

Diverging fates of cells of origin in acute and chronic leukaemia

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The large difference in phenotypes among tumour populations may stem from the stochastic origin of tumours from distinct cells – tumour cells are assumed to retain the phenotypes of the cells from which they derive. Yet, functional studies addressing the cellular origin of leukaemia are lacking. Here we show that the cells of origin of both, BCR/ABL-induced chronic myeloid (CML) and B-cell acute lymphoid leukaemia (B-ALL), resemble long-term haematopoietic stem cells (LT-HSCs). During disease-maintenance, CML LT-HSCs persist to function as cancer stem cells (CSCs) that maintain leukaemia and require signalling by the transcription factor STAT5. In contrast, B-ALL LT-HSCs differentiate into CSCs that correspond to pro-B cells. This transition step requires a transient IL-7 signal and is lost in IL-7R α -deficient cells. Thus, in BCR/ABLp185⁺ B-ALL and BCR/ABLp210⁺ CML, the final phenotype of the tumour as well as the abundance of CSCs is dictated by diverging differentiation fates of their common cells of origin.

INTRODUCTION

The term ‘cells of origin of cancer’ (COCs) refers to the population of cells in which the initial transforming event has occurred, whereas the cancer-propagating ‘cancer stem cells’ (CSCs) describe the cells within an established tumour that sustain tumour growth *in vivo* (Adams & Strasser, 2008; Wang &

Dick, 2005). The phenotype of CSCs varies strongly among cancers—ranging from cells that resemble adult tissue stem cells (Huntly et al, 2004; Passegue et al, 2004), over progenitor-like (Cozzio et al, 2003; Jamieson et al, 2004; Kelly et al, 2007; Krivtsov et al, 2006; Somerville & Cleary, 2006), to mature cells with rearranged B-cell receptors (Barabe et al, 2007; Kelly et al, 2007).

Two major models are employed in CSC biology in order to explain intratumoural heterogeneity (Dick, 2008). The CSC model postulates that cancers are hierarchically organized and that self-renewal is limited to a highly specialized and immature cell fraction that can be distinguished from other tumour cells by its phenotype. This ‘stem cell-like’ population is functionally capable of differentiating into and reconstituting the entire phenotype of the respective tumour (Al-Hajj et al, 2003; Bonnet & Dick, 1997; Lapidot et al, 1994; Ricci-Vitiani et al, 2007; Singh et al, 2004). On the other hand, the stochastic model attempts to describe cancers lacking a functional hierarchy. Nevertheless, these cancers are not mandatory homogenous; some of these cancers may be phenotypically heterogeneous as a result of intrinsic and/or niche factors. These types of cancer may be propagated by most or all tumour cells (Adams & Strasser, 2008; Kelly et al, 2007; Quintana et al, 2008, 2010; Williams et al, 2007).

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Fairly little is known about the COCs from which cancer originally arises. The strong intertumoural diversities led to speculations that tumours may arise stochastically from any cell in a tissue. Thus, the progressing tumour mirrors the phenotype of the cell from which it arose (Visvader, 2011). Accordingly, every cell represents a potential COC and all tumour cells—including CSCs—are derivatives thereof. This concept was recently challenged by the finding that COCs in human prostate cancer (Goldstein et al, 2010) resemble stem/progenitor-like basal cells despite the differentiated appearance of the large bulk of tumour cells.

In leukaemia, both, normal stem and committed progenitor cells, have been implicated as COCs. Whereas murine chronic leukaemia may predominantly originate from HSCs (Huntly et al, 2004; Passegue et al, 2004; Perez-Caro et al, 2009; Somervaille & Cleary, 2006), the situation in acute leukaemia is less clear. MLL-GAS7 acute myeloid leukaemia (AML) arises from c-kit⁺ cells (So et al, 2003), while MOZ-TIF2 (Huntly et al, 2004), MLL-AF9 (Krivtsov et al, 2006) and MLL-ENL (Cozzio et al, 2003) induced acute leukaemia regardless of the target cell population expressing the respective oncogenes.

Our current knowledge relies on leukaemic mouse models and thus, it is currently unclear how well these studies translate into the human disease. So far, the only available experimental system used to define CSCs in human leukaemia is the xenotransplantation into immune-compromised mice (Barabe et al, 2007; Holyoake et al, 1999; Hope et al, 2004). However, recent studies have revealed that significant differences in the frequencies of CSCs may exist, depending on the xenograft model used (Taussig et al, 2008; Vormoor, 2009). For obvious reasons, it is nearly impossible to study COCs in humans. However, one recent study describes the existence of TEL-AML1 pre-leukaemic clones as early as *in utero* (Hong et al, 2008).

Attempts to define CSCs in BCR/ABL-induced disease have obtained conflicting results. BCR/ABL, a constitutively active tyrosine kinase (Konopka & Witte, 1985) most commonly exists in two versions—210 or 185 kDa (Nowell & Hungerford, 1960; Rowley, 1973). In patients, BCR/ABLp210 is associated with chronic myeloid leukaemia (CML), while BCR/ABLp185 is prevalent in B-cell acute lymphoid leukaemia (B-ALL) (Melo, 1996). Here, haematopoietic stem cells (HSCs) (Fialkow et al, 1977; Huntly et al, 2004), haematopoietic progenitors (Jaiswal et al, 2003) as well as aberrant precursor cells (Neering et al, 2007) are discussed as prime targets for transformation. In contrast, BCR/ABL⁺ B-ALL has been reported to arise in committed pro-B cells (Wang et al, 2008; Williams et al, 2006).

To identify the prospective COCs in CML and B-ALL, we dissect the processes of tumour-initiation and tumour-maintenance in BCR/ABLp210- and BCR/ABLp185-induced leukaemia.

RESULTS

Identification of COCs in BCR/ABL⁺ CML and B-ALL

To discover the COC of CML and B-ALL, we isolated (i) unfractionated bone marrow (BM) cells, (ii) purified long-term haematopoietic stem cells (LT-HSCs), (iii) purified common

lympho-myeloid progenitors (CLMPs) and (iv) purified HSC-depleted BM, from wildtype mice (see Supporting Information Fig S1A and B, and Supporting Information Table SI). The obtained cell populations were retrovirally transduced with either BCR/ABLp210-IRES-GFP, or BCR/ABLp185-IRES-GFP or a control vector (empty-IRES-GFP) and injected into lethally irradiated syngeneic wildtype mice. The disease induced by transduction of BCR/ABLp210 into unfractionated BM cells resembled CML ($n=9$ in total), whereas BCR/ABLp185-transduced BM cells conferred a B-ALL ($n=6$ in total; Fig 1A, Supporting Information Fig S1C and D). Strikingly, only the infection of purified LT-HSCs with BCR/ABLp210 or BCR/ABLp185 induced CML or B-ALL in all mice, respectively (Fig 1A, $n=4$ each). Neither the infection of purified CLMPs ($n=4$ and $n=5$, respectively) nor the HSC-depleted BM ($n=5$ and $n=4$, respectively) resulted in leukaemia formation (Fig 1A). The disease incidence and the properties of leukaemia inflicted by the respective oncogenes were similar, regardless whether whole BM or LT-HSCs were infected. This led us to conclude that LT-HSCs represent the COCs of leukaemia induced by both fusion-oncogenes.

As LT-HSCs are at the apex of the haematopoietic system, we expected their mature descendants to carry the introduced constructs. Indeed, GFP⁺ cells were present throughout all mature lineages upon introducing empty-IRES-GFP vector or BCR/ABLp210 (Fig 1B). In contrast, in B-ALL, GFP⁺ cells were predominantly found within the B-lymphoid population, which infiltrated BM, spleen and peripheral blood of the diseased mice (Fig 1B and Supporting Information Fig S1C and D). This population was characterized as CD19⁺/B220⁺/IgM⁻/IL-7R α ⁺ pro-B cells (Supporting Information Fig S1E). GFP⁺ cells were hardly detectable in all other cell lineages.

So far, we relied on the expression of characteristic surface markers to define the lineage compartment of GFP⁺ leukaemic cells. As surface markers may be aberrantly expressed in leukaemia, we related the expression of surface molecules to the expression of known lineage-determining transcription factors (TFs). We compared BCR/ABL-expressing and control cells side by side in FACS-purified cell populations. Neither normal nor leukaemic HSCs from BCR/ABLp210⁺ or BCR/ABLp185⁺ leukaemia expressed significant amounts of lineage-commitment TFs, indicating that these populations are highly immature (Fig 1C and D). In CML, the expression pattern of untransformed Gr-1⁺/Mac-1⁺ cells and the leukaemic Gr-1⁺/Mac-1⁺ cells showed a high degree of overlap in their gene expression pattern (Fig 1C and Supporting Information Fig S1F). In B-ALL, the BCR/ABLp185⁺/CD19⁺ population expressed high levels of PAX5 and EBF1 and corresponded to normal CLPs and CD19⁺ cells (Fig 1D and Supporting Information Fig S1G). Hence, the correlation between surface marker expression and lineage-determining TFs seems unaltered upon expression of BCR/ABL oncogenes. Thus, despite the fact that both oncogenes need to infect LT-HSCs in order to induce disease, only BCR/ABLp210-infected LT-HSCs keep the ability to differentiate into all haematopoietic lineages.

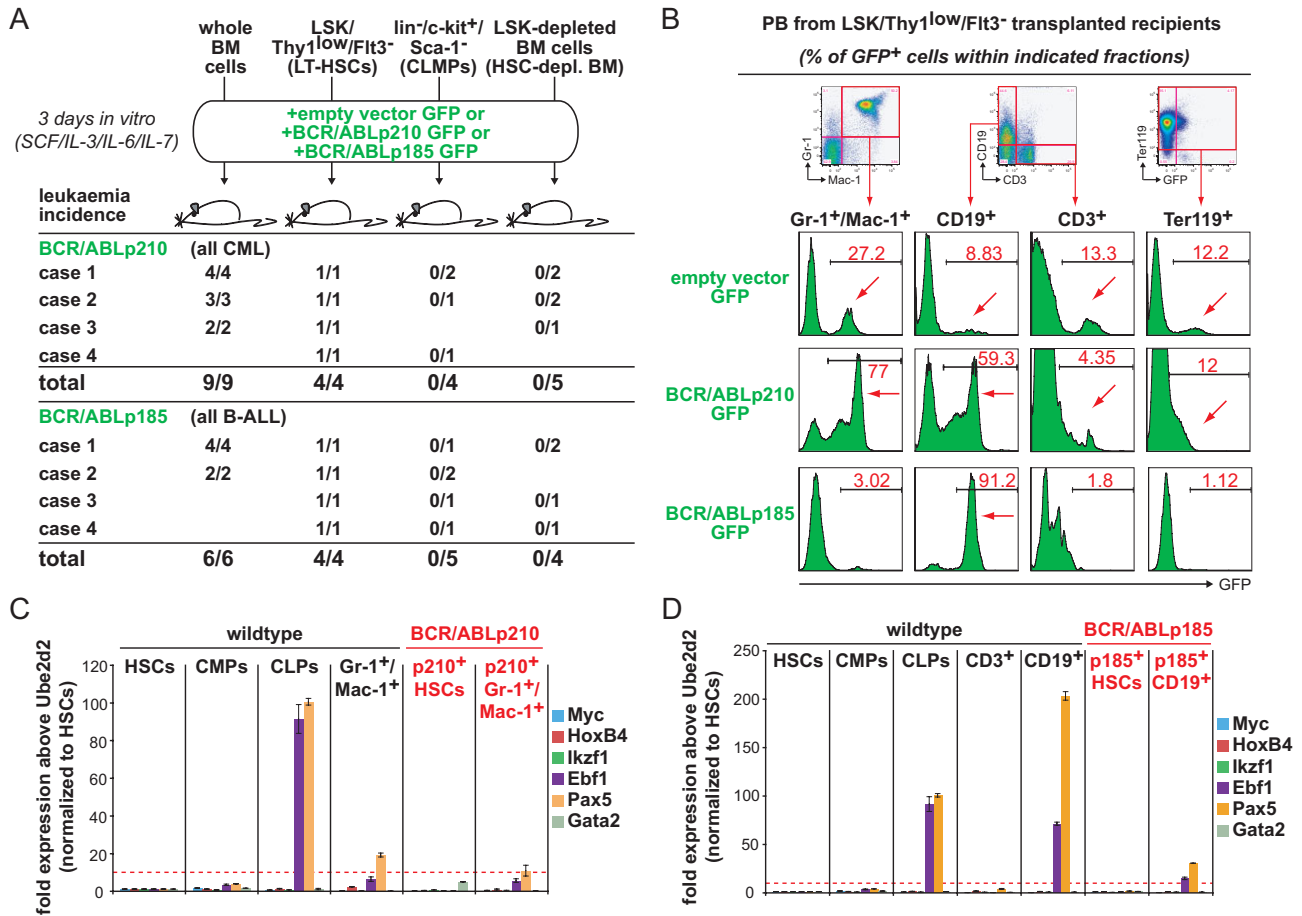


Figure 1. LT-HSCs are cells of origin in BCR/ABLp210 and BCR/ABLp185 leukaemia.

- A.** Whole BM cells and FACS-purified subpopulations were transduced with retroviruses encoding BCR/ABLp210-IRES-GFP, BCR/ABLp185-IRES-GFP or an empty-GFP-vector. Oncogene-infected LT-HSCs—but none of the more mature populations—are able to induce leukaemia with equal frequencies as whole BM cells (see also Supporting Information Fig S1 and Supporting Information Table S1).
- B.** Peripheral blood analysis of mice transplanted with BCR/ABLp210-, BCR/ABLp185- and control vector-transduced LT-HSCs. Relative contribution of leukaemic (GFP⁺) cells to the myeloid, lymphoid and erythroid lineage is indicated. One representative FACS plot from each group is depicted.
- C, D.** Comparison of relative gene expression of lineage-specific TFs in indicated cell fractions purified from wildtype and moribund BCR/ABLp210⁺ mice (C) and wildtype and moribund BCR/ABLp185⁺ mice (D). Data show fold differences compared to an internal control (Ube2d2) and are normalized to the gene expression in wildtype HSCs.

Differentiation fates of BCR/ABLp210 and BCR/ABLp185 infected LT-HSCs

One may envision different scenarios underlying the divergent fates of BCR/ABLp185⁺ and BCR/ABLp210⁺ LT-HSCs: First, the transformed LT-HSCs may constantly self-renew and supply the leukaemic cell pool in the periphery. Alternatively, the transformed LT-HSCs may represent a pre-leukaemic stage and self-renewal occurs after differentiation into a more mature stage (see scheme in Fig 2A). Thus, we analysed the abundance of GFP⁺ LT-HSCs in BM from moribund mice originally transplanted with either BCR/ABLp185- or BCR/ABLp210-infected LT-HSCs. We hardly detected GFP⁺ cells within the HSC-subpopulations in BCR/ABLp185-transplanted mice (Fig 2B, and Supporting Information Fig S2A and B, *n* = 3). In contrast, GFP⁺ cells were readily detectable in all HSC-subpopulations of BCR/ABLp210-transplanted mice

(69.1 ± 5.7% GFP⁺ cells within LT-HSCs, Fig 2B; 78.4 ± 8.4% in ST-HSCs and 29 ± 12.3% in MPPs, Supporting Information Fig S2A). The total numbers of HSCs were ~20 fold increased (Supporting Information Fig S2B, *n* = 3). Notably, we also detected few BCR/ABLp185⁺ cells within the CLMP population, (Fig 2C). In contrast, the vast majority of BCR/ABLp210⁺ CLMPs were GFP⁺ (Fig 2C). Taken together, these data suggest that mature CML is continuously supplied by the COCs, whereas the initial B-ALL COC-population is lost in case of B-ALL.

These findings prompted us to investigate the fates of LT-HSCs expressing either BCR/ABLp185- or BCR/ABLp210 during the initial steps of transformation *in vitro*. We used a cytokine-supplemented medium (SCF, IL-3, IL-6 and IL-7) supporting the outgrowth of lymphoid and myeloid haematopoietic lineages (Fig 2D). Indeed, BCR/ABLp185 LT-HSCs differentiated into CD19⁺/CD3⁺ cells within 11 days (Fig 2D,

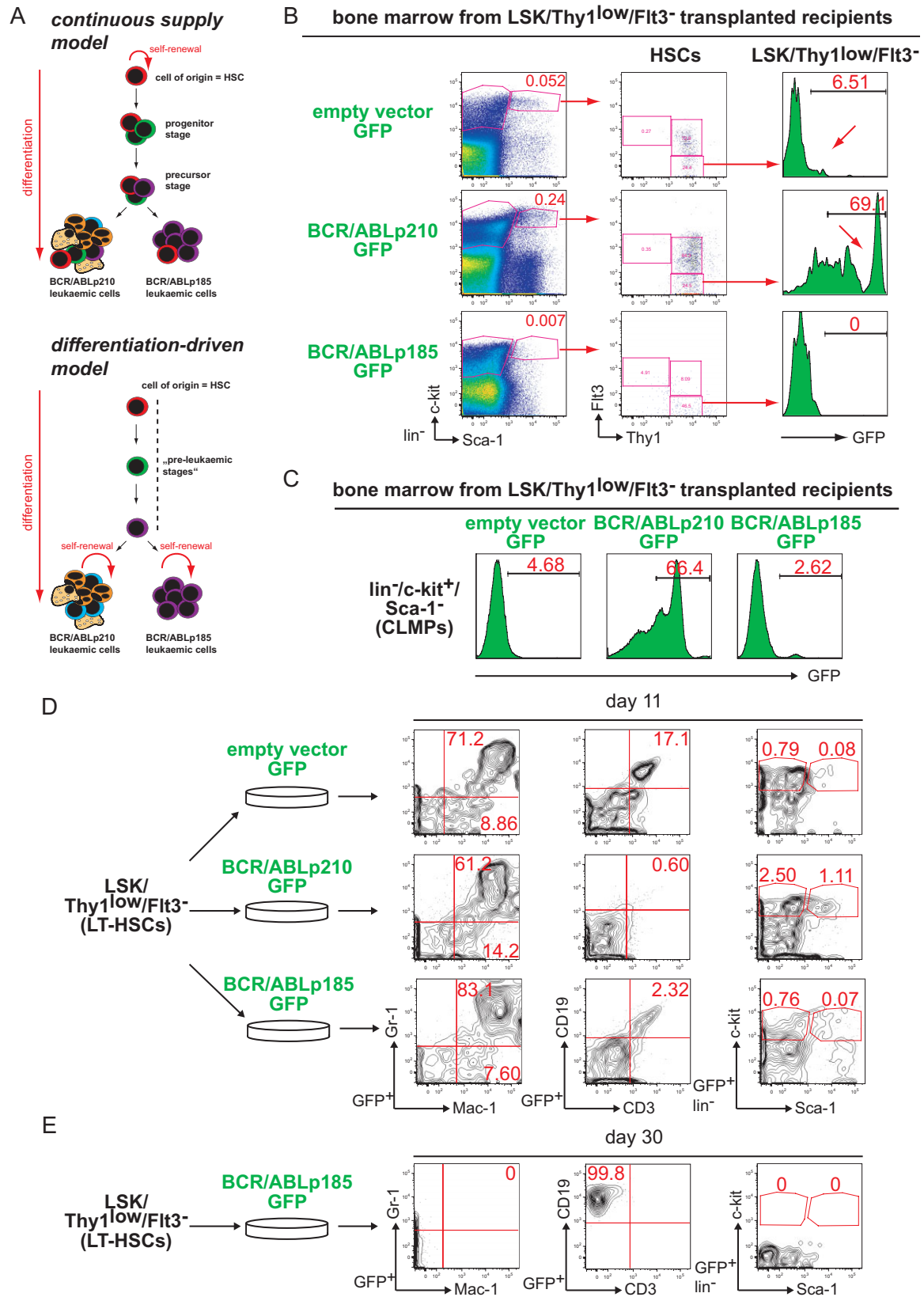


Figure 2.

lower middle panel) and progressed into CD19⁺ cells within 30 days (Fig 2E). Initially, a minor population of GFP⁺ cells with HSC-markers (Fig 2D, lower right panel) was observed that was no longer detectable after 30 days (Fig 2E). In contrast, the majority of BCR/ABLp210⁺ LT-HSC cells differentiated into myeloid Gr-1⁺/Mac-1⁺ cells (Fig 2D, middle left panel). Here, a subpopulation of BCR/ABLp210⁺ LT-HSCs expressed surface markers indicative for HSCs (Fig 2D, middle right panel) and preserved them even after 30 days (data not shown). These results demonstrate that BCR/ABLp185⁺ LT-HSCs differentiate into the B-lineage and that the original COC population is exhausted. In contrast, BCR/ABLp210 supports the self-renewal of LT-HSCs, which may differentiate into Gr-1⁺/Mac-1⁺ cells.

BCR/ABLp185-infected LT-HSCs require IL-7 to induce leukaemia

Transduction of BCR/ABLp210 into LT-HSCs, but not in their more mature populations, induced CML *in vivo* (Huntly et al, 2004). However, propagation of BCR/ABLp210-transduced BM cells on IL-7-producing stromal cells selectively yields weakly tumorigenic pro-B cells (Williams et al, 2006, 2007). To investigate whether BCR/ABLp185 alone—or in combination with microenvironmental cues—accounts for the differentiation of HSCs into pro-B cells, we infected purified HSCs with BCR/ABLp185 in absence of SCF, IL-3, IL-6 and IL-7 (Fig 3A). After 28 days *in vitro*, the resulting cells proliferated and were composed of lin⁻/c-kit⁺/Sca-1⁺ cells, indicative of HSCs (Fig 3A, upper panels). However, when we analysed lin⁻/c-kit⁺/Sca-1⁻ cells (CLMPs) infected under similar conditions, we obtained CD19⁺ pro-B cells after 4 weeks (Fig 3A, lower panels). This indicates that HSCs, but not CLMPs, require additional cytokine signals to induce B-ALL. We next evaluated the potential of these *in vitro* generated cells to form leukaemia in mice. BCR/ABLp185⁺ HSC-like cells failed to induce leukaemia in sub-lethally irradiated mice (Fig 3B, *n* = 5). Interestingly, they contributed to all haematopoietic lineages (Fig 3C). In contrast, BCR/ABLp185⁺ pro-B cells induced B-ALL in recipient mice (Fig 3D, *n* = 4). These results demonstrate that a differentiation signal is required for BCR/ABLp185-transformed HSCs to develop their tumorigenic potential. To test whether IL-7 is the cytokine required, we compared the transforming capacity of BCR/ABLp185 in LT-HSCs, HSCs, CLMPs and HSC-depleted BM cells in the presence and absence of IL-7. BCR/ABLp185 induced a transformation of CLMPs in absence of IL-7 (Fig 3E),

reflecting our findings in Fig 3A. Interestingly, we obtained no colonies from LT-HSCs. To more closely mimic *in vivo* conditions, we next added IL-7 during BCR/ABLp185-transformation. Now, the frequency of factor-independent colonies was the highest after transformation of LT-HSCs and/or HSCs (Fig 3F), which was in line with our results obtained *in vivo* (Fig 1A). Importantly, these differences could not be accounted to different transduction rates in the respective target cell populations (Supporting Information Fig S3A–C). However, we found that about ~2% of all HSCs and 4% of all CLMPs expressed IL-7Rα on their surface (Supporting Information Fig S3D and E). Thus, it is possible that CLMPs are transformable in absence of cytokines (Fig 3A and E) because they might have received IL-7-signals from the BM-niche (Kondo et al, 1997), whereas LT-HSCs might have not. To further substantiate that IL-7-signalling is essential for BCR/ABLp185-transformation, we compared the potential of wildtype, IL-7Rα^{+/-} and IL-7Rα^{-/-} BM cells to form factor-independent colonies. Compared to wild type cells, IL-7Rα^{+/-} BM cells showed a reduction in their transformation ability, whereas the IL-7Rα^{-/-} BM cells completely failed to form colonies *in vitro* (Fig 3G).

To investigate the time frame of the IL-7-dependence in B-ALL, we infected LT-HSCs and HSCs with BCR/ABLp185 in absence of IL-7 and subsequently injected the cells into lethally irradiated mice only 8 h after infection. None of the indicated cell populations was able to induce leukaemia (Fig 3H). Despite the presence of GFP⁺ cells in the peripheral blood of these mice, no signs of leukaemia could be detected, indicating that HSC-infection and repopulation had been successful (data not shown). In contrast, purified LT-HSCs or HSCs infected with BCR/ABLp185 in presence of IL-7 for 8 h induced B-ALL in all transplanted mice (Fig 3H). We therefore conclude that COCs from BCR/ABLp185⁺ B-ALL require instantaneous IL-7 signals to evolve into progressing leukaemia.

STAT5 activation in LT-HSCs suffices to induce a CML-like disease

STAT5 is a critical node in the signalling network downstream of BCR/ABL (Hoelbl et al, 2006, 2010) and overexpression of a constitutively active STAT5 protein (caSTAT5) in BM cells suffices to induce CML, closely resembling a BCR/ABLp210-induced disease (Moriggl et al, 2005). Hence, STAT5 might act as a relevant factor driving self-renewal of haematopoietic cells.

Figure 2. Hierarchy among CML and B-ALL is established by differentiation into classical haematopoietic lineages.

- In the 'continuous supply model', the cells of origin confer their self-renewal capacity to their descendants. The choice of lineage is oncogene-specific. In the 'differentiation-driven model', the cells of origin are pre-leukaemic. Full transformation and self-renewal is achieved after subsequent differentiation at a specific cell stage.
- BM analysis of mice transplanted with BCR/ABLp210-, BCR/ABLp185- and empty vector-transduced LT-HSCs. Relative contributions of leukaemic (GFP⁺) cells to LT-HSCs are indicated. One representative FACS plot from each analysed group is depicted (see also Supporting Information Fig S2).
- BM analysis of mice transplanted with BCR/ABLp210-, BCR/ABLp185- and empty vector-transduced LT-HSCs. Relative contributions of leukaemic (GFP⁺) cells to CLMPs are indicated. One representative FACS plot from each analysed group is depicted.
- Transformation of LT-HSCs with either empty vector, BCR/ABLp210 or BCR/ABLp185, followed by *in vitro* cultivation with SCF, IL-3, IL-6 and IL-7. Contour-plots indicate differentiation into myeloid and lymphoid lineages (left and middle panels) and presence of HSCs (right panel) after 11 days. Numbers indicate percentages of total cells. One representative set of data is depicted (*n* = 3).
- Outgrowing cells from BCR/ABLp185-transduced LT-HSCs on day 30 at same conditions as in (D).

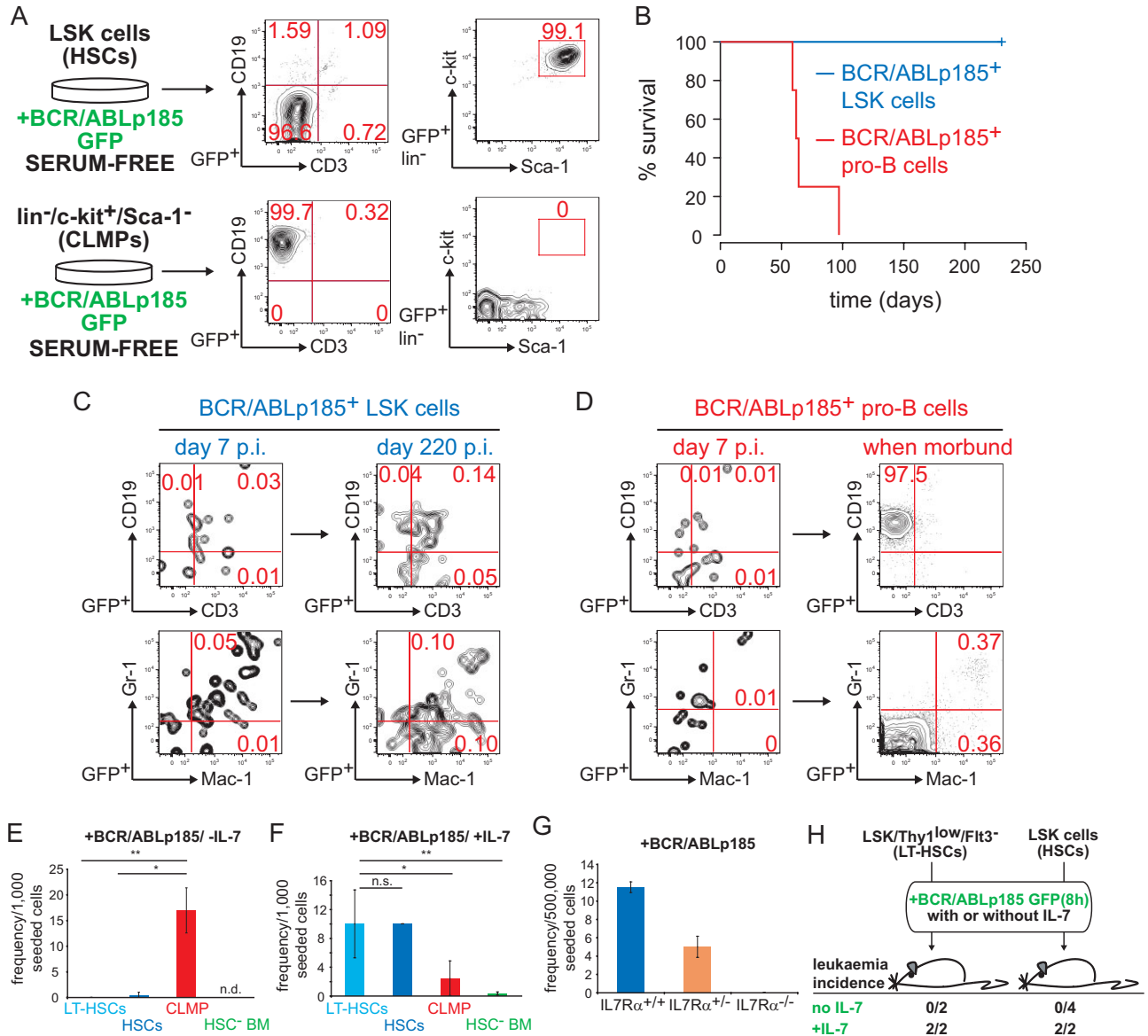


Figure 3. Leukaemogenicity of the cells of origin of B-ALL is dependent on their ability to differentiate.

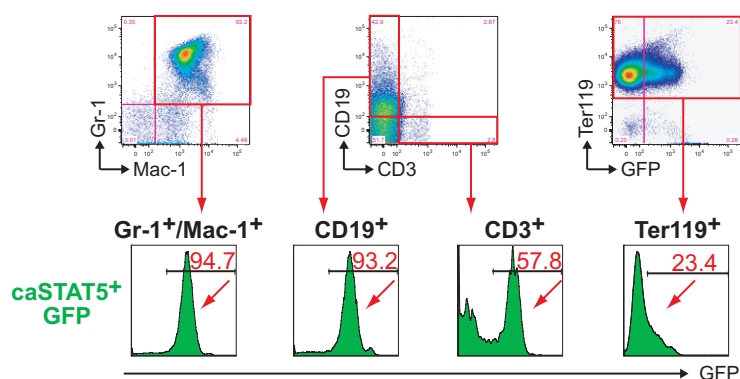
- A.** Without cytokine treatment, BCR/ABLp185-transformed CLMPs differentiate towards the B-cell lineage, whereas BCR/ABLp185-transformed HSCs do not. One representative set of data is depicted ($n = 3$, see also Supporting Information Fig S3).
- B.** Cytokine-free derived BCR/ABLp185⁺ LSK cells are unable to induce leukaemia when injected into sublethally irradiated wildtype mice ($p = 0.0027$).
- C.** Cytokine-free derived BCR/ABLp185⁺ LSK cells show long-term contribution to multiple lineages without B-ALL formation *in vivo*. One representative plot is depicted ($n = 5$), p.i., post injection.
- D.** Cytokine-free derived BCR/ABLp185⁺ pro-B cells form B-ALL *in vivo* and contribute to the B-cell lineage only. One representative plot is depicted ($n = 5$).
- E, F.** Outgrowth of factor-independent colonies after BCR/ABLp185-transduction of indicated BM-subpopulations without IL-7 (**E**) and with IL-7 (**F**).
- G.** Outgrowth of factor-independent colonies after BCR/ABLp185-transduction of wildtype, IL-7Rα^{+/-} and IL-7Rα^{-/-} BM-subpopulations.
- H.** Short-term IL-7-exposure suffices for induction of differentiation. LT-HSC and HSCs were BCR/ABLp185-transduced for 8 h in absence or presence of IL-7 and injected into recipient mice. The numbers indicate the frequency of leukaemia formation observed after 6 months.

Therefore, we asked whether STAT5 signalling is critical within the LT-HSCs or within more mature progenitors for the initiation of CML. Transformation of whole BM and purified LT-HSCs with caSTAT5, both led to induction of CML *in vivo* (Fig 4A), whereas the transformation of CLMPs or HSC-depleted BM failed to do so. Upon transplantation of caSTAT5⁺ LT-HSCs, GFP⁺ cells

were present throughout all haematopoietic lineages and all stages of differentiation, and densely infiltrated BM, spleen and liver (Fig 4B and data not shown). Again, we found augmented numbers of HSCs in the BM of leukaemic mice (Supporting Information Fig S4A), reminiscent to our observations made in BCR/ABLp210 transplants. Furthermore, the vast majority of

leukaemia incidence	whole BM cells	LSK/Thy1 ^{low} /Flt3 ⁻ (LT-HSCs)	lin ⁻ /c-kit ⁺ /Sca-1 ⁻ (CLMPs)	LSK-depleted BM cells (HSC-depl. BM)
caSTAT5	(all CML)			
case 1	4/4	3/3	0/4	0/4
case 2	2/2	1/1	0/3	0/4
total	6/6	4/4	0/7	0/8

B peripheral blood from LSK/Thy1^{low}/Flt3⁻ transplanted recipients (% of GFP⁺ cells within gated fractions)



caSTAT5⁺ ST-HSCs, MPPs and CLMPs were GFP⁺ (Supporting Information Fig S4B). These results indicated that STAT5-activation in LT-HSCs is sufficient to initiate a CML-like disease.

CSCs in BCR/ABLp210 and BCR/ABLp185 induced leukaemia

The COC is defined as the cell in which leukaemia arises. Thus, CSCs may represent differentiated descendants of COCs. Accordingly, CSCs in different types of leukaemia may have distinct phenotypes despite arising from LT-HSCs. To investigate this concept, we first evaluated the abundance of CSCs by performing *in vivo* limiting dilution assays. GFP⁺ cells were sorted from the BM of moribund mice and transplanted into

NOD/SCID/IL-2Rγ^{-/-} (NSG) mice (Risueno et al, 2011; Sanchez et al, 2009; Taussig et al, 2008). In case of BCR/ABLp210, disease was induced only upon transplantation of high numbers of sorted cells which is in line with previous data. These findings indicate that CSCs in BCR/ABLp210 CML follow the CSC model with rare CSCs [leukaemia-initiating cell frequency (95% confidence interval): 1/85,428 (1/55,550–1/131,376)] within the leukaemic cell pool (Table 1, upper panel). In contrast, when transplanting BCR/ABLp185 cells, few cells sufficed to induce disease [leukaemia-initiating cell frequency (95% confidence interval): 1/66 (1/32–1/137)]—as predicted for CSCs in the stochastic model (Table 1, lower panel).

Table 1. BCR/ABLp210 CSCs follow the CSC model, while BCR/ABLp185 CSCs progress through frequent leukaemic clones

BCR/ABLp210								
Leukaemia incidence	Cell dose							
	1,000,000	100,000	10,000	1,000	100			
Case 1	20/20							
Case 2		22/31						
Case 3		1/2	0/3	0/4	0/4			
BCR/ABLp185								
Leukaemia incidence	Cell dose							
	250,000	100,000	50,000	10,000	1,000	100	50	10
Case 1	5/5							
Case 2		7/7	5/5			2/2	2/2	
Case 3		5/5		5/5	2/2	3/6		2/7

In vivo limiting dilution assay of serially transplanted BCR/ABLp210 and BCR/ABLp185 leukaemia. Whole GFP⁺ leukaemic cells were FACS-sorted from moribund primary transplanted mice and injected into NOD/SCID/IL-2Rγ^{-/-} mice in indicated dilutions.

Figure 4. STAT5 is necessary and sufficient for the leukaemogenic activity of cells of origin of CML.

A. Whole BM cells and indicated FACS-purified subpopulations were transduced with retroviruses encoding caSTAT5-IRES-GFP. Infected LT-HSCs were the only population able to induce leukaemia with equal frequencies like whole BM cells (see Supporting Information Fig S5).

B. Peripheral blood analysis of mice transplanted with caSTAT5-transduced LT-HSCs. Relative contributions of leukaemic (GFP⁺) cells to the myeloid, lymphoid and erythroid lineage are indicated. One representative FACS plot from each analysed group is depicted.

In contrast to the CSC model, the stochastic model does not discriminate between pseudo-hierarchical tumours (Quintana et al, 2010) and non-hierarchical tumours (Williams et al, 2007). Hence, we devised three hypothetical scenarios how BCR/ABLp210 CML and BCR/ABLp185 B-ALL progress based on the phenotypical characterization and hierarchical structure of the haematopoietic system (Fig 5A). In the first model, all leukaemic cells are capable to maintain leukaemia irrespective of their phenotype. The second model assumes that only one subtype of phenotypically defined cells act as a CSC. In the third model, rare HSC-like cells exclusively function as CSCs.

To test which of the models is compatible with disease progression, we purified BCR/ABLp210 or BCR/ABLp185 GFP⁺ LT-HSCs from BM of moribund mice and mixed them with HSC-depleted wildtype BM for secondary transplantation. Conversely, wildtype LT-HSCs were mixed with BCR/ABLp210 or BCR/ABLp185 GFP⁺ leukaemic BM cells devoid of all cells expressing HSC markers (Supporting Information Fig S5A and B).

We found that the transplantation of GFP⁺ LT-HSCs purified from BCR/ABLp210 leukaemic pool resulted in a fast CML onset (mean survival of 15 ± 2 days) in all mice analysed ($n = 9$). GFP⁺ leukaemic cells were detected throughout all haematopoietic lineages (Fig 5B, upper panel) indicating that CSCs fully reconstituted the leukaemic phenotype. In contrast, transplantation of HSC-depleted BCR/ABLp210 leukaemic cells (250,000) supplemented with wildtype LT-HSCs failed to inflict disease over a period of 14 months (Fig 5B, lower panel). We next analysed whether LT-HSCs or more mature leukaemic progenitors from caSTAT5-induced CML function as CSCs. Similarly, the leukaemic LT-HSC-fraction expressing caSTAT5 was able to induce a novel disease in serial transplants in 9/9 cases (Supporting Information Fig S5C), whereas leukaemic cells depleted of HSCs failed to do so (0/9 cases). These data show that in BCR/ABLp210, as well as in caSTAT5 induced CML, the CSCs correspond to the LT-HSC-compartment.

The situation in BCR/ABLp185 B-ALL was entirely different. Transplantation of HSC-depleted BCR/ABLp185 leukaemic cells combined with wildtype LT-HSCs rapidly induced a fatal leukaemia in all mice within 14 days ($n = 8$). This disease closely resembled original B-ALL (Fig 5C, lower panel). As GFP⁺ LT-HSCs were hardly available in moribund BCR/ABLp185-transplanted mice (Fig 2A), we chose to inject the entire pool of GFP⁺ and GFP⁻ LT-HSCs. These cells failed to induce leukaemia in secondary recipients over a period of 8 months (Fig 5C, upper panel). These data confirm that BCR/ABLp210

and BCR/ABLp185 leukaemia are maintained by CSCs of distinct developmental stages—LT-HSCs and pro-B cells, respectively.

Amplification and characterization of CML-CSCs *in vitro*

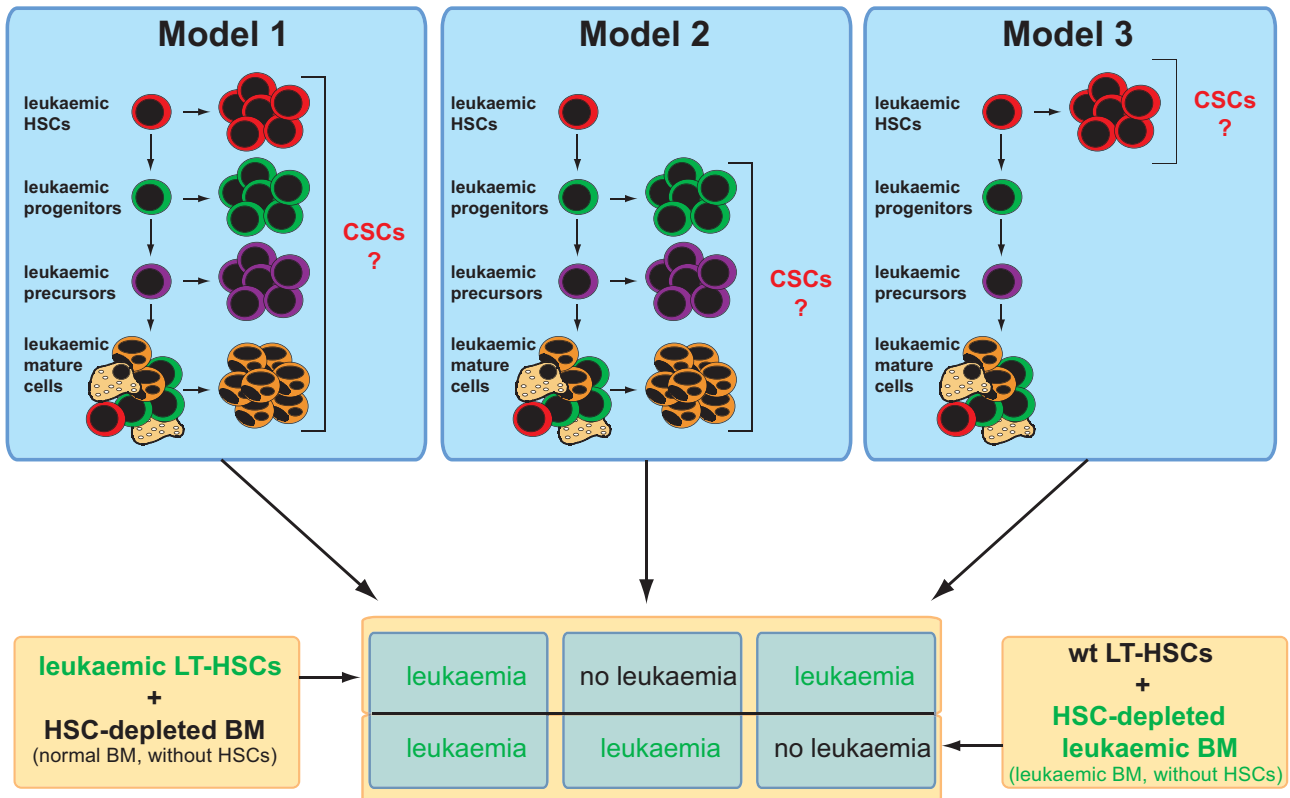
Methods to study CSCs *in vitro* are currently hardly available. Thus, we developed an *in vitro* cultivation system by purifying BCR/ABLp210⁺ LT-HSCs and BCR/ABLp185⁺ pro-B cells from moribund mice and maintaining them under various cell culture conditions. As previously described, BCR/ABLp185⁺ CSCs grew out rapidly using 10% FCS (Supporting Information Fig S6A) (Sextl et al, 2000). These cells kept their ability to induce leukaemia upon serial transplantations with a consistent pro-B cell phenotype (Supporting Information Fig S6B).

Long-term cultivation of CSCs from CML has so far not been reported. When we cultivated *ex vivo* purified BCR/ABLp210⁺ LT-HSCs in serum-free medium plus defined growth factors (SCF/Tpo/IGF-II and FGF-1; Zhang & Lodish, 2005, 2008), the cells formed ‘cobblestone structures’ consisting of adherent and suspension cells (Supporting Information Fig S6C). Efficient cultivation was only achieved when the large (forward scatter, FSC^{high}) cells representing approximately 7–8% of the CSC-pool were re-plated (Supporting Information Fig S6C). Small (FSC^{low}) BCR/ABLp210⁺ CSCs were unable to proliferate *in vitro* (Movies S1 and S2). Using this method, BCR/ABLp210⁺ CSCs were expanded >10¹⁴-fold within 67 days (Fig 6A). Wildtype LT-HSCs increased only about 100-fold and stopped to proliferate after 14 days. BCR/ABLp210⁺ CSCs expressed multiple markers indicative for HSCs (Supporting Information Tables SIIA and SIIB) and retained the ability to induce a fatal CML-like disease ($n = 3$) upon injection into lethally irradiated mice even after 10³-fold expansion (Fig 6B). This CML recapitulated the phenotype observed in primary transplants and was dominated by Gr-1⁺/Mac-1⁺ leukaemic cells (Fig 6B). However, this leukaemogenic capacity was lost after 10⁷-fold expansion (data not shown). We also tested whether our cultivation system extends to CSCs from caSTAT5⁺-induced disease. Purified caSTAT5⁺ LT-HSCs from moribund mice were maintained in SCF/Tpo/IGF-II and FGF-1 under serum-free conditions—enabling sustained proliferation (Supporting Information Fig S6D). Comparable to BCR/ABLp210 CSCs, the caSTAT5⁺ CSCs expressed HSC-surface markers *in vitro* (Supporting Information Tables SIIIA and SIIB) and were able to induce CML in secondary transplants (data not shown). In summary, we show that BCR/ABLp210⁺ and caSTAT5⁺ CSCs may be expanded *in vitro*—retaining key features such as HSC

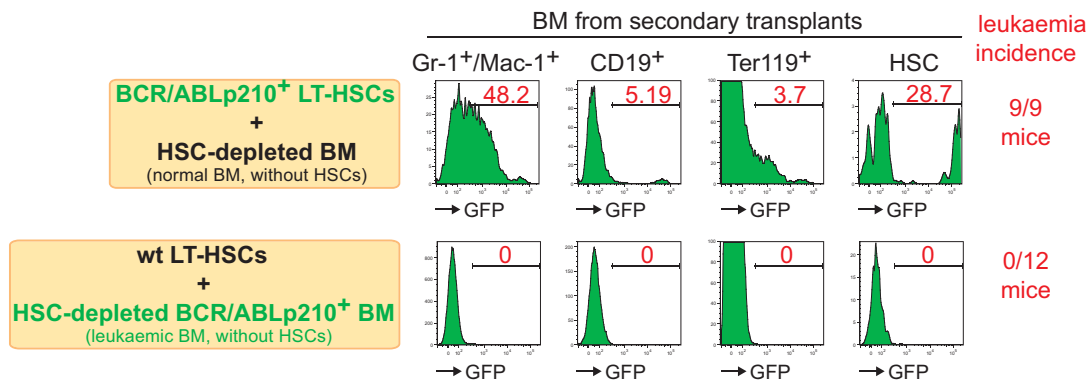
Figure 5. BCR/ABLp210 and BCR/ABLp185 CSCs are distinct in phenotype.

- A.** Models for leukaemia-maintenance. In model 1, all leukaemic cells have the ability to re-initiate leukaemia. Model 2 assumes that leukaemia is maintained either by frequent progenitors, lineage-restricted precursor cells or mature cells. In model 3, the leukaemia-maintaining cell is a rare LT-HSC. Predicted experimental outcomes are indicated for each model.
- B.** CSCs in BCR/ABLp210 leukaemia behave according to model 3. A mixture of BCR/ABLp210 LT-HSCs with wildtype HSC-depleted BM induces a CML-like disease (upper panel) in 9:9 serially transplanted mice. Mixture of wildtype LT-HSCs with frequent, leukaemic BCR/ABLp210 HSC-depleted BM leaves the mice disease-free for 14 months in 12:12 mice (lower panel, see also Supporting Information Fig S4).
- C.** CSCs in BCR/ABLp185 leukaemia behave according to model 2. The mixture of BCR/ABLp185 LT-HSCs with wildtype HSC-depleted BM prevents B-ALL formation in 4:4 mice (upper panel). Mixture of wildtype LT-HSCs with frequent BCR/ABLp185 HSC-depleted BM induces a B-ALL in 8:8 serially transplanted mice (lower panel).

A



B



C

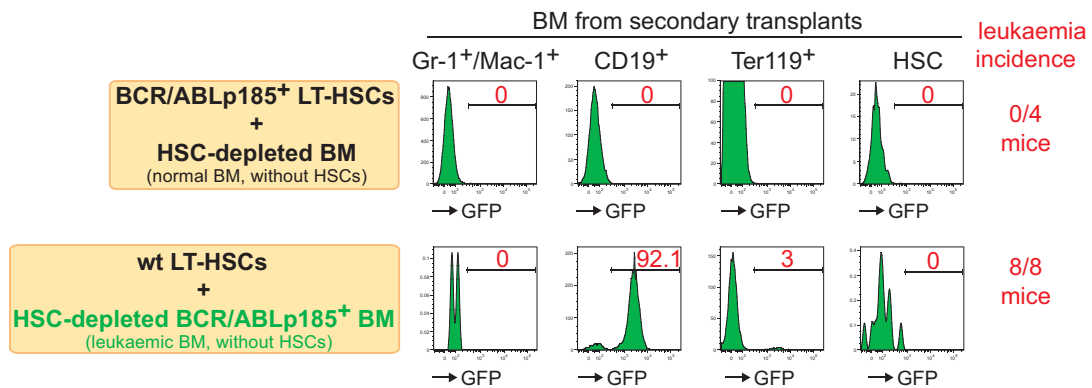


Figure 5.

surface marker expression, self-renewal and the ability to induce leukaemia upon transplantation.

As stem cell-like cancer cells are discussed to be more resistant to chemotherapeutic agents (Oravec-Wilson et al, 2009), we next tested the sensitivity of *in vitro* cultured CSCs to Imatinib treatment. As depicted in Supporting Information Fig S7A, cultivated BCR/ABLp210⁺ CSCs were highly resistant to Imatinib. In contrast, imatinib blocked the proliferation of BCR/ABLp185⁺ CSCs in a concentration-dependent manner (Supporting Information Fig S7B). Recent evidence suggested an involvement of Sonic hedgehog (Shh) signalling in the regulation of self-renewal of CML CSCs (Dierks et al, 2008). However, the Shh-inhibitor cyclopamine efficiently inhibited proliferation of BCR/ABLp185⁺ CSCs (IC₅₀ = 0.96 μM), but failed to inhibit BCR/ABLp210⁺ CSCs (Supporting Information Fig S6C). Similarly, inhibitors directed against JAK kinases (AG490) or STAT3 (Stattic) failed to efficiently inhibit BCR/ABLp210 CSCs (Supporting Information Fig S7D and E). These data are line with a decreased drug sensitivity of CML-CSCs.

Another strategy to eradicate CSCs—is to force them into differentiation. Addition of cytokines that drive terminal differentiation (Zhang & Lodish, 2005) resulted in the conversion of BCR/ABLp210⁺ CSCs into a mixture of CD19⁺, Mac-1⁺ and CD71⁺/TER119^{low} cells after 7 days (Fig 6C). These differentiated cells (Fig 6C, middle panel) were unable to re-initiate leukaemia upon transplantation. No GFP⁺ cells emerged during the observed period of 12 months (Fig 6C, right panel).

Recent and our findings suggest that BCR/ABL⁺ human CML stem cells might not be oncogene-addicted and therefore are not eliminated by imatinib therapy (Corbin et al, 2011). This prompted us to speculate whether the inhibition of STAT5 would allow the eradication of CSCs and thus provide a therapeutic option to treat CML. We addressed this question by deleting STAT5 in CSCs from BCR/ABLp210 CML using an inducible conditional knockout mouse model for STAT5. BM from Mx1Cre⁺STAT5^{+/+} and Mx1Cre⁺STAT5^{fl/fl} mice was transformed with BCR/ABLp210 and transplanted into recipient mice (*n* = 5 each). Upon leukaemia onset GFP⁺ LT-HSCs were purified and treated with IFN-β for 18 h *in vitro* to induce deletion of STAT5 and injected with HSC-depleted wildtype carrier BM into secondary recipients. Four out of five animals that had received IFN-β-pre-treated Mx1Cre⁺Stat5^{fl/fl} LT-HSCs did not develop leukaemia, whereas all mice that had received

Mx1Cre⁺STAT5^{+/+} control LT-HSCs rapidly succumbed to CML (Fig 6D). In one mouse that suffered from leukaemia (Fig 6C), STAT5 deletion was incomplete and thus all remaining leukaemic cells expressed STAT5 (data not shown). Accordingly, the amount of GFP⁺ leukaemic cells was strongly reduced in this animal (Fig 6D, lower panels) when compared to Mx1Cre⁺STAT5^{+/+} transplanted controls (Fig 6D, upper panels).

DISCUSSION

The distinct appearance of BCR/ABLp210-induced CML and BCR/ABLp185-induced B-ALL has led to the belief that CML arises in stem/progenitor cells while B-ALL evolves from precursors restricted to the B-lineage. We show here that BCR/ABLp210- and p185-induced leukaemia arise from a common cellular origin resembling LT-HSCs. The leukaemogenic potential of the infected LT-HSCs depends on the version of BCR/ABL: cells from CML (BCR/ABLp210) are fully capable of inducing leukaemia whereas cells from B-ALL (BCR/ABLp185) are pre-leukaemic and require further differentiation. Thus, not only does evolution from the cell of origin to the CSC depend on a genetic mutation (in our case BCR/ABL), it may also require the presence of additional distinct environmental cues.

For BCR/ABLp210-induced CML, STAT5 signalling is absolutely necessary. Interestingly, caSTAT5 expression in BM cells suffices to induce a multi-lineage leukaemia closely resembling BCR/ABL-induced CML (Moriggl et al, 2005). The similarity is underlined by the fact that in both diseases the COC and the CSC correspond to the LT-HSC compartment. Accordingly, deletion of STAT5 in purified BCR/ABLp210 CSCs suffices to abrogate leukaemia. These findings confirm the role of STAT5 as a critical signalling node in BCR/ABL-induced disease (Hoelbl et al, 2006, 2010). Blocking STAT5 signalling represents a promising novel approach to target CSCs in BCR/ABL⁺ CML. It is currently unclear whether the direct activation of STAT5 by BCR/ABLp210 (Hantschel et al, 2012) suffices to ensure an adequate STAT5 signal or whether additional cues from the microenvironment, such as cytokines, are required. In the case of BCR/ABLp185-transformed LT-HSCs, an additional signal is needed; only in the presence of IL-7 do the cells differentiate into pro-B cells and become capable of causing disease upon transplantation into mice.

Figure 6. Eradication of therapy-resistant BCR/ABLp210 CSCs by enforced differentiation and by STAT5-deletion.

- Cumulative cell number of BCR/ABLp210 LT-HSC and wildtype LT-HSCs grown under conditions as described in Supporting Information Fig S6. Wildtype LT-HSCs die at day 16.
- BCR/ABLp210 LT-HSCs induce a CML-like disease in secondary recipient mice (*n* = 3). Contributions of BCR/ABLp210⁺ LT-HSCs to myeloid and lymphoid lineages are indicated by FACS plots.
- BCR/ABLp210 LT-HSCs from long-term cultures were forced into terminal haematopoietic differentiation by addition of SCF, IL-3, IL-6, IL-11, GM-CSF, M-CSF and Epo to the serum-free medium. The FACS-plots on the left side show cells before addition of cytokines. Cells express myeloid, lymphoid and erythroid lineage markers such as Mac-1, CD19 and Ter119 after 7 days of cultivation (middle panels). FACS-plots from the peripheral blood of secondary transplants are depicted in the right panels. No leukaemia formation was observed (*n* = 4).
- Deletion of Stat5 from primary transplanted leukaemic LT-HSCs (BCR/ABLp210⁺ Mx1Cre⁺Stat5^{fl/fl} LT-HSCs) abrogates leukaemia formation in 4:5 secondary recipients. The one moribund mouse showed strongly reduced leukaemic cell populations (bottom panel) as compared to control BCR/ABLp210⁺ Mx1Cre⁺Stat5^{+/+} LT-HSCs-transplanted mice.

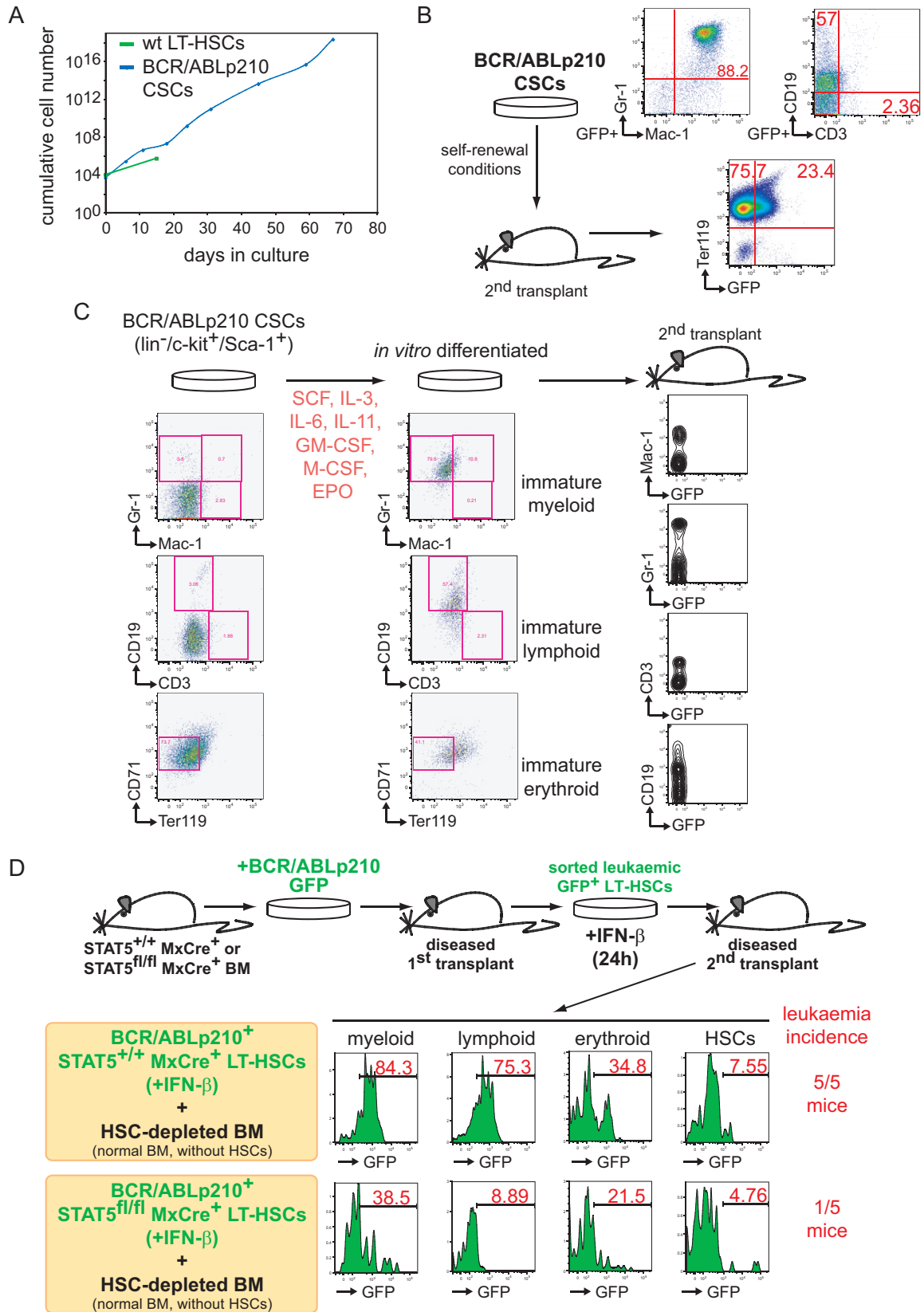


Figure 6.

The paper explained

PROBLEM:

The large diversity of tumour phenotypes may originate in oncogenic mutations arising in the stem, progenitor or precursor cell stages of different tissues. It has been postulated that BCR/ABL⁺ CML arises in stem cells, while BCR/ABL⁺ B-ALL originates from B-cell precursors.

RESULTS:

Both, BCR/ABL⁺ CML and B-ALL originate from LT-HSCs. During disease-maintenance, CML LT-HSCs require STAT5 and persist to

function as CSCs. In contrast, B-ALL LT-HSCs undergo IL-7-dependent differentiation into CSCs that correspond to pro-B cells.

IMPACT:

We show that the ultimate leukaemia phenotype is influenced by the cellular origin as well as the pathway of differentiation. Therefore, two therapeutic challenges must be addressed in certain types of leukaemia—both, the COCs and the CSCs must be eradicated for a successful therapy.

The STAT5 signalling pathway downstream of IL-7 also appears crucial for leukaemogenesis—as BCR/ABLp185-induced B-ALL also requires STAT5 expression for disease initiation and maintenance (Hoelbl et al, 2006, 2010). Our results show that constitutively active STAT5 is not capable of replacing IL-7. Hence, STAT5 activation is required but not sufficient to drive B-ALL development. Intriguingly, our experiments revealed that IL-7R α -deficient BM is resistant to BCR/ABLp185-induced transformation. We found that about 2% of LT-HSCs express the IL-7R α chain, indicating that these immature cells are already IL-7 responsive. It is notable that CLMPs can be transformed *in vitro* in the absence of IL-7, although we cannot rule out that these cells might have received IL-7 from the niche where they reside *in vivo*. Interestingly, recent findings have also implicated the IL7-signalling as being crucial for development (Zenatti et al, 2011) and for progression of childhood T-ALL (Silva et al, 2011). However, our findings on IL7 still need to be treated with caution, as IL7 seems not to play a role during normal human B-lymphopoiesis (Espeli et al, 2006).

It was previously shown that the chemical agent 5-FU may modulate the appearance of leukaemic phenotypes. Pre-treatment of donor BM with 5-FU influenced the outcome of the disease induced by BCR/ABL oncogenes. Recipient mice develop CML upon transplantation of BM cells from 5-FU pre-treated mice, irrespective of whether these are infected with BCR/ABLp210 or with BCR/ABLp185. In contrast, a mixed phenotype (CML and B-ALL) is induced in the absence of 5-FU pre-treatment (Hu et al, 2004; Li et al, 1999; Roumiantsev et al, 2001). This puzzling finding may be explained in the light of our data: both leukaemia originate from the same cellular origin. As 5-FU is known to shift the composition of BM cells, it can be speculated that 5-FU favours the differentiation of COCs into the myeloid lineage.

How may these findings be reconciled with our knowledge of the initiation of leukaemia? Our data raise the question of whether different leukaemia might arise not from distinct haematopoietic lineages but instead from a common cell origin. In this scenario, genetic mutations in stem cells may influence programs for differentiation and/or reinforce programs for self-renewal. In the

case of BCR/ABLp185-induced B-ALL, for example, the COC develops into a homogeneous pro-B cell population.

Several documented examples could be explained by this model. For example, MOZ-TIF2, a fusion-protein that causes AML, transforms HSCs but induces leukaemia consisting of mature myeloid progenitors *in vivo* (Huntly et al, 2004). Similar scenarios might also apply for diseases induced by MLL-AF9 (Krivtsov et al, 2006) and MLL-ENL (Cozzio et al, 2003).

Moreover, CSCs may also change during tumour progression. In the chronic phase of human CML, disease is maintained by a CSC that corresponds to a multipotent stem cell. Upon progression to blast crisis, more differentiated myeloid precursor cells (granulocyte-macrophage progenitor, GMP) are responsible for maintaining leukaemia (Jamieson et al, 2004).

There are currently two models to explain the progression of cancer, the CSC model and the stochastic model (Dick, 2008). The former predicts that a tumour is heterogeneous and hierarchically organized. This model predicts that self-renewing activity can be enriched by sorting stem cells with specific characteristics and distinct phenotypes. In contrast, the stochastic model proposes that tumours are heterogeneous but lack functional hierarchy. In consequence, phenotypic changes would be reversible and all tumour cells would possess both self-renewal and tumour-initiating ability.

Our serial transplantation experiments revealed that CML induced by BCR/ABLp210⁺ LT-HSCs progresses according to the CSC model, whereas B-ALL induced by BCR/ABLp185⁺ LT-HSCs does not. However, the stochastic model, as observed in melanoma (Quintana et al, 2010), is also not fully consistent with our findings as neither a functional nor a phenotypical hierarchy exists in B-ALL induced by BCR/ABLp185⁺ LT-HSCs. In the future, these differences may however turn out to be important considering their therapeutic options, because cells with distinct phenotypical appearances may respond differently to chemotherapeutic agents.

It is important to mention that both models are compatible with the clonal evolution as the CSCs in the CSC model may also progress by clonal evolution, but stochastic tumours should entirely depend on the clonal evolution (Shackleton et al, 2009). Recently, it has been reported that within progressing B-ALL an

evolution of successor mutations is taking place, which occurs in functionally defined CSCs (Anderson et al, 2011; Notta et al, 2011). This may explain why patients contain multiple genetic hits at the same time and why some tumours develop towards more aggressive growth and to a poorer outcome. Most importantly, these studies establish the importance of genetical heterogeneity in obviously homogenous B-ALL samples. Theoretically, such a diversity of B-ALL subclones may also occur in mice, but given the short latency (<4 weeks) and the lack of pressure by the immune system, it remains speculative whether similar mutations can be observed in our murine model of tumour initiation. Given that 83% of our secondarily transplanted mice recapitulated B-ALL (Table 1), it becomes difficult to explain how most leukaemic cells might have acquired mutations within a short period of time. Thus, it is unlikely that murine BCR/ABLp185⁺ B-ALL accumulate secondary mutations at these progression stages. However, it remains possible that aggressive leukaemic clones appear upon numerous serial transplantations. Recently, it has been shown that multiple serial transplantations have led to a reduction of clonality within leukaemia towards few aggressive clones (Barabe et al, 2007; Li et al, 1999).

In the end, our findings have important ramifications for the treatment of leukaemia.

It is obvious that therapies designed to destroy a rare population of CSCs will not be effective in eliminating tumours composed of many CSCs and vice versa. Hence, it is imperative to identify differences between the tumourigenic and the non-tumourigenic populations in all cancers. In BCR/ABLp185⁺ B-ALL, which is sustained by frequent clones, treatment with Imatinib, Shh-inhibitor cyclopamine, Jak2-inhibitor or Stat3-inhibitor is highly effective and destroys CSCs. Perhaps in the future, a combined inhibition will prove to impair the emergence of successor B-ALL clones bearing more aggressive potential. In contrast, CSCs propagating BCR/ABLp210⁺ CML can be indirectly eliminated by forcing the cells to undergo terminal differentiation or by a specific abrogation of the STAT5-signalling. Thus, these two strategies might prove to become a tantalizing new therapy for elimination of CSCs in CML.

MATERIALS AND METHODS

Mice

All mice were kept under sterile conditions in individually ventilated IVC-cages. Animal experiments were performed according to the rules of the Austrian Animal Law 1988, licence no. MA58/1253/03, MA58/001489/2008/12 and 66.009/0065-II/10b/2009.

BM transduction and transplantation

Four- to six-week-old male wildtype or alternatively, Mx1Cre⁺ STAT5^{+/+} and Mx1Cre⁺STAT5^{fl/fl} mice were used as donors for 6-to-8-week old female syngeneic recipient mice. NOD/SCID/IL-2R $\gamma^{-/-}$ (NSG-) mice were used for evaluation of cancer-progression models, as previously described (Taussig et al, 2008).

BM-subpopulations were infected using stable producer cell lines

carrying empty-IRES-GFP, BCR/ABLp210-IRES-GFP, BCR/ABLp185-IRES-GFP and caSTAT5-IRES-GFP as described previously (Moriggl et al, 2005). Unless otherwise mentioned, 2–3 × 10⁶ infected total BM cells were injected via tail-vein into lethally (10 Gy) irradiated recipient mouse. For phenotypical CSC-evaluation, we injected 1000 GFP⁺ leukaemic LT-HSCs supplemented with 250,000 wildtype GFP⁻ HSC-depleted BM cells or 1000 GFP⁻ wildtype LT-HSCs mixed with 250,000 GFP⁺ HSC-depleted leukaemic cells.

FACS analysis and sorting

For lineage-differentiation, cells (BM, spleen and peripheral blood) were stained with fluorescence-conjugated antibodies TER119, CD3 ϵ , Mac-1, Gr-1 and CD19. In addition, BM cells were stained with: (i) lineage markers (B220, TER119, CD3 ϵ , Gr-1 and Mac-1), c-kit, Sca-1, Flt3 and Thy1.2 (or CD150) for detection of all HSC subpopulations (Kim et al, 2006; Passegue et al, 2004), (ii) with lineage markers (B220, TER119, CD3 ϵ , Gr-1 and Mac-1), c-kit, Sca-1 and IL-7R α for detection of lymphoid precursors (Kondo et al, 1997) and (iii) with lineage markers (B220, TER119, CD3 ϵ , Gr-1, Mac-1 and IL7R α), c-kit, Sca-1, CD16/CD32 and CD34—for detection of myeloid precursor cells (Akashi et al, 2000). Flow cytometric analysis of all organs was performed on six-colour BD FACSCanto (BD Biosciences) equipped with 488 and 633 nm lasers. FACS-sorting was performed at 4°C immediately after biopsy using an eight-colour BD FACSAria equipped with 488, 633 and 407 nm lasers.

Author contributions

BK, VS and HB designed the study and wrote the manuscript; BK, AH, MA, CS, KMF and MAK performed experiments; BK, AH, CS and MAK analysed and interpreted the data; GL and GS gave technical support; VS and RM provided reagents, gave conceptual advice and valuable scientific input.

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In Memoriam Hartmut Beug (1945–2011).

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Supporting Information is available at EMBO Molecular Medicine online.

The authors declare that they have no conflict of interest.

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