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In Childhood Acute Lymphoblastic Leukemia, Blasts at Different Stages of Immunophenotypic Maturation Have Stem Cell Properties

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SUMMARY

We examined the leukemic stem cell potential of blasts at different stages of maturation in childhood acute lymphoblastic leukemia (ALL). Human leukemic bone marrow was transplanted intrafemorally into NOD/scid mice. Cells sorted using the B precursor differentiation markers CD19, CD20, and CD34 were isolated from patient samples and engrafted mice before serial transplantation into primary or subsequent (up to quaternary) recipients. Surprisingly, blasts representative of all of the different maturational stages were able to reconstitute and reestablish the complete leukemic phenotype in vivo. Sorted blast populations mirrored normal B precursor cells with transcription of a number of stage-appropriate genes. These observations inform a model for leukemia-propagating stem cells in childhood ALL.

INTRODUCTION

Acute lymphoblastic leukemia (ALL) is the most frequent malignancy of childhood. Despite its overall good response to current treatment protocols and an approximately 80% long-term event-free survival rate (Pui, 2000), there is great demand for new therapies for patients with high-risk and relapsed ALL with a cure rate below 50%.

There is an ongoing debate as to the existence of rare cancer stem cell populations (Clarke et al., 2006; Kelly et al., 2007; Kennedy et al., 2007; Adams et al., 2007), which, if they exist, would provide a key target for novel curative therapies. The presence of leukemic stem cells has been clearly defined in acute myeloid leukemia (AML) by xenotransplantation studies. It has been demonstrated that cells with the ability to reestablish human leukemia in nonobese diabetic/severe combined immunodeficiency (NOD/scid) mice are exclusively present within the $CD34^{+}CD38^{-}$ stem cell fraction (Lapidot et al., 1994; Bonnet and Dick, 1997). Like the normal hematopoietic stem cell compartment, the leukemic stem cell compartment in AML is heterogeneous and organized as a hierarchy with distinct subclasses that differ in their proliferative and self-renewal capacities. With a clonal tracking approach and serial transplantation of candidate leukemic stem cells, it has been possible to define short-term, long-term, and quiescent long-term leukemic stem cells within this hierarchy (Hope et al., 2004). These profound similarities between normal and leukemic hematopoietic stem cells support the hypothesis that AML arises

SIGNIFICANCE

There is an ongoing debate as to whether all malignancies are maintained by a small population of immature cancer stem cells (as for myeloid leukemias) or whether the majority of malignant cells possess some "stemness." This question is of key clinical relevance, as in vivo clonogenic stem cells constitute the primary therapeutic target. We have developed an in vivo assay permitting the analysis of the stem cell potential of human acute lymphoblastic leukemia (ALL) blasts at different stages of maturation. Using this approach, we demonstrate that B precursor blasts mirroring different stages of maturation display self-renewal and possess malleability within their altered B cell developmental program. Thus, leukemic lymphoid progenitors may not lose their self-renewal capability with maturation.

within the normal HSC compartment and retains a hierarchy similar to normal hematopoiesis.

For ALL, the picture is less clear. Our understanding of the hierarchy of childhood B precursor ALL has been limited by the lack of appropriate in vitro and in vivo models. The original hypothesis, as proposed by Mel Greaves, suggested that the success of treatment for childhood ALL is linked to the transformation of a B cell progenitor prone to undergo apoptosis (Greaves, 1993). In contrast, adult and certain types of high-risk ALL may originate in a more primitive stem cell equipped with multiple protective mechanisms to resist chemotherapy. This hypothesis is supported by studies showing that in the most common subtype of childhood ALL, ALL/t(12;21), blasts and preleukemic stem cells harboring t(12;21) are found exclusively in the more mature $CD19^+$ population (Hotfilder et al., 2002; Castor et al., 2005; Hong et al., 2008). Similarly, in high-hyperdiploid ALL, the hyperdiploidy is restricted to lymphoid cells (Kasprzyk et al., 1999). However, the identification of leukemic subclones with unrelated rearrangements of the diversity (D) and joining (J) segments (Stankovic et al., 2000) and of diagnostic cytogenetic abnormalities in lineage marker-negative cells (Quijano et al., 1997) argues for involvement of more primitive cells in certain ALL patients. Moreover, involvement of immature $CD34^+CD19^-$ cells in two types of high-risk ALL, namely infant ALL with a translocation t(4;11) and Philadelphia chromosome-positive ALL (Hotfilder et al., 2005; Castor et al., 2005), highlights the heterogeneity of ALL.

Most importantly, there is a paucity of functional in vivo studies showing successful engraftment of ALL subpopulations in immunodeficient mice. The studies published to date have presented heterogeneous results. In two studies, only cells with an immature stem cell-like immunophenotype (either $CD34^+CD38^-$ or $CD34^+CD19^-$) were able to engraft and reinitiate the leukemia in immunodeficient mice following intravenous injection (Cobaleda et al., 2000; Cox et al., 2004), while two more recent studies demonstrated engraftment of more mature $CD19^+$ lymphoid blasts rather than immature $CD19^-$ cells (Castor et al., 2005; Hong et al., 2008).

These conflicting results indicate that key questions regarding leukemic stem cells in ALL remain unresolved. In which cell does childhood ALL arise? Is there heterogeneity of stem cell involvement in ALL? What is the phenotype of the in vivo propagating leukemic stem cells? Is it a rare cell with a primitive immunophenotype, or do the majority of blasts retain some "stemness"? The aim of this study was to develop a more sensitive and consistent functional assay for self-renewing candidate ALL stem cell populations. Sorted blasts mirroring different stages of B cell maturation were able to fully reconstitute and maintain the human leukemia through serial transplantations. These populations also express a number of stage-appropriate B cell developmental genes.

RESULTS

Flow-Sorted ALL Cells Serially Engraft NOD/scid Mice Following Intrafemoral Injection

To establish a sensitive in vivo assay for self-renewing leukemic stem cell populations, unmanipulated and flow-sorted bone marrow cells from 13 children with ALL were transplanted via intrafemoral injection into NOD/scid mice, and engrafting leukemias were serially transplanted into secondary, tertiary, and quaternary recipients. Seven leukemias were classified as high-risk ALL, and the remaining six leukemias were from standard-risk patients (see the Supplemental Experimental Procedures available online). Overall, cells from 6 out of 7 high-risk (86%) and 2 out of 6 standard-risk (33%) leukemias engrafted. The eight engrafting leukemias were serially transplanted in 199 mice. Of the 185 mice that received at least the minimally engrafting cell dose of 2×10^3 cells (see below), 115 (62%) showed engraftment with more than 5% human leukemic blasts in the bone marrow, and 112 (61%) developed overt leukemia with 20%–100% blasts (mean = 89%, SD = 18%).

The Leukemia in the Mice Recapitulates the Original Disease within the Patient and Remains Stable through Four Serial Transplantations

To confirm that our mouse model recapitulates the original disease, a thorough morphological, flow cytometric, and molecular analysis of the human leukemias in the mice was performed. Human leukemic engraftment was assessed and quantified by flow cytometric analysis of cells within the lymphocyte gate (scatter profile) expressing CD19, CD34, and CD45. A representative comparison of the initial and posttransplantation blasts demonstrates a conserved immunophenotype (Figure S1). In all mice analyzed, the human cells had a B cell precursor phenotype consistent with the original diagnosis. Consistent with the flow cytometry data, bone marrow from leukemic mice showed a massive infiltration of mainly L1 lymphoblasts. Both of these findings remained stable from primary to quaternary transplants (Figure 1). Engraftment of human cells also correlated with the development of splenomegaly, indicating that transplanted mice developed overt leukemia (Figure S2).

Engrafted Human Cells Express the Immunophenotype and Carry the Genetic Markers of the Original Leukemia

As engraftment of normal human myeloid cells is dependent on pretransplant conditioning of the mice by irradiation (except for the transplantation of very high cell doses), coengraftment of normal human hematopoietic cells was not expected in our nonirradiated recipient mice (Spiegel et al., 2004). This was confirmed by flow cytometry, which failed to detect human B lineage-negative cells expressing the myeloid markers CD33 or CD15 (Figure S1). To molecularly confirm engraftment of leukemic rather than normal lymphoid progenitor cells, the proportion of leukemic cells within the different human subpopulations in the mice was quantified with fluorescence in situ hybridization (FISH) probes that detect the leukemia-specific aberrations. Twenty-one leukemic mice transplanted with samples containing the following chromosomal aberrations were selected for this analysis: t(4;11), t(11;19), dup(21q), and t(12;21). Human $CD34^+CD19^-$, $CD34^+CD19^+$, and $CD34^-CD19^+$ cell populations were purified from the marrow of engrafted mice by flow sorting these cells directly onto glass slides. The leukemia-specific chromosomal aberration could be detected in the majority of human cells at a mean frequency of 85% (SD = 12.0%) in each cell fraction (Table 1; Figure S3A). As the cells sorted directly onto the slides were not always nicely spread and were sometimes slightly damaged, FISH analysis was likely to underestimate the percentage of aberrant cells.

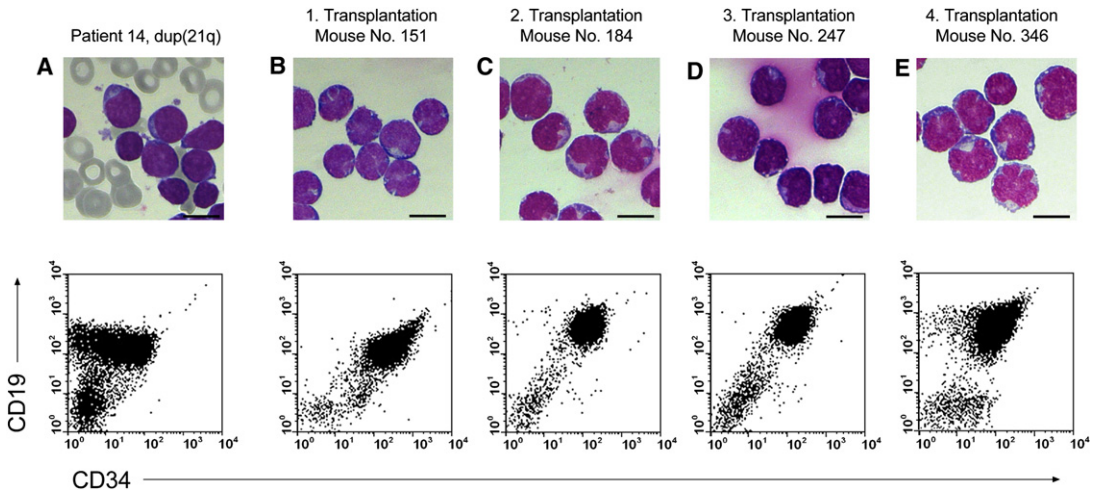

Figure 1. The Morphology and Immunophenotype of the Leukemic Blasts Remain Stable for Four Passages in the Mice

(A) Original patient #14 blast cell morphology and immunophenotype.

(B–E) Bone marrow samples derived from mice serially transplanted with $CD34^+CD19^+$ cells from the patient shown in (A). Blast cell morphology and immunophenotype resemble the original patient sample in all four consecutive transplantations.

Scale bars = 10 μm.

To confirm the leukemic nature of human engraftments from patient samples without a known chromosomal marker, we analyzed unsorted bone marrow of 26 leukemic mice by PCR to detect and quantify patient-specific clonal immunoglobulin heavy chain gene rearrangements. These rearrangements had already been established as a feasible minimal residual disease (MRD) marker within the patient bone marrow after initial diagnosis (#1075/04, Vd2-Dd3 and VH1.2-JH4b; #1002/05, VH1.2-JH4b and DH2.15-JH4b). We detected high MRD loads in 26 leukemic mouse bone marrows transplanted with cells from the two patients. Therefore, consistent with the flow cytometric data, the molecular analysis detects human leukemic engraftment in the mice.

Sorted $CD34^+CD19^-$, $CD34^+CD19^+$, and $CD34^-CD19^+$ Populations All Contain Leukemia-Initiating Cells

$CD34^+CD19^-$, $CD34^+CD19^+$, and $CD34^-CD19^+$ cells were purified from the primary patient sample or most often from engrafted primary or subsequent mice and serially transplanted into primary, secondary, tertiary, and quaternary recipients to identify which population would contain leukemic stem cell activity. To our surprise, all three leukemic subpopulations had the capacity to engraft and reconstitute the leukemia in NOD/scid mice after intrafemoral transplantation. First, we looked at the engraftment of sorted subpopulations from the two patients with standard-risk ALL. One patient was diagnosed with ALL/t(12;21), an ALL subtype that is thought not to involve the $CD34^+CD19^-$ compartment (Hotfilder et al., 2002; Castor et al., 2005). In concordance with these previous studies, $CD19^+$ cells from the two standard-risk ALL patients were able to transfer the leukemia into secondary, tertiary, and quaternary mice (Table 2). Interestingly, this was independent of the expression of CD34. In contrast, two mice transplanted with $CD34^+CD19^-$ cells showed no engraftment. A similar result was seen in mice transplanted with cells from high-risk patients (Table 2): $CD19^+$ cells, irrespective of whether they expressed CD34, were able to transfer the leukemia into recipient mice. In addition, the more immature $CD34^+CD19^-$ population was also shown to contain cells with leukemia-initiating capability at high frequency. Leukemia development after transplantation of the different subpopulations was a consistent and reproducible finding across the 115 mice that engrafted. The mean time to leukemia development was 11.7 weeks (SD 5.4 weeks), regardless of which population was transplanted (Figure S4A). At the point of sacrifice, all three populations had produced mean engraftment levels of >80% human cells (Figure S4B).

To confirm these surprising results, independent xenograft transplantation experiments were initiated at the Northern Institute for Cancer Research in Newcastle using ALL blasts sorted to be $CD19^+CD20^{-/low}$ or $CD19^+CD20^{high}$. Eight primary and five secondary NOD/scid *IL2Rγ*^{null} mice were transplanted with sorted blasts from two ALL patients (#L754 and #L736) and then assessed using diagnostic bone marrow punctures taken between 12 and 20 weeks without being sacrificed (Table 2). Of 7 mice receiving 8×10^4 to 1×10^5 $CD19^+CD20^{-/low}$ cells, 4 showed full engraftment with 7%–34% human leukemic infiltrates, 2 showed low levels of human cells (4%–5%), and 1 did not engraft. One engrafted mouse was harvested and transplanted into secondary mice. Of 4 primary and 2 secondary mice transplanted with 8×10^4 to 1×10^5 $CD19^+CD20^{high}$ cells, 3 have shown good engraftment (8%–19% blasts), 1 shows low levels of engraftment (2.5% blasts), and 2 died at 10 weeks post-transplantation before engrafting (<0.1% blasts). The engraftment of sorted populations again demonstrates recapitulation of the original patient immunophenotype (Figure S5). Ten of these thirteen mice were still alive and appeared to be in good health at the time of manuscript submission (March 26, 2008), and we continue to monitor their engraftment. As both $CD19^+CD20^{-/low}$ and the more mature $CD19^+CD20^{high}$ blasts engraft, these data confirm our observation that leukemic blasts, mirroring different stages of B cell maturation, display leukemic stem cell activity.

Table 1. All Analyzed Engrafted Subpopulations Are Leukemic

Patient	Mouse #	Subpopulation and Dosage	Transplantation (Prim.-Tert.)	Final Engraftment Level (%)	FISH-Positive Human Cells in Mouse (%)		
					$CD34^+CD19^-$	$CD34^+CD19^+$	$CD34^-CD19^+$
#14 (dup(21q))	153	6.0×10^6 unsorted	primary	96	74.1 (unsorted)		
	177	2.0×10^5 $CD34^+CD19^+$	secondary	98	60	84	–
	179	2.0×10^5 $CD34^+CD19^+$	secondary	96	91	74	84
	229	2.0×10^4 $CD34^+CD19^+$	tertiary	97	–	86	–
	232	2.0×10^4 $CD34^+CD19^+$	tertiary	97	–	87	–
	249	1.5×10^4 $CD34^+CD19^-$	tertiary	98	–	80	–
	213	3.0×10^4 $CD34^-CD19^+$	tertiary	97	77	43	72
	219	3.0×10^4 $CD34^-CD19^+$	tertiary	99	90	80	–
#1 (t(4;11))	51	1.0×10^5 $CD34^+CD19^-$	primary	76	69	69	–
	65	6.0×10^4 $CD34^+CD19^+$	secondary	97	–	75	65
#9 (t(4;11))	patient sample	–	–	–	100	98	99
	235	3.0×10^4 $CD34^+CD19^-$	primary	99	89	78	80
	236	2.0×10^5 $CD34^+CD19^+$	primary	99	88	88	90
	239	3.0×10^4 $CD34^+CD19^-$	primary	93	99	97	–
	240	2.0×10^5 $CD34^+CD19^+$	primary	97	–	94	98
#15 (t(11;19))	162	11.5×10^6 unsorted	primary	93	89	95	98
	202	2.0×10^6 $CD34^+CD19^+$	secondary	100	98	94	99
	206	4.0×10^4 $CD34^-CD19^+$	secondary	96	–	87	97
	243	2.0×10^6 $CD34^+CD19^+$	tertiary	98	–	92	90
	244	2.0×10^4 $CD34^+CD19^+$	tertiary	98	–	93	–
#866/06 (t(12;21))	167	2.0×10^5 $CD34^-CD19^+$	secondary	70	–	99	93
	104	7.0×10^6 unsorted	primary	34	–	98	93

High proportions of cells with leukemia-specific chromosomal aberrations were detected by fluorescence in situ hybridization (FISH) analysis in every engrafted subpopulation (mean = 85.4%, SD = 12.2%). Whenever possible, two specimens per engrafted mouse bone marrow were examined. Data represent mean values. –, no data available.

Of the 185 mice in the original experiments, 21 were transplanted with cells directly sorted from the original diagnostic bone marrow sample (patients #1, 9, and 12) (Table S1). Primary mice engrafted with $CD34^+CD19^-$ (n = 2), $CD34^+CD19^+$ (n = 4), and $CD34^-CD19^+$ blasts (n = 3). Moreover, eight additional NOD/scid *IL2Rγ*^{null} mice were transplanted in Newcastle with 1×10^5 $CD19^+CD20^{-/low}$ or $CD19^+CD20^{high}$ blasts directly sorted from the original patient sample (patient #L754). Five of these primary mice had human leukemic blasts in the murine bone marrow at 12 weeks after transplantation (Figure S5). We are therefore confident that there was no difference in engraftment, whether the cells were sorted directly from the original patient sample or from the leukemia recovered from engrafted mice.

Table 2. Engraftment of Populations from High-Risk and Standard-Risk ALL, Sorted Using CD34/19 or CD19/20

Transplanted Subpopulation	Transplanted Mice	Engrafted Mice
Standard-risk ALL (n = 3)		
$CD34^+CD19^-$	2	0 (0%)
$CD34^+CD19^+$	5	4 (80.0%)
$CD34^-CD19^+$	18	11 (61.1%)
$CD19^+CD20^{-/low}$	3	1 (33.3%)
$CD19^+CD20^{high}$	2	1 (50.0%)
High-risk ALL (n = 7)		
$CD34^+CD19^-$	37	15 (40.5%)
$CD34^+CD19^+$	80	58 (72.5%)
$CD34^-CD19^+$	41	27 (65.9%)
$CD19^+CD20^{-/low}$	4	3 (75.0%)
$CD19^+CD20^{high}$	4	2 (50.0%)

The table summarizes all 196 mice that were transplanted with purified ALL subpopulations from the ten engrafting leukemias (patients #1, 9, 12, 14, 15, 862/02, 1075/04, 1002/05, L736, and L754) using a minimal cell dose of 2×10^3 cells.

The Intrafemoral NOD/scid Assay Is a Highly Sensitive and Specific Model for Engraftment of Sorted Leukemic Populations

To evaluate the sensitivity of our mouse model, we performed a limiting dilution assay by transplanting $CD34^+CD19^-$, $CD34^+CD19^+$, and $CD34^-CD19^+$ cells from patient #12 in descending concentrations into NOD/scid mice as follows. $CD34^+CD19^-$ cells: 2 mice each transplanted with 1.0×10^3, 1.0×10^2, and 1.0×10^1 cells; $CD34^+CD19^+$ and $CD34^-CD19^+$ cells: 2 mice each transplanted with 1.0×10^4, 1.0×10^3, and 1.0×10^2 cells. None of the mice transplanted with $CD34^+CD19^-$ cells and one mouse each transplanted with 1.0×10^4 $CD34^+CD19^+$ or $CD34^-CD19^+$ cells engrafted. In addition, 4 out of 7 mice (57%) transplanted with $2–7 \times 10^3$ $CD34^+CD19^-$ cells and 3 out of 6 mice (50%) transplanted with 1.0×10^4 $CD19^+$ cells engrafted. Thus, a minimum cell dose of 2.0×10^3 cells was shown to be sufficient to reestablish the leukemia in

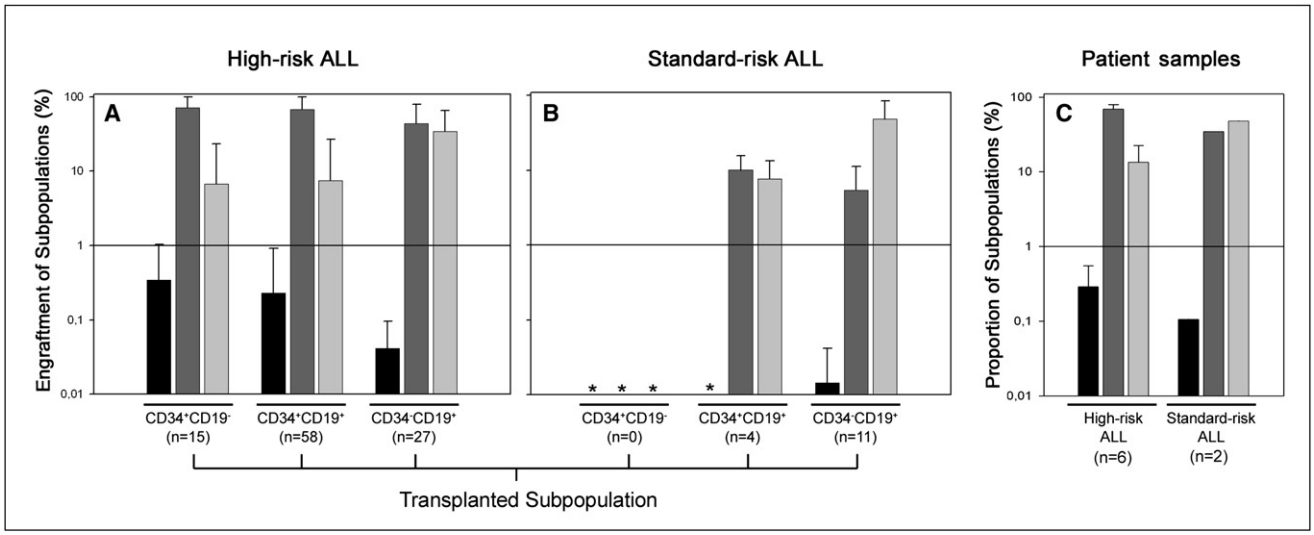

Figure 2. Immunophenotypic Composition of the Leukemic Grafts in the Mice

(A) High-risk ALL. The composition of the human grafts in respect to the presence of $CD34^+CD19^-$ (black), $CD34^+CD19^+$ (dark gray), and $CD34^-CD19^+$ (light gray) cells resembles that of the original leukemias shown in (C). All three subpopulations were able to reconstitute the complete phenotype of the original leukemia. However, mice transplanted with the more mature $CD34^-CD19^+$ immunophenotype engrafted with a slightly lower level of $CD34^+CD19^-$ cells (0.04% versus 0.2% and 0.3%; $p = 0.022$) and higher levels of $CD34^-CD19^+$ lymphoid cells (36% versus 6.3% and 7.4%; $p \leq 0.001$) as compared with mice transplanted with the other two cell fractions.

(B) Standard-risk ALL. The composition of the graft was similar in mice that received $CD34^+CD19^+$ and $CD34^-CD19^+$ cells. Two mice transplanted with $CD34^+CD19^-$ cells did not engraft.

(C) Distribution of the three subpopulations in the original patient samples.

Error bars show one SD (not calculated for two standard-risk patients in [C]).

recipient NOD/scid mice. This compares favorably with previously published xenograft models that required a minimum of 2.0×10^4 $CD34^+CD38^-$ cells (Cobaleda et al., 2000), 5.0×10^4 $CD34^+CD19^-$ cells (Cox et al., 2004), or 5.5×10^5 $CD19^+$ cells (Castor et al., 2005) to engraft transplanted mice and confirms the higher efficacy of intrafemoral as compared with intravenous transplantation (Mazurier et al., 2003).

Sorted cells were reanalyzed by flow cytometry, and the purities of the populations were 95% (SD = 2.5%; n = 9) for $CD34^+CD19^-$ cells, 97% (SD = 3.5%; n = 58) for $CD34^+CD19^+$ cells, and 96% (SD = 7.0%; n = 34) for $CD34^-CD19^+$ cells. Similarly, the sort purities of the $CD19^+CD20^{-/low}$ and $CD19^+CD20^{high}$ blasts were above 96%. Therefore, 1×10^4 $CD34^+CD19^+$ cells with an average purity of 97% may have contained 300 $CD34^+CD19^-$ or $CD34^-CD19^+$ cells and 2×10^3 $CD34^+CD19^-$ cells with an average purity of 95% may have contained only 100 $CD19^+$ cells, well below the minimal cell number necessary for leukemic engraftment. Table S2 summarizes those engraftments where the maximum contaminating population was lower than the minimally engrafting cell dose on 2.0×10^3 cells, confirming that engraftment originates from the bulk population and not contamination.

Flow-Sorted Populations Reestablish the Complete Immunophenotype of the Original Leukemia and Self-Renew

To compare the maturation and self-renewal potential of the different candidate stem cell populations, we analyzed the composition of the grafts in the mice as established by these subpopulations from high- and standard-risk patients (Figure 2). The data show that, independent of the immunophenotype of the transplanted population, the same pattern of surface marker expression as found in the original leukemia was reestablished in the bone marrow of leukemic mice.

Another important question was whether all three populations retain the capacity to reestablish the complete leukemic phenotype in vivo over a series of sequential transplantations. Figure 3 shows a representative series of three transplantations, starting with primary, unsorted mononuclear cells derived from patient #1075/04, followed by subsequent transplantations of sorted populations. This demonstrates that in both primary and subsequent transplants, regardless of immunophenotype of the transplanted population, the human leukemia in the mice very closely recapitulates the immunophenotype in the original patient. All three subpopulations, i.e., $CD34^+CD19^-$, $CD34^+CD19^+$, and $CD34^-CD19^+$ cells, were able to engraft, proliferate, reestablish the complete phenotype, and maintain the leukemia (self-renew) for at least four sequential transplantations, spanning a period of $\geq$12 months.

Sorted Primary Blasts Retain the B Precursor Developmental Program and Express Immunoglobulin Transcripts in a Stage-Appropriate Manner

While B precursor populations expressing all investigated developmental surface markers are able to recapitulate the full leukemic phenotype, we wanted to know whether this is representative of an underlying biological difference. Primary patient samples were sorted by fluorescence-activated cell sorting (FACS) to give blast populations expressing $CD34^+CD19^+$ and $CD34^-CD19^+$, with mean purity of 96.8% (SD 1.98%; data not

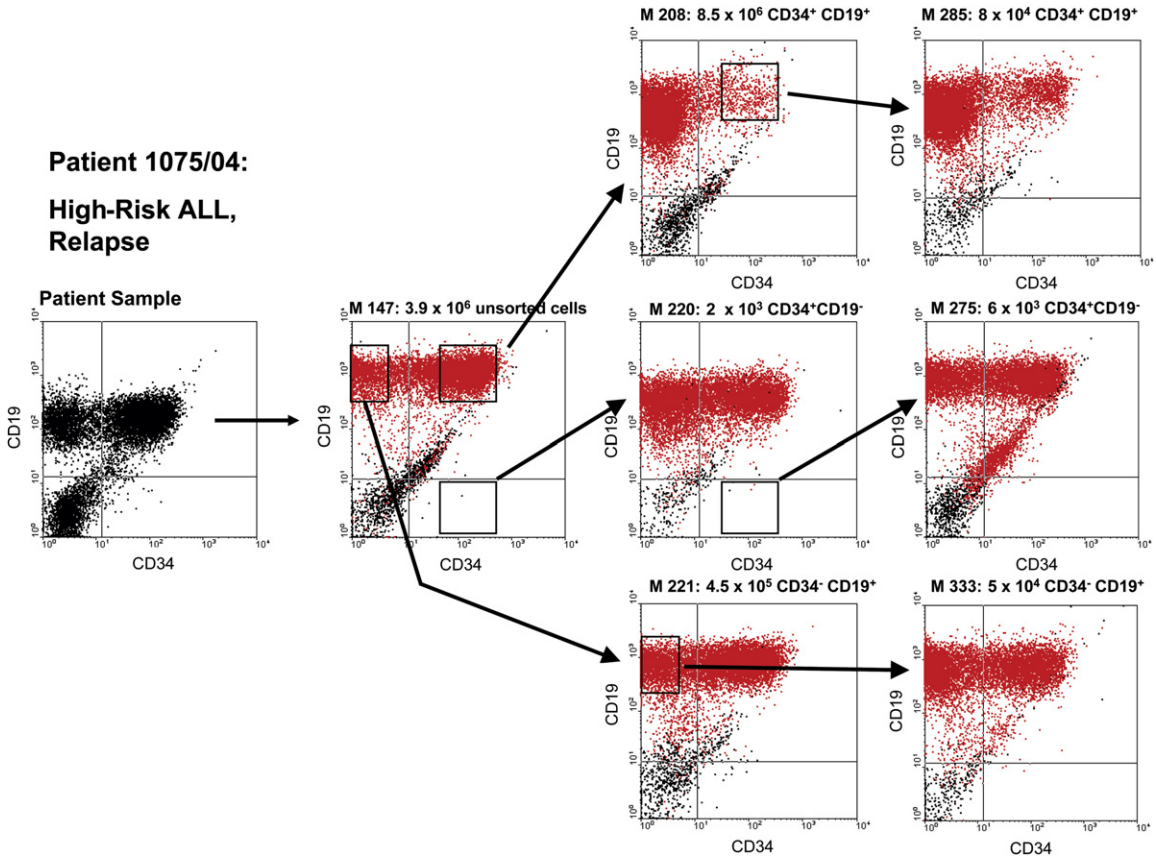

Figure 3. All Three Transplanted Subpopulations Can Self-Renew and Serially Transfer the Leukemia in the Mice

Each dot plot (except for the patient sample) represents one mouse engrafted with human leukemic cells. Sorting gates and arrows indicate the subpopulation each mouse received from its predecessor. Mouse number and the exact cell dose transplanted into that mouse are provided in the heading of each dot plot.

shown), for real-time RT-PCR analysis using a candidate gene approach. Figure 4 shows that, across four patient samples, the expression of transcription factors involved in the B precursor developmental program mirrors that expected in normal B cell development. Principally, both populations express the early transcription factors *E2A*, *EBF1*, *PAX5*, and *LEF1* while only the more mature $CD34^{-}CD19^{+}$ population shows a significant upregulation of *IRF4*, a transcription factor that has a role in making the immunoglobulin light chain loci accessible for recombination and expression in small pre-BII cells (Ma et al., 2006).

Furthermore, genome-wide expression analysis demonstrated that 5 of 13 genes that show >4× upregulation in the $CD34^{-}CD19^{+}$ populations are immunoglobulin loci transcripts (Table 3). Additionally, CD20 and CD34 appropriately show upregulation and downregulation, respectively, in the more mature population.

DISCUSSION

We have been able to characterize candidate human ALL stem cell populations by serial transplantation of flow-sorted subpopulations in immunodeficient NOD/scid mice. Up to now, progress on understanding the hierarchy of childhood B cell precursor ALL has been hampered by the lack of appropriate in vitro and in vivo models. In vitro, only a minority of ALL leukemias are able to survive and proliferate on stromal cell cultures (Nishigaki et al., 1997) and undergo rapid apoptosis. The few studies published to date showing successful engraftment of ex vivo manipulated subpopulations from B cell precursor ALL have yielded conflicting results (Cobaleda et al., 2000; Cox et al., 2004; Castor et al., 2005; Hong et al., 2008). We therefore set out to develop a more sensitive and robust transplantation assay to define the cell populations that are able to maintain the ALL in vivo (leukemia-propagating stem cells).

As has been shown in previous studies (Lapidot et al., 1994; Mazurier et al., 2003; Hope et al., 2004; Castor et al., 2005), the bone marrow microenvironment appears to be sufficiently conserved between mice and men to allow the survival and proliferation of normal and leukemic human stem cell populations that cannot be maintained in vitro. However, clinically aggressive and relapsed leukemias grow better in immunodeficient mice than samples from standard-risk patients (Kamel-Reid et al., 1991; Uckun et al., 1998), and not every human leukemia is able to engraft and proliferate in mice (Bonnet and Dick, 1997). This may be due to the dependency of some leukemias on microenvironmental signals that are not active across species. In accordance with this experience, we saw that cells from 6 out of 7 high-risk (86%) but only 2 out of 6 standard-risk (33%) patients engrafted. Despite this, the NOD/scid mouse model represents the best available functional assay for candidate human

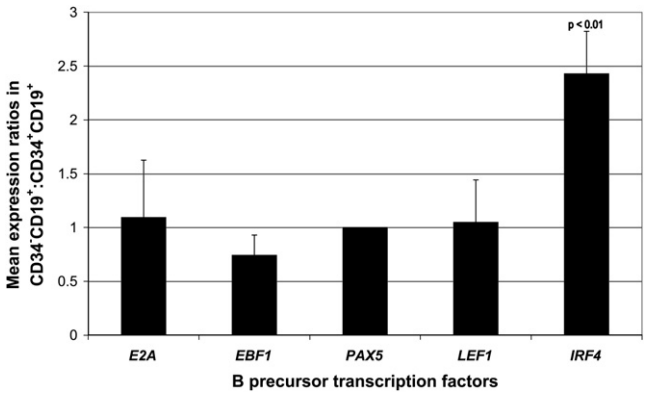

Figure 4. Primary Patient CD34$^-$CD19$^+$ Populations Show Significant Upregulation of the Small Pre-B Cell-Specific Transcription Factor *IRF4*

Each bar represents pooled quantitative PCR data from four (three for *E2A*) patient bone marrow specimens taken at diagnosis and sorted into CD34$^+$CD19$^+$ and CD34$^-$CD19$^+$ blast populations. ΔΔCt values between CD34$^-$CD19$^+$ and CD34$^+$CD19$^+$ populations have been transformed and normalized within a patient specimen against the B cell-specific transcription factor *PAX5*. Expression of *IRF4* shows significant upregulation in the more mature CD34$^-$CD19$^+$ population ($p < 0.01$). Error bars show one SD.

Table 3. Microarray Analysis of CD34$^+$CD19$^+$ and CD34$^-$CD19$^+$ Primary Blast Populations

Array ID	Transcript Product	CD34$^-$:CD34$^+$ Expression Ratio	
		Patient L787	Patient L812
228592_at	MS4A1 (CD20)	11	92
224795_x_at	Immunoglobulin κ locus	14	33
230245_s_at	Hypothetical protein	4	33
221671_x_at	Immunoglobulin κ locus	34	29
217022_s_at	Immunoglobulin heavy constant α 1 and 2	14	22
214677_x_at	Immunoglobulin λ locus	4	16
214836_x_at	Immunoglobulin κ locus	9	15
216834_at	Regulator of G protein signaling 1	10	7
237849_at	Transcribed locus	5	6
233955_x_at	CXXC finger 5	6	5
208178_x_at	TRIO	4	4
209013_x_at	TRIO	4	4
1568983_a_at	cDNA clone	4	4
209543_s_at	CD34	0.16	0.05
212002_at	Chr 1 open reading frame 144	0.24	0.18
243489_at	Transcribed locus	0.04	0.18

Two patient bone marrow specimens taken at diagnosis and sorted by fluorescence-activated cell sorting (FACS) using CD34 and CD19 expression show upregulation > 4× in the expression of immunoglobulin loci IgH, Igκ, and Igλ. *MS4A1* codes for the B cell differentiation marker CD20 and shows upregulation. CD34 shows appropriate downregulation in the CD34$^-$CD19$^+$ population.

leukemic stem cell populations, in which there appears to be only minimal pressure on the human cells to adapt to the murine microenvironment.

The intrafemoral NOD/scid serial transplantation assay described in the present study provides a highly sensitive and specific assay for transplantation of ALL stem cells. It has allowed us to achieve excellent rates of engraftment from a range of donor blast populations without preconditioning, thus avoiding coengraftment of normal hematopoietic cells. Most importantly, the constant immunophenotype and reproduction of clinical disease over serial transplantations and many months, whether the primary transplant involved sorted or unsorted material, provides reassurance that this assay is highly representative of the biology of our target population.

The sequential expression of immunophenotypic markers of B cell differentiation has been well defined (Hystad et al., 2007; Noordzij et al., 2002). Many are present throughout much of normal B precursor development, such as CD10, CD22, and CD38. Others can be used to define the "stages" of development of B precursor cells to a greater degree: CD34 is expressed on hematopoietic stem cells. Downregulation follows IgH locus rearrangement and the appearance of cytosolic IgM heavy chain. CD19 is a transcriptional target of PAX5, forming one of the earliest, B lymphoid-restricted markers. Surface expression correlates with rearrangement of V_H-DJ_H (Zhang et al., 2006). CD20 expression is upregulated in cells undergoing rearrangement of, or cells that have rearranged, the immunoglobulin light chain locus (Noordzij et al., 2002; van Zelm et al., 2005). We therefore chose to use these surface markers as a tool to identify and purify leukemic blasts mirroring subpopulations with differing maturation status.

Surprisingly, and in contrast to previous studies (Cobaleda et al., 2000; Cox et al., 2004; Castor et al., 2005), we were able to show that in high-risk ALL, each of the CD34$^+$CD19$^-$, CD34$^+$CD19$^+$, and CD34$^-$CD19$^+$ populations was able to serially transplant the leukemia. In keeping with previous studies showing that standard-risk ALL is restricted to the CD19$^+$ fractions (Hotfilder et al., 2002; Castor et al., 2005; Hong et al., 2008), low levels of CD34$^+$CD19$^-$ cells could only be purified from two leukemic mice and may not have constituted a real population. As expected, these cells did not engraft subsequent mice. In addition to propagating leukemia, each of the transplanted fractions was able to recapitulate the complete disease immunophenotype, demonstrating the ability of blasts to move back and forth between the different populations. Again, this characteristic was maintained over serial transplantations. These results have been independently replicated by a separate series of experiments at a second institute using CD20 as an alternative marker of differentiation (Figure S5).

These findings raised the question of whether the immunophenotypically defined populations genuinely represent cells with differing biology or whether the immunophenotype is a stochastic phenomenon. The expression patterns of sorted blasts indicate that the different populations display a pattern of B precursor transcription factors similar to that expected in normal B cell development, with upregulation of the late B precursor

transcription factor *IRF4* in the $CD34^{-}CD19^{+}$ population (for review, see Matthias and Rolink, 2005). Furthermore, initial expression array data show that the $CD34^{-}CD19^{+}$ population, which would be expected to contain small pre-BII cells in normal hematopoiesis, upregulates expression of immunoglobulin loci. These findings suggest that the biology of our sorted populations may mirror, at least in part, that of their ''normal'' hematopoietic counterparts.

One potential concern was that serial transplantation might lead to the selection of an in vivo cell line with an altered stem cell biology. However, mice transplanted with cells sorted directly from primary patient material demonstrated a consistent pattern of engraftment over multiple passages. All populations, defined using both CD34/CD19 (Table S1) and CD19/CD20 combinations, were able to reestablish the leukemia in mice. Thus, there appears to be no selection process that alters the human stem cell program during serial transplantations in the mice.

The high sensitivity of this assay may also raise the question of the purity of the flow-sorted cell populations. Transplantation of limiting dilutions with known purity has shown that, for each of the three populations, robust engraftment follows transplantation of very low cell doses at which the contaminant populations are too small to account for the leukemic engraftment. This argues strongly that engraftment originates from the intended populations and not from a minor, contaminating stem cell population.

Although unlikely, our study does not formally rule out that there is a rare leukemic stem cell population present in all of the different cell fractions. We have, however, previously shown that $CD34^{+}CD19^{-}CD117^{+}$ cells do not appear to be part of the leukemic clone, and $CD19^{+}CD117^{+}$ cells were not detectable (Baersch et al., 1999). In addition, the commonly used stem cell marker CD133 is usually not expressed on the most mature leukemic $CD34^{-}CD19^{+}CD20^{+}$ cells (Baersch et al., 1999). Thus, any putative rare ALL stem cell, present in all populations, could not be characterized by the established stem cell markers CD34, CD117, or CD133. The presence of a putative rare leukemic stem cell population therefore seems quite unlikely, also considering the fact that the different populations mirror normal B cell development. Thus, the observed differences between standard- and high-risk ALL, the consistency and reproducibility of the engraftment of all subpopulations, the sensitivity of the assay, and the purity of the flow-sorted cells all indicate that these results reflect the biology of childhood ALL rather than being an artifact of our model system.

In an elegant study, it has recently been shown that *TEL-AML1* affects a $CD19^{+}$ B lymphoid progenitor cell that has partially rearranged its DJ_H locus (Hong et al., 2008). The authors demonstrate that preleukemic stem cells reside only in an abnormal $CD34^{+}CD38^{-/low}CD19^{+}$ population, previously identified in *TEL-AML1* ALL (Castor et al., 2005). However, they performed only limited studies with three patient samples to test whether more mature leukemic $CD34^{+/-}CD38^{+}CD19^{+}$ cells may also have acquired this stem cell phenotype. As our experiments only contain two engrafted mice from one patient with *TEL-AML1*-positive ALL, one transplanted with $CD34^{+}CD19^{+}$ cells and one with $CD34^{-}CD19^{+}$ cells, the question of the leukemia-propagating stem cell populations in this ALL subtype remains unresolved. It may be that complete leukemic transformation is required for B precursors to develop their full stem cell potential. Further studies are therefore required to clarify whether, unlike other leukemic subtypes transplanted in this study, *TEL-AML1*-positive ALL displays stem cell activity only in a restricted population.

Our in vivo data are compatible with existing data showing a surprisingly high percentage of clonogenic cells in ALL samples (up to 20%) that are able to proliferate and self-renew in vitro on stromal cell cultures (Nishigaki et al., 1997). Similarly, in some murine leukemia models, a very high frequency of 1:6 leukemic stem cells can be found (Krivtsov et al., 2006). These data are compatible with our observation that all subpopulations, i.e., cell populations with an immature stem cell-like or a more mature B cell progenitor phenotype, contain leukemia-initiating cells. Due to the sensitivity of ALL blasts ex vivo and their tendency to undergo apoptosis, it is likely that this in vivo assay underestimates the frequency of leukemic stem cells. Therefore, despite the fact that after cell sorting, a minimum of 2×10^3 cells are needed to achieve successful leukemic engraftment, the actual frequency of leukemic stem cells may be higher, approaching that observed in the stromal cell assay.

In conclusion, we have shown that leukemic blasts expressing a range of B lineage differentiation markers are able to engraft immunodeficient mice, reconstitute the complete leukemia phenotype (even cell populations expected to be less mature than the engrafting cells), and continue to engraft further mice with the same diverse populations over four serial transplantations and 12 months. Furthermore, we have early evidence in support of these populations being diverse at a transcriptome level and appearing to mirror their ''normal'' immunophenotypic counterparts. From these findings, we have developed a model of B precursor ALL biology. Figure 5 shows both a schematic of normal B precursor differentiation with the pattern of transcription factors, immunogenetic rearrangements, and immunophenotype (A) and B precursor ALL behavior mirroring this ''normal'' process (B). While normal B precursor development follows a closely ordered pattern dictated by the sequential expression of transcription factors, we show that ALL blasts are able to move back and forth within this narrow window of development, adopting biology mirroring the relevant normal populations. That all populations of lymphoid blasts should be able to initiate a self-renewal program is perhaps not so unexpected given the ability of both large pre-BII cells and mature B cells to undergo clonal expansion. Thus, this model of malleability describes biological characteristics possessed by normal B precursor cells and therefore programs that presumably remain relatively accessible following leukemic transformation.

A key question remaining to be investigated is the role of immature $CD34^{+}CD19^{-}$ cells in drug resistance. Primitive hematopoietic stem cells are thought to protect themselves against DNA damage, e.g., by expression of certain transporter proteins (ABCB1/MDR-1/p-glycoprotein, ABCG2/BCRP) that pump a wide variety of xenobiotic toxins out of the cells. If primitive $CD34^{+}CD19^{-}$ leukemic stem cells express the same stem cell-associated resistance mechanisms, this may contribute to the poor prognosis of ALL with involvement of the primitive stem cell compartment. Even if more mature ALL blasts retain the ability to self-renew, the most primitive $CD34^{+}CD19^{-}$ leukemic stem cells may still provide a reservoir of drug-resistant stem cells

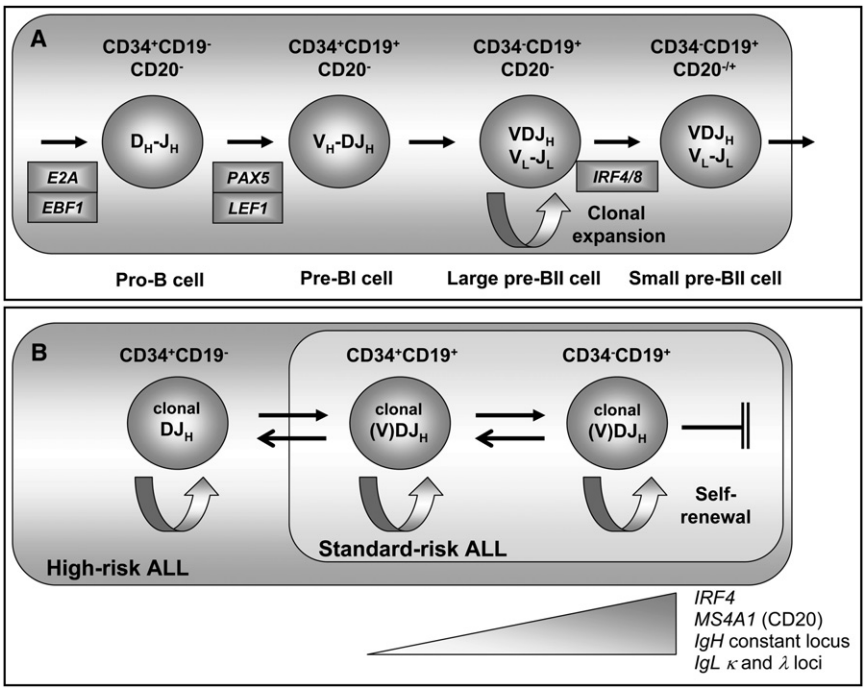

Figure 5. Model of the Malleability and Self-Renewal Seen in B Precursor ALL Blasts

(A) Normal hematopoiesis. The development from $CD34^+CD19^-$ early B progenitor expressing transcription factors *E2A* and *EBF1* through to $CD34^-CD19^+CD20^{+/-}$ small pre-B cells expressing *IRF4* and rearranging their immunoglobulin light chain loci. A period of clonal expansion in large pre-B cells is shown. DJ_H, D_H-J_H segments rearranged; V_H-DJ_H, rearrangement of the V_H-D_H segment; VDJ_H, heavy chain allele rearranged.

(B) ALL blast malleability and self-renewal. The blast compartment for high- and low-risk ALL is shown. Within these relatively narrow compartments, blasts are able to upregulate and downregulate components of the normal developmental program, including accessing the self-renewal program at all stages. There is, however, a block to differentiation beyond the B precursor stage. Genes demonstrated as being upregulated are shown below the schema.

that, unlike the more mature leukemic stem cells, survive chemotherapy and subsequently cause relapse.

This model of leukemia-propagating stem cells may prove instrumental in unraveling the exact developmental hierarchy within the different ALL subtypes, understanding the mechanisms of in vivo chemotherapy resistance, and targeting new drugs against the cells that cause chemotherapy resistance and relapse.

EXPERIMENTAL PROCEDURES

Patient Samples

Bone marrow specimens were taken from children with ALL either at diagnosis or at relapse (one patient, #14). Patients were under the care of the Department of Pediatric Hematology and Oncology, University of Münster, Germany; the Erasmus MC–Sophia Children's Hospital, Rotterdam, The Netherlands; or the Department of Paediatric Oncology, Royal Victoria Infirmary, Newcastle upon Tyne, UK. Further patient information is given in the Supplemental Data.

The study was approved in Münster by the combined Ethics Committee of the Medical Faculty, University of Münster and the regional chamber of physicians (registration number 31 VVormoor) and in Newcastle by the Newcastle and North Tyneside Ethics Committee 2 (REC reference number 06/Q0906/79).

Antibody Staining and Cell Sorting

Bone marrow freshly recovered from transplanted mice and thawed mononuclear cells (1.0×10^7 to 1.6×10^8 cells) from diagnostic bone marrow samples were analyzed by flow cytometry. Cells were washed with RPMI 1640 medium containing 20% fetal calf serum (FCS) and were stained with saturating amounts of monoclonal antibodies (mAbs) against human cell-surface antigens (Table S3) in a total volume of 0.5–1.0 ml for 20 min at 4°C. Flow cytometric analysis of bone marrow samples from patients and engrafted mice was performed on a FACSCalibur flow cytometer (BD Biosciences) using CellQuest Pro software. For cell sorting experiments, cells were stained with anti-CD34 and anti-CD19 mAbs (Beckmann Coulter), and the cell concentration was adjusted to 10^7 cells per 1.5 ml in RPMI 1640 containing 20% FCS. Cell sorting was performed on a FACSVantage SE cell sorter (BD Biosciences). Figure S6A shows the gates used to isolate our populations. An example of the reanalyses of the sorted populations is depicted in Figures S6B–S6D.

For experiments conducted in Newcastle, bone marrow samples were resuspended in 0.2% PBSA solution (BSA, Sigma; DPBS, Cambrex) and stained with saturating amounts of mAbs against human cell-surface antigens (Table S3). In addition, samples were labeled with anti-muCD45, a panleukocyte marker, and anti-muTER119, an erythroid marker, to gate out the majority of the murine background. Flow cytometric analysis was carried out on a FACSCanto II (BD Biosciences) using BD FACSDiva software. For cell sorting experiments in Newcastle, samples were stained with anti-CD19 and anti-CD20 mAbs as well as mAbs against muCD45 and muTER119 and transplanted as described below (for sorting gates, see Figure S5).

Animal Model

NOD/scid mice (JAX Mice stock name NOD.CB17-*Prkdc*^{scid}/J mice) were provided by J.E. Dick (University of Toronto) (with permission from L.D.S.). The NOD/scid mice were bred and maintained in individually ventilated cages at the University of Münster as described previously (Baersch et al., 1997). Due to a complex immunodeficiency involving B and T lymphocytes, natural killer (NK) cells, macrophages, and the complement system, NOD/scid mice are almost completely unable to reject human xenotransplants (Shultz et al., 1995). In general, unconditioned 8- to 16-week-old NOD/scid mice were used for the experiments.

To further increase the sensitivity of the NOD/scid mouse model, during the course of this study we began to NK cell deplete the mice with an anti-CD122 mAb. This treatment was shown to completely eradicate any residual NK cell activity still present in these mice and for the first time allowed engraftment of short-term repopulating human cells (McKenzie et al., 2005). A total of 89 of 199 mice (45%) were treated intraperitoneally with 135 μl of a 1.5 μg/μl anti-CD122 mAb preparation 18–24 hr before transplantation. The anti-CD122 mAb was purified from the hybridoma cell line TM-β1 (gift of T. Tanaka, Osaka University Medical Center, Osaka, Japan) (Tanaka et al., 1991). Purification was performed using High-Trap protein G columns (Amersham Pharmacia). The final preparation was quantified using the Bio-Rad Protein Assay (Bio-Rad) with bovine γ-globulin as standard.

For the additional experiments performed in Newcastle, NOD/scid *IL2Rγ*^{null} mice (JAX Mice stock name NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ) that completely lack any NK cell activity were used as recipients for human leukemic

transplants (Shultz et al., 2005). All experimental manipulations with mice were performed under sterile conditions in a laminar flow hood.

Primary intrafemoral transplantations were usually performed with unsorted patient samples. For serial transplantations, engrafted mice were humanely killed, and both femurs and tibias were flushed with 1.5 ml RPMI medium containing 20% FCS. Recovered bone marrow cells were stained with antibodies, sorted, and transplanted.

Overall, a total of 338 mice were transplanted with human leukemic cells (Münster cohort). 13 mice (4%) had to be excluded for technical reasons (mainly failed intrafemoral injections). 60 mice were transplanted with cells from the five nonengrafting leukemias (patients #2, 1365/01, 291/02, 384/05, and 804/05). 66 mice were transplanted with unseparated bone marrow cells from the eight engrafting leukemias (patients #1, 9, 12, 14, 15, 862/02, 1075/04, and 1002/05). The remaining 199 mice were transplanted with ALL subpopulations purified from engrafted leukemias. These 199 mice, of which 185 received at least the minimally engrafting cell dose of 2×10^3 cells, represent the primary experimental group for this study. For a complete list of all 199 transplantations performed, see Table S1.

The animal experiments in Münster were approved by the animal care committee of the local government (Bezirksregierung Münster, Aktenzeichen 50.0835.1.0 (G 25/2003)), and the transplantations in Newcastle were performed under Home Office license PPL60/3554.

Intrafemoral Sample Injection and Bone Marrow Sampling

All leukemic cell samples were injected directly into the right femur of NOD/scid mice as described previously (Mazurier et al., 2003). In brief, mice were anesthetized with an isoflurane/oxygen gas mix (2.5% isoflurane and 0.5 l O_2 per minute), and 3 μg buprenorphine (Temgesic, Essex Pharma) or 50 ng carprofen (Rimadyl, Pfizer) per 10 g body weight was subcutaneously injected as an analgesic. The right femur was punctured with a 25 or 27G needle, and sample volumes of up to 30 μl were subsequently injected with a 0.5 ml insulin syringe (27 or 30G). To sample bone marrow, primary femoral puncture with a 25 or 27G needle was followed by bone marrow aspiration with a 25G needle into a syringe filled with 400 μl of 10% heparin (Liquemin, Hoffmann-La Roche AG) in PBS buffer.

Preparation of Slides for Fluorescence In Situ Hybridization Analysis and May-Grünwald Giemsa Staining

Bone marrow cells for FISH analysis were prepared as described previously (Hotfilder et al., 2002, 2005). In brief, 600–2000 cells from each population were sorted into 20 μl drops of PBS that were placed on a grease-free glass slide. Settling and adherence of the cells to the glass slide was facilitated by incubating the slides for 10 min in a moist chamber. Excess PBS was carefully removed with a paper towel. Ice-cold methanol/glacial acid (3:1 v/v) was used to fix the cells, and the air-dried slides were analyzed by FISH. The histological analysis of patient samples and mouse bone marrow was performed by May-Grünwald Giemsa staining of cytospin preparations.

FISH analyses for the *TEL-AML1* gene fusions were performed on cytospin preparations of sorted cells using the LSI *TEL-AML1* ES Dual Color Translocation Probe Set (Vysis/Abbott). In addition, for detection of *MLL* rearrangements, the LSI *MLL* Dual Color Break Apart Rearrangement Probe (Vysis/Abbott) was applied. After a 2 min pepsin treatment (50 μg pepsin/ml 0.01 M hydrochloric acid [pH 2.3], 37°C), slides were washed once in PBS for 3 min, fixed in 4% formaldehyde for 10 min at 4°C, and, after two further washes with PBS, dehydrated in a 70%–100% ethanol series. Air-dried slides and probes were codenatured for 8 min on a 78°C heat block and hybridized in a moist chamber at 37°C overnight. Posthybridization washes and analyses of the cells were performed as described previously (Hotfilder et al., 2005).

Analysis of Patient-Specific Clonal Immunoglobulin Heavy Chain Gene Rearrangements

This analysis was performed as described previously (van der Velden et al., 2007). DNA from engrafted mouse bone marrow was extracted with a DNA extraction kit (QIAGEN) (Verhagen et al., 1999). qRT-PCR was performed on a LightCycler (Roche Diagnostics). For comparison and validation, initial diagnostic DNA samples of the patients were analyzed in parallel in the same experiment as positive controls. Identical crossing points of both materials confirmed specificity, origin, and high tumor load of the engrafted human leukemic cells in the mice.

Transcription Factor Expression

Primary patient bone marrow specimens were defrosted, labeled, and sorted as described above. After sorting, cells were immediately resuspended in RLT lysis buffer and homogenized using a 25G needle and syringe. Lysates were stored at −80°C prior to RNA extraction for qPCR and Affymetrix expression analysis.

RNA extraction was performed using RNeasy Plus Micro extraction columns (QIAGEN) according to the manufacturer's instructions. cDNA synthesis was performed using a RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas UK) according to the manufacturer's instructions. The starting reaction mixture contained 50 ng total RNA and random hexamer primers.

cDNA was used immediately for quantitative RT-PCR using an ABI Prism 7900HT (Applied Biosystems). Primers were designed using Primer Express software V2.0 (Applied Biosystems) to produce transcript-specific amplicons for the gene of interest. Primer sequences are listed in the Supplemental Experimental Procedures. Reactions contained 5 μl Platinum SYBR qPCR Supermix-UDG with ROX (Invitrogen Ltd.), 0.3 μl primer mix at 10 mM, 2.7 μl RNase-free water, and 2 μl cDNA. Experiments were run in triplicate. Data were analyzed using SDS 2.0 software (Applied Biosystems).

Affymetrix Expression Microarray Analysis

RNA from sorted cell populations was taken forward for expression microarray analysis using the Affymetrix GeneChip HG-U133 Plus 2.0 array (Geneservice Ltd.). Briefly, this process involves two cycles of cDNA synthesis and linear amplification. cDNA is transcribed in vitro prior to biotinylation of cRNA using the Affymetrix Two-Cycle Target Labeling kit. Biotinylated cRNA is subsequently hybridized to the array chip.

Data were processed by GC robust multiarray average (GCRMA) background adjustment normalization using the affy package supplied by BioConductor (http://www.bioconductor.org). Normalized data were analyzed for genes showing >4× difference in expression between $CD34^+CD19^+$ and $CD34^-CD19^+$ populations.

ACCESSION NUMBERS

The microarray data are available online at ArrayExpress (http://www.ebi.ac.uk/microarray-as/aer/) under the accession number E-MEXP-1522.

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, Supplemental References, six figures, and three tables and can be found with this article online at http://www.cancercell.org/cgi/content/full/14/1/47/DC1/.

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REFERENCES

Adams, J.M., Kelly, P.N., Dakic, A., Nutt, S.L., and Strasser, A. (2007). Response to comment on "Tumour growth need not be driven by rare cancer stem cells". Science *318*, 1722.

Baersch, G., Möllers, T., Hötte, A., Dockhorn-Dworniczak, B., Rübe, C., Ritter, J., Jürgens, H., and Vormoor, J. (1997). Good engraftment of B-cell precursor ALL in NOD-SCID mice. Klin. Padiatr. *209*, 178–185.

Baersch, G., Baumann, M., Ritter, J., Jürgens, H., and Vormoor, J. (1999). Expression of AC133 and CD117 on candidate normal stem cell populations in childhood B-cell precursor acute lymphoblastic leukaemia. Br. J. Haematol. *107*, 572–580.

Bonnet, D., and Dick, J.E. (1997). Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat. Med. *3*, 730–737.

Castor, A., Nilsson, L., Astrand-Grundström, I., Buitenhuis, M., Ramirez, C., Anderson, K., Strömbeck, B., Garwicz, S., Békássy, A.N., Schmiegelow, K., et al. (2005). Distinct patterns of hematopoietic stem cell involvement in acute lymphoblastic leukemia. Nat. Med. *11*, 630–637.

Clarke, M.F., Dick, J.E., Dirks, P.B., Eaves, C.J., Jamieson, C.H., Jones, D.L., Visvader, J., Weissman, I.L., and Wahl, G.M. (2006). Cancer stem cells–perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Res. *66*, 9339–9344.

Cobaleda, C., Gutierrez-Cianca, N., Perez-Losada, J., Flores, T., Garcia-Sanz, R., Gonzalez, M., and Sanchez-Garcia, I. (2000). A primitive hematopoietic cell is the target for the leukemic transformation in human philadelphia-positive acute lymphoblastic leukemia. Blood *95*, 1007–1013.

Cox, C.V., Evely, R.S., Oakhill, A., Pamphilon, D.H., Goulden, N.J., and Blair, A. (2004). Characterization of acute lymphoblastic leukemia progenitor cells. Blood *104*, 2919–2925.

Greaves, M.F. (1993). Stem cell origins of leukaemia and curability. Br. J. Cancer *67*, 413–423.

Hong, D., Gupta, R., Ancliff, P., Atzberger, A., Brown, J., Soneji, S., Green, J., Colman, S., Piacibello, W., Buckle, V., et al. (2008). Initiating and cancer-propagating cells in TEL-AML1-associated childhood leukemia. Science *319*, 336–339.

Hope, K.J., Jin, L., and Dick, J.E. (2004). Acute myeloid leukemia originates from a hierarchy of leukemic stem cell classes that differ in self-renewal capacity. Nat. Immunol. *5*, 738–743.

Hotfilder, M., Rottgers, S., Rosemann, A., Jurgens, H., Harbott, J., and Vormoor, J. (2002). Immature $CD34^{+}CD19^{-}$ progenitor/stem cells in TEL/AML1-positive acute lymphoblastic leukemia are genetically and functionally normal. Blood *100*, 640–646.

Hotfilder, M., Rottgers, S., Rosemann, A., Schrauder, A., Schrappe, M., Pieters, R., Jurgens, H., Harbott, J., and Vormoor, J. (2005). Leukemic stem cells in childhood high-risk ALL/t(9;22) and t(4;11) are present in primitive lymphoid-restricted $CD34^{+}CD19^{-}$ cells. Cancer Res. *65*, 1442–1449.

Hystad, M.E., Myklebust, J.H., Bo, T.H., Sivertsen, E.A., Rian, E., Forfang, L., Munthe, E., Rosenwald, A., Chiorazzi, M., Jonassen, I., et al. (2007). Characterization of early stages of human B cell development by gene expression profiling. J. Immunol. *179*, 3662–3671.

Kamel-Reid, S., Letarte, M., Doedens, M., Greaves, A., Murdoch, B., Grunberger, T., Lapidot, T., Thorner, P., Freedman, M.H., Phillips, R.A., and Dick, J.E. (1991). Bone marrow from children in relapse with pre-B acute lymphoblastic leukemia proliferates and disseminates rapidly in SCID mice. Blood *78*, 2937–2981.

Kasprzyk, A., Harrison, C.J., and Secker-Walker, L.M. (1999). Investigation of clonal involvement of myeloid cells in Philadelphia-positive and high hyperdiploid acute lymphoblastic leukemia. Leukemia *13*, 2000–2006.

Kelly, P.N., Dakic, A., Adams, J.M., Nutt, S.L., and Strasser, A. (2007). Tumour growth need not be driven by rare cancer stem cells. Science *317*, 337.

Kennedy, J.A., Barabe, F., Poeppl, A.G., Wang, J.C.Y., and Dick, J.E. (2007). Comment on "Tumor growth need not be driven by rare cancer stem cells". Science *318*, 1722.

Krivtsov, A.V., Twomey, D., Feng, Z., Stubbs, M.C., Wang, Y., Faber, J., Levine, J.E., Wang, J., Hahn, W.C., Gilliland, D.G., et al. (2006). Transformation from committed progenitor to leukaemia stem cell initiated by MLL-AF9. Nature *442*, 818–822.

Lapidot, T., Sirard, C., Vormoor, J., Murdoch, B., Hoang, T., Caceres-Cortes, J., Minden, M., Paterson, B., Caligiuri, M.A., and Dick, J.E. (1994). A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature *367*, 645–648.

Ma, S., Turetsky, A., Trinh, L., and Lu, R. (2006). IFN regulatory factor 4 and 8 promote Ig light chain κ locus activation in pre-B cell development. J. Immunol. *177*, 7898–7904.

Matthias, P., and Rolink, A.G. (2005). Transcriptional networks in developing and mature B cells. Nat. Rev. Immunol. *5*, 497–508.

Mazurier, F., Doedens, M., Gan, O.I., and Dick, J.E. (2003). Rapid myeloerythroid repopulation after intrafemoral transplantation of NOD-SCID mice reveals a new class of human stem cells. Nat. Med. *9*, 959–963.

McKenzie, J.L., Gan, O.I., Doedens, M., and Dick, J.E. (2005). Human short-term repopulating stem cells are efficiently detected following intrafemoral transplantation into NOD/SCID recipients depleted of $CD122^{+}$ cells. Blood *106*, 1259–1261.

Nishigaki, H., Ito, C., Manabe, A., Kumagai, M., Coustan-Smith, E., Yanishevski, Y., Behm, F.G., Raimondi, S.C., Pui, C.H., and Campana, D. (1997). Prevalence and growth characteristics of malignant stem cells in B-lineage acute lymphoblastic leukemia. Blood *89*, 3735–3744.

Noordzij, J.G., de Bruin-Versteeg, S., Comans-Bitter, W.M., Hartwig, N.G., Hendricks, R.W., de Groot, R., and van Dongen, J.J.M. (2002). Composition of precursor B-cell compartment in bone marrow from patients with X-linked agammaglobulinaemia compared with healthy children. Pediatr. Res. *51*, 159–168.

Pui, C.H. (2000). Acute lymphoblastic leukemia in children. Curr. Opin. Oncol. *12*, 3–12.

Quijano, C.A., Moore, D., 2nd, Arthur, D., Feusner, J., Winter, S.S., and Pallavicini, M.G. (1997). Cytogenetically aberrant cells are present in the $CD34^{+}CD33^{-}38^{-}19^{-}$ marrow compartment in children with acute lymphoblastic leukemia. Leukemia *11*, 1508–1515.

Shultz, L.D., Schweitzer, P.A., Christianson, S.W., Gott, B., Schweitzer, I.B., Tennent, B., McKenna, S., Mobraaten, L., Rajan, T.V., Greiner, D.L., and Leiter, E.H. (1995). Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. J. Immunol. *154*, 180–191.

Shultz, L.D., Lyons, B.L., Burzenski, L.M., Gott, B., Chen, X., Chaleff, S., Kotb, M., Gillies, S.D., King, M., Mangada, J., et al. (2005). Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. J. Immunol. *174*, 6477–6489.

Spiegel, A., Kollet, O., Peled, A., Abel, L., Nagler, A., Bielorai, B., Rechavi, G., Vormoor, J., and Lapidot, T. (2004). Unique SDF-1-induced activation of human precursor-B ALL cells as a result of altered CXCR4 expression and signaling. Blood *103*, 2900–2907.

Stankovic, T., Weston, V., McConville, C.M., Green, E., Powell, J.E., Mann, J.R., Darbyshire, P.J., and Taylor, A.M. (2000). Clonal diversity of Ig and T-cell receptor gene rearrangements in childhood B-precursor acute lymphoblastic leukaemia. Leuk. Lymphoma *36*, 213–224.

Tanaka, T., Tsudo, M., Karasuyama, H., Kitamura, F., Kono, T., Hatakeyama, M., Taniguchi, T., and Miyasaka, M. (1991). A novel monoclonal antibody against murine IL-2 receptor beta-chain. Characterization of receptor expression in normal lymphoid cells and EL-4 cells. J. Immunol. *147*, 2222–2228.

Uckun, F.M., Sather, H.N., Waurzyniak, B.J., Sensel, M.G., Chelstrom, L., Ek, O., Sarquis, M.B., Nachman, J., Bostrom, B., Reaman, G.H., and Gaynon, P.S. (1998). Prognostic significance of B-lineage leukemic cell growth in SCID mice: a Children's Cancer Group Study. Leuk. Lymphoma *30*, 503–514.

van der Velden, V.H.J., Panzer-Grümayer, E.R., Cazzaniga, G., Flohr, T., Sutton, R., Schrauder, A., Basso, G., Schrappe, M., Wijkhuijs, J.M., Konrad, M., et al. (2007). Optimization of PCR-based minimal residual disease diagnostics for childhood acute lymphoblastic leukemia in a multi-center setting. Leukemia *21*, 706–713.

van Zelm, M.C., van der Burg, M., de Ridder, D., Barendregt, B.H., de Haas, E.F.E., Reinders, M.J.T., Lankester, A.C., Revesz, T., Staal, F.J.T., and van Dongen, J.J.M. (2005). Ig gene rearrangement steps are initiated in early human precursor B cell subsets and correlate with specific transcription factor expression. J. Immunol. *175*, 5912–5922.

Verhagen, O.J., Wijkhuijs, A.J., van der Sluijs-Gelling, A.J., Szczepański, T., van der Linden-Schrever, B.E., Pongers-Willemse, M.J., van Wering, E.R., van Dongen, J.J., and van der Schoot, C.E. (1999). Suitable DNA isolation method for the detection of minimal residual disease by PCR techniques. Leukemia *13*, 1298–1299.

Zhang, Z., Espinoza, C.R., Yu, Z., Stephan, R., He, T., Williams, G.S., Burrows, P.D., Hagman, J., Feeney, A.J., and Cooper, M.D. (2006). Transcription factor Pax5 (BSAP) transactivates the RAG-mediated V_H-to-DJ_H rearrangement of immunoglobulin genes. Nat. Immunol. *7*, 616–624.