MicroRNA-34b promoter hypermethylation induces CREB overexpression and contributes to myeloid transformation

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ABSTRACT

MicroRNA-34b down-regulation in acute myeloid leukemia was previously shown to induce CREB overexpression, thereby causing leukemia proliferation *in vitro* and *in vivo*. The role of microRNA-34b and CREB in patients with myeloid malignancies has never been evaluated. We examined microRNA-34b expression and the methylation status of its promoter in cells from patients diagnosed with myeloid malignancies. We used gene expression profiling to identify signatures of myeloid transformation. We established that microRNA-34b has suppressor ability and that CREB has oncogenic potential in primary bone marrow cell cultures and *in vivo*. MicroRNA-34b was found to be up-regulated in pediatric patients with juvenile myelomonocytic leukemia (n=17) and myelodysplastic syndromes (n=28), but was down-regulated in acute myeloid leukemia patients at diagnosis (n=112). Our results showed that hypermethylation of the microRNA-34b promoter occurred in 66% of cases of acute myeloid leukemia explaining the low microRNA-34b levels and CREB overexpression, whereas preleukemic myelodysplastic syndromes and juvenile myelomonocytic leukemia were not associated with hypermethylation or CREB overexpression. In paired samples taken from the same patients when they had myelodysplastic syndrome and again during the subsequent acute myeloid leukemia, we confirmed microRNA-34b promoter hypermethylation at leukemia onset, with 103 CREB target genes differentially expressed between the two disease stages. This subset of CREB targets was confirmed to associate with high-risk myelodysplastic syndromes in a separate cohort of patients (n=20). Seventy-eight of these 103 CREB targets were also differentially expressed between healthy samples (n=11) and *de novo* acute myeloid leukemia (n=72). Further, low microRNA-34b and high CREB expression levels induced aberrant myelopoiesis through CREB-dependent pathways *in vitro* and *in vivo*. In conclusion, we suggest that microRNA-34b controls CREB expression and contributes to myeloid transformation from both healthy bone marrow and myelodysplastic syndromes. We identified a subset of CREB target genes that represents a novel transcriptional network that may control myeloid transformation.

Introduction

MicroRNA (miRNA or miR) are small non-coding RNA molecules that regulate gene expression at a post-transcriptional level. MiRNA expression patterns are regulated during development and differentiation of the hematopoietic system and have an important role in cell proliferation, apoptosis, differentiation and even tumorigenesis.[1] Various miRNA and their functions have been intensively studied in acute leukemia, but the precise mechanisms controlling their expression and critical targets are largely unknown for the majority of aberrantly expressed miRNA.[2] The discovery of miRNA as a new class of post-transcriptional regulators that act via interactions with their target messenger RNA has revealed an important pathway for controlling gene expression.[3,4]

We previously reported that miR-34b regulates the level of cAMP-response-element-binding protein (CREB) expression in myeloid cell lines by directly binding to its 3'untranslated region.[5] MiR-34b/c has been implicated in the oncogenesis of

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colon,[6] ovarian[7] and oral cancers.[8] Here, we further studied the role of miR-34b in the pathogenesis of myeloid malignancies. One mechanism by which miRNA are frequently silenced in human tumors is aberrant hypermethylation of CpG islands that encompass or lie adjacent to their genes.[9,10] DNA methylation is in fact being increasingly recognized as important in the regulation of normal and tumor cells,[11,12] the latter being characterized by specific hypermethylation of CpG islands in the promoters of tumor suppressor genes, resulting in transcriptional repression and gene inactivation.[1,3,4,9-12]

We identified hypermethylation as a cause of decreased miR-34b levels in leukemia cell lines, which in turn directly controls the expression of the proto-oncogene CREB.[5] CREB is known to regulate a wide range of cellular processes such as growth, proliferation, differentiation and apoptosis. Furthermore, it plays a crucial role in normal[13] and neoplastic hematopoiesis.[14-17] CREB knockdown decreases proliferation and survival of myeloid progenitor cells and regulates the differentiation of committed progenitors.[13] CREB is overexpressed in bone marrow from patients with acute myeloid leukemia (AML) and increases AML cell proliferation *in vitro* and *in vivo*, through up-regulation of specific target genes.[13,15] CREB-overexpressing transgenic mice develop a myeloproliferative neoplasm with splenomegaly but not AML, highlighting a causative role for CREB in myeloid cell transformation.[13] However, the consequences of abnormal expression of both CREB and miR-34b in AML are not well defined, and the role of both CREB and miR-34b in transition from myelodysplastic syndrome (MDS) to AML has not been examined.

In this study, we investigated a cohort of patients with juvenile myelomonocytic leukemia (JMML) and MDS as well as *de novo* AML at diagnosis to study CREB and miR-34b. We examined the role of their abnormal expression in the development of a malignant phenotype using *in vitro* and *in vivo* assays.

Design and Methods

Patients

We studied bone marrow (BM) samples from 112 patients with AML at diagnosis enrolled in the AIEOP-2002 AML pediatric protocol. The cohort of 28 pediatric MDS samples was composed of six cases of refractory cytopenia of childhood (RCC), 22 cases of refractory anemia with excess blasts (RAEB) according to the MDS classification proposed for pediatric patients (aged 0-18 years, age mean 10.26±4.43). Seventeen pediatric patients (aged 0-18 years, mean 2.51±3.76) with JMML, according to differential diagnostic criteria, as previously published, were also included in the study. The criteria for enrollment in the study are described in the *Online Supplementary Design and Methods*.[18,19] DNA and RNA were extracted and analyzed for methylation of the miR-34b promoter and for miR-34b levels. $CD19^{-}CD3^{-}$ from healthy BM (HL-BM) from 17 children (aged 0-18 years, mean 11.86±4.84) were used as control samples in the assessment of miR-34b expression in AML.

RNA isolation and SYBR green real-time polymerase chain reaction analysis

Total RNA was isolated using Trizol (Invitrogen). One microgram (μg) of RNA was reverse-transcribed into cDNA using SuperScript II (Invitrogen) according to the manufacturer's instructions. Real-time polymerase chain reaction (RQ-PCR) analysis was performed with the SYBR Green method (Invitrogen) and analyzed on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).[20] Experiments were carried out in triplicate and gene expression, relative to GUS, was calculated by the comparative ΔΔCt method.[21]

RNA isolation and real-time polymerase chain reaction for microRNA analysis

Ten nanograms (ng) of total RNA were used as the starting material for the stem-loop RQ-PCR method to detect the expression level of mature miR-34b (Applied Biosystems). All PCR were run in triplicate and gene expression, relative to RNU6B, was calculated by the comparative ΔΔCt method.[21]

Methylation analysis

One microgram of genomic DNA, extracted from pediatric samples of 112 AML, 28 MDS, 17 JMML, 3 HL-BM, from sorted $CD34^{+}$ of MDS and HL-BM samples according to the manufacturer's instructions (Gentra Autopure LS, Qiagen), was treated with sodium bisulfite using an EZ DNA Methylation-Gold™ Kit (ZYMO RESEARCH). A methylation-specific (MS) and unmethylation-specific (UMS) PCR reaction to detect the methylation status of miR-34b promoter was performed as previously described by Lujambo *et al.*[22] The PCR amplicon obtained by MS-PCR was sequenced by an ABI PRISM™310 Genetic Analyzer sequencer (Applied Biosystems), as described in the *Online Supplementary Design and Methods*.

Gene expression analysis

BM samples from patients with *de novo* AML at diagnosis (n=72), MDS at diagnosis (n=24), AML that evolved from MDS (n=4), and healthy controls (n=11) were subjected to gene expression analysis. This series of *de novo* AML patients comprised 16 patients with core-binding factor aberrations, 23 with MLL-rearrangements, 30 with a normal karyotype and 3 with a complex karyotype. Patients with promyelocytic AML with t(15;17) were excluded *a priori* from this series as they constitute an independent group. RNA quality was assessed on an Agilent2100 Bioanalyzer (Agilent Technologies). The GeneChip Human Genome U133 Plus 2.0 was used for the microarray experiments, as previously described[23-26] (*Online Supplementary Design and Methods*).

Gene set enrichment analysis

Gene expression profiles obtained from 19 out of 72 AML patients at diagnosis were analyzed by using gene set enrichment analysis *(http://www.broadinstitute.org/gsea/index.jsp)* (see the *Online Supplementary Design and Methods*).

Principal component analysis

We studied gene expression in an independent cohort of 20 samples from MDS patients at diagnosis, selecting the top 103 genes among CREB targets. Principal component analysis was performed using Partek Genomic Suite software.

Primary cell culture and transfection

Primary cell cultures were obtained from BM from healthy donors. $CD34^{+}$ sorted cells from human fetal liver were also used.[27] Cell transfection was performed using a Nucleofector (Amaxa Biosystems) according to the manufacturer's guidelines and efficiency was up to 40%. Cell cycle analysis and colony assays were performed (*Online Supplementary Design and Methods*).

Constructs

pEGFP-N1-ΔGFP-CREB plasmid was obtained by cloning between the NotI and EcoRI cloning site a full length cDNA for CREB into the pEGFP-N1 vector (Invitrogen). Lentiviral constructs

were constructed as previously described by Gentner *et al.*[28,29] (*Online Supplementary Design and Methods*).

Western blot analysis

Western blots were performed as previously described.[30] The antibodies used were anti-actin (Sigma-Aldrich), anti-PU.1, anti-GATA1, anti-MEIS1/2, anti-cMET, anti-cMYB (Santa Cruz Biotechnology), anti-cMYC, anti-CDK6 (GeneTex), anti-CREB, and anti-P-CREB; the horseradish peroxidase–conjugated secondary antibody was goat anti-rabbit or mouse IgG (Upstate Biotechnology).

Flank injection xenograft experiments in NOD-SCID interleukin-2 receptor gamma null mice

Ten NOD-SCID interleukin-2 receptor gamma null (NSG) mice between 6 to 8 weeks of age were injected subcutaneously with 5×10^6 HL60-miR-34b/LUC (or EV/LUC as a control) or K562-miR-34b/LUC (or EV/LUC as a control) cells. All mice were euthanized when tumors reached a volume of 1.5cm^3. The mice were treated according to the NIH Guidelines for Animal Care and as approved by the UCLA Institutional Animal Care and Use Committee.

Xenograft experiments by bioluminescence imaging in NSG mice

Ten mice were injected with 5×10^6 HL60-34b/LUC (or EV/LUC as a control) cells through the tail vein. Mice were imaged (IVIS100 bioluminescence/optical imaging system Xenogen) every week to monitor tumor engraftment and growth from 21 days post-transplant. Three milligrams of D-Luciferin (Xenogen) in phosphate-buffered saline were injected intraperitoneally into each mouse 15 min before measurement [in relative intensity units (RIU) = photons/sec/cm^2]. General anesthesia was induced with isoflurane. RIU for regions of interest were measured in triplicate and averaged.

Data analysis

Statistical analyses were performed using the Mann-Whitney or unpaired two-tailed t test. A P value of <0.05 was considered statistically significant.

Results

MicroRNA-34b promoter is hypermethylated in acute myeloid leukemia

We had already found that miR-34b expression was down-regulated in 78 patients with AML at diagnosis.[15] We enlarged this cohort of patients to 112, confirming the down-regulation compared to levels in HL-BM (RQ_{AML}=0.178, $RQ_{HL\text{-}BM}$=1). We previously correlated this decrease in miR-34b expression with promoter hypermethylation in leukemic cell lines.[15] Here, we investigated miR-34b promoter hypermethylation directly in 112 AML patients. MS-PCR revealed that 74/112 (66%) AML patients had miR-34b promoter hypermethylation (Figure 1A). We sequenced the amplicons and confirmed the presence of CpG islands in the patients with miR-34b promoter hypermethylation, whereas the TG nucleotides at the same position were found in unmethylated cases after bisulfite treatment. The 74 patients with hypermethylation of miR-34b promoter had significantly lower miR-34b expression and higher CREB protein levels, compared to the 38 patients without hypermethylation of miR-34b promoter in whom miR-34b was highly expressed (RQ_{Meth}=0.075, RQ_{Unmeth}=0.373, $P<0.05$) and CREB protein was undetectable by western blot analysis. Gene set enrichment analysis was used to study the gene expression profile in 19 AML patients. The results showed that patients with hypermethylation of miR-34b promoter and, therefore, lowered miR-34b levels had positive enrichment of genes predicted to be miR-34b targets (Figure 1B and *Online Supplementary Figure S1*). These results suggest that hypermethylation of miR-34b promoter is a common feature in pediatric AML. We then studied the event-free survival of the cohort of AML patients (74 with miR-34b promoter hypermethylation *versus* 38 without methylation at same promoter). There was a trend towards lower overall survival in AML patients with methylated miR-34b promoter (69% at 4 years) than in those with unmethylated promoter (61% at 4 year), although the difference was not statistically significant (P=0.34). Still, the methylation of miR-34b promoter might have a negative prognostic significance in AML. There was not a significant independent correlation among clinical and biological parameters with the methylation of miR-34b promoter (*Online Supplementary Figure S2*). These findings indicate that cells from a large group of AML patients (66%) have miR-34b promoter hypermethylation,[15] suggesting that this may be contributing to leukemogenesis.

MicroRNA-34b expression is higher in myelodysplastic syndromes and juvenile myelomonocytic leukemia since microRNA-34b promoter is not hypermethylated

We examined miR-34b expression in other rare pediatric hematopoietic disorders such as MDS (22 RAEB, 6 RCC) and JMML (n=17), which can sometimes transform into AML. MiR-34b expression was higher in patients with RAEB (RQ_{mean}=5.74), RCC (RQ_{mean}=8.79) and JMML (RQ_{mean}=8.86) than in patients with AML at diagnosis (RQ=1). We then investigated miR-34b promoter methylation status in this cohort of myeloid malignancies. MS-PCR revealed that miR-34b promoter was not methylated in any of the samples, and sequencing always showed TG nucleotides at the CpG islands after bisulfite treatment (Figure 1C). However, since the expression of miR-34b is heterogeneous and some patients have low miR-34b expression, a nested PCR was performed to increase the sensitivity of the target amplicon detection (sensitivity up to 10^{-6} as calculated with serial dilutions of MS-PCR-positive AML samples). These results confirmed the absence of methylation in all the MDS and JMML samples at diagnosis. Moreover, we sorted CD34$^+$ hematopoietic cells from HL-BM or MDS patients' samples to verify whether the methylation of miR-34b promoter was found only in stem cells and was not, therefore, detected in the whole BM of patients. Neither HL-BM nor MDS sorted CD34$^+$ cells showed hypermethylation of miR-34b promoter (Figure 1C). Furthermore, CREB expression was undetectable in MDS samples (Figure 1D).

MiR-34b promoter hypermethylation occurs during transformation from myelodysplastic syndrome to acute myeloid leukemia

MDS is a disease known to evolve to AML in 30% of cases. We examined miR-34b expression in three patients for whom paired samples were available at diagnosis of MDS and at the time of progression to AML. MiR-34b expression decreased following transformation into AML

($RQ_{meanMDS}$=0.61 *versus* $RQ_{meanAML}$=0.26). MS-PCR on the same paired samples revealed hypermethylation of miR-34b promoter exclusively in the AML specimens (Figure 2A). We then studied the gene expression profile in paired RNA samples taken during MDS and at AML diagnosis which were available for four patients. Using a supervised analysis we found a set of genes (n=175, see *Online Supplementary Table S1*) that were significantly (P<0.05) differentially expressed between the two diseases. To interrogate the transcriptional programs associated with the aberrant expression of miR-34b and CREB, we intersected our gene expression data with CREB-target genes identified by performing ChIP on Chip analysis in human tissue (Table S4 at *http://natural.salk.edu/CREB/*).[31] This analysis revealed that 103/175 (65%) genes differentially expressed were CREB target genes. We used the 103 CREB target genes as a new subset to perform hierarchical clustering analysis. Results revealed that CREB targets were able to distinguish the MDS from their own AML evolution (Figure 2C). Then, by principal component analysis, we validated the 103 CREB target genes in an independent cohort of 20 MDS patients at diagnosis. This analysis showed that two distinct groups of MDS patients were generated although the mean age between the two groups was not statistically significantly different. One included four high-risk MDS patients (whose disease evolved into AML in a median interval between diagnosis and evolution of 225 days, range 59-714 days) and the other included low-risk MDS patients (whose disease had not evolved at the time of the study from 3-10 years after diagnosis)[26] (Figure 2D). Therefore, differential expression of CREB target genes marks MDS, high-risk MDS (MDS that evolved) and AML, supporting the role of miR-34b and CREB in the process of myeloid transformation. To identify the mechanism of miR-34b down-regulation, we performed MS-PCR on the four evolved MDS, finding that miR-34b promoter hypermethylation became evident at the onset of AML, supporting the idea that promoter hypermethylation occurred during the transition from MDS to AML.

CREB overexpression in healthy bone marrow induces dysmyelopoiesis through its targets' up-regulation

To study the effect of CREB overexpression in myeloid transformation, we transiently transfected HL-BM cultures with a full-length plasmid containing CREB cDNA. We confirmed increased protein levels after CREB transfection (Figure 3A). Cell cycle analysis showed a slight increase in S phase cells after CREB over-expression (Figure 3B) (cell apoptosis was similar in cells transfected

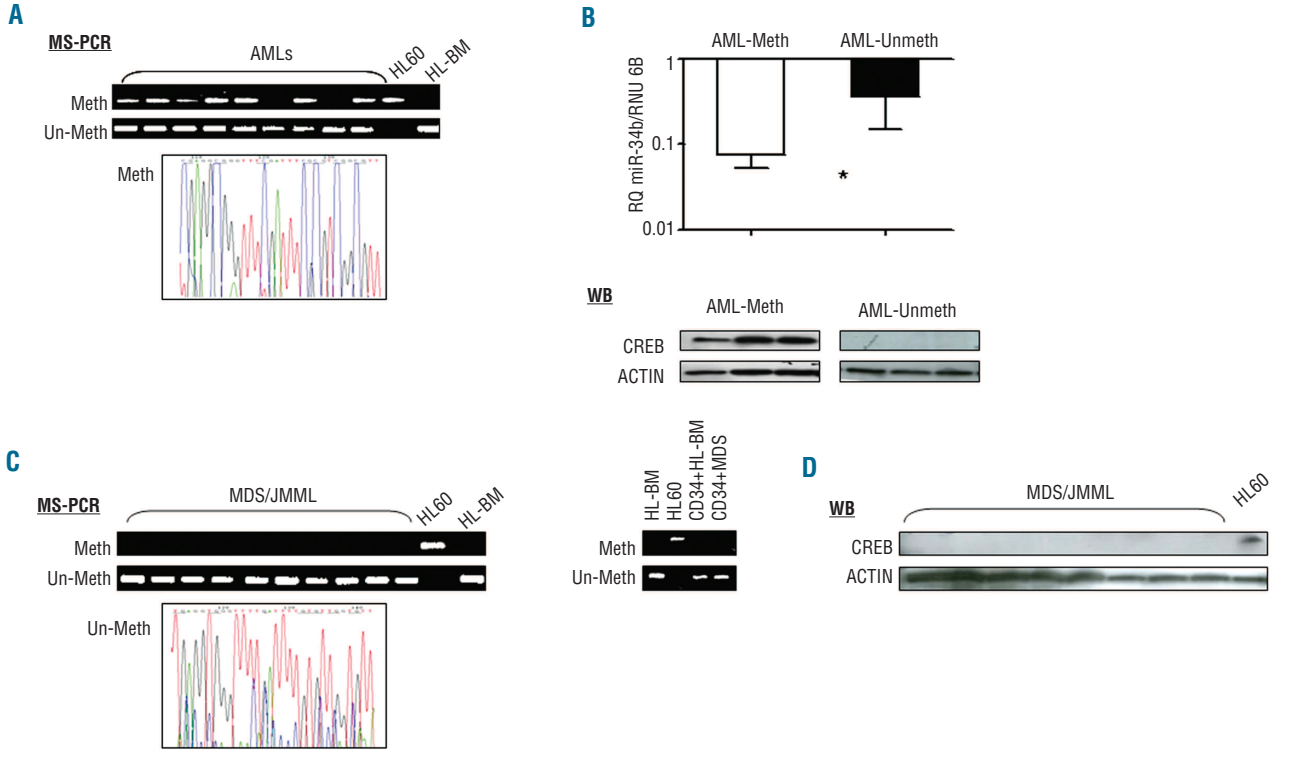

Figure 1. Methylation status of miR-34b promoter in patients with AML, MDS or JMML. (**A**) Methylation-specific PCR (MS-PCR) analysis of the miR-34b/c promoter in a representative set of AML patients' samples is shown. HL60 cell line and healthy bone marrow samples (HL-BM) were used as positive and negative controls, respectively. Direct sequencing of the miR-34b/c promoter amplicon after bisulfite treatment confirmed the methylation of GC nucleotides in the miR-34b promoter of patients. (**B**) RQ-PCR analysis of this group of patients with hypermethylation of the miR-34b promoter (Meth, RQ=0.075) and without hypermethylation of the promoter (Un-Meth, RQ=0.373, *P<0.05). CREB expression by western blotting in AML patients with or without methylated promoter. (**C**) MS-PCR analysis of the methylation of the miR-34b/c promoter CpG island in a representative set of samples from MDS patients. HL60 cell line and HL-BM samples were used as positive and negative controls, respectively. Direct sequencing of the miR-34b promoter amplicon confirmed the unmethylation of GpC islands shown as T nucleotides after bisulfite treatment. HL-BM and MDS $CD34^+$ sorted cells did not show a methylation-specific band. (**D**) Western blotting of a representative cohort of MDS patients shows non-detectable levels of CREB expression in MDS samples. HL60 cells were used as positive controls.

with CREB or empty vector; *data not shown*). The clonogenic growth of BM cells over-expressing CREB was enhanced as measured by methylcellulose colony assays (Figure 3C). By FACS analysis we identified an increased number of erythroid precursors (CD45 negative), an increased number of cells positive for erythroid and megakaryocyte markers (CD36, glycophorin A, and CD61), and for a granulocyte/monocyte marker (CD15) in CREB-overexpressing cells (Figure 3D, *P*=ns). We investigated CREB targets of the myeloid lineage and, by western blot analysis, found that GATA-1 and PU.1 proteins were up-regulated.[32,33] MEIS-1, a known CREB target gene in AML, was also increased.[34,35] (Figure 3E). We performed RQ-PCR for previously confirmed CREB targets[20] finding that most of them were increased in normal BM cultures with enforced CREB expression (Figure 3F). Next, we investigated the expression of a series of CREB target genes that were extrapolated from the analysis of MDS evolved into AML (*Online Supplementary Table S1*). These novel CREB target genes were up-regulated after exogenous CREB transfection (Figure 3G). To better dissect the mechanism of transformation, we compared the gene expression profile of 72 AML patients at diagnosis and that of 11 healthy volunteers. We found 15597 present calls by Affymetrix parameters expressed in all the 11 HL-BM analyzed and intersected this result with the 12407 genes present in the CREB-database (that we previously used in our gene expression analysis[31]). We found 3281/12407 CREB target genes expressed in HL-BM, which represent 26% of expressed genes in normal BM. When we compared gene expression from HL-BM and *de novo* AML, we found 2618 genes differentially expressed (false discovery rate <0.05) between the two groups, and among them 1195/2618 (46%) were CREB targets (previously found by human Chip-chip analysis).[31] The differences highlighted by these probe sets are not due to an age difference between the healthy subjects and AML patients, since their mean age was not statistically different. We, therefore, defined a crucial subset of CREB targets that are expressed at AML onset *versus* HL-BM. We intersected these data with the gene expression data regarding CREB genes in the transition of MDS to AML, in the above section, and found that 78 out of 103 genes (76%) were common to the two data sets (Figure 3H, *Online Supplementary Table S1*). Furthermore, examination of all the genes (CREB targets and non-targets) that are significantly differentially expressed and in common to the two processes showed that CREB targets are enriched (78/131 CREB targets, 60%) compared with non-targets (53/131, 40%).

MicroRNA-34b inhibition phenocopies CREB up-regulation in healthy fetal liver cells

Since decreased expression of miR-34b leads to CREB overexpression, we measured miR-34b levels in whole HL-BM (RQ_{mean}=13.66, n=17), in $CD34^+$ sorted HL-BM cells (RQ_{mean}=9.08, n=3) and $CD34^+$ sorted fetal liver cells (RQ_{mean}=43.14, n=6). Our results demonstrated that miR-34b expression was higher in healthy hematopoietic tissues than in BM from patients with AML (n= 3, *P*<0.05). To study the role of miR-34b in human fetal liver cells, we knocked down miR-34b using a lentiviral sponge vector containing four in tandem miR-34b target sequences.[28,29] Infected cells were sorted and analyzed. MiR-34b expres-

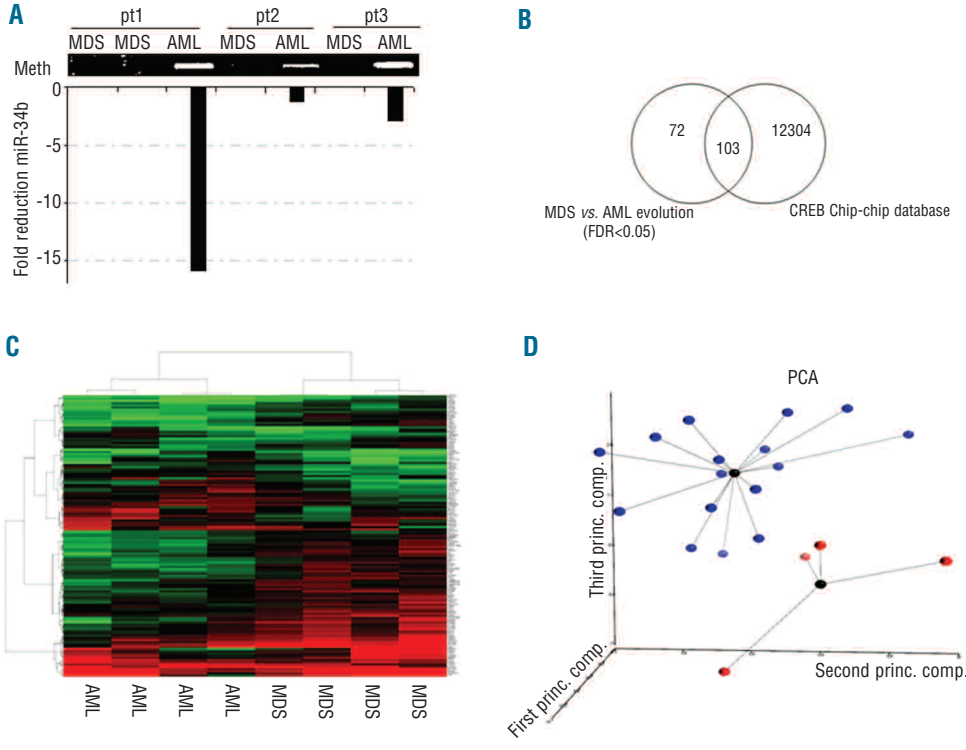

Figure 2. Characterization of MDS patients and their evolution into AML. (A) MS-PCR analysis of the miR-34b promoter in three MDS patients whose disease evolved to AML. Methylation of the promoter is present just at the onset of AML. Fold reduction of miR-34b expression for each patient's samples was 15.9, 1.16, and 2.9. (B) Venn diagram showing the overlap (103 genes) between genes differentially expressed by gene expression profiling and human CREB targets (from the ChIP-chip database,[29]) among the MDS samples and those after evolution into AML. (C) Hierarchical clustering analysis of four pairs of samples at diagnosis of MDS and their evolution into AML using the 103 differentially expressed CREB-target genes divided the cases of MDS from their evolution into two separate clusters. (D) Principal component analysis using 103 CREB target genes distinguishing MDS samples from samples taken after evolution to AML in an independent cohort of 20 MDS patients. Patients are divided between those known to have developed AM (red), and those whose disease has not yet evolved (blue). In black there is the centroid of the two groups of patients.

sion decreased by 75% compared to the negative control (RQ=0.25), whereas CREB levels were increased (RQ=4.48) in addition to CREB target genes known to influence myeloid leukemia (Figure 4A). We next analyzed cell morphology and phenotype of miR-34b knockdown human fetal liver cells. May-Grunwald-Giemsa staining revealed a larger number of myeloid precursors and differentiated cells (examples are highlighted in the 60X square), indicating an aberrant expansion of the myeloid compartment (Figure 4B). A difference in the percentage of myeloid markers by flow cytometry was also documented (Figure 4C). Since decreased miR-34b expression can affect CREB expression, we examined the clonogenic properties in methylcellulose colony assays. Enhanced colony formation was observed in the miR-34b knockdown human fetal liver cells compared to the scrambled miR-target (miR-Neg, Figure 4D). Therefore, miR-34b is critical for normal hematopoietic proliferation and differentiation.

MicroRNA-34b is an acute myeloid leukemia tumor suppressor in vivo

To confirm the hypothesis that miR-34b is a tumor suppressor in AML, we transduced HL60 and K562 leukemic cell lines with lentivirus for miR-34b or miR-neg ($RQ_{miR\text{-}34b}$ up to 10^{-3} fold increase). MiR-34b overexpression decreases CREB expression as shown by Western blot analysis (Figure 5A). The introduction of miR-34b in K562 cells did not affect cell proliferation *in vitro* (*data not shown*). We next injected HL-60 and K562 cells into NSG mice and monitored tumor formation. We observed that cells injected into the flank of NSG mice with K562 + miR-34b developed smaller tumors (0.68 g) 21 days post-injection compared to those injected with K562 + empty vector (1.18 g) (Figure 5B, 10 mice in each cohort, $P<0.05$). The same result was observed for HL60 + miR-34b (0.79 g) compared to HL60 + empty vector (1.39 g) (Figure 5C, 10 mice in each cohort, $P<0.05$). Furthermore, 5×10^6 HL60 + miR-34b or HL60 + empty vec-

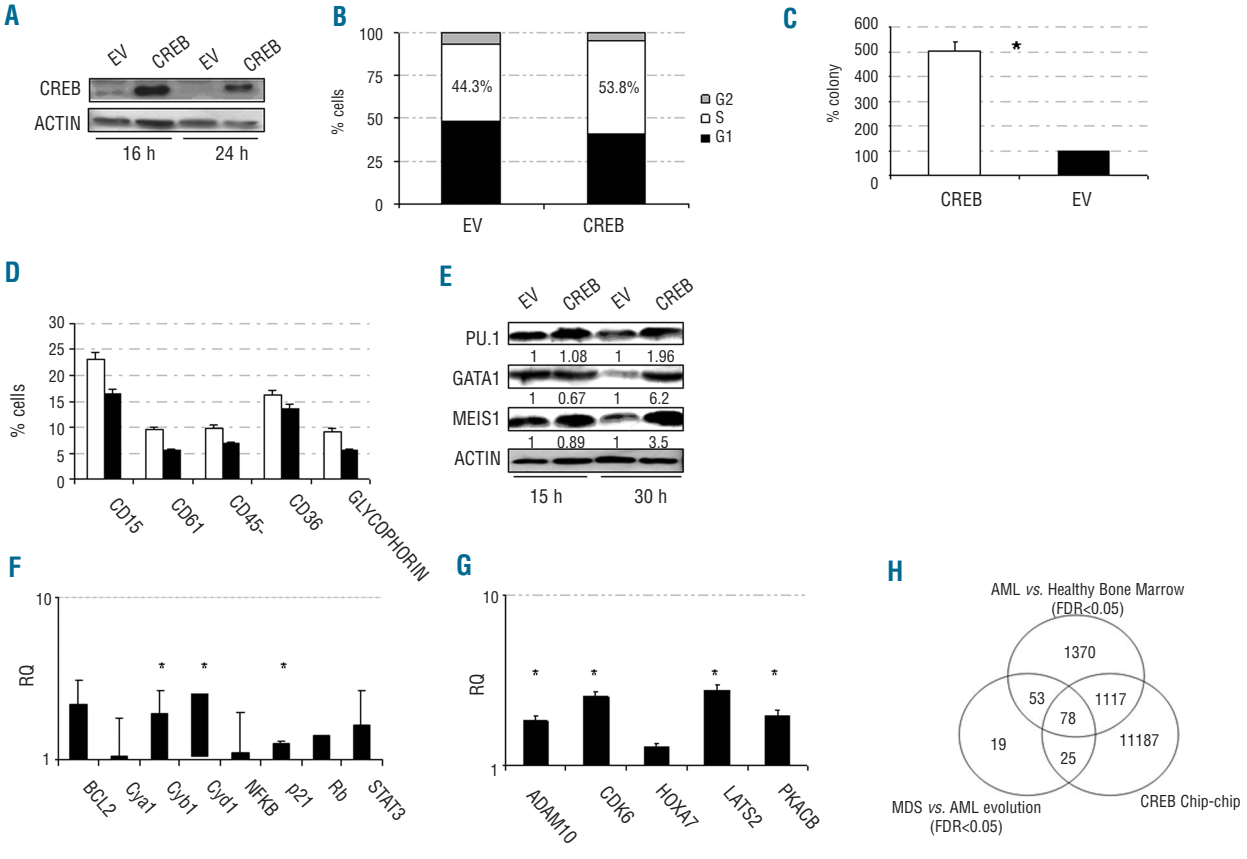

Figure 3. CREB overexpression in normal bone marrow cultures. Healthy bone marrow (HL-BM) transiently transfected with a CREB overexpressing vector (CREB) or a control (EV). (A) Western blot 16 h and 24 h post-transfection: induced CREB protein expression is shown in CREB transfected cells. (B) Cell cycle analysis in transfected healthy bone marrow showed an increased S-phase in CREB-transfected HL-BM (n = 2). (C) Colonies growth in methocult were counted after 14 days of culture and histograms represent the number of colonies formed by CREB-expressing cells (white bar) with respect to those formed by cells transfected with control vector (EV = 100%, black bar,*$P<0.05$). (D) FACS analysis of myeloid and erythroid markers (CD15, 61, CD45, CD36, glycophorin) in healthy primary cultures transfected with CREB (white bar) compared to EV (black bar) showed an increase in all myeloid markers. (E) Western blot of PU.1, GATA-1 and MEIS-1 16 and 30 h post-transfection showed an increase in expression of those proteins after CREB exogenous expression in HL-BM after 30 h. Densitometry measured the expression levels normalized to total amount of actin. (F) Expression of CREB target genes 24 h post transfection was RQ BCL2= 2.20, CyA1= 1.04, CyB1= 1.93, CyD1= 2.4, NFKBp50= 1.11, p21= 1.26, STAT3= 1.63. Each gene quantification was normalized to the RQ of HL-BM transfected with EV (RQ = 1) (n= 3). (G) Expression of genes extrapolated in Figure 2B (see *Online Supplementary Table S1*) was RQ ADAM10= 1.82, CDK6= 2.54, HOXA7= 1.26, LATS2= 2.75, PKACB= 1.97. Each gene quantification was normalized to the RQ of HL-BM transfected with EV (RQ= 1) (n= 3). (H) Venn diagram representation showing the overlap between genes differentially expressed between the samples taken from patients with MDS and the patients after evolution to AML, genes differentially expressed between AML and HL-BM and CREB targets from the ChIP-chip database.[29]

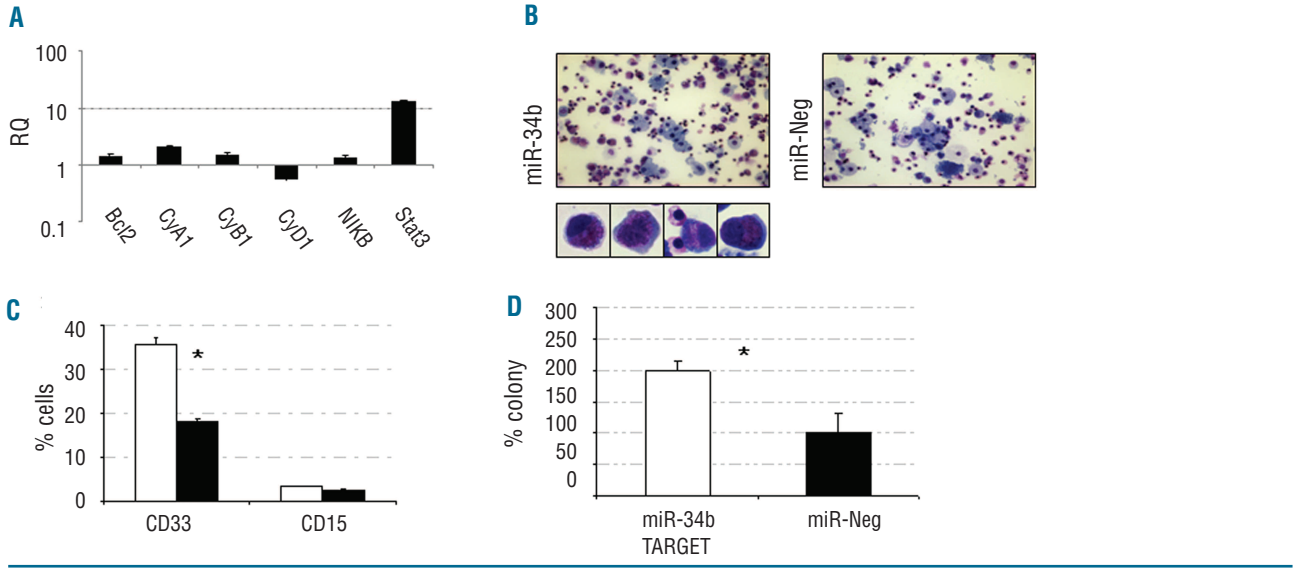

Figure 4. MiR-34b knockdown in CD34$^+$ fetal liver cells. (A) Expression of known CREB targets was BCL2= 1.44, CyA1= 2.09, CyB1= 1.51, CyD1= 0.54, NFKBp50= 1.37, and STAT3= 13.5. Each gene quantification was normalized to the RQ of fetal liver transduced with EV (RQ= 1) (n= 3). (B) Representative microscope field of May-Grünwald-Giemsa (MGG) staining (20X) of fetal liver cells with a knock down in miR-34b shows an increase in all the myeloid populations compared to the EV-treated sample. Example of myeloid cells not found in the controls are illustrated in the enlargement (60X). (C) By FACS analysis we showed an increase in myeloid markers (CD33 and CD15) in cells of fetal liver transduced with miR-TARGET (white bar) compared to control cells (black bar) (CD33= 35.4% *vs.* 18%, CD15= 3.3% *vs.* 2.5%, **P*<0.05). (D) Histogram represents the 2-fold increase in the number of colonies formed after knockdown of miR-34b (white bar) with respect to those formed by cells transfected with miR-NEG (miR-NEG= 100%, black bar,**P*<0.05).

tor cells were injected into the tail veins of five mice per group to analyze the effect of miR-34b overexpression in leukemia cell engraftment and progression. Results showed decreased engraftment and disease progression in mice injected with HL60 + miR-34b compared to the empty vector controls monitored by acquiring *in vivo* bioluminescence 2 and 3 weeks after inoculation. After 2 weeks, the size of the tumor in mice injected with cell lines + miR-34b was not significantly different from that of the mice injected with cell lines + empty vector. However, after 3 weeks, the mice injected with cell lines + miR-34b had significantly decreased tumor progression due to miR-34b over-expression (P<0.01, Figure 5C). Other miR-34b targets, such as C-MYB, CDK6 and MET,[22,36] were not found to be significantly reduced in the transplanted cell lines (*data not shown*). These experiments suggest that miR-34b is a tumor suppressor in AML principally by inhibiting CREB expression.

Discussion

In this study we showed that AML patients have decreased miR-34b expression through aberrant hypermethylation of the miR-34b promoter CpG island. Interestingly, hypermethylation of miR-34b promoter was completely absent in primary samples of HL-BM as well as in patients with MDS or JMML. We considered the childhood MDS because of their propensity to evolve into AML in approximately 30-40% of cases.[19,37,38] Primary BM samples from patients with MDS and the corresponding, transformed AML revealed that cells acquired miR-34b promoter hypermethylation during the evolution to AML. Epigenetic modification of miR-34b promoter results in aberrant CREB levels. We previously showed a direct interaction of miR-34b with the CREB 3'-untranslated region using a reporter assay, with the subsequent reduction of CREB protein levels *in vitro*. We also documented that miR-34b overexpression caused cell cycle abnormalities, reduced colony assay growth, and altered CREB target gene expression in leukemic cell lines.[5] Here, we demonstrate a direct link between miR-34b and CREB expression in pediatric myeloid malignancies. By using an integrative bioinformatic approach, we identified a subset of genes that could potentially mediate this transformation. We extrapolated a series of genes that were able to significantly cluster MDS from their paired AML samples. CREB target genes represented 59% of differently expressed genes, and were also able to distinguish high-risk MDS from low-risk MDS, therefore being a marker of disease evolution into AML. The differentially expressed genes that were non-CREB targets did not predict correctly all the four cases of MDS that evolved into AML, supporting the idea that CREB targets play a main role in triggering disease evolution. To further support the hypothesis that CREB, through its targets, contributes to myeloid transformation, we used *de novo* AML and HL-BM samples and performed gene expression analysis intersecting the results with the CREB target gene database. Fifty percent of CREB target genes, which are 2-fold more than typical CREB targets expressed in normal BM, were found to be differentially expressed between HL-BM and BM from AML patients, suggesting that CREB over-expression activates a pathway that is involved in the pathogenesis of AML *de novo*. We intersected gene expression profiles of MDS evolved into AML and HL-BM into *de novo* AML, extrapolating 78/103 genes in common with known CREB target genes. A relative enrichment of CREB targets with respect to non-targets supports our hypothesis that CREB controls a key pathway involved in myeloid transformation. These results suggest that CREB target genes may

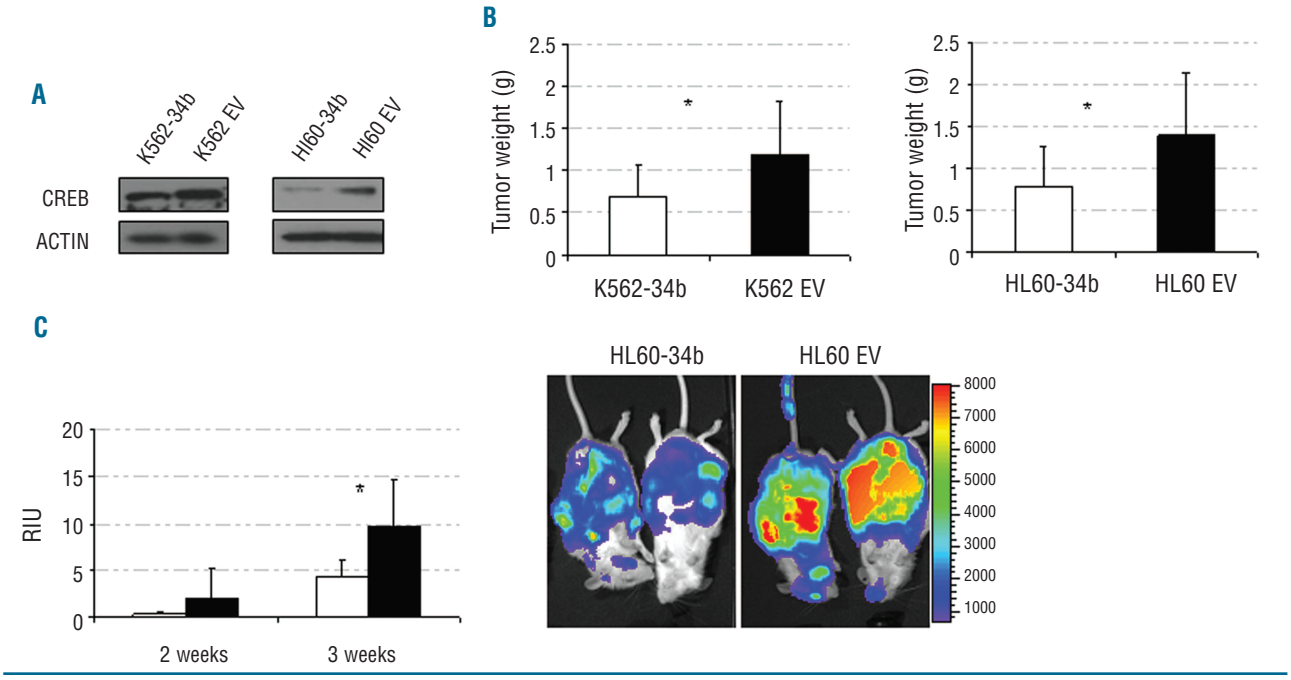

Figure 5. MiR-34b suppresses CREB expression and AML progression in primary cultures and *in vivo*. (A) Western blot analysis confirmed the reduction of CREB protein levels in leukemic cell lines (K562 and HL60) after transfection with Fugw-miR-34b with respect to levels in cells transfected with empty vector (EV). Densitometric analysis: HL-60 = 0.7±0.15 *vs.* 1, and K562 = 0.22±0.24 *vs.* 1, n = 3, $P<0.05$. (B) Tumor weight of the xenograft model obtained with a flank injection of K562 or HL60 leukemic cell lines overexpressing miR-34b in NSG mice was reduced in comparison to tumor weight of mice injected with EV cells. Error bars represent standard deviation. *$P<0.05$. (C) *In vivo* imaging with IVIS 100 bioluminescence/optical imaging system (Xenogen) of mice injected, via tail vein, with $5x10^6$ HL60-34b or EV transduced cells 2 or 3 weeks post-injection. The histogram shows a decrease in the relative intensity units (RIU) of mice injected with cells from a leukemic cell line overexpressing miR-34b compared to those injected with the control cell line (n= 5 each group, $P=0.01$).

represent a common pathway involved during transformation from normal BM, as well as from MDS. Among the targets there were several genes already known to play a role in tumor processes, such as RAB7L1, CDK6, HOXA7 and PKACβ, the catalytic subunit beta of the protein kinase A which posphorylates CREB on Ser133, enhancing its transcriptional activation. We propose that CREB is one of the regulators of the transcriptional program associated with myeloid leukemia, and moreover is critical to the pathogenesis of myeloid transformation. The fact that the CREB-driven expression profile "signature" is also found in MDS samples that are prone to evolve into AML, even if the miR-34b promoter is not methylated, might be due to PKACβ expression. This gene encodes for the catalytic subunit beta of protein kinase A, which leads to CREB phosphorylation, and consequently CREB transcriptional activation. We found that PKACβ expression increased from HL-BM, to MDS to AML. In this scenario, we suppose that it triggers CREB activation during high-risk MDS, this condition is then stabilized and worsened with the hypermethylation of miR-34b promoter at the onset of leukemia leading to AML. Previous studies documented that PKACβ is activated in proliferating cells also through a cAMP-independent mechanism, leading to cell transformation,[39] supporting its role in our model. Nevertheless, our data suggest that future studies may shed light on this mechanism.

Finally, we examined the role of miR-34b inhibition and CREB over-expression *in vitro* and *in vivo*. The inhibition of miR-34b levels in healthy BM and fetal liver cells enhances cell proliferation and clonogenic potential, leading to aberrant myelopoiesis. Our results showed that miR-34b rescue had a minor effect on cell growth in leukemic cell lines. However, miR-34b over-expression suppressed tumor growth *in vivo*, suggesting that the microenvironment may play a role in the tumor suppressive activity of miR-34b. Moreover, in an attempt to demonstrate that miR-34b acts solely through CREB, we rescued CREB expression on K562 cells stably expressing miR-34b. We did not find a significant increase in cell growth after CREB rescue, which may be due to the fact that leukemia cell lines have a high proliferative rate and multiple mutations affecting various signaling pathways. *In vivo* rescue experiments are needed in the future to prove this hypothesis, and eventually to identify other miR-34b targets.

These data implicate CREB overexpression, together with the alteration of its target genes, acting as a driving force in AML transformation from both HL-BM and MDS. This work highlights the oncogenic circuitry that is fed by the hypermethylation of miR-34b, increased CREB levels, and a potential downstream mechanism involved in the pathogenesis of AML transformation. Furthermore, the CREB subset of genes may be considered a novel transcriptional network that controls the leukemia phenotype, which can guide further functional studies and therapeutic opportunities, and represents a novel approach to evaluate risk stratification of MDS patients.

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