Transformation of myeloid progenitors by MLL oncoproteins is dependent on Hoxa7 and Hoxa9

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Transcriptional deregulation through the production of dominant-acting chimeric transcription factors derived from chromosomal translocations is a common theme in the pathogenesis of acute leukemias; however, the essential target genes for acute leukemogenesis are unknown. We demonstrate here that primary myeloid progenitors immortalized by various MLL oncoproteins exhibit a characteristic Hoxa gene cluster expression profile, which reflects that preferentially expressed in the myeloid clonogenic progenitor fraction of normal bone marrow. Continued maintenance of this MLL-dependent Hoxa gene expression profile is associated with conditional MLL-associated myeloid immortalization. Moreover, Hoxa7 and Hoxa9 were specifically required for efficient in vitro myeloid immortalization by an MLL fusion protein but not other leukemogenic fusion proteins. Finally, in a bone marrow transduction/transplantation model, Hoxa9 is essential for MLL-dependent leukemogenesis in vivo, a primary requirement detected at the earliest stages of disease initiation. Thus, a genetic reliance on Hoxa7 and Hoxa9 in MLL-mediated transformation demonstrates a gain-of-function mechanism for MLL oncoproteins as upstream constitutive activators that promote myeloid transformation via a Hox-dependent mechanism.

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with over 40 distinct partner chromosomal loci, resulting in the production of chimeric proteins containing a common N-terminal region of MLL fused to variable portions of each partner protein [Ayton and Cleary 2001]. MLL partner proteins are structurally diverse, but possess motifs suggestive of normal functions associated with either transcriptional regulation within the nucleus or cytoplasmic signaling events. To date, structure/function analyses for multiple nuclear partner proteins suggest that transcriptional activator properties are essential for leukemogenesis [Lavau et al. 1997, 2000a; Slany et al. 1998, DiMartino et al. 2000, 2002, So and Cleary 2002, 2003; Zeisig et al. 2003]. Chimeric MLL proteins behave as dominant-acting oncogenes to promote leukemogenesis [Corral et al. 1996; Lavau et al. 1997, 2000b; Dobson et al. 1999], however, it has been controversial as to whether this results in either MLL loss of function via trans-dominant inhibition or gain of MLL function. Furthermore, although MLL fusion proteins have been proposed to act as deregulated chimeric transcription factors, the target genes that mediate their oncogenic properties remain unknown. Here we used a genetic approach to demonstrate that Hoxa7 and Hoxa9 are required for the full oncogenic activity of an MLL oncprotein. These results define an oncogenic transcriptional pathway in which MLL fusion proteins function as Hox-dependent gain-of-function mutants to induce acute myeloid leukemias.

Results

Myeloid transformation by MLL oncogenes is associated with expression of a specific subset of Hoxa genes

Given the conserved genetic requirement for MLL and its fly ortholog Trithorax in the maintenance of Hox gene expression, we used expression of Hoxa genes as surrogate gene expression markers for the status of MLL function in cells immortalized by MLL oncoproteins. Murine primary myeloid progenitor (MPMP) cell lines immortalized by MLL oncogenes were analyzed for the expression of various Hox genes. These lines were established in vitro clonogenic assays and uniquely represent the earliest stages of MLL-associated acute leukemogenesis [Lavau et al. 1997; for review, see Ayton and Cleary 2001]. Unlike human leukemias and leukemic cell lines, which have sustained multiple mutations associated with tumor initiation and progression, MPMP lines have sustained very few if any secondary mutations and retain cytokine dependence. Reverse transcriptase PCR (RT–PCR) analysis was conducted on RNA isolated from MPMP cell lines immortalized by the MLL fusion genes indicated at the top of the lanes. Primers employed for RT–PCR were specific for various Hox transcripts indicated to the left of the panels. RT–PCR was performed on human cell lines that express MLL fusions with various partner proteins, which are indicated along with the leukemia subtypes at the top of the gel lanes. Cell lines are as follows: HB1119 [lane 1], RS411 [lane 2], MV4-11 [lane 3], ML21 [lane 4], MonoMac6 [lane 5], and THP1 [lane 6].

Selective Hoxb and Hoxc genes, including those whose overexpression results in hematopoietic deregulation or those previously implicated as MLL targets during embryogenesis, were not consistently expressed in MPMP lines [Fig. 1A]. Furthermore, expression of Hox genes was not a general property of myeloid transformation, because MPMP lines immortalized under identical in vitro conditions by E2A-HLF [Hunger et al. 1992] or E2A-PBX1 [Nourse et al. 1990] oncogenes did not express Hoxa, Hoxb, or Hoxc genes [data not shown]. Given the known collaborative roles of Hoxa and Meis genes in myeloid oncogenesis [Nakamura et al. 1996], the expression of three members of the Meis family of Hox cofactors were also analyzed. Meis1, but not Meis2 or Meis3 was expressed in all MPMP lines immortalized by MLL fusion proteins. Similar to MPMP lines, Hoxa9 was also expressed at high levels in all examined human cell lines derived from MLL-associated acute lymphoid, biphenotypic, or myeloid leukemias [Fig. 1B], consistent with previous observations in human ALL cells [Rozowskaia et al. 2001; Armstrong et al. 2002; Drabkin et al. 2002; Yeoh et al. 2002]. Taken together, these data suggest that a specific subset of 5’ Hoxa genes is consistently coexpressed with Meis1 in cells transformed by MLL oncogenes, including cells representing the earliest stages of myeloid progenitor immortalization. These
data provide further evidence to support a gain-of-function mechanism for MLL-associated transformation events.

**MLL fusion proteins are required for the initiation and maintenance of myeloid immortalization in vitro and 5′ Hoxa gene expression**

To determine the relationship between Hoxa gene expression and the myeloid clonogenic activity of hematopoietic progenitors, normal bone marrow (BM) cells were fractionated into populations enriched [lin+] or depleted [lin−] for the expression of lineage-associated antigens and then cultured for 7 d in methylcellulose supplemented with exogenous cytokines that promote myeloid differentiation. Myeloid clonogenic activity resided exclusively in the lin− fraction [Fig. 2A], known to be enriched in progenitors and stem cells. Robust expression of 5′ Hoxa genes including Hoxa7 and Hoxa9 was observed in the lin− clonogenic fraction, we therefore focused on the role of these Hoxa genes in MLL-associated myeloid transformation.

The requirement for MLL fusion protein activity in the continued maintenance of Hoxa gene expression and myeloid transformation in vitro was assessed using conditional MLL fusion proteins. A mutant ligand-binding domain of the human estrogen receptor that is specifically responsive to 4-hydroxy-tamoxifen [4-OHT; Littlewood et al. 1995] was fused to the C terminus of MLL–ENL (MER), or a transformation-defective mutant [Slany et al. 1998] of MLL–ENL (MEC; Fig. 2B). The conditional constructs, as well as MLL–ENL and retroviral vector alone, were transduced into primary BM cells, which were then cultured in an in vitro immortalization assay.

![Figure 2](image-url)

**Figure 2.** Myeloid transformation requires sustained function of MLL–ENL and correlates with maintenance of Hox gene expression. (A) Myeloid clonogenic activity in normal bone marrow is associated with expression of Hoxa cluster genes. CFUs [per 10,000 plated cells] were determined after 7 d of culture in methylcellulose for whole bone marrow mononuclear cells (BM) or cellular fractions enriched based on the expression [Lin+] or absence [Lin−] of lineage markers. RT–PCR results are shown below for the Hox transcripts indicated to the right of each panel. (B) Conditional MLL–ENL proteins are schematically illustrated and consist of ER fusion at the C terminus of transformation-competent MLL–ENL (MER) or a mutant lacking the critical transformation domain of ENL (MEC). (C) Conditional immortalization of myeloid progenitors by MLL–ENL. Results of serial myeloid replating assays are shown for myeloid progenitors transduced with the constructs indicated at the top of the panels, cultured in the presence (+) or absence (−) of 4-OHT. Results of RT–PCR assays performed on cells harvested at day 7 of each plating are shown below for the transcripts indicated on the right. (D) MLL fusion protein activity is continuously required for the maintenance of transformation. A myeloid precursor cell line [MER3] immortalized by MLL/ENL–ER was plated in methylcellulose culture in the presence (+) or absence (−) of 4-OHT, and CFUs [per 10,000 plated cells] were determined on day 7 (lanes 1,2, respectively). Serial replating was performed as illustrated below, and CFUs again determined on day 7 (lanes 3,4, respectively).
in the presence or absence of 4-OHT (Fig. 2C). Under these conditions, transduction with vector alone was unable to sustain clonogenic activity through a second round of plating in methylcellulose and was associated with prior loss of 5′ Hoxa gene expression in first-round cells. Thus, in the absence of an immortalizing oncogene, the culture conditions promoted concomitant loss of both clonogenic activity and 5′ Hoxa gene expression. In contrast, MLL–ENL maintained clonogenic activity throughout serial replating and was associated with maintenance of 5′ Hoxa gene expression [Fig. 2C].

Similar to MLL–ENL, cells transduced with MER maintained clonogenic activity in vitro and 5′ Hoxa gene expression, but only in the presence of 4-OHT. Conversely, the immortalization-defective MEC protein was unable to maintain 4-OHT-dependent clonogenic activity through three rounds of serial replating. Low levels of Hox gene expression were detected in first-round cells transduced by MEC in the presence but not the absence of 4-OHT, reflecting transient residual clonogenic activity in second-round cells, which was eliminated by the third round of culture. Thus, 4-OHT-dependent regulation of an MLL fusion protein results in both conditional myeloid immortalization and associated 5′ Hoxa gene expression.

After three rounds of serial replating, MER-transduced cells were readily adapted to growth in liquid culture dependent upon the presence of cytokine [IL-3 or GM-CSF] and 4-OHT. In a representative MPMP cell line [Fig. 2D, line 3], 4-OHT was absolutely essential for continued clonogenic activity in vitro. Thus, MLL fusion protein activity is required for both initiation and maintenance of the immortalized phenotype, which correlates with 5′ Hoxa gene expression.

Hoxa7 and Hoxa9 independently contribute to efficient MLL-associated myeloid immortalization in vitro

A genetic approach was employed to specifically address the requirement for Hoxa7 and Hoxa9 in myeloid transformation initiated by an MLL oncogene. In vitro serial replating assays were performed using hematopoietic progenitors harvested from wild-type mice or mice homozygous for inactivating mutations of Hoxa7 or Hoxa9, respectively [Chen et al., 1997, 1998]. The enhanced replating potential typically induced by MLL–ENL in wild-type myeloid progenitors was significantly compromised in the absence of Hoxa7 or Hoxa9, despite comparable primary transduction efficiencies [Fig. 3A]. In second- and third-round cultures, colony numbers were reduced, although not eliminated, in transduced Hoxa-deficient BM compared to wild-type BM. Most of the colonies obtained [52%–67%] were small and displayed atypical morphology [Fig. 3B] in contrast to the large, dense, blast-like colonies seen in cultures initiated with transduced wild-type BM [51%–82% blast-like wt colonies versus 12%–25% of Hoxa-deficient]. The transformation potential of Hoxa-deficient progenitors was confirmed by transduction of the unrelated chimeric on-
and \( Hoxa9 \) cotransduction induced fully penetrant rescue of the leukemogenic phenotype with regards to latency, histology, and immunophenotypic criteria (Fig. 4B; data not shown).

At 5 wk posttransplant, recipients of \( MLL–ENL \)-transduced wild-type BM displayed a significant preleukemic phenotype with moderate splenomegaly associated with a 2.7-fold increase in spleen weight and total splenocyte numbers relative to nontransplanted age-matched controls (data not shown). The BM was extremely pale due to myeloid progenitor expansion, as revealed by the abnormal appearance of a Mac-1\(^+\)/Gr-1\(^{\text{int}}\) population (Fig. 5). Comparable myeloid expansion was not present in recipients of \( Hoxa9^{-/-} \) BM transduced with \( MLL–ENL \), despite adequate engraftment, indicating that \( Hoxa9 \) was also essential for premalignant myeloid progenitor outgrowth in vivo.

Discussion

Although many transcription factors have been directly implicated as dominant-acting oncoproteins, the target genes that mediate their oncogenic properties are largely unknown. Gene expression profiling of transformed cells has suggested a number of provisional candidate target genes. However, stringent genetic analyses in combination with biologically relevant functional assays for tissue-specific oncogenesis are essential for the correct assignment of bona fide downstream genes that make crucial contributions to the malignant phenotype. In this study we identified and validated subordinate genes that contribute to induction of AML by an MLL chimeric oncoprotein.

Our studies clearly define early, and apparently independent, roles for \( Hoxa7 \) and \( Hoxa9 \) in mediating the myeloid transforming properties of an MLL fusion protein. These results establish a linear pathway in which MLL oncoproteins function as upstream constitutive regulators to aberrantly maintain the expression of \( 5' \) \( Hoxa \) genes required for transformation through inappropriate self-renewal of myeloid progenitors that cannot terminally differentiate (Fig. 6). Although our in vivo leukemogenesis studies only addressed the requirement for \( Hoxa9 \), it is possible that \( Hoxa7 \) may also be required for AML induction, given the compromised clonogenic properties of transduced \( Hoxa9^{-/-} \) cells in vitro. Future experiments will determine whether other members of the \( Hoxa \) gene cluster also contribute to the MLL-assoc-
associated malignant phenotype by these stringent criteria, and whether other MLL oncoproteins are similarly dependent on Hoxa7/Hoxa9. We note that maintenance of the characteristic Hoxa expression profile was consistently associated with myeloid immortalization by five distinct MLL fusion proteins, including nuclear partner proteins such as ENL, ELL, CBP, and AF10 or the cytoplasmic partner protein AF6, suggesting that deregulated Hoxa gene expression may be a common event in the initiation of MLL-associated leukemogenesis. It is likely that MLL oncoproteins directly deregulate Hox gene expression, as recent studies using chromatin immunoprecipitation have identified Hox gene promoters as direct targets for wild-type MLL during embryogenesis. Differences in the spectrum of Hox genes regulated by either wild-type or mutant MLL proteins may be due in part to differential transcriptional effector properties of the novel partner proteins fused to the N terminus of MLL in leukemias.

Previous retroviral transduction/transplantation studies showed that forced expression of individual 5’ Hoxa genes such as Hoxa9 or Hoxa10 or the NUP98–HOXA9 fusion gene leads to the development of AML after particularly long latencies (Thorsteindottir et al. 1997; Kroon et al. 1998, 2001). In our studies, Hoxa9 did not induce AML in Hoxa9−/− progenitors, consistent with the dosage-dependent nature of the Hox-associated leukemia phenotype. The long latencies in wild-type progenitors contrast with the more rapid induction of leukemias by MLL oncogenes. On this basis, we propose that sustained 5’ Hoxa gene expression is necessary, but not sufficient, for MLL-associated transformation. Interestingly, induction of AML is greatly accelerated if individual Hox genes such as Hoxa9 are coexpressed with the Hox DNA-binding cofactor Meis1 (Kroon et al. 1998). Indeed, Meis1 was originally isolated from retroviral genetic screens as a myeloid transforming oncogene only when aberrantly coexpressed with Hoxa7 or Hoxa9 (Nakamura et al. 1996). Because the latency period for AML

Figure 4. Hoxa9 is essential for the leukemogenic properties of MLL–ENL. [A] Survival curves for cohorts of animals that were transplanted with MLL–ENL transduced progenitors from Hoxa9+/− [n = 15] or Hoxa9−/− [n = 13] BM donors. [B] Coexpression of Hoxa9 in combination with MLL–ENL rescues in vivo leukemogenicity of Hoxa9−/− BM cells. Survival curves are shown for cohorts of animals that were transplanted with Hoxa9−/− progenitors transduced with Hoxa9 alone [n = 10], Hoxa9 + MLL–ENL [n = 10], or cell lines [lines 3 and 4] immortalized by coexpressed Hoxa9 and MLL–ENL. [C] Histology for spleens and livers from mice transplanted with transduced BM cells. Transduced genes and BM genotypes are indicated at the top.
induction by MLL–ENL is similar to that observed upon coexpression of Hoxa9 (or Hoxa10) with Meis1, and MFMP cell lines immortalized by numerous MLL fusion proteins consistently express Meis1, the potent transforming activity of MLL oncoproteins may reside in the consistent simultaneous expression of 5′ Hoxa genes in combination with Meis1. This predicts that MLL oncoproteins may also be dependent on Meis1, a concept that we are currently evaluating using genetic approaches.

Although Hox-dependent lymphoid transformation was not directly addressed in the present study, we and others have observed a characteristic 5′ Hoxa gene expression profile in human lymphoid tumors and cell lines expressing translocation-derived MLL fusion proteins such as MLL–AF4 (Rozovskaia et al. 2001; Armstrong et al. 2002; Drabkin et al. 2002; Yeoh et al. 2002). These results suggest that Hox-dependent MLL-associated transformation is not myeloid-specific but may represent a general unifying feature of deregulated MLL activity regardless of the lineage affected. Furthermore, microarray expression profiles revealed HOXA9 to be the most consistent expression marker for all human AML subtypes (Golub et al. 1999). In light of our current results, widespread HOXA9 expression in human AML may be due to aberrant MLL gain-of-function activity. This possibility warrants further analyses for MLL aberrations in human myeloid tumors that lack 11q23 chromosomal translocations but maintain HOXA9 expression.

During mouse embryonic development, normal MLL function is required for maintenance but not the initiation of expression of Hox genes such as Hoxa7 and Hoxc8 (Yu et al. 1998). These results imply that prior initiation of Hox expression may be an essential prerequisite for MLL-associated myeloid transformation to take place. It is currently unclear whether a similar mechanism operates during hematopoiesis to control MLL-dependent Hox transcriptional regulation, although some evidence lends support to this hypothesis.

Firstly, we observed that cells enriched for the clonogenic progenitors that were used to initiate either in vitro immortalization or in vivo leukemogenesis assays preferentially expressed 5′ Hoxa genes. Secondly, the cellular targets for myeloid transformation by MLL–ENL are restricted to hematopoietic stem cells [HSC], CMP, and GMP subsets that normally express Hoxa9 (A. Cozzio, E. Passegue, P.M. Ayton, H. Karsunky, M.L. Cleary, and I.L. Weissman, in prep.). In contrast, progenitor populations such as the MEP that have down-regulated Hoxa9 expression are refractory to transformation by MLL–ENL. Thus, it is likely that a requirement for MLL-mediated transformation is pre-existent 5′ Hoxa gene expression in susceptible hematopoietic progenitors.

Genetic studies in the mouse indicate that MLL function is required for the correct execution of definitive hematopoiesis (Hess et al. 1997; Yagi et al. 1998). Loss of MLL function results in severe deficits in granulocyte and monocyte clonogenic [CFU-GM] progenitor activity associated with a dramatic reduction in colony number and size [Hess et al. 1997]. Strikingly, it is the same CFU-GM progenitor that is efficiently immortalized by MLL fusion proteins. Reminiscent of the MLL-deficient phenotype, we also observed the abnormal appearance of extremely small CFU-GM in serially replated cultures of Hoxa7−/− or Hoxa9−/− progenitors transduced by MLL–ENL. These observations suggest that 5′ Hoxa gene expression may also contribute to the essential role for normal MLL function during definitive hematopoiesis.

Retroviral transduction/transplantation studies have defined a critical role for multiple MLL fusion proteins in the initiation of in vitro myeloid immortalization and AML in vivo [Lavau et al. 1997, 2000a, b; Slany et al. 1998; DiMartino et al. 2000, 2002; So and Cleary 2002, 2003; So et al. 2003; Zeisig et al. 2003]. Our use of a conditional MLL fusion protein suggests that MLL oncoproteins may also be necessary for the continued maintenance of the immortalized phenotype. To our knowledge, this is the first demonstration that a dominant-acting chimeric transcription factor associated with acute leukemia is required for both initiation and maintenance of transformation. Continuous transcriptional deregulation of critical target genes by chimeric transcription factors may therefore be essential for the
maintenance of the tumorigenic phenotype. These results compare well with other models using tetracycline-regulated transformation of pre-B cells or thymocytes with the BCR-ABL kinase or wild-type c-MYC, respectively (Felsher and Bishop 1999; Huettner et al. 2000).

In summary, the direct functional link between onco-
genome MLL mutants and their specific 
Hox targets repre-
sents a unique entry point to delineate the molecular 
mechanisms of transcriptional deregulation in acute leukemias. Our genetic approach should serve as a useful paradigm for future functional analysis of oncogenic pathways.

Materials and methods

Hox gene expression analysis

Total RNA was isolated from MPMP cell lines or transduced 
myeloid cells using Trizol reagent (Invitrogen), and 1 µg was 
converted to random-primed cDNA using a preamplification kit (Invitrogen) according to the manufacturer's instructions. Resultant cDNA was diluted to a final volume of 100 µL, and 1 µL was used as the template for RT–PCR expression analysis. Nucleotide sequences of oligonucleotide primers used in this study are available upon request.

Retroviral vectors

A fragment of ENL cDNA (encoding amino acid residues 373–559) was ligated in-frame into the 3′ HpaI site of MSCV/neo/ 
5′/HpaI MLL vector (DiMartino et al. 2002) to generate MSCV/MLL–ENLC. MSCV/EGFP was generated by replacement of the PGK-neo sequences with PGK–EGFP. MSCV/puro/MLL–ENLC and 
MSCV/EGFP/MLL–ENLC were constructed by ligation of a 
blunt-ended EcoRI–XhoI fragment of MLL–ENLC cDNA into the HpaI site of either MSCV/puro or MSCV/EGFP. ENL sequences encoding residues 373–558 or an inactive mutant en-
coding ENL residues 373–543 (Slany et al. 1998) were cloned 
into pBS–ER (Littlewood et al. 1995) to generate pBS/ENL–ER fusions. Inserts were subsequently excised with EcoRI, blunt, 
and ligated in-frame into the 3′ HpaI site in MSCV/neo/5′/MLL vector to generate the corresponding transformation-competent or -incompetent MSCV/neo/MER or MSCV/neo/MEC constructs, respectively. MSCV/puro/E2A-HLF [a kind gift from K. Smith (Stanford University, Stanford, CA)] consisted of the 
E2A-HLF cDNA inserted into the EcoRI site of MSCV/puro.

In vitro myeloid immortalization assays

In vitro immortalization assays were performed as described (Lavau et al. 1997). For cotransduction experiments, clonogenic progenitors were further enriched by use of the lin− cell fraction of 5FU-treated BM as described below. 4-hydroxy-tamoxifen (4OHT; Sigma) was used at a final concentration of 1 µM. All experiments with conditional constructs were performed in the presence of either 4OHT or vehicle control (ethanol). Cytokine-
dependent MPMP cell lines were generated by seeding cells from the third round of methylcellulose cultures in RPMI 1640 medium supplemented with 10% serum and 1 ng/mL of either murine IL-3 or GM-CSF (R&D Systems).

In vivo leukemogenesis assays

Donor BM cells were harvested from 4- to 8-week-old C57BL/ 
6-Ly5.1 wild-type or Hoxa9−/− mice 5 d after intraperitoneal 
administration of 150 mg/kg 5FU. Mononuclear cells were 
stained with a cocktail of phycoerythrin (PE)-conjugated antibo-
dies specific for lineage markers B220, CD3, CD4, CD8, 
Mac1, GR1, and Ter119 (Pharmingen). The lineage-negative 
(lin−) cellular fraction of BM was isolated using anti-PE mi-
crobeads (Miltenyi Biotech) and an Automacs cell separator as 
recommended by the manufacturer (Miltenyi Biotech). lin− cells

![Figure 6](https://example.com/figure6.png)
were transduced with retroviruses as previously described (Lavau et al. 1997). Approximately $10^8$ transduced lin− C57BL/6-Ly5.1 cells together with a radioprotective dose of $2 \times 10^5$ con-
genic C57BL/6-Ly5.2 recipient BM cells were transplanted into irradiated [960 rad] congenic C57BL/6-Ly5.2 mice via the retro
orbital plexus. For analysis of hematopoietic reconstitution or tumor immunophenotyping, cells were stained with anti-Ly5.1
antibody directly conjugated to fluorescein isothiocyanate in combination with PE-conjugated antibodies specific for lineage
antigens, and then analyzed using a FACScanCalibur flow cytom-
eter (Becton Dickinson).

Histopathology

Tissues were fixed in 10% buffered formalin, embedded in par-
affin, sectioned (5 µm), and stained with hematoxylin and eosin.

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