HOXA/PBX3 knockdown impairs growth and sensitizes cytogenetically normal acute myeloid leukemia cells to chemotherapy

Glenda J. Dickson,[1] Fabio G. Liberante,[1] Laura M Kettyle,[1] Kathleen A. O'Hagan,[1] Damian P. J. Finnegan,[1] Lars Bullinger,[2] Dirk Geerts,[3] Mary Frances McMullin,[4] Terry R. J. Lappin,[1] Ken I. Mills,[1] and Alexander Thompson[1]

[1]Centre for Cancer Research and Cell Biology, Queen's University Belfast, Northern Ireland, UK; [2]Department of Internal Medicine III, Ulm University, Ulm, Germany; [3]Department of Pediatric Oncology, Erasmus University Medical Center, Rotterdam, Netherlands; and [4]Department of Haematology, Belfast City Hospital, Queen's University Belfast, Northern Ireland, UK

ABSTRACT

The cytogenetically normal subtype of acute myeloid leukemia is associated with an intermediate risk which complicates therapeutic options. Lower overall *HOX/TALE* expression appears to correlate with more favorable prognosis/better response to treatment in some leukemias and solid cancer. The functional significance of the associated gene expression and response to chemotherapy is not known. Three independent microarray datasets obtained from large cohorts of patients along with quantitative polymerase chain reaction validation were used to identify a four-gene *HOXA/TALE* signature capable of prognostic stratification. Biochemical analysis was used to identify interactions between the four encoded proteins and targeted knockdown used to examine the functional importance of sustained expression of the signature in leukemia maintenance and response to chemotherapy. An 11 *HOXA/TALE* code identified in an intermediate-risk group of patients (n=315) compared to a group with a favorable risk (n=105) was reduced to a four-gene signature of *HOXA6*, *HOXA9*, *PBX3* and *MEIS1* by iterative analysis of independent platforms. This signature maintained the favorable/intermediate risk partition and where applicable, correlated with overall survival in cytogenetically normal acute myeloid leukemia. We further showed that cell growth and function are dependent on maintained levels of these core genes and that direct targeting of HOXA/PBX3 sensitizes cytogenetically normal acute myeloid leukemia cells to standard chemotherapy. Together the data support a key role for *HOXA/TALE* in cytogenetically normal acute myeloid leukemia and demonstrate that targeting of clinically significant HOXA/PBX3 elements may provide therapeutic benefit to patients with this subtype of leukemia.

Introduction

Leukemia is associated with genomic aberrations including chromosomal translocations, inversions and deletions.[1] Cytogenetic analysis remains the primary method for prognostic sub-classification of acute myeloid leukemia (AML) at presentation.[2,3] However, the majority (~50%) of patients presenting with AML are cytogenetically normal (CN-AML) with an intermediate prognosis and marked variation in treatment outcome.[1,4] Chemotherapy can induce complete remission in approximately 85% of younger AML patients (aged 16 to 60 years)[5] but many relapse with chemo-resistant disease.[6] Myeloablative conditioning followed by allogeneic stem-cell transplantation is the currently preferred treatment option, although procedural mortality and graft-*versus*-host disease severely affect the long-term benefits in many groups of patients.[3,7] Accurate predictors of clinical outcome and novel targeted therapies are, therefore, needed to improve treatment for patients.

Molecular analysis, including whole exome and targeted sequencing, has mapped the landscape and frequency of CN-AML-associated mutations that correlate with prognosis to define "more-favorable" or "less-favorable" subgroups. Over 50% of CN-AML cases harbor an *NPM1* mutation which, in the absence of *FLT3*/ITD mutations,[8] acts as an independent prognosticator for achieving complete remission and increased overall survival.[9-11] *DNMT3A* mutations are reported to be independently associated with a "less-favorable" outcome in CN-AML.[12] Whatever combination of genetic mutations that emerges as providing the best prognostic value, it is their downstream targets and associated factors that may provide the basis for therapeutic intervention.

Class I homeobox (*HOX*) and three-amino loop extension (*TALE*) homeobox co-factor genes are associated with prognostic subtypes in AML.[13-15] *HOXA* cluster genes are normally expressed an order of magnitude higher than the other *HOX* genes in hematopoietic stem and progenitor cells indicating a particular functional role in hematopoiesis.[16] Ectopic expression of individual *HoxA* genes, in several model systems, resulted in altered hematopoiesis ranging from potentiation of cell proliferation and extended self-renewal for *HoxA6*[17] to the onset of an AML phenotype e.g. *HoxA9*.[18,19] Haploinsufficient *HoxA* cluster mice develop to adulthood but have impaired hematopoietic stem cell function.[16] *HoxA* gene

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Correspondence: alex.thompson@qub.ac.uk

deficiency is also associated with defective myeloid, erythroid and lymphoid development.[20]

The *Meis1* *TALE* gene is a collaborating oncogene to many *Hox* genes.[19,21,22] In several transduction and transplantation models *Pbx1* was used as a control *TALE* gene and was shown to have a limited or negative impact on leukemia initiation.[21,22] Direct functions of other Pbx or Meis proteins or isoforms in hematopoiesis are less well-defined, although complementary roles in multiple HOX:TALE complexes are expected, based on their structural similarity.[23,24] Deletion of *TALE* genes is also associated with impaired hematopoiesis. Both *Meis1* and *Pbx1* are essential for definitive hematopoiesis whereas *Pbx2* is reported to be dispensable for normal hematopoiesis[25] and *Pbx3* deficiency is associated with early death due to central respiratory failure.[26]

We provide evidence that defined *HOX/TALE* gene expression signatures may have specific prognostic value and that targeting clinically relevant HOXA/PBX3 axes may provide therapeutic benefit to patients with CN-AML.

Design and Methods

Samples from patients with acute myeloid leukemia

Diagnostic CN-AML peripheral blood or mononuclear cells were obtained following informed consent and anonymized. All studies adhered to the tenets of the Declaration of Helsinki and had local ethical approval.

Gene expression profiling and validation

Expression profiles were generated for patients with favorable cytogenetic risk (MILE Classes 9-11, n=105) and intermediate risk (MILE Class 13, n=315) according to the reported classification (NCBI Gene Expression Omnibus Accession number: GSE13204).[27] Two independent cohorts were also examined: *de novo* CN-AML samples (n=235) with known *NPM1*-mutation, *FLT3*-ITD and *CEBPα* status[28]; and a custom cDNA array (n=114 patients; Bullinger *et al.*, unpublished). Affymetrix CEL files were imported into the Partek Genomics Suite (St. Louis, MO, USA) as previously reported.[29,30] *HOXA/TALE* genes were examined using TaqMan™ chemistry as previously described.[14,31] Differentially expressed genes were validated in independent cohorts of patients for the *HOXA/PBX3/MEIS1* (n=21) and the *HOXA6/HOXA9/PBX3/MEIS1* core signature (n=37). Relative expression values are represented as copy number equivalents, fold-change or percentage of appropriate controls.

Acute myeloid leukemia cell lines and primary acute myeloid leukemia cultures

Two well-characterized human cell lines representative of CN-AML, namely U937 and OCI-AML3, were maintained as described elsewhere.[32,33] Low passage numbers of DSMZ-authenticated stocks were used throughout. Primary AML cells were rapidly thawed in the presence of DNase and cultured directly in RPMI 10% fetal calf serum (Invitrogen, Paisley, UK).

Targeted knockdown of HOXA/TALE

AML cell lines were nucleofected using the manufacturer's dedicated protocol (Amaxa™ GmbH, Cologne, Germany). Primary cells were nucleofected using the dedicated protocol for $CD34^+$ cells. Target short hairpin (sh)RNA and non-targeting control sequences (*Online Supplementary Table S1*) were obtained commercially or selected following sequential optimization of plasmid delivery and measurable knockdown. Multiple shRNA moieties, specific for widely separated regions along the mRNA sequence targeted, were used throughout. Targeted knockdown was established by quantitative real-time polymerase chain reaction (RQ-PCR) and confirmed by western blot analysis.

Cell cycle, growth, proliferation and caspase 3/7 assays

Samples for cell cycle analysis were processed as previously reported.[17] DNA content was measured with an LSR II flow cytometer and analyzed using FACS Diva™ software (BD Biosciences, Franklin Lakes, NJ, USA). Cell growth and viability were determined by trypan blue exclusion and cell proliferation by CellTiter-Glo®. Caspase activity was quantified using Caspase-Glo® 3/7 (Promega, Madison, WI, USA).

Colony-forming unit assays

AML cells and cell lines were cultured for up to 14 days in MethoCult® Classic (Stem Cell Technologies, Vancouver, Canada). Colony staining with 1 mM 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride (INT, Sigma-Aldrich, Gillingham, UK) enabled visualization of colonies and determination of metabolic activity.

Drug treatment

AML cells, pre-treated with or without shRNA, were plated in liquid culture or methylcellulose with 1 μM cytarabine (cytosine arabinoside)[34] or 100 ng/mL Mylotarg (gemtuzumab ozogamicin).[35]

Statistical analysis

ANOVA and Kaplan-Meier analysis of microarray experiments was implemented using Partek Genomics Suite. Two-way ANOVA or Student *t*-tests were performed on non-microarray-derived data by Graph-Pad Prism software (GraphPad software, Version 5.0, La Jolla, CA, USA) or the SPSS software package (IBM, Portsmouth, UK).

Results

HOXA/TALE expression in favorable/intermediate risk acute myeloid leukemia

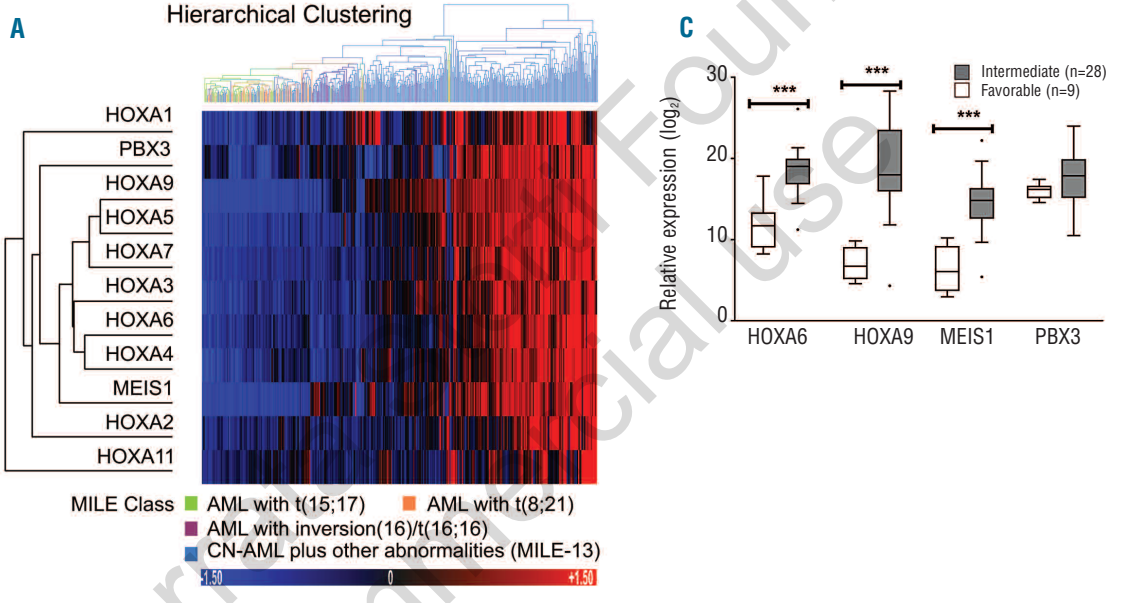
Microarray analysis of a cohort of AML samples (n=420) obtained as part of the MILE study was interrogated by probe-sets for the *HOXA* cluster and *TALE* genes. The cohort was made up of a combined favorable cytogenetic risk group consisting of 40 class 9 [t(8;21)], 37 class 10 [t(15;17)] and 28 class 11 [inv(16)/t(16;16)] samples referred to as MILE Classes 9-11 (n=105) and an intermediate risk group classified as CN-AML plus fewer than three structural abnormalities, excluding 11q23, referred to as MILE-13 (n=315). Favorable/intermediate risk AML was partitioned by unsupervised hierarchical clustering on the basis of nine *HOXA* genes plus *PBX3* and *MEIS1* (Figure 1A); these genes demonstrated significant differences between MILE-13 and combined MILE Classes 9-11 with a false discovery rate-corrected *P*-value of less than 0.0000005 (*Online Supplementary Table S2*). The differential expression of *HOXA* cluster genes (*HOXA1-HOXA11*) with the exception of *HOXA3* was validated in an independent cohort of patients (n=21) by RQ-PCR. The most consistent and highly ranked genes within the Class 13 equivalent, based on corrected C_T values, were *HOXA6*, *PBX3*, *HOXA9* and *MEIS1* (Figure 1B). Differential expression of the four genes was also observed in a more cytogenetically defined independent cohort of patients

(n=37) by RQ-PCR (Figure 1C and *Online Supplementary Table S3*).

Notably, the four-gene code was sufficient to partition an independent cohort (n=235) of *de novo* CN-AML patients (Figure 2A) demonstrating that lower expression of the signature correlated in part with favorable cytogenetic risk as defined by *NPM1*mut/*FLT3*-ITD/*CEBPα* status. To investigate whether the *HOXA/TALE* signature merely reflected known prognostic indicators, data from a custom array applied to a further cohort of CN-AML patients (n=114) with long-term follow up and extensive molecular profiling (Bullinger, personal communication) were examined. Four probes from the custom array corresponding to the *HOX/TALE* code were again capable of partitioning the patients' samples by unsupervised hierarchical clustering (Figure 2B). In this case, four distinct clusters were observed (1-4 right to left in the figure) which did not completely align with *NPM1*, *FLT3-ITD*, *FLT3-TKD*, *CEBPα*, *TET2*, *IDH* or *WT1* status. In addition, the clusters representing four different states namely: (i) $HOX^{Hi}/PBX^{Hi}/MEIS^{Hi}$; (ii) $HOX^{Hi}/PBX^{Hi}/MEIS^{Lo}$; (iii) $HOX^{int}/MEIS^{int}/PBX^{Lo}$; and (iv) $HOX^{Lo}/MEIS^{Lo}/PBX^{Lo}$ correlated with overall survival (P=0.015). Furthermore, $HOX^{Hi}/PBX^{Hi}/MEIS^{Lo}$ (Cluster 2) may be an indicator of poorer prognosis within this intermediate risk subgroup and is distinct both from patients with *NPM1* mutations (mainly located in Cluster 1) and those with *CEBPα* mutations (mainly Cluster 4) (Figure 2C). Together these data indicate an important role for *HOXA6*, *HOXA9*, *PBX3* and *MEIS1* in intermediate risk/CN-AML.

Functional targeting of HOXA/TALE *interactions in acute myeloid leukemia cell lines*

A targeted knockdown approach (shRNA) was taken to disrupt the HOX/TALE axis at the molecular level. We first wished to investigate the potential targetable HOXA/TALE interactions. A series of co-immunoprecipitation and west-



B

Subgroup	(n=)		HOXA1	HOXA2	HOXA3	HOXA4	HOXA5	HOXA6	HOXA7	HOXA9	HOXA11	PBX3	Meis1
		Rank	7	8	ND	10	6	1*	5	3*	9	2*	4*
Intermediate	15	Median CT	23.82	25.57	ND	27.24	22.92	19.15	22.12	21.69	26.83	20.7	23.34
		Range: low - high {	22.28	21.07	ND	21.54	17.81	14.27	18.11	15.98	21.66	17.29	15.32
			27.11	28.55	ND	31.76	32.57	24.37	25.96	32.98	31.34	22.25	26.46
Favorable	6	Median CT	28.84	31.65	ND	27.28	31.15	26.31	20.13	30.79	31.57	24.05	30.2
		Range: low - high {	24.01	27.38	ND	25.09	27.45	20.97	17.57	26.55	28.17	22.71	18.62
			33.04	35.01	ND	32.95	33.24	28.13	23.24	33.02	33.14	25.52	34.82

Figure 1. The ***HOXA/TALE*** signature stratifies favorable and Intermediate risk AML. (A) An unsupervised hierarchical cluster and heatmap depicting partitioning of a cohort of AML patients (n=420) into those with favorable or Intermediate risk by ***HOXA/TALE*** expression using a Euclidean distance metric with average linkage calculation. The favorable risk and intermediate risk groups comprised 105 cases and 315 cases, respectively. Probe-sets for the 11 genes included in the cluster were significantly differentially expressed between Class 13 *versus* Classes 9-11 with a false discovery rate-corrected *P*-value < 0.0000005. (B) Tabulated ranked gene expression levels, median and range, represented as C_T values corrected for *18S* rRNA loading. Color coding indicates overall expression levels: very high expression C_T <20 (red); high expression 20 <C_T <24 (orange); moderate expression 24 <C_T <30 (yellow); low expression 30< C_T <35 (green). The numbers of patients within prognostic subgroups are indicated where appropriate. The highest ranked genes in intermediate AML (***HOXA6***, ***HOXA9***, ***MEIS1*** and ***PBX3***) are highlighted (*). ND not determined; ***HOXA3*** primers not validated. (C) The ***HOXA/TALE*** signature was validated in an independent cohort of patients (n=37), by specific RQ-PCR analysis The favorable group (n=9) comprised five t(8;21) and four t(15;17) patients. Relative expression is displayed as copy number equivalents (log_2 scale) from standard curve values corrected for *18S* rRNA loading.[31] Significant differences between risk groups are denoted by ***P≤0.001.

ern blot analyses confirmed known HOXA9:TALE interactions[36] and demonstrated that HOXA6 can also interact with PBX and MEIS proteins (*Online Supplementary Figure S1*). Endogenous proteins were enriched for by immunoprecipitation in two AML cell lines, U937 and OCI-AML3. High affinity interactions between HOXA6 and PBX proteins were confirmed irrespective of *NPM1* status. Interestingly HOXA6 was also found to interact with MEIS in both AML cell lines, providing additional evidence that HOXA:MEIS interactions are not limited to the Abdominal B-like proteins.

Target shRNA were delivered with high efficiency (>45%) to both cell lines (*data not shown*). Cell lines displayed comparable *HOX/TALE* expression to that of primary CN-AML samples (*Online Supplementary Table S4*). Knockdown of gene expression was demonstrated by RQ-PCR (Figure 3A,B) and specific reduced protein expression was confirmed by western blot analysis of either 293FT cells overexpressing tagged *HOXA/PBX3* cDNA or U937 cells for endogenous MEIS1 (Figure 3C,D). Multiple shRNA moieties for each target were used to demonstrate specific effects. Differentially expressed genes associated with single *HOXA6* or *HOXA9* knockdown in the AML cell lines were obtained using AmpliChip-Leukemia arrays containing 1480 leukemia-associated probe-sets (*Online Supplementary Figure S2A*). Although knockdown of *HOXA6* or *HOXA9* resulted in altered expression of a small subset of common genes, associated with cell viability and differentiation in each cell line, significant differences were largely target gene-specific (*Online Supplementary Table S5*). This suggested that combined knockdown of *HOXA6* and *HOXA9* would have a more potent effect on AML cells than either alone. Targeted *HOXA6/A9* knockdown resulted in minimal compensation by *HOXA10*, but no reduced expression of other HOX genes, in a similar manner to *TALE* family members following knockdown of *PBX3* or *MEIS1* (*Online Supplementary Figure S2B*).

HOXA/TALE knockdown alters viability and growth of acute myeloid leukemia cell lines

Combined *HOXA6+A9* or individual *TALE* knockdown resulted in reduced viability and altered morphology in both AML cell lines compared to non-silencing controls (Figure 3E-F and *Online Supplementary Figure S3*). Targeted *PBX3* knockdown demonstrated a greater effect than *MEIS1*. These data were supported by significantly enhanced caspase 3/7 activities, indicative of cellular apoptosis, within 24 h (Figure 4A). Reduced caspase 3/7 activity

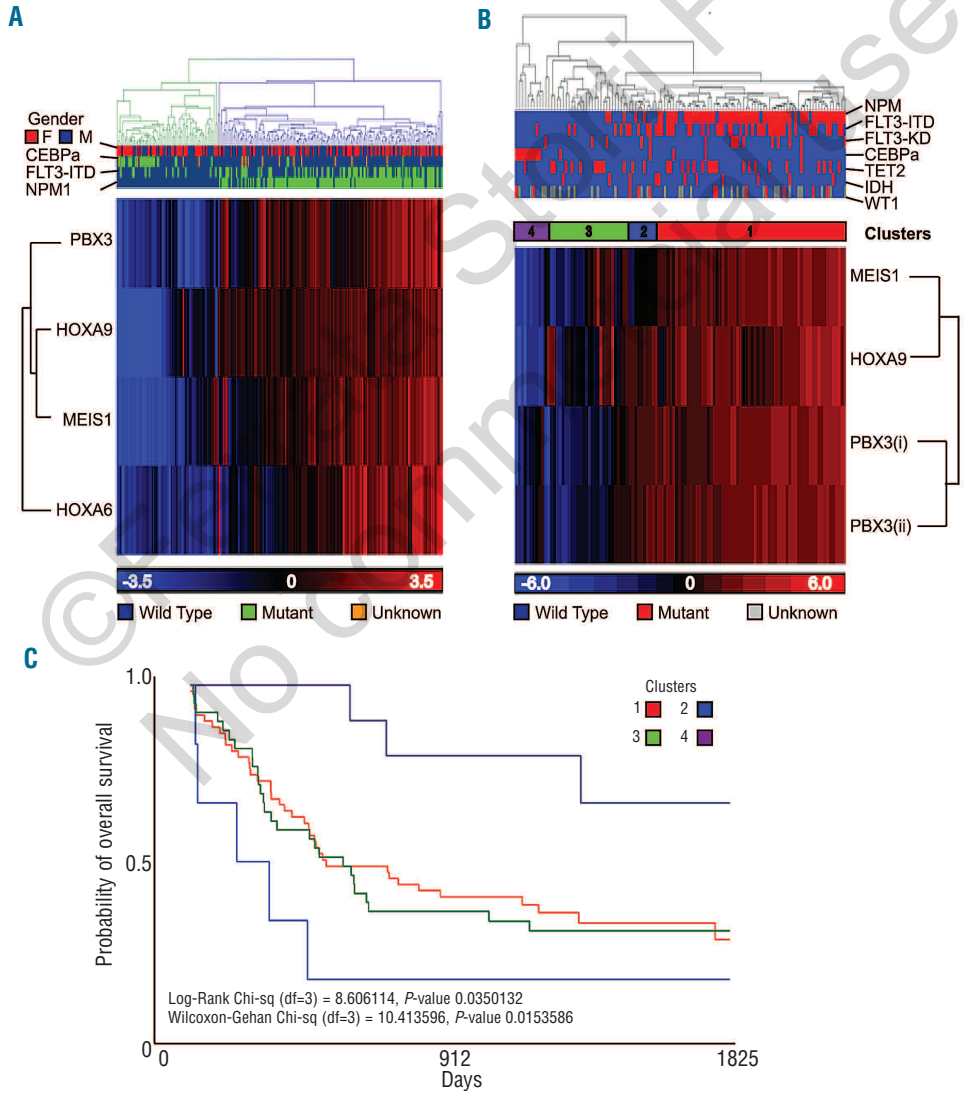


Figure 2. ***HOXA/TALE*** **code partitions CN-AML patients and correlates with overall survival. (A) An unsupervised hierarchical cluster and heatmap depicting partitioning of a cohort of *de novo* CN-AML patients (n=235) by *HOXA6*, *HOXA9*, *PBX3* and *MEIS1* expression. (B) Probe-sets for the four genes were significantly differentially expressed against NPM-1 mut status with a false discovery rate-corrected *P*-value < 0.0000005. Unsupervised hierarchical clustering and heatmap generation showed partitioning of a cohort of CN-AML patients' samples (n=114) with well-defined mutation status based on the probe-sets for *HOXA9*, *PBX3*(i) and (ii) and *MEIS1*. Four distinct gene expression-based clusters 1-4 (right to left) were identified that were not strictly dependent on cytogenetic status. (C) A Kaplan-Meier plot demonstrating correlation between the probe-sets clusters (1-4) and survival (*P*=0.015), with *HOXA*^{Hi}, *PBX3*^{Hi}/*MEIS1*^{Lo} (cluster 2) correlating with worst overall survival and *HOXA*^{Lo}/*PBX3*^{Lo}/*MEIS1*^{Lo} (cluster 4) associated with best overall survival.**

at later time points reflected poor cell state (*Online Supplementary Figure S4*). Furthermore, sub G_0/G_1 events were notably increased following *HOXA6+A9* or *PBX3* knockdown in both cell lines compared to respective controls (Figure 4B) but not for *MEIS1* (*data not shown*). Consistent with these findings, overall cell growth was significantly reduced following *HOXA6+A9* or *PBX3* knockdown but less so after *MEIS1* knockdown in either cell line (Figure 4C).

The liquid culture assays were extended into methylcellulose to investigate the effect of reduced *HOXA/TALE* levels on hematopoietic cluster and colony formation.

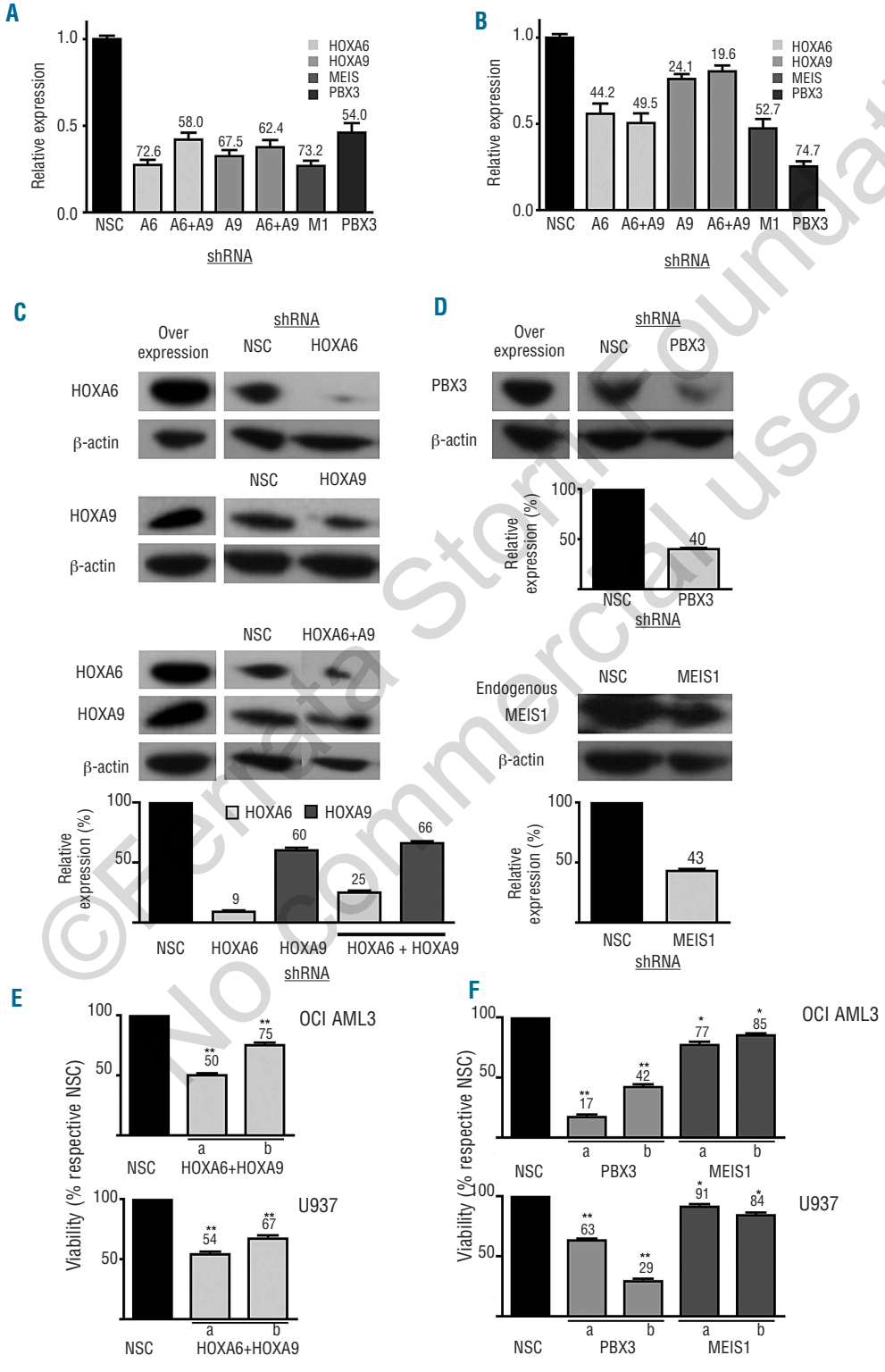


Figure 3. Histograms depicting expression of *HOXA6*, *HOXA9*, *PBX3* and *MEIS1* in (A) OCI-AML3 and (B) U937 cell lines following target gene knockdown, compared to non-silenced controls (NSC). Percentage knockdown values at 72 h of either single genes or combinations as stated are labeled. Mean and standard deviation of three experiments are shown where relative expression was calculated using the $2^{-\Delta\Delta CT}$ method with *18S* rRNA values as the calibrator. Western blot analysis shows reduced protein levels of overexpressed *HOXA/PBX3* constructs or *MEIS1* following incubation with specific shRNA for 48 h in 293FT or U937 cells. Representative data from two or three shRNA sequences per target are shown normalized using β-actin as a loading control and relative to NSC levels (C, D). Reduced viability of (E) HOXA6+HOXA9, PBX3 or (F) MEIS1 knockdown cells (two constructs per gene target a & b) expressed as percentages of respective NSC, measured by trypan blue exclusion with cell counting 24 h post-transfection. Data are representative of n≥4 with mean and standard deviation plotted, $P \leq$ 0.01/0.001 are denoted by */**, respectively.

Significant reductions in cluster formation were observed for both cell lines following knockdown of *HOXA6+A9* (~2-fold) or *PBX3* (~8-fold) (Figure 4D). Notably, reduced MEIS1 levels significantly increased the number of clusters in OCI-AML3 and in U937 cells (>3-fold). Reduced HOXA, PBX3 or MEIS1 levels resulted in reduced cell metabolism and overall colony size in both cell lines as indicated by INT uptake and microscopy (Figure 4E).

HOXA/TALE *knockdown impairs growth of primary cytogenetically normal acute myeloid leukemia cells*

Peripheral blood or bone marrow mononuclear cells from anonymized patients with *de novo* CN-AML (n=5), were directly nucleofected with individual or combined shRNA (two per target) or respective non-silencing control constructs. RQ-PCR analysis of the patients' samples showed comparable levels of expression for the four *HOXA/TALE*

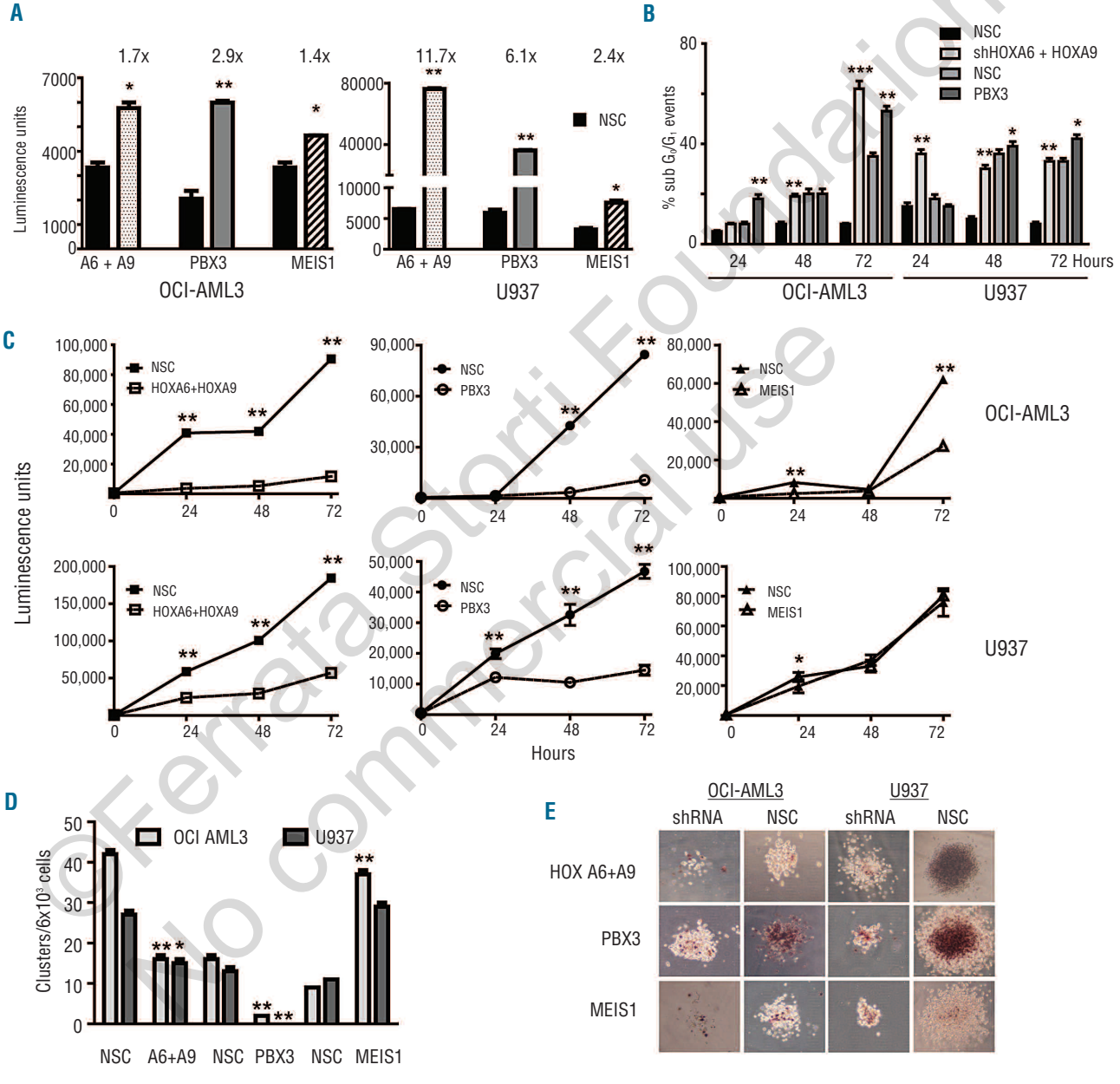


Figure 4. Targeted *HOXA/PBX3* knockdown impairs AML cell growth and function. (A) Histogram plots showing increased caspase 3/7 activity (luminescence) 24 h following shRNA knockdown in U937 and OCI-AML3 cells. (B) Targeted knockdown of HOXA/PBX3 resulted in altered cell cycle dynamics and accumulation of sub G_0/G_1 events measured by propidium iodide staining and flow cytometry analysis. (C) HOXA/PBX3 shRNA reduced overall growth in liquid culture (24-72 h), determined by ATP levels and (D) impaired cluster/colony forming potential following 7 days culture in methylcellulose whereas shMEIS1 resulted in increased clusters compared to respective non-silenced controls (NSC). (E) Reduced cellularity and metabolic activity (INT staining) of colonies following HOXA/TALE knockdown in OCI-AML3 or U937 cells cultured in methylcellulose for 10 days. Graphed data are representative of n≥4 experiments with mean and standard deviation plotted, $P \leq 0.01/0.001$ are denoted by */**, respectively. Images acquired at 200X and presented at 50X are representative of at least two constructs per gene target by lentiviral or retroviral delivery in each cell line.

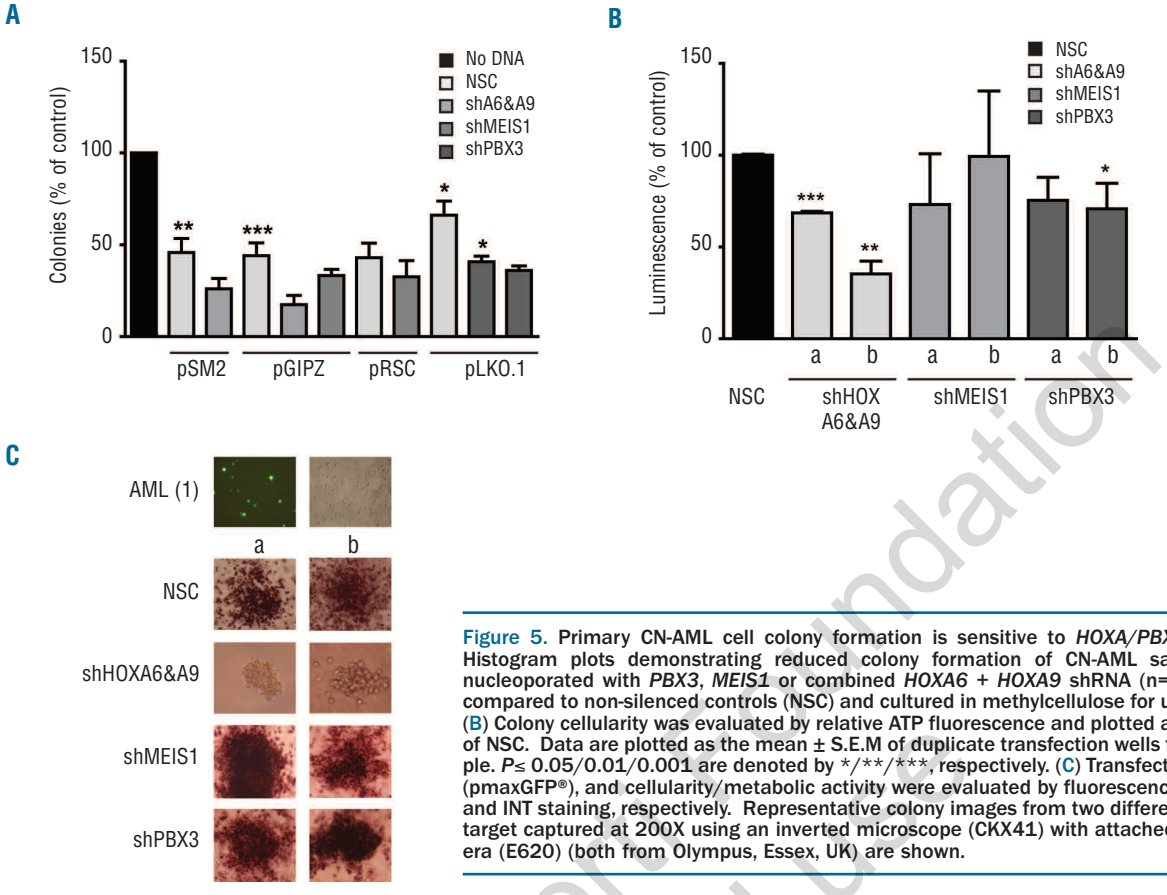


Figure 5. Primary CN-AML cell colony formation is sensitive to *HOXA/PBX3* levels. (A) Histogram plots demonstrating reduced colony formation of CN-AML samples (n=5) nucleoporated with *PBX3*, *MEIS1* or combined *HOXA6* + *HOXA9* shRNA (n=2 per target) compared to non-silenced controls (NSC) and cultured in methylcellulose for up to 14 days. (B) Colony cellularity was evaluated by relative ATP fluorescence and plotted as percentage of NSC. Data are plotted as the mean ± S.E.M of duplicate transfection wells for each sample. *P*≤ 0.05/0.01/0.001 are denoted by *//***, respectively. (C) Transfection efficiency (pmaxGFP®), and cellularity/metabolic activity were evaluated by fluorescence microscopy and INT staining, respectively. Representative colony images from two different shRNA per target captured at 200X using an inverted microscope (CKX41) with attached digital camera (E620) (both from Olympus, Essex, UK) are shown.**

genes (*Online Supplementary Table S4*). Transient knockdown of *HOXA6+A9* and *PBX3* with specific shRNA constructs (n=2 per target) significantly and consistently reduced overall colony number (Figure 5A) and demonstrated reduced overall cellularity as determined by ATP quantification assays (Figure 5B). Transient transfection (~50%) was determined by positivity for green fluorescent protein and knockdown of *HOXA6+A9*, using different shRNA combinations, resulted in reduced cellularity and metabolic activity of colonies compared to non-silenced controls as supported by microscopy with INT staining. Although *PBX3* knockdown resulted in a significant reduction in colony number, *TALE* knockdown did not result in a significant change in colony size or INT staining compared to respective controls (Figure 5C).

HOXA/TALE *knockdown sensitizes acute myeloid leukemia cells to chemotherapy*

We next decided to investigate whether reduced *HOXA/TALE* could sensitize AML cell lines to standard-of-care chemotherapy. Initially, OCI-AML3 and U937 cells were shown to be more sensitive to cytarabine-treatment than Mylotarg using published IC_{50} dosages[34,35] (*Online Supplementary Figure S5*). *HOXA/TALE* knockdown cells demonstrated significantly less growth in liquid culture and a measurable degree of sensitization compared to non-silencing and non-drug-treated controls (Figure 6A and *Online Supplementary Figure S6A*). *MEIS1* knockdown plus cytarabine resulted in a significant reduction in growth of both cell lines (up to 4-fold) compared to non-silenced controls. Cytarabine treatment in combination with *HOXA6+A9* or *PBX3* knockdown resulted in a marked reduction in growth of both OCI-AML3 cells (up to 11-fold) and U937 cells (up to 7-fold) after 24 h of treatment. In colony formation assays cells pre-treated with shRNA for 48 h then exposed to cytarabine or Mylotarg showed dramatically impaired colony-forming capacity (*Online Supplementary Figure S6B*) and cellular metabolism as indicated by reduced INT uptake (Figure 6B) compared to non-silenced controls. Co-treatment with Mylotarg was generally not as cytotoxic as cytarabine. However, *HOXA6+A9* or *PBX3* knockdown in the presence of Mylotarg consistently reduced AML cell growth. Notably, *MEIS1* knockdown plus Mylotarg increased colony formation in both cell lines under these conditions (Figure 6B and *Online Supplementary Figure S6B*). In all shRNA and drug treatment combinations, *HOXA6+A9* or *PBX3* knockdown resulted in impaired cluster/colony formation compared to that of their non-silenced control counterparts.

Discussion

Molecular classification of the heterogeneous CN-AML subgroup is aimed at improving prediction of outcome and response to therapy.[3,4,37] With greater availability of next-generation-sequencing data the list of genetic markers[38] is likely to increase. Accumulation and integration of these data will ultimately lead to greater understanding of complex genetic landscapes that may stratify patients' treat-

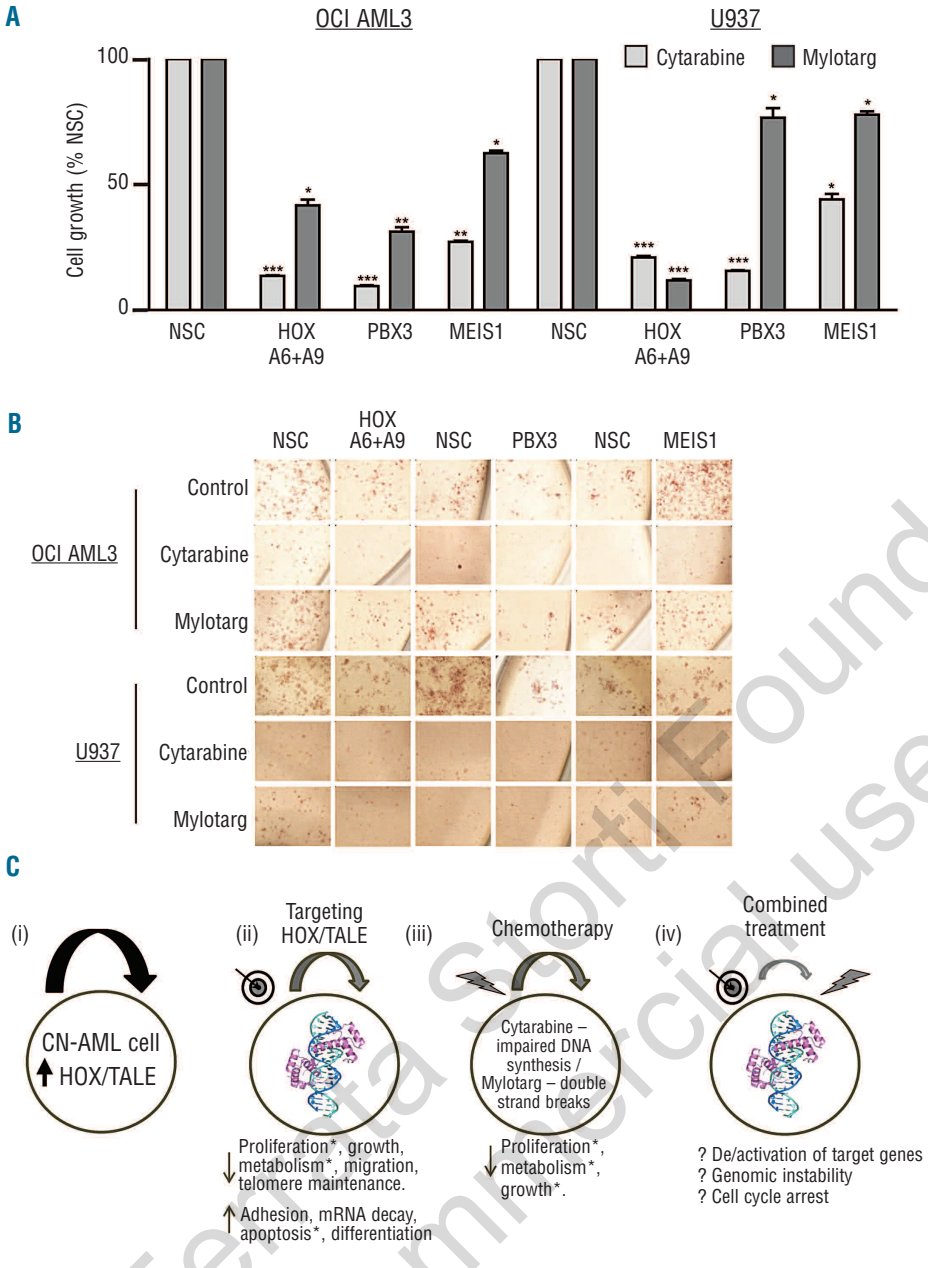


Figure 6. ***HOXA/PBX3*** **knockdown sensitizes AML cells to chemotherapy. (A) OCI-AML3 and U937 cell growth was quantified in liquid culture by ATP levels following pre-conditioning knockdown with the indicated shRNA (48 h) ± cytarabine or Mylotarg treatment for a further 24 h. Data are presented as percentages of the respective non-silenced control cells (NSC).** ***P*** **≤ 0.05/0.01/0.001 are denoted by */**/***, respectively. (B) Pre-conditioned and treated cells were also seeded in methylcellulose ± drug, incubated for 10 days and stained with INT for 16 h prior to microscopy. Representative colony images captured at 200X using an inverted microscope (CKX41) with attached digital camera (E620) (both from Olympus, Essex, UK) are shown. (C)** ***HOXA6***, ***HOXA9***, ***PBX3*** **and** ***MEIS1*** **are a core part of the** ***HOXA/TALE*** **overexpression signature in CN-AML and may contribute to maintenance of leukemia clones (i). We present evidence indicating that targeting specific** ***HOX/TALE*** **complex components reduces growth of primary CN-AML and cell lines via intrinsic processes and cell–cell / matrix interactions identified by altered gene expression and phenotypic assays* (ii). Standard-of-care chemotherapeutics inhibit DNA synthesis and repair pathways (iii) and combined treatment further reduces CN-AML cell survival by mechanisms which may include (epi)genetic differences, altered cell cycle, destabilization of key cellular complexes or a combination of action (iv).**

ment. However, some candidate genes with no demonstrable lesion are dysregulated in AML. Here we investigate whether a subset of homeobox genes can also be used to stratify subgroups of patients and, more importantly, could be candidates for targeted therapy.

A highly statistically significant core *HOXA/TALE* gene signature (*HOXA6, HOXA9, PBX3, MEIS1*) that partitions favorable risk from intermediate risk AML was identified in a large cohort of patients (n=420) and validated by RQ-PCR (Figure 1). The candidate signature was further validated in an independent AML dataset.[28] CN-AML patients (n=114) were clustered based on *HOX/TALE* expression, not dependent on known mutations, and *HOXA*^{Hi}*/PBX3*^{Hi}*/MEIS1*^{Lo} was shown to be associated with poorer overall survival using a custom array (Figure 2). The identification of a four-gene signature with the ability to stratify CN-AML patients and indeed act further as a prognostic marker, irrespective of current molecular markers, may provide for higher throughput screening in a clinical setting in which large scale analysis is prohibitive.

Knockdown of *HOXA6* or *HOXA9* resulted in minimal compensation from the *HOX* network, with only a moderate increase in *HOXA10* and decrease in *HOXB2* expression noted (*Online Supplementary Table S5*). Combined *HOXA6* plus *HOXA9* knockdown, or targeted *PBX3* knockdown alone, activated apoptotic pathways and resulted in markedly reduced AML cell growth and colony formation independently of *NPM1* or *DNMT3A* mutations (Figures 3, 4, and 5 and *Online Supplementary Figure S4*). Notably, targeted knockdown of MEIS1 in primary cells or cell lines enhanced colony formation, indicating a dose-dependent role in AML colony formation or maintenance congruent with the *HOX*^{Hi}*/PBX3*^{Hi}*/MEIS1*^{Lo} phenotype. MEIS1 is known to have a role in colony formation[22,39] and is essential

for the induction and maintenance of MLL leukemogenesis. The data presented here suggest that further investigations will be required to understand the complex role of MEIS1 in CN-AML.

AML cell lines primed by *HOXA/PBX3* knockdown demonstrated greater sensitivity to standard chemotherapies than mock-treated lines or those primed with non-silencing controls (Figure 6A,B and *Online Supplementary Figure S6*). In methylcellulose models, directly evaluating stem/progenitor cell colony-forming potential, the combination of *HOXA/PBX3* knockdown and Mylotarg treatment proved highly effective in reducing cluster formation, particularly in OCI-AML3 cells. The difference in response may be due to mutation status or the level of expression of CD33, the primary route for Mylotarg internalization. The *NPM1*-mut and *DNMT3A*-mut OCI-AML3 cells are reported to have elevated levels of CD33-antigen compared to U937 cells.[40,41]

In a recent study Li *et al.* reported that *mir181b* overexpression inhibited an MLL model of leukemia in part by decreasing *HOXA7*, *A9*, *A11* and *PBX3* levels.[42] A follow-up study indicated that the HOXA/PBX3 interaction is critical for MLL-induced leukemia[43]. The data presented herein demonstrate that CN-AML cells are also sensitive to *HOXA/PBX3* levels and indicate that clinically practical approaches resulting in defined targeting of the HOXA-PBX3-MEIS1 axis alone, as next generation therapy, or in combination with standard chemotherapy (Figure 6C), may provide benefit to intermediate risk, CN-AML patients.

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