated with longer overall survival: median of 22.9 *versus* 7.3 months in patients with or without induction of fetal hemoglobin, respectively [hazard ratio=0.2 (95% confidence interval: 0.1-0.9); P=0.03]. *In vitro* decitabine treatment of two bi-potential myeloid leukemia cell lines (K562 and HEL) resulted in induction of an erythroid (not megakaryocytic) differentiation program, and of fetal hemoglobin mRNA and protein, associated with GATA1 gene demethylation and upregulation. In conclusion, fetal hemoglobin may provide a useful dynamic biomarker during hypomethylating agent therapy in patients with myelodysplastic syndrome or acute myeloid leukemia.

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Introduction

DNA-hypomethylating agents (HMAs) alter gene expression of malignant cells by gene reactivation, e.g. of epigenetically silenced tumor suppressor genes resulting in induction of apoptosis or senescence,[1-4] or of cancer/testis antigens and retroviral sequences, thus eliciting an immune response against cancer cells,[5,6] and by reduction of oncogene overexpression.[7] In cell line models of acute myeloid leukemia (AML), they also induce granulocytic maturation although this effect has yet to be validated *in vivo*.[8]

Probably the earliest proof of therapeutic gene reactivation by an HMA was the demonstration of induction of fetal hemoglobin (HbF) in a patient with severe beta-thalassemia through demethylation and transcriptional activation of the gamma-globin gene locus,[9] the developmental regulation of which is governed by DNA methylation. This ability of HMAs to de-repress epigenetically silenced gamma-globin expression in (polyclonal) erythropoietic precursors has also been observed in patients with solid tumors[10,11] and has prompted clinical trials on the use of HMAs in hemoglobinopathies.[12,13]

Several groups have shown that in patients with myelodysplastic syndrome (MDS) or AML receiving azacitidine, HbF is induced and may be a marker of treatment effect.[14,15] Very recently, we observed elevated HbF levels also in MDS/AML patients treated with decitabine.[16] We now dissected the kinetics of the *in vivo* induction of HbF in these patients with high-risk MDS or AML, in order to evaluate the prognostic value of HbF induction. We observed that HbF levels after two courses of treatment were of prognostic value regarding hematologic responses of the different lineages, and, in MDS, for overall survival from this time point on [hazard ratio (HR)=0.2; 95% confidence interval (95% CI): 0.1-0.9; *P*=0.03]. The cellular source of HbF in responders appeared to be predominantly non-malignant erythroid precursors. To model HbF reactivation *in vitro*, we investigated two transformed leukemia cell lines for the induction of an erythroid differentiation program that also encompassed HbF protein synthesis induced by decitabine.

Methods

Patients, treatments and response evaluations

Details on the patients, the treatments given and evaluation of responses are provided in the *Online Supplement*. Both the therapeutic studies and the translational investigations were approved by the institutional review board (ethics committee) of the University of Freiburg Medical Center. Patients (all treated at the Freiburg study site) provided their written informed consent to be included in the respective clinical studies, and to related translational investigations according to the Declaration of Helsinki.

Hemoglobin quantification by high-performance liquid chromatography

HbF levels were measured by high-performance liquid chromatography (HPLC) before treatment and after the end of each treatment course (every 6 weeks) as described previously.[17] Patients were grouped by normal (HbF ≤1%) or elevated HbF levels (HbF >1%), in accordance with reference values for the University of Freiburg Medical Center Central Laboratory.

Statistical methods

We used non-parametric Spearman correlation coefficients (r_s) to assess the association between HbF levels and other variables. To address the hypothesis that HbF after course 2 of decitabine treatment may predict hematologic responses after course 4, we employed linear regression models with HbF levels as the independent variable, and platelet count, hemoglobin concentration, neutrophil counts, and bone marrow blasts as dependent variables. Results are displayed graphically. The evaluation of possible outliers was based on studentized residuals.[18] No potential outliers were excluded from the statistical analyses. The HbF value distributions between responders and non-responders were compared using the Wilcoxon two-sample test. Before-after differences in binary variables such as elevated HbF (yes/no) were assessed statically using the McNemar test.

Time-to-event endpoints comprised overall survival, progression-free survival, and AML-free survival. We performed landmark analyses starting at the time of HbF measurements after two cycles of decitabine treatment in order to avoid the so-called immortal-time bias.[19] Thus, they were relevant only for patients who reached this time point. The Kaplan-Meier method was used to estimate distributions of overall, progression-free and AML-free survival according to HbF value (> *versus* ≤1%), and to compute median estimations. A log-rank test was used to assess the prognostic importance of HbF value, and a Cox model to estimate the hazard ratio (HR) and its 95% confidence interval (95% CI).

Statistical analyses (all conducted at the CTU Freiburg, Germany) were performed with SAS 9.2 (SAS Institute Inc., Cary, NC, USA), and IBM SPSS Statistics 22.

Cell lines and *in vitro* treatment

K562 and HEL cells (DMSZ, Braunschweig, Germany) were cultured in RPMI1640 containing 10% heat-inactivated fetal calf serum. Cells were treated with three 24-h pulses of 100 and 20 nM decitabine (Sigma Aldrich) and harvested 144 h after the first treatment, as described previously.[20] Hemin (Sigma Aldrich), a positive control for induction of erythroid differentiation,[21] was added to the culture medium at a final concentration of 50 μM.[22] Cell viability was tested by 0.4% trypan blue staining. Megakaryocytic differentiation was induced by exposure to 5 nM phorbol ester (PMA, Sigma Aldrich) for 48 h, as described previously.[23] .

Further information on the methods, including transcriptome profiling, immunoblotting, and methyl-CpG immunoprecipitation sequencing, is provided in the *Online Supplement.*

Results

Kinetics of *in vivo* induction of HbF in patients with myelodysplastic syndrome/acute myeloid leukemia receiving decitabine

In 40 patients (15 with higher-risk MDS, 25 with AML), HbF was measured serially twice or more during treatment (in a single patient, HbF before treatment was not available). The patients' characteristics and those of their diseases are presented in Table 1.

When analyzing HbF kinetics over time (Figure 1A, *Online Supplementary Figure S1*), the first on-treatment time point with robust HbF induction was at the end of the second course of decitabine treatment. Compared to the median pre-treatment HbF of 0.8%, we observed overall induction to 1.1% after course 2. The median fold-change of HbF concentration at the end of the second course of decitabine treatment as compared to pre-treatment levels was 1.67 (*P*=0.088).

By grouping patients according to pre-treatment HbF levels (normal *versus* elevated) and subsequent HbF levels (normal *versus* increased), four different patterns could be discerned. In patients with normal pre-treatment HbF, HbF was either induced to levels above the upper limit of normal (group A, n=11) or remained within the normal range (group B, n=12); among patients with elevated pre-treatment levels, HbF either remained elevated also in subsequent measurements (group C, n=13, in most cases after an initial drop following the first treatment course) or dropped to normal levels, without subsequent elevation above the upper limit of normal (group D, n=3). Two vignettes representative of each of the four different patterns of HbF kinetics are shown in Figure 1B. The number of treatment courses varied between these four groups: median numbers were 5, 2, 7 and 3 courses in groups A, B, C and D, respectively (range: 2-11, 2-6, 2-23, and 2-6 courses, respectively). Consequently, more patients in groups A and C attained hematologic responses over time, including complete remission (with suppression of the abnormal clone in patients with initial cytogenetic abnormalities), compared to patients in groups B and D. HbF levels were elevated in significantly more patients during treatment (groups A+C: n=24, 61.5%) than prior to treatment (groups C+D: n=16, 41.0%; *P*=0.033).

Early HbF induction by decitabine has predictive value for subsequent hematologic stabilization and response

Among patients achieving complete or partial remission as their best overall response, a median HbF of 1.9% (1.9% in MDS, 2.0% in AML) was observed at this time point compared to a median of 0.8% (1.0% and 0.6%, respectively) in patients not attaining complete or partial remission (*P*=0.015) (Figure 1C, *Online Supplementary Figure S2*).

Given that robust HbF induction during decitabine treatment was first observed after two cycles of treatment, we chose this time point for further analyses regarding the potential value of HbF induction for predicting subsequent responses. Using linear regression models and correlation analyses, we assessed the association between HbF levels after course 2 of decitabine treatment and responses in the different hematopoietic lineages after course 4 in all 40 MDS/AML patients (3 patients did not receive more than 2 courses, but had peripheral blood counts available 3 months after course 2: one underwent hematopoietic stem cell transplantation, one changed to a different study after course 2, and one received thalidomide and best supportive care at that point).

Higher HbF levels after two courses were associated with significantly higher platelet counts after four courses (r_s=0.49, *P*=0.01) (Figure 2A). As for neutrophil counts, a trend towards higher counts after course 4 in patients with elevated HbF levels after course 2 was noted (r_s=0.35, *P*=0.08) (Figure 2B). A borderline significant association was also observed between HbF levels after two courses of treatment and hemoglobin levels after four courses (r_s=0.36, *P*=0.08) (Figure 2C). Interrogating the potential prognostic value of HbF induction for bone marrow blast suppression after four courses in the entire cohort of 21 MDS/AML patients with available blast counts, an association was noted (r_s=-0.48, *P*=0.03) (Figure 2D). When looking at MDS and AML patients separately, the overall results were similar in both cohorts, particularly for blast suppression (*Online Supplementary Figure S4A-D*). Moreover, similar results were observed when restricting the same kind of analysis to MDS and AML patients who had received more than four courses of decitabine (*data not shown*).

In patients achieving platelet doubling already after one course of decitabine, the median HbF after course 2 was 1.9%, *versus* 0.8% in patients without this platelet response (*P*=0.006) (Figure 2E). In contrast, no correlation was observed between HbF levels and platelet counts before treatment initiation: r_s=-0.13 (*P*=0.63) in MDS (n=16) and r_s=0.02 (*P*=0.91) in AML patients (n=36).

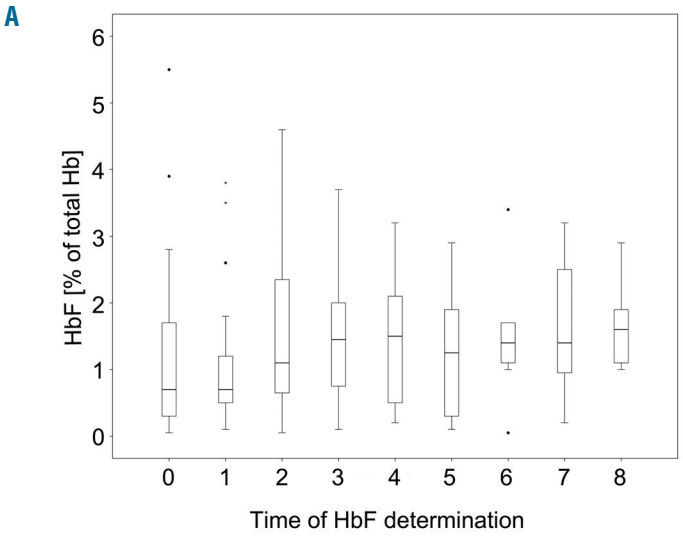
Association of HbF induction by decitabine and survival outcomes in patients with myelodysplastic syndrome/acute myeloid leukemia

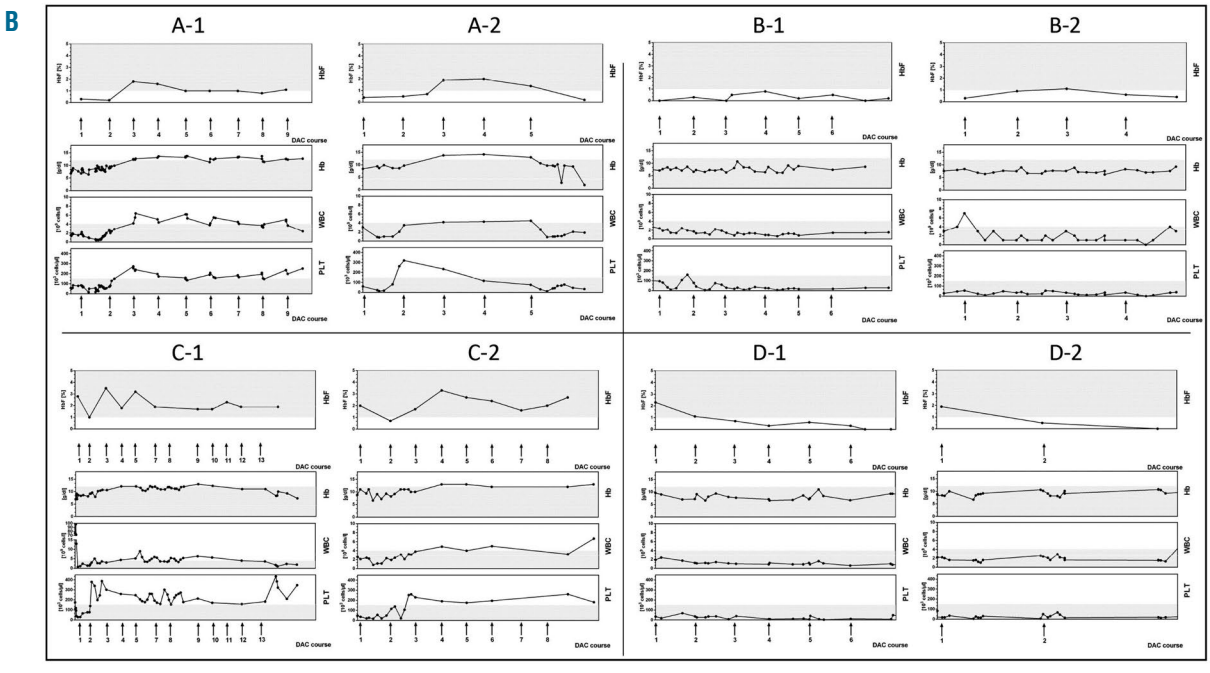
Overall, progression-free and AML-free survival were measured from the time of HbF determination after completion of two courses of decitabine treatment. In the MDS cohort, overall survival was significantly longer in the nine patients in whom HbF was elevated above the upper limit of normal at that time point than in the six patients with HbF in the normal range: median 22.9 *versus* 7.3 months, respectively (HR=0.21; 95% CI: 0.05-0.87; *P*=0.03) (Figure 3A). Censoring the six patients who had

Table 1. Characteristics of MDS and AML patients and their disease at baseline.

	MDS	AML
Age (years; median and range)	74 (66 - 77)	73 (62 - 82)
Sex		
Male	8	15
Female	7	10
FAB subtype		
	RA: 1 RAEB: 8 RAEB-t: 4 CMMoL: 2	M1: 4 M2: 2 M4: 5 M5: 1 M6: 3 N/A: 10
WBC (x 10^9/L; median and range)	2.4 (0.8 - 18.8)	2.2 (0.5 - 27.1)
ANC (x 10^9/L; median and range)	0.93 (0.16 - 2.40)	0.33 (0 - 14.09)
Peripheral blood blasts (%; median and range)	1 (0 - 13)	19 (0 - 96)
Bone marrow blasts (%; median and range)	18 (2 - 30)	55 (10 - 95)
Hb (g/dL; median and range)	8.4 (5.0 - 11.3)	8.7 (5.6 - 12.4)
PLT (x 10^9/L; median and range)	35 (10 - 151)	34 (7 - 229)
sLDH (U/l; median and range)	235 (102 - 842)	271 (134 - 1276)
Cytogenetics		
	Normal: 3 Sole 5q-: 1 Sole -7: 1 Complex: 6 No metaphases: 4	Intermediate risk: 7 Poor risk: 6 < 10 normal metaphases: 4 No metaphases: 1 N/A: 7

MDS: myelodysplastic syndrome; AML: acute myeloid leukemia. RA, refractory anemia; RAEB: refractory anemia with blast excess; RAEB-t: refractory anemia with blast excess in transformation (i.e. AML according to the World Health Organization classification); CMMoL: chronic myelomonocytic leukemia. FAB (French-American-British) subtypes: M1: acute myeloblastic leukemia with minimal maturation; M2: acute myeloblastic leukemia with maturation; M4: acute myelomonocytic leukemia; M5: acute monocytic leukemia; M6: acute erythroid leukemia. N/A: not assessed; WBC: white blood cells; ANC: absolute neutrophil counts; Hb: hemoglobin; PLT: platelets; sLDH: serum lactate dehydrogenase.





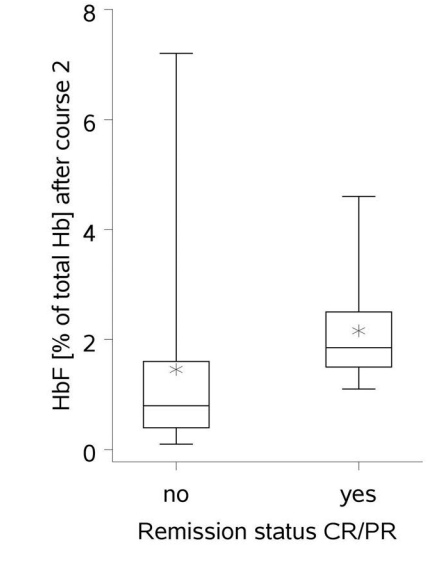


Figure 1. HbF kinetics in patients with myelodysplastic syndrome/acute myeloid leukemia during treatment with decitabine. (A) Box plot of HbF values prior to treatment with decitabine and after each of the first eight treatment courses in patients with myelodysplastic syndrome (n=15) or acute myeloid leukemia (n=25) for whom there was more than one measurement of HbF level. HbF was measured prior to treatment (= cycle 0) in 39 of these patients, after courses 1 to 8 (= cycles 1, 2, 3, etc.) in 38, 32, 24, 18, 14, 10, 7, and 9 of these patients, respectively. Outliers are plotted as individual points, extreme values as stars. For each of cycles 0, 1, and 3, one extreme value is not displayed in the diagram, and one outlier is not shown for cycle 2. (B) Two representative vignettes of each of the four groups of HbF responses to decitabine treatment are shown: initially normal HbF induced to levels above (group A) or remaining within the normal range during treatment (group B); already elevated pre-treatment HbF remaining elevated (group C) or decreasing to normal levels (group D). Shaded areas within the charts indicate values outside the normal range. The end of each decitabine treatment course is indicated with a numbered arrow. Hb: hemoglobin; HbF: fetal hemoglobin; PLT: platelets; WBC: white blood cells. (C) Box plot of HbF values at the end of the second course of decitabine treatment in 32 MDS/AML patients showing higher HbF levels in patients achieving complete or partial remission (CR, PR) as best overall response (right) compared to patients who did not attain CR/PR (left).

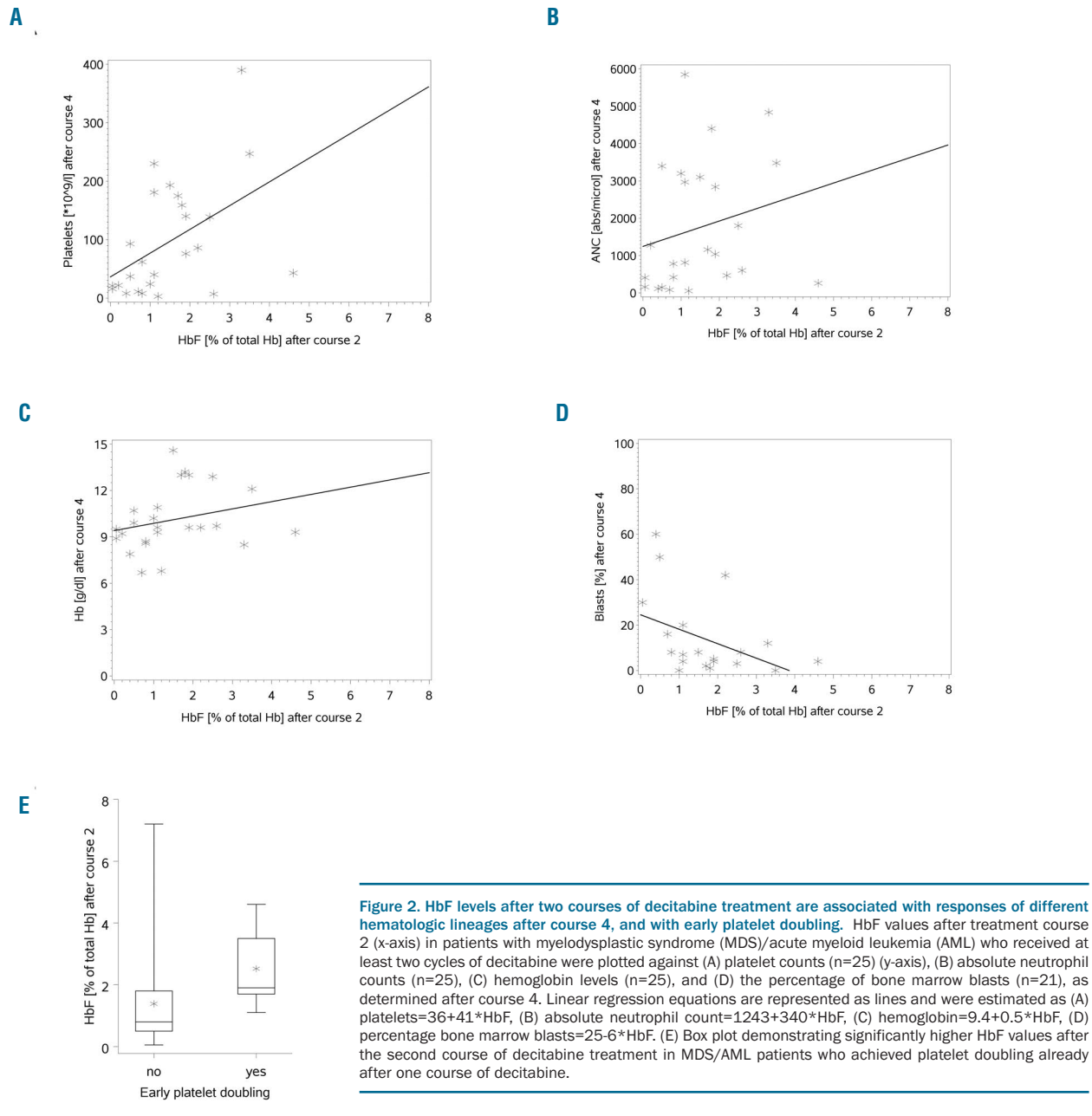


Figure 2. HbF levels after two courses of decitabine treatment are associated with responses of different hematologic lineages after course 4, and with early platelet doubling. HbF values after treatment course 2 (x-axis) in patients with myelodysplastic syndrome (MDS)/acute myeloid leukemia (AML) who received at least two cycles of decitabine were plotted against (A) platelet counts (n=25) (y-axis), (B) absolute neutrophil counts (n=25), (C) hemoglobin levels (n=25), and (D) the percentage of bone marrow blasts (n=21), as determined after course 4. Linear regression equations are represented as lines and were estimated as (A) platelets=36+41*HbF, (B) absolute neutrophil count=1243+340*HbF, (C) hemoglobin=9.4+0.5*HbF, (D) percentage bone marrow blasts=25-6*HbF. (E) Box plot demonstrating significantly higher HbF values after the second course of decitabine treatment in MDS/AML patients who achieved platelet doubling already after one course of decitabine.

undergone hematopoietic stem cell transplantation at the time of their transplant, this difference lost statistical significance (P=0.098) (*Online Supplementary Figure S6*). The secondary endpoints, progression-free survival and AML-free survival were also investigated and showed a trend to a more favorable outcome for patients with elevated HbF: the median progression-free survival was 7.7 months *versus* 2.4 months (HR=0.32; 95% CI: 0.10-1.10; P=0.07) (Figure 3B) and the median AML-free survival was 13.1 months *versus* 7.6 months (HR=0.42; 95% CI: 0.13-1.38; P=0.15) (Figure 3C).

In the AML cohort, 17 patients had HbF determinations available after two courses of treatment and could be included in a survival analysis. There was no significant difference in overall survival between those with elevated HbF (n=9) and those with normal HbF (n=8): the median overall survival was 17.3 and 11.6 months, respectively (HR=0.68; 95% CI: 0.23-1.96; P=0.47) (Figure 3D).

Elevated HbF levels are observed in decitabine-treated patients with myelodysplastic syndrome/acute myeloid leukemia with bone marrow blast clearance, and decline at relapse

To investigate whether malignant cells or normal, emerging erythroid precursors synthesize the elevated HbF in response to HMAs, linear regression analyses were performed (Figure 4A). This showed an association between HbF induction after four treatment courses and a decreasing percentage of blasts at this time point in 17 MDS/AML patients.

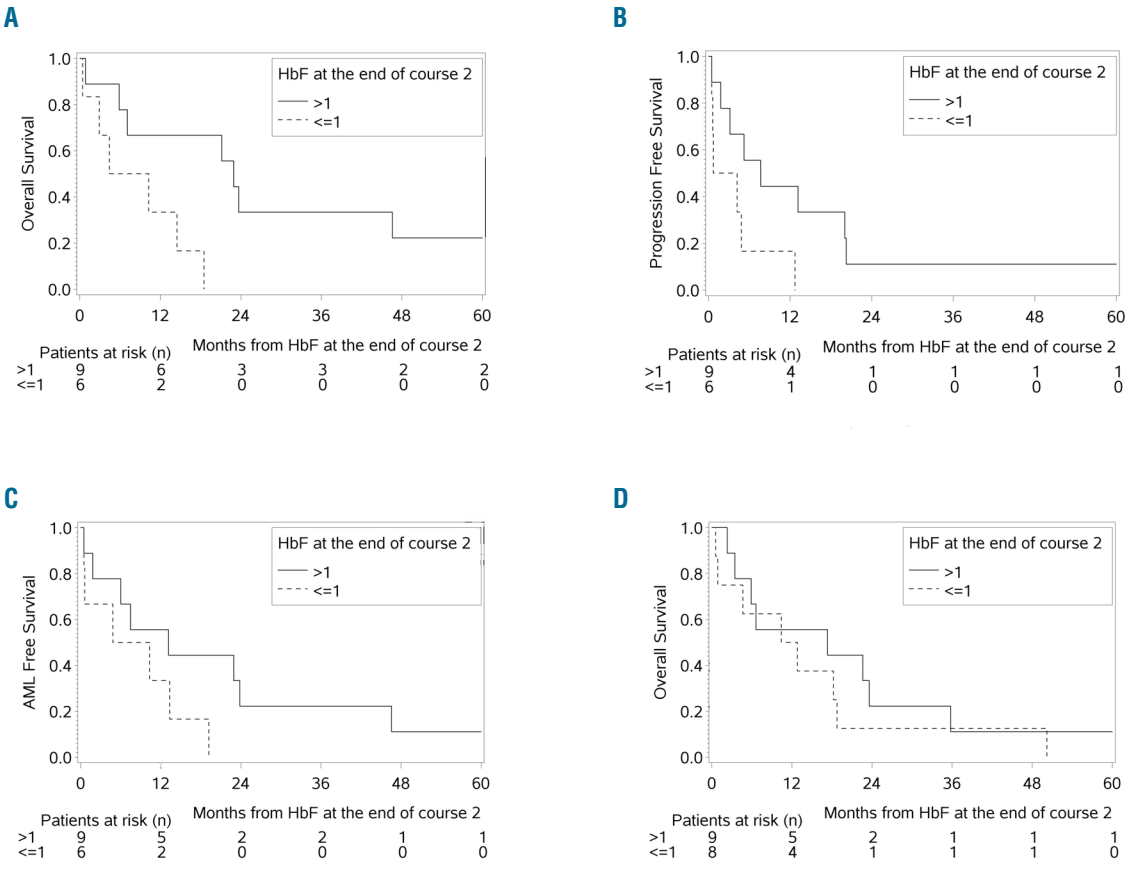


Figure 3. Elevated HbF after course 2 of decitabine treatment is associated with improved survival in patients with myelodysplastic syndrome. Kaplan-Meier survival estimates were determined according to whether HbF after course 2 of decitabine treatment was in the normal range (i.e. 0-1.0% of total hemoglobin) or elevated (i.e. >1.0%). (A) Overall survival of 15 patients with myelodysplastic syndrome (MDS), two in the group with elevated HbF censored at 100 months (still alive in remission following allografting). (B) Progression-free survival of 15 MDS patients, one in the group with elevated HbF censored (still alive in remission following allografting). (C) Acute myeloid leukemia (AML)-free survival of 15 MDS patients, one in the group with elevated HbF censored (still alive in remission following allografting). (D) Overall survival of 17 AML patients, one in the group with elevated HbF censored (still alive in remission following allografting).

Looking in more detail at the subgroup of 18 patients who received more than four courses of treatment, it was noted that six attained not only a multilineage hematologic response but also bone marrow blast clearance. Interestingly, in all of them HbF levels were, or became, elevated at the time of remission. In four of these patients, the complete remission status could be confirmed by cytogenetics: chromosomal abnormalities present at baseline were no longer detectable at that point (cytogenetic remission; in several of these patients, suppression of the abnormal clone was confirmed by fluorescence *in situ* hybridization).

Taken together, these analyses strongly suggested that HbF induction observed after four courses of treatment occurred preferentially in the emerging non-clonal erythroid cells, in which epigenetically silenced beta-globin-like genes were reactivated by treatment with the HMA.

For seven MDS/AML patients, HbF measurements were obtained both after the end of decitabine course 2 and at the time of hematologic relapse. Relative to an elevated HbF level after course 2 (median 1.9%; range, 1.5-4.6%), a decrease was observed in all seven patients at the time of relapse (median 1.1%; range, 0.2-1.9%). In two of these patients (both of whom had a complex karyotype), the decline in HbF level preceded the hematologic relapse, implicating it as a potential early predictor of relapse in a subgroup of patients. Notably, the initial increase after course 2 compared to pre-treatment HbF levels (available for 6 patients: median 1.2%; range, 0.3-3.9%) became reversed at relapse in five of the six patients (Figure 4B).

Decitabine triggers an erythroid but not a megakaryocytic maturation program in bi-potential myeloid leukemia cells

To model the effects of decitabine *in vitro*, two myeloblastic cell lines (K562, HEL) with bi-lineage differentiation potential were treated with decitabine at non-toxic concentrations. Striking morphological changes included polyploidy and cytoplasmic maturation indicative of partial erythroid differentiation (Figure 5A), confirmed by benzidine staining in K562 cells: decitabine- and hemin- but not PMA-treated cells became hemoglobin-positive (Figure 5B). K562 cells treated with PMA developed morphological changes indicative of megakaryocytic differentiation and induction of surface CD41/61 whereas lack of CD41 detection by FACS analysis after decitabine

and hemin indicated absence of megakaryocytic differentiation (Figure 5C).

Transcriptome profiling by mRNA expression arrays in K562 cells revealed the greatest similarity between hemin- and decitabine-treated cells (Figure 6A,B). Notably, transcriptome changes of PMA-treated K562 cells were most extensive and clustered most distantly from those of all other treatments (Figure 6A,B). In HEL cells, variable probes also differed considerably between decitabine and PMA treatment (*Online Supplementary Figures S8* and *S9*). Gene ontology analyses of upregulated transcripts consistently identified terms related to erythropoiesis and iron metabolism among the top regulated transcript groups in decitabine-treated cells whereas terms related to megakaryocyte lineage differentiation did not show significance (*data not shown*).

Among the transcripts upregulated by decitabine, almost all major globin genes could be identified (Figure 6C). Decitabine-treated K562 showed a >7-fold induction of alpha-1/alpha-2-globin transcription whereas PMA treatment did not alter alpha-globin transcription. Gamma-globin transcription was induced by all three treatments, with decitabine exhibiting the most pronounced effect. A strong induction was also noted for zeta-globin transcription upon decitabine and hemin, but not PMA, treatment. Similar patterns were noted for HEL (*data not shown*).

To prove induction of functionally competent HbF molecules in K562 cells by decitabine treatment, we quantified hemoglobin tetramers by cation-exchange chromatography. In two independent experiments, a reproducible time- and dose-dependent (albeit modest) induction of HbF tetramers was noted (Figure 6D).

Decitabine-induced globin transcription in K562 cells is associated with *GATA1* gene demethylation and upregulation

To determine whether transcriptional upregulation of both the hemoglobin gamma 1 (*HBG1*) and 2 (*HBG2*) gene was a consequence of direct DNA methylation changes at the locus control region or at direct upstream regulatory regions of both genes, DNA methylation was assessed globally by methyl-CpG immunoprecipitation, as previously described.[24] No enrichment could be demonstrated in untreated or in decitabine-treated K562 cells, indicating absence or only very low levels of DNA methylation at the *HBG1* and *HBG2* promoter and at the locus control region (*Online Supplementary Figure S10*). The gamma-globin locus is known to be devoid of a CpG island,[25] and was shown to be unmethylated when selected CpG sites were interrogated for their methylation status by using methylation-sensitive restriction enzymes.[26]

The presence of activating H3K4me3 and H3K9ac histone marks (from K562 data sets of the ENCODE consortium[27]) and absence of the repressive mark H3K27me3 (*data not shown*) indicated that transcriptional activity was already present in untreated cells at both loci, which could be demonstrated by the elevated *HBG1/HBG2* probe intensity in the mRNA expression array data (Figure 6C).

GATA1 has been identified as a key regulatory factor which binds prominently to the locus control region of the beta-globin locus (*Online Supplementary Figure S10*). In order to understand how HMA treatment might contribute to the transcriptional upregulation of *HBG1* and *HBG2* without altering pre-existing low levels of local DNA methylation in K562 cells, we assessed DNA methylation at the *GATA1* gene locus. Upon decitabine treatment, DNA methylation was substantially reduced in K562 cells at the GATA1 promoter region and at an upstream regulatory region (Figure 7A). Western blot analysis of K562 cells after 3 days of decitabine treatment demonstrated a dose-dependent, 2- to 3-fold upregulation of GATA1 protein at day 3 (Figure 7B). After 6 days of treatment, this effect was less pronounced. In contrast, PMA treatment strongly repressed GATA1 expression, an effect only modestly antagonized by decitabine.

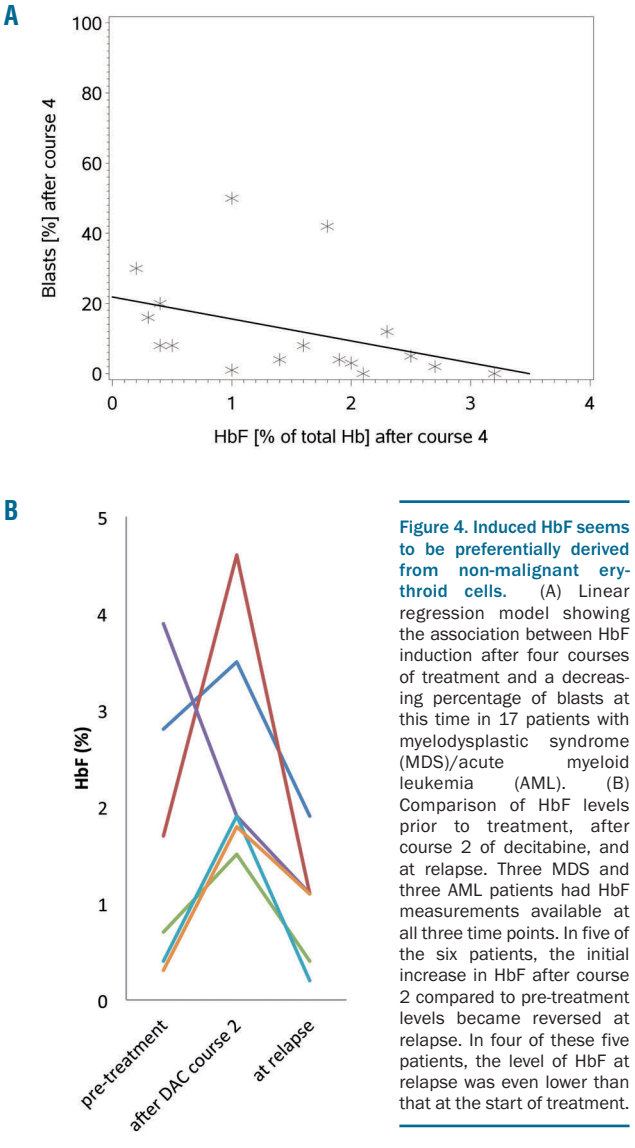


Figure 4. Induced HbF seems to be preferentially derived from non-malignant erythroid cells. (A) Linear regression model showing the association between HbF induction after four courses of treatment and a decreasing percentage of blasts at this time in 17 patients with myelodysplastic syndrome (MDS)/acute myeloid leukemia (AML). (B) Comparison of HbF levels prior to treatment, after course 2 of decitabine, and at relapse. Three MDS and three AML patients had HbF measurements available at all three time points. In five of the six patients, the initial increase in HbF after course 2 compared to pre-treatment levels became reversed at relapse. In four of these five patients, the level of HbF at relapse was even lower than that at the start of treatment.

Discussion

Clinically meaningful pharmacological induction of developmentally silenced HbF gene expression[9,28] is a prime example of what has been termed epigenetic therapy. However, the broad clinical application of this approach in hemoglobinopathies has been limited by concerns of long-term mutagenic effects in these chronic disorders. The recent advent of DNA-hypomethylating treatment using azanucleoside DNA methyltransferase

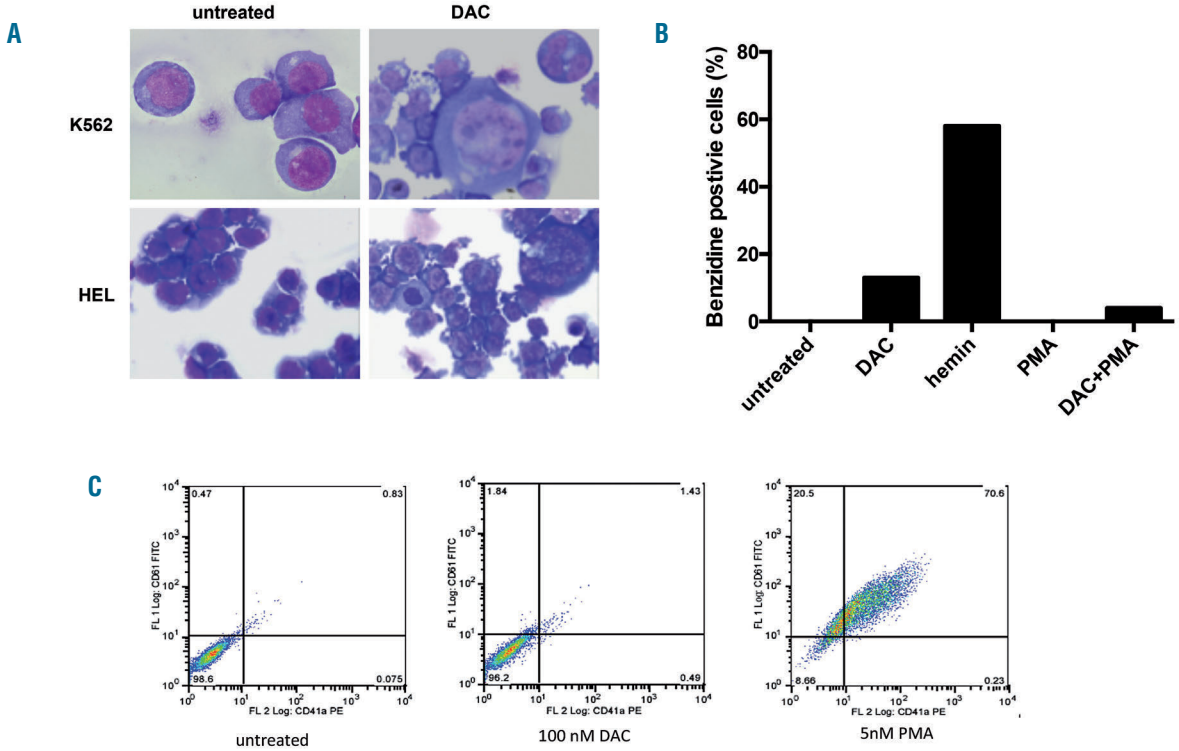


Figure 5. Decitabine induces erythroid differentiation and hemoglobin synthesis in K562 and HEL early myeloid progenitor cells. (A) K562 and HEL cells were treated with three 24 h pulses of 100 nM decitabine. Morphological signs of erythroid differentiation upon decitabine (DAC) treatment were determined by cytospin preparation and May-Grünwald staining. (B) Hemoglobin synthesis in K562 cells was determined by benzidine staining. Cells were treated with DAC (three 24 h pulses, 100 nM), hemin (50 μM for 48 h), phorbol 12-myristate 13-acetate (PMA, 5 nM) and a combination of DAC and PMA at these concentrations. For each experimental point, 300 cells were counted, with blue cells being considered hemoglobin-positive. (C) Flow cytometry analysis of CD41/61 surface markers (highly specific for megakaryocytic differentiation) showed differentiation with PMA but not DAC treatment.

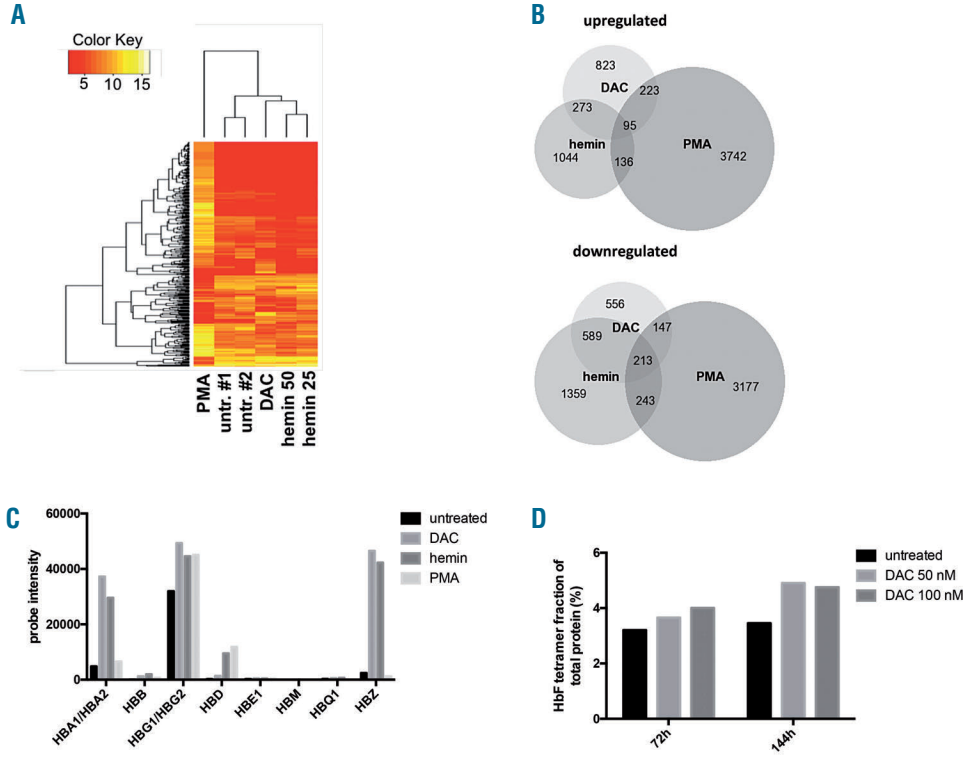


Figure 6. Decitabine treatment induces an erythroid transcriptome signature, globin chain expression and fetal hemoglobin assembly in K562 cells. (A) Transcriptome analyses were performed in K562 cells upon treatment (as described in the legend to Figure 5) with decitabine (DAC, 100 nM), hemin (25 and 50 μM), phorbol 12-myristate 13-acetate PMA (PMA, 5 nM) or without treatment (untreated #1 – no treatment, untreated #2 – phosphate-buffered saline as a vehicle control) using mRNA microarrays (HG-U133plus 2.0 gene chip, Affymetrix). The most variable probes (relative standard deviation among all samples ≥2) are displayed as a heatmap with unsupervised hierarchical clustering (Euclidian distance). (B) Venn diagram displaying unique and shared ≥2-fold upregulated and ≥2-fold downregulated probes between the three treatments in K562 cells. (C) Relative mean probe intensities representing expression of globin chain transcripts: HBA1/HBA2, alpha-globin 1 and 2; HBB, beta-globin; HBG1/HBG2, gamma-globin 1 (G-gamma) and 2 (A-gamma); HBD, delta-globin; HBE1, epsilon-globin 1; HBM, mu-globin; HBQ1, theta-globin, HBZ, zeta-globin) in untreated K562 cells and upon DAC, hemin and PMA treatment, respectively. (D) Increase of functional HbF tetramers determined by high-performance liquid chromatography as a fraction of total cellular protein upon DAC treatment (50 and 100 nM) after 72 and 144 h, respectively. Note the time-dependent incremental rise in HbF (dose-dependent at 72 h).

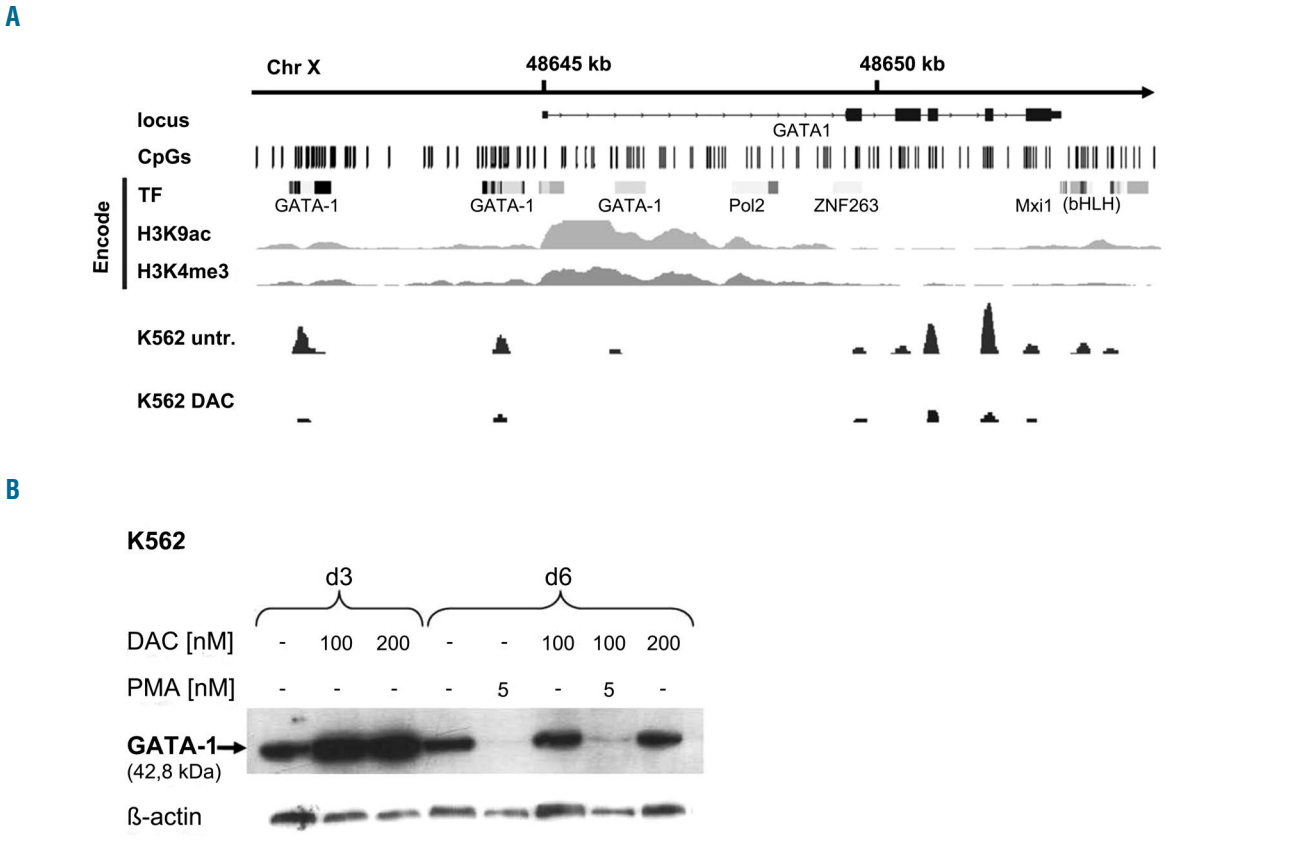


Figure 7. Decitabine induces gene demethylation and expression of the erythroid-specific transcription factor GATA1 in K562 cells. (A) Schematic representation of the *GATA1* gene locus. The *GATA1* gene is depicted as a black line from 5' (left) to 3' (right) with exons represented as boxes. CpG density (CpGs) is indicated by vertical bars. Transcription factor (TF) binding sites were determined through combined TF chromatin immunoprecipitation sequencing (ChIP-seq) experiments from different tissues/cell lines published by the ENCODE consortium. Likewise, activating histone marks H3K9ac and H3K4me3 are taken from ChIPseq experiments in untreated K562 cells which were published and made publicly available from the ENCODE consortium. DNA methylation was assessed by enrichment through methyl-CpG immunoprecipitation sequencing experiments in untreated K562 cells (K562 untr.) and in K562 cells treated with decitabine (K562 DAC). (B) K562 cells were treated with three 24 h pulses of 100 nM or 200 nM decitabine and harvested on days 3 and 6. PMA 5 nM was added to the culture media for the final 48 h. Immunoblotting of whole cell lysates with rat anti-GATA1. Immunoblotting with mouse anti-beta-actin was performed to control for lane loading.

inhibitors in MDS and AML has re-kindled an interest in HbF regulation *in vivo*. Specifically, we and others have recently described HbF induction in patients with MDS or AML receiving the DNA methyltransferase inhibitors azacitidine or decitabine.[14-16,29]

Since HMAs have to be administered over repeated treatment cycles in order to induce responses (which then occur only in about half of the patients), early markers predicting subsequent outcome are urgently needed in order to better advise patients whether to continue HMA treatment or switch to alternative therapy.

In order to investigate induction of HbF as a potential predictor of outcome, we chose the time point at the end of two courses of decitabine treatment, i.e. approximately 12 weeks after starting treatment, as the most meaningful: it disclosed the first robust HbF induction (Figure 1A), and was still early enough to be useful for predicting later outcome (whereas information at later time points would be a "self-fulfilling prophecy" regarding response and survival). Indeed, higher HbF at this time point was associated with overall objective responses and, particularly in MDS patients, with improvement of the different hematopoietic lineages after four courses of treatment. Notably, MDS patients with elevated HbF after two course of decitabine treatment had longer overall survival and trends to longer progression-free and AML-free survival. For AML patients, the median overall survival was also nominally longer in this group, but the difference was not statistically significant (possibly because at that time point, half of the AML patients had already died). Time point optimization in AML patients therefore appears necessary, particularly in view of the presently used decitabine treatment schedules of drug administration 5 or 10 days every 4 weeks. It was encouraging to note that higher HbF levels after two courses of treatment were associated with platelet doubling after one course, one of the few established early dynamic predictors of outcome with HMAs.[30,31]

In order to assess whether these results are applicable in clinical practice, and given the fact that a different decitabine dose is commonly used in MDS and AML patients at present, these results warrant validation in a larger cohort of patients treated with current dosing schemes. For this purpose, serial measurements of HbF have recently been performed in decitabine-treated AML patients randomized within the "inDACtion *versus* induction" Intergroup AML trial 1301 of the EORTC Leukemia Group (NCT02172872).

With the perspective of exploiting HbF as a potential biomarker for response to HMA therapy, it might be used for selecting a cohort of MDS/AML patients who exhibit a significant increase in HbF after two cycles of decitabine treatment as candidates for a future clinical trial. Such a trial could have the objective to increase the effectiveness of HMA therapy by dose-schedule modifications and/or in combination with other epigenetic or other agents to increase the duration of response.

In practical terms, HbF measurement during HMA treatment needs to be compared in different laboratories (at this study's central laboratory, HPLC quantification of different rare Hb species, including HbF, has been optimized for linear quantification also of smaller amounts). However, baseline HbF is also a promising predictor of HMA response and outcome, as recently demonstrated in the same cohorts of patients.[16] At first sight it appears counter-intuitive that pre-treatment high expression may be biologically linked to outcome. However, as already discussed,[16] elevated pre-treatment HbF levels may reflect incomplete silencing by methylation (and possibly other epigenetic mechanisms) of the gamma-globin locus, providing a potential surrogate marker for higher *de novo* sensitivity to HMAs. Interestingly, Cross *et al.*[32] made a similar observation studying long interspersed element (LINE)-1 methylation in AML patients treated with azacitidine: lower LINE-1 methylation prior to treatment, but not early hypomethylation under treatment, predicted hematologic response to this *in vivo* hypomethylation.

What is the cellular source of HbF during the different treatment phases in MDS and AML patients receiving HMAs? In untreated MDS patients clonal erythroid progenitors,[33] and in untreated AML patients residual normal, non-clonal erythroid progenitors may be the source of HbF-containing erythrocytes.[34] In contrast, in patients attaining a complete or cytogenetic remission, it is more likely that the target cell of HMAs (presumably achieving hypomethylation and transcriptional de-repression of the beta-globin-like gene locus) is part of the non-clonal erythropoiesis, as in patients with solid tumors who show HbF induction during HMA treatment.[10,11] The increase in platelet count after decitabine treatment indicates that decitabine reduced or eliminated the suppressive action of the malignant cells on normal hematopoiesis, with subsequent expansion of normal hematopoietic stem cells, which undergo differentiation. At the time of hematologic relapse, the decline in HbF levels might be due to the recurrence of the malignant clone. This contrasts with the model of increased HbF levels in juvenile myelomonocytic leukemia resulting from epigenetic dysregulation of beta-like-globin genes in leukemic cells.[35] Serial immunohistochemical bone marrow studies for HbF expression in MDS/AML patients receiving HMAs are warranted to determine the cell of origin of HbF production during the different phases of treatment.

Modeling the effects of decitabine on the erythroid *versus* megakaryocytic lineage in two bi-potential myeloid cell lines, activation of an erythroid but not megakaryocytic gene expression program was observed, including induction of gamma-globin expression and induction, albeit modest, of HbF tetramer formation. We could demonstrate that decitabine treatment regulated many of the genes also regulated by hemin (including mRNA for erythroid-specific transcription factors and beta-like-globin genes), and induced GATA1 at the protein level, concomitantly with demethylation at several *cis*-regulatory regions known to be important for the regulation of this gene.[36,37] Notably, the overlap between the transcriptome changes induced by decitabine *versus* hemin was more marked in the downregulated genes compared to the upregulated ones. Despite demonstrating *GATA1* gene demethylation following decitabine treatment, we are unable to conclude that GATA1 induction is a direct consequence of demethylation or is occurring during erythroid differentiation triggered via other factors. Here, a similar DNA methylation analysis of K562 cells treated with hemin instead of decitabine would address this "cause or consequence" question.

Taken together, the cell line experiments suggested that increased levels of HbF can also occur because of the effects of decitabine on malignant cells of the erythroid lineage. However, since a cell line is not a good model of the normal functional hematopoietic hierarchy, no conclusion can be drawn as to why erythroid rather than megakaryocytic differentiation was observed.

Is there a clinical relevance of induction of HbF beyond HMA treatment? Very recently, in a preclinical study a novel, specific inhibitor of histone deacetylase 1/2 also demonstrated a strong propensity to induce HbF.[38] Furthermore, it is well established that inhibitors of the first histone lysine-specific demethylase (LSD)1, including novel, highly specific LSD1/KDM1A inhibitors such as RN-1, are able to reactivate a silenced beta-globin-like gene locus.[39] Furthermore, UNC0638, a selective inhibitor of the histone methyltransferases EHMT1 and EHM2, has the ability to induce HbF expression, and this potency is enhanced when the drug is combined with decitabine or the histone deacetylase inhibitor entinostat.[40] Very recently, pomalidomide was also shown to be able to induce HbF in patients with multiple myeloma.[41] Thus, serial HbF measurements in these different clinical settings may be interesting in order to determine whether the kinetics of this parameter is predictive of treatment response.

In conclusion, the technically simple test of assaying HbF levels warrants further prospective studies since the time to best response in patients treated with HMAs is often in the range of 4-6 months. Earlier tailoring of treatment is, therefore, highly desirable. It will be of interest to determine the predictive value of HbF levels compared to other, already established predictors of HMA response such as hematologic parameters, genetic and DNA methylation markers.

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