High levels of the adhesion molecule CD44 on leukemic cells generate acute myeloid leukemia relapse after withdrawal of the initial transforming event

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Multiple genetic hits are detected in patients with acute myeloid leukemia (AML). To investigate this further, we developed a tetracycline-inducible mouse model of AML, in which the initial transforming event, overexpression of HOXA10, can be eliminated. Continuous overexpression of HOXA10 is required to generate AML in primary recipient mice, but is not essential for maintenance of the leukemia. Transplantation of AML to secondary recipients showed that in established leukemias, ~80% of the leukemia-initiating cells (LICs) in bone marrow stopped proliferating upon withdrawal of HOXA10 overexpression. However, the population of LICs in primary recipients is heterogeneous, as ~20% of the LICs induce leukemia in secondary recipients despite elimination of HOXA10-driven overexpression. Intrinsic genetic activation of several proto-oncogenes was observed in leukemia-resistant cells to inactivation of the initial transformation event. Interestingly, high levels of the adhesion molecule CD44 on leukemic cells are essential to generate leukemia after removal of the primary event. This suggests that extrinsic niche-dependent factors are also involved in the host-dependent outgrowth of leukemias after withdrawal of HOXA10 overexpression event that initiates the leukemia.

Keywords: acute myeloid leukemia; homeobox; HOXA10; CD44; leukemia addiction; leukemia-initiating cells

Introduction

Leukemia is a heterogeneous disease in which hematopoietic stem and progenitor cells acquire aberrant regulation of prominent transcription factors or genetic lesions that lead to a block in differentiation, increased self-renewal and dysregulated proliferation. The HOX family of Homeobox genes encodes transcription factors that regulate hematopoiesis and are expressed in specific compartments of the hematopoietic hierarchy.1–3 Several HOX genes have been directly implicated in hematological malignancies as supported by the frequently observed elevation of HOXA gene expression in acute myeloid leukemia (AML) patients.4–6 A more direct involvement for HOX genes in leukemia is supported by their frequent fusion to the nucleoporin gene NUP98 in leukemia patients.7,8 Transgenic and retroviral transplantation mouse models have established that deregulation of HOXA genes9–11 or NUP98-HOXA fusion genes12,13 initiates AML. HOX proteins contain a highly conserved DNA-binding homeodomain flanked by variable sequences that influence the DNA-binding specificity, by coordinating interactions to cofactors such as PBX and MEIS.14,15 The cofactor MEIS1 has been described to synergize with multiple native HOX and NUP98-HOXA fusion genes in promoting a differentiation block of HOXA-transformed myeloid progenitors accelerating the initiation of the leukemia in mice.9,11,16

Several AML mouse models have recently identified a role for oncogenes in survival/maintenance of leukemia in addition to the initiation of leukemogenesis. This suggests that the growth of leukemic cells in vivo depends on continued activity of the oncogene and that efficient targeting of this activity may be a promising avenue to cure AML. HOX9 has been described to be required for survival in human mixed lineage leukemia (MLL)-rearranged acute leukemias.17 Similarly, a mouse model showed that AML induced by conditional expression of MLL-ENL can be cured by oncogene ablation, despite additional acquisition of complex genetic abnormalities.18

HOXA10 is expressed in the most primitive hematopoietic cell compartment in normal hematopoiesis, and its overexpression increases proliferation of human hematopoietic progenitor cells.19 By using a tet-operator mouse model where the expression level of HOXA10 can be tightly regulated, we recently characterized the role of HOXA10 as a critical regulator for normal hematopoietic stem cells and erythroid/megakaryocyte development in vivo.20 In addition, it is known that deregulation of HOXA10 initiates AML, as a significant proportion of recipient mice transplanted with retroviral vector-transduced HOXA10-overexpressing grafts developed AML with a latency of 19–50 weeks.11 Therefore, we asked whether our tet-operator system-driven HOXA10 mice would develop leukemia after long-term treatment with doxycycline to force expression of HOXA10. The development of leukemia was not observed even after doxycycline treatment for 1 year, suggesting that the HOXA10 expression level generated in these mice might not be optimal for leukemic transformation in vivo.20 To develop a mouse model of AML using the tet-operator system-driven HOXA10 mice, we transduced the HOXA10-inducible bone marrow (BM) cells with the cofactor MEIS1 and showed that MEIS1 can potentiate leukemogenesis induced by moderate expression of HOXA10. However, the long latency period required to induce leukemia suggests that the cooperation with MEIS1 is not sufficient to transform cells, but that additional hits might be required for full-blown AML to develop.

Recently, a mouse model using inducible expression of the MLL-ENL oncprotein was used to show that withdrawal of induction of this powerful oncprotein leads to elimination of the malignant clone because it depends entirely on this protein.18 Similarly, a recent report shows that another powerful
oncoprotein, HOXA9, is required for survival in human MLL-rearranged acute leukemias, suggesting that targeting HOXA9 may be a therapeutic option. Even if oncoproteins have the potential to drive hematopoietic cells into full malignant progression, the latency period to induce AML can be several months, indicating that additional events are required for leukemogenesis. Combination/cooperation of specific genetic events is known to be involved in the multi-step evolution of premalignant cells to full-blown AML. This concept is supported by clinical evidence from a variety of leukemias, in which multiple genetic hits are often diagnosed in AML patients. Moreover, mouse transplantation models have shown that several genetic changes collaborate to induce AML and addressed whether elimination of the initial oncogenic event (HOXA10 overexpression) caused by an oncoprotein that has a relatively weak activity will lead to elimination of established leukemia in vivo or whether acquisition of secondary events by leukemic cells renders them resistant to the removal of the oncoprotein overproduction that initiated the disease. The findings show that in 20% of cases, secondary mutations in additional proto-oncogenes and overproduction of the adhesion molecule CD44 contribute toward rendering the leukemic cells resistant to inactivation of the initial transformation event.

Materials and methods

Generation and screening of transgenic mice

The generation of the Rosa26-rtTA-nls/tetO-HOXA10 mouse model and its characterization has been described previously. Genomic DNA was isolated from tail biopsies and analyzed by polymerase chain reaction (PCR) for the detection of tetO-HOXA10 and Rosa26-rtTA-nls. Lund University’s Ethical Committee approved all animal experiments.

Isolation of BM cells

Femurs and tibias were harvested from 7- to 12-week-old Rosa26-rtTA-nls/tetO-HOXA10-positive mice and littermate control mice (Ly5.2). Bones were crushed, the cell suspension was filtered through a 100-μm pore size Cell Strainer filter (Falcon; Becton Dickinson, Franklin Lakes, NJ, USA) and the lineage-negative (Lin−) population was depleted from BM cells, with a cocktail of antibodies: rat anti-mouse-B220, -Gr1, -Mac1, -CD4, -CD5, -CD8 and -Ter119 (Becton Dickinson). Anti-rat immunoglobulin G-conjugated immunomagnetic beads (Dynal Biotech, Oslo, Norway) were used for enrichment of the Lin− population. For prestimulation before the retroviral transduction, cells were cultured in X-Vivo medium for 48 h, supplemented with 100 U/ml PenStrep (Sigma, St Louis, MO, USA), 1% glutamine, 1% bovine serum albumin, 50 ng/ml murine stem cell factor, 10 ng/ml murine interleukin-3 and 50 ng/ml human interleukin-6.

Retroviral transduction and BM transplantation

The MEIS1 retroviral vector expressing both the MEIS1 cofactor together with green fluorescent protein (GFP) was provided by Dr Corey Largman (Department of Medicine, University of California, San Francisco, CA, USA) and Dr Jeffrey Lawrence (UCSF, Roche Molecular Systems, San Francisco, CA, USA). Vectors were transfected into Phoenix Amphi cells (Nolan Lab, Stanford University, Palo Alto, CA, USA), and supernatants were harvested for transduction of Rosa26-rtTA-nls/tetO-HOXA10 Lin− cells. A total of 2 × 105 transduced Lin− cells (Ly5.2) were mixed with 2 × 105 support BM cells (Ly5.1) and transplanted into the tail vein of lethally irradiated (900 cGy) C57BL/6 × B6SJL recipient mice (Ly5.1/Ly5.2). Recipient mice were continuously fed doxycycline (0.2 g/ml) in the drinking water to induce the expression of HOXA10 or ciproxine as a negative control. For the limiting dilution assay, leukemic cells (Ly5.2) were transplanted in escalating doses: 1, 2 or 8 × 103 cells with 2 × 105 support BM cells (Ly5.1) into lethally irradiated secondary recipient mice. To study the role of CD44, 2 × 104 leukemic cells were transplanted with 2 × 105 support BM cells into lethally irradiated secondary recipient mice. Different populations of the CD44 surface marker of leukemic cells from primary recipient mice were sorted (DiVo; Becton Dickinson) and sorted cells were injected in the tail vein of lethally irradiated secondary recipient mice fed doxycycline or ciproxine. For inhibition of CD44, leukemic cells were incubated during 20 min with high dose of the anti-CD44 phycoerythrin (PE) (20 μg/ml) or control anti-immunoglobulin G2a PE (20 μg/ml) before transplantation.

Flow cytometry

Engraftment and expansion of the transduced population (Ly5.2) was monitored by gating Ly5.1-negative cells using fluorescence-activated cell sorting (FACS) with PE-CD45.1 antibody and measuring the proportion of GFP+ cells. The phenotype of the leukemia was analyzed in peripheral blood or BM cells by staining with Mac1 adenomatus polyposis coli and c-Kit PE antibodies (Becton Dickinson). 7-Amino-actinomycin D was used for the detection of non-viable cells (Sigma). Stained cells were run throughout a FACS Calibur (Becton Dickinson Biosciences, Sparks, MD, USA) and the subsequent data were analyzed with the FlowJo software (Tree Star, Ashland, OR, USA). For the lineage staining analysis, the Lin− population from BM cells was stained with a cocktail of antibodies: rat anti-mouse -B220, -Gr1, -Mac1, -CD4, -CD5, -CD8 and -Ter119 (Becton Dickinson) and goat anti-rat Tricolor antibody (Caltag Lab, Burlingame, CA, USA). Sca-1 PE-Cy5.5 and c-Kit PE antibodies were used to gate granulocyte–monocyte progenitors, common myeloid progenitors and megakaryocyte–erythroid progenitors within the Lin− population. For cell cycle analysis, bromodeoxyuridine adenomatus polyposis coli anti-body and 7-AAD were used using Bromodeoxyuridine Flow Kit (Becton Dickinson Pharmingen, San Diego, CA, USA). For CD44 surface glycoprotein expression, anti-CD44 PE antibody (Becton Dickinson) was used.

Hematology and histology

For in vivo studies, peripheral blood from the tail vein was collected in heparin (LEO Pharma, Thornhill, ON, USA) and put in a 96-well U-bottom plate. Following centrifugation, the supernatant was poured off, erythrocytes were lysed with ammonium chloride (NH₄Cl; Stem Cell Technologies, Vancouver, BC, Canada) and the cells were stained with conjugated antibodies for FACS analysis. Hematological parameters of peripheral blood were monitored to control the development of leukemia (XX-21N; Sysmex, Mundelein, IL, USA). For histology, smears of peripheral blood were generated and cells from BM, spleen and liver were subjected to cytospin onto glass slides, and subsequently stained with May–Grünwald Giemsa for microscopic examination.
Western blot analysis

One million cells were lysed in 50 µl Laemmli buffer (BioRad, Hercules, CA, USA). Proteins were separated by electrophoresis (10% SDS-polyacrylamide gel electrophoresis) and transferred onto nitrocellulose membrane. Specific proteins were detected by an anti-HOXA10 antibody (Sc-17158; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and an anti-matrix metalloproteinase 9 (MMP9) antibody (ab38898; Abcam, Cambridge, UK). For CD44 staining, an anti-CD44 (14-0441; eBioscience, San Diego, CA, USA) was used on PNGase F1 deglycosylated and heat-denatured proteins. Anti-actin (Ab-5; Becton Dickinson Biosciences) was used as a loading control. After blotting with a horseradish peroxidase-conjugated secondary antibody, chemiluminescence activity was detected using the enhanced chemiluminescence Advance Western blot detection kit (GE Healthcare, Piscataway, NJ, USA). Gel images were analyzed using Image J (Wright Cell Imaging Facility, www.umnresearch.ca/wcil) for quantification.

Microarray assay

Total RNA from BM cells of ciproxine- and doxycycline-receiving recipient mice suffering from leukemia was extracted from BM cells (Qiagen, Valencia, CA, USA). RNA concentration and integrity were, respectively, determined by NanoDrop spectrophotometry (NanoDrop Technologies, Wilmington, DE, USA) and a Bioanalyzer (Agilent Technology, Waldronn, Germany). Reverse transcription was carried out using Illumina TotalPrep RNA Amplification Kit (Applied Biosystems/Ambion, Austin, TX, USA) and hybridization using Mouse WG-6 Expression v1. v1.2 BeadChips (Illumina, San Diego, CA, USA) interrogating more than 45,000 transcripts were performed by the Svergene Center for Integrative Biology at Lund University (SCIBLU). Signal intensity data was acquired with a BeadArray Scanner (Illumina) and experimental quality was performed using the BeadStudio Software (Illumina). The raw expression values mRNA abundance were submitted to the European Bioinformatics Institute (EMBL-EBI) ArrayExpress repository (accession no. E-MEXP-2491). Validation of proto-oncogene transcriptional modulation was performed by real-time PCR (LightCycler; Roche Diagnostic, Mannheim, Germany) with primers described in Supplementary Table 1.

Mass spectrometry

The experimental procedure has been described in detail.30 Mass spectrometer (ThermoFisher Scientific, Bremen, Germany) was used to collect proteomics data from tryptic digests of leukemic whole-cell lysates. Identification of proteins by mass spectrometry was performed by matching experimental mass spectra against calculated spectra of all possible peptides in a protein database.

Statistical analyses

All data are expressed as the means ± s.e.m. We assessed differences between the groups by Student’s unpaired t-test. Statistical analysis of survival curves was performed using the Mantel–Haenszel log-rank test.

Results

Generation of leukemia in mice following MEIS1 and HOXA10 expression

HOXA10 transgenic mice were crossed with Rosa26-rtTA-nls mice to generate the Rosa26-rtTA-nls/tetO-HOXA10 mouse model.20 With this tet-operator system, the expression level of HOXA10 can be tightly regulated in hematopoietic cells using doxycycline.20 These cells were then transplanted together with support cells to lethally irradiated recipients that were given doxycycline (0.2 g/ml) in the drinking water continuously for induced expression of HOXA10 or ciproxine as a negative control for HOXA10 induction (Figure 1a). All mice were then monitored for the development of leukemia. Induced expression of HOXA10 mediated by a tet-operator system does not transform BM cells in vivo, as doxycycline-treated recipients of inducible HOXA10 BM cells that had been transduced with the empty control vector remained healthy throughout a whole year (Figure 1b). This is in agreement with previous observations,20 that failed to detect leukemia in these same mice after long-term induction of HOXA10. The failure to develop leukemia could be due to the level of expression in the in vivo setting that might not be optimal for leukemic transformation. To create a leukemia model, we harvested BM cells from the Rosa26-rtTA-nls/tetO-HOXA10 mice and transduced them with an MEIS1 expressing vector. Seventy percent of recipient mice that received MEIS1 and doxycycline developed leukemia, from around 12 weeks following transplantation. The average survival for mice in this group was 26 weeks (Figure 1b). We have therefore confirmed previous findings showing that MEIS1 interacts with the HOXA10 protein in leukemogenesis and we show here that MEIS1 is required to initiate leukemia following tet-promoter-inducible expression of HOXA10. The disease has characteristics of acute leukemia, with a greater than eight-fold increase of total white blood cells in peripheral blood of moribund animals compared with healthy animals that have been fed ciproxine and killed 50 weeks after the transplantation (Figure 1c). In addition, the leukemic mice show lower red blood cell, hemoglobin and platelets levels, probably due to the outgrowth of leukemic cells and suppression of normal hematopoiesis. Symptomatic mice had severe splenomegaly (Figure 1d) and infiltrations of myeloid progenitors in different hematopoietic tissues, peripheral blood, BM, spleen and non-hematopoietic tissues like the liver (Figure 1e). Recipient mice that are given doxycycline continuously (HOXA10-4) and have leukemia exhibit a clear upregulation of HOXA10 protein levels in BM cells (Figure 1f), whereas only endogenous levels of HOXA10 protein are detected in mice transplanted with Rosa26-rtTA-nls/tetO-HOXA10 cells that are given ciproxine (HOXA1038). Furthermore, no leakiness of the tet-promoter was observed in the HOXA1039 situation, as the HOXA10 protein levels detected was similar to the endogenous HOXA10 level found in unmanipulated wild-type mice (Figure 1f). FACs analysis of BM cells from moribund mice developing leukemia revealed a large accumulation of myeloblastic cells (Mac1c-Kitc-), which correspond to a substantial expansion (>80%) of donor Ly5.1tetO-HOXA10 cells (Figure 1g), and furthermore confirmed transformation of BM with a complete disappearance of LSK stem cells (Lin-c-Kitc+Sca-1c-) and a total reconstitution with BK progenitors (Lin-c-Kitc+Sca-1c-), which in turn largely consist of granulocyte–monocyte progenitors (CD34cFcγRc) (Figure 1h). Twenty percent of the LICs induce leukemias in secondary transplant mice upon withdrawal of HOXA10 overexpression

Leukemia-initiating cells (LICs) are capable of limitless self-renewal and are responsible for the maintenance of leukemia. Therefore, we asked whether all clones among the population of LICs are dependent on continuous expression of the HOXA10
transgene or whether the LICs’ population is heterogeneous with respect to the dependence on the primary event that has generated the leukemia.

We previously described that the HOXA10 transgene transcription and protein production is inducible and reversible in this model and can be terminated upon withdrawal of doxycycline.20 GFP+ leukemic cells from two independent AML donor mice (nos. 1 and 2) were injected into lethally irradiated secondary recipients that were fed either doxycycline for continuous induced expression of HOXA10 or ciprofloxacin to eliminate the expression (Figure 2a). We asked how many LICs per recipient were required to cause disease by transplanting limiting dilutions of the leukemic cells derived from mice, which continuously received doxycycline together with support cells. We tested the repopulative capacity of LICs from two independent donors. Time to onset of leukemia following BM transplantation and the number of mice that developed AML were quite dependent on the quantity of leukemic cells injected (Figure 2b). After enumerating the LICs per donor and testing the proportion of mice that developed leukemia in secondary transplant recipients, we could determine that the percentage of leukemic cells that represent LICs capable of generating

Figure 1  Generation of AML in primary recipients following MEIS1 transduction requires continuous induction of HOXA10 overexpression. (a) Illustration of the method used to generate AML in primary recipient mice. Mice were transplanted with BM cells from the Rosa26-rtTA-nls/tetO-HOXA10 mice, transduced either with an MEIS1-expressing vector (MEIS1) or an empty control vector (mig). Following transplantation, mice were continuously fed doxycycline (doxy) in the drinking water (HOXA10OFF) or doxycycline transiently during 5 weeks (transient doxy). (b) Kaplan–Meier plot of recipient survival over time; n = 10 mice for doxy + MEIS1 and n = 6 mice for all the other groups. P-value is measured by Mantel–Haenszel log-rank test. (c) Analysis of peripheral blood parameters, white blood cell (WBC), red blood cell (RBC), hemoglobin (HGB) and platelets (PLT) observed in moribund mice from the doxycycline group (HOXA10OFF), n = 6) and healthy mice killed 50 weeks after the transplantation from the control group (HOXA10OFF, n = 6). (d) Severe splenomegaly in mice developing AML, representative pictures of two spleens of each group and weight (n = 5). (e) Giemsa stainings showing infiltration of myeloid progenitors in hematopoietic organs, in the peripheral blood (PB), bone marrow (BM), spleen and liver in mice with AML. Data are representative of five mice. (f) Western blot of BM cells showing induced expression of the HOXA10 protein in moribund mice receiving doxycycline (HOXA10OFF), in contrast to healthy mice killed 50 weeks after the transplantation from the ciprofloxacin group (HOXA10OFF) or wild-type control mice (Wt). Representative western blot (left panel) and presentation of the relative increase (right panel, n = 5 for each group). (g) FACS analysis showing robust reconstitution of BM with the donor tetO-HOXA10 mice (Ly5.1−) expressing MEIS1 (GFP+) for mice receiving continuous doxycycline (HOXA10OFF), whereas no accumulation of donor cells is observed in mice given ciprofloxacin in the drinking water (HOXA10OFF). (h) FACS analysis showing the transformation of Lin− cells for mice suffering from AML. Representative FACS (left panel) and pooled data from five mice (right panel). In this figure, data show means ± s.e.m. *P<0.01, as measured by unpaired t-test. n.s., nonsignificant variation.
leukemias was 0.117% for no. 1 and 0.082% for no. 2 in the HOXA10ON condition. The percentage of LICs that can generate secondary leukemias was 0.023% for no. 1 and 0.019% for no. 2 in the HOXA10OFF condition. In conclusion, approximately 20% of LICs (average of two donors; Figure 2c and Supplementary Table 2) were able to cause secondary leukemias after removal of the primary events.

Maintenance of HOXA10 overexpression is not essential for growth of resistant leukemic cells in vivo

When we analyzed further mice transplanted with 2000 leukemic cells, ~80% (n = 21/26) of the ciproxine-treated control mice stayed healthy over the 30 weeks they were monitored, showing that a large majority of LICs from the primary recipient mice require continuous overexpression of HOXA10 to develop leukemia in secondary recipients. Using FACS analysis, we could not detect a significant expansion of the donor cells in healthy mice, and there were no detectable GFP+ cells in the BM after 30 weeks (Figure 2d). The total clearance of the LICs in BM was moreover confirmed since the re-administration of doxycycline in the drinking water of these healthy mice during 10 weeks did not re-activate expansion of the LICs. This was confirmed by western blot showing expression of the HOXA10 protein in BM of healthy mice and by analysis of secondary recipient mice BM transplanted with 2000 leukemic cells showing expansion of GFP+ cells only for ~20% of mice that developed AML. Increase in the WBC level (means ± s.e.m; *P < 0.001 compared with doxycycline (OFF)) recipients. Quantification of transplanted leukemia to induce 50% of leukemic mice, when HOXA10ON (EC50ON) or HOXA10OFF (EC50OFF) recipients. The percentage of LICs in the population of leukemic cells is calculated for the HOXA10ON (LICON) and the HOXA10OFF (LICOFF) conditions as 1 LIC / total number of cells injected to induce EC50. The percentage of LICs resistant upon withdrawal of the initial HOXA10-overexpression event is calculated as percent of LICON compared with LICOFF (total LICs). 

Figure 2 Twenty percent of the LICs generate a leukemia after withdrawal of HOXA10-induced expression in secondary transplanted recipients. (a) Schematic overview of the transplantation strategy into secondary recipient mice. Leukemic cells were transplanted into lethally irradiated mice that were given either doxycycline for continuous expression of HOXA10 (HOXA10ON) or ciproxine as a negative control (HOXA10OFF). (b) Kaplan–Meier plot of survival mice over time when mice were transplanted with 1000, 2000 and 8000 leukemic cells. *P-value is measured by Mantel–Haenszel log-rank test. (c) Limiting dilution assay for quantification of transplanted leukemic cells to induce leukemia in 50% of the mice in HOXA10ON (EC50ON) or HOXA10OFF (EC50OFF) recipients. Quantification of transplanted leukemia to induce 50% of leukemic mice, when HOXA10ON (EC50ON) or HOXA10OFF (EC50OFF) recipients. The percentage of LICs in the population of leukemic cells is calculated for the HOXA10ON (LICON) and the HOXA10OFF (LICOFF) conditions as 1 LIC / total number of cells injected to induce EC50. The percentage of LICs resistant upon withdrawal of the initial HOXA10-overexpression event is calculated as percent of LICON compared with LICOFF (total LICs). 

(a) Representative western blot showing expression of the HOXA10 protein in BM of healthy mice and by analysis of secondary recipient mice BM transplanted with 2000 leukemic cells showing expansion of GFP+ cells only for ~20% of mice that developed AML. Increase in the WBC level (means ± s.e.m; *P < 0.001 compared with doxycycline (OFF)) recipients. Quantification of transplanted leukemia to induce 50% of leukemic mice, when HOXA10ON (EC50ON) or HOXA10OFF (EC50OFF) recipients. The percentage of LICs in the population of leukemic cells is calculated for the HOXA10ON (LICON) and the HOXA10OFF (LICOFF) conditions as 1 LIC / total number of cells injected to induce EC50. The percentage of LICs resistant upon withdrawal of the initial HOXA10-overexpression event is calculated as percent of LICON compared with LICOFF (total LICs). (d) FACS analysis of secondary recipient mice BM transplanted with 2000 leukemic cells showing expansion of GFP+ cells only for ~20% of mice that developed AML. Increase in the WBC level (e) and enlarged spleen size (f) in leukemic mice. HOXA10ON (ON; n = 10), HOXA10OFF (OFF; n = 3, donor no. 1, n = 2, donor no. 2) and healthy mice from the ciproxine group killed at week 30 (n = 10). (g) Analysis of cell cycle stages showing a reduction in the proportion of cells in the S phase and an increased percentage of cells in the G0/G1 phase in AML from mice given ciproxine (HOXA10OFF) compared with doxycycline (HOXA10ON). Representative FACS analysis is shown (left) and pooled data from four mice per group are displayed (right). (h) Representative western blot showing expression of the HOXA10 protein in BM of healthy mice.

Leukemia
Figure 3 Leukemias generated upon withdrawal of HOXA10 overexpression exhibit elevated expression of several proto-oncogenes. (a) Microarray scatter plots showing that the global RNA expression profile of leukemic cells from the HOXA10OFF secondary recipient mice are similar to leukemic cells generated from primary donors. (b) Microarray scatter plots showing upregulation of several proto-oncogenes in leukemias from HOXA10OFF secondary recipient mice when compared with leukemic cells from HOXA10ON secondary recipient mice. Scatter plots show normalized raw data. Line represents a fold change value of 5. (c) Validation of the transcriptional activation of several proto-oncogenes observed in resistant leukemic cells by real-time PCRs. Data are expressed in arbitrary units (AU): real-time PCRs from HOXA10OFF are normalized to HOXA10ON.

(Figure 2e) and splenomegaly (Figure 2f). Using FACS to detect cycling and non-cycling leukemic cells ex vivo, we observed that HOXA10OFF leukemic cells had an increased proportion of cells in the G0/G1 phase and a decreased proportion of cells in the S phase (Figure 2g). This clearly shows that the cell cycling capacity is greatly reduced when overexpression of HOXA10 is stopped, but this does not affect outgrowth capacity of resistant leukemic cells. High levels of the HOXA10 protein were observed in leukemic mice under the administration of doxycycline, whereas only endogenous expression of the HOXA10 protein was detected in BM from leukemic mice fed ciproxine (Figure 2h). This clearly provides evidence that maintenance of HOXA10 overexpression is not essential for the growth of resistant leukemic cells.

Leukemias generated upon withdrawal of HOXA10 overexpression exhibit elevated expression of several proto-oncogenes

A clonality analysis of BM cells was carried out by linear amplification-mediated PCR to identify if resistance might be explained by the expansion of specific leukemic cells owing to insertional mutagenesis caused by retroviral integration in genomic DNA. In secondary recipient mice, we observed that leukemias of the HOXA10ON and the HOXA10OFF groups were both induced by expansion of LICs generated in primary recipient mice (Supplementary Figure 1). Assignment of sequencing data to mouse chromosomes shows that the retroviral integration sites are at the 5’ side of the eukaryotic translation elongation factor 1 alpha 1 (EEF1A1) gene for donor no. 1 and at the 5’ side of RAB1, a member of RAS oncogene family, for donor no. 2. However, integration did not seem to affect expression of these target genes, as transcription of EEF1A1 and RAB1 genes was not affected in leukemias upon withdrawal of HOXA10 expression. Very high levels of MEIS1 were still detected in those leukemic cells (Figure 2h). High levels of MEIS1 can theoretically cooperate with dysregulation in the expression of other Hox genes, but we never observed dysregulation of several tested Hox genes in those samples (Hoxa1, Hoxa5, Hoxa7 and Hoxa9 and endogenous murine Hoxa10; Supplementary Figure 1). Next, the cytogenetic status of leukemic cells resistant upon withdrawal of the HOXA10 expression was characterized by molecular karyotyping using a murine comparative genomic hybridization array. Interestingly, we could not detect any difference in whole chromosome gain or loss, unbalanced subtelomeric rearrangements or
HOXA10 overexpression, we selected genes showing great degree of dysregulation (fold-change value > 5). We found that Jun, Fos and Egr1 genes were activated in different samples of the HOXA10\textsuperscript{OFF} group, with variability among the three tested donors (Figure 3a). Dysregulation of these proto-oncogenes was furthermore confirmed by real-time PCRs to be donor specific (Figure 3c).

High levels of CD44 on leukemic cells correlate with AML in secondary transplant mice upon withdrawal of the initial HOXA10 transforming event

To further investigate if another possible mechanism is involved in leukemia, upon withdrawal of the primary oncogenic event, we performed proteomic analysis using mass spectrometry to define proteins differently expressed. Interestingly, we observed that leukemias generated upon removal of the primary event produced high levels of several proteins involved in cell–cell and cell–matrix interactions (Figure 4a). The CD177 and CD44 proteins expressed on the cell surface participate in cell–cell and cell–matrix interactions (Figure 4a).

High levels of CD44 on leukemic cells correlate with AML in secondary transplant mice upon withdrawal of the initial HOXA10 transforming event

Figure 4 High levels of the CD44 transendothelial marker correlate with increased leukemia rate generated by LICs after withdrawal of the initial HOXA10 transforming event. (a) Functional clustering of proteins detected by mass spectrometry comparing leukemic cells from HOXA10\textsuperscript{ON} secondary recipient mice to leukemic cells from the HOXA10\textsuperscript{OFF} group (two donors are tested: nos. 1 and 2). The secondary leukemias show high expression of subset of proteins involved in transendothelial migration, whereas proteins from other functional clusters do not exhibit a difference. (b) Western blot showing high levels of the CD44 protein in leukemic cells from the HOXA10\textsuperscript{OFF} mice. A representative western blot is displayed using recipients of cells from donors: nos. 1 and 2. (c) Transcriptional analysis of CD44 and MMP9 genes. Results are expressed in arbitrary units (AU); raw data from HOXA10\textsuperscript{ON} microarrays are normalized to HOXA10\textsuperscript{OFF}. Data show means ± s.e.m. of three donors.

Figure 3a-c Western blots showing high levels of CD44 protein in leukemic cells from the HOXA10\textsuperscript{OFF} mice. A representative western blot is displayed using recipients of cells from donors: nos. 1 and 2. (c) Transcriptional analysis of CD44 and MMP9 genes. Results are expressed in arbitrary units (AU); raw data from HOXA10\textsuperscript{ON} microarrays are normalized to HOXA10\textsuperscript{OFF}. Data show means ± s.e.m. of three donors.
Leukemic cells are sorted by FACS and transfected into lethally irradiated mice that were given either doxycycline for continuous expression of HOXA10 (HOXA10ON) or ciprofloxacin as a negative control (HOXA10OFF). (b) Homing of GFP^+ leukemic cells in BM from HOXA10ON and HOXA10OFF mice 24 h after transplantation (n = 5), with or without inhibition with anti-CD44 antibody. (c) Outgrowth of leukemic cells in BM 2 weeks after transplantation (top panel, representative FACS of one mouse for each group) and Kaplan–Meier plot of survival over time (bottom panel) displaying findings from mice transplanted with the different population and that were given doxycycline or ciprofloxacin. P value is measured by Mantel–Haenszel log-rank test and compared with mice transplanted with CD44^high leukemic cells (n = 5, each group). (d) Kaplan–Meier plot of survival over time displaying findings from mice transplanted with leukemic cells following CD44 inhibition. Leukemic cells were incubated during 20 min with high dose of the anti-CD44 PE (20 μg/ml) or control anti-immunoglobulinG2a PE (20 μg/ml) before tail vein injection (n = 7, each group).

Withdrawal of the initial HOXA10 transforming event promotes outgrowth of leukemic cells expressing high levels of CD44 in secondary transplantation settings

To determine whether an increase in CD44 is a key mechanism by which LICs are resistant, we performed a functional test by FACS sorting leukemic cells generated in primary donors and transplanted 20,000 cells expressing different levels of the CD44 surface marker (CD44^low, CD44^medium and CD44^high), in the tail vein of lethally irradiated secondary recipient mice fed doxycycline or ciprofloxacin (Figure 5a). When we analyzed engraftment 24 h after the transplantation, we observed that CD44^low leukemic cells have strong potential to home and engraft, as there is only a slight decrease in their capacity to engraft compared with CD44^high leukemic cells (Figure 5b). There was furthermore no difference in homing between mice fed with doxycycline or ciprofloxacin; therefore, when HOXA10 overexpression was turned off within 24 h, homing of leukemic cells was not affected.

When we monitored mice for the occurrence of leukemia (Figure 5c), outgrowth of leukemic cells was not dependent on CD44 level of expression in the HOXA10ON condition. Consistent with this, onset of leukemia was not delayed for mice transplanted with CD44^low leukemic cells when mice were fed with ciprofloxacin to turn off HOXA10 overexpression (Figure 5c), all mice injected with CD44^high leukemic cells developed leukemia, whereas all mice injected with CD44^low leukemic cells remained healthy (n = 5 for each group, P < 0.005) and no detectable GFP^+ leukemic cells were observed in the BM of those healthy mice 2 weeks after the transplantation. In conclusion, we confirmed that withdrawal of the initial HOXA10 oncogene promotes the outgrowth of leukemic cells expressing high levels of CD44. To rule out that the differences in the leukemicogenic capacity of transplanted cells was not owing to secondary effects, we performed detailed immunophenotypic analysis of the three CD44 fractions by FACS and could not find a significant difference in the

surface of HOXA10OFF cells (Supplementary Figure 4). The neutrophil cytosol factor 4 protein is involved in leukocyte transendothelial migration.

We decided to focus on the CD44 surface marker, as this surface glycoprotein mediates cell adhesion, migration and homing of hematopoietic cancer cells.31,32 Using western blot analysis (Figure 4b), we confirmed that the outgrowth of cells from HOXA10OFF leukemias is restricted to a population of cells expressing high levels of the CD44 adhesion protein on the surface, despite unaltered transcription levels of the CD44 gene (Figure 4c). Even if different variants of CD44 are correlated to different risk in human AML,33 resistance cannot be explained by the outgrowth of a population of leukemic cells expressing a variant of CD44. Using RT-PCR, we observed that HOXA10OFF-resistant leukemias as well as leukemic cells generated in primary and secondary recipient mice fed with doxycycline were expressing the same CD44 transcript isoform (variant 3; NM_001039151) (Supplementary Figure 5).
expression of several other surface markers expressed on leukemic cells (Supplementary Figure 6).

We furthermore inhibited CD44 with high concentration of anti-CD44 antibody preceding transplantation of secondary mice and provided evidence that targeting CD44 dramatically affect leukemogenesis of HOXA10OFF cells (Figure 5d). In conclusion, CD44 represents the major driving force of the growth of leukemic cells upon withdrawal of the initial transforming events.

**Increased expression of several proto-oncogenes and high levels of CD44 on leukemic cells correlate with relapsed leukemias in primary transplant recipients after withdrawal of HOXA10 overexpression**

We next asked whether upregulation of HOXA10 is required for maintenance of the leukemia in primary transplant recipients, or alternatively, whether the presence of ∼20% of resistant LICs among the pool of LICs could affect relapse to leukemia after withdrawal of the primary oncogene. In primary transplantation settings, doxycycline administration was stopped immediately after increase of the white blood cell count above 30 x 10⁶ cells per ml. Peripheral blood was subsequently monitored to test whether abolition of HOXA10 might lead to a reversal of the leukemia (Figure 6a). In the positive control group, all mice were subject to continuous administration of the doxycycline, resulting in a dramatic increase of total white blood cells characteristic of acute leukemia (Figure 6b). In the group of mice where HOXA10 expression was turned off, a stabilization of the number of leukemic cells in the blood was observed 2 weeks after withdrawal of the doxycycline, followed by relapse that occurs 2 weeks later with a substantial increase of myeloid cells in peripheral blood. Recipient mice with relapsed leukemias 4 weeks after withdrawal of doxycycline exhibit a clear downregulation of HOXA10 in BM cells (Figure 6c). Activation of several proto-oncogenes was frequently observed in relapsed samples after removal of the HOXA10 expression (Figure 6d). Furthermore, relapsed leukemic cells were also restricted to a

![Figure 6](image-url)
population of cells expressing high levels of the CD44 adhesion protein on the surface (Figure 6e). This finding suggests that complete remission cannot be achieved in the primary transplant mice despite withdrawal of HOXA10, because secondary mutations in additional proto-oncogenes and overproduction of the adhesion molecule CD44 contribute toward rendering the leukemic cells resistant to inactivation of the initial transformation event.

Discussion

Retrovirus-mediated overexpression of HOXA10 in murine BM cells followed by transplantation has been shown to induce AML in a significant proportion of mice, with a latency of 19–50 weeks. However, induced expression of HOXA10 mediated by a tet-operator system is not leukemogenic neither for the original transgenic animals nor when BM from these animals is transplanted into recipients. There is experimental evidence that retroviral integration induces genetic hits that have an important contribution in leukemogenesis of AML in mice, but we never observed the development of leukemia in mice receiving transplants of inducible HOXA10 BM cells transduced with the empty control vector. It is therefore likely that the oncogene levels may be critical for the development of the disease phenotype. In whole BM cells from in vivo induced Rosa26-rtTA-nls/tetO-HOXA10 mice receiving doxycycline in the drinking water, the HOXA10 transgene transcription was induced ~6-fold compared with uninduced control recipients receiving ciprofloxacin, whereas the retroviral approach is well known to induce a constant activation of the transgene, at several proviral insertion sites in DNA, leading to very high expression levels of the transgene, often >20-fold. In agreement with this, retroviral vector-mediated overexpression of HOXA10 in murine BM cells induces AML in vivo with outgrowth of clones expressing extremely high levels of the HOXA10 transgene. Therefore, our model has many advantages since the expression can be induced upon demand, although the absolute expression levels are somewhat lower.

These findings show that moderate levels of HOXA10 have a restricted capacity to transform cells. To develop a mouse model for leukemia, we had to transduce HOXA10-inducible BM cells with the cofactor MEIS1, which generated AML in 70% of transplanted recipients and showed that MEIS1 can potentiate the leukemogenesis induced by moderate expression of HOXA10. The long latency period of 15–30 weeks to induce leukemia suggests that the cooperation with MEIS1 is not sufficient to transform cells. Rather, additional hits may occur later during the leukemogenesis in vivo. Combination of specific genetic events is involved in the multi-step evolution of premalignant cells to full-blown AML. In several AML mouse models, sequential genetic hits have been shown to collaborate to induce aggressive leukemias. This concept is supported by clinical evidence as well since multiple genetic hits are often diagnosed in AML patients.

The oncoproteins BCR-ABL and MLL-ENL have been investigated as chronic myeloid leukemia and AML models, respectively, using conditional expression in mice. In both cases, the leukemic cells remained dependent on oncogene expression, whereas ablation of the transgene resulted in remission. Complete reversion of leukemias upon inactivation of the primary event suggests important implications for the design of therapy. It is however important to note that both BCR-ABL and MLL-ENL are potent oncogenes that can probably generate leukemia by themselves. The HOXA10 oncogene is less potent and needs additional genetic alterations for transformation. The reversal of a ‘weak’ initiating oncogene leads only to a partial remission with a short period of remission because the clone is genetically unstable and has acquired many other alterations, whereas removal of a potent oncogene leads to elimination of the malignant clone because it is merely depending on that oncogene. The MLL-ENL fusion gene is one of the few genetic alterations that probably need very few secondary events to initiate AML, as the mouse model shows that the disease penetrance is high and the latency short, arguing that very few additional hits are needed. Not all models of MLL fusion-induced leukemia have short latencies, whereas the latency period required for the onset of acute leukemia in vivo is variable and partner protein dependent. For example, a mouse model with AML-ETO overexpression does not generate AML when the oncogene is expressed alone, but AML develops when combined with dysregulation of additional oncogenes.

Our findings show that the majority of LICs are sensitive to ablation of the primary oncogenic event (HOXA10 overexpression), whereas ~20% of the LICs cause leukemic relapse after withdrawal of HOXA10 overexpression in a secondary transplantation setting. Activation of several oncogenes may compensate for reduction in HOXA10 overexpression and confer additional advantage to a subpopulation of LICs, as the disease evolves and relapses. The set of commonly dysregulated proto-oncogenes (Jun, Fos and Egr1) we observed in relapsed leukemias have been previously described to occur in murine AML, and are found significantly activated in relapsing AML patients. Our findings show that activation of these proto-oncogenes is not owing to subchromosomal changes or retroviral vector integration in DNA flanking or within the proto-oncogenes. In most cases, we observed dysregulation of additional oncogenes in relapsed leukemias.

By performing proteomic analysis using mass spectrometry, we detected an important increase in the levels of specific proteins involved in invasiveness of LICs in the microenvironmental BM niche. By transplanting leukemic cells expressing different levels of the adhesion protein CD44, we clearly provide mechanistic evidence that relapse of the leukemia may be niche dependent, as only leukemic cells expressing high levels of CD44 cause leukemias upon withdrawal of the HOXA10 oncogene. Although the bulk of the tumor failed to grow upon inactivation of the initiating HOXA10 oncogene, our findings show that LICs are not totally eliminated by withdrawal of HOXA10 overexpression. Rather, it seems that a population of LICs are resistant because proteins involved in cell–cell and cell–matrix interactions are highly expressed on the cell surface, modulating homing, motility and invasive properties of the leukemic cells. Among the proteins that we found increased in leukemic cells resistant upon withdrawal of HOXA10 were MMP9 and CD44. The MMP9 protein is an important factor for tumor-associated tissue remodeling and metastasis, and several MMPs have been linked to leukemic survival rates. CD44 correlates with poor prognosis in AML, facilitating motility and tumor growth, and may be a good target to eradicate human AML stem cells. Here we show the highly aggressive nature of leukemic cells expressing high levels of CD44 and conversely show the impaired outgrowth of leukemic cells expressing low levels of this surface marker.

In conclusion, our murine model of inducible HOXA10 expression recapitulates many of the features of human AML and is helpful in analyzing the oncogene addiction and unravel the basic mechanisms involved in the initiation and maintenance of leukemia, and to study whether adhesion molecules...
expressed on the surface of leukemic cells are important factors for leukemic relapse in the microenvironmental niches of the BM. Our findings support the notion that cell intrinsic genetic events are not the only factors causing leukemic relapse, but suggest that host-dependent extrinsic factors in the BM niche may also play a fundamental role in the mechanism mediating leukemic relapse.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)