

## Down-regulation of MicroRNAs 222/221 in Acute Myelogenous Leukemia with Deranged Core-Binding Factor Subunits<sup>1,2</sup>

Matteo Brioschi<sup>\*,3</sup>, John Fischer<sup>†,3</sup>,  
Roberto Cairoli<sup>‡</sup>, Stefano Rossetti<sup>†</sup>, Laura Pezzetti<sup>§</sup>,  
Michele Nichelatti<sup>‡</sup>, Mauro Turrini<sup>‡</sup>,  
Francesca Corlazzoli<sup>\*</sup>, Barbara Scarpati<sup>‡</sup>,  
Enrica Morra<sup>‡</sup>, Nicoletta Sacchi<sup>†</sup> and  
Alessandro Beghini<sup>\*</sup>

\*Dipartimento di Biologia e Genetica per le Scienze Mediche, Facoltà di Medicina, Università degli Studi di Milano, Milan, Italy; †Cancer Genetics Program, Roswell Park Cancer Institute, Buffalo, NY, USA; ‡Department of Oncology, Niguarda Hospital, Milan, Italy; §Department of Hematology, Niguarda Hospital, Milan, Italy

### Abstract

Core-binding factor leukemia (CBFL) is a subgroup of acute myeloid leukemia (AML) characterized by genetic mutations involving the subunits of the core-binding factor (CBF). The leukemogenesis model for CBFL posits that one, or more, gene mutations inducing increased cell proliferation and/or inhibition of apoptosis cooperate with CBF mutations for leukemia development. One of the most common mutations associated with CBF mutations involves the KIT receptor. A high expression of KIT is a hallmark of a high proportion of CBFL. Previous studies indicate that microRNA (MIR) 222/221 targets the 3' untranslated region of the KIT messenger RNA and our observation that AML1 can bind the MIR-222/221 promoter, we hypothesized that MIR-222/221 represents the link between CBF and KIT. Here, we show that MIR-222/221 expression is upregulated after myeloid differentiation of normal bone marrow AC133<sup>+</sup> stem progenitor cells. CBFL blasts with either t(8;21) or inv(16) CBF rearrangements with high expression levels of KIT (CD117) display a significantly lower level of MIR-222/221 expression than non-CBFL blasts. Consistently, we found that the t(8;21) AML1-MTG8 fusion protein binds the MIR-222/221 promoter and induces transcriptional repression of a MIR-222/221-LUC reporter. Because of the highly conserved sequence homology, we demonstrated concomitant MIR-222/221 down-regulation and KIT up-regulation in the 32D/WT1 mouse cell model carrying the AML1-MTG16 fusion protein. This study provides the first hint that CBFL-associated fusion proteins may lead to up-regulation of the KIT receptor by down-regulating MIR-222/221, thus explaining the concomitant occurrence of CBF genetic rearrangements and over-expression of wild type or mutant KIT in AML.

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Abbreviations: AML, acute myeloid leukemia; BFU-E, erythroid burst-forming units; BM-MNC, bone marrow mononuclear cells; CBFL, core-binding factor leukemia; CFU-GM, granulocyte/monocyte colony-forming unit; EPO, erythropoietin; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte/macrophage colony-stimulating factor; HSPCs, hematopoietic stem/progenitor cells; non-CBFL, non-core-binding factor leukemia

Address all correspondence to: Alessandro Beghini, PhD, Dipartimento di Biologia e Genetica per le Scienze Mediche, Facoltà di Medicina, Università degli Studi di Milano, via Viotti 3/5, 20133 Milano, Italy. E-mail: alessandro.beghini@unimi.it

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<sup>3</sup>These authors contributed equally to this work.

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## Introduction

The multistep model of acute myeloid leukemia (AML) pathogenesis postulates the cooperation between class I mutations, which confer a proliferative and antiapoptotic advantage to leukemic cells, and class II mutations, which impair cell differentiation [1]. Core-binding factor leukemia (CBFL) defines a subgroup of AML characterized by class II cytogenetic mutations involving the master hematopoietic transcription factor CBF [1]. CBF consists of two subunits, CBF $\alpha$  and CBF $\beta$ , both critical for proper transcriptional activation of CBF target genes. Whereas the CBF $\alpha$  (AML1/RUNX1) is the actual DNA-binding subunit, CBF $\beta$  is necessary to strengthen AML1 DNA binding [2]. The two most common leukemia-associated CBF rearrangements are the t(8;21)(q22;q22) and inv(16)(p13;q22), which affect the CBF $\alpha$  and CBF $\beta$  subunit, respectively. Knock-in mice models harboring either the fusion protein AML1-MTG8 (AML1-ETO/RUNX1-RUNX1T1), consequent to the t(8;21)(q22;q22), or the CBF $\beta$ -MYH11 fusion protein, consequent to the inv(16)(p13;q22), were used to demonstrate that other mutations are necessary, in addition to the mutant CBF fusion proteins, for the development of overt leukemia [3–5]. The class I mutations, so far identified, that would cooperate with CBF fusion proteins in the leukemogenic process include mutations of *KIT*, *CSF1R* (*c-FMS*), *FLT3*, *N-Ras* and *K-Ras* genes [6–10]. Specifically, we and others found that the frequency of mutations involving the *KIT* gene, which encodes the receptor for the steel factor or stem cell factor (SCF) receptor, is significantly higher in both adult and childhood CBFL than in non-CBFL [11–13]. Furthermore, the expression level of both *KIT* mRNA and proteins is much higher in t(8;21) AML, with either wild type or mutant *KIT*, than in leukemia cells negative for t(8;21) [14]. Despite these observations, it is not yet clear whether there is a mechanistic link between CBF fusion proteins and overexpression of wild type and mutant *KIT* receptors.

MicroRNAs (MIRs) have been recently found to play an important role in the circuits that regulate the lineage differentiation fate of hematopoietic cells by modulating the expression of known oncogenes or tumor suppressors [15–20]. Human *MIR-222/221*, on chromosome X, has been predicted to target the 3' untranslated region (3'UTR) of *KIT* mRNA [16]. By performing *in silico* analysis of the promoter region of the *MIR-222/221* gene, we identified a few conserved AML1 consensus sequences. By chromatin immunoprecipitation (ChIP), we found that AML1 indeed binds these AML1-binding sites.

The promoter of the myelopoiesis-regulator *MIR-223*, a MIR on chromosome X, contains an AML1-consensus sequence, and its expression is epigenetically silenced by the t(8;21) CBFL-specific fusion protein AML1-MTG8 [21]. Thus, we hypothesized that *MIR-222/221* is another direct transcriptional target of AML1 and that down-regulation of *MIR-222/221* expression by AML1 fusion proteins is a potential mechanism leading to *KIT* overexpression. Reporter gene experiments showing that the expression of exogenous AML1-MTG8 can repress *MIR-222/221*-luciferase expression supported this hypothesis. To further tackle our hypothesis, we analyzed the expression of *MIR-222/221*, along with the expression of the myeloid-specific *MIR-223*, in different contexts: 1) normal bone marrow mononuclear cells (BM-MNCs) expressing or not expressing the glycosylated CD133 epitope (AC133), a hallmark of primitive progenitors and stem cell populations [22]; 2) AML samples characterized for the presence or absence of the most common CBF chromosome rearrangements, namely, t(8;21) and inv(16), and for the expression of the *KIT* receptor; and 3) a 32D mouse model of a rare CBFL characterized by the t(16;21) rearrangement [23]. Here, we show that *MIR-222/221* expression levels are

lower in AC133-positive (AC133<sup>+</sup>) cells relative to AC133-negative (AC133<sup>-</sup>) cells but are sharply upregulated in the course of AC133<sup>+</sup> granulocyte/monocyte differentiation. Significantly, we detected lower levels of *MIR-222/221* and *MIR-223* expression in CBFL, in correlation with a higher *KIT* expression, relative to non-CBFL samples. Lower levels of mouse *MIR-222/221* and mouse *MIR-223* as well as a higher level of mouse *KIT* (CD117) expression were also detected in the 32D/WT1 cell model of AML1-MTG16, the CBF fusion protein resulting from the t(16;21) [24].

The overall findings suggest that CBFL-related fusion proteins are capable of inducing the concerted down-regulation of both *MIR-223* and *MIR-222/221*, thus leading to the concerted block of myeloid differentiation and *KIT* overexpression.

## Materials and Methods

### *In Silico Analysis of the MIR-222/221 Gene Cluster*

Human MIRs sequences were obtained from the miRBase Sequence Database Release 8.1 (<http://microrna.sanger.ac.uk/sequences/ftp.shtml>; Griffiths-Jones). The ENSEMBL Database (<http://www.ensembl.org/index.html>) provided full-length DNA sequences of the MIRs genes on chromosome X and the sequence of the 3'UTR of the *KIT* gene. To identify the transcription start site, potential control elements, and consensus sites of *MIR-222/221* cluster gene sequence, the upstream pri-MIRs sequence was analyzed by MAPPER (<http://tftargetmapper.erasmusmc.nl/>), which is a platform for the computational identification of transcription factor-binding sites in multiple genomes. It uses an innovative technique that combines TRANSFAC and JASPAR data with the search power of profile hidden Markov models. A “good” match usually has a score greater than 0.8 and an *E* value less than 20. The greater the score, the better the match between the hit and the model is. A more stringent set of parameters was used for the query by setting the score greater than 1. The *E* value, computed with respect to the number of the sequences in the database queried, is a measure of the expected number of false-positives that will have scores equal to or larger than the score of the hit. The smaller the *E* value, the more significant the hit is [25].

### *Cell Lines and Culture Conditions*

The human leukemic monocyte lymphoma cell line U937 was cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Thermo Fisher Scientific, Waltham, MA). The t(8;21) leukemia patient-derived SKNO-1 cell line (kindly provided by Dr. Shujun Liu, Ohio State University) was cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (HyClone) and 10 ng/ml human granulocyte/macrophage colony-stimulating factor (GM-CSF; PeproTech, Rocky Hill, NJ). Clones derived from the mouse myeloid 32D/WT1 cell line, ectopically expressing human granulocyte colony-stimulating factor receptor (G-CSFR) [26] and infected with either AML1-MTG16 (RUNX1-CBFA2T3) (A16 clones), or the empty vector pLNCX2 (PL clones) were previously described [23]. Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (HyClone), and 10 ng/ml of mouse interleukin 3 (IL-3; PeproTech), adjusting the cell density to  $2 \times 10^5$  cells/ml daily. Granulocyte differentiation was induced by replacing IL-3 with 10 ng/ml human G-CSF (Amgen, Thousand Oaks, CA). Granulocytic differentiation was microscopically evaluated after Giemsa staining of cytospin preparations.

### Isolation and Culture of AC133<sup>+</sup> Hematopoietic Stem/Progenitor Cells

MNCs were isolated according to standard procedures using Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) from the BM drawn from the posterior iliac crest of a healthy donor, after obtaining informed consent, as per the Niguarda Hospital's institutional review board guidelines. The AC133<sup>+</sup> cell fraction was isolated by immunomagnetic separation after labeling with CD133/1 (AC133)-biotin antibody and anti-biotin MicroBeads on LS columns and Midi MACS separator (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of the AC133<sup>+</sup> fraction, evaluated by flow cytometry analysis, was greater than 97%. BM-MNCs ( $2 \times 10^4$  per 35-mm dish) and AC133<sup>+</sup> cells ( $1 \times 10^3$  per 35-mm dish) were grown in semisolid culture using ready-made MethoCult GF H 4534 CE-IVD medium (StemCell Technologies, Inc, Vancouver, British Columbia, Canada), which contains human recombinant GM-CSF, IL-3, and SCF, with or without erythropoietin (EPO). Erythroid burst-forming units (BFU-E) and granulocyte/monocyte colony-forming units (CFU-GM) were identified based on their morphology and counted after 14 days of culture.

### Flow Cytometry Analysis

Unselected BM-MNC, CD133/1-positive (AC133<sup>+</sup>), and CD133/1-negative (AC133<sup>-</sup>) cells were incubated for 20 minutes at room temperature in the dark with the appropriate monoclonal antibody (mAb) mixture, at a concentration deriving from specific titration experiments. MAbs were directly conjugated with the fluorochromes fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridinin chlorophyll protein (PerCP), and allophycocyanin (APC), and combined for four-color analysis. Each sample was incubated with the following mAbs panels: CD34-FITC/CD133/1-PE/CD45-PerCp/CD38-APC and CD34-FITC/CD133/2-PE/CD45-PerCp/CD38-APC. Unselected BM-MNC from AML patients were also incubated with the mAbs panel CD34-FITC/CD117-PE/CD45-PerCp/CD14-APC to test for KIT (CD117) expression. At the end of incubation, red blood cells were lysed for 10 minutes at room temperature by adding 3 ml of ammonium chloride. Cells were centrifuged at  $800 \times g$  for 8 minutes, and the cell pellet was resuspended in 500  $\mu$ l of PBS for flow cytometry analysis. All measurements were performed on a dual-laser FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA) and contained 10,000 to 50,000 cells, adjusted to the leukocyte subpopulations in the CD45/side scatter plot. Data acquisition was performed with the CellQUEST software, whereas both CellQUEST and Paint-a-Gate (Becton Dickinson) were used for analysis. Multiparameter analysis including logical gates on forward scatter, side scatter, FL1, FL2, FL3, and FL4 was used to evaluate cell populations. To assess mouse KIT expression in the 32D cell model, cells were incubated with antimouse CD117-PE (Miltenyi Biotec) as per manufacturer's instructions, fixed in 4% paraformaldehyde, and analyzed by using a FACScan flow cytometer (Becton Dickinson) and FCSExpress software. The results were expressed as geometrical mean of the fluorescence intensity of the selected markers.

### AML Samples

Leukemic MNC cells were isolated from the BM of 39 patients affected by *de novo* AML (samples were obtained as per the Niguarda Hospital's institutional review board guidelines). AML samples were classified according to the French-American-British classification. Twenty-five AML samples showed cytogenetic evidence of involvement of

the CBF factor, including 11 samples with t(8;21)(q22;q22) and 14 with inv(16)/t(16;16). The remaining samples included 12 samples with an apparently normal karyotype and 2 samples with a complex karyotype, with three to five chromosome abnormalities in at least one clone but negative for t(8;21)(q22;q22) and inv(16)/t(16;16).

### Mutation Analysis

All the AML samples, previously screened for the presence of KIT mutation in the entire coding region [11], were screened for this study for mutations in the 3'UTR of the *KIT* gene and in the genomic region where MIR-222/221 are mapped. A 235-bp sequence of the 3'UTR *KIT* region was amplified by standard polymerase chain reaction (PCR) with 3'UTR *KIT* forward primer (5'-CTC CTC TTT TAG CTG ATG AAC-3') and 3'UTR *KIT* reverse primer (5'-AGA TAC TGG CCC GGT GTC C-3'), whereas a 438-bp sequence within the MIR-222/221 genomic region in the chromosome X (chrX) was amplified with chrX forward primer (5'-TCT GGT TTA CTA GGC TGG TG-3') and chrX reverse primer (5'-GTT GGT AGT AGG TAA GTC CC-3'). Direct DNA sequencing of the PCR fragments was performed by using Thermo Sequence Dye Terminator sequencing reaction and an ABI Prism 3100 sequencing analyzer (Applied Biosystems, Foster City, CA).

### Stem-loop Reverse Transcription and Real-time PCR

Total RNA from leukemic blasts isolated by Ficoll-Hypaque density-gradient centrifugation was extracted using TRIzol (Invitrogen, Karlsruhe, Germany), according to the manufacturer's instructions and treated with DNase I (Ambion, Austin, TX). Total RNA (200 ng) was reverse-transcribed by using ImProm-II Reverse Transcription System (Promega, Madison, WI) and 10  $\mu$ M of stem-loop reverse transcription (RT) primer. Stem-loop RT primers for human MIR-221 (5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG AAA CCC-3'), human MIR-222 (5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG AGA CC-3'), and human MIR-223 (5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACG GGG TAT TT-3') were used for multiplex RT reactions under the following conditions: 30 minutes at 16°C, 30 minutes at 42°C, and 15 minutes at 70°C and then held at 4°C. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used for normalization of the RNA samples, was reverse-transcribed with a linear primer (5'-CAG TGT AGC CCA GGA TGC-3') by using ImProm-II Reverse Transcription system (Promega).

Complementary DNA (cDNA) obtained by stem loop RT were quantified by real-time PCR performed on an iQ5 Multicolor Real-time PCR detection system (Bio-Rad, Hercules, CA) by using Premix Ex Taq (Perfect Real Time; Takara, Shiga, Japan), and primers/probes were designed using the Beacon Designer software (Bio-Rad). The reaction was performed by using TaqMan probe 5'-FAM-TTC GTC GTA TCC AGT GCG AAT ACC T-3'/BHQ1, forward primer 5'-AGC TAC ATT GTC TGC TGG-3', and reverse primer 5'-GTA TCC AGT GCA GGG TCC-3' for MIR-221; TaqMan probe 5'-HEX-CTC GTC GTA TCC AGT GCG AAT ACC T-3'/BHQ1, forward primer 5'-AGC TAC ATC TGG CTA CTG G-3', and reverse primer 5'-GTA TCC AGT GCA GGG TCC-3' for MIR-222; TaqMan probe 5'-FAM-CCG TCG TAT CCA GTG CGA ATA CCT-3'/BHQ1, forward primer 5'-GTG TCA GTT TGT CAA ATA C-3' and reverse primer 5'-GTA TCC AGT GCA GGG TCC-3' for MIR-223; and TaqMan probe 5'-FAM-CCT CCG ACG CCT GCT TCA CCA-3'/BHQ1, forward

primer 5'-ACC TGC CAA ATA TGA TGA CAT C-3', and reverse primer 5'-GTA TCC AGT GCA GGG TCC-3' for human GAPDH. The reactions, run in triplicate in a 96-well plate, were incubated at 95°C for 3 minutes, followed by either 40 cycles at 95°C for 5 seconds, 56°C for 20 seconds, and 72°C for 10 seconds (for MIR-221) or 40 cycles at 95°C for 5 seconds and 60°C for 1 minutes (for MIR-222, MIR-223, and GAPDH). The level of the MIR transcripts was normalized to the level of the GAPDH transcripts and quantified by the threshold cycle  $C_t$  method. The ability of the TaqMan MIR assays to discriminate MIRs that differ by as little as a single nucleotide was tested with synthetic MIR-221 and MIR-222. Each MIR assay was examined against synthetic MIR-222 and MIR-221. Detection specificity was calculated from  $C_t$  differences between perfectly matched and mismatched targets, assuming 100% efficiency for the perfect match between target MIR and TaqMan probe. Very low levels of nonspecific signals were observed ranging from 0% to 0.17%, respectively (Table W1). We tested also the sensitivity of MIRs detection using synthetic MIR-221, MIR-222, and MIR-223 at decreasing concentrations. The TaqMan MIR assay showed a good linearity between synthetic RNA input and  $C_t$  value, demonstrating that  $C_t$  value correlates to the MIRs copy number (data not shown).

### Quantitative ChIP

ChIP was performed using reagents purchased from Millipore (Billerica, MA) following the manufacturer's protocol. Occupancy of endogenous AML1 or AML1-MTG8 at the AML1-consensus sites in the MIR-222/221 and MIR-223 promoters was assessed by ChIP with either anti-AML1 (C-19X; Santa Cruz Biotechnologies, Santa Cruz, CA), recognizing the AML1 N-terminus, or anti-MTG8 [27], recognizing the MTG8 C-terminus, respectively. ChIPs without antibody were performed as control. The immunoprecipitated DNA was amplified by real-time PCR with primers specific for the following regions: MIR-222/221 region no. 1 (sense: 5'-TGACCACACTAAACCCTTGCC-3'; antisense: 5'-AGTGTGGTTAGCTCTTGGTGG-3'), MIR-222/221 region no. 2 (sense: 5-CACAGCAAAGGATTCTAAGACG-3'; antisense: 5'-CCTGGCATTTGAGTGGATTCC-3'), MIR-223 promoter (sense: 5'-GGGAGAATTGAGAAGAGGGA-3'; antisense: 5'-GATAAGCAGG-TAAAGCCCGA-3') [21], and control region (sense: 5'-GGT-GCGTGCCAGTTGAACCA-3'; antisense: 5'-AAAGAA-GATGCGGCTGACTGTGCGAA-3'). The DNA relative enrichment was calculated by using the  $\Delta\Delta C_t$  method. The PCR signals obtained for each gene region were normalized to the PCR signal obtained from the input DNA (total chromatin fraction). Significance was calculated by using the Student's  $t$  test on three independent determinations.

### Luciferase Assay

U937 cells grown in a 24-well plate ( $2 \times 10^5$  cells/well) were transfected by using Lipofectamine LTX (Invitrogen) with 20 ng of pRL-TK and the indicated amounts of (-1600) MIR-222/221-Luc (kindly provided by C. Croce, Ohio State University), alone or in combination with either pCMV5-AML1B (Addgene, Cambridge, MA) or pcDNA3.1-AML1-MTG8-V5 [27]. Luciferase activity was measured 48 hours after transfection by using Dual Glow Luciferase Assay System (Promega) and was normalized to Renilla Luciferase expression.

### Statistical Analysis

All data were analyzed with usual descriptive statistical technique, after checking their distribution with the Shapiro-Wilk test. Quantita-

tive expression of MIR among genotypes (wt *vs* inv(16) *vs* t(8;21)) were compared using the Kruskal-Wallis test; in case of statistical significance ( $P < .05$ ), the pairwise evaluations were carried out by means of the Mann-Whitney  $U$  test, adjusted with the Bonferroni method for multiple comparisons. Differences in expression among CBFL *versus* non-CBFL, non-CBFL *versus* inv(16) *versus* t(8;21) were checked by the Mann-Whitney  $U$  test. Subject variations in AC133<sup>+</sup> and AC133<sup>-</sup> were analyzed by the Wilcoxon signed rank test.

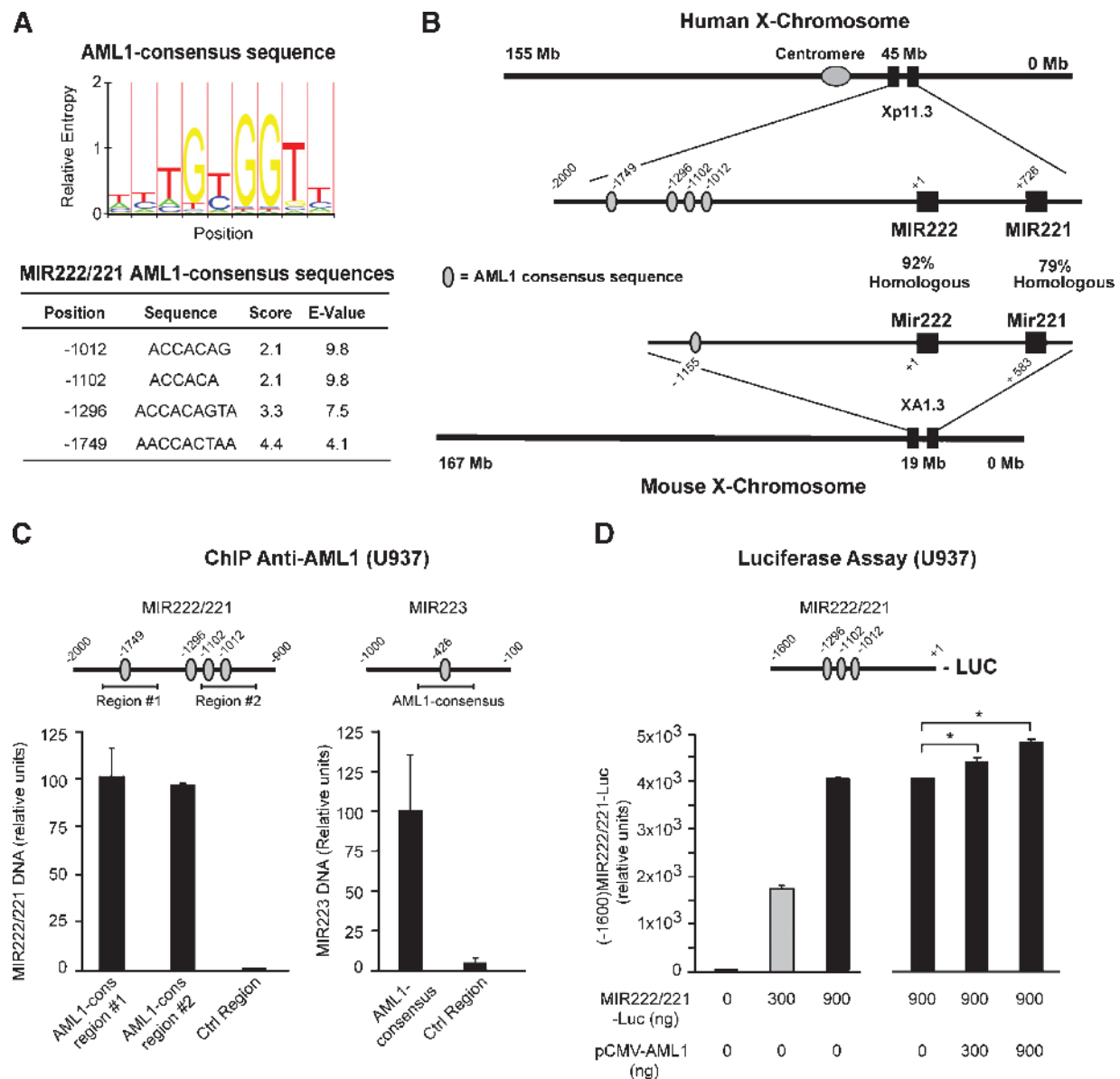
## Results

### *AML1 Is Implicated in the Transcriptional Control of the MIR-222/221 Gene Cluster*

The CBF transcription factor regulates the transcription of critical hematopoietic genes by binding the consensus sequence TG(T/C)GGT through its CBF $\alpha$  (AML1/RUNX1) subunit [28]. MIR-222 and MIR-221 are clustered on chromosome X and transcribed from the minus strand into a common precursor. MIR-222/221 transcription is driven from the same promoter region, which spans approximately 1.6-kb upstream of the transcription start site [29]. The 2-kb region upstream of the MIR-222/221 gene cluster transcription start site was searched for the presence of AML1-consensus sequences by using the MAPPER program. This program identified the most probable combinations of bases for AML1-binding sites within the context of the MIR-222/221 promoter (Figure 1A). Specifically, four AML1-consensus sequences were identified: three canonical AML1-consensus sequences (at -1012, -1102, and -1296) and one non-canonical AML1-consensus sequence (at -1749; Figure 1B). One of the canonical AML1-consensus sequences is conserved also in the mouse MIR-222/221 promoter (at -1155; Figure 1B). To establish whether AML1 plays a role in MIR-222/221 transcriptional regulation, we tested whether endogenous AML1 can bind one, or more, of the MIR-222/221 AML1-consensus sequences by ChIP analysis. To this end, we chose U937 cells, in which endogenous AML1 was previously shown to bind an AML1-consensus sequence in the MIR-223 promoter [21]. ChIP with an anti-AML1 antibody shows that endogenous AML1 binds two regions containing AML1-consensus sequences in the MIR-222/221 gene (Figure 1C, *left*) as well as the previously described AML1-consensus sequence in the MIR-223 promoter (included as a positive control; Figure 1C, *right*) but does not bind a negative control region lacking AML1-binding sites (Figure 1C). Further, we tested whether AML1 affects MIR-222/221 transcription by using a reporter construct carrying the luciferase gene under the control of the MIR-222/221 promoter (from -1600 to +1) [29]. This construct could be efficiently expressed in a dose-dependent manner when transiently transfected in U937 cells (Figure 1D, *left*), and its expression was significantly ( $P < .05$ ) enhanced by cotransfection with increasing amounts of AML1 (Figure 1D, *right*). Altogether, these results implicate AML1 as one of the transcriptional regulators of MIR-222/221.

### *Up-regulation of MIR-222/221 in AC133<sup>+</sup> Hematopoietic Stem/Progenitor Cells versus AC133<sup>-</sup> Cells*

To evaluate MIR-222/221 expression in different hematopoietic cell contexts, we set up a real-time stem-loop RT-PCR assay [30] that allowed us to detect with high efficiency and specificity the two MIRs (Table W1). We used this method to define the expression



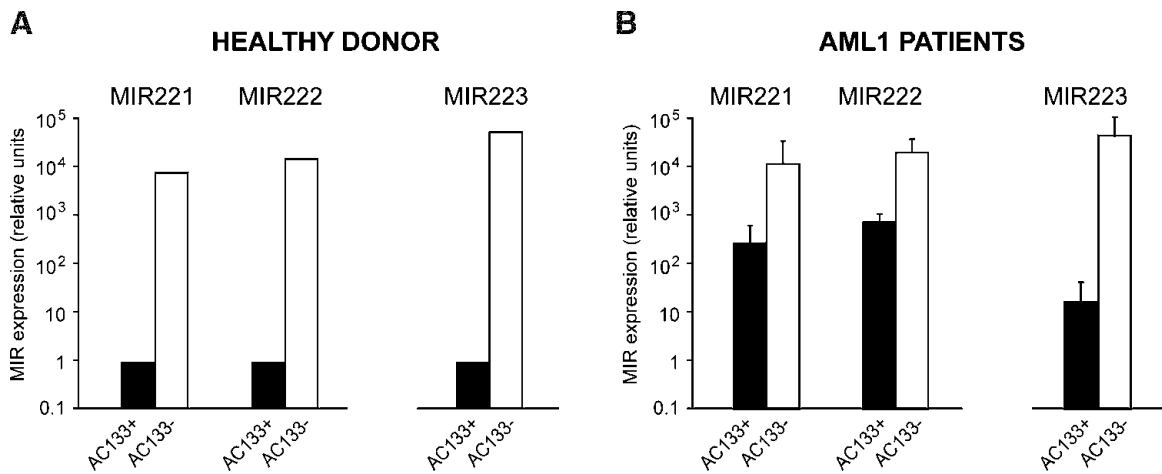
**Figure 1.** AML1 is implicated in the transcriptional control of MIR-222/221. (A) "Logo" representation of the most probable nucleotide combinations of the AML1-consensus sequence (top). Four AML1-consensus sequences could be identified on the minus strand (the transcribed strand) of the 2-kb region upstream of the MIR-222/221 transcription start site (bottom). (B) Scheme showing the relative position of the putative AML1-binding sites identified by *in silico* analysis of the human and mouse MIR-222/221 cluster. (C) ChIP with anti-AML1 showing that endogenous AML1 is bound significantly more to the AML1-consensus sequence-containing regions present in the MIR-222/221 and MIR-223 promoter relative to a control region in U937 cells. (D) Reporter assay showing that luciferase expression driven by the MIR-222/221 promoter (from -1600 to +1, see top panel) was induced in U937 cells (left) and was significantly enhanced by the expression of exogenous AML1 (right).

profile of MIR-222/221 and the myelopoiesis-regulator MIR-223 in different hematopoietic maturation stages *in vivo*. We analyzed two cell fractions isolated by immunomagnetic separation from BM-MNCs: the AC133<sup>+</sup> fraction enriched for hematopoietic stem/progenitor cells (HSPCs), and the AC133<sup>-</sup> fraction, enriched for more differentiated cells. In a healthy donor, the AC133<sup>-</sup> cells displayed significantly higher transcript levels of both MIR-222/221 and MIR-223 relative to the AC133<sup>+</sup> HSPCs (Figure 2A), suggesting that all three MIRs are up-regulated during normal myelopoiesis. In contrast, in AML patients, the up-regulation of MIR-221/222/223 in AC133<sup>+</sup> (99.6% blast cells, RSD 0.4%) versus AC133<sup>-</sup> (88% blast cells, RSD 11.25%) cells was

less pronounced than the one observed in the healthy donor (Figure 2B). This could be due, in part, to the cellular composition of AC133<sup>+</sup> fraction, which is particularly enriched for leukemic blasts expressing myeloid-associated differentiation antigens.

#### Up-regulation of MIR-222/221 during In Vitro Granulocyte/Macrophage Differentiation

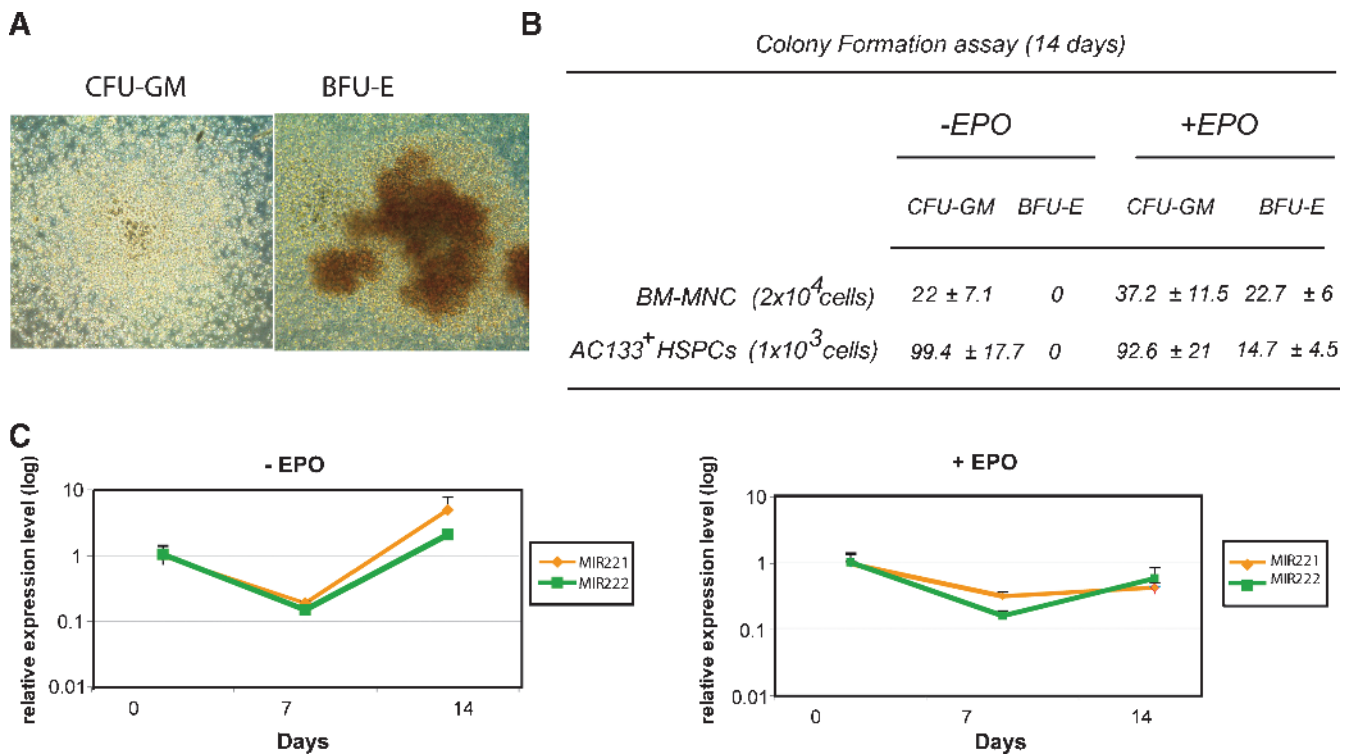
To determine whether MIR-222/221 expression is modulated in the course of normal myelopoiesis, we evaluated their transcript levels during *in vitro* cell differentiation of AC133<sup>+</sup> hematopoietic progenitor cells obtained from the BM-MNC cells of a healthy donor. AC133<sup>+</sup>



**Figure 2.** Up-regulation of MIR-221/222/223 in AC133<sup>+</sup> versus AC133<sup>-</sup> cells is more pronounced in the healthy donor than in AML patients. Stem-loop RT-PCR showing MIR-222/221 and MIR-223 expression levels in the AC133<sup>+</sup> and AC133<sup>-</sup> cell fractions isolated from BM-MNC of either a healthy donor (A) or non-CBFL patients (shown is the average of five patients) (B). The data represent the mean (±SD) of three replicates from one representative experiment of three performed.

cells were grown in a semisolid medium containing growth factors that induce the formation of colonies of differentiated cells after 7 to 14 days. The differentiating potential of AC133<sup>+</sup> cells was compared with the one of nonsorted BM-MNCs grown under the same conditions. In

the absence of EPO, the growth factors present in the medium (including GM-CSF) stimulate the formation of granulocyte/monocyte colonies (CFU-GM), which reach full differentiation within 14 days. A representative CFU-GM is shown in Figure 3A (left). When EPO is



**Figure 3.** Up-regulation of MIR-222/221 during AC133<sup>+</sup> HSPCs *in vitro* granulocyte/macrophage differentiation. (A) Representative CFU-GM (left) and BFU-E (right) colonies formed by AC133<sup>+</sup> HSPCs isolated from the BM-MNCs of the healthy donor, after 14 days in a colony formation assay in the absence or presence of EPO, respectively. (B) Colony quantitative/qualitative analysis shows that AC133<sup>+</sup> cells form significantly more CFU-GM colonies than BM-MNC after 14 days of culture in the presence or absence of EPO. (C) CFU-GM induction of AC133<sup>+</sup> cells followed by real-time stem-loop RT-PCR shows up-regulation of MIR-222/221 after 14 days (left), whereas no significant differences in MIR-222/221 expression could be detected when BFU-E and CFU-GM were concomitantly induced by culturing AC133<sup>+</sup> cells for up to 14 days in the presence of EPO (right).

added to the other growth factors, cells are induced to form BFU-E besides CFU-GMs. A representative BFU-E, characterized by EPO-induced hemoglobinization, is shown in Figure 3A (right). After 14 days of culture, the AC133<sup>+</sup> cells formed five and three times more CFU-GMs than BM-MNCs, when grown in the absence and presence of EPO, respectively (Figure 3B), indicating that the AC133<sup>+</sup> fraction has a stronger granulocyte/macrophage differentiation potential relative to the unsorted BM-MNC cells.

Next, we analyzed the MIR-222/221 expression profiles during AC133<sup>+</sup> *in vitro* differentiation. The expression of MIR-222/221 was induced about seven times on CFU-GM induction after 14 days of culture (Figure 3C, left). In contrast, no significant effect on MIR-222/221 expression was observed in the presence of EPO (Figure 3C, right). Because down-regulation of MIR-222/221 is known to occur during EPO [16], MIR-222/221 up-regulation in CFU-GM may be masked by MIR-222/221 down-regulation in the BFU-E colonies induced by EPO.

#### MIR-222/221 Transcriptional Repression by the t(8;21)-CBFL Fusion Protein AML1-MTG8 (AML1-ETO)

AML1 target genes, including MIR-223 [21], have been reported to be repressed in CBFL patient samples and CBFL cell lines. To test whether CBFL rearrangements can induce repression of MIR-222/221, which is a *bona fide* AML1 target gene (Figure 1), we chose the AML1-MTG8 protein, derived from the t(8;21)-CBFL translocation. AML1-MTG8 is known to exert a repressive action on the transcription of several AML1 target genes [31]. ChIP analysis with an anti-MTG8 antibody of the t(8;21)-positive cell line SKNO-1 showed significantly more binding of endogenous AML1-MTG8 to the AML1-consensus sequences of the MIR-222/221 promoter and the MIR-223 promoter (positive control) relative to a control region without AML1-binding sites (Figure 4A). Further, transient expression of exogenous AML1-MTG8 in U937 induced a significant ( $P < .05$ ), dose-dependent repression of MIR-222/221-luciferase (Figure 4B). Apparently, AML1-MTG8 can directly repress MIR-222/221 transcription.

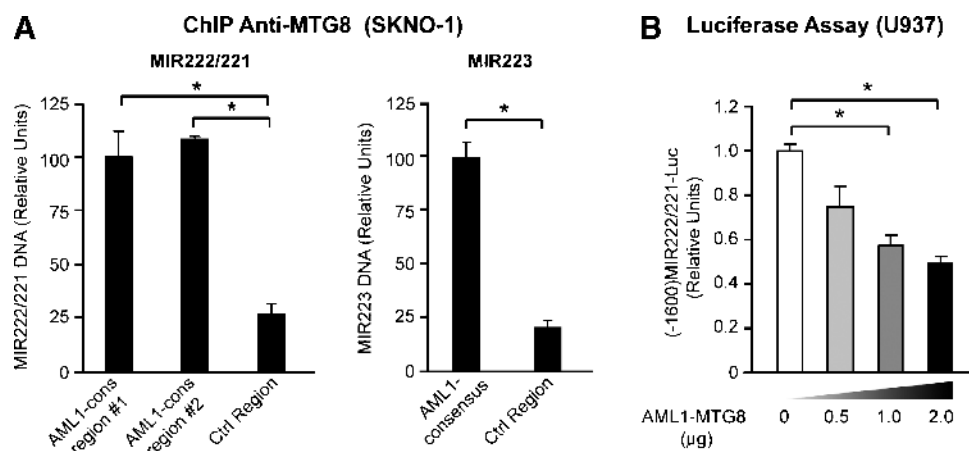
#### Down-regulation of MIR-221/222 in CBFL Overexpressing KIT Antigen

CBFL progression has been reported to be associated with activating mutations and/or over expression of the tyrosine kinase receptor KIT [11–14]. Because *KIT* mRNA is a known target of MIR-221 and MIR-222 [16], we tested whether *KIT* overexpression in CBFL samples is associated with either *KIT* mutations that may impair MIR-mRNA binding or defects in MIR-222/221 expression in CBFL samples.

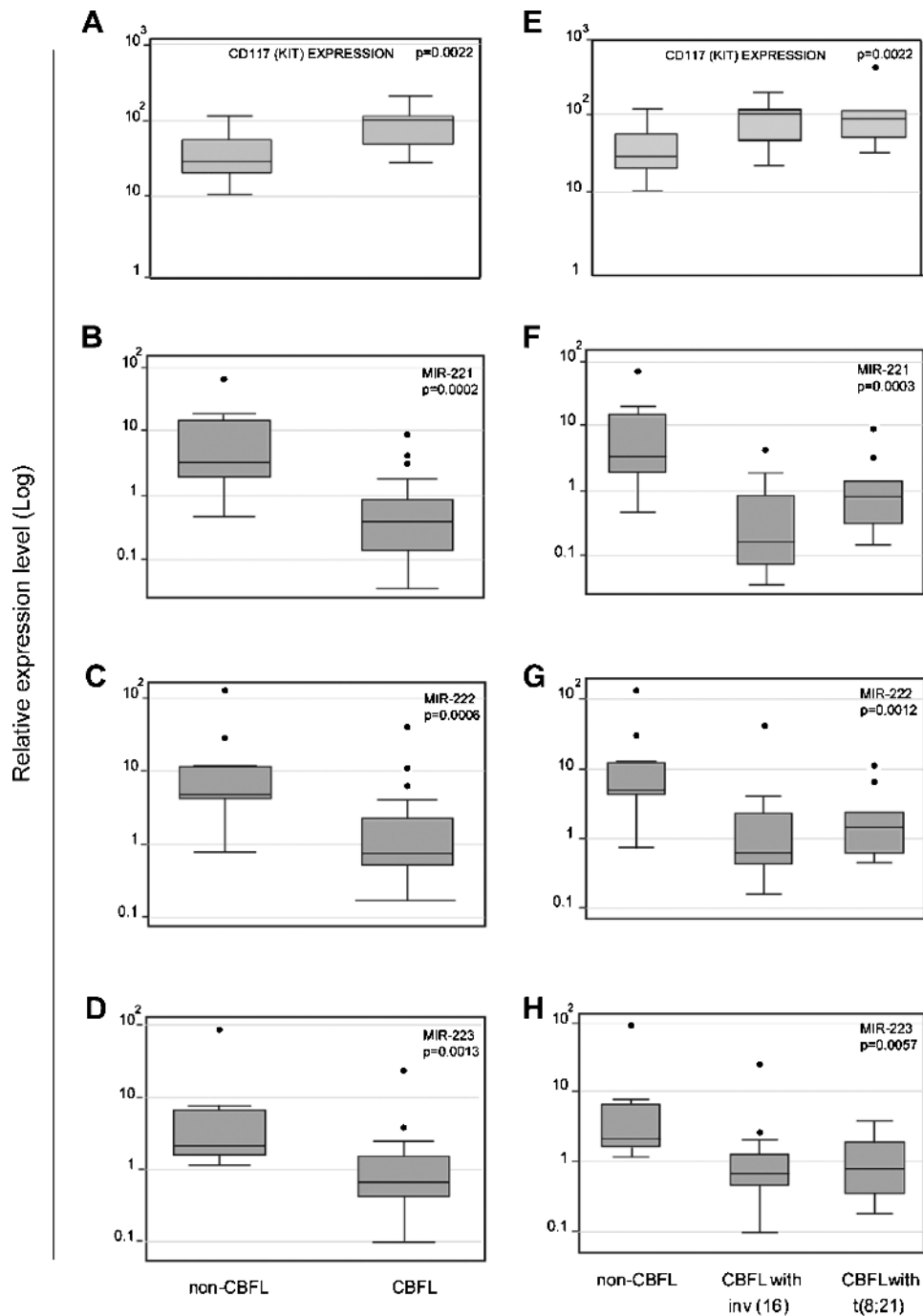
We analyzed 26 CBFL samples, which had been tested at diagnosis both for the presence of mutations in the KIT coding region and for the expression of the KIT receptor in BM-MNC cells (Table W2 based on Beghini et al. [11]), and 13 non-CBFL samples. The CBFL samples displayed higher incidence of KIT mutations (Table W2) and significantly higher KIT expression (CD117 antigen) relative to non-CBFL samples (Figure 5A). Further, we could detect by stem-loop RT-PCR lower expression levels of the CBF MIR target MIR-223 [21] in CBFL samples relative to non-CBFL samples (Figure 5D).

When we tested the same samples for MIR-221 and MIR-222 expression levels, we detected significant down-regulation of MIR-221 and MIR-222 in CBFL *versus* non CBFL (Figure 5, B and C). The observed MIR-222/221 down-regulation correlated with KIT/CD117 overexpression. Comparison of KIT (CD117) and MIR expression in either inv(16) or t(8;21) CBFL samples *versus* non-CBFL samples showed that KIT (CD117) overexpression (Figure 5E) was associated with MIR-221, MIR-222, and MIR-223 down-regulation (Figure 5, F–H) in both inv(16) and t(8;21) samples relative to non-CBFL samples. Apparently, both AML1-MTG8 (AML1-ETO) and CBF $\beta$ -MYH11 seem to exert a comparable repressive effect on the transcription of both MIR-223 and MIR-222/221.

By sequence analysis, we did not detect any mutations in both the *KIT* 3'-UTR and the pri-MIR-222/221 genomic sequences of CBFL samples (data not shown). Thus, MIR-222/221 down-regulation does not seem to be due to the lack of MIR-222/221 binding to KIT 3' UTR. On the basis of the evidence gathered so far, KIT (CD117)



**Figure 4.** MIR-222/221 transcriptional repression by the CBFL fusion protein AML1-MTG8. (A) ChIP with anti-MTG8 showing that endogenous AML1-MTG8 is bound significantly more to the AML1-consensus sequence-containing regions present in the MIR-222/221 and MIR-223 promoter relative to a control region in the t(8;21)-positive SKNO-1 cell line. (B) Reporter assay showing that luciferase expression driven by the MIR-222/221 promoter (from –1600 to +1) is significantly repressed by expression of exogenous AML1-MTG8 in U937 cells.



**Figure 5.** Down-regulation of MIR-222/221 and MIR-223 in CBFL-overexpressing KIT. (A) Flow cytometry analysis of the CD117 antigen (KIT) in non-CBFL patients and CBFL patients with either inv(16) and t(8;21). (B-D) Stem loop RT-PCR showing MIR-221, MIR-222, and MIR-223 expression levels in non-CBFL patients and CBFL patients with either inv(16) and t(8;21). (E) Flow cytometry analysis of the CD117 antigen (KIT) in BM-MNCs of non-CBFL patients, CBFL patients with inv(16), and CBFL patients with t(8;21). (F-H) Stem,loop RT-PCR showing MIR-221, MIR-222, and MIR-223 expression levels in non-CBFL patients, CBFL patients with inv(16), and CBFL patients with t(8;21). The median values for each sample group are indicated by the black line ( $\pm$  SD) in the box plots. Mann-Whitney  $U$  test was used to calculate the  $P$  value;  $P < .05$  was considered statistically significant.

overexpression in CBFL may be traced, at least in part, to MIR-222/221 down-regulation induced by CBF fusion proteins.

#### *Ectopic Expression of a CBF-Related Fusion Protein Leads to Down-regulation of MIR-221/222/223*

To test whether the down-regulation of MIR-222/221 and KIT overexpression observed in CBFL samples can indeed be traced to

the action of CBF fusion proteins, we exploited a mouse CBFL cell model that we previously described [23]. This model consists of 32D/WT1 cells ectopically expressing AML1-MTG16 (RUNX1-CBFA2T3), the CBF fusion protein of t(16;21)-positive CBFL. AML1-MTG16 is almost identical to the AML1-MTG8 protein because the wild type MTG16 and MTG8 mainly differ in their N-terminal region, and this region is lost on the fusion of MTG16 to AML1



(Figure 6A and Rossetti et al. [32]). Two clones expressing AML1-MTG16 (A23 and A24) and two clones carrying the control empty retroviral vector (PL4 and PL5) were used in this study. Although control clones are induced to differentiate into granulocytes by treatment with G-CSF, AML1-MTG16-positive clones, cultured under the same conditions, do not undergo granulocytic differentiation (Figure 6B). The expression of both MIR-221 and MIR-222 was significantly downregulated in AML1-MTG16-positive clones relative to control clones, both in the absence and the presence of G-CSF (Figure 6C). Similarly, MIR-223, which is highly conserved between human and mouse (Figure W2), was significantly downregulated in AML1-MTG16-positive clones after induction by G-CSF (Figure 6D).

Next, we tested whether there was a correlation between down-regulation of MIR-222/221 and level of KIT expression by cytofluorimetric analysis of the mouse CD117 antigen. Interestingly, we found that the two AML1-MTG16-positive clones showed a significantly ( $P < .05$ ) higher KIT level relative to both wild type 32D cells, and a control 32D clone (Figure 6E). Apparently, ectopic AML1-MTG16 expression leads to both increased KIT expression and down-regulation of MIR-222/221 transcription.

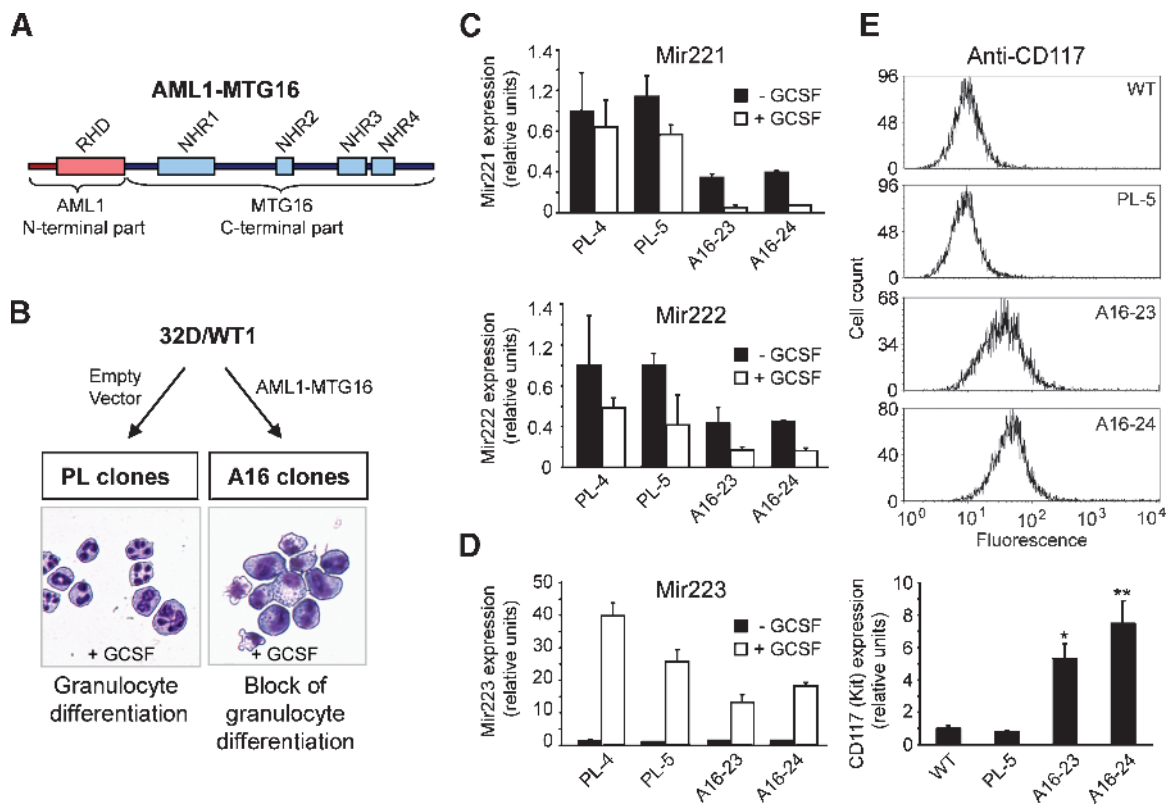
## Discussion

This study extends previous studies, including ours, showing that there is a significant association between rearrangements involving

the CBF subunits and overexpression of either wild type or mutant KIT receptor [11–14]. Whether the rearranged CBF subunits are directly involved in the overexpression of (wild type or mutant) KIT has been an open question.

We hypothesized that MIR-222/221 could be the molecular link between rearranged CBF subunits and KIT receptor up-regulation in CBF because there was evidence that MIR-222 and MIR-221 can act as regulators of KIT protein expression by targeting the 3'UTR of *KIT* mRNA [16] and because we found that the MIR-222/221 promoter harbors conserved consensus sequences for AML1, the CBF $\alpha$  subunit. The t(8;21) CBF fusion protein AML1-MTG8 (AML1-ETO) was shown to be a direct transcriptional regulator of MIR-223, a regulator of myeloid differentiation, capable of inducing MIR-223 epigenetic down-regulation [21]. We demonstrate here that MIR-222/221 is an AML1-regulated MIR cluster and that AML1-MTG8 can bind AML1-consensus sequences of the MIR-222/221 promoter and induce transcriptional repression of a MIR-222/221-luciferase reporter gene. This observation strengthened our hypothesis that CBF rearrangements, by down-regulating MIR-222/221, can induce overexpression of the KIT receptor.

We set up a stem-loop RT-PCR assay, which was specific and sensitive to detect a differential expression of MIR-221 and MIR-222 in AC133<sup>+</sup> and AC133<sup>-</sup> fractions from BM-MNC cells of a healthy donor. The AC133<sup>-</sup> cell fraction, enriched for more differentiated cells, displayed a higher level of both MIR-221 and MIR-222 expression



**Figure 6.** Ectopic expression of a CBF-related fusion protein leads to concomitant MIR-222/221 down-regulation and KIT up-regulation. (A) Scheme of the CBF-related fusion protein AML1-MTG16. (B) 32D/WT1 cells stably expressing AML1-MTG16 are unable of proper granulocytic differentiation in response to treatment with G-CSF for 6 days. (C) Two representative AML1-MTG16-positive clones (A16) display down-regulation of MIR-221/222 relative to two representative control clones (PL), both in the presence and absence of G-CSF. (D) G-CSF-induced MIR-223 expression is downregulated in AML1-MTG16-positive clones relative to control clones. (E) Cytofluorimetric analysis with PE-labeled anti-CD117 antibody (representative plots are shown on top) showing that two representative AML1-MTG16-positive clones express significantly higher KIT levels than wild type and control cells.

relative to the AC133<sup>+</sup> cell fraction, which is enriched for stem/progenitor cells and positive for stem cell antigens, including CD117. The stem-loop RT-PCR assay let us also detect an increasing expression of MIR-222/221 in the course of AC133<sup>+</sup> granulocyte/monocyte differentiation, which results in a decrease of CD117-positive cells (data not shown; and Ruzicka et al. [33]).

Next, we searched for an association between the level of MIR-222/221 expression and expression of CD117 KIT receptor antigen in leukemic samples with CBF rearrangements. By comparing samples of CBFL and non-CBFL with significant differences in the expression level of the KIT CD117 antigen, we found a significant difference in the expression of MIR-223, known for being downregulated by the CBF fusion protein AML1-MTG8 (AML1-ETO) [21]. Further, we found a significantly lower level of expression of both MIR-221 and MIR-222 in the CBFL group *versus* the non-CBFL group, showing that there is a significant correlation between down-regulation of MIR-221 and MIR-222 and the expression of different CBF fusion proteins. Interestingly, both the t(8;21)-positive CBFL group and the inv(16)-positive CBFL group showed comparable down-regulation in the expression of MIR-223 as well as MIR-221 and MIR-222. Thus, MIR-223 and MIR-221/222 down-regulation does not seem dependent on a specific CBF subunit rearrangement. How rearrangements of different CBF subunits exert similar repressive activity on the promoter regions of both MIR-223 and MIR-222/221 remains to be investigated. It is interesting to note that the promoter regions of both MIR-223 [17] and MIR-222/221 (data not shown) also contain a putative CEBPA-binding sequence and that both AML1-MTG8 and CBF $\beta$ -MYH11 can interfere with CEBPA expression at the transcriptional and translational levels, respectively [34–36]. Thus, it is possible that the down-regulation observed for all these MIRs are due to direct targeting of the fusion proteins at AML1 sites in the MIR promoter regions and/or indirectly by the fusion proteins affecting CEBPA-mediated regulation of the MIRs.

Because of the high conservation between the mouse and human MIR-223 and MIR-222/221 promoters, we were able to assess whether a CBF-related fusion protein (AML1-MTG16) can concomitantly induce both MIR-221/222/223 down-regulation and KIT up-regulation in the mouse myeloid cell model 32D/WT1. AML1-MTG16, like AML1-MTG8, maintains the DNA-binding domain of AML1 (the Runt domain), and the same four repressive domains of the MTG8 protein [37]. We found that AML1-MTG16 leads to down-regulation of MIR-223, MIR-221, and MIR-222 in the course of mouse granulocytic differentiation. Thus, AML1-MTG16, as AML1-MTG8, in addition to directly targeting and downregulating the expression of hematopoietic protein-coding genes containing AML1 consensus sequences [23–33,37], can target MIR genes important for myelopoiesis, such as *MIR-223*, as well as *MIR-222/221*, involved in the regulation of the KIT receptor.

In conclusion, this study supports a model in which CBF genetic abnormalities can lead to the overexpression of (wild type or mutated) KIT receptor by direct down-regulation of CBF-regulated MIRs. This mechanism would explain, at least in part, the concerted contribution of class I and class II mutations to the pathogenesis process of CBFL.

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**Table W2.** Features of Leukemia Samples.

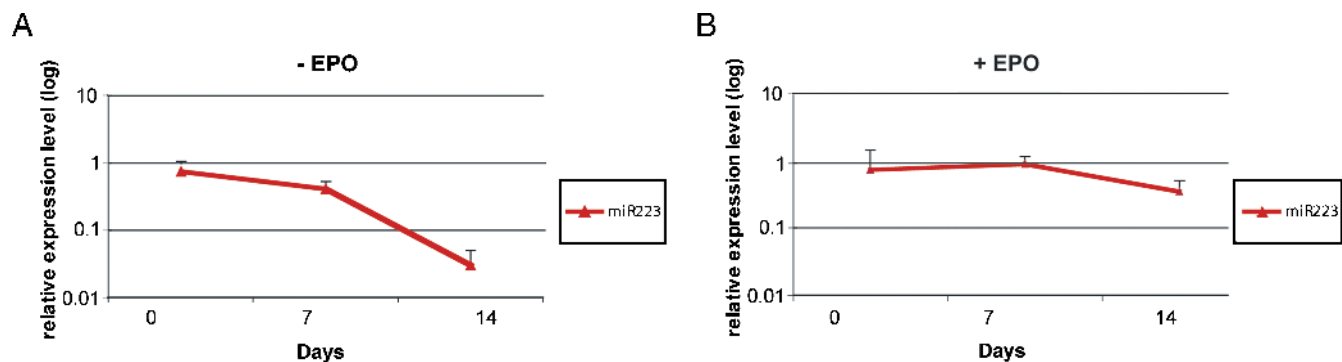
Patient No.	Sex	Age (years)	FAB	Karyotype	c-KIT
1	F	47	M4	46,XX	wt
2	M	59	M1	46,XY	wt
3	M	68	M2	46,XY	wt
4	M	72	M1	46,XY	wt
5	M	65	M1	46,XY	wt
6	F	68	M1	46,XX	wt
7	F	63	M1	46,XX	wt
8	M	58	M1	45,X,-Y	wt
9	M	62	M0	46,XY,+13	wt
10	M	62	M2	47,XY,+11	wt
11	F	68	M4	46,XX,-5,-17, tas(13;?) (pter;?),+mar,50dim	wt
12	F	65	M1	Complex Karyotype	wt
13	F	55	M2	46,XX,t(8;21)	D816V
14	F	31	M2	46,XX,t(8;21)	D816V
15	F	41	M2	46,XX,t(8;21)	D816V
16	M	51	M2	46,XY,t(8;21)	wt
17	M	49	M2	45,X,-Y,t(8;21)	D816V
18	M	48	M2	45,X,-Y,t(8;21)	wt
19	M	66	M2	45,X,-Y,t(8;21)	wt
20	M	25	M2	45,X,-Y,t(8;21)	wt
21	M	40	M2	46,XY,t(8;21)	D816V
22	M	16	M2	47,XY,t(8;21),+13	D816V
23	M	39	M2	49,XY,t(8;21),+4,+6,+19	D816V
24	M	67	M4Eo	46,XY,inv(16)	wt
25	M	64	M4Eo	46,XY,inv(16)	D816Y
26	M	36	M4Eo	46,XY,inv(16)	wt
27	F	56	M4Eo	46,XX,inv(16)	wt
28	M	40	M4Eo	46,XY,inv(16)	wt
29	M	37	M4Eo	46,XY,inv(16)	wt
30	M	36	M4Eo	46,XY,inv(16)	wt
31	M	45	M4Eo	46,XY,inv(16)	wt
32	F	40	M4Eo	46,XX,inv(16)	wt
33	M	49	M4Eo	46,XY,inv(16)	wt
34	M	15	M4Eo	46,XY,inv(16)	wt
35	F	32	M4Eo	46,XX,inv(16)	wt
36	F	28	M4Eo	46,XX,inv(16)	D816V
37	F	58	M4Eo	46,XX,inv(16)	D816V
38	F	62	M4Eo	45,X0,inv(16)	wt
39	M	60	M4Eo	48,XY,inv(16),+22,+9	wt

FAB indicates French-American-British classification.

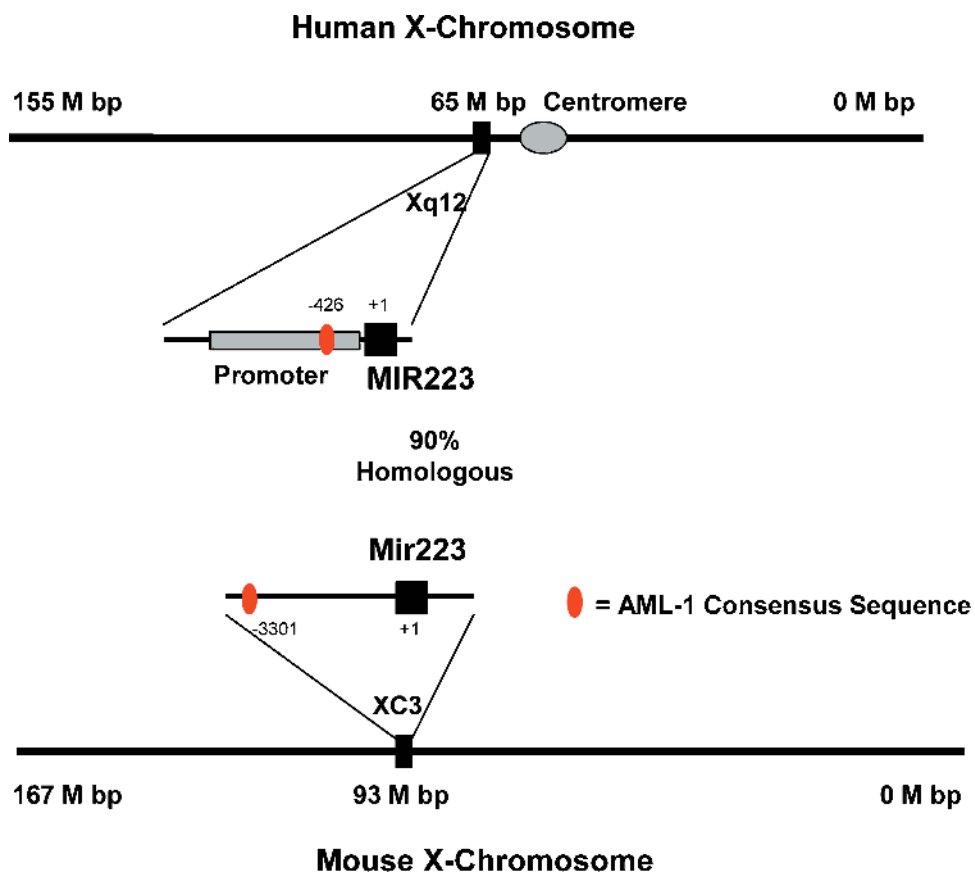
**Table W1.** Discrimination Power of 222/221 MIR Assay.

	Synthetic MIR Target		Relative Detection (%)
	221	222	
MIR assay	221	100.0	0.0
	222	0.17	100.0
MIR-221 5'-AGCUACAUGUCUGCUGGGU <u>UUC</u> -3'			
MIR-222 5'-AGCUACAUCUGGCUACUGGGU <u>CUC</u> -3'			

Relative detection (%) calculated based on  $C_t$  difference between perfectly matched and mismatched targets (red). A total of  $8.4 \times 10^8$  copies of synthetic RNA were added to the RT reaction.



**Figure W1.** MIR-223 expression modulation during CFU-GM and BFU-E induction of AC133<sup>+</sup> HSPCs in a colony-forming cell (CFC) assay. (A) CFU-GM induction of AC133<sup>+</sup> cells at 7 and 14 days followed by real-time quantification showed a downregulation of MIR-223 while (B) Concomitant BFU-E and CFU-GM induction of AC133<sup>+</sup> cells at 7 and 14 days followed by real-time quantification showed a weak modulation of miRNAs expression.



**Figure W2.** Scheme showing the position of the putative AML1-binding sites identified by *in silico* analysis of the human and mouse MIR-223.