Lhx2 expression in hematopoietic progenitor/stem cells *in vivo* causes a chronic myeloproliferative disorder and altered globin expression

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Background and Objectives. Chronic myeloproliferative disorders (CMDs) are thought to be due to mutation(s) in a single clone at the level of the hematopoietic stem cell (HSC). Such mutations and additional mutations causing progression of the disease are largely unknown. Chronic myeloid leukemia (CML) is a CMD characterized by a chromosomal translocation between chromosomes 9 and 22 creating the fusion protein BCR-ABL. This translocation has also been suggested to cause mis-expression of the LIM-homeobox gene *Lhx2* in hematopoietic cells. We have previously shown that *Lhx2* expression in mouse HSC generates cytokine-dependent stem cell-like cell lines that can produce long-term repopulation in stem cell-deficient mice.

Design and Methods. Since the consequences of *Lhx2* expression in hematopoietic cells *in vivo* were unknown, mice engrafted with the stem cell-like cell lines were analyzed in detail for any pathologic changes.

Results. Expression of *Lhx2* was maintained *in vivo* and most engrafted mice developed a myeloproliferative disorder characterized by splenomegaly, extramedullary hematopoiesis and anemia. The disorder was transplantable and the *Lhx2*-expressing cells could also cause acute leukemia. The anemia was due to both a reduced number of circulating erythrocytes and a reduced mean corpuscular hemoglobin concentration (MCHC).

Interpretation and Conclusions. These observations suggest that constitutive expression of Lhx2 in hematopoietic cells causes CMD, and also that a novel cell-autonomous mechanism can contribute to anemia.

Key words: anemia, chronic myeloproliferative disorders, *Lhx2*.

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The chronic myeloproliferative disorders (CMD) are a group of hematologic disorders including chronic myeloid leukemia (CML), essential thrombocythemia (ET), polycythemia vera (PV) and myelofibrosis with myeloid metaplasia (MMM). One of the characteristics in common to all these disorders is a progressive increase in the number of cells of one or several lineages in the hematopoietic system. CMD are suggested to be caused by mutation(s) in a single clone at the level of the hematopoietic stem cell (HSC).[1-5] Although a few specific mutations have been associated with ET and PV,[6-9] the underlying molecular mechanisms responsible for the initiation and progression of CMD are unknown.

CML starts with a chronic phase characterized by high peripheral white blood cell (WBC) counts, particularly neutrophilic granulocytes and myeloid precursors, extramedullary hematopoiesis and splenomegaly. CML usually accelerates into a terminal phase, referred to as the acute phase or blast crisis, during which there is accumulation of immature hematopoietic cells arrested at an early stage.[10] CML was the first neoplastic disorder in which a specific genetic aberration was identified.[11] The genetic alteration observed in CML involves a reciprocal translocation between chromosomes 9 and 22 [t(9;22)(q34.1;q11.21)].[12] This translocation results in a shortened chromosome 22, which is the cytogenetic hallmark of CML and is referred to as the Philadelphia chromosome (Ph[1]). At the fusion point on chromosome 22, the c-abl proto-oncogene from chromosome 9 and the breakpoint cluster region (bcr) gene on chromosome 22 generate a fusion protein denoted BCR-ABL.[13] Mice expressing the BCR-ABL protein in HSC develop a CML-like syndrome,[14-18] and specific inhibitors of the BCR-ABL protein improve the disease in humans,[19] supporting the notion that the BCR-ABL protein is involved in the pathogenesis of the disease. Whether expression of the BCR-ABL protein is solely responsible for the development of the myeloid hyperplasia during the chronic phase in CML is not clear since the bcr-abl transcript can also be detected in occasional blood cells of a significant fraction of healthy individuals.[20,21] Molecular and cellular characterization of additional genetic changes in CML cells would, therefore, increase our understanding of disease progression in CML and in CMD in general.

It has been shown that human CML cells express the gene *Lhx2* (previously *hLH-2*) whereas expression of this gene could not be detected in normal hematopoietic

cells.[22] The *Lhx2* gene is located close to the c-abl gene on chromosome 9 and the mis-expression of *Lhx2* was suggested to be due to *cis*-acting effects at the reciprocal translocation point caused by the chromosomal translocation.[22,23] The *Lhx2* gene belongs to the LIM-homebox gene family which is characterized by a homeodomain and a cysteine-rich LIM-domain first recognized in the homebox genes *lin-11*, *isl-1* and *mec-3*.[24-26] The LIM-homebox family of genes appears to be involved in many important developmental processes such as asymmetric cell division, tissue specification and differentiation of specific cell types.[24,26-31] *Lhx2* was first identified as a gene specifically expressed in pre-B lymphoid cell lines and was independently isolated as a factor that bound to the pituitary glycoprotein hormone α-subunit promoter.[32,33] *Lhx2* is expressed in liver, forebrain, optic stalk, neuronal retina, olfactory epithelia and the progress zone of developing limbs during embryonic development.[32,34] A critical function of *Lhx2* in the development of most of these tissues, including the hematopoietic system, has been revealed either by gene targeting in mice or by a dominant negative mutation in the chick system.[35,36]

The observation that *Lhx2* is expressed in many different organ systems during their expansion in embryonic development, together with its critical function in the development of the fetal hematopoietic system, prompted us to analyze the effect of *Lhx2* expression in hematopoietic stem/progenitor cells. By expressing *Lhx2* in embryonic stem (ES) cells differentiated *in vitro* we were able to establish immortalized and cytokine-dependent pluripotent hematopoietic progenitor cell (HPC) lines.[37] Likewise, expression of *Lhx2* in bone marrow (BM) cells derived from adult mice allowed for the generation of immortalized cytokine-dependent bone marrow-derived hematopoietic progenitor/stem cell (BM-HPC) lines. The BM-HPC lines were pluripotent since they were clonal and generated myeloid, erythroid, B lymphoid and T lymphoid cells *in vivo* upon transplantation into lethally irradiated animals. The BM-HPC lines provided long-term repopulation of stem cell-deficient mice and could generate a large proportion of the circulating erythrocytes in primary, secondary and tertiary recipients for at least 18 months.[38]

Since normal hematopoietic progenitor/stem cells do not express *Lhx2* whereas human CML cells do,[22] we analyzed the animals that were long-term repopulated with *Lhx2* transduced cells in detail. Expression of *Lhx2* from the retroviral vector was maintained *in vivo* and most engrafted mice developed a myeloproliferative disorder resembling human CMD. Serial transplantation of the BM-HPC lines indicated that this CMD-like syndrome was transplantable and in a small fraction of recipients the cells also caused acute leukemia. Moreover, animals with a large proportion of erythrocytes derived from *Lhx2*-expressing HSC were anemic due to both reduced red blood cell (RBC) counts and decreased mean corpuscular hemoglobin concentration (MCHC), suggesting that *Lhx2* might interfere with erythropoiesis and/or globin expression in a cell-autonomous manner.

Design and Methods

Mice

Mice used in these studies were obtained from Jackson Laboratory and maintained at the animal facility at Umeå University under pathogen-free conditions. Cell lines were generated from the C57BL/6-cast (B6-cast) mouse strain (genotype *Gpi1^a/Gpi1^a*). The c-kit-kinase-deficient C57BL/6-*W^{41}/W^{41}* (B6W) mouse strain (*Gpi1^b/Gpi1^b*) was used as the recipient mouse strain in the transplantation experiments. All animal experiments carried out in this study were approved by the ethical committee at Umeå University.

Southern blot analysis, Northern blot analysis and in situ hybridization

Genomic DNA was prepared by standard procedures and subsequently digested with *BamHI*, separated on 1% agarose gel, blotted onto a Zeta-Probe GT blotting membrane (Bio-Rad, Hercules, CA, USA) and hybridized to radioactively labeled Neo-probe. Total RNA was prepared from bone marrow and spleen using the RNAgents system (Promega, Madison, WI, USA). Ten micrograms of total RNA were separated on a 1% formaldehyde agarose gel, blotted onto a Zeta-Probe GT blotting membrane and hybridized to radioactively labelled probes according to standard procedures.[39] All membranes were analyzed in a Phosphosimager (Molecular Dynamics, Sunnyvale, CA, USA). For analysis of spleen sections, the tissue was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) at 4°C over night. The spleens were subsequently transferred to 30% sucrose solution in phosphate buffer and incubated for at least 6 hours at 4°C. The spleens were mounted in O.C.T. Tissue Tek and sectioned into 8 μm slices and collected on superfrost slides (Fischer). Linearized cDNA clones of 1.3 kb containing the whole mouse *Lhx2* coding region, or 1 kb containing the *Lhx2* gene were transcribed with T3 or T7 RNA polymerase and digoxigenin labeling mix to generate antisense strands of *Lhx2* and Neo, respectively (Boehringer, Ingelheim, Germany). *In situ* hybridization was performed on sections as described elsewhere.[40] The labeled cells were detected with anti-digoxigenin antibodies using the DIG Nucleic Acid Detection Kit (Boehringer).

Histologic and morphologic analysis

For histologic analysis the spleens, fixed and mounted as described above, were cut into 8 µm sections, collected on superfrost slides and stained with hematoxylin-eosin (SIGMA, St. Louis, MO, USA). For morphologic analysis, single cell suspensions of bone marrow and spleens were prepared. Following osmotic shock to eliminate erythrocytes, the cells were cytospun and stained with May Grünwald-Giemsa stain (Merck).

Transplantation of BM-HPC lines and serial transplantation of bone marrow cells

Three million cells were transplanted by intravenous injection into sublethally irradiated (4-5 Gy) B6W mice as previously described.[38] For serial transplantation total bone marrow cells were harvested from primary B6W recipients and $1\text{-}2\times10^6$ nucleated cells were injected into sublethally irradiated B6W mice.

Glucose phosphate isomerase (Gpi) assays

Cells from bone marrow and spleens were collected and single cell suspensions were prepared. Following osmotic shock to eliminate erythrocytes, nucleated cells were lysed in 7 mg/mL EDTA solution and subjected to Gpi analysis. B6W mice have the *Gpi1*^{b} allele at the Gpi locus. The *Gpi1*^{b} type forms a band that migrates more rapidly from positive to negative on electrophoresis then does the band from the *Gpi1*^{a} type. The procedures for electrophoresis and detection of the Gpi types were adapted from Eppig *et al*. and Harrison *et al*.[41,42]

Progenitor (clonal) assays

The progenitor assays were carried out in Iscove´s modified Dulbecco´s medium (IMDM, Gibco-BRL, Paisley, Scotland) containing 1% methylcellulose (Fluka, Neu-Ulm, Switzerland) supplemented with L-glutamine, 300 µg/mL iron saturated transferrin (Boehringer) and 10% plasma derived serum (PDS, Antech Inc. Tyler, TX, USA). The cells were plated in a final volume of 1.25 mL in 35-mm Petri dishes (Falcon 1008) in triplicate. The following growth factors were used at a predetermined optimal concentration: steel factor 100 ng/mL, thrombopoietin (Tpo) 20 ng/mL, interleukin (IL)-6 10 ng/mL, granulocyte colony-stimulating factor (G-CSF) 20 ng/mL, granulocyte-macrophage colony-stimulating factor (GM-CSF) 10 ng/mL, monocyte colony-stimulating factor (M-CSF) 20 ng/mL, erythropoietin (Epo) (Eprex-Cilag, Sollentuna, Sweden) 4 U/mL and IL-3, used at 1% final concentration. IL-3 was obtained from medium conditioned by X63Ag8-653 myeloma cells transfected with a vector containing murine IL-3 cDNA.[43] Except where indicated all growth factors were obtained from R&Dsystems (Abingdon, United Kingdom).

Flow cytometry

The monoclonal antibodies used in this study were direct conjugates with phycoerythin (PE), fluorescein isothiocyanate (FITC) or biotin. The following antibodies were purchased from Pharmingen (San Diego, CA, USA): anti-Gr-1 (RB6-8C5), anti-Mac-1 (M1/70), anti-CD3 (145-2C11), anti-CD19 (1D3), and TER119. Most analyses were carried out on cell populations from which the erythrocytes had been eliminated by osmotic shock; the exceptions were when the erythroid/erythroid precursor specific TER119 antibody was used. Unspecific antibody binding was prevented by incubating cells on ice in supernatant from the 2.4G2 hybridoma 15 min prior to all antibody labeling. The cells were incubated with specific antibodies on ice for 20 min, washed twice and subsequently incubated with PE-conjugated streptavidin (Southern Biotechnology, Birmingham, AL, USA). Labeled cells were washed twice before being analyzed in a FACScan (Becton Dickinson, San José, CA, USA).

Analyses of blood parameters

Blood was collected into tubes containing EDTA and subsequently analyzed in an automated blood analyzer (Sysmex, SE-9000).

Statistical methods

The Mann-Whitney U-test was used for group comparisons. The reduction of MCHC in the engrafted (high) group was significantly different (p=0.001) from that of all other groups. However, the most relevant comparison was between this group and the B6-cast control group since these two groups had red blood cells derived from progenitor/stem cells of the same genetic background. Hence, only the p value for this comparison is indicated in Table 2.

Results

Expression of Lhx2 from the provirus is maintained in vivo

We have previously shown that the expression of *Lhx2* in BM cells allowed the generation of clonal and cytokine-dependent HSC-like cell lines denoted BM-derived hematopoietic progenitor cell (BM-HPC) lines.[38] Independent BM-HPC lines reconstituted stem cell-deficient C57BL/6-W^{41}/W^{41} (B6W) mice for at least 1 year. Serial transplantation into secondary and tertiary B6W recipients showed that BM-HPC lines could produce a significant proportion (up to 100%) of the circulating erythrocytes for 18 months. Primary, secondary and tertiary recipients were engrafted with cells having the same retroviral insertion site as the original BM-HPC line.[38] To determine whether the retroviral vector was also transcriptionally active *in vivo*, we

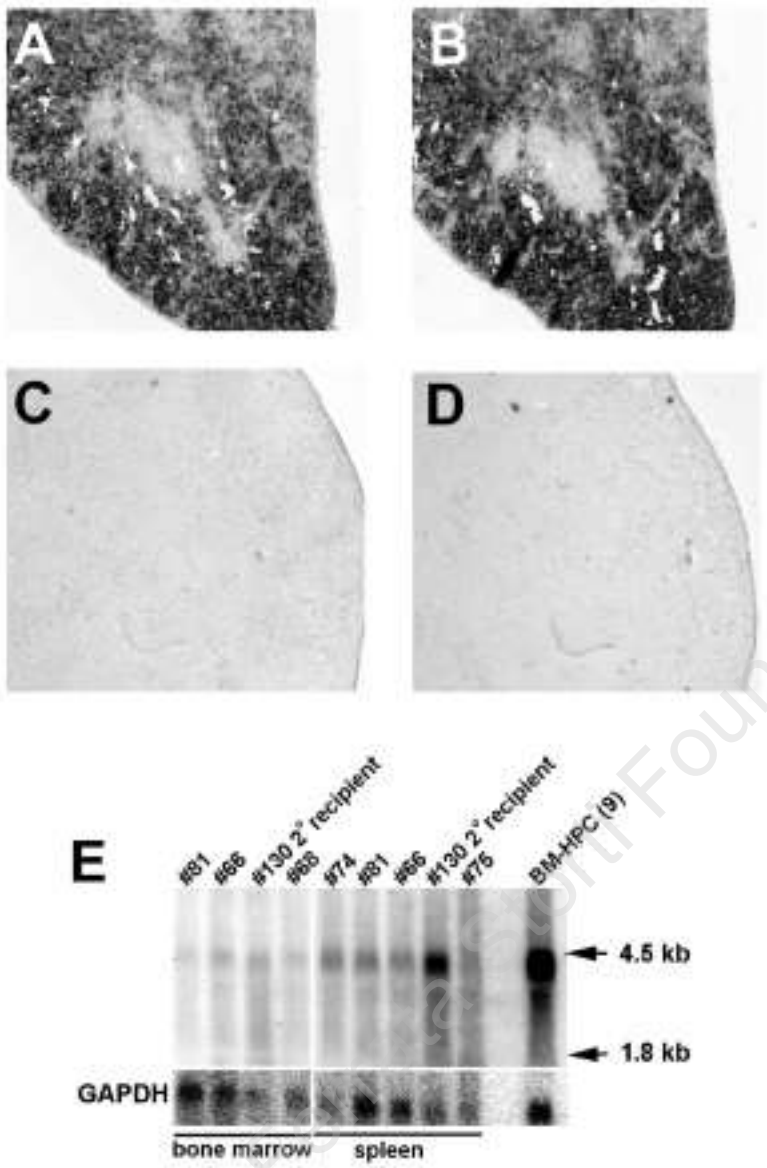

Figure 1. The retroviral vector is transcriptionally active in vivo. In situ hybridization of a spleen from an engrafted mouse (A and B) compared to a control spleen (C and D). An Lhx2 antisense probe was used in A and C and a Neo antisense probe was used in B and D. Original magnification A-D × 10. (E). Lhx2 expression is analyzed by Northern blot analysis of total RNA derived from bone marrow cells and spleen cells prepared from primary and secondary (2°) recipient animals. Sizes indicate locations of the 28S and 18S rRNA. The same blot was stripped and re-hybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

analyzed BM and spleen of a number of recipients for *Lhx2* expression. *In situ* hybridization revealed that a significant proportion of the cells in the spleen of engrafted animals expressed both Lhx2 and Neo (Figure 1A and B), whereas no expression was detected in control spleens (Figure 1C and D). The fraction of cells expressing *Lhx2 INeo* roughly corresponded to the fraction of donor cells as determined by Gpi assays (*ref. 38 and data not shown*). Northern blot analysis of spleen and BM of these mice showed that the size of the transcript was similar to that in the original cell line (Figure 1E). This observation, together with the observation that the cells had the same retroviral integration site as the BM-HPC lines,[38] suggest that the integrity of the proviral sequence is maintained *in vivo* and that most, if not all, *Lhx2* expression originates from the provirus. Thus, mice transplanted with BM-HPC lines are engrafted with cells continuously expressing *Lhx2*, a gene found to be misexpressed in human CML cells. These mice would, therefore, be useful for studying the *in vivo* consequences of *Lhx2*-expressing hematopoietic cells.

Mice engrafted with Lhx2-expressing cells develop splenomegaly and disrupted architecture of the spleen

While analyzing the engrafted mice, it became apparent that almost all of the mice engrafted with independent BM-HPC lines developed spleno-

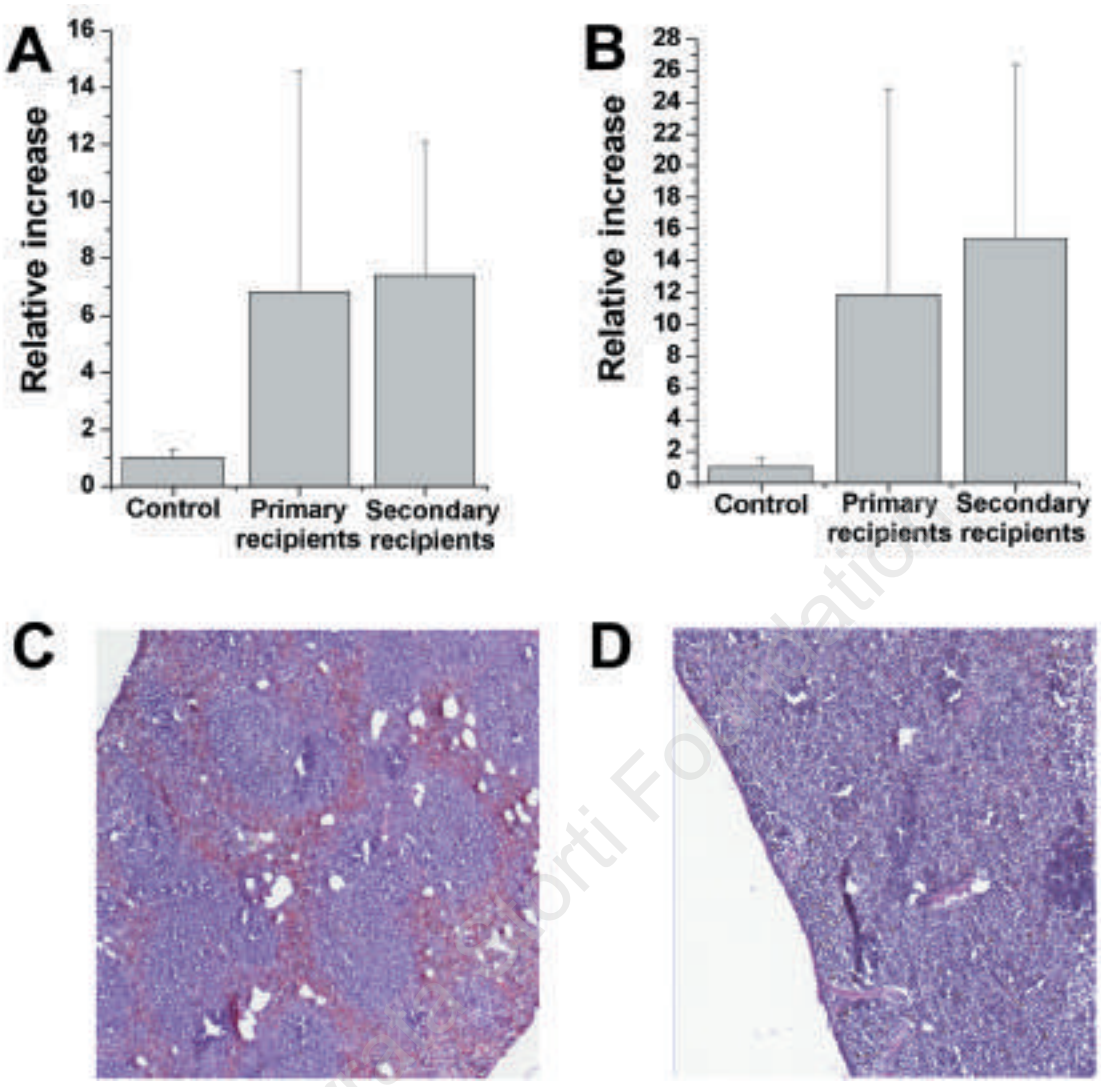

Figure 2. Mice engrafted with BM-HPC lines developed splenomegaly and disrupted architecture of the spleen. The relative sizes of the spleens were determined both by weight (A) and by cellularity (B). The size of control spleens was arbitrarily set as 1. The average size of the spleen from 22 primary recipients, 15 secondary recipients and 6 age-matched control mice are compared. Hematoxylin-eosin staining of a section of spleen from a control animal (C) and from an animal with splenomegaly (D). Original magnification C-D × 10.

megaly over time. The splenomegaly became obvious 3-4 months post-transplantation and progressed with time, most recipients showing severe splenomegaly 7-12 months post-transplantation. The average weight and cellularity of the spleen of primary recipients analyzed 8-12 months post-transplantation were 6.8 times and 11.9 times greater, respectively, than those of control spleens (Figure 2A and 2B). The cellularity of the BM in engrafted mice was not significantly different from that in the control mice (*data not shown*). There was no obvious correlation between the severity of the splenomegaly and the fraction of *Lhx2*-expressing cells in the spleen, since both a moderately and a severely enlarged spleen could contain 30-90% of *Lhx2*-expressing cells (*data not shown*). This finding suggests a cell non-autonomous component of the splenomegaly. However, the phenotype was transplantable since all the secondary and tertiary B6W recipients also developed splenomegaly to a similar degree, excluding the possibility that the splenomegaly was caused by a pure reactive myeloproliferation (Figure 2A and 2B, and *data not shown*). Histologic sections of the spleen

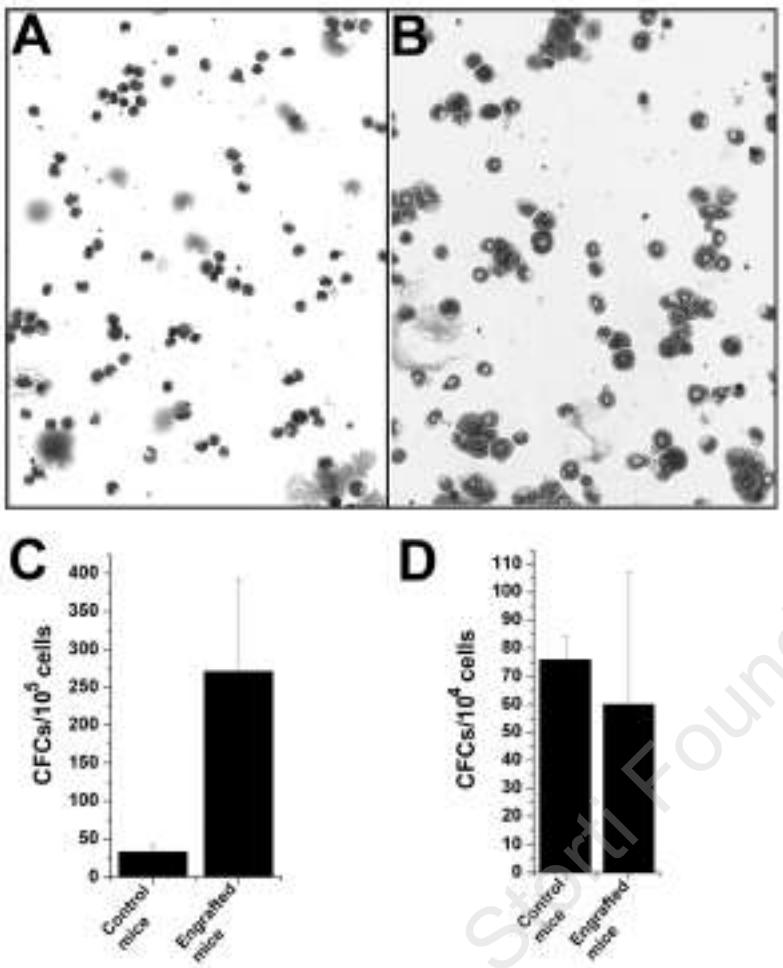

Figure 3. Extramedullary hematopoiesis developed in the spleen of mice with splenomegaly. May Grünwald-Giemsa staining of cytospun cells derived from the spleen of a control mouse (A) and from a mouse with splenomegaly (B). Original magnification A-B x 60. Clonal assays of cells derived from the spleen (C) and the bone marrow (D) of control mice and engrafted mice. The average frequency of colony-forming cells (CFC) of 5 control mice and 8 engrafted mice (primary and secondary recipients) is shown.

revealed that infiltrating cells disrupted the normal architecture of the spleen since no white and red pulp could be distinguished in engrafted animals (Figure 2C and 2D). Approximately 5% of the engrafted animals showed slight hepatomegaly that was due to infiltrating Lhx2$^+$ cells, but Lhx2$^+$ cells were rarely observed in the parenchyma of other organs (lungs, kidney, intestine, heart, brain). Thus, most engrafted mice developed severe splenomegaly and disrupted architecture of the spleen.

Cells of the myeloid lineage accumulate in the spleen of engrafted animals

We analyzed the cell types present in the spleen of engrafted mice by morphology and flow cytometry in order to characterize them further. The most common observation among the engrafted animals was the presence of cells in most stages of neutrophil development and usually some proerythroblast-like cells (Figure 3B), which were rarely present in control spleens as these mostly contained lymphoid cells (Figure 3A). This observation was confirmed by flow cytometry revealing a higher proportion of Gr-1$^+$, Mac-1$^+$ and TER119$^+$ cells in the spleen of engrafted animals than in the control spleens (Table 1). Most engrafted animals also

Table 1. Average fraction of different cell types in the bone marrow and spleen of control and engrafted animals.

Tissue	*Cell type*	*Control animals (n=3)#*	*Engrafted animals (n=4)*
Bone marrow	CD3$^+$	6±4	2±1
	CD19$^+$	12±7	1±2
	Gr-1$^+$	62±10	33±16
	Mac-1$^+$	77±8	59±23
	TER119$^+$	57±2	46±44
Spleen	CD3$^+$	24±3	7±2
	CD19$^+$	57±18	5±6
	Gr-1$^+$	8±4	39±12
	Mac-1$^+$	15±7	58±23
	TER119$^+$	26±8	48±13

#Number of mice analyzed.

showed lower proportions of lymphocytes (CD19+ and CD3+ cells) in both BM and spleen than did control animals (Table 1). In some engrafted individuals the myeloid cells (Gr-1+ and Mac-1+ cells) were expanded at the expense of the erythroid cells (TER119+ cells) in the BM, hence the large variability of these markers (Table 1). Other cell surface markers tested, such as Sca-1, c-kit, CD16/32, CD41, CD44, CD29, H-2k^b, or morphologic analysis, did not reveal any major differences between the BM of control and engrafted animals (*data not shown*). The increases of myeloid cells and their precursors in the spleen are indicative of extramedullary hematopoiesis.

Increase of clonable progenitor cells in the spleen of engrafted animals

To test whether hematopoietic activity was increased in the spleen we carried out clonal assays on cells from the spleen to determine the frequency of colony-forming cells (CFC). The frequency of CFC in the spleen of primary recipient mice was 8 times greater that in control spleens (Figure 3C). Since the cellularity of the spleen in engrafted animals was also increased (Figure 2B), the absolute number of CFCs in the spleen was increased by an average of 54-fold compared to that in control animals. The frequency of CFC in the spleen of engrafted mice was comparable to the frequency of CFC in the BM of control mice, but there was no significant difference between the frequency of CFC in the BM of control mice and engrafted animals (Figure 3D) (76±8 per 10^4 cells vs. 53±41 per 10^4 cells, respectively). The fraction of donor cells among the CFC generally correlated to the fraction of donor cells in the whole organ[38] (*and data not shown*). The substantial increase of the total number of CFC in the spleen further supports the idea that the engrafted animals developed extramedullary hematopoiesis.

Engrafted animals are anemic and can have leukocytosis

CMD are often associated with leukocytosis, anemia and erythrocytes with morphologic abnormalities such as poikilocytosis or anisocytosis[44,45] To examine this issue, we analyzed the peripheral blood parameters of mice 8-12 months post-transplantation. Based on Gpi-assays the donor contribution to nucleated cells in the BM and spleen of all engrafted mice analyzed was at least 50% (*data not shown*). All engrafted mice analyzed, irrespective of whether the donor cells contributed to a large proportion (80-100%) or a low proportion (0-10%) of the red blood cells (RBC) were anemic to varying degrees as reflected by a low hemoglobin concentration (Hb) in the peripheral blood (Table 2). The former group of animals had a lower Hb value than the latter, despite comparable RBC counts,

Table 2. Peripheral blood parameters in control and engrafted mice.

Mice	*WBC* ($\times10^9$/L)	*RBC* ($\times10^{12}$/L)	*MCV* (fL)	*Hb* (g/L)	*MCHC* (g/L)
B6-cast (n=4)+	7.8 (6.7-8.8)	9.5 (9.2-9.8)	54 (53-55)	147 (145-151)	286° (281-305)
B6W control (n=4)	9.5 (7.1-9.6)	7.5 (7.0-8.3)	61 (59-62)	132 (125-143)	287 (285-291)
Engrafted (high)* B6W (n=9)	15.0 (9.5-23.6)	6.9 (6.6-8.2)	54 (51-56)	85 (77-111)	227° (223-242)
Engrafted (low)# B6W (n=5)	7.5 (5.0-46.2)	7.0 (3.7-7.0)	62 (61-77)	120 (68-122)	277 (238-282)

*Median values are displayed and minimum-maximum values are presented within brackets. +number of mice analyzed in each group; *engrafted animals in which donor contribution to RBC was high (80-100%); #engrafted animals in which donor contribution to RBC was low (0-10%); °p=0.001.*

suggesting defects at the single cell level. The reduction in RBC counts (93% of B6W control) correlated directly to the reduction in Hb (91% of B6W control) in engrafted animals which had a low fraction of donor RBC, as compared to B6W control mice (Table 2). However, in the group of mice in which the donor contribution to RBC was high, the Hb value was 58% of that in B6-cast control mice (85 and 147 g/L, respectively), whereas the RBC count was 73% of that in control mice (6.9×10^{12} and 9.5×10^{12}/L, respectively) (Table 2). Hence, the reduction of RBC counts could not account for the total reduction in Hb. Furthermore, in this group of animals, the mean corpuscular hemoglobin concentration (MCHC) was 79% of that in control B6-cast mice (227 and 286 g/L, respectively, $p = 0.001$) (Table 2). The reduced RBC counts together with the reduced MCHC values accounted for the whole reduction of Hb in this group. No obvious morphologic abnormalities could be seen in blood smears from engrafted animals with a high donor contribution to RBC (*data not shown*), and indeed the mean corpuscular volume (MCV) in this group of engrafted animals was identical to that in control B6-cast animals (Table 2), the mouse strain from which the BM-HPC lines were derived. The WBC counts were highly variable in the engrafted groups. Median WBC counts show no to moderate (two-fold) increase but individual mice could show up to a five-fold increase in WBC. Thus, mice engrafted with *Lhx2*-expressing cells developed an anemia that was due to both decreased RBC counts and defects at a single cell level, and some, but not

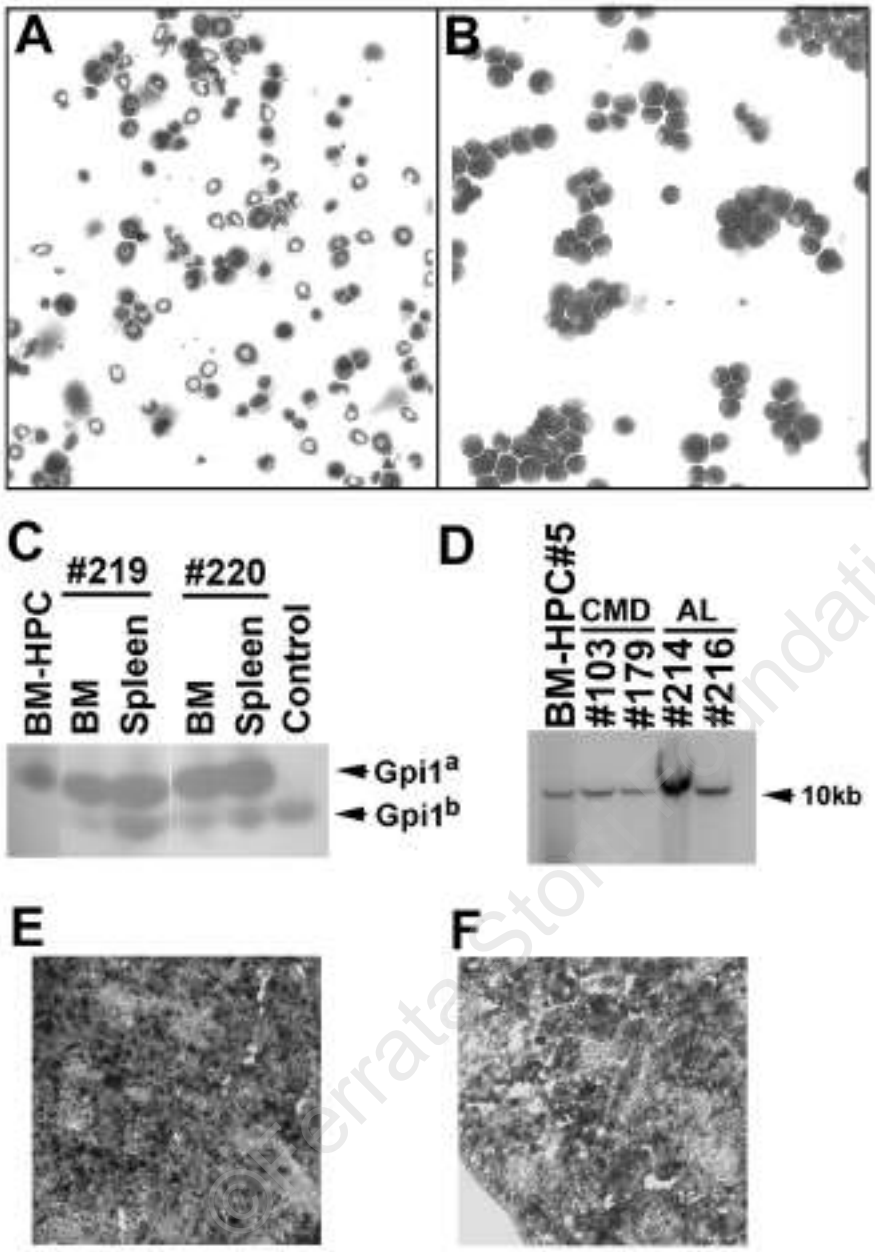

Figure 4. The Lhx2-expressing cells can cause acute leukemia. May Grünwald-Giemsa staining of cells derived from normal control BM (A) and from the BM of a representative recipient from the group of animals that became seriously ill and started to die within 3 months post-transplantation (B). Gpi analysis of the BM and spleen of two of the recipients (#219 and #220) developing acute leukemia (C). Bands corresponding to the donor Gpi isoform ($Gpi1^a$) and recipient Gpi isoform ($Gpi1^b$) are indicated. The control was a B6W mouse not transplanted with BM-HPC. (D). Southern blot analyses of genomic DNA derived from BM-HPC line #5 and the spleen of mice engrafted with this cell line. Mice #103 and #179 are representative of the group that developed CMD and mice #214 and #216 are representative of the group that developed acute leukemia (AL). In situ hybridization of spleen from two mice that developed acute leukemia using Lhx2 as a probe (E and F). Original magnification A-B × 60 and E-F × 20.

all, also had leukocytosis. These data indicate that expression of *Lhx2* in HSC interferes with normal erythropoiesis and/or globin expression.

The CMD-like disorder can transform into an acute leukemia

In some CMD, particularly in CML, the disease progresses from a relatively benign chronic phase into an acute phase, or blast crisis, in which immature cells accumulate in hematopoietic organs.[44] In one group of secondary recipients, several mice became seriously ill and died within 3 months post-transplantation. BM and spleen smears from the mice that we were able to analyze (4/9) contained almost exclusively blast cells. The morphology of the blast cells was identical in all cases and compatible with acute myeloid leukemia (Figure 4A and 4B), indicating that 9 of 62 engrafted individuals (15%) developed acute leukemia. However, it should be noted that all individuals that developed acute leukemia had received BM cells from the same donor. The vast majority of the cells in BM and spleen of the mice with acute leukemia were of BM-HPC origin (Figure 4C) and expressed *Lhx2*

(Figure 4E and 4F). The cells causing the acute leukemia in this group of mice and the cells causing the CMD-like disease in the other groups of mice had the same retroviral insertion site as the original cell line that was transplanted into the respective animals (Figure 4D). Using a panel of different molecular markers for hematopoietic cells, we have thus far been unable to distinguish the acute leukemia cells from those present during the myeloproliferative stage. Markers used included PU.1, EpoR, GATA-1, myeloperoxidase and CD41. These data suggest that the cells causing the myeloproliferative disorder are also responsible for the progression into an acute leukemia.

Discussion

We previously demonstrated that stem cell-deficient B6W mice transplanted with HSC immortalized by *Lhx2*, had sustained engraftment, as shown by these cells' continuous contribution to a significant fraction of the circulating erythrocytes for at least 18 months.[38] The engrafted mice were analyzed in detail because human CML cells express *Lhx2* whereas normal hematopoietic cells do not express this gene.[22] The retroviral vector remained transcriptionally active *in vivo* since *Lhx2* and Neo expression derived from the provirus was detected in the spleen and BM of engrafted mice. Most primary, secondary and tertiary recipients of BM-HPC lines developed severe splenomegaly and showed signs of extramedullary hematopoiesis in the spleen. All individuals in one group of secondary recipient animals transplanted with BM cells from the same primary recipient developed acute leukemia, suggesting that the malignant transformation occurred in that primary recipient prior to transplantation. The clonal relationship (e.g. same retroviral insertion site) between the cells causing the chronic phase and the acute phase strongly suggest that the chronic phase can convert into an acute phase, as in human CML. Since most mice developed the CMD-like disease whereas a minority of the mice developed acute leukemia, it is unlikely that the retroviral insertion *per se* causes acute leukemia. Rather, it suggests that *Lhx2* expression causes CMD and that the cells accumulate random mutations during the chronic phase *in vivo* which eventually lead to a malignant transformation of the *Lhx2*-expressing cells. Thus, hematopoietic stem/progenitor cells expressing *Lhx2 in vivo* cause a myeloproliferative disorder which transforms into acute leukemia. These features resemble human CML in which a chronic phase, characterized by myeloid hyperplasia, precedes the acute phase, or blast crisis, characterized by acute leukemia.[10] The characteristics of the murine disorder described here fulfil the criteria for myeloproliferation (genetic) as described in the recently published proposals for classification of non-lymphoid neoplasms in mice.[46] All recipient animals developed anemia to varying degrees, as determined by a low Hb level in peripheral blood. The low Hb level in mice in which the donor contribution to circulating erythrocytes was high, was due to both decreased RBC counts and to decreased MCHC, suggesting a novel mechanism whereby mis-expression of disease-associated genes can contribute to anemia in a cell-autonomous manner.

The molecular hallmark of human CML is the expression of BCR-ABL fusion proteins in hematopoietic cells.[13] The BCR-ABL protein is clearly contributing to the myeloid hyperplasia in humans and causes a CML-like disease when ectopically expressed in mouse HSC.[14-19] Although it is unknown whether *Lhx2* is involved in the development of human CML, there are several features in common between ectopic expression of BCR-ABL and *Lhx2* in the mouse hematopoietic system that suggest that *Lhx2* might contribute to disease pathogenesis. Firstly, expression of BCR-ABL and *Lhx2* during *in vitro* differentiation of ES cells generates immortalized hematopoietic progenitor cell lines. The major differences between the resulting cell lines are that BCR-ABL-induced cell lines are cytokine-independent and cause a CML-like disease when transplanted into immunocompromised adult mice,[47] whereas the *Lhx2* -induced cell lines are cytokine-dependent and unable to engraft immunocompromised adult mice.[37,48] Secondly, expression of BCR-ABL or *Lhx2* in hematopoietic cells in adult mice causes a myeloproliferative disorder which can convert from a chronic phase to an acute leukemia, or acute phase, as does human CML. However, BCR-ABL usually promotes a more rapid onset and more severe disease progression than does *Lhx2*.[14-18,49,50] Thirdly, expression of BCR-ABL or *Lhx2* in hematopoietic cells in adult mice can cause leukocytosis.[14,16] Again, as compared to *Lhx2*, BCR-ABL consistently causes a more severe leukocytosis with myeloid precursors in the circulation in both CML patients and in the murine models.[51,52] It is suggested that BCR-ABL promotes proliferation and/or differentiation, induces anti-apoptotic pathways and alters adhesive properties of cells.[51,52] We have observed that the immortalization event induced by *Lhx2* is relatively specific to immature hematopoietic progenitor/stem cells.[37,38] These similar and complementary molecular mechanisms suggest that synergistic effects in the pathogenesis of the disease could be expected if BCR-ABL and *Lhx2* were to be co-expressed in the correct cellular context. Whether BCR-ABL and *Lhx2* act via overlapping molecular pathways or whether their respective pathways are independent of each other remains to be resolved.

The observation that a reduced MCHC contributes significantly to the anemia in recipients

with a high fraction of donor erythrocytes, suggests that *Lhx2* interferes in a cell-autonomous manner with globin expression and/or erythroid development. The *Lhx2* gene encodes a LIM-homedomain transcription factor and LIM-domain-containing and LIM-domain-interacting transcriptional regulators are known to be involved in erythroid development.[53,54] Since the *Lhx2* protein is most likely present during erythroid differentiation from an early stage,[37,38,48] its LIM-domain might physically interfere with these protein interactions and hence cause dominant negative effect(s). This mechanism might contribute to the fluctuations in donor erythropoiesis previously reported in mice engrafted with BM-HPC lines.[38] In human CML it has been reported that patients may present with anemia even though the number of erythroid precursors can be increased.[55] This finding is somewhat unexpected since expression of BCR-ABL in human hematopoietic progenitors appears to promote erythropoiesis,[56] and BCR-ABL can rescue erythroid development both from progenitor cells lacking the erythropoietin receptor (EpoR) and progenitor cells defective in EpoR downstream signaling.[57,58] This discrepancy could be explained by the expression of genes encoding proteins that interfere with erythroid development and/or globin expression in the transformed clone. If the transformed clone contributes to a major part of hematopoietic system, such a cell-autonomous mechanism would explain the development of anemia despite an increase in erythroid precursors.

Lhx2 is involved in the expansion of different organ systems during embryonic development, such as the forebrain, eyes, limbs and the hematopoietic system.[35,36] *Lhx2* appears to play an important role during the development of the hematopoietic system since it is expressed in the fetal liver where the number of HSC is increasing,[59,60] and *Lhx2*-deficient mice die *in utero* due to a severe anemia.[35] The anemia in the mutant mice is caused by a cell-dependent defect,[35] and *Lhx2* immortalizes HSC by a cell-dependent mechanism since *Lhx2*-induced proliferation *in vitro* is strictly dependent on Steel factor and additional secreted molecule(s) provided by the *Lhx2*-expressing cells themselves.[48] As the cells retain *Lhx2* expression upon transplantation a similar mechanism can be envisaged *in vivo*. The *Lhx2*-expressing cells might have an increasing proliferative advantage compared to host cells as these cells accumulate since the host (C57BL/6-W^{41}/W^{41}) cells are defective in c-kit signaling.[61] This idea is further supported by the observation that *Lhx2*-expressing cells do not accumulate in mice with normal c-kit signaling.[38] A similar mechanism might also be responsible for the accumulation of abnormal cells in different types of human CMD, in which the abnormal cells can be stimulated via an autocrine mechanism and the stimulatory signal preferentially triggers the abnormal cells. Autocrine loops involving IL-3, GM-CSF and G-CSF have been suggested for both human BCR-ABL$^+$ progenitor cells and murine progenitor cells expressing BCR-ABL,[47,62,63] although the relevance of this in the pathogenesis of the disease is not clear.[63,64] We have no evidence for the involvement of any of these growth factors in the proliferation of *Lhx2*-expressing cells (at least *in vitro*),[38,48] suggesting that distinct and putatively novel stimulatory signals are involved. Thus, elucidation of the genes regulated by *Lhx2* could give insights into the molecular mechanisms involved in HSC physiology as well as HSC pathology and the pathogenesis of myeloproliferative disorders.

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Contributions

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