Immortalization and leukemic transformation of a myelomonocytic precursor by retrovirally transduced HRX–ENL

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A subset of chromosomal translocations in acute leukemias results in the fusion of the trithorax-related protein HRX with a variety of heterologous proteins. In particular, leukemias with the t(11;19)(q23;p13.3) translocation express HRX–ENL fusion proteins and display features which suggest the malignant transformation of myeloid and/or lymphoid progenitor(s). To characterize directly the potential transforming effects of HRX–ENL on primitive hematopoietic precursors, the fusion cDNA was transduced by retroviral gene transfer into cell populations enriched in hematopoietic stem cells. The infected cells had a dramatically enhanced potential to generate myeloid colonies with primitive morphology *in vitro*. Primary colonies could be replated for at least three generations *in vitro* and established primitive myelomonocytic cell lines upon transfer into suspension cultures supplemented with interleukin-3 and stem cell factor. Immortalized cells contained structurally intact HRX–ENL proviral DNA and expressed a low-level of HRX–ENL mRNA. In contrast, wild-type ENL or a deletion mutant of HRX–ENL lacking the ENL component did not demonstrate *in vitro* transforming capabilities. Immortalized cells or enriched primary hematopoietic stem cells transduced with HRX–ENL induced myeloid leukemias in syngeneic and SCID recipients. These studies demonstrate a direct role for HRX–ENL in the immortalization and leukemic transformation of a myeloid progenitor and support a gain-of-function mechanism for HRX–ENL-mediated leukemogenesis.

Keywords: HRX–ENL/immortalization/MLL/myeloid leukemia/retroviral vector

Introduction

Chromosomal translocations constitute important mechanisms for the activation of cellular proto-oncogenes with essential roles in leukemogenesis (Cleary, 1991; Rabbitts, 1991). A subset of acute leukemias carries translocations of chromosome band 11q23 with reciprocal partners located at 30 or more cytogenetically diverse loci (for a review, see Waring and Cleary, 1997). Chromosomal abnormalities of 11q23 are seen in B-lineage acute lympho-

blastic leukemias (ALL) and myelomonocytic and monocytic subtypes of acute myeloid leukemias (AML). They result in structural interruption of the *HRX* gene (also called *MLL*, *ALL1* or *Htrx*) which encodes a very large (430 kDa) protein with limited amino acid similarity to the *Drosophila* trithorax protein (Djabali *et al.*, 1992; Gu *et al.*, 1992; Tkachuk *et al.*, 1992). Genetic analyses in flies and mice suggest that both trithorax and HRX are involved in the maintenance, but not initiation, of *HOX* gene expression during embryonic development (Breen and Harte, 1993; Kennison *et al.*, 1993; Yu *et al.*, 1995). Since HRX-associated leukemias often express markers of both lymphoid and myeloid lineages, it is possible that HRX translocations may occur at the level of a multipotent hematopoietic progenitor or hematopoietic stem cell (HSC), leading to the disruption of normal genetic programs for terminal differentiation.

Chromosomal translocations involving HRX result in its in-frame fusion with a variety of heterologous proteins, several of which have now been cloned and characterized (Waring and Cleary, 1997). Some HRX partners are structurally related to each other, and many contain motifs previously associated with proteins involved in transcriptional regulation. One of the more common HRX fusion partners is ENL (Tkachuk *et al.*, 1992), located at chromosome band 19p13.3. The ENL locus is involved in t(11;19) translocations in both B-lineage ALLs and myelomonocytic AMLs (Rubnitz *et al.*, 1996). Leukemias bearing t(11;19) express HRX–ENL fusion proteins comprised of an amino-terminal portion of HRX, containing its DNA-binding A–T hook motifs, and the carboxy-terminal portion of ENL, which displays weak transcriptional activation potential (Rubnitz *et al.*, 1994). ENL displays amino acid similarity to AF9, which is fused to HRX following t(9;11) translocations in another subset of AMLs (Iida *et al.*, 1993; Nakamura *et al.*, 1993). Both ENL and AF9 are distantly related to the yeast Anc1 protein (Welch and Drubin, 1994) which has been shown to be a component of two basal transcription complexes (TFIID and TFIIF) and the SWI/SNF complex (Henry *et al.*, 1994; Cairns *et al.*, 1996). It is not yet clear what biochemical role Anc1 plays in these transcription complexes or whether ENL or AF9 are components of the comparable mammalian complexes. Although available data suggest a role in transcriptional regulation, *in vitro* biological assays are necessary to establish a link between the transcriptional properties of ENL/AF9 and the oncogenic properties of their respective HRX fusion proteins.

The genetic mechanisms by which mutations of HRX contribute to leukemogenesis are not yet clear. Consistently observed, in-frame fusions of the amino-terminal portions of HRX with heterologous proteins suggest an important role for 3' portions of each fusion partner and support a potential gain-of-function mechanism. However, the

Fig. 1. Design and expression of MSCV constructs and experimental strategy for transduction of primitive hematopoietic cells. (A) Schematic illustrations of the retroviral vectors employed. (B) Western blot analysis of proteins expressed from the MSCV constructs shown in (A). Arrows indicate exogenously expressed proteins detected with the specific antibodies indicated beneath each panel. The anti-ENL antiserum (Butler *et al.*, 1997) detects endogenous ENL proteins in Bosc23 cells whose level of expression is considerably lower than that observed in cells transfected with MSCV/ENL. (C) Experimental scheme employed for transduction of primitive hematopoietic cells.

diversity of observed HRX fusion partners, which lack any apparent unifying functional contribution, has led to counter-proposals for loss-of-function models. Furthermore, in a subset of myeloid leukemias, HRX is not fused to a heterologous protein but undergoes self-fusion, resulting in internal duplication of its amino-terminal portions (Schichman *et al.*, 1994). Thus, a critical transforming event may be inactivating mutations of HRX resulting in its haplo-insufficiency. Another possibility is that translocations involving HRX contribute two oncogenic events by simultaneously creating both a gain-of-function mutation on the translocated allele as well as haplo-insufficiency for wild-type HRX (Yu *et al.*, 1995).

In an effort to address these mechanistic issues, we developed an *in vitro* model to characterize the effects of HRX-ENL on primitive murine hematopoietic cells by employing retroviral gene transfer. Following retroviral transduction, HRX-ENL increased the self-renewal and proliferative capacity of hematopoietic progenitors with *in vitro* clonogenic potential and resulted in the immortalization of early myelomonocytic cells. Hematopoietic cells transduced with HRX-ENL induced myeloid leukemias upon transplantation into syngeneic mice. *In vitro* transformation required fusion of HRX with ENL and was not observed with wild-type ENL or the 5' portion of HRX alone. These data demonstrate a direct role for HRX-ENL in the immortalization and leukemic transformation of myeloid precursors, and support a simple gain-of-function mechanism for its oncogenic activity.

Results

Structure and expression of HRX-ENL retroviruses

A cDNA encoding the HRX-ENL fusion transcript (Tkachuk *et al.*, 1992) was inserted under the transcrip-

tional control of the long terminal repeat (LTR) of the murine stem cell virus (MSCV) retroviral vector (Hawley *et al.*, 1994) (Figure 1A). This vector was employed because it has previously been used successfully with several genes to induce transformation of either myeloid or lymphoid cells (Hawley *et al.*, 1995), suggesting that its LTR is capable of directing gene expression in multiple hematopoietic lineages. The MSCV/HRX-ENL vector also encodes a neomycin resistance gene (*neo*) under the control of the phosphoglycerate kinase (PGK) internal promoter and enables selection of infected cells on the basis of resistance to G418. High titer retroviral stocks for MSCV/HRX-ENL and MSCV/neo were produced by transient transfection into the ecotropic retroviral packaging cell line Bosc23 (Pear *et al.*, 1993). Appropriate expression of HRX-ENL in transfected Bosc23 cells was confirmed by Western blot analysis (Figure 1B).

Murine hematopoietic cells transduced with HRX-ENL exhibit a growth advantage *in vitro*

To determine if HRX-ENL could induce the leukemic transformation of primitive HSCs, we targeted its expression to two different populations of enriched murine bone marrow (BM) cells by retroviral infection (Figure 1C). In experiment 1, BM was harvested from mice 2 days after 5-fluorouracil (5-FU) treatment and enriched by depletion of mature cells expressing lineage-associated antigens, followed by fluorescence-activated cell sorting (FACS) to isolate a fraction of Thy-1^{lo}Sca-1⁺H-2K^bhi cells shown previously to be highly enriched (one per 55 cells) in competitive long-term repopulating units (Szilvassy *et al.*, 1996a). In experiment 2, BM was harvested from donor mice 5 days after 5-FU treatment, and primitive hematopoietic cells were enriched simply by immunomagnetic

depletion of terminally differentiated (Lin^+) cells. Enriched hematopoietic cells were infected with MSCV/HRX-ENL retrovirus by spinoculation and cultured in methylcellulose medium supplemented with interleukin (IL)-3, IL-6, granulocyte-macrophage colony-stimulating factor (GM-CSF) and stem cell factor (SCF) with or without G418 selection. In the absence of G418, an average of 10% (experiment 1) and 2% (experiment 2) of the cells infected with either MSCV/HRX-ENL or MSCV/neo viruses formed colonies of mature myeloid cells *in vitro*. Culture in G418 did not significantly diminish the cloning efficiency of hematopoietic cells infected with MSCV/neo [80 and 100% of colony-forming cells (CFCs) were infected in experiments 1 and 2, respectively] but resulted in an ~10-fold reduction in the number of colonies generated by both cell populations infected with MSCV/HRX-ENL. This difference in the efficiency of gene transfer into clonogenic progenitors probably reflected the lower titer of the MSCV/HRX-ENL viral stocks compared with the parental vector as measured on NIH3T3 cells ($1\text{--}5 \times 10^5$ versus $1\text{--}5 \times 10^6$ G418^R U/ml, respectively). G418-resistant colonies derived from progenitors infected with either vector were heterogeneous in size and exhibited a range of morphologies similar to colonies obtained from normal or mock-infected cells (data not shown).

To determine if hematopoietic progenitors infected with HRX-ENL might display some perturbation in their normal self-renewal or differentiation potential not apparent in the primary colony assays, whole methylcellulose cultures were harvested 8–10 days after plating and a proportion of the cells was seeded into secondary methylcellulose cultures without G418. The total number of colonies generated in the secondary assays was similar for both HRX-ENL- and neo-infected cells: ~30 per 10^4 cells plated for experiment 1, and ~90 per 10^4 cells plated for experiment 2 (Figure 2). However, these secondary colonies displayed striking differences in their morphology, noticeable as early as 6 days after plating. Virtually all of the colonies derived from MSCV/neo-infected progenitors were very small (<30 cells) and diffuse, consistent with the limited self-renewal and proliferative potential of CFCs in normal BM. In contrast, secondary colonies derived from MSCV/HRX-ENL-infected cells were much larger (~300 cells) and exhibited three distinct morphologies. Most (50–80%) were extremely compact and resembled colonies generated by primitive hematopoietic cells (Figure 3A). A smaller subset (20–40%) had a compact center with a diffuse halo of differentiating cells (Figure 3B) and a third minor subset (<15%) was comprised of large diffuse colonies (Figure 3C). Wright-Giemsa staining revealed that the very compact colonies consisted of immature myeloid cells while the colonies with a diffuse component also included differentiated macrophages (data not shown).

After 10 days of culture, when HRX-ENL colonies consisted of $>10^3$ cells, secondary assays were harvested and tertiary assays initiated with MSCV/neo- or MSCV/HRX-ENL-transduced cells (10^4 cells/dish). Very few tertiary colonies were generated by MSCV/neo-infected cells, demonstrating that CFCs present in the secondary assays had exhausted virtually all proliferative capacity by the second generation. In contrast, progenitors expressing HRX-ENL exhibited a greatly enhanced proliferative

Fig. 2. Effect of HRX-ENL on the replating efficiency of hematopoietic progenitors. Each point represents the mean number of colonies generated per 10^4 cells seeded ($\pm$ SEM) from three independent transduction experiments (one conducted on day 2 post-5-FU Thy-1^{lo}Sca-1⁺H-2K^{hi} BM cells, and two conducted on lineage-depleted day 5 post-5-FU BM cells as described in experiments 1 and 2, respectively, in Materials and methods).

potential in a third (1–7%) and fourth (10–19%) round of serial replating (Figure 2). The third and fourth generation HRX-ENL colonies conserved the distribution of morphologies noted in the secondary assays above. Furthermore, when 5–10 secondary colonies of each type were picked and replated according to morphology, all three colony types regenerated tertiary daughter colonies of all three morphologies at proportions equivalent to that produced by unselected secondary colonies (data not shown). It was noted, however, that the diffuse colonies had a low frequency of replating (only one of five colonies tested successfully replated), which correlates with the finding that these colonies consisted predominantly of macrophages. Taken together, these data demonstrate that HRX-ENL markedly enhances the self-renewal capacity and proliferative potential of clonogenic hematopoietic progenitors *in vitro*.

Progenitors expressing HRX-ENL establish long-term myeloid progenitor cell lines

To study further the properties of HRX-ENL-transduced progenitors *in vitro*, tertiary colonies described above were pooled and propagated in suspension cultures containing IL-3, IL-6 and SCF. Two cell lines were established by continuous passage of the expanding cells: line A from ~250 pooled colonies derived from day 2 post-5-FU Thy-1^{lo}Sca-1⁺H-2K^{hi} cells, and line B from ~100 colonies arising from lineage-depleted day 5 post-5-FU BM. After a few passages, IL-6 was omitted from the medium and the cells maintained in IL-3 and SCF for >7 months.

The integration pattern of MSCV/HRX-ENL proviruses in each cell line was assessed by Southern blot analysis with an HRX probe. The presence of intact proviral DNA was confirmed in both cell lines by detection of two

Fig. 3. Morphology of secondary colonies generated by MSCV/HRX-ENL-infected hematopoietic cells. (A) Typical compact colony representing 50–80% of total HRX-ENL colonies. (B) Colonies with a dense center surrounded by a halo of migrating cells (20–40% of colonies). (C) Diffuse colonies of mobile differentiating cells (<15% of colonies). Bar = 1 mm.

predicted bands of 5.8 and 3.0 kb in size following digestion with *KpnI* (which cuts once in each LTR and in the *HRX-ENL* cDNA) (Figure 4A). The number of proviral integrants in the infected cells was determined by Southern blot analysis of DNA digested with *BamHI* (which cuts once in the provirus to generate fragments whose size is dependent on the site of integration). A single fragment of ~8.0 kb was found in line A, suggesting its derivation from a single retrovirally infected progenitor cell. In line B, a major band of ~25 kb and a fainter band of ~6 kb (not apparent in Figure 4A) were detected. Southern blot analysis of subclones of line B confirmed that the 6 kb fragment corresponds to an independent integration site and that this line is composed of at least two marked

Fig. 4. Integration and expression of retrovirally transduced *HRX-ENL* sequences in progenitor lines A and B. (A) Southern blot analysis of genomic DNA digested with *KpnI* or *BamHI* and hybridized with a human *HRX* probe. *KpnI* cleaves once within each LTR and once in the *HRX-ENL* cDNA, thus generating two fragments of 3.0 and 5.8 kb which span the proviral genome. The 5.6 kb band also observed in genomic DNA from NIH3T3 cells represents the endogenous murine *HRX* gene which cross-hybridizes with the human *HRX* probe. *BamHI* cleaves once within the MSCV/HRX-ENL vector and allows detection of proviral DNA fragments whose size varies with the site of integration. The 9.0 kb *BamHI* fragment represents the endogenous *HRX* gene. (B) RT-PCR analysis for *HRX-ENL* transcripts. DNA-free RNA was converted to cDNA and amplified with primers flanking the fusion site of *HRX-ENL*. The predicted product of 373 bp is observed for MSCV/HRX-ENL-infected cells from line A and the t(11;19)-bearing control cell line HB1119, but not in negative control lanes in which reverse transcriptase (RT) was omitted from the amplification reaction. The lower band in HB1119 cells is a differentially spliced form of the *HRX-ENL* fusion transcript (Tkachuk *et al.*, 1992).

clones (data not shown). Southern blot analyses of proviral integrations were also performed on pooled and expanded tertiary colonies that originated from individually plucked secondary colonies. Unique hybridization patterns were observed in nine of 12 cultures (data not shown). *HRX-ENL* transcripts were detected in lines A and B by RT-PCR analysis employing oligonucleotide primers flanking the *HRX-ENL* fusion site (Figure 4B and data not shown). Thus, the lines were of monoclonal or oligoclonal origin

Fig. 5. Morphology and phenotype of cells immortalized by *HRX-ENL*. (A) and (B) Wright-Giemsa-stained cytopsin preparation of lines A and B, respectively. Bar = 14 μm. (C) Flow cytometric analysis of surface antigen expression by line A. Black lines represent staining obtained with PE-conjugated antibodies specific for the indicated hematopoietic cell surface antigens. Gray lines represent the signal obtained with corresponding isotype control antibodies. (D) Wright-Giemsa-stained cytopsin preparation and flow cytometric analysis demonstrating modulation of Mac-1 and c-kit expression of line A after culture in medium containing G-CSF.

and harbored transcriptionally active MSCV/HRX-ENL proviral genomes, confirming that HRX-ENL is directly responsible for immortalization of CFCs *in vitro*.

Both cell lines were composed predominantly of cells with morphologic features of immature myelomonocytic cells, but also contained some cells with more advanced stages of nuclear segmentation (Figure 5A and B). Flow cytometric analysis demonstrated that 100% of the cells were Mac-1⁺, Gr-1⁺, TER-119⁺, B220⁺ and Thy-1⁺ (Figure 5C). Both lines also expressed CD43 and c-kit, which are

found on primitive hematopoietic precursors, but were Sca-1⁺ (data not shown). Line A expressed slightly higher levels of Mac-1 and CD43 than line B (data not shown), suggesting that line A may be further engaged in the myeloid differentiation pathway (Moore *et al.*, 1994). Like the tertiary colonies from which they were derived, both cell lines displayed a very high cloning efficiency in methylcellulose cultures supplemented with IL-3, IL-6, GM-CSF and SCF (80% for line A and 40% for line B). Most colonies had the typical compact appearance

Fig. 6. Proliferation of lines A and B in liquid cultures supplemented with recombinant hematopoietic growth factors. Cells (10^4 per well) from each line were cultured with the indicated cytokines and cell expansion measured after 5 days by adding Alamar blue substrate. Shown are the mean number of arbitrary fluorescence units ($\pm$ SEM) obtained from triplicate wells from four independent experiments.

observed previously and were comprised of immature myeloid cells and monocytes/macrophages. Consistent with these findings, both lines expanded ~ 25 -fold after 5 days of culture in medium containing either IL-3, granulocyte colony-stimulating factor (G-CSF) or GM-CSF alone (Figure 6) and could be sustained for >20 days in these cytokines without SCF. The only significant difference noted between the two lines was the 2-fold lower proliferation of line B in G-CSF alone. Lower, but significant, proliferation of each line was also observed in IL-6 or SCF alone, and the two factors together had an additive effect on cell output (Figure 6). When both lines were stimulated with G-CSF, c-kit expression was lost while Mac-1 expression increased and terminally differentiated granulocytes were generated (Figure 5D and E). We were unable to demonstrate B lymphoid or erythroid differentiation potential (assessed by B220 or TER-119 expression, respectively) of HRX-ENL-infected progenitors upon culture with SyS-1 stromal cells in medium containing IL-7, or in liquid or methylcellulose cultures containing erythropoietin. Therefore, HRX-ENL-infected progenitors retained some capacity for terminal differentiation, but this was restricted to the myeloid lineage.

Hematopoietic progenitor cells transduced with HRX-ENL induce acute myeloid leukemias *in vivo*

To determine whether immortalized progenitor cells were capable of generating tumors *in vivo*, cells of line B were injected i.v. into sub-lethally irradiated syngeneic (B6.SJL) mice. Over an observation period of 7 months, all 10 mice injected with line B cells succumbed to AMLs (Figure 7). Histologic and cytologic analyses showed that $>90\%$ of BM cells had morphologic features of blasts similar to

Fig. 7. Development of leukemia in mice transplanted with virus-infected cell lines or sorted BM cells. A total of 10^6 cells from line A ($\bullet$) or line B ($\circ$) were injected into 10 non-irradiated SCID or 10 sub-lethally irradiated syngeneic B6.SJL mice, respectively. For experiments with freshly infected BM cells, lethally irradiated B6.SJL mice were transplanted with 200 Thy-1^{lo}Sca-1⁺H-2K^{hi} day 2 post-5-FU BM cells (Ly-5.2⁺) transduced by MIN/HRX-ENL [17 mice ($\blacklozenge$)] or MIN/neo (nine mice), together with 10^5 twice serially transplanted Ly-5.1 BM cells to provide radioprotection.

those displayed by cells of line B. Leukemic cells effaced the normal splenic architecture, and in the liver they were present as massive diffuse periportal and sinusoidal infiltrates. Southern blot analysis of DNA from the spleens of two animals revealed intact MSCV/HRX-ENL proviral DNA which confirmed that the tumor cells were derived from line B (data not shown). Notably, in two cases where explanted leukemia cells were analyzed, these remained dependent on IL-3 for *in vitro* growth. Similar results were obtained with line A. Following its injection into non-irradiated SCID mice (10^6 cells i.p. per recipient), nine of 10 recipients died 72–84 days later with AMLs (Figure 7).

Leukemic transformation of freshly isolated hematopoietic progenitor cells infected with MIN/HRX-ENL

To determine whether expression of HRX-ENL in freshly isolated BM cells induced their leukemic transformation *in vivo*, lethally irradiated B6.SJL (Ly-5.1⁺) mice were transplanted with 200 Thy-1^{lo}Sca-1⁺H-2K^{hi} day 2 post-5-FU BM cells (Ly-5.2⁺) after infection with the MSCV-derived vector MIN/HRX-ENL (see Materials and methods). All mice were also co-transplanted with 10^5 twice serially transplanted Ly-5.1 BM cells to provide radioprotection (Szilvassy *et al.*, 1990). Nine control mice injected with HSCs infected with MIN/neo exhibited a mean of 69% donor-derived (Ly-5.2⁺) peripheral blood cells but remained healthy up to 4 months after transplantation. In contrast, 17 mice injected with MIN/HRX-ENL-infected HSCs exhibited a mean of 78% donor blood cells, and eight animals (47%) developed myeloid leukemia with latencies of 73–118 days (Figure 7 and Table I). Elevated white blood cell counts and abnormal increases in the ratio of myeloid to lymphoid cells were detected in most animals before death or sacrifice. Histological analysis of the spleen, liver, kidney and BM of diseased animals revealed infiltration by large numbers of mitotically active, immature myeloid cells (Figure 8A). The leukemia cells present in peripheral blood and BM displayed a range of

Table I. Characteristics of leukemias induced in mice transplanted with HRX-ENL-infected hematopoietic cells

Animal	Latency (days)	Terminal blood counts ($\times 10^{-3}/\mu\text{l}$)			Mac1 ⁺ cells in PB (%)	Blasts in PB (%)	Marrow blasts ^a	Tumor clones	Secondary transplantation
		WBC	RBC	PLT					
1	81	140	7	137	66	18	++	2	5 (36–44 days)/5
3 ^b	116	11	9	625	nd	nd	nd	nd	
4	82	222	3	79	76	25	nd	nd	
5	117	72	7	149	nd	22	+	2	1 (98 days)/4 (>209 days)
6	119	3	2	36	nd	4	+++	1	
7 ^c	73	25	7	268	nd	10	nd	nd	
8	109	55	6	109	nd	25	++	1	4 (37–103 days)/5 (>153 days)
16	104	192	2	55	86	26	++	1	
Normal ^d	na	9	10	1048	11	0	–	na	1 (45 days)/3 (>158 days)

^aBone marrow blasts were quantitated as: –, <10%; +, 10–19%; ++, 20–29%; and +++, 30–40%.

^bBlood smear and cell counts were performed 18 days before death.

^cBlood smear and cell counts were performed 11 days before death.

^dMean of data obtained from three normal control mice; na, not applicable.
nd, not determined.

morphologic features from immature myelomonocytic cells to cells with more advanced stages of nuclear segmentation (Figure 8B). Southern blot analysis of *KpnI*-digested DNA isolated from the spleens of five animals showed intact MIN/HRX-ENL retroviral DNA, and *Bam*HI digests demonstrated one or a few clones in each case (Figure 8C). Upon explantation, only one leukemia was observed to have acquired factor independence for *in vitro* growth. Two others were dependent upon IL-3 for optimal viability and proliferation. Of the nine animals which remained healthy even after 4 months, only two contained proviral DNA in peripheral blood cells, indicating that the absence of leukemias in most of these animals was likely to be due to poor efficiency of transduction of reconstituting HSCs by MIN/HRX-ENL.

Fusion of HRX to ENL is necessary to induce the deregulated proliferation of hematopoietic progenitors

To begin to address the mechanisms by which HRX-ENL contributes to the leukemic transformation of early hematopoietic cells, mutated forms of HRX-ENL were assessed for their ability to affect the proliferative capacity of progenitors *in vitro*. Mutant constructs were engineered to remove all ENL sequences from HRX-ENL (MSCV/HRXdel) or to express ENL without associated HRX sequences (MSCV/ENL) (Figure 1A). Expression of the appropriate proteins was confirmed by Western blot analysis of transfected Bosc23 cells (Figure 1B). Lineage-depleted day 5 post-5-FU BM cells were transduced with MSCV vectors encoding these proteins as well as with MSCV/HRX-ENL, MSCV/neo and MSCV/as (containing HRX-ENL in the antisense orientation), which served as controls. Hematopoietic cells infected with all viruses except MSCV/HRX-ENL generated equivalent numbers of colonies when plated into methylcellulose assays containing G418 (97–120 colonies per 10^4 cells) (Figure 9). When primary colonies were harvested and replated into secondary, tertiary and quaternary assays, no effect of HRXdel or wild-type ENL alone on the replating capacity of the transduced cells was observed. Like progenitors transduced with MSCV/neo, proliferative potential was essentially exhausted after three successive rounds of

replating (representative experiment shown in Figure 9). These data demonstrate that the ability of HRX-ENL to induce the sustained proliferation of myeloid progenitor cells *in vitro* requires the fusion of HRX with ENL and is not a simple consequence of HRX truncation or ENL overexpression.

Discussion

The studies reported here demonstrate that HRX-ENL functions as a transforming protein for myeloid progenitors, and suggest potential mechanisms for its oncogenic effects. HRX-ENL was capable of immortalizing early myelomonocytic cells, resulting in their enhanced replatability in methylcellulose assays and long-term propagation in liquid culture. These immortalized cells, as well as freshly infected BM cells enriched for hematopoietic stem and progenitor cells, were capable of inducing myeloid leukemias with high frequencies upon transplantation into syngeneic recipients. The leukemias observed in mice displayed similarities with human leukemias carrying t(11;19) translocations encoding HRX-ENL fusion proteins in which maturational arrest at the level of myelomonocytic progenitors is frequently observed (so-called M4 AML). However, t(11;19) translocations are also associated with human leukemias displaying lymphoid characteristics or features of a common lymphoid/myeloid progenitor. This phenomenon was not observed in our studies, despite efforts to specifically target primitive progenitors or HSCs by stringent enrichments of the target cells. The observed preferential immortalization of myeloid progenitors was unlikely to have resulted from a failure to express retroviral HRX-ENL at earlier stages of hematopoietic cell development since the MSCV vector has effectively targeted HoxB4 expression to the most primitive HSC compartment (Sauvageau *et al.*, 1995). Although our culture conditions undoubtedly influenced the myeloid phenotype of the cells transformed *in vitro*, a similar bias is less likely for the *in vivo* reconstitution experiments which also yielded myelomonocytic leukemias, suggesting that cells at this stage of myeloid differentiation may be particularly susceptible to the effects of HRX-ENL. This conclusion is consistent with the observa-

Fig. 8. Characteristics of leukemias induced by HRX-ENL. (A) Histological analysis showing leukemia cells infiltrating sinusoids of liver. (B) Wright-Giemsa-stained preparation of peripheral blood showing leukemia cells displaying morphologic features ranging from blasts to early stage myelomonocytic cells with immature nuclear segmentation. (C) DNA isolated from spleens of moribund animals was subjected to Southern blot analysis employing a human *HRX* probe. *KpnI* digests (left panel) generated two fragments of 3.0 and 5.0 kb (arrows), confirming intact MIN/HRX-ENL proviral DNA in all cases. *BamHI* digests (right panel) showed one or two clonal bands in each case whose migration differed from the 9 kb fragment (dash) representative of the endogenous *HRX* gene.

tion that mice chimeric for an *MLL-AF9* knock-in gene developed AMLs exclusively in spite of expression of the *MLL* knock-in allele in a wide variety of cell types (Corral *et al.*, 1996).

Our studies are the first to demonstrate the oncogenic potential of an HRX fusion protein by retroviral gene transfer. As such, they allowed an assessment of the effects of HRX-ENL on primary BM cells in the absence of confounding events such as loss of wild-type HRX function on the translocated allele (as occurs in human leukemias or knock-in mouse models). The rapid emergence of immortalized cells in CFC assays suggests that

Fig. 9. Fusion of HRX to ENL is necessary for the transformation of hematopoietic progenitor cells *in vitro*. Lineage-depleted day 5 post-5-FU BM cells (6×10^4) were infected by spinoculation with the parental vector (MSCV/neo), the MSCV vector containing a deleted version of HRX present in HRX-ENL (HRXdel), the HRX-ENL fusion gene in antisense orientation (MSCV/as) or wild-type ENL (MSCV/ENL). Transduced cells were plated into methylcellulose assays containing IL-3, IL-6, GM-CSF and SCF (7.5×10^3 cells per dish). Shown are the mean number of day 8 colonies per 10^4 cells plated in duplicate dishes for a representative experiment.

secondary events are not required *in vitro*. Under these conditions, HRX-ENL primarily induced an enhanced self-renewal capacity with a concomitant partial block in myeloid differentiation. These effects on differentiation were reversible, as evidenced by the normal terminal differentiation of the immortalized cells in G-CSF *in vitro* and the abundant differentiated myeloid cell types arising from infected primary HSCs. Significantly, the latter is not a notable feature of human t(11;19)-bearing leukemias and may reflect the lower levels of HRX-ENL expression achieved in our experiments compared with those observed in t(11;19)-bearing cell lines. In contrast, progression of HRX-ENL-immortalized cells to a malignant state *in vivo* most probably requires additional genetic alterations. This is suggested by (i) the relatively long and variable disease latencies in animals injected with HRX-ENL-immortalized cell lines, (ii) the monoclonal origin of leukemias resulting from infection of primary BM cells, and (iii) our observation, in at least one case, that explanted leukemia cells had become cytokine independent for *in vitro* growth.

Several findings suggest that low-level expression of HRX-ENL may be an important requirement for its observed effects on the primary myeloid progenitors transduced in our studies. Although *HRX-ENL* transcripts were detected in immortalized cells by RT-PCR, they were below the threshold of detection by Northern blot analyses. Similarly, HRX-ENL protein was not detected in these cells by Western blot analyses using a highly specific monoclonal antibody or polyclonal antisera directed against HRX. Taken together, these observations indicate that the actual levels of HRX-ENL expression in

immortalized and primary myeloid cells are very low. Such low level expression is not a general feature of MSCV vectors, suggesting that high levels of HRX–ENL expression may in fact be deleterious or incompatible with stable transduction. This is consistent with observations that HRX–ENL cannot be stably expressed under control of a constitutive promoter in a variety of cultured cells (R.Slany and M.L.Cleary, unpublished observations). The basis for this has not been determined but may result from growth arrest induced by high-level HRX–ENL expression similar to that reported for MLL–AF4 (Caslini *et al.*, 1996). It is likely that in our CFC assays, as well as in our *in vivo* reconstitution experiments, there was selection for proviral insertions yielding low but physiologically significant levels of HRX–ENL expression, perhaps accounting for the low number of marked clonal cell populations (1–2 on average) observed in each transduction experiment.

Genetic studies indicate that HRX and its *Drosophila* homolog trithorax positively regulate expression of *HOX* genes, whose products play critical roles in specifying cell fates along body axes during embryonic development (Breen and Harte, 1993; Kennison *et al.*, 1993; Yu *et al.*, 1995). *HOX* genes have also been shown to have important lineage-specific functions in the hematopoietic system. They are differentially expressed in subsets of hematopoietic cells, and hematopoietic abnormalities are observed in *HOX* gene transfer and ablation studies (Lawrence and Largman, 1992). Gene transfer studies implicate HoxB4 as an important regulator of early but not late hematopoietic cell proliferation, since it induced selective expansion of primitive populations *in vitro* and *in vivo* without associated anomalies in the peripheral blood or leukemic transformation (Sauvageau *et al.*, 1995). HoxB8, on the other hand, induced the conditional immortalization of myelomonocytic, megakaryocytic and mast cells (Perkins and Cory, 1993) which could be established as long-term cell lines only in the presence of very high concentrations of IL-3. Leukemias developed after long latencies and commonly showed secondary activation of IL-3 gene expression, underscoring the cooperative effects of IL-3 and HoxB8 as demonstrated by gene transfer studies (Perkins *et al.*, 1990) and by their co-activation following IAP targeting in WEHI-3B tumor cells (Ymer *et al.*, 1985; Blatt *et al.*, 1988; Kongsuwan *et al.*, 1989). Overexpression of *HoxA10* by retroviral transfer into BM cells resulted in increased numbers of myeloid progenitors *in vitro* and *in vivo*, and the majority of transplanted mice developed AMLs (Thorsteinsdottir *et al.*, 1997). The non-Hox homeodomain protein Hox11 undergoes translocations in a subset of T-cell leukemias and is capable of immortalizing hematopoietic precursors, but does not induce myeloid leukemias (Hawley *et al.*, 1994).

The *HOX* gene transfer studies indicate that class I Hox proteins display a spectrum of oncogenic potentials (HoxA10>HoxB8>HoxB4). HRX–ENL, by comparison, appears most similar to *HOXA10* in its potent ability to immortalize and transform myeloid progenitors. In other settings, the closely related *HOXA9* gene is a target of chromosomal translocations in a subset of human myeloid leukemias (Borrow *et al.*, 1996; Nakamura *et al.*, 1996a), whereas *Hox-a7* and *Hox-a9* are targeted by proviral insertions in myeloid leukemias in BXH-2 mice

(Nakamura *et al.*, 1996b). Therefore, altered expression of 5' genes in the *HOXA* cluster frequently are associated with myeloid leukemias. Recent studies have shown that during hematopoietic differentiation, expression of 3' *HOX* genes is extinguished earlier than 5' *HOX* genes, which remain transcriptionally active into the committed progenitor stage and are inactivated as cells leave the CD34⁺ compartment (Sauvageau *et al.*, 1994). Furthermore, in leukemic cell lines, *HOXA10* displays an apparent specificity of expression predominantly in those of myelomonocytic derivation (Lowney *et al.*, 1991). Given our observations that HRX–ENL specifically perturbs the self-renewal and differentiation properties of myelomonocytic progenitors, a potential mechanism for its oncogenic effects may be to prevent the down-regulated expression of *Hox-a10* and/or other 5' *Hox* genes that accompany terminal myeloid differentiation.

The ability of HRX–ENL to induce the sustained proliferation of myeloid progenitors *in vitro* required the fusion of HRX with ENL and was not a simple consequence of HRX truncation or ENL overexpression. These results provide compelling support for a gain-of-function mechanism for the oncogenic effects of 11q23 translocations. Our findings do not rule out a possible contribution of reduced dosage of wild-type HRX in human leukemias due to loss of function on the translocated allele, as first suggested by the observation that knock-out mice heterozygous for a null *MLL* allele display homeotic morphologic abnormalities (Yu *et al.*, 1995). However, our findings exclude loss of function as the sole oncogenic event, and indicate that HRX fusion results in a gain of function dependent on contributions from the HRX fusion partner. In the case of HRX–ENL, these are likely to be transcriptional in nature, based on the similarity of ENL to the yeast protein Anc1 which is a component of two different basal transcription complexes, TFIID and TFIIF (Henry *et al.*, 1994). Anc1 is also a component of the yeast SWI/SNF complex (Cairns *et al.*, 1996) which is a subunit of the RNA polymerase II holoenzyme that alters chromatin structure and may assist in counteracting the repressive effects of chromatin on transcription initiation (Hirschhorn *et al.* 1992). ENL displays weak, promoter-specific transcriptional activation properties *in vitro*, suggesting that it serves an undefined transcriptional role in mammals as well (Rubnitz *et al.*, 1994). The only HRX fusion partner for which a biochemical function has been ascribed is ELL (Thirman *et al.*, 1994; Mitani *et al.*, 1995), which functions as a transcription elongation factor in *in vitro* assays (Shilatfard *et al.*, 1996). However, it is not known whether elongation activity is retained by ELL following fusion with HRX or is required for the oncogenic activity of HRX–ELL. *In vitro* assays that read out the transforming properties of HRX fusion proteins should, therefore, prove very useful for more refined structure–function studies to correlate their transcriptional and transforming contributions.

Materials and methods

Design and production of retroviral constructs

A 6 kb fragment of the *HRX–ENL* cDNA (Tkachuk *et al.*, 1992) was inserted into the retroviral vector MSCV (Hawley *et al.*, 1994) in sense and antisense orientations, using the *EcoRI* site located downstream

from the proviral LTR to generate the MSCV/HRX-ENL and MSCV/ as constructs, respectively. MIN/HRX-ENL was obtained by inserting an identical HRX-ENL coding sequence into the MIN vector derived from MSCV as follows: the 1.3 kb *XhoI*-*Clal* fragment of MSCV encoding the PGK-neo cassette was replaced by a 1.6 kb *XhoI*-*NaeI* fragment encoding the human encephalomyocarditis virus (Jang *et al.*, 1989) internal ribosomal entry site (IRES) fused in-frame to the extracytoplasmic and transmembrane portions of the low affinity nerve growth factor receptor (NGFR) (Johnson *et al.*, 1986) to which a universal termination *XbaI* linker (Biolabs, Beverly, MA) was added. HRXdel was created by digesting the HRX-ENL cDNA with *ApaI* which cleaves at the fusion site (position 4330). The resulting 5' HRX fragment was inserted into pMSCV to generate plasmid pMSCV/HRXdel. Plasmid pMSCV/ENL was created by cloning the ENL cDNA into the *EcoRI* site of pMSCV.

Bosc23 packaging cells were transfected with retroviral vectors as described previously (Pear *et al.*, 1993). Supernatants were collected 2, 3 and 4 days later, filtered and used for infection of enriched hematopoietic cells. Viral titers were determined by transfer of neomycin resistance to NIH3T3 cells after infection with day 2 retroviral supernatants as described previously (Lavau *et al.*, 1996).

Western blot analysis of protein expression

Expression of HRX-ENL, HRXdel and ENL proteins was verified by Western blot analysis. Nuclear extracts of transiently transfected Bosc23 cells were prepared by extraction with high salt buffer [500 mM NaCl; 20 mM HEPES pH 7.5; 0.5 mM EDTA; 0.1% Triton X-100; 0.5 mM Na vanadate; 2 mM NaF; 2 mM dithiothreitol (DTT); 0.2 mM phenylmethylsulfonyl fluoride (PMSF); 40 µg/ml aprotinin; 20 µg/ml leupeptin; and 40 µg/ml pepstatin A] and centrifugation. Protein samples (40 µg per lane) were separated in a 5% SDS-polyacrylamide gel and blotted onto polyvinylidene difluoride membranes (Immobilon-P, Bedford, MA) by tank blotting using an alkaline transfer buffer (10 mM CHAPS pH 11.0; 0.1% SDS; 1% methanol). Membranes were blocked in 5% non-fat dry milk in phosphate-buffered saline (PBS) with 0.2% Tween-20, and HRX fusion proteins were detected by staining with the monoclonal anti-HRX antibody HRX107 (Butler *et al.*, 1997) followed by a secondary horseradish peroxidase-conjugated goat anti-mouse IgM antibody (Sigma, St. Louis, MO) in a standard chemiluminescent Western blotting protocol (ECL, Amersham, Arlington Heights, IL). Rabbit anti-ENL antisera (Butler *et al.*, 1997) were employed for detection of ENL.

Enrichment and infection of primitive hematopoietic progenitors

Four-week-old C57BL/Ka.AKR/JSys-*Ptpr^a*-*Thy-1^a* (known as B.A1) mice (Thy-1.1, H-2^b, Ly-5.2) were injected i.v. with 150 mg/kg 5-FU in PBS. BM cells were harvested 2 (experiment 1) or 5 (experiment 2) days later and enriched for primitive hematopoietic precursors as follows. In experiment 1, hematopoietic stem and progenitor cells expressing low levels of Thy-1.1 and high levels of Sca-1 and H-2K^b antigens (Thy-1^{lo}Sca-1⁺H-2K^{hi}) were enriched by FACS as described previously (Szilvassy *et al.*, 1996b). In experiment 2, 5-FU-treated BM cells were stained with a cocktail of antibodies directed against antigens expressed on mature lymphoid or myeloid cells [CD3, CD5, CD8a, CD11b (Mac-1), Gr-1 and B220] and enriched for immature cells by immunomagnetic depletion as described previously (Szilvassy *et al.*, 1996b). Subsequently, 1–3 × 10³ (experiment 1) or 5–15 × 10⁴ (experiment 2) enriched hematopoietic cells were infected with retroviruses by centrifugation (spinoculation) for 4 h at 2500 g at 33°C in 1–2 ml of retroviral supernatant supplemented with 4 µg/ml polybrene (Bahnon *et al.*, 1995). The medium was then discarded and replaced with fresh culture medium [RPMI 1640, 10% fetal calf serum (FCS), 2 mM L-glutamine] supplemented with IL-3 (10 ng/ml), IL-6 (10 ng/ml) and SCF (100 ng/ml) and the cells allowed to 'rest' for ~20 h. The spinoculation procedure was performed once daily for three consecutive days and, following the third round, the infected cells were either plated into methylcellulose cultures or injected into irradiated mice.

CFC assays and generation of immortal progenitor cell lines

Retrovirally infected hematopoietic cells were divided so that an equivalent of 500 of the initially sorted Thy-1^{lo}Sca-1⁺H-2K^{hi} population (experiment 1) or 10⁴ of the initial lineage-depleted cells (experiment 2) were plated into duplicate 35 mm Petri dishes (Nunc) in 1.1 ml of Methocult M3330 methylcellulose medium (StemCell Technologies Inc., Vancouver) containing 10 ng/ml recombinant murine IL-3, IL-6, GM-CSF (R&D Systems, Minneapolis, MN) and 2% supernatant from COS cells expressing murine SCF (provided by M.Kilpatrick, SysTemix, Inc.)

with or without 1–1.3 mg/ml G418. Cultures were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air, and colonies consisting of >50 cells were scored after 9–11 days. Secondary CFC assays were performed by harvesting all of the G418-resistant cells from the primary cultures after colony enumeration, and replating them into new assays (10³–10⁴ cells/dish) under identical conditions without further G418 selection. Secondary colonies were scored as above, and tertiary and quaternary assays then conducted in the same way. Immortalized progenitor cell lines were generated by culturing ~250 (experiment 1) or ~100 (experiment 2) pooled tertiary colonies in RPMI 1640 medium containing 10% FCS plus supplements (50 U/ml penicillin G, 50 µg/ml streptomycin, 2 mM L-glutamine and 0.05 mM 2-mercaptoethanol). Cells were cultured initially in the presence of 10 ng/ml recombinant IL-3, IL-6 and 2% COS SCF supernatant, but IL-6 was omitted from the medium after three passages.

Cell proliferation assays

Immortalized cells were seeded in triplicate into 96-well plates (10⁴ cells per well) in 100 µl of supplemented RPMI 1640 medium containing various combinations of recombinant murine IL-2 (100 U/ml) (Genzyme Diagnostics, Cambridge, MA), IL-3, IL-6, IL-7, G-CSF, GM-CSF (all at 10 ng/ml) and SCF (100 ng/ml) (R&D Systems). Cell proliferation was measured 5 days later by staining with Alamar blue substrate (Alamar Biosciences, Sacramento, CA) and quantitation of fluorescence on a CytoFluor plate reader (Perseptive Biosystem, Pharmingham, MA) (Pagé *et al.*, 1993). Results represent the mean value from triplicate wells, and the assay was repeated four times at 1 week intervals.

Analysis of differentiation potential

The granulocytic differentiation potential of immortalized cells was measured by culturing them for 10 days in supplemented RPMI 1640 medium containing 10% FCS and 10 ng/ml recombinant murine G-CSF. B-lymphoid potential was assayed by culturing immortalized cells (100 per well) in 96-well tissue culture plates containing pre-established monolayers of unirradiated murine SyS-1 stromal cells (Whitlock *et al.*, 1987) in supplemented RPMI 1640 medium containing 5% FCS and 10 ng/ml recombinant murine IL-7. One half of the culture medium was replaced after 6 days and the cells harvested for analysis of morphology and B220 expression on days 10–12. Erythroid differentiation potential was assessed by culturing cell lines in methylcellulose medium or in liquid cultures containing 3 U/ml recombinant human erythropoietin-α (Amgen Inc., Thousand Oaks, CA) followed by immunophenotyping with TER-119 antibody on day 12.

Phenotyping and morphological analysis

Cytoprecipitated cells were stained with Wright-Giemsa stain to assess cell morphology. Immunophenotypic analysis was performed on cells collected in Hank's balanced salt solution containing 2% FCS, 0.02% sodium azide (HFN medium) and saturating amounts of purified anti-Fcγ receptor (clone 2.4G2) antibody to block non-specific staining. Cells were labeled with phycoerythrin (PE)-conjugated monoclonal antibodies directed against B220 (clone RA3-6B2), Gr-1 (clone RB6-8C5), CD11b/Mac-1 (clone M1/70), Thy-1.1 (clone His51), TER-119, CD43/Ly-48 (clone S7), c-kit receptor/CD117 (clone 2B8) and H-2K^b (clone AF6-88.5) antigens or with IgG_{2a}-PE or IgG_{2b}-PE isotype controls (Pharmingen Inc., San Diego, CA). Stained cells were washed and resuspended in HFN containing 2 µg/ml propidium iodide (PI) and analyzed with a FACScan instrument (Becton-Dickinson, Mountain View, CA) following exclusion of dead cells by high PI staining and forward light scatter.

Tumorigenicity and long-term in vivo reconstitution assays

Ten-week-old C.B17^{scid/scid} (SCID) or 6- to 12-week-old (C57BL/6J.SJL/J)Sys-*Ptpr^b*-*Thy-1^b* (known as B6.SJL) mice (Ly-5.1) were administered a sublethal dose of 5.25 Gy total body γ irradiation (¹³⁷Cs) from a Nordian Gamma Cell 40 machine (J.L.Shepard & Associates, San Fernando, CA) at a dose rate of 0.81 Gy/min. All mice were bred and maintained in the animal facility at SysTemix, Inc. SCID and B6.SJL recipients were injected i.p. or i.v., respectively, with 10⁶ immortalized cells, and the growth of tumors was monitored for 8 (SCID) and 4 (B6.SJL) months. To study the developmental potential *in vivo* of freshly isolated hematopoietic cells infected with HRX-ENL retrovirus, lethally irradiated (two doses of 5.25 Gy total body irradiation administered 3 h apart) B6.SJL mice were injected i.v. with a fraction of infected cells corresponding to 200 of the initial Thy-1^{lo}Sca-1⁺H-2K^{hi} cells subjected to the transduction, together with 10⁵ twice serially transplanted B6.SJL BM cells to provide radioprotection (Szilvassy *et al.*, 1990). Peripheral

blood was collected from the retro-orbital plexus of anesthetized mice 5 weeks after transplantation, and reconstitution by donor (Ly-5.2⁺) SCs assessed for each recipient by flow cytometric analysis of samples stained with anti-Ly-5.2-FITC (clone ALI4A2) as described previously (Szilvassy and Cory, 1993). Blood smears were stained with Wright-Giemsa to assess morphology. Circulating leukocyte, erythrocyte and platelet counts were determined by analysis of 40 µl of blood using a System 9000 Hematology Series Cell Counter (Sero Diagnostics Inc., Allentown, PA) with mouse-specific discriminator settings. Sick mice were euthanized by CO₂ inhalation and their tissues fixed in buffered formalin, sectioned and stained with hematoxylin and eosin for histological analysis. Transplantability of tumors was analyzed by injecting 10⁶ nucleated BM cells from primary diseased mice i.v. into 3–5 non-irradiated secondary B6.SJL recipient mice.

DNA analysis and RT-PCR

DNA was prepared from tissues or immortalized cell lines, and Southern blot analyses were performed using standard procedures. The hybridization probe consisted of a 2.2 kb *Hind*III *HRX* cDNA fragment (encompassing the *Kpn*I site) labeled with [α -³²P]dCTP using a random primer DNA labeling kit (Boehringer, Indianapolis, IN). Total RNA was isolated from cell lines using RNAzol (Tel-Test Inc., Friendswood, TX) as recommended by the manufacturer. Residual DNA was removed from the samples by digestion with RNase-free DNase, and poly(A)⁺ RNA was isolated from 100 µg of total RNA using an Oligotex mRNA isolation kit (Qiagen Inc., Chatsworth, CA). After reverse transcription with oligo(dT) primers and Superscript II reverse transcriptase (Gibco Life Technologies, Gaithersburg, MD), one-tenth of the resulting cDNA was subjected to PCR. The *HRX-ENL* fusion gene was amplified in 30 PCR cycles (30 s at 94°C, 1 min 10 s at 72°C) using primers (5'-gcaaacagaaaaagtgcctcccg-3'; 5'-accatccagtcgtgagtgaccct-3') that generated a product spanning 373 bp across the breakpoint of the fusion cDNA. Reactions without reverse transcriptase and with buffer alone served as controls.

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