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Cancer induction by restriction of oncogene expression to the stem cell compartment

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In human cancers, all cancerous cells carry the oncogenic genetic lesions. However, to elucidate whether cancer is a stem cell-driven tissue, we have developed a strategy to limit oncogene expression to the stem cell compartment in a transgenic mouse setting. Here, we focus on the effects of the *BCR-ABLp210* oncogene, associated with chronic myeloid leukaemia (CML) in humans. We show that CML phenotype and biology can be established in mice by restricting BCR-ABLp210 expression to stem cell antigen 1 (Sca1)$^+$ cells. The course of the disease in Sca1-BCR-ABLp210 mice was not modified on STI571 treatment. However, BCR-ABLp210-induced CML is reversible through the unique elimination of the cancer stem cells (CSCs). Overall, our data show that oncogene expression in Sca1$^+$ cells is all that is required to fully reprogramme it, giving rise to a full-blown, oncogene-specified tumour with all its mature cellular diversity, and that elimination of the CSCs is enough to eradicate the whole tumour.

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Introduction

An axiom in the treatment of tumours is that the remission is, in general, more difficult to achieve with each relapse. Despite a better understanding of the biology of tumour cells, the treatment of most cancers has not significantly changed for the past three decades and the decreasing mortality has been mostly the result of early detection and prevention rather than the consequence of effective therapeutics (Etzioni *et al*, 2003; Chabner and Roberts, 2005; Huff *et al*, 2006).

A new hypothesis in cancer biology postulates that tumours are hierarchically structured as abnormal tissues, which are maintained by cancer stem cells (CSCs) (Reya *et al*, 2001; Perez-Caro and Sanchez-Garcia, 2006; Dalerba *et al*, 2007a; Sánchez-García *et al*, 2007). This theory provides a rationale to explain the failure of many currently used antitumoral strategies, because just a small population of CSCs resistant to the therapy would be enough to maintain the whole tumour mass (Etzioni *et al*, 2003; Chabner and Roberts, 2005; Huff *et al*, 2006). Evidences supporting this model have been recently published for human leukaemias (Bonnet and Dick, 1997; Cobaleda *et al*, 2000; Cox *et al*, 2004, 2007; Hope *et al*, 2004) and solid tumours (Al-Hajj *et al*, 2003; Singh *et al*, 2004; Collins *et al*, 2005; Kim *et al*, 2005; Xin *et al*, 2005; Bao *et al*, 2006a, b; Dalerba *et al*, 2007b; Li *et al*, 2007; O'Brien *et al*, 2007; Prince *et al*, 2007; Ricci-Vitiani *et al*, 2007). However, this emerging concept has not been fully validated yet in an experimental model system. If validated, the CSC hypothesis will have far-reaching consequences for our understanding of cancer biology and for the development of new strategies to improve cancer treatment. A major obstacle to elucidate the contribution that CSCs make to the development and maintenance of cancer and their suitability as a target is the lack of a system to limit oncogene expression to the CSC compartment.

Since the discovery of oncogenes in human tumours, many efforts have been made to elucidate the causal role that these oncogenes have in cancer development. These previous studies have shown that oncogene expression is not only required for initiation of cancer but also for the maintenance of the disease (Chin *et al*, 1999; Huettner *et al*, 2000; Boxer *et al*, 2004). In mouse models where oncogene expression is driven by tissue-specific promoters, tumours arise at high frequency, but disappear again when the inducing stimulus is switched off (Chin *et al*, 1999; Huettner *et al*, 2000; Boxer *et al*, 2004). However, it has not been biologically proven that cancer is a hierarchically organized tissue that can be created and maintained similar to a normal stem cell-based tissue. If that is indeed the case, then cancer should be created and maintained similarly to any other normal stem cell-driven tissue, similar to the haematopoietic system. In a normal

stem cell-driven tissue, genetic programming of stem cells is all that is required to (re)constitute all differentiated cells forming the tissue and the genetic information responsible for the stem cell programming is not present anymore within the differentiated cells that form the tissue. Thus, we reasoned that a similar organization could be happening for cancer formation.

To address these biological questions, we have used the *BCR-ABLp210* oncogene associated with chronic myeloid leukaemia (CML) in humans as a model (Koeffler and Golde, 1981; Melo and Barnes, 2007). CML is a paradigmatic stem cell disorder that begins as a protracted chronic phase, characterized by high leukocyte counts and enlarged spleen and liver. In all patients, the chronic phase of CML eventually converts to a blast crisis that is indistinguishable from acute leukaemia. The specific BCR-ABL inhibitor STI571 can prolong the remission times of CML patients because it is able to eliminate the BCR-ABL-expressing differentiated cells that constitute the bulk of the tumour (Druker *et al*, 2001). However, it cannot eliminate BCR-ABL-expressing CSCs, even though it can penetrate into the cells, and these CSCs eventually repopulate the tumour with STI571-resistant mature cells (Graham *et al*, 2002; Hu *et al*, 2006; Primo *et al*, 2006; Jiang *et al*, 2007).

Here, we show that CML phenotype and biology can be established in mice in which we limited BCR-ABLp210 expression to stem cell antigen 1 (Sca1)$^+$ cells. These model mice mimic CML human pathology. The course of the CML disease in the Sca1-BCR-ABLp210 mice was not modified on STI571 treatment. However, we show that BCR-ABLp210-induced CML is reversible through the unique elimination of the CSCs. To our knowledge, this study demonstrates for the first time that limited oncogene expression in CSCs is all that is required to fully reprogramme it, giving rise to a full-blown, oncogene-specified tumour with all its mature cellular diversity, and that CSC elimination is enough to eradicate the whole tumour.

Results

Derivation of Sca1-BCR-ABLp210 mice

The mouse Ly-6E.1 promoter (Miles *et al*, 1997) was used to drive Sca1-directed expression of a human BCR-ABLp210 (Sca1-BCR-ABLp210) or TK-IRES-BCR-ABLp210 transgene (Sca1-TK-IRES-BCR-ABLp210) in C57BL/6 × CBA mice (Figure 1). This promoter is known to have heterologous expression in haematopoietic stem cells (HSCs) (Miles *et al*, 1997), hence transgene expression is restricted to a limited percentage of Sca1-expressing cells. The BCR-ABLp210-over-expressing animals had normal gestation and were viable. Three independent lines were obtained and used to examine the phenotype further (Table I). As c-kit is known from earlier studies to be downregulated in leukaemia stem cells (Blair and Sutherland, 2000; Neering *et al*, 2007), our functional definition of stem cell in this study does not include c-kit as a surface marker. Quantitative RT–PCR of BCR-ABLp210 and/or TK messenger mRNA confirmed that expression was largely confined to Sca1$^+$ cells with little or no ectopic expression in Sca1$^-$ cells (Figure 1). In line with these results, neither BCR-ABL protein nor downstream signalling was detected in Sca1$^-$Lin$^+$ cells of Sca1-BCR-ABLp210 mice (Supplementary Figure 1 online). As reported earlier in transgenic reporter lines with the same promoter (Miles *et al*, 1997; Ma *et al*, 2002a, b), BCR-ABLp210 was expressed in a limited percentage of Sca1$^+$ cells (25–50%) as measured by single-cell RT–PCR (Supplementary Figure 2 online). This enables cell transformation to occur in a similar manner as it happens in humans, where cells expressing the oncogene are present along with non-expressing cells. The phenotype described here is therefore due primarily to the expression of BCR-ABLp210 in Sca1$^+$ cells.

CML development in Sca1-BCR-ABLp210 mice

The expression of BCR-ABLp210 in Sca1$^+$ cells led to significant increases in the numbers of white blood cells (WBCs) and in the percentages of neutrophils (Table I) in the peripheral blood (PB) of Sca1-BCR-ABLp210 animals (Figure 2A). Flow cytometry of bone marrow (BM) cells demonstrated significantly increased numbers of cells staining positively for the myeloid markers Gr1 and Mac-1, comprising up to 75–90% of the cells in the BM of transgenic animals (Figure 2A). However, the percentage of Sca1$^+$Lin$^-$ cells in BM did not increase (data not shown), similarly as it happens in human CML (Jamieson *et al*, 2004). Macroscopic analysis of these animals showed splenomegaly (Table I) and hepatomegaly (79% of the animals). The histologic examination of the spleen and liver demonstrated the presence of megakaryocytes as markers of myeloid metaplasia (Figure 2B) and expansion of the splenic red pulp by predominantly granulocytic myeloid cells (Figure 2C). This was confirmed by flow cytometric analysis (Figure 2A). In addition, infiltration of myeloid cells was also seen in the liver, lymph nodes and lung (Figure 2C). The major cause of death in animals that had progressive disease was related to myeloid infiltration of extramedullary organs and kidney failure (30%), or the evidence for progression to blast crisis (70%). In addition, targeting Sca1$^+$ cells in non-haematopoietic tissues gave rise to carcinoma development (lung adenocarcinoma 10%, hepatocarcinoma 3%, GIST 2%, osteogenic sarcoma 2% and Sertoli cell tumour 2%; data not shown). Altogether, the survival time of mice expressing BCR-ABLp210 in Sca1$^+$ cells ranged from 4 to 18 months (Figure 1E; Table I). Mice surviving myeloid infiltration of extramedullary organs progressed spontaneously to a blast crisis characterized by the appearance of blasts (myeloid or lymphoid) in PB, BM, spleen and liver (Figure 2D and E). Similar to the human disease, this secondary disorder appeared in the context of a myeloproliferative disease (Figure 2F). The time of transition to blast crisis has been described to be reduced with increased levels of *BCR-ABL* in human CML cells (Melo and Barnes, 2007; Modi *et al*, 2007). Similarly, when compound Sca1-BCR-ABLp210 transgenic mice were generated (Figure 1), leukaemia was detectable at 1–2 months of age (Figure 1; Table I). Thus, the limited expression of BCR-ABLp210 to Sca1$^+$ cells is able to mimic human CML, characterized also by a progression from chronic towards an acute phase (blast crisis), which is also invariably fatal in Sca1-BCR-ABLp210 mice and that is dependent on the dose of the oncogene expression in the Sca1$^+$ cells.

The fusion protein responsible for CML has been shown to cause genome instability in humans (Giehl *et al*, 2005; Melo and Barnes, 2007). We examined Sca1-BCR-ABLp210 mice for evidence of secondary genetic changes. First, we examined the overall DNA methylation status in tumour cells of

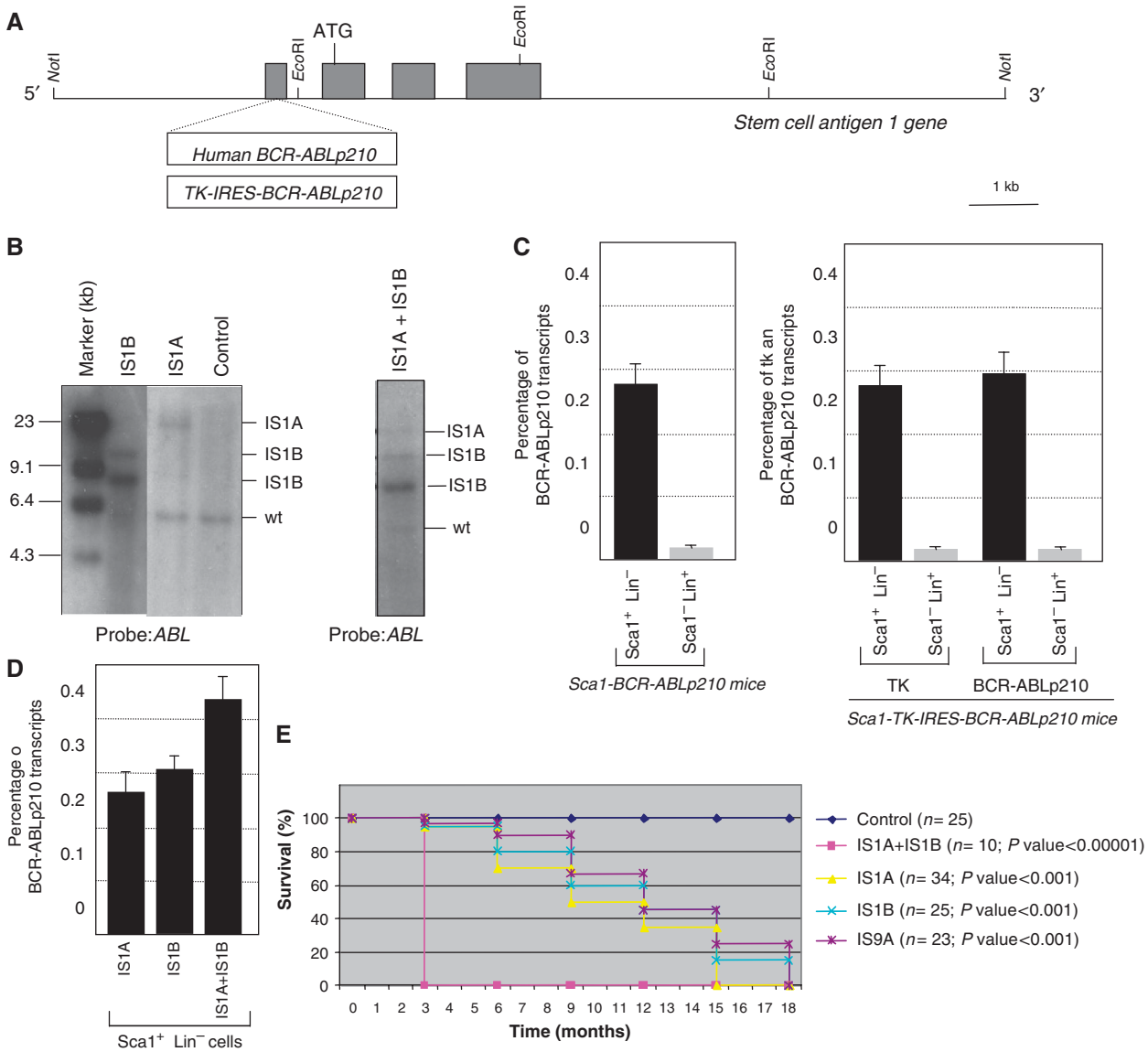


Figure 1 Sca1-BCR-ABLp210 and Sca1-TK-IRES-BCR-ABLp210: transgene constructs, expression and survival. (**A**) Schematic representation of the genomic structure of the mouse *Sca1* locus and the Sca1-BCR-ABLp210 and Sca1-TK-IRES-BCR-ABLp210 transgenic vectors used in this study. The HSV-TK/BCR-ABLp210 fusion gene construct is a bicistronic construct consisting of the herpes simplex thymidine kinase (TK) cDNA separated from BCR-ABLp210 by the picornaviral internal ribosome entry site (IRES) sequence. *Not*I sites used to excise the transgene fragments and *Eco*RI sites used to examine Southern blots are indicated. (**B**) Identification of the transgenic mice by Southern blot analysis of tail snip DNA after *Eco*RI digestion. Human *ABL* cDNA was used for the detection of the transgene. Sca1-BCR-ABLp210 and the endogenous c-Abl are indicated. Lines IS1A and IS1B are two different Sca1-BCR-ABLp210 transgenic lines. (**C**) Quantification of *BCR-ABLp210* expression in Sca1-BCR-ABLp210 mice and quantification of thymidine kinase (TK) and *BCR-ABLp210* expression in Sca1-TK-IRES-BCR-ABLp210 mice by real-time PCR in $Sca1^+Lin^-$ and $Sca1^-Lin^+$ cells. Percentage of *TK* and *BCR-ABLp210* transcripts with reference to β-actin is shown. (**D**) Quantification of *BCR-ABLp210* expression by real-time PCR in $Sca1^+Lin^-$ cells of Sca1-BCR-ABLp210 mice (line IS1A or IS1B) and of double Sca1-BCR-ABLp210 mice (line IS1A × IS1B). (**E**) Kaplan–Meier survival plots of Sca1-BCR-ABLp210 mice (line IS1A or IS1B), double Sca1-BCR-ABLp210 mice (line IS1A × IS1B) and Sca1-TK-IRES-BCR-ABLp210 mice (line IS9A). The total number of mice analysed in each group is indicated. Statistical analysis was performed using the χ^2 test, and the corresponding *P*-values are given in parentheses.

Sca1-BCR-ABLp210 mice by immunolocalization of 5-methylcytosine to give qualitative information on nuclear distribution. We performed similar analyses in human $CD34^+$ CML cells. Although control cells present unmethylated DNA, an aberrant DNA methylation pattern was observed in tumour cells of human CML ($n=3$) and Sca1-BCR-ABLp210 mice ($n=8$) (see Supplementary Figure 3 online). As genetic instability and centrosome defects are common and early detectable features in CML (Melo and Barnes, 2007), we sought to investigate whether centrosome aberrations occur in Sca1-BCR-ABLp210 mice. We examined 14 CML samples including $Sca1^+$ cells of 9 newly diagnosed mice (chronic phase) and 5 blast crisis specimens by using a centrosome-specific antibody to pericentrin. Centrosome abnormalities were detected in 23.6 ± 5.1% of chronic phase cells and in 61.6 ± 5.3% of blast crisis cells, but in only 3.1 ± 0.9% of controls (see Supplementary Figure 3 online). The similarities between the secondary genetic changes observed in humans and Sca1-BCR-ABLp210 mice suggest that similar mechanisms contribute to CML progression in both species.

Table I Incidence and age of CML onset in Sca1-BCR-ABLp210 and Sca1-TK-IRES-BCR-ABLp210 mice

Transgenic line	Mice autopsied[a]	Mice with tumour (%)[b]	Age in months at tumour onset	Haematopoietic tumour type (%)	WBC/μl, neutrophils (%)	Hgb (g/100 ml)	Spleen weight (mg)
IS1A (Sca1-BCR-ABLp210)	34	34 (100)	8–12	CML–BC (100)	12 900 ± 1750 (43 ± 4)	11.5 ± 1.3	285 ± 36
IS1B (Sca1-BCR-ABLp210)	25	25 (100)	4–10	CML–BC (100)	11 850 ± 1650 (38 ± 5)	12.7 ± 1.1	217 ± 29
IS1A + IS1B	10	10 (100)	1–2	Leukaemia	ND	13.2 ± 1.3	ND
IS9A (Sca1-TK-IRES-BCR-ABLp210)	23	23 (100)	6–10	CML–BC (100)	13 300 ± 230 (41 ± 8)	11.1 ± 1.2	261 ± 23

ND, not determined.
[a]Number of mice during or after the period of cancer.
[b]Number of mice with CML and percentage of tumour incidence. Normal range of WBC counts was 4000–10 000/μl. Mean neutrophil percentage of control animals was 10 ± 4% WBCs, and the neutrophil percentage in control animals did not exceed 20%. Thus, neutrophilia is defined as more than 20% neutrophils. Hgb indicates haemoglobin (normal range, 12–16 g/100 ml). Normal spleen weight in control animals is 78–92 mg ($n = 25$). The WCC estimations were made when the mice were premorbid. CML–BC indicates mice spontaneously progress to blast crisis. $P < 0.001$ for Sca1-BCR-ABLp210 mice versus control mice.

Nature of the leukaemogenic cell in the Sca1-BCR-ABLp210 model

We next examined the nature of the leukaemogenic cell in the Sca1-BCR-ABLp210 model. To determine whether the $Sca1^+Lin^-$ population contains CSC, we sorted $Sca1^+Lin^-$ and $Sca1^-Lin^+$ cells from mice that developed CML. Transplantation of purified fractions of cells into sublethally irradiated syngeneic recipient mice was used to assess leukaemogenesis *in vivo*. Each of the mice transplanted with $Sca1^+$ Lin^- cells developed CML that was phenotypically identical to the primary disease (Table II). Importantly, $Sca1^-Lin^+$ cells did not show leukaemic engraftment into secondary recipients or induced leukaemia, even at 10 times higher concentrations. Overall, these data indicate that the CSCs reside in the $Sca1^+$ cell compartment. All these facts indicate that restricting oncogene expression to the stem cell compartment is sufficient to generate all the CML cell types, including the most differentiated ones, which do not express the oncogene and are unable to propagate the disease.

Characterization of CSCs in Sca1-BCR-ABLp210 mice

Having prospectively purified a population highly enriched for CSC, we used gene expression to identify the genes that are associated with BCR-ABLp210 reprogramming of stem cells (Supplementary Table I online; Figure 3). We performed a supervised analysis of the transcriptional profiles of CSCs purified from Sca1-BCR-ABLp210 versus those from control mice. The data showed that a total of 293 genes are reproducibly regulated in Sca1-BCR-ABLp210 versus control HSC with a false discovery rate (FDR) $\leqslant 0.07\%$ (Supplementary Table I online). Human CML has been characterized earlier according to gene expression profile (Kronenwett *et al*, 2005). Remarkably, the gene expression patterns of $Sca1^+Lin^-$ cells from Sca1-BCR-ABLp210 mice were similar to those from the human CML $CD34^+$ cells (Figure 3A), thereby further validating the model by reflecting the similarities in the transcriptome between the two populations. Thus, BCR-ABLp210-dependent, stem cell-driven murine CML shares the molecular features of human CML.

The CSC hypothesis represents a modern-day interpretation of the proposal made by Rudolph Virchow and Julius Cohnheim that cancer results from the activation of dormant embryonal-rest cells (Virchow, 1855; Cohnheim, 1867). Accordingly, we next proceeded to examine, in CSCs from Scal-BCR-ABLp210 mice, the expression of embryonic surface markers, the presence of which has been identified earlier in undifferentiated mouse embryonic stem cells (Nunomura *et al*, 2005) (Figure 3B). We could identify reproducible upregulation of gene signals corresponding to 55 embryonic stem cell surface proteins in the CSCs from Sca1-BCR-ABLp210 mice (FDR $\leqslant 1\%$). These results show that CSCs in Sca1-BCR-ABLp210 present embryonic figures that could represent attractive targets for selective CSC removal.

STI571 treatment does not modify the survival of Sca1-BCR-ABLp210 mice

We also examined the effect of STI571 treatment in Sca1-BCR-ABLp210 mice. STI571 treatment began 1 day after leukaemia was confirmed by PB analysis. Mice were monitored clinically and by serial PB count for evidence of leukaemia. STI571 did not prolong the survival of these mice (Figure 4A) and Sca1-BCR-ABLp210 mice treated with STI571 did not demonstrate a marked reduction in WBC and spleen weight (Figure 4B). This idea is in agreement with the insensitivity of the human leukaemic stem cells to STI571 (Graham *et al*, 2002; Hu *et al*, 2006; Primo *et al*, 2006; Jiang *et al*, 2007). Thus, we next examined whether CSCs from STI571-treated Sca1-BCR-ABLp210 mice propagates CML disease to secondary recipients. Equal numbers of $Sca1^+Lin^-$ cells (5×10^3) from the BM of STI571-treated animals failing therapy were used to reconstitute sublethally irradiated recipients. All reconstituted animals ($n = 5$) developed CML disease with an average latency of only 24 days, confirming that persistent BCR-ABLp210 expression is not required to maintain the malignant phenotype of CML CSCs.

CSC ablation eradicates CML in Sca1-BCR-ABLp210 mice

We next examined whether CSC ablation implies elimination of CML *in vivo* following tumour formation in a whole animal. For this purpose, we generated Sca1-TK-IRES-BCR-ABLp210 mice. These animals developed disease similarly to the Sca1-BCR-ABLp210 ones (Figure 1; Table I). Diseased Sca1-TK-IRES-BCR-ABLp210 mice were injected daily with ganciclovir (GCV) as described in the Materials and methods section. During this period, mice were monitored for evidence of leukaemia. GCV treatment clearly reduced the number of mice developing tumours (Figure 5A), with the majority of animals demonstrating a marked reduction in splenomegaly and, in some cases, complete normalization of their peripheral WBC (Figure 5B) and lack of solid tumours (although we could not confirm their presence before treat-

ment). So GCV treatment cures CML in previously sick transgenic animals. Although all animals exhibited a prolonged survival with GCV treatment, approximately 23% of the animals died of leukaemia during the treatment with the drug. To test the possibility that CSCs would remain within Sca1-TK-IRES-BCR-ABLp210 mice after GCV treatment, we highly purified the Sca1$^+$Lin$^-$ sub-population from BM and transplanted it into sublethally irradiated syngeneic recipients ($n=5$). Sca1$^+$Lin$^-$ cells did not show leukaemic engraftment into secondary recipients or induced leukaemia, indicating that leukaemogenic cells had been removed from this population by the GCV treatment. To our knowledge, this study demonstrates for the first time that killing CSCs is an effective therapeutic strategy for cancer treatment.

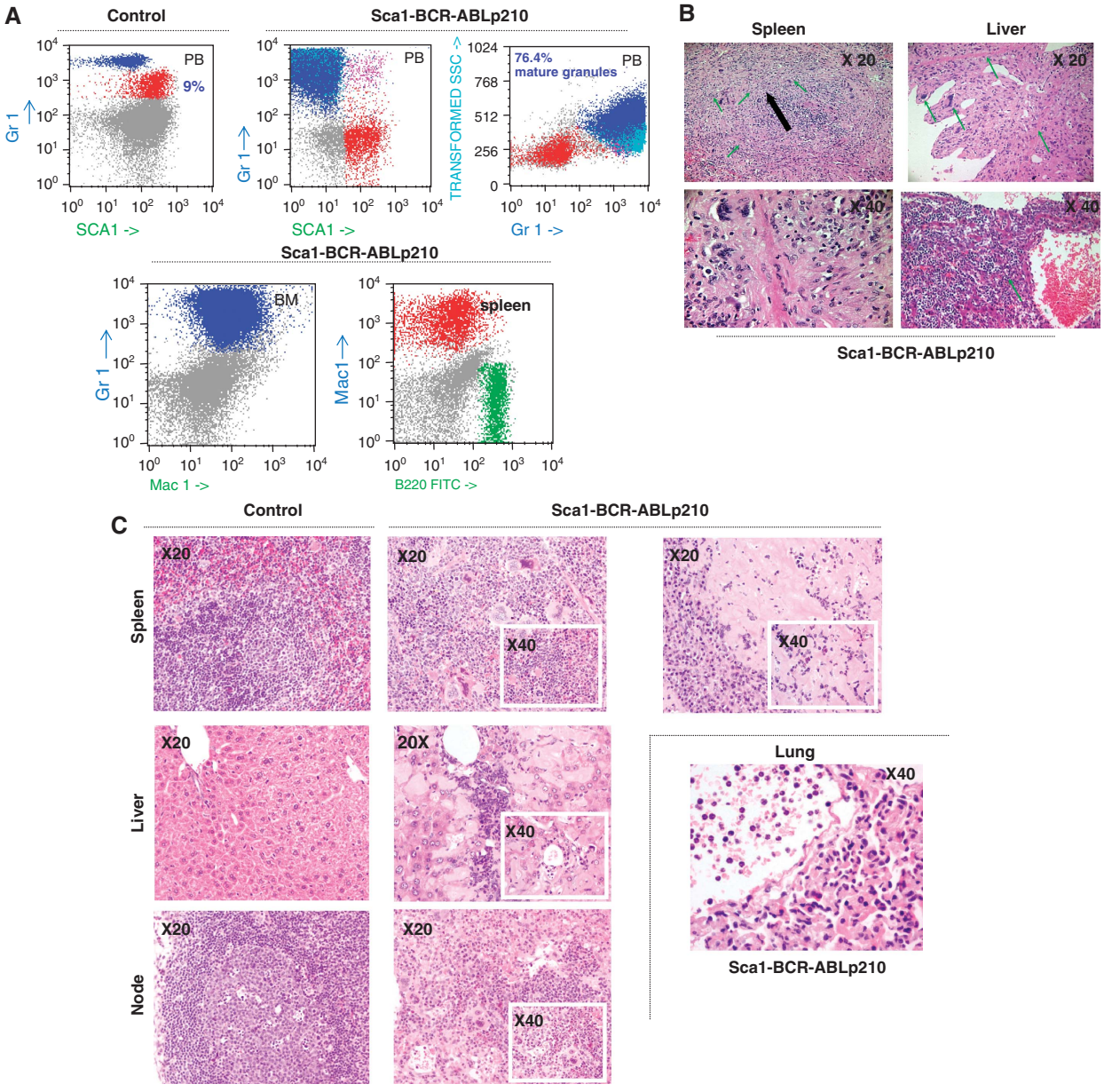


Figure 2 Sca1-driven BCR-ABLp210 expression induces CML. (**A**) Cells from PB of Sca1-BCR-ABLp210 mice were analysed by flow cytometry. Representative FACS analysis demonstrating accumulation of mature myeloid cells in PB and spleen, and an increase in the myeloid fraction within BM. PB, peripheral blood; BM, bone marrow. (**B**) Representative histologic appearance of liver and spleen of diseased Sca1-BCR-ABLp210 mice after haematoxylin–eosin staining. Megakaryocytes in spleen and liver define myeloid metaplasia and are indicated by arrows. (**C**) Organ infiltration by myeloid cells. Haematoxylin–eosin-stained sections of the spleen (megakaryocytes, myeloid blasts and mature myeloid cells), liver (perivascular infiltration of the liver by blasts and mature myeloid cells), peritoneal lymph node (with myeloid metaplasia) and lung (infiltration of the lung by blasts and mature myeloid cells). (**D**) Phenotypic characteristics of cells from peripheral blood (PB) as determined by flow cytometry. Blast cells but not mature granulocytes are present in PB from two different leukaemic Sca1-BCR-ABLp210 mice. Note that the B-cell leukaemia is characterized by the presence of blast cells co-expressing Mac-1 and B220. (**E**) Liver haematoxylin–eosin-stained sections showing blast infiltration and histologic appearance of blood smears (Giemsa staining) in Sca1-BCR-ABLp210 mice in blast crisis. Blast cells infiltrate both liver and PB. (**F**) Representative histologic appearance of liver and spleen tissue sections stained by Masson's trichrome of Sca1-BCR-ABLp210 mice in blast crisis. The presence of fibrosis (green colour) and blast infiltration in the liver and the spleen of Sca1-BCR-ABLp210 mice demonstrate that the blast crisis takes place in the context of a myeloproliferative disease.

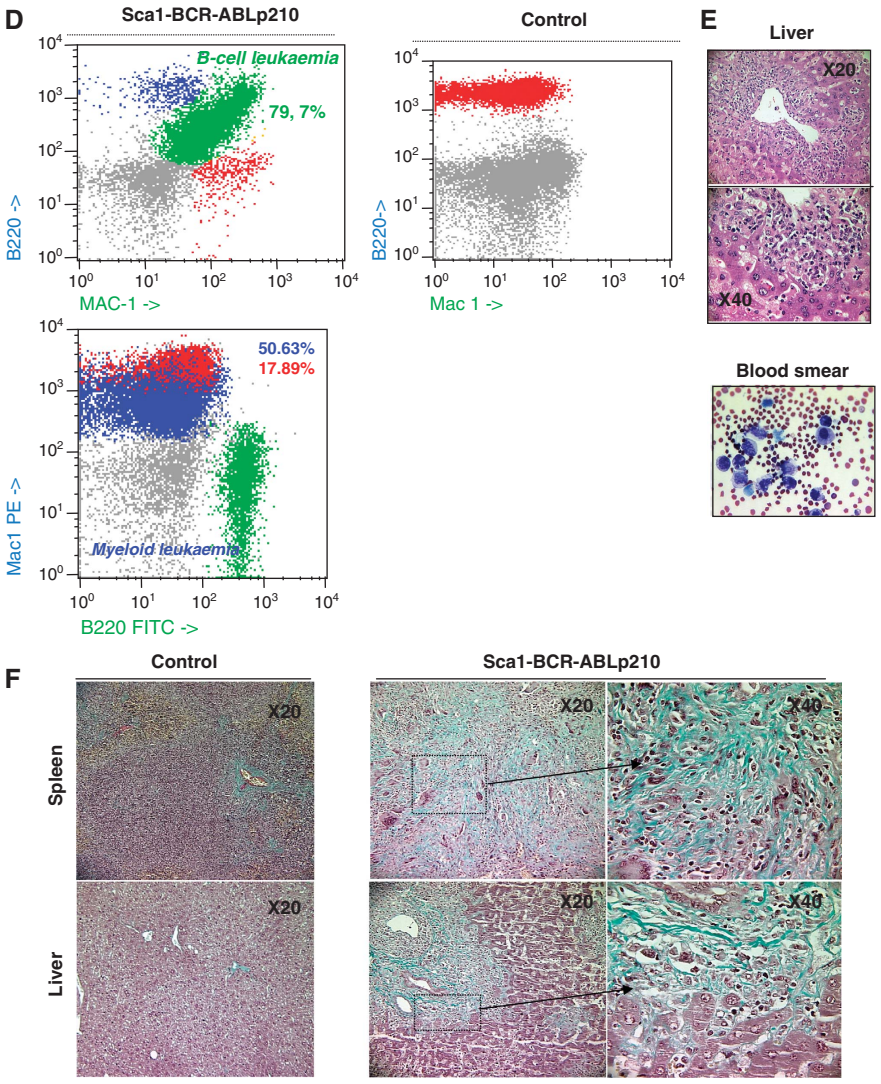


Figure 2 Continued.

Table II CML disease is readily transplantable to secondary recipients

Sorted cells	Number of transplanted cells	Transplanted animals	Incidence of CML (%)	Latency of disease (days ± s.d.)
$Sca1^+Lin^-$ (BM, Sca1-BCR-ABLp210 mice)	10.000	8	100	69 ± 14
	1.000	8	100	85 ± 11
$Sca1^+Lin^-$ (BM, control mice)	10.000	8	0	NA
	1.000	8	0	NA
$Sca1^-Lin^+$ (BM, Sca1-BCR-ABLp210 mice)	1×10^5	8	0	NA
	1×10^6	8	0	NA
$Sca1^-Lin^+$ (BM, control mice)	1×10^5	8	0	NA
	1×10^6	8	0	NA

NA, not applicable.
Eight irradiated syngenic recipients mice per cohort were transplanted with the indicated number of cells. The diagnosis of CML was confirmed by histological and immunophenotypic examination of the ill recipient mice.

Discussion

The elucidation of the molecular mechanisms that underlie tumour development not only remains a tremendous challenge for basic science but also represents an essential step in the development of new and more potent drugs, in particular with the emergence of cancer-specific, targeted therapies. The origin of human cancer within a particular tissue is often impossible to determine, due to the advanced stages of many tumours when patients enter the clinic. Our knowledge about

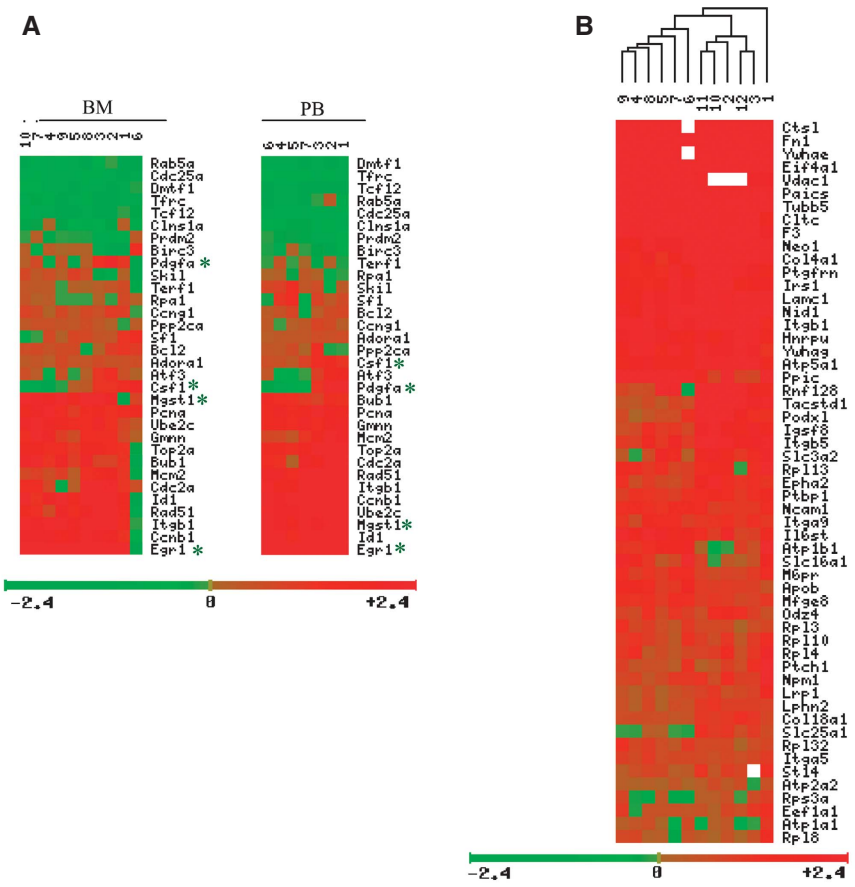
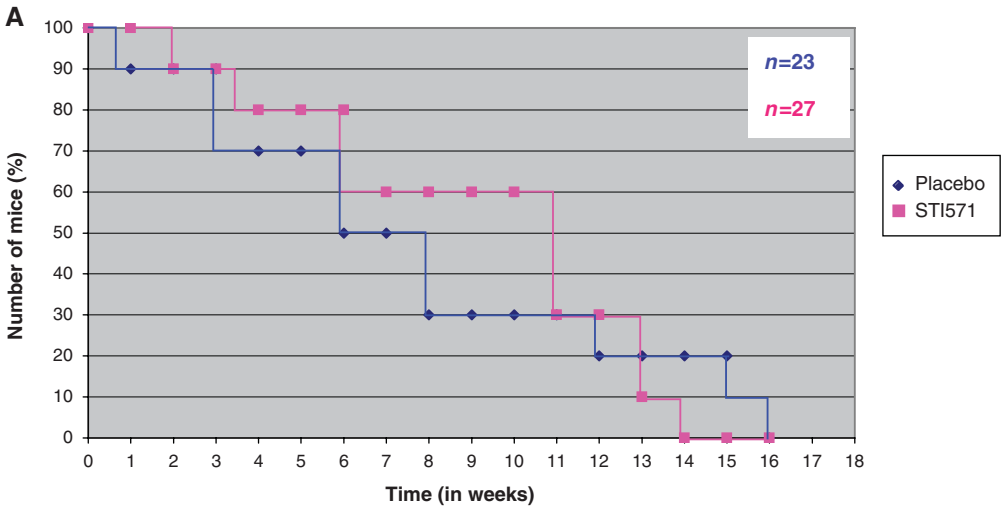


Figure 3 Identity of cancer stem cells in Sca1-BCR-ABLp210 mice. To identify genes associated with BCR-ABLp210-induced reprogramming of stem cells, we have compared the gene expression profiles of purified CSC populations versus normal HSCs. Both CSCs and HSCs were isolated as $Sca1^+Lin^-$ cells. (**A**) Comparison of the mouse and human CSCs at the molecular level. Graphical description of the expression pattern in CSCs ($Sca1^+Lin^-$ cells) from BM ($n=10$) and PB ($n=7$) of Sca1-BCR-ABLp210 mice of genes that have been previously published to be significantly regulated in the human CML $CD34^+$ fraction (Kronenwett *et al*, 2005). The genes were similarly regulated in CSCs of Sca1-BCR-ABLp210 mice. We referred the ratios of the CSCs to the control haematopoietic stem cells ($Sca1^+Lin^-$ cells purified from control mice). Green indicates that the genes are downregulated in CSCs versus HSCs and red indicates upregulation. Green asterisks mark genes that are downregulated in human $CD34^+$ CML. The remaining genes behave similarly in both human and mouse stem cells. (**B**) Embryonic surface proteins in CSCs of Scal-BCR-ABLp210 mice. Graphical description of the expression pattern in CSCs ($Sca1^+Lin^-$ cells) from BM ($n=12$) of Sca1-BCR-ABLp210 mice of previously identified upregulated cell surface markers in undifferentiated mouse embryonic stem cells (Nunomura *et al*, 2005). We identified genes for which signal intensities were upregulated (threshold ± 2) in CSCs and in at least seven CSC samples. We referred the ratios of the CSCs to the control haematopoietic stem cells ($Sca1^+Lin^-$ cells purified from control mice). Each gene (identified at right) is represented by a single row of coloured boxes; each experimental mouse is represented by a single column. Data are displayed by a colour code. Red fields indicate higher values than the median, green fields indicate lower values than the median.

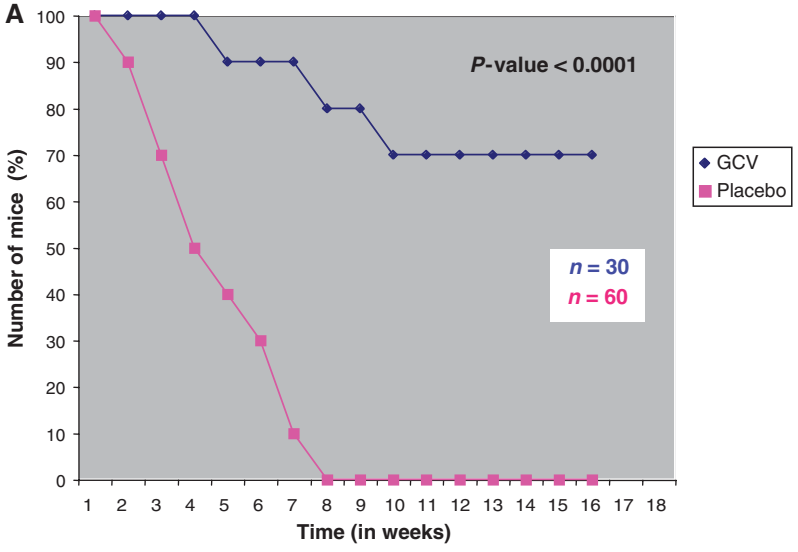
the mechanism of cancer development and progression is therefore derived from animal models that recapitulate human disease as accurately as possible. In contrast to the situation in humans, the genotype–phenotype correlations found in human cancer processes (where a given cancer-inducing event usually associates specifically with a given type of cancer) have not always been established in mouse models of cancer. This phenotypic variability is most likely to be related to the fact that the generation of current mouse models of cancer has only taken into account the genetic defects identified in human cancers without taking into account where the initiating mutation occurred. An emerging concept in tumour biology suggests that tumours are maintained by CSCs (Reya *et al*, 2001; Perez-Caro and Sanchez-Garcia, 2006; Dalerba *et al*, 2007a). However, it has not been biologically proven that cancer is formed and maintained similar to a normal stem cell-based tissue. In a normal stem cell-driven tissue, genetic programming of stem cells is all that is required to (re)constitute all differentiated cells forming the tissue and the genetic information responsible for the stem cell programming is not present anymore within the differentiated cells that form the tissue. Thus, we reasoned that a similar organization could be happening for cancer formation. To overcome these limitations and create a tractable model to study the contribution of CSCs to cancer development and maintenance, we have generated a transgenic mouse model in which oncogene expression is limited to $Sca1^+$ cells in mice. As a universally accepted model of human stem cell disorder, we focused our studies on the *BCR-ABLp210* oncogene associated with CML in humans (Melo and Barnes, 2007). This Sca1-BCR-ABLp210 model was able to reproduce genetically and physiologically the human CML, providing a unique opportunity, both for the identification of mechanisms contributing to the myeloproliferative disease and the progression to blast crisis, and ultimately to design effective therapies to interfere with these molecular events



B Sca1-BCR-ABLp210 mice treated with STI571 do not demonstrate a marked reduction in white blood count and spleen weight.

	STI571-treated*	Placebo
WBC/μl, neutrophils (%)	11,930 ± 467 (35±7)	12,508 ± 211 (40 ± 4)
Spleen weight (mg)	247±75	298±37

Figure 4 STI571 treatment in Sca1-BCR-ABLp210 mice. (**A**) STI571 treatment does not modify the survival of Sca1-BCR-ABLp210 mice. Mice were randomized to treatment with either STI571 or placebo to study the *in vivo* efficacy in three independent experiments. The survival curve depicts the percentage of animals alive at the indicated time point. The number of mice in each arm (*n*) is also shown. (**B**) Sca1-BCR-ABLp210 mice treated with STI571 do not demonstrate a marked reduction in white blood count and spleen weight. Standard errors are depicted. *The data depicted are from mice in (A) and are representative of at least three independent experiments.



B

Sca1-TK-IRES-BCR-ABLp210 mice treated with GCV show a marked reduction in white blood count and spleen weight.

	GCV-treated*	Placebo
WBC/μl, neutrophils (%)	8156±529 (12±6)	11,608 ± 303 (44 ± 9)
Spleen weight (mg)	109 ± 22	361±54

Figure 5 GCV treatment in Sca1-TK-IRES-BCR-ABLp210 mice. (**A**) Survival of Sca1-TK-IRES-BCR-ABLp210 mice following GCV administration. Diseased Sca1-TK-IRES-BCR-ALp210 mice were randomized to treatment with either GCV injected daily or placebo (saline solution treatment) to address the question of whether selective ablation of $Sca1^+$ cells can be used as a therapeutic target in cancer. The survival curve depicts the percentage of animals alive at the indicated time point. The number of mice in each arm (*n*) is also shown and corresponds to three independent experiments. $P<0.0001$ for treated versus untreated animals. (**B**) Sca1-TK-IRES-BCR-ABLp210 mice treated with GCV show a marked reduction in white blood count and spleen weight. Standard errors are depicted. *The data depicted are from mice in (A) and are representative of at least three independent experiments.

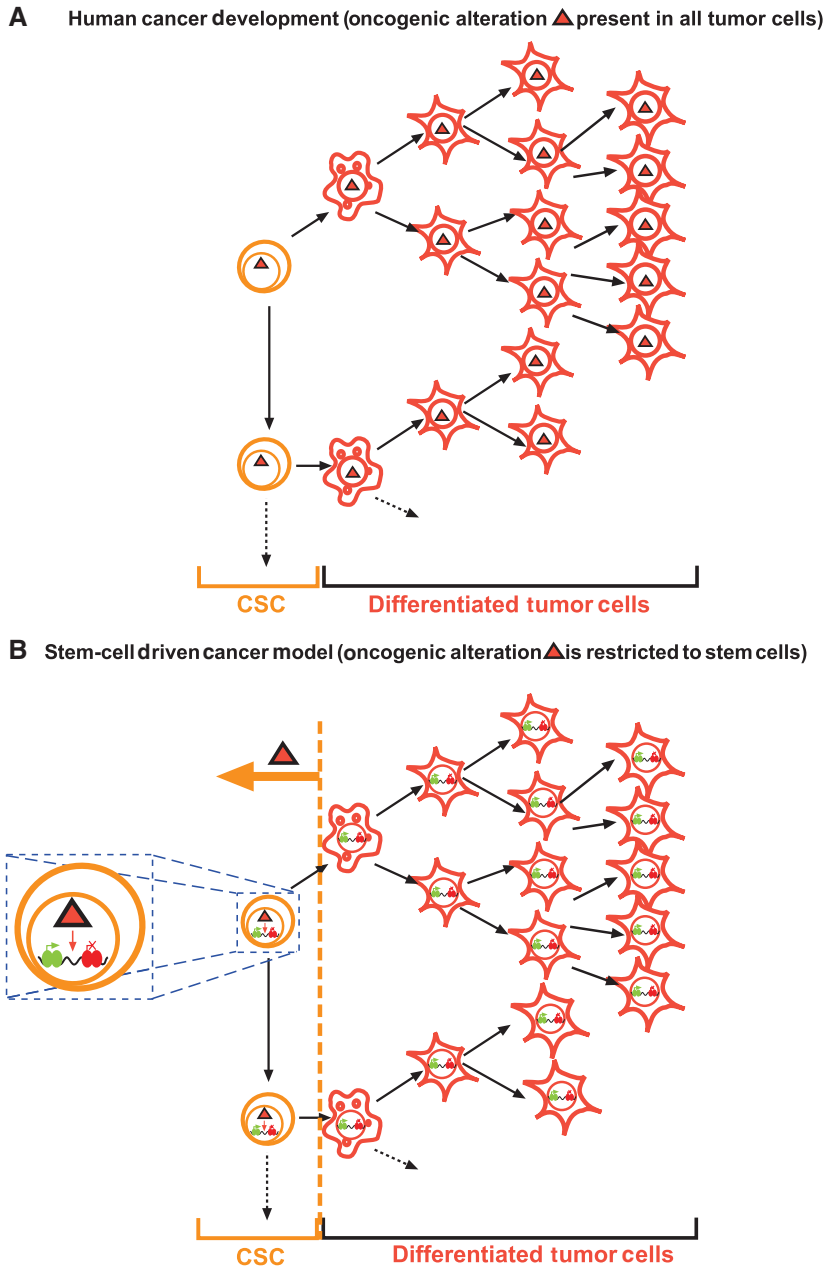


Figure 6 Generation of stem cell-driven cancer in the mouse. (**A**) In human pathologies and in most animal models of cancer, the oncogenic alteration(s) is(are) present in all the cellular types that compose the tumoral tissue, from the cancer stem cells to the more differentiated types. (**B**) In our stem cell-driven cancer model, the expression of the oncogenic alteration is restricted to the progenitor compartment but is nevertheless capable of generating a full-blown tumour with all its differentiated cellular components. This model implicitly relies on the fact that the oncogenic presence in the cancer stem cell compartment originates (epi)genetic latent alterations, which are responsible for the posterior appearance of the tumoral phenotype. These alterations are represented here as repressing (red) or activating (green) marks in the chromosome, induced by the oncogene in the CSCs and inherited in the lower compartments where the oncogene is no longer expressed, so that these descendant cells are not normal and constitute a non-clonogenic leukaemic progeny.

and to study the contribution of CSCs to disease development and maintenance.

From the point of view of the basic understanding, the demonstration that cancer development can be established in mice by limiting oncogene expression to Sca1$^+$ cells questions our view of the early steps of carcinogenesis. Results presented here suggest that the oncogene imposes a gene regulatory state in stem cells that somehow persists during haematopoiesis and which imposes a tumour phenotype reflective of the usual CML that cannot be modified through oncogene inactivation, an observation that could be applied to other cancer-initiating gene defects (Figure 6). This fact also brings up the distinction between the direct consequences of expression of oncogenic BCR-ABL, such as engagement of signalling pathways that require ongoing BCR-ABLp210 protein expression and will cease with subsequent differentiation of Sca1$^+$ stem cells and the indirect consequences of BCR-ABLp210 activity such as DNA methylation, centrosome abnormalities (or aneuploidy subsequent to abnormal centrosome function) or other secondary genetic

changes that may be inherited by subsequent generations regardless of continued BCR-ABLp210 expression. Our findings show that BCR-ABL appears to cause epigenetic and/or genetic changes in tumour-maintaining cells that could render them insensitive to BCR-ABL inactivation. Thus, *BCR-ABL* oncogene inactivation cannot change this epigenetic/genetic context in differentiated tumour cells in agreement with the common occurrence of tumour relapse by which tumours evolve to escape oncogene dependence. Therefore, we hypothesize that BCR-ABL mediates tumorigenesis through epigenetic/genetic modification of target genes that remain in this modified state in the mature tumour even in the absence of BCR-ABL. As an added level of complexity, this effect of BCR-ABL in stem cells could be mediated by a kinase-independent mechanism and therefore it would be Gleevec insensitive. The model provides evidence that the oncogenic proteins expressed in stem/progenitor cells can have selective impacts that depend on their intrinsic molecular properties. This result in turn provides a rationale for the striking associations between different chromosome translocations, unique fusion genes and cancer phenotypes. Importantly, a small subset of mice developed additional solid tumours, indicating that BCR-ABL stem cell-driven oncogenesis is not only specific to haematopoietic tissues but also represents a broader mechanism for deregulation of stem cell differentiation, providing a paradigm that can be applied to solid-organ cancers. Thus, these results support the view of cancer as a disease of cell differentiation rather than multiplication, asking for a redefinition of the role of oncogenes.

Overall, our findings indicate that BCR-ABLp210 appears to reprogramme stem cells rendering them insensitive to posterior BCR-ABLp210 inactivation. This evidence that BCR-ABLp210 inactivation could not stop tumour growth is in apparent conflict with earlier studies showing that BCR-ABLp210 is required for the persistence of tumoral characteristics in cellular systems (Szczylik *et al*, 1991; Choo *et al*, 1994; Skorski *et al*, 1994; Cobaleda and Sanchez-Garcia, 2000; Huettner *et al*, 2000). However, we should consider that the effects of BCR-ABLp210 inactivation will depend on the mechanisms by which BCR-ABLp210 is actually contributing to the tumorigenic phenotype, which are likely to vary according to the genetic and cellular context. When BCR-ABLp210 causes CML *in vivo*, as described here and observed in humans, its inactivation cannot eliminate CML cell progenitors. By contrast, when BCR-ABLp210 is just transforming a cell line into tumorigenic, its inactivation would result in tumour regression (Szczylik *et al*, 1991; Choo *et al*, 1994; Skorski *et al*, 1994; Cobaleda and Sanchez-Garcia, 2000; Huettner *et al*, 2000). So, as mentioned before, it is very possible that the molecular mechanisms of action of BCR-ABLp210 at the CSC level are different from those acting at later stages of tumoral cell differentiation (i.e. direct versus indirect BCR-ABL effects as discussed above).

The data presented here further show for the first time the *in vivo* physiological relevance of the CSC suppression using a model system representing *in vivo* biology of the human CML disease. Specific CSC ablation is able to eradicate CML in our model, although these cells were insensitive to Gleevec-mediated BCR-ABLp210 inactivation, suggesting that the reprogramming imposed by BCR-ABLp210 renders the cells oncogene independent. We conclude that limited oncogene expression in a CSC is all that is required to fully reprogramme it, giving rise to a full-blown, oncogene-specified tumour with all its mature cellular diversity, and that BCR-ABLp210-induced CML is reversible through the unique elimination of the CSC. Further experiments should confirm that this observation could be applied to other cancer-initiating gene defects. However, are there targets that can be exploited to eradicate CSCs without affecting healthy stem cells? The characterization of the CSCs in the Sca1-BCR-ABLp210 mice showed that CSCs were different from normal stem cells exhibiting cell surface embryonic figures that could be used to target CSCs. Thus, in agreement with earlier studies (Yilmaz *et al*, 2006; Liu *et al*, 2007; Ito *et al*, 2008), these data provide a rationale for the development of diagnostic and therapeutic strategies targeting CSCs without affecting normal stem cells. The hope is to translate this discovery into new tools for a better diagnostic and therapeutic management of the human disease.

Materials and methods

Generation of transgenic mice and BM transplantation

The Ly-6E.1 gene has been shown earlier to direct heterologous gene expression in HSCs of the adult BM and to specify copy number-dependent and integration site-independent expression (Miles *et al*, 1997). In this study, we have exploited the fact that this transgene is not expressed in all Sca1-positive cells for the study of cancer. The Sca1-BCR-ABLp210 and Sca1-TK-IRES-BCR-ABLp210 vectors were generated as follows. The 6 kb *Eco*RI–*Eco*RI fragment, containing the human BCR-ABLp210 cDNA, and the 9 kb *Eco*RI–*Eco*RI TK-IRES-BCR-ABLp210 cassette were inserted into the *Cla*I site of the pLy6 vector (Miles *et al*, 1997), resulting in Sca1-BCR-ABLp210 and Sca1-TK-IRES-BCR-ABLp210 vectors, respectively. The transgene fragments (Figure 1A) were excised from its vectors by restriction digestion with *Not*I, purified for injection (2 ng/μl) and injected into CBA × C57BL/6J fertilized eggs. Transgenic mice were identified by Southern blot analysis of tail snip DNA after *Eco*RI digestion. Human *ABL* cDNA was used for detection of the transgene. A total of 82 transgenic animals and 25 control animals were used to define the phenotype. Three independent transgenic lines were generated and analysed and similar phenotypic features were seen in both Sca1-BCR-ABLp210 lines and Sca1-TK-IRES-BCR-ABLp210 mice. IS1A line was crossed with IS1B transgenic mice to generate compound heterozygotes and increase BCR-ABL dosage (Figure 1B and D).

To determine the nature of the leukaemogenic cell, $Sca1^+Lin^-$ and $Sca1^-Lin^+$ cells were isolated and highly purified from the BM of a leukaemic primary mouse before or after different treatments or from control mice. The sorting purity of these cells was re-analysed with the fluorescence-activated cell sorting (FACS) and determined to be over 98%. In each cohort, these cells were injected intravenously into sublethally irradiated (4 Gy) recipient mice. Diseased mice were killed and assessed for leukaemia development. Mice that did not develop disease within 20 weeks post-transplantation were killed and tested for engraftment.

Histological analysis

All mice included in this study were subjected to standard necropsy. All major organs were examined under the dissecting microscope, and samples of each organ were processed into paraffin, sectioned and examined histologically. All tissue samples were taken from homogenous and viable portions of the resected sample by the pathologist and fixed within 2–5 min of excision. Haematoxylin–eosin-stained sections of each tissue were reviewed by a single pathologist (TF). Demonstration of fibrosis in spleen and liver of diseased Sca1-BCR-ABLp210 mice (green colour) was carried out by Masson's trichrome staining. For comparative studies, age-matched mice were used.

Analysis and monitoring of disease

PB was collected from retro-orbital plexus with a heparinized capillary tube, and total WBC and differential counts were performed twice a week. The number of WBCs was determined

with a haemocytometer after lysis of enucleated red blood cells with RCLB lysis buffer (0.15 M NH_4Cl; 1 mM $KHCO_3$; 0.1 mM Na_2-EDTA, pH 7.4).

Flow cytometry

Nucleated cells were obtained from total BM (flushing from the long bones), PB, thymus, liver and spleen. To prepare the cells for flow cytometry, contaminating red blood cells were lysed with RCLB lysis buffer and the remaining cells were then washed in PBS with 2% fetal calf serum (FCS). After staining, all cells were washed once in PBS with 2% FCS containing 2 μg/ml propidium iodide (PI) to allow dead cells to be excluded from both analyses and sorting procedures. Monoclonal antibodies were obtained from Pharmingen and included: lineage markers (CD45R/B220, for B lineage staining; CD4, CD8 and CD3 for T-cell lineage; CD11b and Gr1 for myeloid lineage and TER119 for erythroid lineage) and Sca1 (E13-161.7) for stem cells. Single cell suspensions from the different tissue samples obtained by routine techniques were incubated first with purified anti-mouse CD32/CD16 (Pharmingen) prior to the addition of other antibodies, to block binding through Fc receptors and then with an appropriate dilution of the different antibodies at room temperature or 4°C, respectively. The samples and the data were analysed in a FACSCalibur using CellQuest software (Becton Dickinson). Specific fluorescence of FITC and PE excited at 488 nm (0.4 W) and 633 nm (30 mW), respectively, as well as known forward and orthogonal light scattering properties of mouse cells were used to establish gates. For each analysis, a total of at least 5.000 viable (PI−) cells were assessed.

Cell purification

For cell sorter separation, BM cells were incubated with anti-Sca1 and anti-lineage marker antibodies (CD3, CD4, CD8, B220, TER119, Gr1 and Mac-1). $Sca1^+Lin^-$ and $Sca1^-Lin^+$ cells were isolated and highly purified from the BM of leukaemic primary mice or control mice by FACS (FACSVANTAGE; Becton Dickinson). c-kit (CD117) was not used for stem cell isolation, as earlier studies of human and mouse specimens have described downregulation of c-kit as a feature of leukaemia stem cells (Blair and Sutherland, 2000; Neering *et al*, 2007). Sorted cells were then re-analysed for purity with the FACS and determined to be over 98%.

RNA extraction

Total RNA was isolated in two steps using TRIzol (Life Technologies Inc., Grand Island, NY) followed by RNeasy Mini-kit (Qiagen Inc., Valencia, CA) purification following the manufacturer's RNA clean-up protocol with the optional On-column DNase treatment. The integrity and the quality of RNA were verified by electrophoresis and its concentration was measured.

Lineal RNA amplification

T-7-based RNA amplifications and preparations of cDNA probes were performed. Briefly, a maximum amount of 5 μg of total RNA was converted to double-stranded cDNA using the superscript choice system (Life Technologies Inc.) using oligo-dT primer containing a T7 RNA polymerase promoter. The double-stranded cDNA was cleaned up, and T7 *in vitro* transcription was performed using Megascript T7 *in vitro* transcription kit (Ambion, Austin, TX) following the manufacturers' instructions.

Microarray procedures

Second round amplified RNA (2.5 μg) from each sample was directly labelled with cyanine 3 (Cy3)-conjugated dUTP, whereas 2.5 μg of second round amplified RNA from the Universal Mouse Reference RNA (Stratagene) was labelled with cyanine 5 (Cy5)-conjugated dUTP as a reference. For all the microarray studies, the CNIO MouseChip was used and hybridizations were performed as described (Bermejo-Rodriguez *et al*, 2006). After washing, the slides were scanned using a Scanarray 5000 XL (GSI Lumonics Kanata, Ontario, Canada) and images were analysed with the GenePix 4.0 program (Axon Instruments Inc., Union City, CA).

Data analysis

Data obtained from each hybridization were stored in a database for analysis. The Cy3/Cy5 ratios were normalized to the median ratio value of all of the spots in the array. After normalization, spots with intensities for both channels (sum of medians) less than that of the local background were discarded. The ratios of the remaining spots were log transformed (base 2), and duplicated spots on the MouseChip were averaged to the median. To obtain the expression profile of CSCs ($Sca1^+Lin^-$ cells purified from Sca1-BCR-ABLp210 mice), we referred the ratios of the CSCs to the control HSCs ($Sca1^+Lin^-$ cells purified from control mice).

Real-time PCR quantification

cDNA for use in quantitative PCR studies was synthesized using reverse transcriptase (Access RT–PCR System; Promega, Madison, WI). Second round amplified RNA (2 μl) was transcribed. Primers and probes used for quantitative PCR are commercially available (TaqMan Assays-on-Demand Gene Expression Products; Applied Biosystems, Foster City, CA). In addition, the probes were designed so that genomic DNA would not be detected during the PCR. The sequences of the specific primers and probes were as follows: *BCR-ABL*^{p210}, sense primer 5′-TTCTGAATGTCATCGTCCACTCA-3′, antisense primer 5′-AGATGCTACTGGCCGCTGA-3′ and probe 5′-CCAC TGGATTTAAGCAGAGTTCAAAAGCCC-3′; c-Abl, sense primer 5′-CACTCTCAGCATCACTAAAGGTGAA-3′, antisense primer 5′-CGTTT GGGCTTCACACCATT-3′ and probe 5′-CCGGGTCTTGGGTTATAAT CACAATG-3′.

Immunolocalization of 5mC and pericentrin

Human and mouse cells grown on coverslips were stained with DAPI (blue) and mouse monoclonal antibodies against 5mC (red) (kindly provided by Alain Niveleau, Universite Claude Bernard, Lyon, France) as described earlier (Habib *et al*, 1999). Images of the nuclear immunolocalization of 5 mC were obtained in a Leica DMRA fluorescence microscope coupled to a Leica DC200 digital camera and captured with the Adobe Photoshop software. Control and Sca1-BCR-ABLp210 $Sca1^+$ cells were stained with antibodies against pericentrin (green; Berkeley ref. PRB-432C) to label centrosomes and with DAPI (blue) to label nuclei.

STI571 (Gleevec)

STI571 treatment regimens were based on earlier pharmacokinetic studies of STI in BCR-ABL tumour-bearing mice (le Coutre *et al*, 1999; Druker *et al*, 2001; Perez-Caro *et al*, 2007). Effectivity of BCR-ABLp210 suppression by STI571 was confirmed by assaying the survival of Ba/F3 cells expressing BCR-ABLp210 24 h after STI571 treatment and by the treatment of BCR-ABL transgenic mice according to previously published studies (Perez-Caro *et al*, 2007) (data not shown). For the animal studies, stock solutions of 5 and 10 mg/ml were prepared fresh in water, sterile filtered and administered to mice in a volume of 250 μl by gavage twice a day. Mice were started on STI571 or placebo (the same volume of diluent water) beginning 1 day after leukaemia was confirmed (day 0) by means of an STI571 regimen of 50 mg/kg every morning and 100 mg/kg every evening by gavage. STI571 was administered in a volume of 250 μl sterile water by means of straight or curved animal feeding needles. Mice tolerated the therapy well and no interruption of therapy was necessary. Mice were followed clinically three times a week, and periodic PB counts were obtained by tail vein blood draw as indicated. For the survival analysis portion of this study, the death end point was determined either by spontaneous death of the animal or by elective killing of the animal because of signs of pain or suffering according to established criteria.

GCV treatment

Sca1-TK-IRES-BCR-ABLp210 mice were used to test the effectiveness of GCV-induced cell in CSCs that express BCR-ABL. Expression of TK induces conversion of the prodrug nucleoside GCV to its drug form as a phosphorylated base analogue. The phosphorylated GCV is incorporated into the DNA of replicating cells causing irreversible arrest at the G2/M checkpoint followed by apoptosis (Rubsam *et al*, 1998). GCV, after preliminary testing, was administered at a dose of 100 mg/kg/day by i.p. injection for 14 days. This dose has been reported to kill cells expressing *TK* in transgenic mice (Bush *et al*, 1998). Dosing started when the mice were leukaemic. A control group was given injections of normal saline.

Statistical analysis

The χ^2 test was used to compare leukaemia incidence in Sca1-BCR-ABLp210 mice versus control mice. Significance analysis of microarrays proposed by Tusher *et al* (2001) was used to assess statistical significance of our differentially expressed genes.

Western blot analysis

Western blot analysis of BCR-ABLp210 expression and presence of phospho-CrkL (Tyr207) was carried out in $Sca1^{-}Lin^{+}$ cells of control and Sca1-BCR-ABLp210 mice. Tyr207 in CrkL is the BCR-ABL phosphorylation site. As positive controls, we used the following cell lines: Ba/F3, a murine haematopoietic precursor IL-3-dependent cell line (Palacios and Steinmetz, 1985), Ba/F3 + BCR-ABLp210, a Ba/F3 cell line expressing human BCR-ABLp210 (Sánchez-García and Grütz, 1995), and K562 cells that were the first human immortalized myelogenous cell line to be established derived from a 53-year-old female CML patient in blast crisis (Lozzio and Lozzio, 1975). All cells were maintained in DMEM with 10% FCS. When required, 5% of WEHI-3B-conditioned medium was included as a source of IL-3. Extracts were normalized for protein content by Bradford analysis (Bio-Rad Laboratories Inc., Melville, NY, USA) and Coommasie blue gel staining. Lysates were run on a 10% SDS–PAGE gel and transferred to a PVDF membrane. After blocking, the membrane was probed with the following primary antibodies: BCR (Ab-2) (Oncogene Science), Phospho-CrkL (Tyr207) (Cell Signaling) and actin (I-19; Santa Cruz Biotechnology). Reactive bands were detected with an ECL system (Amersham).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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