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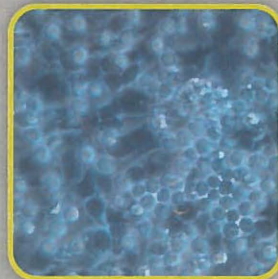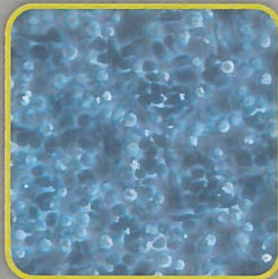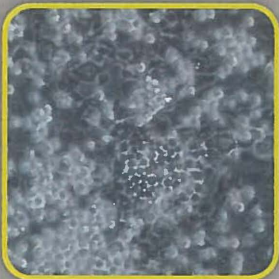# THE POLYCOMB GENE BMI1 IN NORMAL HEMATOPOIESIS AND LEUKEMIA

Aleksandra Rizo

The polycomb gene BMI1 in normal hematopoiesis and leukemia

Aleksandra Rizo-Crcareva

Stellingen behorde bij het proefschrift

The polycomb gene BMI1 in normal hematopoiesis and leukemia

1. The polycomb-group protein BMI1 is a cell intrinsic regulator of human stem/progenitor cell self-renewal. *This thesis*

2. Enforced expression of BMI1 is a powerful mediator of maintenance and self-renewal of human hematopoietic stem and progenitor cells. *This thesis*

3. Elevated levels of BMI1 act as a collaborating event with BCR-ABL to induce leukemic transformation of human cells. *This thesis*

4. BMI1 is an attractive candidate for targeting CML patients in blast crisis. *This thesis*

5. A stem cell is as good as its microenvironment.

6. What is a stem cell today may not be one tomorrow.

7. To the individual who devotes his/her live to science nothing can give more happiness than when the results find practical application. There are not two sciences. There is science and the application of science, and these two are linked as the fruit is to the tree. *Luis Pasteur*

8. If I have seen further than others, it is by standing upon the shoulders of giants. *Isaac Newton*

9. There is no harm in doubt and skepticism, for it is through these that new discoveries are made. *Richard Feynman*

10. If you want to kiss the sky, better learn how to kneel. *Bono*

Aleksandra Rizo, 24 mei 2011

RIJKSUNIVERSITEIT GRONINGEN

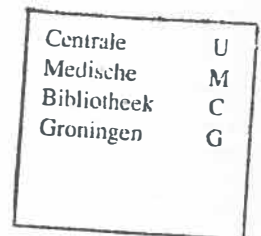**The polycomb gene BMI1 in normal hematopoiesis and leukemia**

Proefschrift

ter verkrijging van het doctoraat in de
Medische Wetenschappen
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Aleksandra Rizo

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GENERAL INTRODUCTION

HEMATOPOIETIC AND LEUKEMIC STEM CELLS

Hematopoietic stem cells (HSCs) are rare multipotent cells which posses the ability to self-renew as well as the ability to differentiate into all lineages of mature blood cells. Hematopoietic stem cells (HSCs) were first identified by Till and McCulloch in 1961.[1] Since then, they are the most extensively studied adult stem cells that have been isolated.[2] These studies have indicated that the mouse HSC is contained within the lineage negative (Lin⁻), Sca1⁺, Kit⁺, CD34⁻ and side-population (SP, cells that have the ability to efflux the DNA-binding dye Hoechst 33342) compartment. Furthermore, Morrison and colleagues identified that SLAM markers could be used to further purify mouse HSCs as cells that are CD150⁺ and CD48⁻.[3] The isolation of true human HSCs still remains elusive and additional studies are needed in order to identify specific markers which will allow its purification. A great progress in the study of human HSCs has been made since the development of NOD/SCID, NOD/SCID-β2-microglobulin⁻/⁻ and NSG (IL2γ⁻/⁻) xenotransplantation assays.[4-8] These assays are based on the lympho-myeloid repopulating capacity of the transplanted human HSC, termed as SCID-repopulating cell (SRC), and these SRCs are contained within the Lin⁻CD34⁺CD38⁻ population in the bone marrow.[4,6] These cells reside on the top of a tightly controlled hierarchical system, giving rise to differentiated progenies called multipotent progenitors (MPP). MPPs give rise to more committed common lymphoid progenitors (CLP) from which differentiated lymphoid cells are produced, and common myeloid progenitors (CMP) which will further produce two different progenies, granulocyte-macrophage progenitors (GMPs) and megakaryocyte-erythroid progenitor cells (MEP) (Fig. 1).

The process of stem cell self-renewal and differentiation needs to be tightly controlled, and hematopoietic disorders such as leukemias may occur when this control is lost. Similar to the normal hematopoietic system, leukemias are built up as a hierarchy as well, whereby leukemic stem cells (LSCs) reside at the top and give rise to new LSCs as well as leukemic daughter cells that have lost this capacity. Leukemic stem cells (LSCs) also often share cell surface characteristics with normal HSCs such as CD34⁺/CD38⁻, as has been identified by transplantation into NOD-SCID mice.[9,10] Nevertheless, there are clear differences between HSCs and LSCs as well. Over the past years, a number of membrane proteins have been indicated to be differentially expressed between HSCs and LSCs, including CD123, CD33, CD44, CD47,
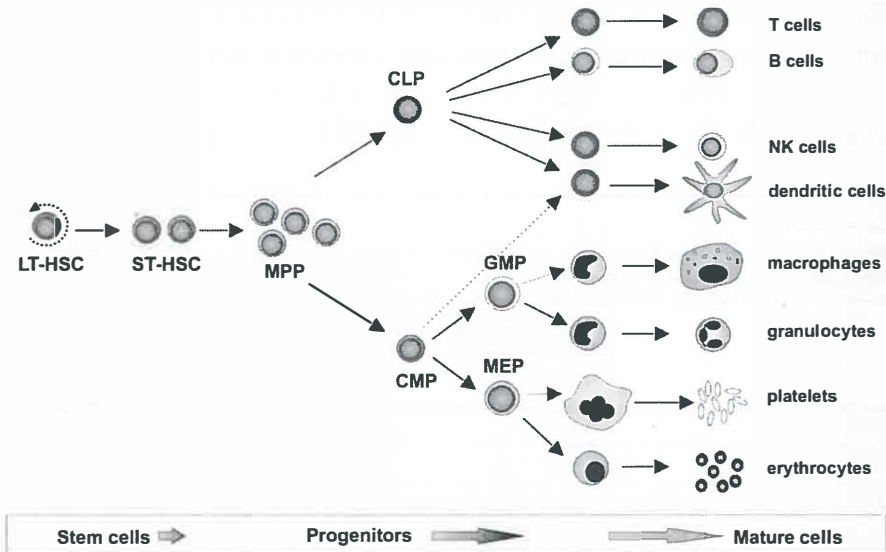
Figure 1. Hematopoiesis. Hierarchical representation of human and mouse hematopoiesis that shows the development of mature blood cells from hematopoietic stem cells. LT-HSC: long-term hematopoietic stem cell; ST-HSC: short-term hematopoietic stem cell; MPP: multipotent progenitor; CMP: common myeloid progenitor; CLP: common lymphoid progenitor; GMP: granulocyte/ macrophage progenitor; MEP: megakaryocyte-erythroid progenitor.

CLL1 and CD96.[11-16] For instance, it has been demonstrated that AML LSCs preferentially express CD96, contrary to normal cells and CD96+ AML cells can indeed engraft in *Rag2* deficient recipient mice.[11] CD33 is another molecule which has been identified to be expressed higher in AML cells than in HSCs and better engraftment was reported from the CD33+ fraction of AML cells in NOD-SCID mice.[16] CD123 has been identified to be differentially expressed on LSCs and its overexpression can give growth advantage of these cells over the normal HSCs.[14] In vitro analysis of the transmembrane protein CD47 showed higher expression in LSCs from patient material and that these patients had worse disease outcome compared to patients with lower CD47 levels.[12,17] These dissimilarities between normal and leukemic cells might serve as good starting points for the manipulation and ultimately the eradication of LSCs.

Acute myeloid leukemia (AML)

The most common leukemia that affects adults is acute myeloid leukemia (AML) and its incidence increases with age. It progresses rapidly and is typically fatal within weeks or months if left untreated. It is considered to be a clonal disease characterized with growth of immature myeloid blast-like cells which accumulate in the bone marrow and disrupt the process of

normal hematopoiesis. The classical view that leukemias are predominantly monoclonal was recently challenged by studies from the Greaves and Enver labs in childhood B-cell acute lymphoblastic leukemia (ALL) that indicated a remarkable clonal heterogeneity.[18] Whether this will also be true for

Description				% of AML cases
AML with recurrent genetic abnormalities				
	Cytogenetic abnormalities		**Genes involved**	
AML with	t(8;21)(q22;q22)		RUNX1-RUNX1T1	5
AML with	inv(16)(p13.1q22) or t(16;16) (p13.1q22)		CBFB-MYH11	5-8
APL with	t(15;17)(q22;q12)		PML-RARA	5-8
AML with	t(9;11)(p22;q23)		MLLT3-MLL	2
AML with	t(6;9)(p23;q34)		DEK-NUP214	0.7-1.8
AML with	inv(3)(q21;q26.2) or t(3;3)(q21;q26.2)		RPN1-EVI1	1-2
AML (megakaryoblastic) with	t(1;22)(p13;q13)		RBM15-MKL1	<1
Provisional entity: AML with mutated NPM1				27-35
Provisional entity: AML with mutated CEBPA				6-15
AML with myelodysplasia-related changes				24-35
Therapy-related myeloid neoplasms				10-20
AML, not otherwise specified (NOS)			**FAB**	
AML with minimal differentiation			M0	<5
AML without maturation			M1	5-10
AML with maturation			M2	10
Acute myelomonocytic leukemia			M4	5-10
Acute monoblastic/monocytic leukemia			M5	<5
Acute erythroid leukemias			M6	<5
Pure erythroid leukemia				
Erythroleukemia, erythroid/myeloid				
Acute megakaryoblastic leukemia			M7	<5
Acute basophilic leukemia				<1
Acute panmyelosis with myelofibrosis				rare
Myeloid sarcoma				
Myeloid proliferation related to Down syndrome				
Transient abnormal myelopoiesis				
Myeloid leukemia associated with Down syndrome				
Blastic plasmacytoid dendritic cell neoplasms				

Table 1. World Health Organization (WHO) classification of AML (Adapted from thesis by Lina Han, UMCG, 2010).

myeloid leukemia or adult leukemias remains to be determined, but clearly the possibility that also in these types of leukemias clonal heterogeneity might exist will have to be considered. Various translocations, mutations and molecular aberrations have been identified in patients with AML, and an overview is presented in Table 1. According to the differentiation phenotype and the morphological features AMLs have been classified by the FAB classification system in 8 different subtypes. However, the recently proposed WHO classification has better prognostic value and appears to be clinically more relevant as it categorizes the disease by genetic, clinical and immunophenotypic features (Table 1).[19]

Chronic myeloid leukemia
CML is myeloproliferative disorder characterized by accumulation of immature and mature myeloid cells. More than 90% of the patients are diagnosed in a relatively early stage of the disease known as chronic phase (CP). It is generally accepted that acquisition of the BCR-ABL translocation t(9;22) is the initiating event in the CML CP.[20,21] It is believed that this acquisition initially occurs in a single HSC that gains proliferative advantage and/or aberrant differentiation capacity over the normal cells, giving rise to expansion of the myeloid compartment.[22,23] Before the discovery of the tyrosine kinase inhibitors (TKIs), all patients with CML-CP progressed spontaneously to advanced disease in a median of 5 years. This phase is divided into an accelerated phase (AP) followed by a blast crisis (BC).[24] The molecular mechanisms underlying this disease progression are still not entirely understood, but it is likely that they involve activation of oncogenic factors and inactivation of tumor suppressors. The phenotype of the self-renewing leukemic stem cells that maintain CML remains obscure. In CML-CP, LSCs reside within Lin⁻CD34⁺38⁻ fraction, suggesting that the first cell that gains the BCR-ABL translocation is a stem cell or immature progenitor cell.[25-27] Furthermore, in contrast to other oncogenes like MOZ-TIF2 and MLL-ENL, BCR-ABL cannot confer self renewal properties on committed progenitor cells, again suggesting that an immature stem/progenitor cells is most likely the cell of origin in CML.[28-30] Upon progression to BC-AML, it has been shown that the phenotype of the leukemia-maintaining stem cell changes and starts to resemble the phenotype of granulocyte/macrophage progenitors (GMPs).[31]

Epigenetic gene regulation in normal and malignant hematopoiesis

The traditional view of cancer is that it is a genetic disease driven by the sequential acquisition of mutations, leading to activation of proto-oncogenes and loss-of-function of tumor suppressor genes. In the recent years, however, it has become evident that tumor development also involves patterns of altered gene expression that are mediated by mechanisms that do not affect the primary DNA sequence, so called 'epigenetic changes'.

Drosophila proteins	Human proteins	Mouse proteins
Esc-E(z)/PRC2 initiation complex		
Esc	EED	Eed
E(z)	EZH1	Ezh1/Enx2
	EZH2	Ezh2/Enx1
Su(z)12	SUZ12	Suz12
PRC1 maintenance complex		
Pc	CBX2/HPC1	Cbx2/M33
	CBX4/HPC2	Cbx4/Mpc2
	CBX7	Cbx7
	CBX8/HPC3	
Ph	EDR1/HPH1	Edr1/Mph1/Rae28
	EDR2/HPH2	Edr2/Mph2
	EDR3/HPH3	
dRing	RING1/RNF1/RING1A	Ring1/Ring1a
	RNF2/RING1B	Rnf2/Ring1b
Esc	BMI1	Bmi1
	RNF110/ZFP144	Rnf110/Zfp144/Mel18
	ZNF134	Znf134/Mblr
Pho	YY1	Yy1
Scm	SCML1	Scml1
		Scml2
Pcl	PHF1	

Table 2 Polycomb repression complex members (Adapted from [33]).

These changes include genome-wide losses or regional gains of DNA methylation, as well as altered patterns of histone modification.[32] The structural units of the chromatin are termed nucleosomes, which constitute of histones and DNA. Perturbations in chromatin structure can cause inappropriate gene expression and genomic instability, resulting in cellular

transformation and malignant outgrowth. Therefore proteins that control chromatin organization play key roles in cancer pathogenesis. One class of such epigenetic regulators is the Polycomb group (PcG) of proteins, which function as transcriptional repressors that silence specific sets of genes through chromatin modification.

PcG protein complexes: PRC1 and PRC2

The PcG gene family is highly conserved throughout evolution. Originally PcG proteins were discovered in Drosophila as repressors of Hox genes, which is necessary for establishment of the body plan and segmentation during development.[34] In mammals this function is conserved, with several Polycomb mutants exhibiting skeletal malformations.[35-38] At the molecular level, PcG proteins are classified into two groups that form the Polycomb Repression Complex 1 (PRC1) or PRC2 (Table 2).[33] PRC2, also referred to as the "initiation complex", is composed of the Drosophila melanogaster proteins Enhancer of Zeste (EZ; mammalian homologue is EZH1/2), Suppressor of Zeste 12 (SUZ12) and Extra Sex Combs (ESC; mammalian homologue is EED). This complex is involved in initiation of silencing and contains histone deacetylases and histone methyltransferases that can methylate histone H3 lysine 9 and 27, marks of silenced chromatin, and histone H1 lysine 26.[39-42] The more diverse PRC1, also termed "maintenance complex", is implicated in stable maintenance of gene repression and recognizes the H3 lysine 27 mark of PRC2.[43] This complex comprises of Polycomb (PC; CBX2, 4, 7, 8), Polyhomeotic (PH; HPH1-3), Posterior Sex Combs (PSC, BMI1, MEL18) and Sex Combs Extra (SCE/RING1/2).

The current model proposes an initial recruitment of the PRC2 complex to Polycomb repressive elements (PREs) located in the chromosomal DNA, either within or in the vicinity of the gene(s) to be silenced.[44] Once recruited, PRC2-associated histone deacetylases remove the acetylation of H3, and allow histone methyl transferases (such as EZH2) to methylate K27.[45] This methylation establishes a binding site for the chromodomain present in the amino-terminus of Polycomb proteins,[46] leading to the recruitment of the PRC1 maintenance complex. Once recruited, PRC1 can repress gene expression by blocking transcription initiation.[47]

The existence of only two PcG complexes is an oversimplification as recent data indicates that heterogeneous protein complexes of various compositions can be formed even within one cell. For example EZH2 can associate with EED thereby determining the specificity of the histone methyltransferase activity

toward histone H3 lysine 27 or H1 lysine 26.[48] Also, it has been shown that the relative amounts of BMl1 within the PRC1 complex can determine its biochemical and biological functions.[49] It has been suggested that the presence of BMl1 and MEL18, as well as CBX7 and CBX8 in the complex is mutually exclusive.[50,51] Although the composition of PRC1 and PRC2 complexes can vary between specific cell types, little is known on how this is regulated and how the composition of PRC complexes affects silencing of specific subsets of genes in a specific cell type. BMl1 is expressed in HSCs, but its expression decreases upon maturation.[52-54] In contrast, other PRC1 components, including MEL18, CBX and Mph1/Rea28 are expressed at rather low levels in HSCs, but increase upon differentiation.[53,54] This appears contradictory, as it is proposed that all PRC1 subunits are required for appropriate chromatin remodeling by PRC1.

BMI1

The BMl1 protein contains a RING finger domain which is essential for its ability to modulate cell proliferation. No enzymatic activity has been described for BMI1. The most critical BMI1 target is INK4a/ARF. This locus encodes two structurally different proteins, p16INK4a and ARF (referred as p14ARF in humans and p19Arf in mouse).[55] While p16INK4a is cycline dependent kinase inhibitor that activates the RB pathway through cycline D complexes, ARF induces the p53 pathway by inhibiting MDM2 function.[55-57]

Mice in which Bmi1 has been ablated exhibit postnatal defects in skeletal patterning, hematopoiesis, and neurological functions.[38] These defects can, at least in part, be corrected by concomitant ablation of the Ink4a/Arf locus.[58] Fibroblasts (MEFs) isolated from Bmi1-null embryos undergo rapid senescence caused by acute upregulation of p16Ink4a and p19Arf. Conversely, overexpression of Bmi1 results in decreased p16Ink4a and p19Arf levels, leading to an extension of cellular lifespan and an apparent relaxation of the tumor-suppressing functions of the locus.[58,59]

Polycomb target genes were mapped in genome-wide screens in human embryonic fibroblasts,[60] murine embryonic stem cells[61,62] and Drosophila melanogaster.[63,64] In embryonic fibroblasts, PRC1, PRC2 and H3K27-3me co-occupied >1000 genes with a strong bias for embryonic development and cell fate decisions. In ES cells, Suz12, Eed and H3K27-3me co-occupied 653 genes, which generally associated with an underexpression of those genes. Target genes included many homeotic genes found in the HOX clusters and included most members of the DLX, IRX, LHX and PAX gene families, which

regulate early developmental steps in neurogenesis, hematopoiesis, tissue patterning and cell fate specification. In Drosophila, PcG proteins were observed to preferentially bind to developmental genes involved in signal transduction pathway regulation, including Wingless, Hedgehog, Notch and Delta. Less is known about PcG targets in human hematopoietic stem cells, and no genome-wide analysis of leukemic stem cells have been performed till date.

BMI1 in HSCs

BMI1 is expressed in hematopoietic stem cells (HSCs) and its expression goes down upon differentiation toward myeloid or erythroid cells, but is retained within the B-cell and T-cell lymphoid compartments.[53,65] In human hematopoietic cells isolated from cord blood, BMI1 is highest expressed within the HSCs, and its expression gradually decreases upon maturation toward common myeloid progenitors (CMPs), granulocyte/ macrophage progenitors (GMPs) a megakaryocyte/erythroid progenitors (MEPs).[53,65,66] Targeted deletion of Bmi1 in murine HSCs impaired the competitive repopulation capacity, while overexpression of Bmi1 enhanced the self-renewal of HSCs and shifted the balance towards more symmetric stem/ progenitor cell divisions.[65,67] In the human hematopoietic system, we have classified BMI1 as a strong intrinsic regulator of human stem cells as BMI1 expressing cells engrafted efficiently in the NOD/SCID mice even after in vitro culturing.[66] However, the mice did not show signs of disease suggesting that BMI1 is not sufficient to induce leukemia by itself in human cells (unpublished observations and [66]), comparable to what has been observed in murine cells. The molecular mechanisms by which BMI1 contributes to stem cell self-renewal are far from completely understood. In senescence screens it was observed that p16INK4a/p19ARF locus is repressed by BMI1, which was required to bypass senescence of embryonic fibroblasts.[58] Also in hematopoietic cells, targeted deletion of BMI1 resulted in an increase in the expression of p16 and p19. Deletion of p16/p19 in Bmi1-/- HSCs partly restored self-renewal, but not completely, and overexpression of Bmi1 could still increase progenitor levels in the absence p16/p19, indicating that other BMI1 targets must exist as well.[68] Recently, it was demonstrated that Bmi1 also regulates mitochondrial function and participates in the DNA damage response pathway and these metabolism pathways might be important targets of Bmi1 in HSCs as well.[69]

BMI1 in LSCs

The first evidence for a possible involvement of BMI1 in the development of hematological malignancies came from a provirus integration screen in which BMI1 was identified as a cooperating factor with Myc in the induction of B cell lymphomagenesis.[70] Sauvageau and colleagues demonstrated that Bmi1 not only determines the proliferative capacity of normal stem cells, but also of leukemic stem cells.[71] In a mouse model in which coexpression of the oncogenes HoxA9 and Meis resulted in a quick onset of myeloid leukemia, no disease was observed in secondary recipients in a Bmi1-deficient background.[71] These data indicate that Bmi1 is essential for the maintenance of HoxA9-Meis1 leukemic HSCs in vivo. The oncoprotein E2a-Pbx1 enhances the expression of Bmi1, which results in repression of the Ink4a-Arf locus and is a prerequisite for hematopoietic transformation.[72] In K562 cells, downmodulation of Bmi1 by RNAi significantly impaired the growth.[73] Sall4, an oncogene that is expressed in AML and induces leukemia in transgenic mice[74] also strongly upregulates Bmi1 expression.[75]

Interestingly, expression of BMI1 alone is not sufficient to induce leukemia. Self-renewal and repopulation activity is enhanced in both mouse[65] and human cells (our preliminary data), but it is plausible that secondary mutations are required for a full leukemic phenotype. A recent paper indeed documented that BMI1 expression alone could extend the life-span of mammary epithelial cells, but only after subsequent introduction of hTert efficient immortalization was observed.[76] In a tumor model in which the oncogene TLS-ERG is introduced into human hematopoietic progenitors, in a limited number of cases the transduced cells underwent a step-wise transformation and immortalization in which upregulation of BMI1 was identified as one of the cooperating hits.[77] In mouse models, it has been shown that BMI1 can collaborate with H-RAS to induce aggressive breast cancer with brain metastasis.[78] In one patient who was transplanted with retrovirally-transduced CD34$^+$ bone marrow cells, the vector had integrated near the BMI1 gene, leaving open the possibility that its upregulation might have been the reason for the development of T-cell leukemia in this patient.[79] BMI1 expression is elevated in a variety of hematological tumors, including non-Hodgkin lymphomas and acute myeloid leukemias (AMLs).[80-83] By making use of intracellular fluorescence-activated cell sorting (FACS) analyses to determine BMI1 expression in AML patient samples, it was shown that the expression level of BMI1 was sufficient to predict overall survival, relapse-free survival and remission duration in a multivariate analysis.[80] In

chronic myeloid leukemia (CML), BMI1 expression increases with disease progression and high levels of BMI1 correlate with reduced overall survival.[84] A small study performed on myelodysplastic syndrome (MDS) samples demonstrated that high BMI1 expression correlated with poor overall survival in those patients as well.[85] In chronic lymphocytic leukemia (CLL) and mantle cell lymphoma (MCL), BMI1 expression was also elevated, which in about 10% of the MCL cases was due to BMI1 gene amplification.[86]

Scope of the thesis

In this thesis we aimed to obtain further insight into the role of BMI1 in normal human hematopoietic stem cells as well as in the mechanisms by which BMI1 might contribute to leukemic transformation. In chapter two we reviewed intrinsic and extrinsic mechanisms that can regulate stem cell self-renewal, and we discuss whether differences might exist in bone marrow niche dependency between normal and leukemic stem cells. In chapter three we introduced BMI1 into human cord blood CD34+ cells and determined the effects on long-term expansion and self-renewal of stem/ progenitor cells in vitro and in vivo. In chapter 4 we downmodulated BMI1 expression in normal hematopoietic stem cells as well as in primary CD34+ cells from acute myeloid leukemia patients using a lentiviral approach and determined the effects on long-term growth and self-renewal. In chapter 5 we describe a human xenograft leukemia model in which co-expression of BMI1 together with BCR-ABL induces a serially transplantable lymphoid leukemia in vivo. In vitro, both myeloid as well as lymphoid transformed cell lines could be established. In chapter 6, the experimental work described in this thesis is summarized and discussed, and possible future directions are evaluated.

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Signaling pathways in self-renewing hemato-poietic and leukemic stem cells: do all stem cells need a niche?

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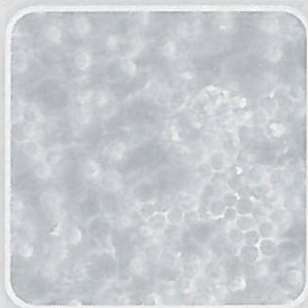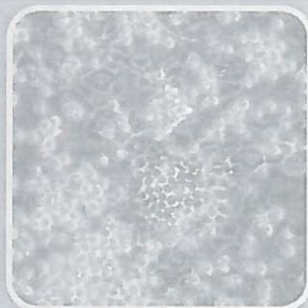
ABSTRACT

Many adult tissue stem cells, such as the cells of the hematopoietic system, gastrointestinal epithelium, brain, epidermis, mammary gland and lung have now been identified, all of them fulfilling a crucial role in supplying organisms with mature cells during normal homeostasis as well as in times of tissue generation or repair. Two unique features characterize adult stem cells: the ability to generate new pluripotent stem cells (to self-renew) and the ability to give rise to differentiated progeny that has lost its self-renewal capacity. Our understanding of the mechanisms that determine whether, where and when a stem cell will self-renew or differentiate is still limited, but recent advances have indicated that the stem cell microenvironment, or niche, provides essential cues that direct these cell fate decisions. Moreover, loss of control over these cell fate decisions might lead to cellular transformation and cancer. This review addresses the current understanding of the molecular mechanisms that regulate hematopoietic stem cell self-renewal in the niche, and how leukemic transformation might change the dependency of leukemic stem cells on their microenvironment for self-renewal and survival.

HEMATOPOIETIC AND LEUKEMIC STEM CELLS

Hematopoietic stem cells (HSCs) were first identified by Till and McCulloch in 1961 (1). Since then, HSCs are the most extensively studied adult stem cells that have been isolated (2). Nevertheless, HSCs are a rare population representing less than 0,01% of the cells in the bone marrow. This has complicated HSC research, and it is indeed surprising to realize how limited our current knowledge still is regarding mechanisms that determine whether, where, and when hematopoietic stem cells undergo self-renewal divisions. The most reliable assays to detect and enumerate HSCs are in vivo repopulation experiments, and advances in technologies have greatly helped to purify mouse HSCs (3-5). These studies have indicated that the mouse HSC is contained within the lineage negative (Lin-), Sca1+, Kit+, CD34-, side-population (SP, cells that have the ability to efflux the DNA-binding dye Hoechst 33342) compartment. Recently, it was reported that combinatorial expression of cell surface receptors of the SLAM family members precisely distinguishes mouse stem from progenitor cells (6). Thus, most of the gained knowledge from mouse HSCs in the past years relies on the possibility to isolate and quantitatively measure stem cell activity in in vivo assays. The isolation of true human HSCs still remains more elusive and additional studies need to be performed in order to identify specific hematopoietic markers that will allow purification of human stem cells to homogeneity. A great progress in the study of human HSCs has been made since the development of NOD/SCID and NOD/SCID-b2-microglobulin-/- xenotransplantation assays (7-9). These assays are based on the lympho-myeloid repopulating capacity of the transplanted human HSC, termed as SCID-repopulating cell (SRC), and these SRCs are contained within the Lin-CD34+CD38- population in the bone marrow (7,8). The NOD/SCID and NOD/SCID-b2-microglobulin-/- model systems have also greatly facilitated the identification of the leukemic stem cell (LSC), or SCID leukemia-initiating cell (SL-IC) (10,11). As in the normal hematopoietic system, it has been recognized that in Acute Myeloid Leukemia (AML) the developing malignant clone is comprised of a heterogeneous group of cells that differ in their differentiation status and only the rare SL-IC population was capable of initiating and sustaining leukemic growth in vivo in SCID or NOD-SCID mice (10,11). These SL-ICs have self-renewal capacity as demonstrated in serial transplantation experiments, but heterogeneity exists in the

self-renewal potential of different classes leukemic stem cells, which further supports the hypothesis that they are derived from normal HSCs (12).

The interaction of the stem cell with specific microenvironmental elements is thought to be a key regulatory mechanism in maintenance of its self-renewal and differentiation capacities. The concept of a 'stem cell niche' was first proposed in the late '70s (13) but the HSC niche was identified in mice almost three decades later independently by two groups of researchers (14,15). Detailed information on the structure, composition and exact localization of the niche is only beginning to be revealed, and particularly in the human system knowledge is only emerging. Nevertheless, a number of different types of signaling and adhesion molecules that have a role in the regulation of stem cell quiescence, self-renewal and cell fate decisions have been reported (discussed in more detail below). The stem cell niche has been attributed functions such as maintenance of stem cell quiescence by providing proliferation inhibiting cues. However, the niche should also provide proliferation or differentiation inducing signals in times that high numbers of progenitors are needed that quickly can give rise to all committed cell lineages. Ultimately, the niche needs to ensure a life-long reserve of stem cells, which requires tightly controlled stem cell self-renewal divisions. It is therefore no surprise that deregulation of self-renewal of stem cells is the main cause for neoplastic or cancer diseases. Stem and cancer cells share certain signaling pathways that regulate their self-renewal which suggests that normal stem cells can in fact give rise to cancer cells. This notion has fed the concept that tumors contain rare 'cancer stem cells' that maintain tumor growth because of their indefinite proliferative and self-renewal potential (reviewed in (16,17)). Understanding the genetic and molecular regulations of the self renewal program and an appreciation of how perturbations in these regulations initiate proliferative diseases such as leukemia is a major challenge of the medical research.

In this review we will discuss how normal and leukemic stem cells interact with their microenvironment, which mechanisms have been reported to be involved and how these correlate with processes such as self-renewal. Do the hematopoietic and leukemic stem cells share the same niche or do leukemic cells interact differently with their own microenvironment? Do these cells home only in the bone marrow or also in other niches? Indeed, do leukemic stem cells need a niche at all?

HEMATOPOIETIC STEM CELLS AND THEIR MICROEN-VIRONMENT

Physical association of the stem cell with the niche

HSC quiescence, self-renewal and differentiation are regulated by both intrinsic and extrinsic mechanisms. Intrinsic mechanisms include those that affect the epigenetic state of HSCs as it is controlled by chromatin remodellers, such as polycomb group proteins. Extrinsic mechanisms include those changes in stem cell fate that are dictated by the environment, i.e. the niche. In such a model, direct physical interaction between HSCs and their niche could be mediated by integrins and cadherins. Once localized within the niche, HSCs are in close proximity of locally secreted or membrane-bound cytokines and growth factors that dictate HSC fate by initiating specific signal

Figure 1. Extrinsic and intrinsic mechanisms that determine stem cell fate

HSC quiescence, self-renewal and differentiation is regulated by both intrinsic and extrinsic mechanisms. Extrinsic mechanisms include changes in stem cell fate that are dictated by the environment i.e. niche. Once physical association between the osteoblast and the HSC has occurred, release of different growth factors will initiate diverse signal transduction pathways that will initiate expression of downstream target genes. Intrinsic mechanisms are niche-independent and for e.g. they can affect the epigenetic state of HSC as it is controlled by chromatin remodellers. (HCS - hematopoietic stem cell, TF-transcription factors)

transduction within the HSC. A schematic presentation of these regulatory mechanisms and there complementary interaction is shown in Figure 1.

The stem cell niche is an anatomical unit located in the endosteum within the bone marrow cavity that is composed of osteoblasts, osteoclasts and stromal fibroblasts. Various studies have shown that osteoblasts are important players in providing HSCs with extrinsic cues. Figure 2 provides an overview of molecules that have been implicated in HSC-osteoblast interactions, and these molecules will be discussed in detail this section.
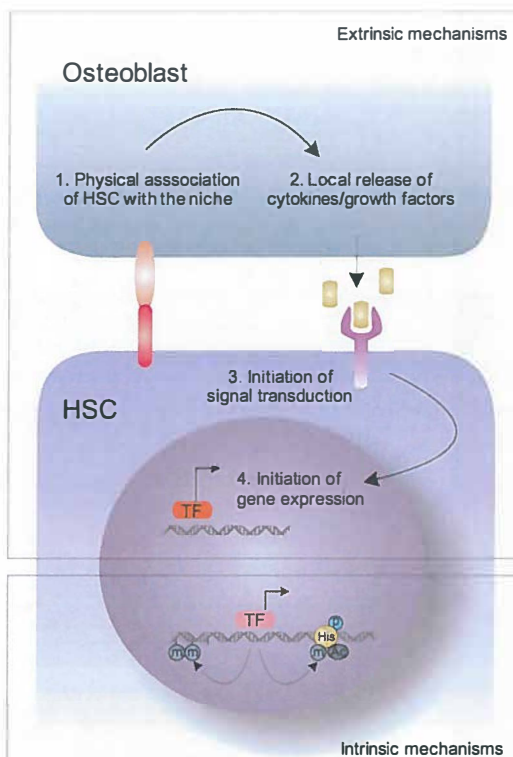
Figure 2. Signal transduction pathways in the hematopoietic stem cell niche

A graphical representation of signaling pathways involved in the hematopoietic stem cell fate in the niche. In this model, direct physical interaction between the HSC and osteoblast could be mediated by cadherins and integrins such as VLA4 and VLA5 (upper left section) which are involved in processes like adhesion and migration of the stem cell. Once appropriately localized within the quiescent niche, processes such as self-renewal, maintenance of quiescence, or exit from the niche followed by proliferation and differentiation are highly controlled by growth factors and cytokines that are secreted locally by osteoblasts and stromal cells. Examples of such molecules are TGF-β which is a negative regulator of the cell growth, Ang-1 responsible for the stem cell quiescence or Wnts and FGF-1 which promotes stem cell expansion (upper right section). These factors can now dictate HSC fate by triggering specific signaling downstream modulators within the HSC, such as Myc, β-Catenin, Stats, Smads or C/ebpα. The possible intrinsic i.e. epigenetic regulators are represented in the lower part of figure.

It is only recently that two groups of researchers independently revealed the spatial organization of the stem cells within the niche (14,15). Calvi and colleagues demonstrated that activation of parathyroid hormone-related protein receptors by feeding mice parathyroid hormone (PTH) resulted in an increase in the number of osteoblasts in endosteum, thereby enlarging the HSC niche which allowed an increase in HSC numbers (14). These osteoblasts expressed elevated levels of Jagged-1, the ligand for Notch receptors. Activation

of Notch1 has been shown to result in enhanced HSC self-renewal, possibly by inducing a set of self-renewal genes including Hes1 (18) (Fig.2). Conditional deletion of Bmpr1 also leads to increased numbers of N-cadherin⁺CD45⁻ osteoblastic (SNO) cells and subsequently to higher stem cell numbers (15). Thus, Bmp signaling via the BmpR type IA complex controls the number of HSCs by regulating the niche size. N-cadherin is not only expressed on osteoblasts, but also on a subpopulation of murine LSK cells (15) where it interacts and forms an adherence complex with β-Catenin (19). Wnts, as potent inducers of β-catenin signaling, have been implicated in the maintenance and expansion of murine HSCs (20,21). How N-cadherin-β-catenin interactions can affect HSC fate within the niche is currently unclear. It is possible that under certain circumstances metalloproteinases such as Mmp9 or Adam10 result in cleavage of membrane-bound N-cadherin, thereby increasing the cytoplasmic β-catenin pool that is now available to act in the canonical Wnt pathway to induce expression of target genes, including CyclinD1 and c-Myc (22). Alternatively, the sole function of cadherins and integrins might be to physically associate HSC with osteoblasts or extracellular matrix (ECM) at the appropriate positions within the bone marrow niche (upper left part of Fig.2). Integrins such as Vla4 and Vla5 are efficient activators of the small GTPase family members Rac1 and Rac2. Targeted deletion of both Rac1 and Rac2 results in mobilization of stem cells, indicating their importance in appropriate positioning of HSCs within the bone marrow microenvironment (23,24). In line with these observations, lack of Vla4 expression restricts competitive repopulation activity and self-renewal potential of HCSs (25). Further support for the view that stem cells need to be physically associated with their bone marrow microenvironment arises from studies that demonstrated that in the absence of membrane-bound Kit Ligand (mKL) in the niche HSC are not maintained (26,27). It furthermore has been shown that metalloproteinase-9 (MMP-9)-mediated cleavage of mKL into soluble KL (sKL) results in the translocation of HSCs from the quiescent endosteal niche towards vascular-enriched niches (discussed in more detail in the next section) favoring differentiation and HSC mobilization into the peripheral circulation (28). Recently, it was demonstrated that HSCs sense relatively high [Ca^{2+}] levels via a seventransmembrane-spanning calcium-sensing receptor (CaR) which is expressed on HSCs. This receptor is required to retain HSCs in close proximity to the endosteal surface of the bone, possibly by mediating the association of HSCs with collagen I (29). It has been reported recently that Muc1 can initiate calcium signaling through

association with its ligand ICAM-1 (30), and as Muc1 can mediate transendo-thelial migration of breast cancer cells (31) these data suggest that Muc1-mediated cytoskeletal rearrangements might be involved in the interaction between HSCs and the niche as well.

The role of growth factors and cytokines in the stem cells niche

Once localized within the niche, locally secreted cytokines and growth factors can dictate stem cell fate by initiating specific signal transduction within the HSC (upper right section of Fig.2). Transforming growth factor - β (Tgf-β) is one of the few known negative regulators of HSCs. It maintains the stem cells in a slow cycling or quiescent state partly by blocking the cell surface expres-sion of cytokine receptors like c-Kit, Flt3, Mpl and IL-6R (32). The cell cycle arrest of human HSCs requires up-regulation of p57 which is a known tumor suppressor (33). Whether Tgf-β is a negative regulator under physiological in vivo conditions it is not clear yet, as HSCs with deleted Tgf-β type I receptors have normal differentiation abilities and a normal cell-cycle distribution (34). In addition, the angiopoietin-1 (Ang-1) produced by stromal cells enhances the ability of HSCs to become quiescent through interaction with its tyrosine ki-nase receptor Tie2 (35). The mechanism of cell-cycle inhibition by Tie-2 still remains to be identified, but the cyclin dependent kinase inhibitor p21 has been shown to be important for maintenance of HSCs quiescence (36). Inter-estingly, the gene encoding p21 is directly repressed by c-Myc (37), another key regulator in the stem cell niche. While resting HSCs are characterized by high p21 levels and an absence of c-Myc expression, increasing levels of c-Myc activity have been linked to reduced p21 expression and a more active state of the HSC (38).

Quite different from the growth inhibiting effects induced by Tie-2 or Tgf-β, growth promoting factors have also been identified. We have reported a role of Fibroblast growth factor -1 (Fgf-1) in stem cell self-renewal and expansion (39). Upon binding to its receptors, FgfR1-4, a variety of signal transduction pathways can be activated, including the MAPK pathway, Stats and PI-3K (40). Prolonged cultures of murine bone marrow cells supplemented with sol-uble FGF-1 resulted in robust expansion of HSCs (41) and these cells represent excellent targets for retrovirus-mediated gene delivery (42).

Intrinsic epigenetic modulators

Epigenetic modification of the chromatin structure underlies the differentiation of pluripotent HSCs into their committed progenies. Recent evidence indicates that members of the Polycomb group (PcG) protein complex play key roles in normal and leukemic hematopoiesis (lowest segment of Fig.2). Notable examples are Ezh2 and Bmi-1 (43). Ezh2 controls gene repression through recruitment of histone deacetylases followed by chromatin deacetylation and methylation of histone H3 at residue lysine 27 (44). On the other hand, Bmi-1 is recruited to methylated histone H3 lysine 27 and has a role in maintenance of the epigenetic memory (45,46). We have recently reported that Ezh2 prevents hematopoietic stem cell senescence which also links chromatin remodeling to aging (47). Bmi-1 has been implicated in the maintenance of hematopoietic cells. Loss of function analysis reveals defects in HSCs renewal (48) and enforced expression leads to ex-vivo expansion of mouse HSCs (49). Bmi-1 regulates senescence and cell proliferation through p19ARF and p16INK4A (50). Genome-wide analysis of PcG complexes in Drosophila, mouse and human embryonic stem cells revealed that they repress developmental regulators, including the Hox gene cluster, and that their target genes are predominantly regulatory genes that control major differentiation pathways (51-54). Mounting evidence suggests that Hox proteins fulfill a critical role in hematopoiesis. HoxB4-defficient mice exhibit mild proliferation defects in HSCs and have normal hematopoiesis (55). Retroviral overexpression of HoxB4 results in increased proliferation of murine stem cells. However overexpression of HoxB4 in human CD34+ cells was reported to either increase proliferation (56) or to promote myeloid differentiation (57). Molecular mechanism and target genes responsible for the HoxB4 induced expansion remain to be elucidated. HoxA9, HoxA7 and Meis1 are expressed in CD34+ cells and are downregulated upon differentiation (58). Disruption of HOXA9 in mice leads to decreased numbers of progenitors and competitive defects of HSCs (59). On the other hand, enforced expression of HoxA9 promoted proliferative expansion of both HSCs and progenitor cells (60).

LEUKEMIC STEM CELLS AND THEIR MICROENVIRONMENT

Similarities and differences between HSCs and LSCs

Whether leukemic stem cells also depend on the niche for self-renewal is currently unclear. Although there are clear differences between normal and

leukemic stem cells, there are also striking similarities. A graphical presenta-tion of the leukemic stem cell niche and some of the important regulatory molecules is shown in Figure 3 and will be discussed in this section of the re-view. Most of the available data suggests that leukemia is a stem cell disease, in which the original stem cell self-renewal mechanisms are preserved but in which the tight control is lost due to transformation events. This appears to be true for most of the leukemic 'hits' that have been identified, apart from a few exceptions including PML-RARα (61), MLL-ENL (62) and MOZ-TIF2 (63). While these last examples are capable of imposing self-renewal characteris-tics on committed progenitors, the majority of genetic defects is observed or need to be experimentally introduced in primitive HSCs in order to create a transplantable leukemia. Thus, it seems reasonable to assume that many mo-lecular mechanisms that enable self-renewal are shared amongst normal and leukemic stem cells. Indeed, both normal as well as leukemic HSCs depend on SDF-1-mediated CXCR4 signaling for homing and mobilization (64). Wnt-in-duced β-catenin signaling has been implicated in the maintenance and expan-sion of murine HSCs (20,21), while inhibition of β-Catenin by overexpression of Axin severely impaired the self-renewal capacity of progenitors in CML (65). The adhesion of normal CD34+ stem/progenitor cells to bone marrow stroma and fibronectin is mediated by the integrins Vla-4 and Vla-5 (66-68), and a similar role is fulfilled by integrins in leukemic cells (69-71). Similar to mouse studies, we have shown that Rac is also required for the interaction of human HSCs with bone marrow stroma and their self-renewal. Moreover, leukemic stem cell self-renewal and expansion on MS5 stroma also depends on Rac activity (manuscript submitted). Thus, many of the molecules that mediate the interaction between stem cells and the bone marrow niche are utilized by both normal as well as leukemic HSCs.

Yet, important differences exist as well. At diagnosis, most of the leukemic cells will be found in the BM or the blood, like the normal HSCs, and only in rare cases in non-hematopoietic organs. It is plausible that LSCs spread through-out the bone marrow and metastasize other sites there. Thus, they are able to escape the growth-inhibiting signals that are provided by the primary niche that maintain stem cell quiescence and prevent unlimited proliferation or self-renewal of normal HSCs. Recently has been shown that stem cells can home to "vascular niches" (28) and one can speculate that exactly these nich-es can serve as a "home" to the metastatic stem cells. Clearly, elucidating the localization of LSCs in the bone marrow and the identification of mechanisms

Figure 3. Signal transduction pathways in the leukemic stem cell niche

A graphical representation of signaling pathways involved in the leukemic stem cell fate in the niche. Whether leukemic stem cells are also depending on the niche for self-renewal is currently unclear. There are certain similarities between normal and leukemic stem cells, as well as striking differences. It seems reasonable to assume that many molecular mechanisms that allow self-renewal are shared amongst normal and leukemic stem cells. The differences in the signaling are most likely initiated by chromosomal translocations that are frequently observed in leukemias, such as Bcr-Abl and Aml1-Eto, or mutations in Ras or tyrosine kinase receptors such as Flt3 and c-Kit.

by which LSCs escape the self-renewal cues of normal HSCs might provide a therapeutic window in which the leukemic stem cell can specifically be targeted. How leukemic stem cells might differ from normal HSCs in their interactions with the niche is discussed below.

Interaction of the leukemic stem cell with its niche

The dissimilarities between normal and leukemic cells that potentially allow us to manipulate and specifically eliminate the leukemia most likely find their origin in differences in signal transduction. These differences in signaling routes are in most cases initiated by chromosomal translocations that are frequently observed in leukemias, such as Bcr-Abl and Aml1-Eto, or mutations in Ras or tyrosine kinase receptors such as Flt3 and c-Kit (Fig.3). As a conse-

quence of these genetic lesions often a constitutive downstream signaling is initiated. For example, it has been observed that NF-kB is highly activated in leukemic cells but not in hematopoietic stem cells (72), which is frequently mediated by the Ras pathway (73). The anti-apoptotic effects of the Bcr-Abl oncogene can also be mediated via activation of NF-kB (74). Consequently, NF-kB inhibition can be a possible selective apoptotic therapy for patients with acute and chronic leukemias ((72,75) and PNAS 2005). Several parallel signaling pathways are activated by Bcr-Abl, including the Ras, PI3-K/Akt, c-Myc, and Stat5 pathways (76,77). Also, Bcr-Abl is associated with f-actin and a variety of cytoskeletal proteins, particularly with focal-adhesion associated adaptor proteins including Paxillin, Fak and Vinculin (78). Rho GTPases including Rac1 are activated by Bcr-Abl and several reports have indicated that cells expressing Bcr-Abl have an increased potential to associate with bone marrow stromal cells or ECM proteins such as fibronectin (79,80). Moreover, it was demonstrated that Bcr-Abl-mediated leukemogenesis requires the activity of Rac as homing to the bone marrow was impaired by expression of dominant negative RacN17, while the survival of mice transplanted with Bcr-Abl and RacN17 double transduced cells was improved (79). By retroviral introduction of Bcr-Abl into human CD34+ cells we have observed that the interaction with bone marrow stromal cells is strongly increased which was associated with increased clonogenic activity (unpublished observations), similarly as described by others (81,82). These data may imply that leukemic stem cell self-renewal driven by Bcr-Abl involves an enhanced interaction with the bone marrow microenvironment, but further studies are required to substantiate this hypothesis, particularly since data from primary human CML progenitor cells suggested that adhesion to stroma and extracellular matrix was reduced due to the expression of Bcr-Abl (83). Furthermore, Stat5 signaling has been implicated downstream of Bcr-Abl in leukemic transformation (84). More recently it was demonstrated that Stat5 is required for the efficient induction and maintenance of Bcr-Abl-induced CML in mice (85).

Other arguments for an improved leukemic stem cell interaction with the niche coupled to elevated self-renewal properties came from studies in which Flt3-Internal Tandem Duplications (Flt3-ITDs) or activating mutants of Stat5A were overexpressed in human cord blood-derived CD34+ cells. A constitutive activation of Stat5 is observed in the majority of AML cases, quite often due to Flt3-ITDs that are one of the most frequent mutations found in AML patients (86). Enforced activation of Stat5A in human CD34+ cells resulted

in elevated self-renewal which was associated with an improved interaction with the microenvironment (87). Similar observations were made using murine HSCs (88). Also, expression of Flt3-ITDs in human CD34+ cells resulted in elevated clonogenic activity and CAFC frequencies (89), which depends at least in part on the activity of STAT5 (unpublished observations). Stat5 signaling has also been implicated in various other chromosomal translocations that are observed in AML, including those resulting from fusions of the Fgf receptor 1 with Bcr and the zinc finger gene Znf198 (90-92). Particularly, cellular transformation of Znf198-FgfR1 fusions of BaF3 cells depends on STAT5 activity (93) (Fig.3).

Recent understandings of leukemogenesis imply that at least two mutations complement each other to exert the AML phenotype (reviewed in (94)). Typically, one mutation impairs the hematopoietic differentiation while the other promotes proliferation and/or survival. As examples, such "collaborative" hits have been reported between activated Flt3 mutations and PML-RARα (95), MLL-SEPT6 (96), AML-ETO (97), and most recently NUP98-HOXD13 and NUP98-HOXA10 fusions (98). In addition it has been recently shown that NUP98-HOXA9 cooperates with BCR/ABL in mouse models of CML blast crisis (99,100). Whether the leukemic cell needs collaborative hits to improve its interactions with the extrinsic stem cell niche remains an open question. One possibility is that LSCs have a much more active migration machinery as compared to normal HSCs that allows them to escape growth inhibition or quiescence promoting signals induced by osteoblasts and stromal cells in the niche. A number of membrane-associated ligands are normally present in the niche, and it has been shown that they can be cleaved by metalloproteinases. The expression levels of MMPs such as MMP9 are often elevated in AML blasts (101). It is tempting to speculate that these metalloproteinases might increase soluble concentrations of e.g. KL to enable leukemic self-renewal or expansion outside the niche. Furthermore, epigenetic modulation of the leukemic stem cell by promoter methylation or histone modifications might result in a block in differentiation and elevated self-renewal, even in the absence of a microenvironment. Such examples could include Bmi1, which contains methyltransferase activity and has been shown to be involved in the regulation of self-renewal of HSCs (48,49,102), possibly by alleviating HSC senescence by downmodulation of p16INK4A and p19ARF (50). Also, the PcG protein Ezh2 can directly methylate DNA (103) and prevents stem cell exhaustion (47). Whether and how chromosomal translocation products

can affect the epigenetic state of the LSC is currently unclear, but it is striking that e.g. CB CD34$^+$ expressing AML1-ETO proliferate in liquid cultures in the absence of stroma for over 7 months while maintaining self-renewal of an immature population of cells that retains lymphoid and myeloid potential, suggesting that AML1-ETO affects the leukemic (stem) cell self-renewal in a microenvironment independent manner (104).

CONCLUDING REMARKS

In conclusion, while a number of similarities exist between normal and leukemic stem cells in terms of molecular mechanisms that regulate self-renewal and interactions with the bone marrow microenvironment, clear differences exist as well. Future studies will be focused on purification procedures in order to be able to isolate LSCs to better homogeneity, on the localization of LSCs within the bone marrow compartment, and on the differences between molecular mechanisms that LSCs utilize for self-renewal and niche interactions. These differences could be used as guidance for further studies to determine whether therapeutic windows exist in which small molecule inhibitors can specifically target the leukemic stem cell in a clinical setting.

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LONG-TERM MAINTENANCE OF HUMAN HEMATOPOIETIC STEM/PROGENITOR CELLS BY EXPRESSION OF BMI1

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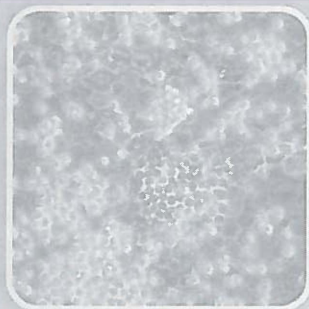
ABSTRACT

The polycomb group (PcG) gene BMI1 has been identified as one of the key epigenetic regulators of cell fates during different stages of development in multiple murine tissues. In a clinically relevant model, we demonstrate that enforced expression of BMI1 in cord blood CD34$^+$ cells results in long-term maintenance and self-renewal of human hematopoietic stem and progenitor cells. LTC-IC frequencies were increased upon stable expression of BMI1 and BMI1-transduced cells engrafted more efficiently in NOD-SCID mice. Week 5 CAFCs from stromal cocultures could be serially replated to give rise to secondary CAFCs. Serial transplantation studies in mice revealed that secondary NOD-SCID engraftment was only achieved with cells overexpressing BMI1. Importantly, BMI1-transduced cells could proliferate in stroma-free cytokine-dependent cultures for over 20 weeks while a stable population of about 1-5% of CD34$^+$ progenitor cells was preserved that retained the capacity to form CFCs. While control cells lost most of their NOD-SCID engraftment potential after 10 days of *ex vivo* culturing in absence of stroma, NOD-SCID multilineage engraftment was retained by overexpression of BMI1. Thus, our data indicate that self-renewal of human hematopoietic stem cells is enhanced by BMI1 and we classify BMI1 as an intrinsic regulator of human stem/progenitor cell self-renewal.

INTRODUCTION

Cellular memory induced by chromatin modifications can be maintained through subsequent cell divisions by the opposing effects of transcriptional activators of Trithorax (Trx) proteins and repressors of the polycomb proteins (PcG)[1]. Two functionally different PcG complexes have been identified. Polycomb repressive complex (PRC) 2, also termed as the "initiation" complex, consists of several subunits, including EED, SU(Z)12 and EZH2, and functions as a histone methyltransferase that specifically trimethylates Histone 3 on lysine residue 27 (H3K27) resulting in compaction of the chromatin and subsequent gene silencing[2]. PRC1 which is referred to as "maintenance" complex consists of various subunits including BMI1, RING1A/B, CBX, MEL18 and MPH/RAE28[2]. This complex does not possess methyltransferase activity itself, and the only observed enzymatic activity is ubiquitinylation of lysine 119 of H2A via its RING subdomains[3]. However, it has recently been demonstrated that DNA methyltransferase (Dnmt1) is required for proper assembly of PRC1 complex and that it can associate with BMI1 via Dmap1 and thus regulate DNA and histone methylation[4;5]. These observations suggest that not only PRC2, but also PRC1 might affect the epigenetic state of cells via methylation in a more direct fashion.

Polycomb target genes were mapped in genome-wide screens in human embryonic fibroblasts[6], murine embryonic stem cells[7;8] and Drosophila melanogaster[9;10]. In embryonic fibroblasts PRC1, PRC2 and H3K27-3me co-occupied genes that tend to be involved in embryonic development and cell fate decisions[6]. In ES cells, SU(Z)12, EED and H3K27-3me co-occupied genes that appear to regulate early developmental stages of neurogenesis, hematopoiesis and cell fate specification[7;8]. In Drosophila, PcG preferentially bind to developmental genes such as Wingless, Hedgehog and Notch[9;10]. In HSCs it has been demonstrated that PcG proteins are involved in the regulation of stem cell self-renewal. We have recently reported that EZH2 prevents murine stem cell exhaustion[11]. Others have reported that targeted deletion of BMI1 in murine HSCs impaired their competitive repopulation capacity while its overexpression enhanced symmetrical cell divisions of HSCs and consequently led to augmented HSC self-renewal[12-14]. In senescence screens it was observed that the p16INK4a/p19ARF locus is repressed by BMI1, which was required to bypass senescence of embryonic fibroblasts[15]. Targeted deletion of BMI1 in HSCs resulted in an increase in the expression of p16 and p19[16]. Deletion of p16 and p19 in *bmi1-/-* HSCs partially,

but not completely, restored the self-renewal. In contrast, overexpression of BMI1 could increase progenitor levels in absence of p16 and p19, indicating that other targets of BMI1 exist as well[13;16]. The expression of the PcG genes in mouse HSCs varies throughout the hematopoietic hierarchy. EZH2 and EED are more ubiquitously expressed[17], MEL18, CBX and MPH1/REA28 are expressed at rather low levels in HSCs but increase upon differentiation, and BMI1 is selectively expressed in HSCs[17;18]. This is suggestive for the notion that BMI1 can participate in the self-renewal and growth mechanisms at the earliest stages of hematopoiesis.

Although these data are intriguing, they are based on murine experimental systems and whether they reflect functional properties of the Polycomb group of genes in the human system is unclear. Several genes that have been shown to induce self-renewal in mouse HSCs have no or very modest effects in human stem cells. Treatment of cells with WNT3a protein or overexpression of modified β-catenin can expand mouse cells more than 100-fold, yet no significant effects on human HSCs have been observed[19;20]. *Ex vivo* expansion and self-renewal of murine long-term repopulating HSCs was observed when the *hoxb4* gene was overexpressed in murine cells or when the TAT-HOXB4 fusion protein was developed[21;22]. However, *ex vivo* and *in vivo* expansion of human CB CD34+ cells by HOXB4 overexpression or direct delivery of HOXB4 had remarkably lower effects compared to the murine cells[23-25].

In a clinically relevant model we wished to assess whether BMI1 could induce expansion of human cord blood cells. We stably introduced BMI1 in stem and progenitor cells derived from umbilical cord blood and we assayed the biologic effects on the hematopoiesis *ex vivo* and *in vivo*. We show that constitutive expression of BMI1 in human cord blood cells results in prolonged maintenance of the stem cell pool and enhances self-renewal of human stem and progenitor cells.

MATERIALS AND METHODS

Primary cell isolation
Neonatal cord blood was obtained after informed consent from healthy full-term pregnancies from the obstetrics departments of the Sophia Hospital in Zwolle, the University Medical Center in Groningen and the Martini

Hospital, Groningen. After ficoll separation of mononuclear cells, CD34+ cells were enriched using magnetically activated cell sorting (MACS) CD34 progenitor kit (Miltenyi Biotech, Nijmegen, The Netherlands). The purity of the CB CD34+ cell ranged between 90-95%, as assessed by flowcytometry.

Cell lines and ex-vivo culture of primary cells

293T human embryonic kidney cells and PG13 cells were grown in DME medium (BioWhittaker, Veriers, Belguim) supplemented with 10% FCS (Sigma, Zwijndrecht, The Netherlands), 200mM glutamine, and penicillin and streptomycin (all from Sigma). MS-5 murine stromal cells were grown in αMEM (BioWhittaker) supplemented with 10% FCS, 200mM glutamine, and penicillin and streptomycin. For the MS-5 co-culture experiments and long-term culture-initiating (LTC-IC) assays cells were grown in 12.5% heat-inactivated FCS, 12.5% heat-inactivated horse serum (Sigma), penicillin and streptomycin, 200mM glutamine, 57.2µM β-mercaptoethanol and 1µM hydrocortisone (Sigma). The stroma-independent culture assays were performed in IMD medium (PAA Laboratories, Pasching, Austria) supplemented with 20% FCS, 200mM glutamine, penicillin and streptomycin, and the following cytokines: SCF (100ng/ml), Flt3 Ligand (100ng/ml; both from Amgen, Thousand Oaks, USA), TPO (100ng/ml; Kirin, Tokyo, Japan), IL3 (10ng/ml) and IL6 (10ng/ml; both from Gist-Brocades, Delft, the Netherlands)

Immunoblotting and cytospins

$5x10^5$ cells were lysed to prepare whole cell extracts. Western blot analysis was performed by standard protocols and as previously described[29]. Antibody against BMI1 (Upstate, CA, USA) was used in a 1:1000 dilution and anti-GFP antibody (Santa Cruz, Heerhugowaard, The Netherlands) was used in a 1:300 dilution. May-Grünwald Giemsa staining was used to analyze cytospins. Pictures were taken on Olympus BX50 microscope (Olympus Nedelrand BV, Zoeterwoude, the Netherlands) with 40x/0.60 objective.

RNA extraction and real time PCR analysis

Total RNA was isolated from $1x10^5$ cell using RNeasy kit from Qiagen (Venlo, the Netherlands) and was reverse transcribed using M-MuLV reverse transcriptase (Fermentas, St Leon-Roth, Germany) according to the manufacturer's instructions. Aliquots of cDNA were then real-time amplified

using iQ SYBR Green mix (Bio-Rad, CA, USA) on a MyIQ thermocycler (Bio-Rad) and quantified using MyIQ software (Bio-Rad). HPRT expression was used to calculate relative expression levels. Sequences and conditions are available on request.

Retroviral vector construct and transduction of cord blood cells

For all transduction experiments, the MiGR1 retroviral expression vector was used which contained murine stem cell virus (MSCV) long-terminal repeats (LTRs) and an internal ribosomal entry site (IRES2) upstream of the enhanced green fluorescent protein (EGFP) as marker gene. The BMI1 gene was cloned from cDNA of cord blood CD34$^+$ cells and a hemagglutinin tag was added to the N-terminus using the following primers: forward 5'-ATGTACCCATACGATGTTCCAGATTACGC TCATCGAACAACGAGAATCAA-3' and reverse 5'-TCAACCAGAAGAAGT TGCTGATG-3'. The gene was cloned into the pCR4 cloning vector (Invitrogen, CA, USA), cut using XhoI-MunI sites and subcloned into XhoI-EcoRI sites of MiGR1. The construct was verified by sequencing.

Stable PG13 virus producer cell lines were generated by first transiently transfecting 293T cells with 2μg MiGR1 or MiGR1-BMI1 plasmid DNA and 2μg pCL-Eco ecotropic packaging plasmid using Fugene 6 (Roche Diagnastics, Mannheim, Germany). Supernatants from the 293T cells were used to transduce PG13 cells in the presence of 8 μg polybrene (Sigma). Retroviral supernatants from the PG13 cells were used to transduce cord blood CD34$^+$ cells pre-stimulated for 48-72h in serum-free HPG medium (Lonza, Walkerswille, MD, USA) supplemented with 100ng/ml SCF, TPO and Flt3L. Three consecutive transduction rounds of 8-12 hours were performed on retronectin-coated plates (Takara, Tokyo, Japan) prior the start of different assays.

Colony-forming Cell (CFC), secondary CFC, LTC-IC and secondary Cobblestone Area-Forming Cell (CAFC) assay

CFC and LTC-IC assays on MS-5 stromal cells were performed as previously described[29]. For the CFC assays, 1000 GFP$^+$-sorted cells were plated directly after transduction and 10.000 GFP$^+$ cells were used at later time points. For the colony re-plating experiments, two weeks after the primary plating, the colonies from one plate were collected, washed three times with PBS, and the cells were plated in new methylcellulose for an additional two weeks. For the LTC-IC assays, transduced GFP$^+$ cells were sorted on MS5 stromal cells

in limiting dilutions from 10 to 810 cells per well in 96-well plates. Cultures were weekly demidepopulated and fed with new medium. After 5 weeks of culture the wells containing cobblestone areas were scored after which the medium from the wells was aspirated and replaced with methylcellulose containing cytokines. After an additional two weeks of culture wells were scored as positive or negative to yield the LTC-IC frequency. For the secondary CAFC assay, day 35 cells were harvested by trypsinization of the adherent cell population, replated on new MS5 stromal cells and maintained for additional 5 weeks.

Primary and secondary transplantations into NOD-SCID mice

Eight to ten weeks old female NOD/SCID mice (NOD.CB17-Prkdcscid/J) were purchased from Charles River Laboratory-Netherlands and were maintained in specific pathogen free conditions. Prior to transplantations mice were sublethally irradiated with 3Gy. One group of mice was injected with $6x10^5$ non-sorted CD34$^+$ cells into the retro-orbital vein immediately after transduction and 6 weeks later the bone marrow cells from the recipients was used to assess the presence of GFP$^+$ human CD45$^+$ cells. Another cohort of mice received $3.8x10^6$ cells cultured for 7 days after transduction in presence of: SCF (100ng/ml), TPO (100ng/ml), Flt3 Ligand (100ng/ml), IL3 (10ng/ml) and IL6 (10ng/ml). Bone marrow cells from the NOD/SCID mice were used to flow cytometrically analyze human cell engraftment after 8 weeks of transplantation. A third group of mice was injected with $5.1x10^5$ CD34$^+$ transduced cells, analyzed for presence of human GFP$^+$ cells 8 weeks later and BM from an individual chimeric primary recipient was injected into an individual secondary NOD/SCID recipient without re-purification of human cells. Prior to the secondary transplantations mice were treated with single intraperitoneal injection (200µg/mouse) of anti-CD122 TM-β1 (BD Bioscience, PharMingen) within 4 hours after 3Gy of total body irradiation in order to eliminate residual murine natural killer cell activity. Mouse BM was analyzed for the presence of GFP$^+$ CD45$^+$ cells 8 weeks after transplantation.

Flow cytometry analysis and sorting procedures

All antibodies were obtained from Becton Dickinson (Alphen /d Rijn, the Netherlands). Staining of the cells was performed for 45 minutes at 4°C. Mouse bone marrow cells were blocked with anti-Fcγ antibody for 15 minutes at 4°C to avoid non-specific binding. Sorting of the cord blood cells into stem and progenitor fractions was performed on the basis of the

combinatorial expression of cell surface antigens as previously reported[30]. HSC were defined as CD34+38-, common myeloid progenitors (CMP) as CD34+38+IL3Rα+CD45RA-, megakaryocyte-erythroid progenitors (MEP) as CD34+38+IL3Rα-CD45RA- and granylocyte-macrophage progenitors (GM) as CD34+38+IL3Rα+CD45RA+. The fluorescence activated cell sorting analyses were performed on a FACS Calibur (Becton Dickinson) and sorting of the cells was performed on MoFlo (Dako Cytomation, Carpinteria, CA, USA). Data were analyzed using WinList 3D (Topsham, ME, USA) and FlowJo (Tri Star, Inc, Ashland, OR, USA) software.

RESULTS

Retroviral introduction of BMI1 in CD34+ cells

In order to determine the normal expression of BMI1 in immature human hematopoietic cells we sorted cord blood (CB) cell into different progenitor and stem cell fractions (Fig.1A). We observed that BMI1 is highly expressed in the CD34+38- fraction (HSCs) as compared to the committed progenitor populations (Fig. 1B). There was no significant difference in the expression levels of BMI1 in the different sorts for CMP, MEP or GM progenitors. We next designed control MiGR1 or BMI1 retroviral vectors (Fig. 1C) which were used to transduce CB CD34+ cells (hereafter referred to as MiGR1 or BMI1-expressing cells respectively). Transduction efficiencies were determined by FACS analysis on the basis of the GFP expression in the cells and ranged from 50-65% in the MiGR1 and 45-70% in BMI1-transduced cells (Fig. 1D). Expression of BMI1 protein was confirmed by Western blotting in the PG-13 stable virus producers and in the transduced cord blood CD34+ cells (Fig. 1E). Cord blood cells express endogenous BMI1 but upon retroviral transduction with BMI1 vector an approximately 4-fold increase in expression was observed. These data were further confirmed by qPCR analysis (Fig. 1F). Using qPCR analysis, we further checked whether overexpression of BMI1 affected expression of known downstream target genes and we indeed observed that p16 and p19 were downmodulated (Fig. 1F).
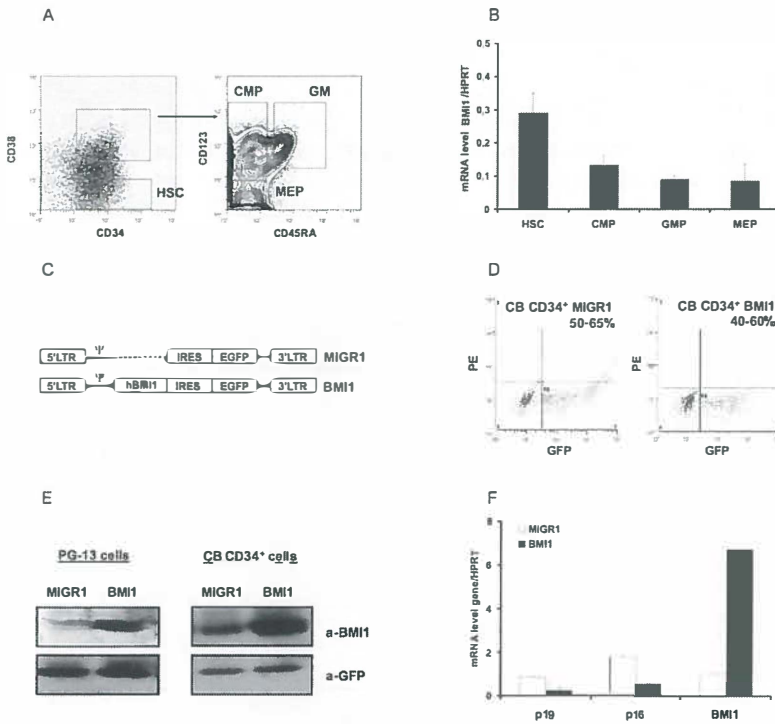
Figure 1. Retroviral introduction of BMI1 in human cord blood (CB) CD34+ cells. (A) Sorting strategies of non-transduced CB stem and progenitor fractions (B) mRNA expression of BMI1 in human CB cells. Cells analyzed are: HSCs (CD34+CD38), CMPs (CD34+38+IL3Rα+CD45RA), MEPs (CD34+38+IL3Rα CD4SRA), GMs (CD34+38+IL3Rα+CD4SRA+). (C) Schematic representation of the MiGR1 (control) and BMI1 retroviral vectors used in this study. (D) CB CD34+ cells were pre-stimulated for 48 hours in HPGM supplemented with KL, Flt3L and TPO followed by three transduction rounds in the next 48h with MiGR1 or BMI1 retroviruses and transduction efficiencies were determined on the basis of GFP expression by FACS. (E) Western blot analysis of total cell extracts from PG13 virus producer cells or CB CD34+ transduced cells. Membranes were probed with anti-BMI1 or anti-GFP antibodies. (F) q-RT-PCR analysis of transduced cells for known downstream target genes of BMI1.

Long-term maintenance of human HSCs and progenitor cells by expression of BMI1

Transduced control MIGR1 or BMI1 cells were GFP-sorted and plated in cytokine-driven stroma-free cultures for weekly analysis. In Fig. 2A weekly cell counts of demidepopulated cultures are shown. While MiGR1 cells typically expanded transiently over a 3-5 week period, BMI1 cells maintained to proliferate over 20 weeks, resulting in a strong expansion of >2x10⁶ fold, shown in Fig. 2B as cumulative cell expansion. Even after 16 weeks some of the cells retained a primitive morphology resembling blast-like cells (Fig. 2C), while the control cells became terminally differentiated myeloid cells at earlier time points (data not shown). Phenotypical FACS analysis
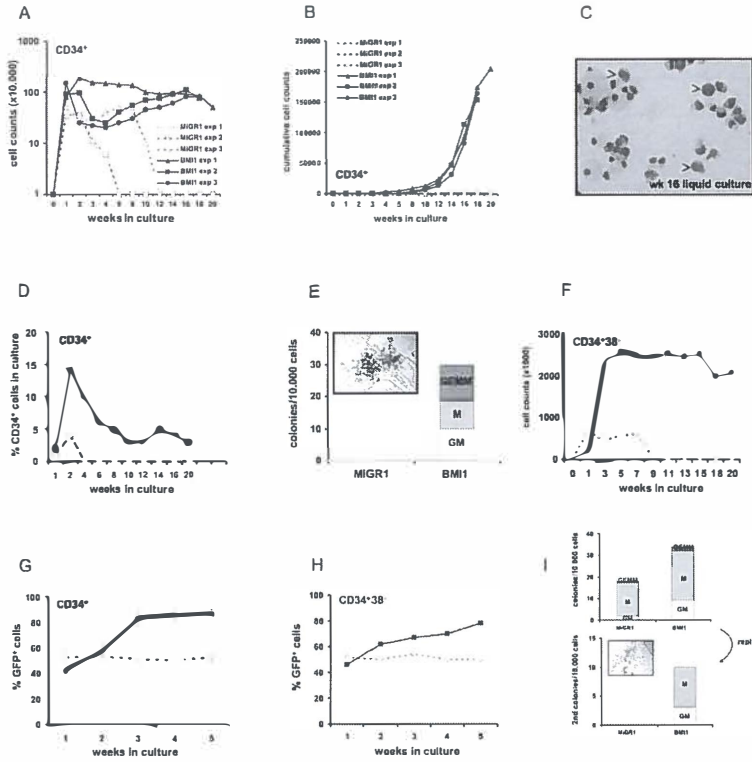
Figure 2. BMI1 promotes long-term in vitro expansion of human CD34⁺ progenitor cells. (A) Cord blood CD34⁺ cells were transduced with either control (open symbols) or BMI1 (closed symbols) vectors and grown in stroma-free liquid cultures using a mix of cytokines as described in the Material and Methods section. Cells were counted weekly and three independent experiments are shown. Week 0 represents the number of transduced cells that were plated. (B) Cumulative expansion of the same cultures is shown. (C) The May-Grünwald Giemsa stained picture shows a cytospin of 16-week cultured cells where monocytes, macrophages, granulocytes and some blasts-like cells can be observed, indicated by the arrows. (D) A representative experiment where the percentage of CD34⁺ cells was maintained at around 4% over a period of 20 weeks in BMI1 overexpressing cells is shown. (E) After 16 weeks cultures were analyzed for progenitor content in CFC assays in methylcellulose by plating 10.000 cells from the culture without further re-sorting. Progenitors were only detected in BMI1-expressing cells. A CFU-GEMM colony from a representative CFC experiment is shown in the inset. (F) Transduced and GFP⁺ sorted CB CD34⁺38⁻ cells were propagated in the same cytokine-driven liquid culture conditions as in (A) for 20 weeks. (G) After transduction unsorted CD34⁺ cells were grown in co-cultures on MS5 stromal cells. The cultures were weekly demidepopulated and analyzed for GFP expression by FACS. (H) Experiment as in (G), but now transduced CD34⁺CD38⁻ cells were plated on MS5 stroma. (I) Non-adherent cells from week 5 co-cultures were used to perform CFC assays (top panel). After 2 weeks, CFCs were harvested and replated into new methylcellulose assays (bottom panel). Only the BMI1-expressing cells contained re-plating capacity in 2ⁿᵈ methylcellulose CFC assays and a colony is shown in the inset. A representative experiment out of 4 performed experiments is shown.

revealed that 1-5% of the cultured BMI1 cells remained CD34⁺ over a period of 20 weeks (Fig. 2D), whereas the other differentiation markers for myeloid (CD11, CD14, CD15) and erythroid (CD71 and GPA) lineages did not reveal any differences between the two groups (data not shown). We used the cells from the culture (without further re-sorting) to perform CFC assays weekly and as

depicted in Fig. 2E overexpression of BMI1 resulted in colony formation even after 16 weeks of culture, whereas the MiGR1 cells lost their capacity to form colonies between 3-5 weeks (data not shown). Remarkably, one third of the BMI1 colonies at this time point were CFU-GEMMs (inset in Fig. 2E), which represent the most primitive type of progenitors, arguing that immature progenitors have been maintained for 16 weeks. We further wanted to test whether BMI1 would have the same effect on the more primitive cells and we sorted CD34$^+$38$^-$ cells and analyzed their growth in cytokine-driven liquid cultures as described above. BMI1 cells were again maintained to proliferate up to 20 weeks, while the MiGR1 cells were exhausted after 7-9 weeks (Fig. 2F).

Thus, we can conclude that introduction of BMI1 in human CD34$^+$ and CD34$^+$38$^-$ cells allows their continued and extensive proliferation *in vitro* in cytokine-driven stroma-free cultures conditions for up over 20 weeks while retaining their progenitor activity throughout this long-term culture period.

BMI1 provides a proliferative advantage of cord blood cells on stroma
To further asses the effects of BMI1 overexpression on the self-renewal and differentiation potential of HSCs, transduced CB CD34$^+$ as well as the most primitive CD34$^+$38$^-$ cells were cultured on MS5 murine stromal cells, and co-cultures were demidepopulated weekly for analysis. Plating of non-sorted cells on MS5 allowed analysis of relative contributions of GFP$^+$ and GFP$^-$ cells to the culture. As indicated in Fig. 2G, overexpression of BMI1 provided a proliferative advantage of the cells as the GFP$^+$ population of the nonadherent cells in the CD34$^+$ cultures increased from 41% to 80%, whereas the MiGR1 population remained constant between 50-55%. The same trend was observed in the cultures when CD34$^+$38$^-$ cells were used (Fig. 2H). Phenotypical FACS analysis revealed marginal differences of the differentiation markers between the two groups, while no morphological differences were observed in the cytospin preparations (data not shown). We have not propagated BMI1 cells on MS5 until week 20 yet to determine whether blast-like CD34$^+$ cells persist under these condition as we have observed in our cytokine-driven liquid-culture conditions. The number of progenitors in the MS5 cocultures was evaluated in colony assays. Transduced CB CD34$^+$ cells were either plated directly for CFC assays or after expansion and weekly demidepopulation (weeks 1-5) on MS5 cells. As depicted in the top panel of Fig. 2I where non-adherent cells from the week 5 co-culture were used, overexpression of BMI1 resulted in production

of more CFUs than control MiGR1 cells (36±9 vs. 19±4 colonies per 10000 cultured cells, respectively). Similar results were obtained using non-adherent suspension cells from weeks 1-4 (data not shown). Two weeks after the primary plating of week 5 cells, the colonies were collected and the cells were plated in new methylcellulose for an additional two weeks to address self-renewal properties of progenitors. While MiGR1 control cells did not give rise to any secondary colonies, BMl1 overexpressing cells did contain replating capacity (Fig. 2I, bottom panel).

Taken together these data indicate that BMl1 provides a proliferative advantage of human CD34$^+$ and CD34$^+$38$^-$ cells in stromal co-cultures with increased numbers of progenitors that contain self-renewal properties.

BMl1 promotes self-renewal of progenitors

In the co-culture studies, we noted that the colony forming units from the BMl1 expressing cells have replating capacity. However, in these experiments bulk CD34$^+$ transduced cells were used. To further discriminate which fraction of the cells may be responsible for these effects, CB CD34$^+$ cells were sorted 48h after the start of the transduction period on the basis of cell surface marker expression into HSCs, CMPs, MEPs and GMs as described in Figure 1A. The cells were plated in methylcellulose and colonies were scored on the basis of their morphology. Figure 3A demonstrates that the sorting procedure yielded relatively pure populations in the MEP and GM fractions in MiGR1 control cells, while, as expected, HSC and CMP population
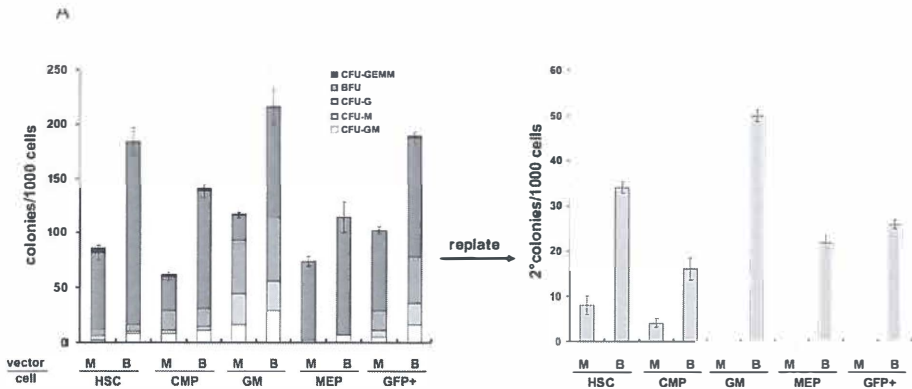

Figure 3. BMl1 promotes self-renewal of progenitors. (A) After transduction of CB CD34$^+$ cells with either control or BMl1 vectors, the cells were sorted into HSC, CMP, MEP and GM fraction and analyzed for CFC content. Two weeks after the primary plating, colonies were scored on the basis of morphology as CFU-Mix, BFU-E, CFU-G, CFU-M or CFU-GM, and secondary re-plating was performed in new methylcellulose assays. The cells were cultured for additional two weeks and colonies were scored.

contained both erythroid and myeloid progenitors. Notably, regardless of the cell subset, in all of the groups where BMI1 was overexpressed, a higher plating efficiency was observed. Secondary replating was performed in new methylcellulose medium and the cells were cultured for an additional two weeks, after which colonies were scored again. Remarkably, overexpression of BMI1 in the stem and all progenitor cell subsets resulted in colony-forming units at a frequency of 22 to 50 colonies per 1000 initial cells. The control cells had replating capacity with a frequency of 4 to 7 colonies per 1000 initial cells, but only in the most primitive cell subsets of HSCs and CMPs. In conclusion, overexpression of BMI1 promotes self-renewal of both immature as well as more committed progenitor subtypes.

BMI1 overexpression results in enhanced stem cell frequencies and elevates their self-renewal potential

To address the effects of BMI1 on HSC frequency and self-renewal, we performed *in vitro* Long-Term Culture Initiating Cell (LTC-IC) assays and *in vivo* transplantation studies in NOD-SCID mice. CD34+ cells were transduced with either MiGR1 or BMI1 vectors, GFP sorted, and were plated in limiting dilutions in 96-well plates onto MS5 bone marrow stroma. Cobblestone area forming cells (CAFCs) were scored at week 5 and methylcellulose was added to the wells. After two additional weeks, the wells containing progenitors were scored as positive. These experiments revealed that the LTC-IC frequencies were 5.7-fold higher by overexpression of BMI1 (1/2077 and 1/361 in the control v.s. BMI1 respectively) (Fig. 4A). It has been previously reported that the LTC-IC frequency in non-manipulated CB CD34+ is 0.2-1/100[29;31-33]. In our experiments, during the manipulation i.e. transduction of the cells, the stem cell frequencies dropped ~3.5-fold in the BMI-1 and 20-fold in the MiGR1 group. These data suggest that the overexpression of BMI1 did not impose a net expansion of HSCs when compared to non-manipulated cells, but rather maintained a proportion of the manipulated cells in a primitive state. To further test the *in vivo* repopulating capacity of these cells, 6×10^5 CD34+ cells were injected directly after transduction into sublethally irradiated NOD-SCID recipients. In the mice transplanted with BMI1-expressing cells, 3.1-fold higher engraftment levels were achieved when compared to the control mice (Fig. 4B). Hence, BMI1 expression increases the number of cells with repopulating ability in NOD-SCID mice.

To test the effect of BMI1 expression on the most immature stem cell fraction, MiGR1 and BMI1-transduced cells were sorted into the immature
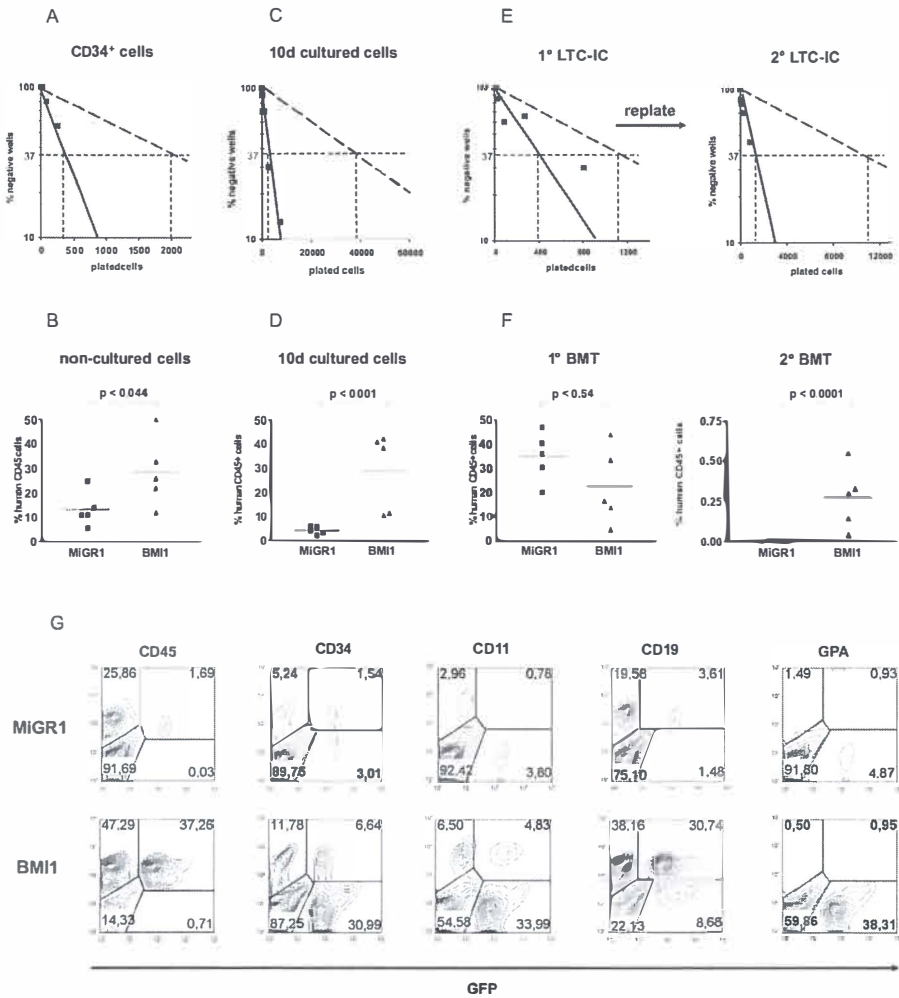
Figure 4. BMI1 overexpression results in enhanced stem cell frequencies and elevates their self-renewal potential. Cord blood CD34+ cells transduced with MiGR1 (open symbols) and BMI1 (closed symbols) were sorted in limiting dilutions and used to enumerate LTC-IC frequencies (A) or were injected into sublethally irradiated NOD-SCID mice (n=5 per group (B)). Human CD45+ chimerism levels were determined 6 weeks post transplantation in the bone marrow of the transplanted mice (B). (C) After transduction GFP+ CD34+38- cells were sorted, cultured for 10 days in stroma free cytokine-driven liquid culture conditions and after which LTC-IC frequencies were determined on MS5 bone marrow stroma in limiting dilution (D) MigR1 and BMI1-transduced CD34+ cells were cultured for 10 days in stroma-free conditions and 3.8x10⁶ cells were injected into sublethally irradiated NOD-SCID mice (n=5 per group). Eight weeks after transplantation human bone marrow engraftment was evaluated on the basis of human CD45+ expression. (E) Transduced and sorted cord blood CD34+38- cells were used to determine CAFC day 35 frequencies (left panel) and after 5 weeks the cultures were harvested and plated on new MS5 stroma to determine secondary LTC-IC frequencies (right panel). (F) Transduced CD34+ cells were used to perform transplantations into sublethally irradiated NOD-SCID mice (n=5 per group) and engraftment levels after 8 weeks were determined (left panel). The bone marrow from the primary recipients was used to perform secondary bone marrow transplants and chimerism levels after an additional 8 weeks are shown in the right panel. (G) Multilineage engraftment of a representative mice transplanted with 10 days cultured cells is shown.

CD34$^+$38$^-$ HSC population, and cells were cultured for 10 days in stroma-free cytokine-driven cultures. LTC-IC assays were performed using the cultured cells and frequencies were scored 7 weeks later. Whereas only few positive wells could be scored in the MiGR1 control group, indicating that few stem cells were maintained during the 10 day culture period, expression of BMI1 did result in stem cell maintenance as LTC-IC frequencies were significantly higher (1/41111 versus 1/2491 for MiGR1 and BMI1 cells, respectively) (Fig. 4C). These results tempted us to test whether constitutive expression of BMI1 could also maintain the most primitive stem cell fraction under prolonged *ex vivo* culture conditions as assessed in the *in vivo* NOD-SCID model. Lethally irradiated NOD-SCID recipients (n=5/group) were injected with 3.8x10^6 transduced 10-day *ex vivo* cultured cells as described above, and chimerism levels were analyzed in the bone marrow (BM) of recipient mice 8 weeks later (Fig. 4D). It was observed that engraftment efficiencies ranged between 2.17-5.68% in the control group while engraftment efficiencies in the BMI1 group were significantly higher, ranging between 10.61-42.36% (Fig. 4D). Further analysis revealed that the donor-derived BMI1 GFP$^+$ cells were CD34$^+$, CD11b$^+$, CD19$^+$ and only few GPA$^+$, indicating that multilineage engraftment was achieved with BMI1 *ex vivo* cultured cells (Fig. 4G and Table 1).

	CD 45	CD34	CD11	CD19	GPA
MiGR1 #1	2.7	1.03	0.46	3.63	0.54
MiGR1 #2	2.14	0.47	0.92	1.89	1.51
MiGR1 #3	5.33	2.07	2.07	5.17	2.31
MiGR1 #4	1.56	0.51	0.48	1.79	0.56
MiGR1 #5	1.69	1.54	0.78	3.61	0.93
BMI1 #1	11.67	1.65	0.92	9.88	0.55
BMI1 #2	46.22	6.55	5.84	37.33	1.45
BMI1 #3	47.14	10.36	6.01	35.3	1.03
BMI1 #4	13.23	2.18	1.26	10.01	0.58
BMI1 #5	37.26	6.64	4.83	30.74	0.95

Table 1. Percent of human chimerism in each lineage in mice transplanted with cells cultured for 10 day in stoma-free cytokine dependent conditions

Self-renewal of HSCs was addressed by the capacity of the transduced CD34+38--sorted cells to form secondary CAFCs in serial replating experiments. MiGR1 and BMI1 transduced cells were plated in LTC-IC assays on MS5 cells. Week 5 CAFCs were scored (Fig. 4E, left panel), harvested and replated onto new MS5 stroma. Cultures were maintained for another 5 weeks, after which methylcellulose was added and plates were scored after an additional 2 weeks of culture (Fig. 4E, right panel). While control MiGR1 cells had a very low replating frequency (1/11690), BMI1 overexpressing cells were capable of forming new CAFCs in the secondary cultures with much higher frequencies (1/1211), indicative for self-renewing capacity. Finally, a third cohort of sub-lethally irradiated NOD-SCID mice were transplanted with $5.1x10^5$ cells transduced with MiGR1 or BMI1. Eight weeks after primary transplantation, the mice were sacrificed and bone marrow was collected for analysis and secondary transplantation experiments. In this experiment both control as well as BMI1-transduced cells engrafted very efficiently without a significant difference in their engraftment levels (Fig. 4F left panel and Table 2). It appears that when initial engraftment levels are high, additional expression of BMI1 does not further increase the engraftment within a primary transplantation setting, although under more stringent conditions, such as secondary transplantation, BMI1-expressing cells did perform better than control mice. Chimerism of the secondary recipients was analyzed 8 weeks later when BMI1 engraftment levels ranged from 0.04-0.55% while no secondary engraftment was observed with MiGR1
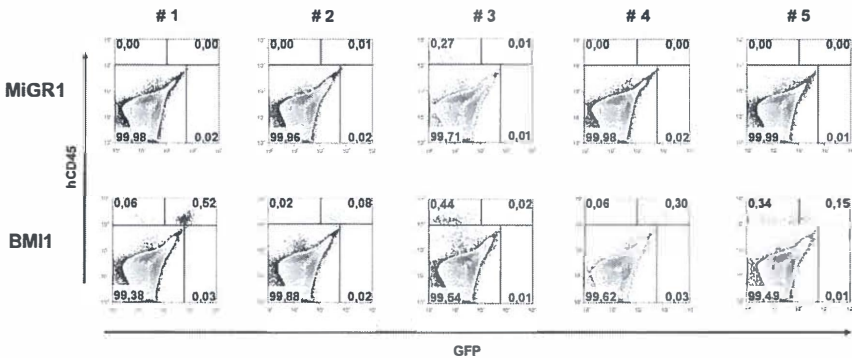
Supplementary Fig. 1 NOD-SCID mice were transplanted with $5.1x10^5$ cells transduced with MiGR1 or BMI1 and 8 weeks after primary transplantation the mice were sacrificed and bone marrow was collected in order to perform secondary transplantation experiments. Engraftment levels in 5 secondary recipients transplanted with BM from primary mice that were transduced with MiGR1 of BMI1 vectors is shown.

control cells (Fig. 4F, right panel and Supplementary Figure 1). Three of the five transplanted BMI1 mice showed multilineage engraftment (Table 2).

	CD45	CD11	CD19	GPA
BMI1 #1	0.56	0.06	0.61	0.04
BMI1 #2	0.14	0	0.1	0
BMI1 #3	0.04	0.01	0.01	0
BMI1 #4	0.33	0.1	0.26	0.03
BMI1 #5	0.3	0	0.3	0

Table 2. Percent of human chimerism in each lineage in secondary mice

In conclusion, these data indicate that overexpression of BMI1 promotes *ex-vivo* maintenance of human stem/progenitor cells and promotes their self-renewal.

BMI-1 prevents apoptosis and maintains quiescence of CD34+38- cells

As our *in vivo* and *in vitro* data suggested that BMI1 expression maintains stem cell characteristics of CB CD34+ cells, we wished to study proliferation and survival at the single cell level of the most immature CD34+38- population. After 48h of prestimulation, MoFlo-sorted CB CD34+CD38- cells were transduced with MiGR1 or BMI1 virus for another 48 hours. Following transduction, single cells were deposited in 96-well plates and cultured in stroma-free conditions in medium supplemented with a cocktail of cytokines. The wells were scored for the presence of living cells at different time points (Fig. 5). When a single-deposited cell was seen in the well after culture, it was referred to as a quiescent cell and when two or more cells were counted in the well they were considered to be proliferating cells. No differences were detected between MiGR1 and BMI1 groups within 24 hrs after single cell deposition (Fig. 5A). Interestingly, under such stringent culture conditions for stem cell growth where more than 75% of the control cells died after 5 days, BMI1 expressing cells were able to prevent apoptosis as ~50% of the cells remained alive after 5 and up to 14 days of culture (Fig.5 B, C). However, the most striking difference was the observation that in the BMI1 group around 30% of the wells contained a single cell that remained
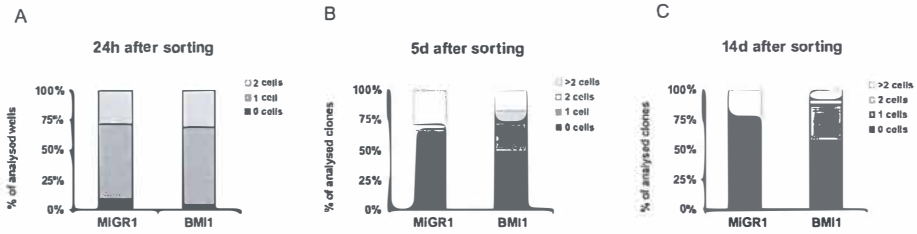
Figure 5. BMI1 prevent apoptosis and maintains quiescence of CD34⁺38⁻ cells.

After 48h of prestimulation, MoFlo-sorted CB CD34⁺CD38⁻ cells were transduced with MiGR1 or BMI1 virus for another 48 hours. Following transduction, single cells were deposited in 96-well plates and cultured in stroma-free conditions in IMDM supplemented with 20% FCS and KL, Flt3L, TPO, IL3, IL6 and EPO. Wells were evaluated for presence of alive and/or proliferating cells at 24 hours (A), 5 days (B) and 14 days (C) after sorting. The data shown is the average of three independent experiments where 210 individual clones were analyzed per group.

quiescent over time while the number of wells with proliferating cells was higher in the MiGR1 group. In conclusion, these data suggest that expression of BMI1 reduces apoptosis and increases survival of individual cells under stress conditions and maintains the CD34⁺38⁻ population quiescent.

DISCUSSION

For reasons unknown, several mouse stem cell genes play no, or only a marginal role in human HSCs. These include WNT3a[19;20], HOXB4[23-25] and FGF-1 (A.Rizo, unpublished data). These observations suggest that self-renewal in human cells maybe intrinsically different than in mouse cells. Loss-of-function and gain-of-function analyses in mouse models have implicated BMI1 as one of the key regulators of stem cell self-renewal. Severe hematopoietic defects attributed to impaired HSCs self-renewal are reported in *bmi*^{-/-} mice [12;13] while overexpression studies have demonstrated increased self-renewal of mouse HSCs[14]. However, to what extent BMI1 regulates human HSC self-renewal has remained elusive.

In this study, we show that persistent activation of BMI1 in human cord blood CD34⁺ cells results in long-term maintenance of hematopoietic stem and progenitor cells. An important component of the phenotypes observed by expression of BMI1 is the proliferative advantage over control MiGR1 cells in stroma-free conditions. In such *ex vivo* expansion cultures, normal cord blood CD34⁺ cells only transiently expand over a period of about 5 weeks after which cultures are exhausted and have lost the ability to give rise

to cells with colony-forming capacity. When BMl1 was overexpressed, the cultures could be maintained for over 20 weeks and retained colony forming capacity up to 16 weeks after culture. Interestingly, we observed in our single cell assays that the MiGR1 CD34$^+$CD38$^-$-transduced cells displayed a higher proliferation rate as compared to BMI1-expressing CD34$^+$CD38$^-$ cells, and thus it is conceivable that cells expressing low levels of BMl1 exhaust faster. Since there is a limited reservoir of quiescent cells that would potentially sustain the formation of progeny at later time points, the control cultures collapse over time. In contrast, a "controlled proliferation" occurs in the BMl1 cultures where the presence of quiescent cells that maintain their primitive phenotype over a period of time is a source of relatively slow-cycling cells that retain the long-term capacity to generate progeny. In line with these considerations, our data indicate that a small population of BMl1-transduced cells indeed continued to express CD34 antigen and retained clonogenic ability for over 16 weeks.

Cell-intrinsic properties are not the only determinants of stem cell fate. HSCs are tightly regulated by their microenvironment. In our study, we report a moderate *ex vivo* proliferative advantage upon BMl1 overexpression in co-culture conditions with MS5 cells. In contrast to our previous studies in which overexpression of Flt3-ITDs[34;35] or activating mutants of STAT5[29] induced a "hyperactive stem cell state", a much more "controlled proliferation" phenotype was observed with BMl1 overexpression. Stem cell frequencies were significantly increased by overexpression of BMl1, and these HSCs contained self-renewal potential. It is interesting to note that self-renewal phenotypes imposed on HSCs by the oncogenes Flt3-ITD and STAT5 required interaction with bone marrow stromal cells, and no maintenance of HSCs was observed in liquid culture. In contrast, we showed that BMl1 overexpression was sufficient to maintain the multipotent characteristics of stem cells in liquid culture conditions, which even resulted in multilineage engraftment in NOD-SCID mice. Thus, BMl1 can be classified as a cell intrinsic regulator of human stem/progenitor cell self-renewal.

The mechanisms by which BMl1 alters the behavior of human stem and progenitor cells are currently unknown. In mouse models, the effects have so far been explained by BMl1-mediated suppression of the Ink4a/Arf cell cycle inhibitory proteins, p16 and p19[15;36]. However, p16 and p19 do not account for all BMl1 actions, and a number of other possible downstream targets have recently been reported. E4F1, a transcriptional regulator, has been reported to mediate at least some of the effects of BMl1 in

hematopoietic progenitors as a factor that directly binds to BMI1[37]. Fasano and colleagues recently provided functional evidence for the role of p21 downstream of BMI1 in neural stem cells[38], and upregulation of p21 mRNA has been noted in *bmi* knockout mice[36]. Our data indicate that indeed p16 and p19 are downmodulated by BMI1 in human CD34^{+} cells. Studies are under way to identify additional BMI1 target genes in human CD34^{+} cells, which should provide further insight into the mechanisms by which BMI1 exerts its effects on human HSCs.

Besides the functions that BMI1 has in normal hematopoiesis, constitutive activation of BMI1 has been observed in a variety of non-hematological and hematological malignancies. The first evidence for a possible role of BMI1 in the development of hematological malignancies came from a provirus integration screen in which BMI1 was identified as cooperating factor of c-MYC in the induction of B cell lymphomas[39]. Later it was demonstrated that BMI1 not only determines the proliferative capacity of normal, but also leukemic cells[12;39]. We have observed in primary AML CD34^{+} cells that BMI1 is expressed at much higher levels as compared to normal BM CD34^{+} cells in the majority of investigated cases[40]. Others have also reported that BMI1 expression is elevated in many hematological malignancies [41-45]. New data may provide some important insights into the possible role of BMI1 in the development of leukemic transformation. In a mouse model in which co-expression of the oncogenes HOXA9 and MEIS1 resulted in a quick onset of myeloid leukemia, no disease was observed in secondary *bmi-1-*deficient recipients[12], suggesting that BMI1 is essential for the maintenance of HOXA9-MEIS1 leukemic stem cells. Smith and colleagues reported that the oncoprotein E2a-PBX1 enhanced the expression of BMI1 as a consequence of which the Ink4a-Arf locus was repressed, and this condition was a requirement for hematopoietic transformation[46]. Recently, it has been shown that SALL4, an oncogene that is expressed in AML and induces leukemia in transgenic mice[47], can also strongly upregulate BMI1 expression [48]. However, our data suggest that BMI1 is not sufficient to induce leukemia by itself. Self-renewal and repopulation activity is enhanced in mouse[14] and human cells (our data), but it is plausible that secondary mutations are required to induce a full leukemic phenotype. Warner and colleagues described a leukemia model in which the oncogene TLS-ERG was introduced into human hematopoietic CD34^{+} cells, and in a few cases the transduced cells underwent a step-wise transformation and immortalization whereby upregulation of BMI1 was identified as cooperating hit[49]. By bypassing

senescence and maintaining the lifespan of stem cells as well as increasing their self-renewal by promoting symmetrical cell divisions, the stem cell pool may be more prone to acquire additional mutations that can ultimately result in leukemia, and this could be one of the possible mechanisms by which BMI1 may be involved in leukemogenesis.

In conclusion, our data characterize BMI1 as a potentially powerful mediator of maintenance and self-renewal of human hematopoietic stem cells and provide a platform to further elucidate the mechanisms of human hematopoietic stem cell fate decisions. These *in vitro* and *in vivo* models facilitate the study of the molecular mechanism underlying the BMI1-induced stem cell maintenance in human cells as well as provide a valuable tool to build human leukemia models in which the relative contribution of BMI1 to induce disease can be evaluated. Finally, insight into BMI1 induced self-renewal of human hematopoietic stem cell may provide future possibilities to expand cord blood cells prior to clinical stem cell transplantations.

AUTHOR CONTRIBUTIONS

A.R. designed and performed research, collected, analyzed and interpreted data and wrote the manuscript. B.D. performed animal research. E.V. designed research and interpreted data. G.d.H., designed research, interpreted data and contributed to writing of the manuscript. J.J. S. designed research, performed research, analyzed and interpreted data, drafted and wrote the manuscript.

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Repression of BMI1 in normal and leukemic human CD34+ cells impairs self-renewal and induces apoptosis

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ABSTRACT

High expression of BMl1 in acute myeloid leukemia (AML) cells is associated with an unfavorable prognosis. Therefore, the effects of downmodulation of BMl1 in normal and leukemic CD34$^+$ AML cells were studied using a lentiviral RNAi approach. We demonstrate that downmodulation of BMl1 in cord blood CD34$^+$ cells impaired long-term expansion and progenitor forming capacity, both in cytokine-driven liquid cultures as well as in bone marrow stromal cocultures. In addition, Long Term Culture-Initiating Cell (LTC-IC) frequencies were dramatically decreased upon knock-down of BMl1, indicating an impaired maintenance of stem and progenitor cells. The reduced progenitor and stem cell frequencies were associated with increased expression of p14ARF and p16INK4A and enhanced apoptosis, which coincided with increased levels of intracellular reactive oxygen species (ROS) and reduced FOXO3A expression. In AML CD34$^+$ cells downmodulation of BMl1 impaired long-term expansion, whereby self-renewal capacity was lost as determined by the loss of replating capacity of the cultures. These phenotypes were also associated with increased expression levels of p14ARF and p16INK4A. Together our data indicate that BMl1 expression is required for maintenance and self-renewal of normal and leukemic stem and progenitor cells and that expression of BMl1 protects cells against oxidative stress.

INTRODUCTION

BMI1 is a member of the Polycomb group (PcG) genes, which are transcriptional repressors that play essential roles in the maintenance of appropriate gene expression during development.[1-5] Two distinct multiprotein PcG complexes have been identified, the Polycomb Repression Complex (PRC) 1 and PRC2.[1] The PRC2 complex is involved in initiation of silencing and contains histone deacetylases and methyltransferases that can methylate H3 lysine 9 and 27 (H3K27).[6] Deletion of PRC2 genes in mice results in embryonic lethality, emphasizing their importance in development.[7-9] PRC1 is implicated in stable maintenance of gene repression and recognizes the methylation marks set by PRC2.[10; 11] Mice mutant for most PRC1 genes survive until birth as result of partial functional redundancy provided by their homologues, but developmental defects do arise thereafter as is e.g. the case in the hematopoietic compartment after deletion of BMI1.[12-14] Targeted deletion of BMI1 has shown that although the numbers of fetal liver-derived HSCs is normal in these mice, their proliferative and self-renewal capacity is severely impaired.[15; 16] In adult BMI1-deficient mice, the HSCs are less frequent and display an impaired competitive repopulation capacity.[16; 17] Gain-of-function studies demonstrated enhanced self-renewal of murine HSC and with a shift in balance towards more symmetric stem cell divisions.[17] We have demonstrated that constitutive expression of BMI1 in human cord blood cells results in prolonged maintenance of the stem cell pool and enhances self-renewal of human stem and progenitor cells.[18] BMI1 is potent negative regulator of the Ink4a/Arf locus in embryonic fibroblasts.[19] This locus encodes the cell cycle regulators and tumor suppressor p16 and p19/p14. Increased expression of these genes was observed in the BMI1-deficient mice.[12; 16; 19; 20] However, INK4A/ARF-independent BMI1-targets must exist as well since overexpression of BMI1 in p16/p19-deficient cells still altered HSC self-renewal phenotypes.[21]

In addition to functions in normal hematopoiesis, BMI1 has been suggested to play a role in leukemogenesis as well.[15; 22] In a murine model of leukemia, self-renewal of HSCs by expression of HOXA9 and MEIS1 was severely impaired in Bmi1^{-/-} cells.[15] Although functional studies have not been performed in primary human leukemic cells, we and others have shown that BMI1 expression is elevated in variety of hematological tumors, including Non-Hodgkin Lymphomas and Acute Myeloid Leukemias (AML).[23-26] In chronic myeloid leukemia (CML), BMI1 expression increases with disease

progression and high levels of BMI1 correlate with reduced overall survival.[27] A small study performed on AML and myelodysplastic syndrome (MDS) patients demonstrated that high BMI1 expression correlated with poor overall survival.[28] These data suggest that (epigenetic) changes induced by BMI1 in the hematopoietic stem cell likely contribute to the development or maintenance of the leukemic phenotype and require further studies to determine whether pharmaceutical targeting of BMI1 will have beneficial effects on the treatment of patients with acute leukemia.

Here, we provide evidence that downmodulation of BMI1 using a lentiviral RNAi approach impairs self-renewal of both normal human CB CD34+ cells as well as primary AML CD34+ cells from patients. Impaired HSC self-renewal was associated with increased expression of the INK4A/ARF locus, increased apoptosis in conjunction with increased levels of ROS and reduced FOXO3A expression.

MATERIALS AND METHODS

Primary cell isolation
Neonatal cord blood was obtained after informed consent from healthy full-term pregnancies from the obstetrics departments of the University Medical Center in Groningen (UMCG), Martini Hospital Groningen and Sophia Hospital in Zwolle. Peripheral blood and bone marrow from untreated patients diagnosed with AML at the UMCG were studied after informed consent and protocol approval by the Medical Ethical Committee of the UMCG. After ficoll separation of mononuclear cells, CD34+ cells were enriched using a magnetically activated cell sorting (MACS) CD34 progenitor kit (Miltenyi Biotech, Nijmegen, The Netherlands).

Lentiviral virus production and infection
Lentiviral vector expressing short hairpins against human BMI1 (CS-H1-shRNA-EF-1α-EGFP) and scrambled lentiviral vectors were a kind gift from Dr. Iwama (Chiba University, Japan).[29] Lentiviral particles were produced by co-transfection of 293T cells with 0.7 µg pcDNA3-VSVg-REV, 3 µg pMDLg-RRE and 3 µg CS-H1-scrambled RNAi or CS-H1-BMI1 RNAi. The lentiviral supernatants were collected 24h later and were either used directly or stored at −80°C until further use. Cord blood CD34+ cells were cultured for 16h in HPGM supplemented with SCF (100ng/ml), Flt3 Ligand (100ng/ml;

both from Amgen, Thousand Oaks, USA) and TPO (100ng/ml; Kirin, Tokyo, Japan) and subsequently transduced on retronectin (Takrara, Tokyo, Japan) coated plates in two consecutive rounds of 8 and 12 hours with lentiviral supernatant supplemented with the same cytokines and 4µg/ml polybrene. AML CD34+ blasts were transduced as previously described.[30-33] Briefly, the cells were pre-stimulated for 4h in RPMI supplemented with 10% FCS, 20ng/ml IL3 (Gist-Brocades, Delft, the Netherlands), G-CSF (Rhone-Poulenc Rorer, Amstelveen, The Netherlands) and TPO and afterward transduced on retronectin coated plates in three consecutive rounds of 8 and 12h with lentiviral supernatants containing cytokines and polybrene as indicated above.

Ex-vivo culture of primary cells, Colony-forming Cell (CFC) and Long Term Cell-Initiating Cell (LTC-IC) assays

Cord blood stroma-free cultures were either propagated in serum-free HPG medium supplemented with SCF, Flt3L and TPO (all 100ng/ml) or in IMD medium supplemented with 10% FCS and IL3 (10ng/ml) and SCF (100ng/ml). For the cord blood MS-5 co-culture experiments and long-term culture-initiating (LTC-IC) assays cells were grown in αMEM (BioWhittaker) supplemented with 12.5% heat-inactivated FCS, 12.5% heat-inactivated horse serum (Sigma), penicillin and streptomycin, 2 mM glutamine, 57.2 µM β-mercaptoethanol and 1 µM hydrocortisone (Sigma). AML blast cells were expanded on MS5 cells using the same co-culture medium as for the cord blood cells, but supplemented with 20ng/ml IL3, G-CSF and TPO as described previously.[23] The cultures were kept on 37°C and in 5% CO_2.

CFC and LTC-IC assays on MS-5 stromal cells were performed as previously described.[33] Briefly, for the CFC assays, 1000 GFP+-sorted cells were plated in methylcellulose directly after transduction and 10.000 GFP+ cells were used at later time points. For the LTC-IC assays, GFP+ cells were sorted on MS5 stromal cells in limiting dilutions from 90 to 7290 cells per well in 96-well plates. Five weeks later, the wells containing cobblestone areas were scored after which the medium from the wells was aspirated and replaced with methylcellulose containing cytokines. After an additional two weeks of culture wells were scored as positive or negative to yield the LTC-IC frequency.

Western blotting, immunohistochemistry, quantitative real time PCR and Flow cytometry analysis

Western blot analysis was performed using standard protocols. Antibody against BMI1 (Upstate, CA, USA) was used in a 1:1000 dilution and anti-GFP antibody (Santa Cruz, Heerhugowaard, The Netherlands) was used in a 1:300 dilution. Antibody against FOXO3 (Cell Signaling) was used in 1:200 dilution and anti-actin (C4) (ICN Biomedical, Zoetermeer, The Netherlands) in 1:1000 dilution. Anti-rabbit Cy3 (Jackson ImmunoRes) secondary antibody was used in 1:1000 dilution. Slides were analyzed on Leica microscope (Leica DM RXA).

Total RNA was isolated from $1x10^5$ cell using the RNeasy kit from Qiagen (Venlo, the Netherlands) and was reverse transcribed using M-MuLV reverse transcriptase (Fermentas, St Leon-Roth, Germany) according to the manufacturer's instructions. Aliquots of cDNA were then real-time amplified using iQ SYBR Green mix (Bio-Rad, CA, USA) on a MyIQ thermocycler (Bio-Rad) and quantified using MyIQ software (Bio-Rad). HPRT expression was used to calculate relative expression levels. Sequences and conditions are available on request.

The fluorescence activated cell sorting analyses were performed on a FACS Calibur (Becton Dickinson (BD), Alphen /d Rijn, the Netherlands) and sorting of the cells was performed on MoFlow (Dako Cytomation, Carpinteria, CA, USA). Data were analyzed using WinList 3D (Topsham, ME, USA) and FlowJo (Tri Star, Inc, Ashland, OR, USA) software. Antibodies were obtained from BD and staining of the cells was performed by standard procedures.

Measurement of intracellular ROS levels

Intracellular ROS levels were determined by staining with the probe for 2',7'-dichlorodihydro-fluorescein diacetate (H2DCFDA; Invitrogen, Carlsbad, CA). H2DCFDA was added to the cell suspension of transduced cells to a final concentration of 10 µM, followed by incubation at 37°C for 20 minutes. The cell pellet was resuspended in 500 µl cold PBS and kept on kept on ice until analyzed on an LSR-II (BD, Alphen /d Rijn, The Netherlands). Where indicated, cells were treated with 100 µM NAC (A9165, Sigma).

RESULTS

Downmodulation of BMI1 expression in cord blood CD34⁺ cells impairs long-term expansion and reduces progenitor and stem cell frequencies.

In order to further elucidate the role of BMI1 in human stem and progenitor cells, we used a lentiviral transduction approach to downmodulate the expression of BMI1. Cord blood CD34⁺ cells were transduced with efficiencies ranging from 20-45% (data not shown), which resulted in a decrease in BMI1 expression of approximately 75% at the RNA level (Fig.1A) and protein level
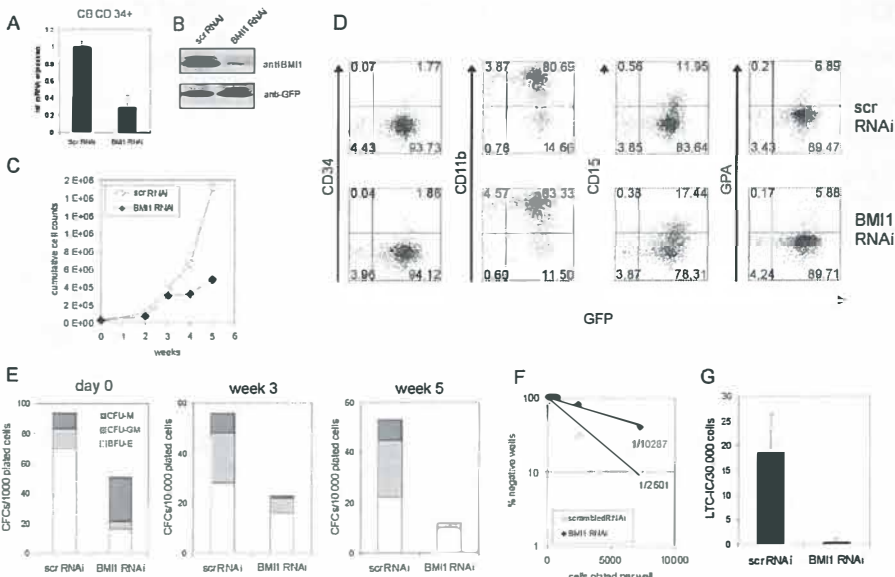
Figure 1. Downmodulation of BMI1 by lentiviral RNAi in human cord blood CD34⁺ cells impairs proliferation and reduces CFC and LTC-IC frequencies.

(A) CB CD34+ cells were transduced with control (scrambled) scr RNAi or BMI1 RNAi particles, sorted and mRNA was isolated and BMI1 expression was analyzed by quantitative RT-PCR analysis. (B) As in A, but now total lysates were prepared and analysed by Western blotting. (C) Transduced CB cells were grown in long-term cocultures on MS5 bone marrow stromal cells. Cultures were weekly analyzed and the growth curve represents cumulative cell numbers during the culture period. A representative experiment out of three independent experiments is shown. (D) Hematopoietic differentiation was analyzed by FACS on suspension cells from MS5 cocultures at week 1. (E) Suspension cells were harvested from MS5 cocultures as described in C and progenitor content was determined by CFC assays in methylcellulose. (F, G) Lentiviral transductions as in A, but now stem cell frequencies were determined in limiting dilution (F) or in bulk T25 flasks (G).

(Fig.1B). Transduced cells were cultured on MS5 bone marrow stroma to study expansion and hematopoietic differentiation. As shown in Fig.1C, the

expansion was severely impaired in BMI1 RNAi cells over a culture period of 5 weeks. No effects were observed on the hematopoietic differentiation program (Fig.1D). The presence of progenitor cells was evaluated by plating suspension cells from MS5 cocultures in CFC assays in methylcellulose. Downmodulation of BMI1 resulted in a significant reduction in the number of progenitors already immediately after transduction (Fig.1E, day 0). Upon expansion on MS5, the reduction in progenitors was even further pronounced (Fig.1E). Stem cell frequencies were determined by LTC-IC assays. Both in limiting dilution assays (Fig.1F) as well as assays in bulk where 30.000 transduced cells were plated on T25 flasks (Fig.1G) a strong reduction in stem cell frequency was observed upon downmodulation of BMI1.

Besides MS5 bone marrow stromal cocultures, stroma-independent cultures were also initiated. Similar results were obtained, whereby downmodulation of BMI1 resulted in an even more dramatic decrease in expansion in cytokine-driven liquid cultures (Fig.2A) and progenitor frequencies (Fig.2B).
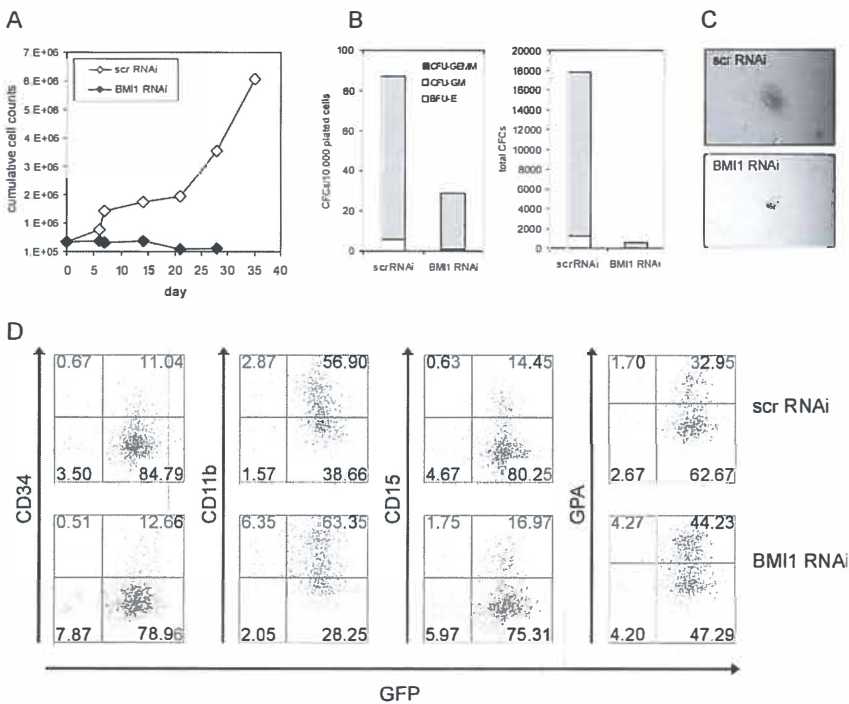
Figure 2. Knockdown of BMI1 reduces cell growth and CFC formation in liquid culture conditions.
(A) CB CD34+ cells were transduced with control (scrambled) scr RNAi or BMI1 RNAi particles, sorted and plated in stroma-free liquid conditions (IMDM supplemented with 10% FCS, 10 ng/ml IL3 and 100 ng/ml TPO). Cumulative expansion is shown of a representative experiment out of three independent experiments. (B) Progenitor content was determined by CFC assays in methylcellulose at week 2 of liquid culture. CFCs per 10.000 cells (left panel) as well as the total amount of generated CFCs (right panel) is shown. (C) Representative micrographs of colonies in methylcellulose displaying a reduction in colony size after BMI1 knock down in cells from week 2 liquid cultures. (D) Hematopoietic differentiation was analyzed by FACS on suspension cells from MS5 cocultures at week 1.

Not only the number of CFCs was strongly reduced, also the size of colonies was reduced upon BMI1 downmodulation (Fig.2C). No significant effects were observed on hematopoietic differentiation upon downmodulation of BMI1 (Fig.2D).

Taken together, these data indicate that downmodulation of BMI1 in human CD34+ cells reduces their proliferative capacity and leads to impaired maintenance of stem and progenitor cells.

BMI1 downmodulation increases apoptosis and ROS accumulation.

Since both in stroma-free and co-culture experiments BMI1 knockdown resulted in impaired proliferation and reduced progenitor and stem cell frequencies, we wanted to determine whether these effects were due to increased apoptosis. To address this question we first single cell-sorted CD34+/CD38- transduced Scr-RNAi and BMI1-RNAi cells in 96-well plates in liquid culture supplemented with SCF and IL3 and monitored each well microscopically. The number of cells per well were enumerated after 1 and 5 days of culture. If a well contained a single cell, it was classified as "quiescence", if multiple cells were observed it was classified as "proliferation", and if no cells were seen, it was classified as "apoptosis". Within 24 hrs after plating the number of apoptotic cells was significantly higher in BMI1 RNAi-transduced cells, while fewer proliferating and quiescent cells were observed (Fig.3).
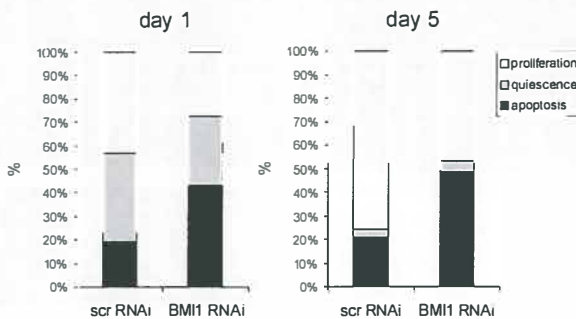
Figure 3. BMI1 knockdown induces apoptosis in CD34+38- cells.
CB CD34+ cells were transduced with control (scrambled) scr RNAi or BMI1 RNAi particles, sorted, and single cells were deposited in 96-well plates and cultured in stroma-free conditions (IMDM supplemented with 10% FCS, 10 ng/ml IL3 and 100 ng/ml TPO). Wells were evaluated microscopically 1 day and 5 day after plating, and wells were classified as "quiescence" if 1 live cell was observed, if multiple cells were observed it was classified as "proliferation", and if no cells were seen, it was classified as "apoptosis". A representative experiment out of three independent experiments is shown, whereby individual 96 wells per group were analyzed.
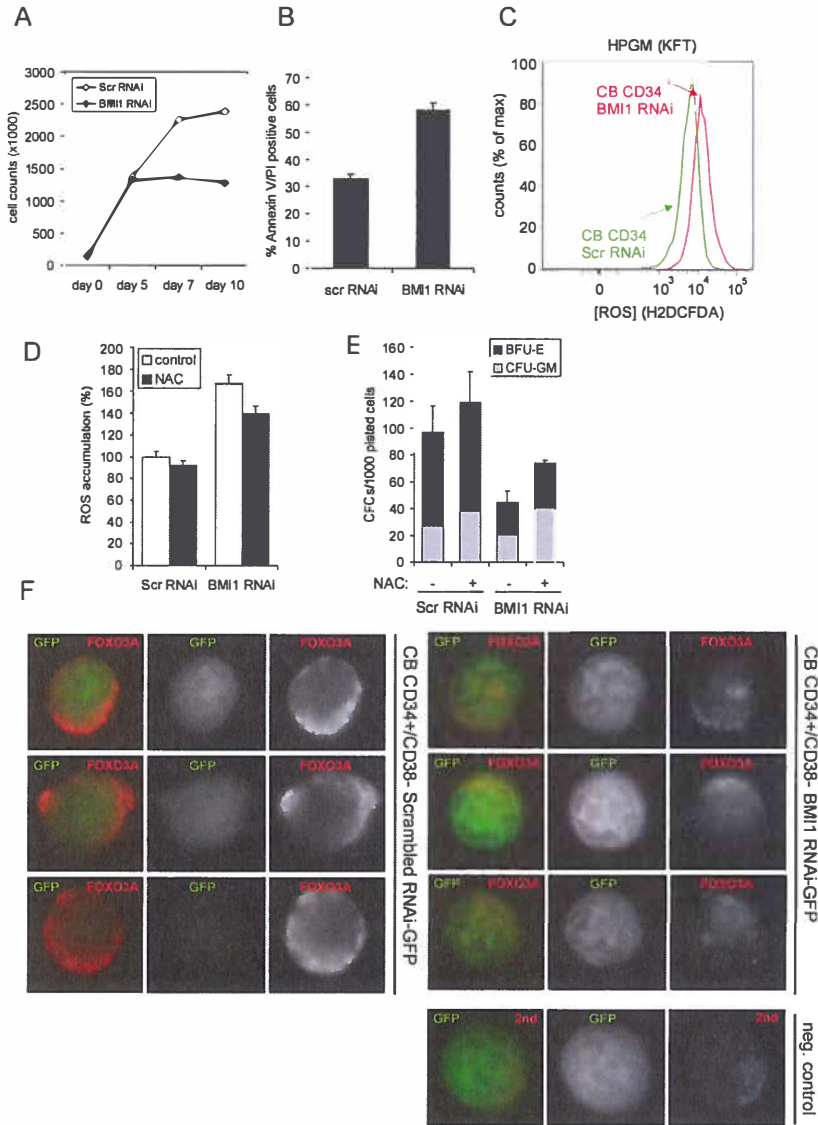
Figure 4. Apoptosis induced by BMI1 downmodulation coincides with elevated ROS accumulation and reduced FOXO3A expression.

(A) Transduced CB CD34+ cells were cultured in serum-free conditions (HPGM supplemented with SCF, Flt3L and SCF) for 10 days and expansion was monitored. (B) The percentage of apoptotic cells at day 10 was determined by FACS staining for AnnexinV and PI. (C) Transduced cells were cultured in conditions described in (A) and at day 10 were stained with H2DCFDA to determine the intracellular levels of ROS by FACS. (D) Transduced cells were cultured in the absence or presence of 100 μM NAC for 9 days after which ROS accumulation was determined by FACS. (E) CFC assays were performed with transduced cells in methylcellulose cultures in the absence or presence of 100 μM NAC. (F) CB CD34+ cells were transduced with control (scrambled) scr RNAi or BMI1 RNAi and CD34+ 38- GFP+ cells were sorted on glass slides. Immunohistochemical staining was performed using antibodies against FOXO3A.

After 5 days, the majority of quiescent cells had started to proliferate in both scr-RNAi as well as BMI1-RNAi transduced cells, whereby the number of apoptotic wells remained significantly higher in the cells in which BMI1 was downmodulated. These data suggest that under stringent stroma-free conditions in human HSCs apoptosis is more dominantly affected than quiescence upon depletion of BMI1.

Next, we cultured Scr-RNAi and BMI1-RNAi-transduced CB CD34$^+$ cells in serum-free conditions (HPGM supplemented with SCF, Flt3L and SCF) for 10 days. A strongly reduced expansion was observed upon downmodulation of BMI1 (Fig.4A) which was associated with increased levels of apoptosis as determined by PI/AnnexinV staining (Fig.4B). Furthermore, intracellular ROS levels were determined by FACS using H2DCFDA and these studies revealed that downmodulation of BMI1 in CB CD34$^+$ cells results in increased ROS accumulation (Fig.4C). Elevated ROS levels in BMI1 RNAi-transduced cells could be partially restored by treatment with 100 μM NAC (Fig.4D). This coincided with a partial, but not complete, restored progenitor frequency as determined by methylcellulose assays (Fig.4E). Since ROS production and the regulation of apoptosis have been tightly associated with FOXO3A signaling in murine HSCs, we studied FOXO3A expression levels in transduced CB cells. Scr-RNAi and BMI1-RNAi-transduced CD34$^+$/CD38$^-$ cells were sorted onto microscopy slides and stained for FOXO3A expression. As shown in Fig.4F, CB CD34$^+$/CD38$^-$ cells expressed FOXO3A, which was mostly localized in the cytoplasm after 4 days of prestimulation and transduction in HPGM supplemented with SCF, Flt3-L and TPO. Downmodulation of BMI1 resulted in a strongly reduced expression of FOXO3A (Fig.4F).

Downmodulation of BMI1 in AML CD34$^+$ cells impairs their long-term expansion

Previous experiments by us and others have revealed an increased expression of BMI1 within CD34$^+$ cells in the peripheral blood and bone marrow from AML patients.[23; 24] We were interested in the effects of downmodulation of BMI1 on proliferation and self-renewal in this population. CD34$^+$ cells were isolated from the peripheral blood or bone marrow of AML patients of various FAB classifications and risk groups (n=9, Table 1) since this fraction is enriched for leukemia-initiating cells, followed by transduction with Scr-RNAi or BMI1-RNAi vectors. Transduction efficiencies ranged from 25-50% for both groups (data not shown). Real-time PCR analysis was performed to determine BMI1 expression levels in transduced cells and efficient
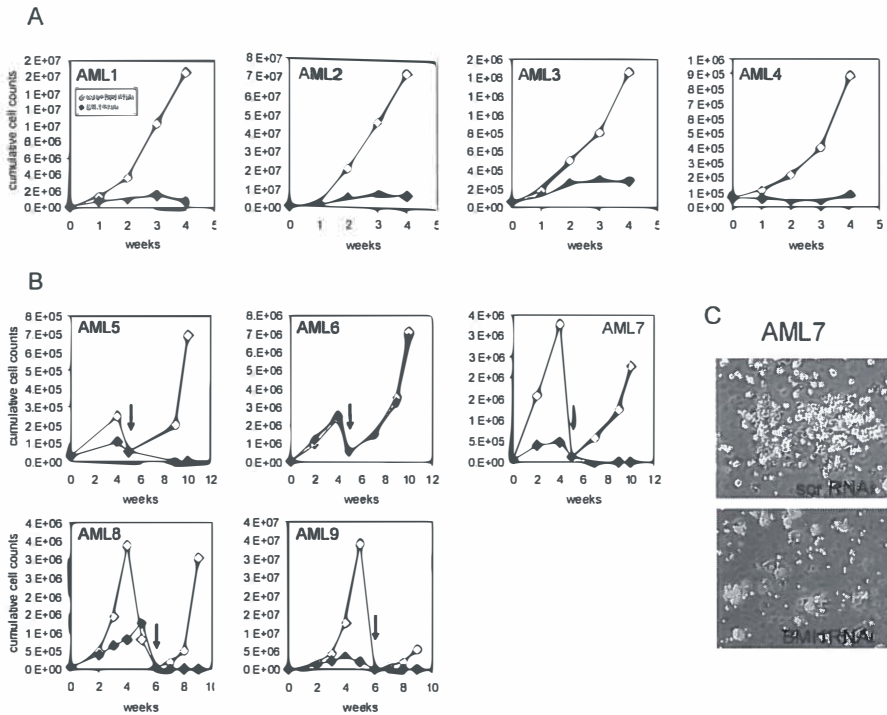
A

B

C

AML7

Figure 5. BMI1 is required for long-term growth and self renewal of acute myeloid leukemia (AML) CD34⁺ cells.
(A) AML CD34+ cells from different FAB subclassification were transduced with scrambled RNAi or BMI1 RNAi lentiviral vectors and long-term cultures on MS5 bone marrow stromal cells were performed. Expansion was monitored weekly and cumulative cell counts are shown. (B) Experiment as in A, but now transduced AML CD34+ cells were cultured on MS5 for a period of 5-6 weeks after which human CD45+ cells were harvested and replated onto new MS5 cells, followed by an additional culturing period of 4-5 weeks. (C) Representative micropgraphs of cobblestone area forming cells present in MS5 cocultures at week 4 initiated with AML CD34+ cells transduced with scrambled RNAi or BMI1 RNAi lentiviral vectors.

patient ID	% CD34	BM/PB	FLT3 ITD	Karyotype	Risk group
1	70	PB	-	+3q;-7;-10	poor
2	26	PB	-	bcr-abl, inv16	poor
3	5	BM	+	normal	intermediate
4	70	BM	-	5q-; trisomy 6	poor
5	86	BM	+	normal	intermediate
6	54	BM	+	normal	Intermediate
7	30	BM	-	normal	intermediate
8	91	BM	-	normal	intermediate
9	85	PB	+	normal	Intermediate

Table 1. Patient characteristics

downmodulation of BMI1 was established in almost all cases (Fig.6A and data not shown). Proliferation of transduced cells was determined in long-term MS5 cocultures. In all but one case expansion was severely impaired

upon downmodulation of BMI1 (Fig.5A and B). In AML 6 we were not able to efficiently downregulate BMI1 expression levels (data not shown). Within these MS5 cocultures we typically observe the formation of cobblestone areas underneath the stroma within 2-5 weeks after plating and these leukemic cobblestone areas (L-CAs) contain self-renewing properties and can be harvested and replated to give rise to new long-term expanding cultures and L-CAs.[23] As depicted in Fig.5B and 5C, scr-RNAi transduced cultures could be harvested and replated to give rise to long-term expanding 2nd cocultures and L-CAs, indicative for self-renewal properties. In contrast, no 2nd cultures could be established from the BMI1-RNAi-transduced AML CD34+ cultures, indicating that self-renewal was severely impaired (Fig.5B and C). Replating could be established in the transduced AML 6, but as mentioned above, we did not succeed in efficient downmodulation of BMI1 in this AML case.
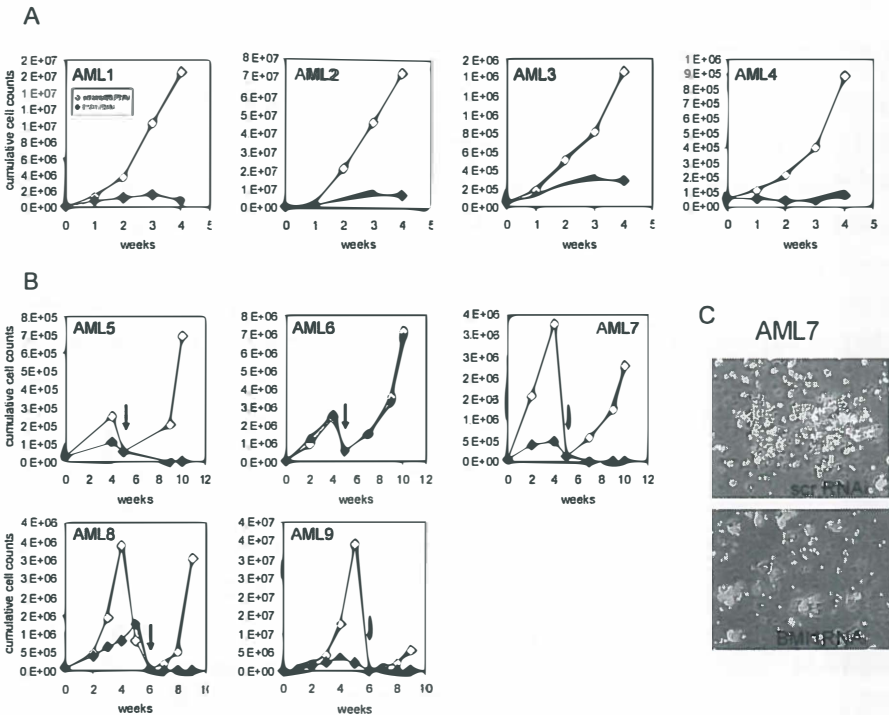
Figure 6. Downregulation of BMI1 expression in CB or AML CD34+ cells results in derepression of p14ARF and p16INK4a.

CB or AML CD34+ cells were transduced with scrambled RNAi or BMI1 RNAi lentiviral vectors, sorted, and RNA was isolated. Quantitative RT-PCRs were performed to determine the expression levels of BMI1, p14ARF and p16INK4a. As controls, BMI1 was overexpressed in CB CD34+ cells and Q-RT-PCR analysis was performed (right panels).

To determine whether repression of the INK4A/ARF locus was relieved upon downmodulation of BMI1, Q-PCR analysis was performed on transduced CB and AML CD34+ cells. As shown in Fig.6, downmodulation of BMI1 in both normal as well as leukemic CD34+ cells resulted in a rapid increase in p14, but most notably p16 expression, although the levels to which the expression were elevated varied between AML cases. Reversely, overexpression of BMI1 in CB CD34+ cells resulted in a decrease in p16 and p14 expression, as demonstrated previously.[18] Together, these data indicate that expansion and self-renewal of primary leukemic AML CD34+ cells depends on BMI1 expression, which coincides with repression of the cell cycle regulators p16 and p19/p14.

DISCUSSION

PcG proteins have been implicated in the regulation of self-renewal in a range of different stem cell systems. In mouse models it has been shown that BMI1, a component of the PRC1 complex, is required for the maintenance of self-renewing hematopoietic stem and progenitor cells.[15-17] Our studies using human models have demonstrated that enforced expression of BMI1 is a powerful mediator of maintenance and self-renewal of human hematopoietic stem and progenitor cells as well.[18] Various reports have described that BMI1 is expressed in a variety of tumor populations.[22; 34; 35] We and others have observed that the expression of BMI1 is often increased in leukemic peripheral blood and bone marrow CD34+ cells.[23; 24] However, it has remained elusive whether the expression of BMI1 is required to maintain human stem and progenitor cells, in particular those that belong to the leukemic clone.
In the present study utilizing efficient lentiviral loss-of-function assays, we first examined the effect of BMI1 downmodulation in cord blood CD34+ cells. Our data show that the proliferative capacity of CD34+ cells was significantly impaired. Specifically, progenitor and stem cell frequencies were strongly reduced as determined by CFC and LTC-IC assays, suggesting that it is particularly the immature hematopoietic compartment that is affected. The bone marrow microenvironment has been attributed with protective effects on the stem/progenitor cell compartment, and we therefore performed experiments in both bone marrow stromal cocultures as well as in more stringent cytokine-driven liquid culture conditions. We observed reduced progenitor and stem cell frequencies in both liquid cultures as well as

bone marrow stromal cocultures, although the effects under liquid culture conditions were more pronounced. Thus, these data suggest that cell-intrinsic pathways are affected by BMl1 depletion, but that the presence of a protective microenvironment can compensate for these effects to some extent. Importantly, we find that expansion of human leukemic stem and progenitor cells also depends heavily on the expression of BMl1. Lentiviral downmodulation of BMI1 in AML CD34+ cells severely impaired their long-term growth in MS5 bone marrow stromal cocultures and the formation of leukemic cobblestone areas was reduced. While leukemic cocultures initiated with AML CD34+ cells can readily be expanded and serially replated onto new bone marrow stroma, indicative for self-renewal properties, we failed to initiate 2nd MS5 cocultures with BMI1 RNAi-transduced AML CD34+ cells. A number of molecular mechanisms could underlie our observed phenotypes. In murine HSCs, overexpression of BMl1 results in enhanced symmetric cell divisions.[17] Although the mechanisms involved are not elucidated yet, it might be associated with a symmetric distribution of cell fate determinants such that the stem cell pool can be expanded. Reversely, loss of BMl1 resulted in loss of HSC self-renewal as determined in competitive repopulation assays.[15-17] Furthermore, in murine Bmi1-/- HSCs self-renewal was impaired which was at least in part mediated via a derepression of p16-INK4A and p19-ARF.[21] We also find that p19/p14 and p16 expression is elevated in both normal as well as leukemic human CD34+ cells upon downmodulation of BMl1, suggesting a direct link between BMl1 expression and repression of this locus in human AML. Derepression of the INK4A and ARF genes might result in premature senescence, as has been shown in mouse embryonic fibroblasts,[19] and it is plausible that this might be involved in the impaired long-term expansion phenotypes that we observed in our cultures as well. However, we also observed rather immediate effects upon downmodulation of BMl1 in the hematopoietic compartment. Within a few days after transduction, strongly reduced cell counts were observed, particularly under more stringent liquid culture conditions. A strong increase in PI/AnnexinV+ apoptotic cells was observed upon downmodulation of BMl1 in CB CD34+ cells, and also in the most immature CD34+/CD38- stem cell population transduced with BMl1 RNAi we observed increased rates of apoptosis. Previously, in mouse Bmi1-/- BM slightly enhanced apoptosis was noted, although in CD34-KSL clonal cultures no signs of apoptosis were observed upon depletion of BMl1.[17] These observations might reflect differences between mouse and human stem/progenitor cells, but it is perhaps more likely that the conditions under which

the cells were studied might have been less stringent, as we also observe the highest apoptosis rates under more stringent conditions. Our data are in line with our previously reported gain-of-function analyses, where enforced expression of BMI1 led to a proliferative advantage and increased stem cell and progenitor frequencies of cord blood CD34+ and CD34+38- cells, which was associated with reduced levels of apoptosis.[18] Also, a recent study in the NB4 cell line indicated that downmodulation of SALL4, an upstream regulator of BMI1, resulted in increased apoptosis, which could be reversed by re-introduction of BMI1.[36]

Although further studies are required to reveal the underlying molecular mechanisms, it is intriguing that the BMI1 RNAi-induced apoptosis coincides with increased levels of ROS accumulation and a reduction in FOXO3A expression. In mouse studies it has been shown that upregulation of p16 and p19 leads to increased ROS production in Atm-/- mice, which resulted in a loss of the HSC pool.37 These data suggest that HSCs and progenitors contain lower levels of ROS as compared to their mature progeny, and that these differences are critical for maintaining stem cell function. As we indeed find elevated expression levels of p16 and p14 by BMI1 knockdown, these data might suggest that derepression of p16 and p14 could account for the impaired self-renewal of the BMI1 RNAi cells mediated by an increase in ROS accumulation. Furthermore it has been demonstrated that FOXO3A is essential for ATM expression and that loss of FOXO3A leads to defects in the hematopoietic stem cells.38; 39 FoxO3A-/- HSCs are defective in their competitive repopulation capacity, associated with an elevation of ROS levels.39 Although in the FoxO3A-deficient mice no effects were observed on apoptosis, mice in which FOXO1, 3 and 4 were deleted a significant increase in apoptosis was noted, both in the HSC as well as myeloid progenitor compartment.[39; 40] In human stem and progenitor cells, we have now coupled loss of BMI1 expression to enhanced apoptosis, possibly mediated via downregulation of FOXO3A resulting in accumulation of ROS. Thus, BMI1 might be required to protect hematopoietic stem/progenitor cells from apoptosis induced by oxidative stress conditions. Treatment with NAC was able, at least in part, to restore progenitor frequencies in BMI1 RNAi-transduced cells. Interestingly however, progenitor frequencies were not completely restored to control levels by NAC treatment. This might be due to the fact that ROS accumulation in BMI1 RNAi-transduced cells was only partially restored by NAC treatment, or that ROS-independent pathways still play a role as well in the induction of apoptosis. Previously,

we have observed that overexpression of BMI1 in human CD34+ cells results in HSC maintenance as determined by NOD-SCID engraftability, even when cells are cultured under high oxygen conditions outside of the bone marrow microenvironment.[18] Although further evidence needs to be provided, it is tempting to speculate that in human leukemias, besides facilitating symmetric stem cell divisions, the leukemic stem cell might utilize enhanced expression of BMI1 as a mode to protect itself from oxidative stress. Our observations are in line with a recent paper indicating that absence of BMI1 impairs mitochondrial function and the DNA damage response pathway.[41] Thus, since BMI1 is frequently overexpressed in human leukemias,[23; 27] it will be interesting to determine whether a therapeutic window exists for the targeting of BMI1 as a treatment modality in AML.

ACKNOWLEDGEMENTS

AUTHORSHIP CONTRIBUTIONS

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Chapter 4

BMI1 collaborates with BCR-ABL in leukemic transformation of human CD34+ cells

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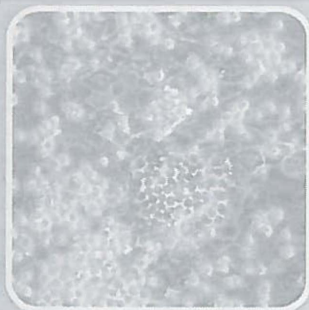
ABSTRACT

The major limitation for development of curative cancer therapies has been incomplete understanding of the molecular mechanisms driving cancer progression. Human models to study development and progression of chronic myeloid leukemia (CML) have not been established. Here, we show that BMI1 collaborates with BCR-ABL in inducing a fatal leukemia in NOD-SCID mice transplanted with transduced human $CD34^+$ cells within 4-5 months. The leukemias were transplantable into secondary recipients with a shortened latency of 8-12 weeks. Clonal analysis revealed that similar clones initiated leukemia in primary and secondary mice. In vivo, transformation was biased towards a lymphoid blast crisis, and in vitro both myeloid as well as lymphoid long-term self-renewing cultures could be established. Retroviral introduction of BMI1 in primary chronic phase $CD34^+$ cells from CML patients elevated their proliferative capacity and self-renewal properties. Thus, our data identify BMI1 as a potential therapeutic target in CML.

INTRODUCTION

Chronic myeloid leukemia (CML) was the first cancer to be associated with a defined chromosomal abnormality, the Philadelphia chromosome (Ph[+]), which occurs as a consequence of reciprocal translocation of chromosome 9 and 22 (t9;22)(q34;q11).[1-3] In the pre-tyrosine kinase inhibitor (TKI) era, CML was characterized by distinct clinical stages evolving from a chronic phase (CP) through accelerated phase (AP) into blast crisis (BC).[4,5] The BCR-ABL oncoprotein is necessary and sufficient for initiating the chronic phase of the disease when preferential expansion of myeloid progenitors and differentiated progeny is observed.[6-8]

While overexpression of BCR-ABL in murine bone marrow was sufficient to induce transplantable leukemias in almost all recipient mice,[9] attempts to develop human CML models did not result in overt hematological disease. When cord blood CD34^{+} cells were retrovirally transduced with p210 BCR-ABL and transplanted into NOD/SCID mice, enhanced numbers of erythroid and megakaryocytic cells were observed, but after 5 months only few mice developed signs of a myeloproliferative disease.[10] When primary CML samples were injected into NOD/SCID mice in an attempt to model the disease, recipients showed accumulation of abnormal populations of cells that recapitulated the onset of the disease rather than the blast crisis, and no reproducible human hematopoietic malignancies were generated sofar.[11] Various pathways downstream of BCR-ABL have been implicated in the transformation process, including activation of β-Catenin,[12,13] STAT5,[14-16] Rac GTPases,[17,18] and MEK/ERK.[19] Even though many of these pathways are activated by BCR-ABL and are required for the onset of disease, little is known about the molecular mechanisms that cooperate with BCR-ABL in the transition from chronic phase to blast crisis CML. Pathways that might cooperate with BCR-ABL in the transition from CP-CML into BC-CML include the Wnt and Hedgehog pathways.[12,20,21] Recent findings report that the expression of the polycomb gene BMI1, which is implicated in normal and leukemic stem cell proliferation,[22,23] is significantly higher expressed in patients with advanced disease than in patients in CP.[24] BMI1 has been linked to leukemogenesis since its discovery as a cooperating partner of c-Myc in the induction of B-cell lymphomas.[25] When HOXA9 and MEIS1 were co-expressed in *bmi1-/-* cells, leukemia did not occur in secondary transplanted animals, suggesting that BMI1 has an important role in the maintenance of leukemic stem cells in this model.[22] We have classified BMI1 as a strong

intrinsic regulator of human stem cells as BMI1 expressing cells engrafted efficiently in the NOD/SCID mice even after in vitro culturing.[26] However, recipient mice did not show signs of disease, documenting that BMI1 by itself is not sufficient to induce leukemia in human cells (unpublished observations,[26]), comparable to what has been observed in murine cells.[27]

Here, we report that co-expression of BMI1 and BCR-ABL in human cord blood (CB) CD34+ cells is sufficient to induce transplantable leukemia in NOD-SCID mice. In vitro, both myeloid and lymphoid transformed cell lines could be established depending on extrinsic cues. Retroviral introduction of BMI1 in primary chronic phase CD34+ cells from CML patients elevated their proliferative capacity and self-renewal properties. In conclusion, our data indicate that BMI1 collaborates with BCR-ABL in leukemic transformation, and our human-based system should provide a useful model to study the pathology of leukemias and test new drug entities.

METHODS

Primary cell isolation
CB and CML patient samples were obtained after informed consent and protocol approval by the Medical Ethical Committee of the UMCG. All analyzed CML samples were obtained at the time of diagnosis prior to treatment. The percentage of CD34+ cells ranged from 6-45%, and all cells were Ph+ as determined by karyotyping. After ficoll separation of mononuclear cells, CD34+ cells were enriched using the magnetically activated cell sorting (MACS) CD34 progenitor kit (Miltenyi Biotech, Nijmegen, The Netherlands).

Cell lines and ex vivo culture of primary cells.
Propagation of 293T, PG13, and MS5 cells, and myeloid-driven MS5 co-culture experiments were performed as previously described.[28-30] Lymphoid-driven MS5 co-cultures contained essentially the same components as in the myeloid-driven cultures, with the exception of hydrocortisone and horse serum and presence of 50μg/ml ascorbic acid (Sigma). The CD34+ cells from chronic phase CML patients were cultured on MS5 cells in myeloid supportive Gartners medium supplemented with IL3 (Gist-Brocades, Delft, the Netherlands), IL6 (Gist-Brocades), G-CSF (Rhone-Poulenc Rorer, Amstelveen, The Netherlands) (20 ng/ml each) and SCF and FLT3-L (Amgen,

Thousand Oaks, USA) (100ng/ml each). For the sequential plating of MS5 co-cultures, suspension and adherent cells were harvested by trypsinization, human CD45$^+$ cells were obtained using a magnetically activated cell sorting (MACS) CD45 progenitor kit (Miltenyi Biotech, Nijmegen, The Netherlands) after which cells were re-plated on new MS5 stromal cells. The stroma-independent culture assays were performed in IMD medium (PAA Laboratories, Pasching, Austria) supplemented with 20% FCS, 2 mM glutamine, penicillin and streptomycin, SCF and IL3 (20ng/ml each).

CFC and LTC-IC assays.

Primary and secondary CFC and LTC-IC assays on MS5 stromal cells were performed as previously described.[30] Briefly, for the CFC assays, 5000 GFP+-sorted cells were plated directly after transduction and 25.000 GFP+ cells were used at later time points. For the colony re-plating experiments, two weeks after the primary plating, the colonies from one plate were collected, washed three times with PBS, and all the cells were plated in new methylcellulose for an additional two weeks. For the lymphoid CFC assays, 10,000 cells were plated in methylcellulose containing 4 ng/ml IL-7. Cultures were scored using a Leica DM-IL inverted microscope (Leica Microsystems, Rijswijk, the Netherlands) at a total magnification of 40x.

For the LTC-IC assays, newly transduced cells were sorted on MS5 stromal cells in limiting dilutions from 10 to 7290 cells per well in 96-well plates. Cultures were weekly demi-depopulated and fed with new medium. After 5 weeks of culture the wells containing cobblestone areas were scored after which the medium from the wells was aspirated and replaced with methylcellulose containing cytokines. After an additional two weeks of culture wells were scored as positive or negative to yield the LTC-IC frequency. Stem cell frequencies were calculated using L-Calc software (Stemsoft, Vancouver, British Colombia).

Immunoblotting and cytospins

Antibodies against BMI1 (Upstate, CA, USA), Abl and STAT5 (Santa Cruz, Heerhugowaard, The Netherlands) were used in a 1:1000 dilution. Anti-Actin antibody (Santa Cruz, Heerhugowaard, The Netherlands) was used in 1:2000 dilution and anti-β-Tubulin antibody (Roche Diagnostics) was used in a 1:1000 dilution. May-Grünwald Giemsa staining was used to stain

cytospins. Cytospin preparations were evaluated and photographed using a Leica DM3000 microscope (Leica Microsystems, Rijswijk, the Netherlands) equipped with a Leica DFC420C digital camera at a total magnification of 400x.

RNA extraction and real time PCR analysis

RNA was isolated from $1x10^5$ cell using RNeasy kit from Qiagen (Venlo, the Netherlands), reverse transcribed using M-MuLV reverse transcriptase (Fermentas, St Leon-Roth, Germany) and real-time amplified using iQ SYBR Green mix (Bio-Rad, CA, USA) on a MylQ thermocycler (Bio-Rad) and quantified using MylQ software (Bio-Rad). HPRT expression was used to calculate relative expression levels. Sequences and conditions are available on request.

Retroviral and lentiviral transduction of cord blood CD34+ cells

The MSCV-BMI1-IRES-GFP (MiGR1 BMI1) vector was cloned as previously reported.[26] The MSCV-BCR-ABL-IRES-GFP (MiGR1 BCR-ABL, expressing p210 BCR-ABL) vector was kindly provided by Dr. M.A.S. Moore (Memorial Sloan-Kettering Cancer Center, New York, USA) and the BCR-ABL gene was removed from this vector and inserted into MSCV-IRES-NGFR (MiNR1) vector by digestion with EcoRI. MiNR1 infected cells were stained with anti-NGFR-PE antibody (Becton Dickinson (Alphen a/d Rijn, the Netherlands) for analysis. Transductions of CB CD34+ cells were performed as described previously.[29-31] The CD34+ cells from chronic CML patients were pre-stimulated for 48h in the presence of IL3, IL6, G-CSF, SCF and FLT3-L. Retroviral supernatants were concentrated in Centriprep YK tubes (Millipore, Billerica, MA) at 3000g, 30 minutes on 4°C. Four consecutive rounds of 8-12 hours were performed.

Lentiviral vector expressing short hairpins against human BMI1 (CS-H1-shRNA-EF-1α-EGFP) and scrambled lentiviral vectors were a kind gift from Dr. Iwama (Chiba University, Japan). Lentiviral particles were produced by co-transfection of 293T cells with 0.7 μg pcDNA3-VSVg-REV, 3 μg pMDLg-RRE and 3 μg CS-H1-scrambled RNAi or CS-H1-BMI1 RNAi. The lentiviral supernatants were collected 24h later and were either used directly or stored at −80°C until further use.

In vivo transplantations into NOD-SCID mice

Eight to ten weeks old female NOD/SCID mice (NOD.CB17-Prkdcscid/J) were purchased from Charles River Laboratory-Netherlands. Prior to transplantations mice were sub-lethally irradiated with 3Gy. Two independent transductions and transplantation experiments were performed. In the first experiment the mice were injected with 4.6×10^5 and in the second experiment with 3.8×10^5 non-sorted CD34$^+$ cells into the retro-orbital vein immediately after transduction. Human cell engraftment was analyzed in the BM, PB, spleen and liver by flow cytometry after 6-25 weeks of transplantation. The bone marrow (two femurs) from two leukemic mice in experiment 2 (m5 and m7) was used for secondary transplantations into sub-lethally irradiated NOD-SCID mice in duplicate, whereby one-third of leukemic bone marrow cells of the primary mice was transplanted into each secondary recipient.

Flow cytometry analysis and sorting procedures

Antibodies were obtained from Becton Dickinson (Alphen /d Rijn, the Netherlands). Sorting of the CB into stem/progenitor fractions was performed as previously reported.[32,33] To deplete CLPs from the HSC fraction anti-CD2, CD3, CD4, CD7, CD8, CD10, CD19, CD20, and CD56 were included in our lineage cocktail. Analyses were performed on a FACS Calibur (Becton Dickinson) and sorting was performed on a MoFlo (Dako Cytomation, Carpinteria, CA, USA). Data were analyzed using FlowJo software (Tri Star, Inc, Ashland, OR, USA).

Ligation-mediated (LM) PCR

LM-PCR was performed as described[34]. PCR products were analyzed on a 2% agarose gel, gel-purified using the QIAquick Gel Extraction Kit (Qiagen) and sequence analyses was performed using the rvLTRIII primer.

RESULTS

BMI1 collaborates with BCR-ABL in inducing leukemia in NOD-SCID mice

Cord blood CD34$^+$ cells were transduced with retroviral BMI1-IRES2-EGFP and BCR-ABL-IRES2-tNGFR vectors and transplantations into sub-lethally irradiated NOD-SCID mice were performed. A control group of cells was co-transduced with MiGR1 and MiNR1 empty vectors. Efficiencies of double
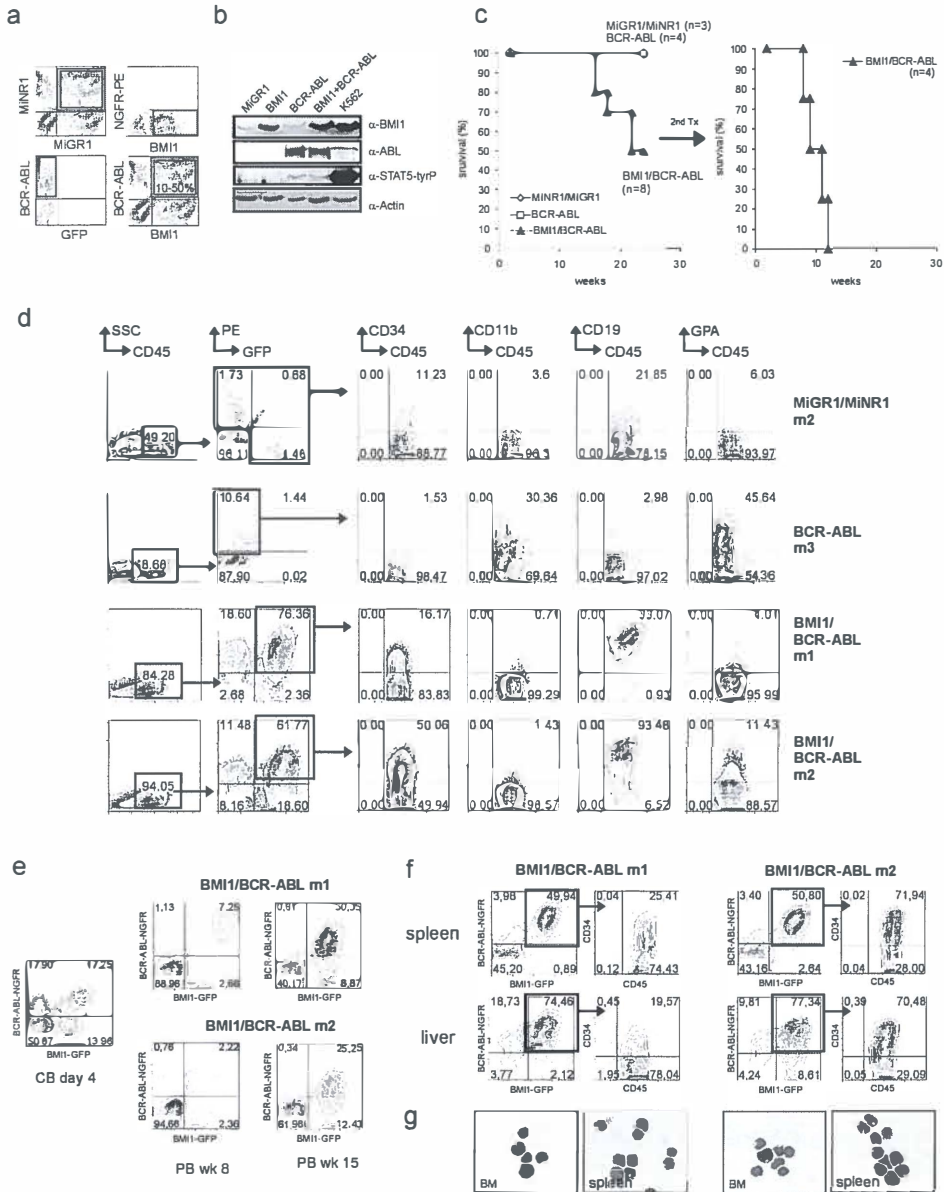
Figure 1 Introduction of BCR-ABL and BMI1 in human cord blood CD34+ cells induces lymphoid leukemia upon transplantation in NOD-SCID mice. (A) Cord blood CD34+ cells were transduced with MiGR1 and MiNR1 empty vector controls, with MiGR1 BMI1 or MiNR1 BCR-ABL vectors, or co-transduced with both vectors (BMI1/BCR-ABL). Efficiencies after 3 transduction rounds are shown. (B) Expression of BMI1, BCR-ABL and phosphorylated STAT5 was analyzed by Western blotting of transduced CB CD34+ cells. (C) Transduced CB CD34+ cells were transplanted into sub-lethally irradiated NOD-SCID mice and Kaplan Meier survival curves are shown. BM of two mice was used for 2nd transplantation into sub-lethally irradiated NOD-SCID mice in duplicate, and Kaplan Meier survival curves of these mice are shown as well. (D) FACS analysis of BM of engrafted NOD-SCID mice at week 16. (E) Transduced CB CD34+ cells were transplanted into sub-lethally irradiated NOD-SCID mice and the percentage of double positive cells was evaluated directly after transduction and at later time points in the PB of the recipient mice. (F) FACS analysis of the spleen and the liver of the diseased mice showed infiltration of the organs with primitive CD34+ human cells. (G) May-Giemsa-Grünwald staining of cytospins from BM and spleen of diseased mice.

transduced cells typically ranged from 10-50% (Figure 1A) and Western blot analysis of transduced cells confirmed expression of the introduced proteins (Figure 1B). Sub-lethally irradiated NOD-SCID mice received transplants of unsorted transduced cells in two independent experiments (n=8 for BMI1/BCR-ABL, n=4 for BCR-ABL mice and n=3 for control MiNR1/MiGR1 mice, Table 1).

sample	Chimerism (%)			phenotype
	BM	spleen	liver	
MiNR1/MiGR1 m1	0.7	nd	nd	no leukemia
MiNR1/MiGR1 m2	1.1	nd	nd	no leukemia
MiNR1/MiGR1 m3	2.1	nd	nd	no leukemia
BCR-ABL m1	1.5	nd	nd	no leukemia, lymphoid/erythroid engraftment
BCR-ABL m2	2.2	nd	nd	no leukemia, erythroid engraftment
BCR-ABL m3	10.6	nd	nd	no leukemia, myeloid/erythroid engraftment
BCR-ABL m4	1.8	nd	nd	no leukemia, lymphoid/erythroid engraftment
BMI1/BCR-ABL m1	76.4	49.9	74.5	leukemia, lymphoid and erythroid engraftment
BMI1/BCR-ABL m2	61.8	50.8	77.3	leukemia, lymphoid and erythroid engraftment
BMI1/BCR-ABL m3	1.2	nd	nd	no leukemia
BMI1/BCR-ABL m4	0.5	nd	nd	no leukemia
BMI1/BCR-ABL m5*	53.8	59.2	63.8	leukemia, lymphoid and erythroid engraftment
BMI1/BCR-ABL m6	5.6	nd	nd	no leukemia
BMI1/BCR-ABL m7*	89.4	59.1	59.3	leukemia, lymphoid and erythroid engraftment
BMI1/BCR-ABL m8	4.3	nd	nd	no leukemia

nd=not determined; *=used for 2nd transplantation

Table 1. Summary of NOD-SCID engraftment studies

The recipients of cells transduced with control MiNR1/MiGR1 retroviruses displayed no overt pathology (Figure 1C, 1D and Supplementary Figure 1). The recipients of BCR-ABL-transduced CB cells remained healthy throughout the observation time of 25 weeks. The engraftment efficiencies ranged

between 1.5-10.6%, and differentiation of donor-derived cells was skewed towards the erythroid lineage (Figure 1D and Supplementary Figure 2), in line with previously published observations.[10] Our previous studies indicated that BMl1 expression by itself was also not sufficient to induce leukemia in mice, in line with data published by others.[26,27] In contrast, four out of eight mice that received co-transduced BMI1/BCR-ABL cells died 16-22 weeks after transplantation (Figure 1C, 1D and Supplementary Figure 3). Mice appeared sick and lethargic, with strongly elevated peripheral nucleated white blood cell counts, moderate splenomegaly, lymphadenopathy and more than 20% poorly differentiated lymphoblasts in the bone marrow as revealed by May-Grünwald-Giemsa staining and phenotyping (Figure 1D, 1G). A strong proliferative advantage of BMI1/BCR-ABL cells was observed when compared to single-transduced populations as the percentages of double transduced cells in the PB and BM increased from 10-20% at the beginning of the experiment to 60-80% at week 18 (Figure 1E). FACS analysis of BM, spleen and liver confirmed the lymphoblastic phenotype as the leukemic cells were highly positive for CD34 (16-91%) as well as CD19 (93-99%) antigens (Figure 1D, 1F, 1G and Supplementary Figure 3). Within the timeframe of our experiments no signs of leukemic development were observed in 4 mice, possibly due to relatively low levels of engraftment of double transduced cells.

To determine whether self-renewing leukemic stem cells were still present within the leukemic graft in the primary recipients, secondary transplantations were performed. Bone marrow of BMI1/BCR-ABL mice 5 and 7 was transplanted into sub-lethally irradiated NOD-SCID recipients. Robust engraftment was observed in all transplanted animals, resulting in a fatal leukemia within 8-12 weeks (Figure 1C) with high infiltration of lymphoid CD34+/CD19+ blast cells in BM, spleen and liver (Supplementary Figure 3). Retroviral integration site analyses were performed by LM-PCR in primary and secondary recipients, and these studies indicated that different clones from 1 transduction experiment gave rise to a similar leukemia in primary mice (nrs 5 and 7, Supplementary Figure 4). Comparison of integration sites between primary and secondary recipients revealed that similar clones could give rise to leukemia upon serial transplantation (5 versus 5.1 and 5.2 and 7 versus 7.1 and 7.2, Supplementary Figure 4). Sequence analysis of a number of integration sites showed no integrations in close proximity to known oncogenes (data not shown). Deletions of Ikaros gene are frequently observed in BCR-ABL-induced lymphoblastic leukemia.[35] However, we did

not detect Ikaros deletions in the leukemic cells isolated from secondary recipient animals (Supplementary Figure 5). Expression levels of BMI1 and BCR-ABL in the BM of primary and secondary recipients is provided in Supplementary Figure 6A and B. Taken together, these data show that constitutive expression of BMI1 and BCR-ABL can functionally collaborate to induce acute lymphoid leukemia.

Co-expression of BMI1 and BCR-ABL in human CD34+ cells promotes long-term myeloid or lymphoid expansion and stem cell self-renewal

To better understand the mechanism underlying the leukemic transformation we performed ex vivo long-term experiments. BMI1, BCR-ABL, or double-transduced CB CD34+ cells were cultured on MS5 stromal cells under myeloid growth conditions. After 5 weeks both suspension and adherent human CD45+ cells were harvested from the cultures and the cells were seeded onto new stroma to evaluate their replating capacity (Figure 2A, 2B). Cells transduced with MiGR1/MiNR1 only transiently expanded over a 3-5 week period but could not be replated (Figure 2B). BMI1-transduced cells could be re-plated once after the first 5 weeks of culture on MS5, in agreement with our previous observations,[26] but 3rd replating did not result in the establishment of new expanding self-renewing cultures. While the BCR-ABL-transduced cells initially displayed a very strong proliferative potential, secondary cultures could not be established. In strong contrast, the double transduced BMI1/BCR-ABL cells displayed a strong proliferative advantage (Figure 2A) and expanding cultures could be maintained for over 20 weeks by sequential replating (Figure 2B). Four independent BMI1/BCR-ABL lines were generated, displaying a doubling time of about 1.5 days (Figure 2A). Phase-dark Cobblestone Area Forming Cells (CAFCs) appeared within three days after plating onto MS5 and reappeared upon each replating of BMI1/BCR-ABL cells (Figure 2C). These CAFCs represent an immature population of hematopoietic stem/progenitor cells and have been shown to contain self-renewal potential based on their serial replating potential, particularly in case when oncogenes are overexpressed.[30] Progenitors were maintained in BMI1/BCR-ABL cultures as determined by CFC assays using suspension cells from MS5 co-cultures at week 9 (Figure 2D). While in previous studies we have observed that CB CD34+ cells transduced with BMI1 were able to maintain progenitors for up to 16 weeks in liquid culture, this was not observed in MS5 co-cultures.[26] This is in line with a more moderate long-term proliferative advantage of BMI1-transduced cells
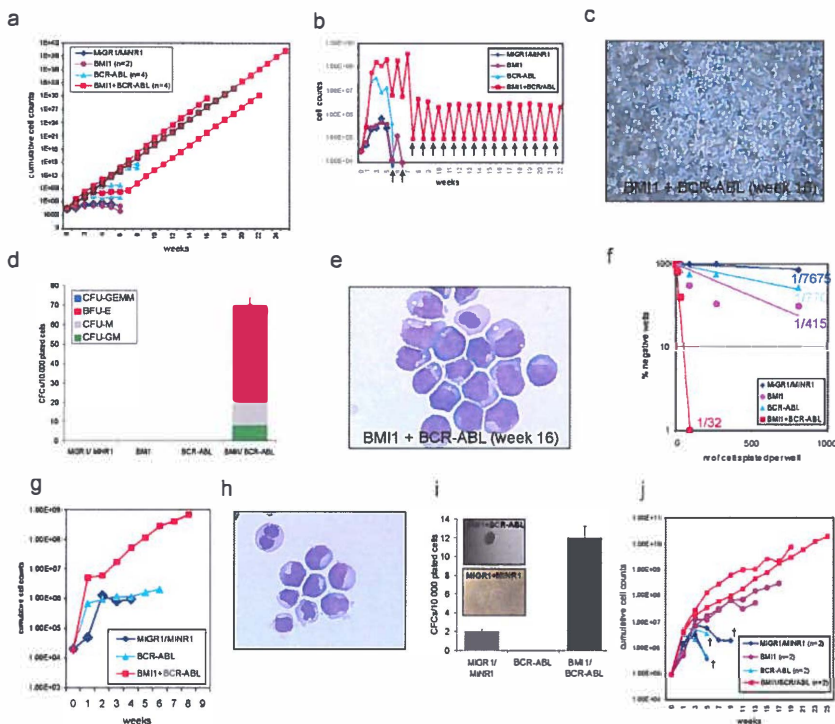
Figure 2 Co-expression of BCR-ABL and BMI1 in human CD34^{+} cells promotes long-term stem cell self-renewal and myeloid and lymphoid expansion in vitro. (A) BMI1/BCR-ABL co-expression results in immortalization of human CB CD34^{+} cells on MS5 stroma under myeloid conditions. The difference in proliferative capacity between the groups is shown. (B) A detailed representation of the experiment in Fig. 2a is shown. After 5 weeks both suspension and adherent human CD45^{+} cells were harvested from the cultures and the cells were seeded onto new stroma to evaluate their replating capacity. Arrows indicate time points when replating was performed. (C) An example of week 18 phase-dark Cobblestone Area Forming Cells (CAFCs) that could be harvested and replated to initiate new expanding co-cultures. (D) The number of hematopoietic progenitors was evaluated in colony forming cell (CFC) assays in methylcellulose. A representative experiment is shown where transduced CB CD34+ cells were analyzed after 9 weeks expansion on MS5 bone marrow stroma. (E) Cytospin preparations of suspension cells from co-cultures described in A at week 16 indicating the presence of blast-like cells as well as erythroid and myeloid precursor cells. (F) Stem cells frequencies were determined in LTC-IC assays using freshly transduced CB CD34^{+} cells. (G) Long-term expansion of the transduced cells under lymphoid culture conditions. (H) Representative cytospin at week 8 from double transduced cultures. (I) Clonogenic assays in methylcellulose were performed to evaluate the capacity to form progenitor colonies in the presence of IL7. Microscopic images of the colonies are shown as the insets in the graph. (J) Immediately after transduction the cells were plated in stroma-free liquid cultures in the presence of IL3 and SCF. Long-term expanding BMI1/BCR-ABL cultures could be maintained for over 23 weeks.

in MS5 stromal cocultures compared to liquid cultures we have observed in our previous studies and described here.[26] FACS analysis of long-term BMI1/BCR-ABL cultures revealed expression of CD45 and CD117 as well as myeloid and erythroid markers (CD33, CD71 and GPA) (Supplementary Table 1). Cytospin preparations showed the presence of blast-like cells as well as erythroid and myeloid precursor cells (Figure 2E). Despite the presence of cells with an immature morphology, expression of CD34 was lost upon long-

term culturing under myeloid conditions. This indicates that a population of cells which lack CD34 expression but most likely express CD117 are able to maintain long-term self-renewal properties under these conditions. Long-Term Culture-Initiating Cell (LTC-IC) assays using freshly transduced CB CD34$^+$ cells indicated that stem cell frequencies in the BMI1/BCR-ABL double-transduced cells were ~240-fold higher compared to controls (Figure 2F).

Since the in vivo leukemic transformation in NOD-SCID mice was biased towards lymphoid leukemia, we questioned whether ex vivo long-term cultures could also be established under lymphoid-permissive conditions. Single or double-transduced CB CD34$^+$ populations were expanded on MS5 under lymphoid growth conditions. A proliferative advantage was particularly observed in the BMI1/BCR-ABL double-transduced cells, and long-term self-renewing cultures could only be established by sequential replating of BMI1/BCR-ABL cultures (Figure 2G). Morphological analysis revealed generation of immature lymphoblastic cells (Figure 2H) which was confirmed by FACS analysis showing the presence of CD34 and CD19-positive cells (Supplementary Table 2). Clonogenic growth in methylcellulose in the presence of IL7 was observed with BMI1/BCR-ABL cells, but not with BCR-ABL cells and only to a limited extent in the control groups (Figure 2I). To determine the necessity of a bone marrow microenvironment, we performed stroma-free liquid cultures driven by IL3 and SCF. While control cells were able to expand over a limiting period of 5-9 weeks, the BMI1/BCR-ABL cells could initiate cultures that maintained proliferative activity for over 25 weeks, resulting in strong expansion of >1×10^{10} fold (Figure 2J). BMI1 cells were able to expand for ~13-20 weeks, as previously reported.[26] Importantly, although BCR-ABL-transduced cells displayed a strong enhanced initial expansion at week 1 as compared to control cultures, no long-term propagating cultures could be established, suggesting that BCR-ABL$^+$ cells still depend on additional cues from the bone marrow microenvironment. Phenotypical FACS analysis of BMI1/BCR-ABL cultures revealed bi-phenotypic expression of erythroid and myeloid differentiation markers over a period of 15 weeks (Supplementary Table 3).

Together, these data indicate that co-expression of BMI1 and BCR-ABL in human CD34$^+$ cells results in a strong proliferative advantage and elevated long-term self-renewal potential under lymphoid as well as myeloid-permissive conditions.

HSCs are the most effective target cells for long-term expansion and self-renewal induced by co-expression of BMI1 and BCR-ABL.

To further distinguish which population of cells may be responsible for the initiation of long-term cultures, transduced HSCs, CMPs, MEPs and GMPs were sorted as illustrated in Figure 3A. Purity of progenitor sorts was determined by CFC assays (Figure 3B). All stem and progenitor
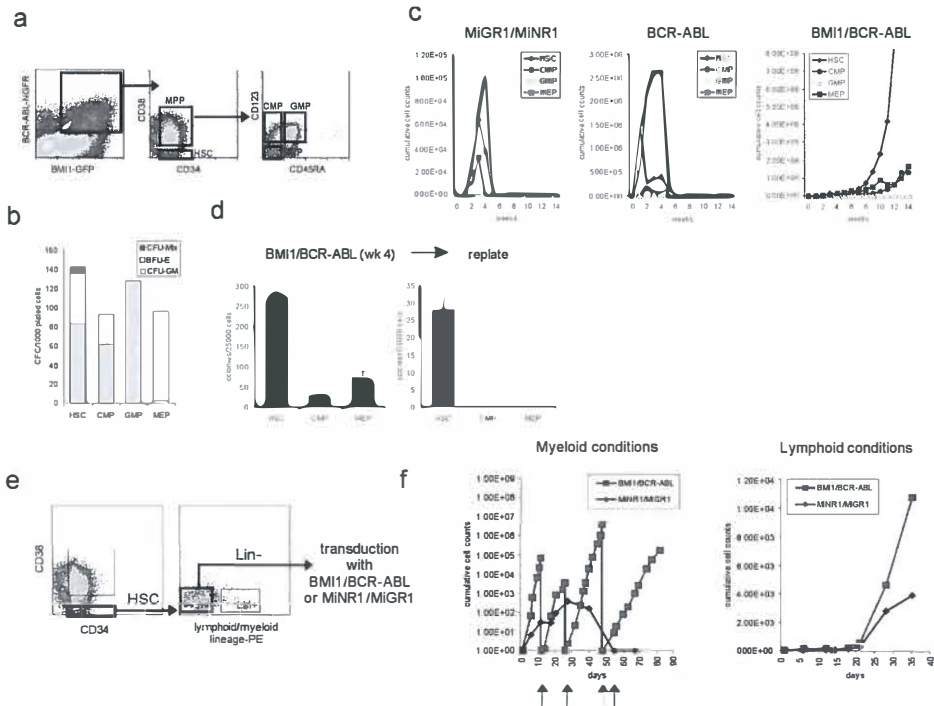

Figure 3 Co-expression of BCR-ABL and BMI1 results in long-term expansion and self-renewal of HSCs but not CMPs, GMPs, or MEPs. (A) Sorting strategies of the transduced cells on the basis of combinatorial expression of cell-surface markers. Four different populations of cells were sorted from each group as indicated. (B) CFC assay from the MiGR1/MiNR1 group revealed high purity of the sorted populations. (C) HSCs, CMPs, GMPs and MEPs from all the groups were grown on MS5 stroma to evaluate their proliferative capacity. Maximal expansion was observed in cultures initiated with HSCs, and moreover only the BMI1/BCR-ABL HSCs could be maintained long-term. (D) Cells were plated weekly in methylcellulose and colonies were scored on the basis of their morphology, a representative example at week 4 is shown. The HSC fraction yielded highest progenitor frequencies and only this fraction contained self-renewal properties as determined by secondary replating assays. (E) Sorting scheme for Lin- HSCs that were used in transduction experiments. (F) Transduced Lin- HSCs were cultured on MS5 bone marrow stromal cells under myeloid or lymphoid conditions. Arrows indicate time of replating.

compartments transduced with the control MiNR1/MiGR1 vectors generated progeny in MS5 co-cultures, whereby maximal expansion was observed in cultures initiated with HSCs. However, none of the cultures could be maintained beyond 5 weeks (Figure 3C). Interestingly, MEPs

transduced with BCR-ABL showed a strong, but transient proliferative capacity. Co-expression of BMI1 and BCR-ABL conferred long-term self-renewal and expansion to the HSC population but much less efficiently to the progenitor subpopulations (Figure 3C). After 4 weeks of MS5 coculture, only the cultures initiated with BMI1/BCR-ABL-transduced HSCs contained self-renewing progenitors (Figure 3D).

To further deplete CLPs from the HSC fraction, we sorted CD34+/CD38-/Lin- cells using an extended lineage cocktail containing antibodies against CD2, CD3, CD4, CD7, CD8, CD10, CD19, CD20 and CD56 (Figure 3E). After MoFlo sorting, cells were transduced with MiNR1/MiGR1 or BMI1/BCR-ABL and plated onto MS5 under myeloid and lymphoid coculture conditions, and similar results were obtained as in previous experiments (Figure 3F and Supplementary Table 4).

Enhanced proliferation and self-renewal of BCR-ABL+ chronic phase CML CD34+ cells by retroviral introduction of BMI1

Finally, we isolated CD34+ cells from CML patients in chronic phase that expressed relatively low endogenous BMI1 as compared to blast crisis CML (Figure 4A), and asked whether retroviral introduction of BMI1 in BCR-ABL+ cells would affect long-term growth and self-renewal. Upon over-expression of BMI1 we observed increased proliferation capacity of the transduced CML cells on MS5 stromal cells under myeloid conditions, and cultures could be replated for an additional 4 weeks in two out of four CML patients (Figure 4B). As depicted in Figure 4C, when non-adherent cells from week 5 co-cultures were analyzed for the presence of progenitors, over-expression of BMI1 resulted in the production of more CFUs which had retained self-renewing ability as determined by sequential replating of the colonies in methylcellulose assays. Progenitor replating activity was also studied in three additional CP-CML samples directly after transduction with BMI1 or MiGR1 control vectors, and these studies further confirmed that over-expression of BMI1 enhances self-renewal of CP-CML progenitor cells (Figure 4D). Although increased expansion and self-renewal was observed in MS5 co-cultures, maturation of the progeny was not impaired and no signs of blast crisis were observed within this timeframe of the experiment (data not shown). In line with these observations, the CD34 percentage was not maintained but declined throughout the culture period under myeloid conditions.

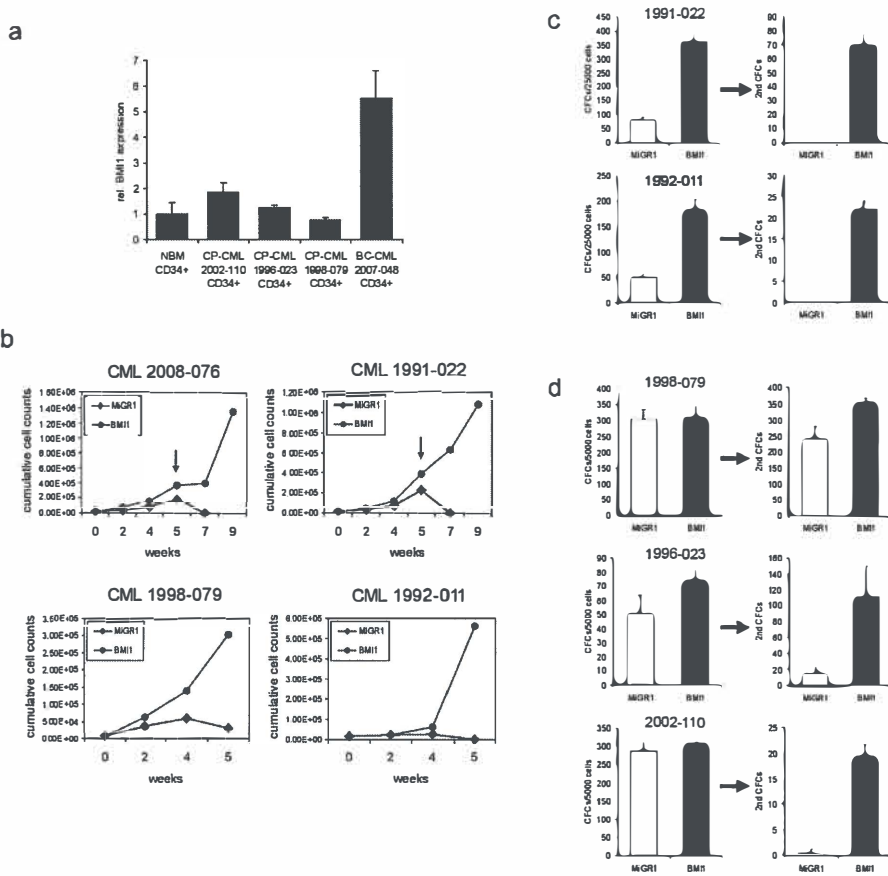In order to study the necessity of BMI1 expression for long-term expansion of

Figure 4 Enhanced proliferation of BCR-ABL+ chronic phase CML CD34+ cells by retroviral introduction of BMI1. (A) RNA was isolated from CD34+ cells of CP-CML patients, one BC-CML patient and normal bone marrow (NBM) and BMI1 expression was determined by Q-RT-PCR. (B) CD34+ cells from CP-CML patients were transduced with control or MiGR1 BMI1 vectors and cells were cultured on MS5 stromal cells and cumulative cell counts in suspension are shown. Where indicated by the arrows, cultures were harvested and replated onto new stroma. (C) Suspension cells from week 5 co-cultures of transduced CP-CML samples were used to perform CFC assays. Progenitor self-renewal was analyzed by replating the cells in secondary CFC assays and only the BMI1 over-expressing cells had re-plating ability. (D) CD34+ cells from CP-CML patients were transduced with control or MiGR1 BMI1 vectors, transduced cells were sorted by MoFlo, and CFC frequencies as well as CFC replating capacity were determined in mehylcellulose assays. (E) CD34+ cells from a BC CML sample were transduced with lentivirus encoding shRNA sequences against BMI1 or scrambled and then cultured on MS5 stromal cells.

BC CML samples, we transduced CD34+ cells isolated from a BC CML patient with lentiviral vectors encoding short-hairpin RNAi sequences against BMI1 or scrambled, and performed long-term co-cultures on MS5 stroma. As shown in Figure 4E, long-term expansion of was severely impaired by down-modulation of BMI1.

Taken together, these data suggest that BMl1 might collaborate with BCR-ABL in progression of the disease of the CML patients.

DISCUSSION

While it is clear that in over 95% of the CML cases leukemia is induced by the p210 BCR-ABL oncogene, it is highly likely that additional mutations or alterations are required to induce the transition from chronic phase into blast crisis. Our current data indicate that elevated levels of the polycomb group protein BMI1 can act as a collaborating event with BCR-ABL to induce leukemic transformation of human cells. Interestingly, a recent report indicated that BMI1 expression levels were significantly higher in patients with advanced-phase than in patients with chronic phase CML.[24] In addition, the level of BMI1 at diagnosis correlated with the time to transformation into blast crisis, and low BMI1 expression levels were associated with an improved overall survival.[24] Some previous reports have also indicated that BMI1 can act as a collaborating factor in the process of cellular transformation. In a provirus integration screen, BMI1 was originally identified as a co-operating factor with Myc in the induction of B cell lymphomagenesis.[25] BMI1 appears not to be sufficient to induce leukemia by itself as we and others observed previously.[26,27] Even though we cannot exclude the possibility that BMI1 might enhance the life-span of lymphoid cells, in our current studies in which non-sorted BMI1, BCR-ABL and double transduced BMI1/BCR-ABL cells were injected into NOD-SCID mice, only double transduced populations were capable of initiating a transplantable leukemia. A number of reports have highlighted the role that BMI1 fulfills in both normal as well as leukemic stem cell self-renewal.[22,23,26,27] Thus, it appears likely that BMI1 contributes to the process of leukemic transformation by acting as a stem cell self-renewal factor in CML as well.

Although the mechanisms by which BMI1 contributes to BCR-ABL-induced leukemia remain to be elucidated, it is well documented that the p16INK4a/p19ARF locus, which is required to bypass senescence of embryonic fibroblasts, is repressed by BMI1.[36] Also in hematopoietic cells, targeted deletion of BMI1 resulted in increased expression of p16 and p19[37]. Deletion of p16/p19 in $bmi1^{-/-}$ HSCs only partly restored self-renewal and over-expression of BMI1 could still increase progenitor levels in the absence of p16/p19, indicating that other BMI1 targets must exist as well.[37] Indeed, we also observed a strong down-regulation of p16 and p14 (human homologue of p19) in BMI1/BCR-ABL transduced cells (data not shown). Although direct downstream targets have not been described yet, forced expression of BMI1 promotes symmetric cell divisions of HSCs and expansion of immature

multipotent progenitors.[27] Thus, BMI1 might contribute to tumorigenesis by bypassing senescence and maintaining the life-span of stem cells, as well as increasing self-renewal by allowing symmetric cell divisions. This would also imply that the BMl1-expressing stem cells might be more prone to acquire yet additional mutations that ultimately aid in the development of leukemia. Recently, we observed that downmodulation of BMl1 in human CB CD34$^+$ cells impairs long-term expansion and self-renewal.[38] This was associated with enhanced levels of apoptosis, and coincided with elevated levels of ROS accumulation. In line with these observations, it was recently shown that BMl1$^{-/-}$ mice are characterized by elevated levels of ROS, impaired mitochondrial function and impaired DNA damage responses.[39] These data suggest that protection against oxidative stress might be one of the functions of BMl1 in HSCs and leukemic stem cells.

Deletion of both the p16INK4a/p19ARF locus, as well as deletion of IKAROS and PAX5 have been implicated in the acute lymphoid, but not myeloid, leukemias initiated by BCR-ABL.[35,40,41] We have currently no indications that IKAROS or PAX5 expression levels are changed in BMl1/BCR-ABL-transduced cells, but since we did observe a strong bias towards a lymphoid leukemia in our NOD-SCID mouse model it is possible that BMl1-mediated repression of the p16INK4a/p14ARF locus drives transformation along the lymphoid lineage. A recent murine model demonstrated that in the absence of p19ARF a lymphoid progenitor served as the target for transformation by BCR-ABL.[42] Another study showed that the growth and survival of aged lymphoid progenitors was increased by BMl1-mediated repression of p16 and p19.[43] In light of these findings we have questioned whether a lymphoid progenitor rather than an HSC is the target of transformation in our model. However, our data pinpoint to a multipotent progenitor or HSC as the most efficient target cell, although we can not exclude the possibility that in vivo the lymphoid progenitor might also be a target cell of transformation. In vitro, both myeloid as well as lymphoid long-term self-renewing cultures could be established, most efficiently from HSC-transduced cell populations. Although in vivo we observed a strong lymphoid bias in transplanted NOD-SCID animals, it will be interesting to study whether mouse models that allow more myeloid engraftment, such as was recently elegantly used to study MLL-induced transformation,[44] also allow in vivo transformation along the myeloid lineage of BMl1/BCR-ABL-transduced cells.

Treatment of CML patients with the inhibitor Imatinib leads to response rates of over 95%.[45] Yet, the leukemia-initiating cells are not targeted

efficiently [46,47], and patients might need to stay on therapy life-long. A significant proportion of patients develops resistance to therapy, often due to mutations in the kinase domain of BCR-ABL.[48,49] Thus, identification of additional targets that facilitate the eradication of BCR-ABL+ leukemia-initiating cells is needed. Our data indicate that self-renewal of Ph+ cells is enhanced by over-expression of BMI1, both in CB model systems as well as in primary CP-CML patient samples. Since in CML patients BMI1 levels also increase upon progression towards blast crisis and correlate with a poor prognosis,[24] BMI1 appears to be an attractive candidate for targeting. Indeed, we observed that lentiviral downmodulation of BMI1 in a BC CML sample was sufficient to impair long-term expansion on MS5 stroma. Our model system allows the identification of BMI1-downstream pathways that collaborate with BCR-ABL-induced leukemic transformation. Inhibitors against BMI1 (or downstream targets) are currently being evaluated, and these should also be tested for their efficacy in eradication of the BCR-ABL+ LSC in CML.

ACKNOWLEDGEMENTS

AUTHOR CONTRIBUTIONS

A.R. and S.J.H performed experiments and analyzed data, S.O. provided technical assistance, B.D., A.A. and R.v.O. assisted in NOD-SCID engraftment studies, V.v.d.B. performed LM-PCR studies, E.V. and G.d.H. analyzed and

discussed data, A.R. and J.J.S. designed the experiments, analyzed and discussed data, and wrote the manuscript.
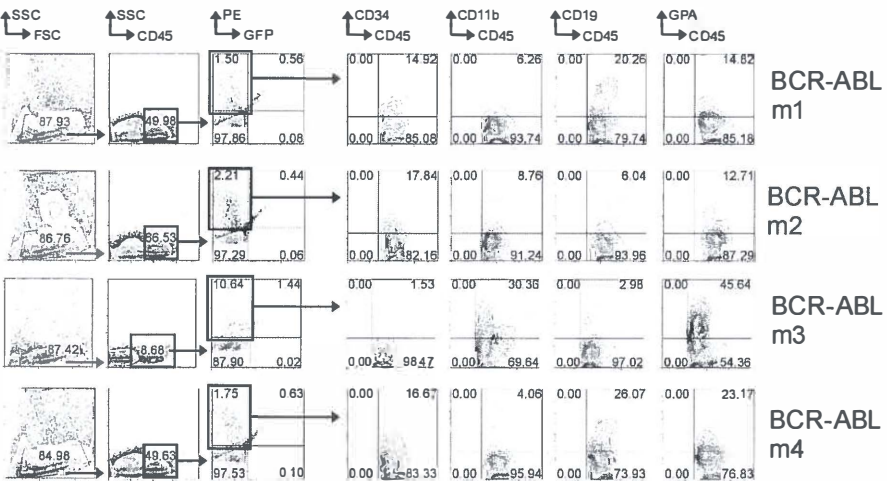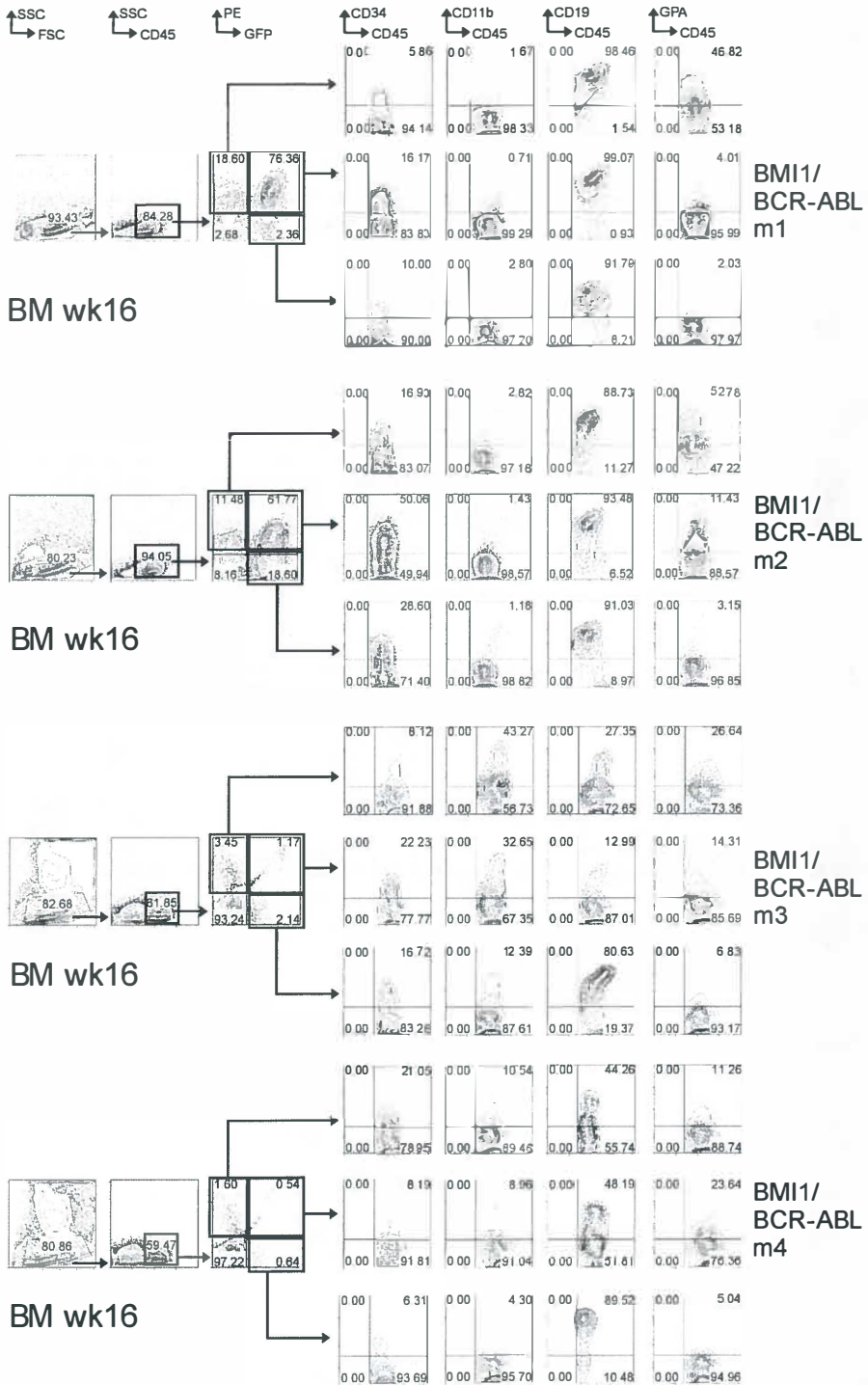
SUPPLEMENTARY TABLES AND FIGURES
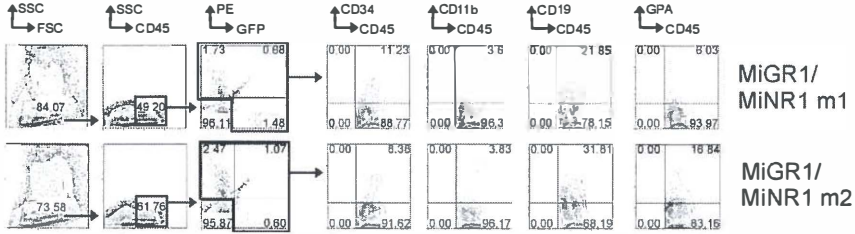
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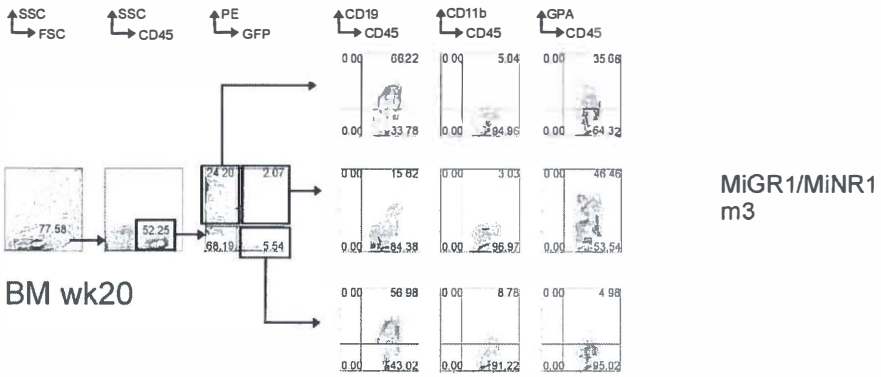
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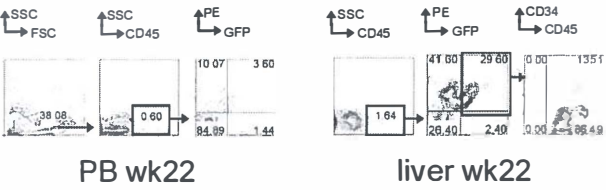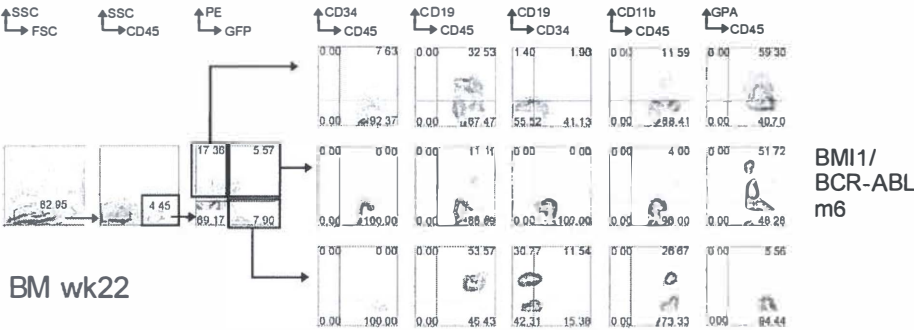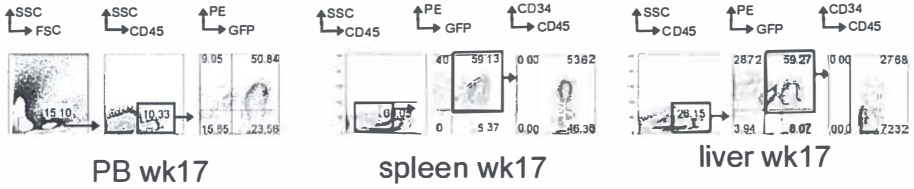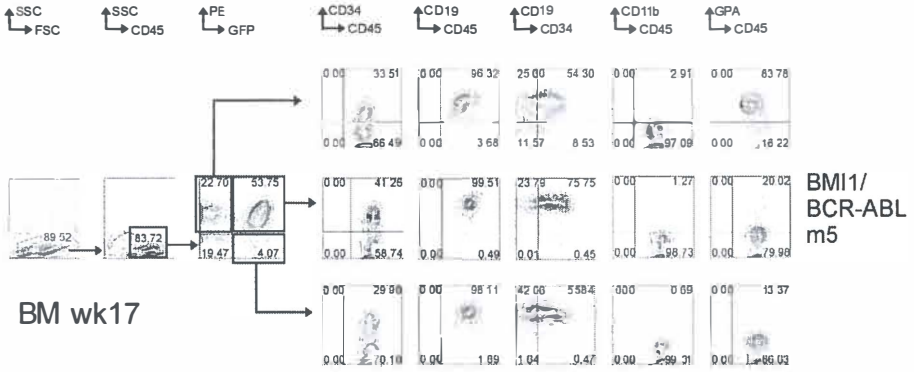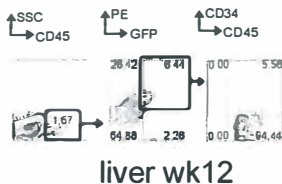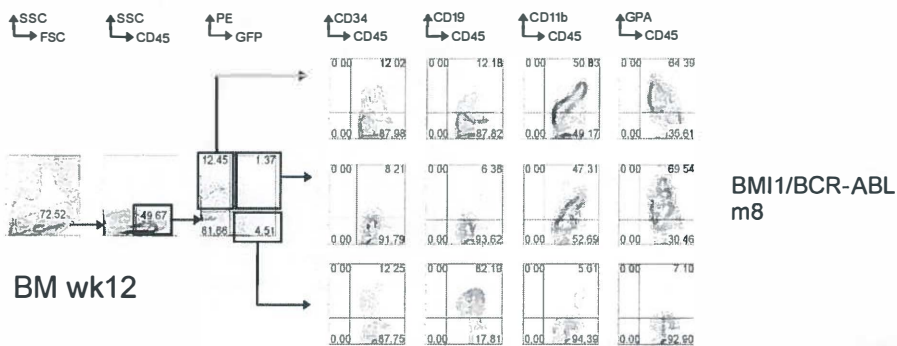**Supplementary Figure 1.** Analysis of MiGR1/MiNR1 control mice

BM wk16

Supplementary Figure 2. Analysis of BCR-ABL mice.
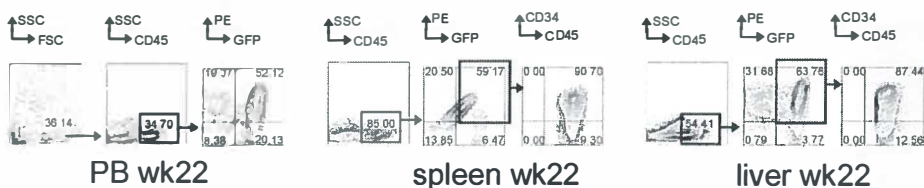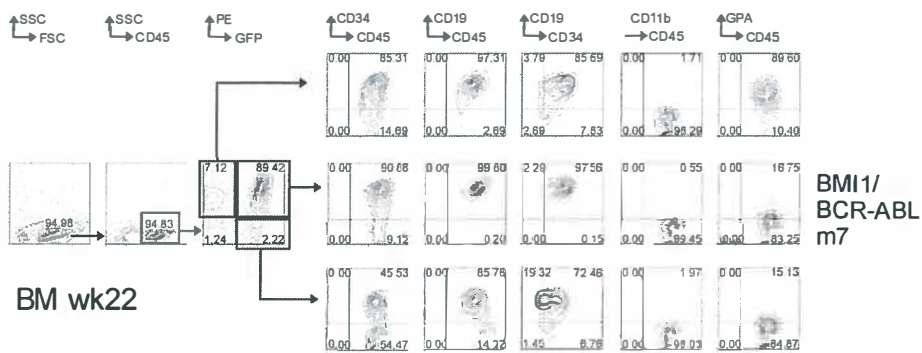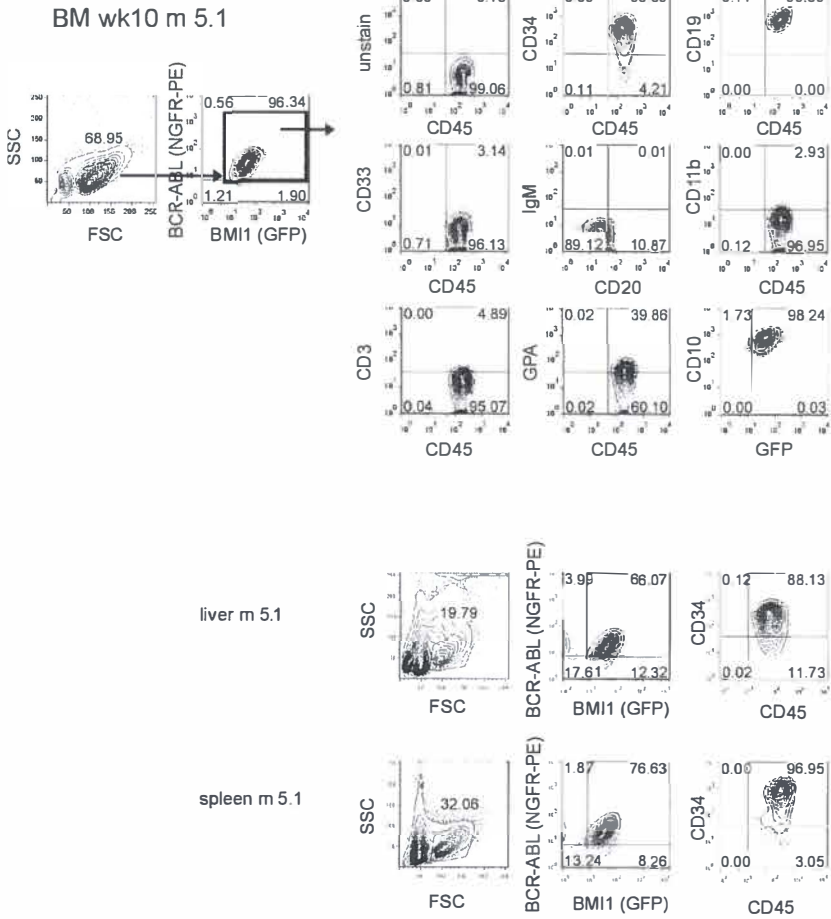
Supplementary Figure 3. Analysis of BMI1/BCR-ABL mice

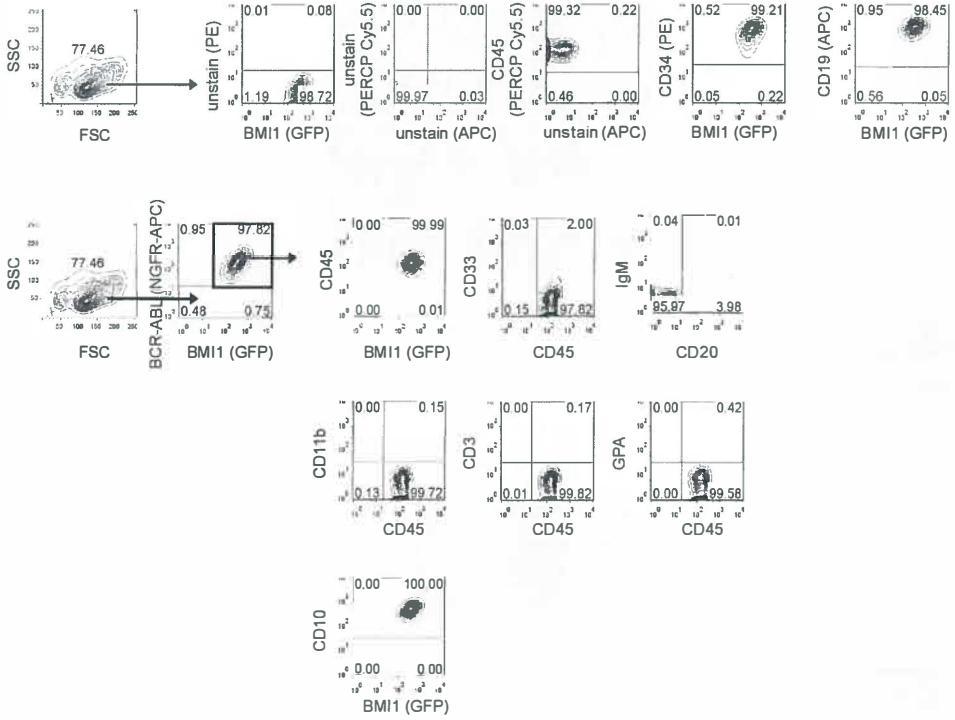**Supplementary Figure 3 continued.** Analysis of BMI1/BCR-ABL mice

Supplementary Figure 3 continued. Analysis of BMI1/BCR-ABL mice

Supplementary Figure 3 continued. Analysis of 2nd BMI1/BCR-ABL mice

BM wk11 m7.1

SPLEEN m7.1
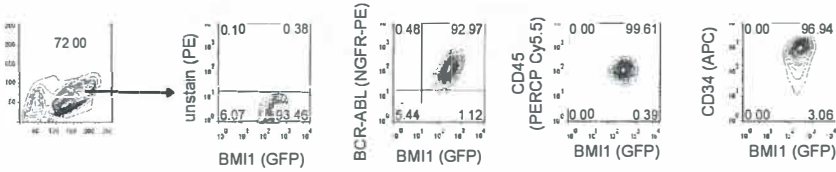
LIVER m7.1
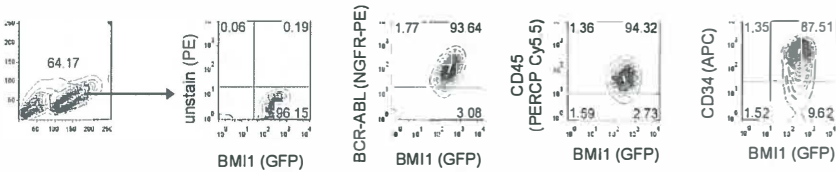
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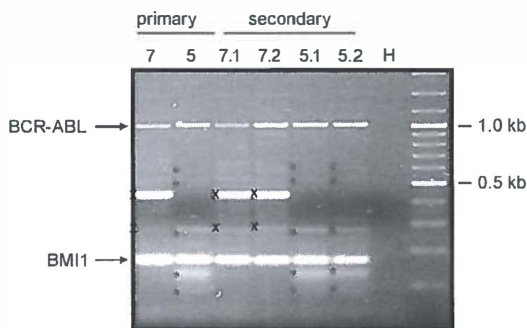
Supplementary Figure 4. LM-PCR on primary and 2nd transplanted NOD-SCID mice. Bone marrow from primary mice 5 and 7 was used for transplantation into 2nd recipients (5.1, 5.2, 7.1 and 7.2). Genomic DNA was isolated from the BM of leukemic primary and secondary transplanted mice, and LM-PCR was preformed.
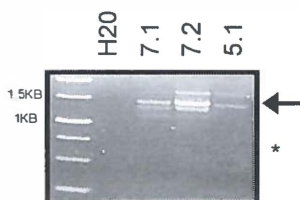
Supplementary Figure 5. Ikaros is not deleted in 2nd transplanted NOD-SCID mice. Arrow indicates normal isoforms, Ikaros deletions would have shown up at the asterisk.

marker	Week 4 (%)	Week 15 (%)
CD34	9	0
CD45	100	50
CD117	nd	100
CD19	nd	0
CD22	nd	5
CD3	nd	0
CD14	3	0
CD13	nd	0
CD33	nd	100
GPA	64	100
CD71	81	90

Supplementary Table 1. FACS profiles of BMI1/BCR-ABL-transduced CB CD34+ cells in MS5 cocultures under myeloid conditions

	Week 2 (%)			Week 4 (%)		
	MiGR1	BCR-ABL	BMI1/BCR-ABL	MiGR1	BCR-ABL	BMI1/BCR-ABL
CD34	4	0	1	0	0	53
CD11b	20	3	30	38	3	31
CD71	13	80	98	15	80	87
CD19	0	0	2	19	0	32
CD20	0	0	0	0	0	0

Supplementary Table 2. FACS profiles of transduced CB CD34+ cells in MS5 cocultures under lymphoid conditions

marker	Week 4 (%)	Week 15 (%)
CD34	1	0
CD45	100	100
CD117	nd	80
CD19	nd	0
CD22	nd	0
CD3	nd	0
CD14	5	0
CD13	nd	30
CD33	35	50
GPA	58	100
CD71	60	81

Supplementary Table 3. FACS profiles of BMI1/BCR-ABL-transduced CB CD34+ cells under liquid culture conditions

marker	myeloid conditions Week 4 (%)	lymphoid conditions Week 4 (%)
CD11b	7	26
CD14	0	4
CD15	94	34
CD71	97	55
GPA	66	14
CD19	nd	20

Supplementary Table 4. FACS profiles of BMI1/BCR-ABL-transduced CB CD34+ HSCs depleted of CLPs

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SUMMARIZING DISCUSSION AND FUTURE PERSPECTIVES
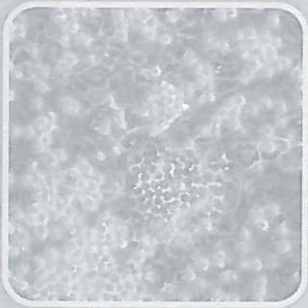
SUMMARY

Hematopoietic stem cells fulfill a crucial role in supplying an organism with sufficient mature cells during normal homeostasis as well as in times of tissue generation or repair. Two unique features characterize hematopoietic stem cells: the ability to generate new pluripotent stem cells (to self-renew) and the ability to give rise to differentiated progeny that has lost its self-renewal capacity. Our understanding of the mechanisms that determine whether, where and when a stem cell will self-renew or differentiate is still limited, but recent advances have indicated that both stem cell intrinsic mechanisms as well as extrinsic mechanisms provide essential cues that direct these cell fate decisions. Moreover, loss of control over these cell fate decisions might lead to cellular transformation and cancer. In **chapter 2**, we discuss that HSC quiescence, self-renewal and differentiation can be regulated by these intrinsic and extrinsic mechanisms. Extrinsic mechanisms include changes in stem cell fate that are dictated by the bone marrow microenvironment, also termed the stem cell the niche. Direct physical association between HSCs and niche cells such as osteoblasts and stromal cells via for instance cadherins and integrins such as VLA4 and VLA5 mediate processes like adhesion and migration of the stem cell. Once appropriately localized within the quiescent niche, processes such as self-renewal, maintenance of quiescence, or exit from the niche followed by proliferation and differentiation are highly controlled by growth factors and cytokines that are secreted locally by osteoblasts and stromal cells. Examples of such molecules are TGF-β which is a negative regulator of the cell growth, ANG1 responsible for the stem cell quiescence or WNTs and FGF-1 which promotes stem cell expansion. These factors can now dictate HSC fate by triggering specific signaling downstream modulators within the HSC, such as Myc, β-Catenin, STATs, SMADs or C/EBPα. Intrinsic mechanisms are niche-independent and these might for instance affect the epigenetic state of HSC. These intrinsic factors might include chromatin remodelers and Polycomb Repression Complex (PRC) members.

In **chapter 3**, we demonstrated that enforced expression of BMI1 in cord blood CD34+ cells resulted in long-term maintenance and self-renewal of human hematopoietic stem and progenitor cells. LTC-IC frequencies were increased upon stable expression of BMI1 and BMI1-transduced cells engrafted more efficiently in NOD-SCID mice. Week 5 CAFCs from stromal cocultures could be serially replated to give rise to secondary CAFCs. Serial transplantation

studies in mice revealed that secondary NOD-SCID engraftment was only achieved with cells overexpressing BMI1. Importantly, BMI1-transduced cells could proliferate in stroma-free cytokine-dependent cultures for over 20 weeks while a stable population of about 1-5% of CD34$^+$ progenitor cells was preserved that retained the capacity to form CFCs. While control cells lost most of their NOD-SCID engraftment potential after 10 days of ex vivo culturing in absence of stroma, NOD-SCID multilineage engraftment was retained by overexpression of BMI1. Thus, our data indicate that self-renewal of human hematopoietic stem cells is enhanced by BMI1 and we classify BMI1 as an intrinsic regulator of human stem/progenitor cell self-renewal.

Since high expression of BMI1 in acute myeloid leukemia (AML) cells is associated with an unfavorable prognosis, we studied the effects of downmodulation of BMI1 in normal and leukemic CD34$^+$ AML cells using a lentiviral RNAi approach in **chapter 4**. We demonstrate that downmodulation of BMI1 in cord blood CD34$^+$ cells impaired long-term expansion and progenitor forming capacity, both in cytokine-driven liquid cultures as well as in bone marrow stromal cocultures. In addition, Long Term Culture-Initiating Cell (LTC-IC) frequencies were dramatically decreased upon knock-down of BMI1, indicating an impaired maintenance of stem and progenitor cells. The reduced progenitor and stem cell frequencies were associated with increased expression of p14ARF and p16INK4A and enhanced apoptosis, which coincided with increased levels of intracellular reactive oxygen species (ROS) and reduced FOXO3A expression. In AML CD34$^+$ cells downmodulation of BMI1 impaired long-term expansion, whereby self-renewal capacity was lost as determined by the loss of replating capacity of the cultures. These phenotypes were also associated with increased expression levels of p14ARF and p16INK4A. Together our data indicate that BMI1 expression is required for maintenance and self-renewal of normal and leukemic stem and progenitor cells and that expression of BMI1 protects cells against oxidative stress.

The major limitation for development of curative cancer therapies has been incomplete understanding of the molecular mechanisms driving cancer progression. Human models to study development and progression of chronic myeloid leukemia (CML) had not been established, and expression of only BCR-ABL in human CD34$^+$ cells is not sufficient to induce leukemia in xenograft models. In **chapter 5**, we show that co-expression of BMI1 with BCR-ABL is sufficient to induce a fatal leukemia in NOD-SCID mice

transplanted with transduced human CD34^{+} cells within 4-5 months. The leukemias were transplantable into secondary recipients with a shortened latency of 8-12 weeks. Clonal analysis revealed that similar clones initiated leukemia in primary and secondary mice. In vivo, transformation was biased towards a lymphoid blast crisis, and in vitro both myeloid as well as lymphoid long-term self-renewing cultures could be established. Retroviral introduction of BMI1 in primary chronic phase CD34^{+} cells from CML patients elevated their proliferative capacity and self-renewal properties. Thus, our data identify BMI1 as a potential therapeutic target in CML.

FUTURE PERSPECTIVES

Our studies as well as others have shown that BMI1 fulfills important roles in both normal human hematopoietic stem cells as well as in leukemic CD34^{+} cells. While overexpression of BMI1 is sufficient to sustain stem cell self-renewal in vitro and in vivo, downmodulation of BMI1 impairs long-term expansion and self-renewal. BMI1 is frequently overexpressed in various hematological malignancies, and downmodulation of BMI1 in AML CD34^{+} cells is sufficient to impair their long-term growth in vitro. Also, co-expression of BMI1 can act as a collaborating hit with oncogenes such as BCR-ABL. However, various aspects regarding polycomb signaling in normal and tumor cells still need to be unraveled.

It is clear that repression of the INK4A/ARF locus is one mode of action by which BMI1 exerts its actions on hematopoietic stem/progenitor cells. In senescence screens it was observed that BMI1-mediated repression of the INK4A/ARF locus is required to bypass senescence of embryonic fibroblasts.[1] Also in hematopoietic cells, targeted deletion of BMI1 resulted in an increase in the expression of p16 and p19.[2] However, deletion of p16/p19 in *Bmi1*^{-/-} HSCs partly restored self-renewal, but not completely, and overexpression of BMI1 could still increase progenitor levels in the absence p16/p19, indicating that other BMI1 targets must exist as well.[2] Which other genes are under the direct control of BMI1 in hematopoietic stem cells still needs to be elucidated. Also, it is currently unclear whether similar or different target genes are under the control of BMI1 in normal versus leukemic stem cells.

Protection against oxidative stress and apoptosis have emerged as an important BMI1-downstream pathways as well, either by reducing p53 levels

via BMI1-mediated repression of the INK4A/ARF locus, or via modulation of the oxidative stress response in an INK4A/ARF-independent manner. Downmodulation of BMI1 resulted in an accumulation of ROS levels, both in knockout mouse models as well as in human CD34^{+} cells transduced with lentiviral BMI1 RNAi vectors.[3,4] The induction of ROS in the absence of BMI1 could be counteracted by treatment with antioxidants such as n-acetylcysteine (NAC), but appeared to be independent of INK4A/ARF in hematopoietic cells.[3] In *Bmi1^{-/-}* mice, the increase in ROS coincided with an increase in DNA damage and an activation of the DNA damage repair pathways, and treatment with NAC or targeting of Chk2 at least partially restored some the phenotypes.[3] A number of genes that have been described to regulate intracellular redox homeostasis were found to be de-repressed in *Bmi1^{-/-}* mice.[3] In human CD34^{+} cells, downmodulation of BMI1 coincided with decreased expression of FOXO3.[4] *FoxO3A^{-/-}* HSCs are defective in their competitive repopulation capacity, lost their quiescence and displayed elevated ROS levels.[5] Thus, BMI1 might be required to protect hematopoietic stem/progenitor cells from apoptosis or loss of quiescence induced by oxidative stress conditions but additional studies are required to further substantiate these findings.

A direct role for BMI1 in the process of DNA repair was recently demonstrated as well. It was observed that BMI1 and RING2 are recruited to sites of DNA double-strand breaks (DSBs) where they contribute to the ubiquitylation of gamma-H2AX.[6] Another study indicated that BMI1 was enriched at the chromatin after irradiation and colocalized and copurified with ATM and the histone gammaH2AX in CD133^{+} glioblastoma cells.[7] Thus, these data suggest a novel function for BMI1 in maintaining genomic stability, and it will be interesting to determine whether such a role for BMI1 is also important in the process of leukemic transformation.

While gain of BMI1 function might be involved in extending the lifespan of normal and leukemic stem cells by bypassing senescence, more direct control over the fate of hematopoietic stem cell divisions appears to exist also. Although the molecular mechanisms remain to be elucidated, the symmetry of cell division of HSCs is directed towards a more symmetric mode of cell division upon overexpression of BMI1.[8] While under normal homeostasis HSCs might divide asymmetrically, resulting in one new HSC and one daughter cell that has lost stem cell integrity and will differentiate, high BMI1 levels might dictate a more symmetric distribution of specific proteins, mRNAs or other metabolites during mitosis whereby stem cell

integrity is maintained in both daughter cells. How BMl1 would be involved in such processes remains unclear.

While the composition of PRC1 and PRC2 complexes can vary between specific cell types, little is known on how this is regulated and how the composition of PRC complexes affects silencing of specific subsets of genes in a specific cell type. BMl1 is expressed in HSCs, but its expression decreases upon maturation.[9-12] In contrast, other PRC1 components including Mel18, Cbx and Mph1/Rea28 are expressed at rather low levels in HSCs but increase upon differentiation.[10,11] This appears contradictory, as it is proposed that all PRC1 subunits are required for appropriate chromatin remodeling by PRC1. Although very little is currently known on how PRC1 complex composition is linked to the regulation of specific subsets of genes, it has been suggested that the relative amounts of BMl1 within the PRC1 complex determine its biochemical and biological functions.[13] Recently, it was suggested that the presence of BMl1 and MEL18, as well as CBX7 and CBX8 in the PRC1 complex is mutually exclusive[14,15], although no information is currently available on the functional consequences of these differences in PRC1 composition. Challenges for the future lie in the further unraveling of gene networks that are under the control of BMl1, and how regulation of these genes affects the fate of normal hematopoietic and leukemic stem cells. It is becoming clear that PRC1 complex composition is not static, and it will be interesting to determine whether complex composition is different between hematopoietic stem cells and progenitors, or between leukemic and normal stem cells, and how complex composition might relate to specific target gene regulation.

Signaling pathways that regulate the expression of BMl1 have not been extensively studied. SALL4, an oncogene that is expressed in AML[16] is capable of upregulating BMl expression by direct binding to its promoter.[17] Moreover, transgenic mice that constitutively express Sall4 have increased levels of BMl1 expression, and levels increase even further upon progression from a preleukemic myelodysplastic stage towards a more acute myeloid leukemic phenotype.[17] Hedgehog signaling is also capable of upregulating Bmi1 expression.[18] Furthermore, BMl1 expression appears to be under the control of miRNAs, including miR128[19] and miR200c[20], although the potential involvement of this regulation in the development of leukemia still needs to be determined. BMl1 is regulated at the post-translational level as well, since it has been demonstrated that PTEN can bind to BMl1, thereby inhibiting its function.[21]

It has become clear that BMI1 expression can participate in the development of hematological malignancies, for instance as a cooperating factor with Myc in the induction of B cell lymphomagenesis[22], as an essential self-renewal factor for the maintenance of HoxA9-Meis1 LSCs *in vivo*[23] or as a collaborating factor with BCR-ABL in inducing a fatal transplantable leukemia in human xenograftmodels.[24] Furthermore, BMI1 expression is elevated in variety of hematological tumors, including Non-Hodgkin Lymphomas and Acute Myeloid Leukemias (AML)[25-28] and it was shown that the expression level of BMI1 was sufficient to predict overall survival, relapse-free-survival and remission duration in a multivariate analysis in AML.[25] In chronic myeloid leukemia (CML), BMI1 expression increases with disease progression and high levels of BMI1 correlate with reduced overall survival.[29] A small study performed on myelodysplastic syndrome (MDS) samples demonstrated that high BMI1 expression correlated with poor overall survival in those patients as well.[30] In chronic lymphocyte leukemia (CLL) and mantle cell lymphoma (MCL) BMI1 expression was also elevated, which in about 10% of the MCL cases was due to *BMI1* gene amplification.[31] Currently, drugs are being developed that inhibit the function of BMI1, and it will be interesting to determine whether such inhibitors will be beneficial in the treatment of hematological malignancies.

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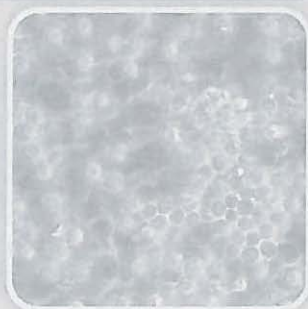
NEDERLANDSE SAMENVATTING

De verschillende bloedcellen worden gemaakt in het beenmerg in een hiërarchisch georganiseerd proces genaamd hematopoïese. Aan de basis van deze hiërarchie staan de hematopoïetische stamcellen (HSC), de "moedercellen" van alle bloedcellen. De stamcellen kunnen delen tot meer stamcellen, door middel van een proces dat zelfvernieuwing genoemd wordt. Of ze vormen voorlopercellen, tijdens het proces dat differentiatie genoemd wordt. Hematopoïese is nauwkeurig gereguleerd en hematopoïetische aandoeningen, zoals leukemieën, treden op wanneer deze regulatie verloren gaat. Een aantal verschillende soorten van extracellulaire en intracellulaire signalering alsmede adhesie moleculen zijn geïdentificeerd die een rol spelen bij de regulering van deze processen. Het identificeren van de genetische en moleculaire factoren die deze moleculen beïnvloeden is een belangrijke uitdaging voor het medisch onderzoek. Onze kennis van de mechanismen die bepalen of, waar en wanneer een stamcel zich zal vernieuwen of differentiëren is nog beperkt, maar recente ontwikkelingen hebben aangegeven dat de stamcellen van hun omgeving, de zogenaamde micro-omgeving of niche, essentiële signalen krijgen die deze beslissingen aansturen. In hoofdstuk 2 wordt ingegaan op het huidige begrip van de moleculaire mechanismen die zelfvernieuwing van hematopoïetische stamcellen reguleren in de niche. Ook werd onderzocht hoe leukemische transformatie van stamcellen hun afhankelijkheid van de micro-omgeving voor zelfvernieuwings- en celoverlevingsprocessen verandert. De traditionele opvatting over de ontwikkeling van de kanker is dat het een genetische ziekte is, die veroorzaakt wordt door het stapsgewijs verkrijgen van mutaties die leiden tot activering van oncogenen (genen die tot kanker leiden) en/of verlies-van-functie van tumor suppressor genen (genen die normaal aanwezig zijn in de cel om de groei van kankercellen te stoppen). Echter, onlangs is duidelijk geworden dat de ontwikkeling van tumoren ook veroorzaakt kan worden door veranderde genexpressie ten gevolge van mechanismen die niet bepaald worden door mutaties in het DNA. Dit zijn zogenaamde 'epigenetische veranderingen ". Deze mechanismen omvatten verlies van DNA-methylatie in het gehele genoom of kleine regionale toenames in DNA-methylatie op specifieke plaatsen in het DNA. Ook veranderingen in patronen van methylatie van histon-eiwitten spelen een rol. Een voorbeeld van een dergelijke epigenetische regulator is het Polycomb

eiwit BMI1. In dit proefschrift onderzochten we de rol van BMI1 in normale humane hematopoïetische stamcellen. Tevens werden de mechanismen bestudeerd waarmee BMI1 zou kunnen bijdragen aan transformatie tot leukemie.

Een manier om de effecten van een bepaald gen in stamcellen te bestuderen is het gebruik van virussen zoals virale vectoren om het gen van belang in de cel te introduceren. In hoofdstuk 3 laten we resultaten zien van experimenten waarbij BMI1 werd geïntroduceerd in CD34+ cellen uit menselijk navelstrengbloed, dat volwassen stamcellen en voorlopercellen bevat. We vonden dat de stamcelpopulatie langduriger behouden kon blijven wanneer BMI1 verhoogd tot expressie gebracht werd en daarnaast dat er sprake was van een toegenomen zelfvernieuwing van de menselijke stam- en voorlopercellen. Dit werd gevonden in celkweken (in vitro) maar ook in vivo nadat cellen getransplanteerd werden naar immuun-deficiënte muizen die fungeren als "gastheer" voor de humane stamcellen (in vivo xenograft modellen). We hebben twee verschillende types van in vitro culturen uitgevoerd. Het eerste type is het kweken van CD34+ cellen waarin het gen BMI1 is geïntroduceerd zonder "omliggende cellen" in zogenaamde stroma-vrije culturen. Opzienbarend was dat deze cellen meer dan 16 weken kunnen groeien en we konden tevens aantonen dat deze cellen op dit tijdstip nog steeds voorlopercel-activiteit hadden door het uitvoeren van kolonie-assays. Het andere type kweek was op stromale cellen, die dienen als "omliggende cellen" en een micro-omgeving vormen. In deze zogenaamde co-culturen hebben de cellen met het BMI1 gen ook een groeivoordeel en behouden hun voorlopercel-activiteit, maar niet in die mate als in de stroma-vrije culturen. Tot slot hebben we de cellen van de stroma-vrije culturen getransplanteerd in immuun-deficiënte muizen en bewezen hiermee dat BMI1 inderdaad de stamcellen en voorlopercellen in stand kunnen houden wanneer ze worden gekweekt in de stroma-vrije culturen.

Een andere mogelijkheid om de rol van een gen te bestuderen is om "knock-out" of "downmodulatie" van het gen te bewerkstelligen in de cel en het gedrag van de cellen te volgen zonder dat gen aanwezig is. In hoofdstuk 4 hebben we downmodulatie van BMI1 expressie in normale hematopoïetische voorloper- en stamcellen uit navelstrengbloed bestudeerd, maar ook in CD34+ cellen van acute myeloïde leukemie patiënten. We hebben dezelfde typen van culturen uitgevoerd zoals beschreven in hoofdstuk 3. We hebben kunnen aantonen dat de downmodulatie resulteert in verminderde

zelfvernieuwing van de twee celtypen, wat betekent dat zowel de normale als leukemische cellen BMI1 nodig hebben voor hun groei. Verminderde zelfvernieuwing ging gepaard met een verhoogde expressie van het locus INK4/ARF wat door BMI1 gereguleerd wordt. Ook werd een toename van celdood gevonden in combinatie met verhoogde spiegels van reactieve zuurstof radicalen en verminderde FOXO3A expressie.

Om de pathologie van leukemieën te bestuderen en om nieuwe geneesmiddelen tegen leukemie te testen, zijn pre-klinische diermodellen nodig die de ziekte imiteren. In hoofdstuk 5 is daarom het effect van BMI1 bij chronische myeloïde leukemie (CML) bestudeerd. De patiënten met CML hebben het BCR-ABL oncogen in hun (leukemie) cellen. We hebben BMI1 samen met BCR-ABL ingebracht in CD34+ cellen uit navelstrengbloed en getransplanteerd in immuun-deficiënte muizen. Na 16-22 weken werden de muizen ziek en stierven aan leukemie, wat niet het geval was als alleen BMI1 of alleen BCR-ABL werd ingebracht en de cellen geïnjecteerd in de muizen. Dit was een bewijs dat de twee genen beiden nodig zijn voor de ontwikkeling van de leukemie. Sterker nog, introductie van BMI1 in primaire CD34+ cellen van CML patiënten in chronische fase verhoogde hun proliferatieve capaciteit en zelfvernieuwing eigenschappen. Tot slot, uit onze gegevens blijkt dat BMI1 samen met BCR-ABL verantwoordelijk is voor leukemische transformatie, en ons systeem van transplantatie van humane cellen in immuun-deficiënte muizen, kan een zeer bruikbaar model worden om de pathologie van leukemie te onderzoeken en om nieuwe drugs te testen.

In hoofdstuk 6 is het experimentele werk van dit proefschrift samengevat en besproken, en worden mogelijke toekomstige richtingen geëvalueerd. Onze studies hebben aangetoond dat BMI1 een belangrijke rol speelt in zowel de normale humane hematopoïetische stamcellen als in leukemische CD34+ cellen. Terwijl verhoogde expressie van BMI1 voldoende is om stamcel zelfvernieuwing te handhaven in vitro en in vivo, belemmert downmodulatie van BMI1 de groei op lange termijn en de zelfvernieuwing van stamcellen. BMI1 komt vaak tot verhoogd tot expressie in diverse hematologische maligniteiten, en downmodulatie van BMI1 in AML CD34+ cellen bleek voldoende om hun groei op lange termijn in vitro te voorkomen. Ook kan co-expressie van BMI1 fungeren als een tweede hit en samenwerken met oncogenen zoals BCR-ABL om leukemie te veroorzaken. Momenteel zijn geneesmiddelen in ontwikkeling die de functie van BMI1 remmen, en het zal interessant zijn om te bepalen of dergelijke remmers ten goede zal komen bij de behandeling van hematologische maligniteiten.

APPENDIX
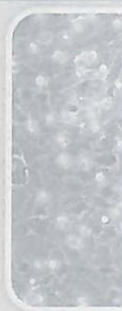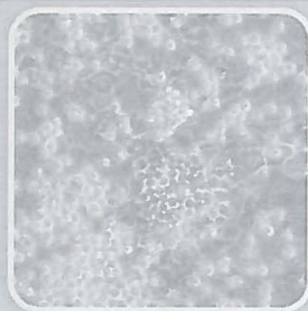
ACKNOWLEDGEMENTS

This has been a long journey. I feel relieved that it's over, but am I am also glad it was actually lengthy – it gave me a chance to learn a lot – bad and good. I learned a lot of science, but I gained a lot of live experience as well – I guess this is all about doing a PhD. I would like to take the chance to thank the people who helped me along the way.

Foremost, I want to thank my promoters, Prof. Gerald de Haan, Prof. Jan Jacob Schuringa and Prof. Edo Vellenga. Dear Gerald – it has been amazing the way we've met, the time I spent in your lab and how much I learned from you. You thought me a lot of science, but equally important, you thought me that science is not only researching, but also networking, talking to people and having fun. Thank you for all the support, independence and trust you gave me. Dear JJ – writing the two words would be trivial how much I appreciate your guidance in the past years. You are probably the most enthusiastic and motivating person I've ever met. After each scientific discussion we've had I felt like running back in the lab and doing the experiment, no matter how many times I've failed before. Dear Edo – your intriguing clinical questions on every graph I've shown you thought me to never forget the patients and always look at the potential clinical relevance of the obtained lab data. Thank you for your supervision and honest discussions.

I would also like to thank the members of the reading committee, Prof. dr. Tariq Enver, Prof. dr. Paul Coffer and Prof. dr. Ody Sibon, for their willingness and time to review my thesis.

My dearest colleagues, I owe so many "THANK YOU'S" to all of you. Dear Bert – your help had been instrumental for the quality of my publications. I feel happy and proud that we were able to establish the NOD/SCID transplantation model and I want to thank you for coming to the lab even after your official retirement day. Sandra – your energy and enthusiasm and the ability to perform experiments as accurate as possible is something I want to thank you for. We've teamed so well that the big and tiresome experiments we'd plan seemed easy and full of fun. Sarah – I cannot thank you enough for finishing the experiments for the last paper we've published. It was comforting to know that the work will be done in the best possible way once I am not in the lab. Thank you for all the time and efforts you put into the preparation of my promotion, despite the fact that you were on the crossroad yourself. I am happy to have you as a colleague and paranypmh, but even more as a fond friend. Dearest Alice – your support and friendship

throughout the years meant a lot to me. Thank you for being always there for me, whether it was an experiment that needed to be done or just a discussion that had to happen "at that moment".

My dear roommates - I enjoyed sharing the room with you. Ronald – having such an experienced scientist next to my desk was a real luck. I enjoyed our discussions and your scientific advices have been valuable to me. A huge "thank you" for all the translations in Dutch for the documents needed to be included in this thesis. Anita, Karen and Martha - it was fun to talk with you on other but scientific topics. Karen – I hope to get your booklet soon!

Dear Bertien and Ellen – once Bert left the lab I had the chance to briefly work with you. Thank you for the help with the animal experiments, it meant a lot especially when huge experiments were planed. Lenya – in your own peculiar way you thought me good science. Thank you for this. Brad – your arrival in the lab was a real boost. I enjoyed the long brainstorming discussions with you and your questions and suggestions have been of a great value to me. I hope you made a good use of the single-cell technique. Joyce, Leone and Vincent – we didn't have the chance to work together, but It was great to have you around and chat just about anything. And then comes the "younger" generation of SCB – Marta, Olya and Genia – good luck with all the research and learn the lesson from me – do not start your next job before your finish the writing of your thesis.

Bart-Jan – do you remember that you helped me with the very first PCR I performed to clone the BMI1 gene? Look what came and is still coming out of that! Thank you very very much for this. Hein – with the magic touch you gave to the BCR-ABL-NGFR PG13 cells I was able to perform all of the experiments in chapter 5 – thanks a bunch! Dear Djoke – your desire to help to just about everyone is amazing. You've helped me at many instances and I want to thank you for that. Marjan – thank you for showing me the way around in the very first months in the lab. Every beginner would greatly appreciate this. Gerwin, Szabolcs, Lina, Carolien, Liendsy, Karen, Annet, Monika, Ingrid, Marcel, Annelies, colleagues from the Pediatric Oncology – it has been great experience to work and to share the lab with you. Thank you for the questions and suggestions on the Monday morning meetings.

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In these years, coincidentally or not, I had the chance to again visit Japan and spend one month in the lab of Prof. Hiro Nakauchi at the Univeristy of Tokyo

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Dear mami i tato - I only hope that Pedro and I will be as good parents to Isabel and the newcomer, as you've been to Biljana and me. Thank for teaching me that continuous education is the key to success in live. I love you from the bottom of my heart. Dear Mara and Hernan Rizo, thank you for all the caring and support in the past years regardless of the distance that has been between us.

My dearest Biljana - my sister, my best friend, my best colleague....I adore you. Together with Gjole, enjoy the coming big steps in your lives.

Cielo......what's next?:)

LIST OF PUBLICATIONS

1. **Rizo A*,** Horton SJ*, Olthof S, Dontje B, Aussema B, van Os R, van den Boom V, Vellenga E, de Haan G, Schuringa JJ. BMI1 collaborates with BCR-ABL in leukemic transformation of human CD34+ cells. Blood, 2010; 116(22):4621-30.
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3. **Rizo A**, Dontje B, Vellenga E, de Haan G, Schuringa JJ. Long-term maintenance of human hematopoietic stem/progenitor cells by expression of BMl1. Blood, 2008 (5):2621-30.
4. Gosliga D, Schepers H, **Rizo A,** Kolk D, Vellenga E, Schuringa JJ. Establishing long-term cultures with self-renewing Acute Myeloid Leukemia stem/ progenitor cells. Exp Hem, 2007 (10):1538-49.
5. **Rizo A,** Vellenga E, de Haan G, Schuringa JJ. Signalling pathways in self-renewing hematopoietic and leukemic stem cells: do all stem cells need a niche? Hum Mol Genet, 2006;15:R210-9
6. **Crcareva A**, Saito T, Kunisato A et al. Hematopoietic stem cells expanded by fibroblast growth factor-1 are excellent targets for retrovirus-mediated gene delivery. Exp Hem 2005; 33(12):1459-69

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1. **Rizo A,** et al.; BMI1 collaborates with BCR-ABL in leukemic transformation of human CD34$^+$ cells. 3rd Annual Dutch Haematology Meeting; Jan 2009, Papendal, the Netherlands. (oral presentation)
2. **Rizo A,** et al.; BMI1 collaborates with BCR-ABL in leukemic transformation of human CD34$^+$ cells. 50th Annual Meeting of ASH; Dec 2008, San Francisco, CA. (poster presentation)
3. **Rizo A,** et al.; The polycomb gene Bmi-1 collaborates with BCR-ABL in leukaemic transformation of human cord blood cells. 6th Annual Meeting of the ISSCR, June 2008, Philadelphia, PA (poster presentation)
4. **Rizo A,** et al.; The polycomb gene Bmi-1 collaborates with BCR-ABL in leukaemic transformation of human cord blood cells. Keystone Symposia, Feb 2008, Vancouver, Canada. (poster presentation)
5. **Rizo A,** et al.; The polycomb gene Bmi-1 collaborates with BCR-ABL in leukaemic transformation of human cord blood cells. 2nd Annual Dutch Haematology Meeting; Jan 2008, Papendal, the Netherlands. (oral presentation)
6. **Rizo A,** et al.; Enforced expression of BMI-1 facilitates ex-vivo expansion of human cord blood CD34$^+$ cells. 36th Annual Meeting of ISEH; Sept 2007, Hamburg, Germany. (poster presentation)
7. **Rizo A,** et al.; Ex-Vivo Expansion of Human Cord Blood CD34$^+$ Cells by Overexpression of Bmi-1. 1st Annual Dutch Haematology Meeting; March 2007, Pappendal, the Netherlands. (oral presentation)
8. **Rizo A,** et al.; Ex-Vivo Expansion of Human Cord Blood CD34$^+$ Cells by Overexpression of Bmi-1. 48th Annual Meeting of ASH; Dec 2006, Orlando, FL. (poster presentation)
9. **Crcareva A,** et al.; Successful retroviral gene transfer to hematopoietic stem cells highly expanded by FGF-1. 34th Annual Meeting of ISEH; July 2005; Glasgow, Scotland. (poster presentation)
10. **Crcareva A,** et al.; Long-term and multilineage reconstitution with retrovirus-transduced hematopoietic stem sells expanded in non-serum condition with FGF1. 45th Annual Meeting of ASH, Dec 2003; San Diego, CA. (poster presentation)
11. Saito T, Ichikawa M, **Crcareva A,** et al.; Notch2 is indispensable for marginal Zone B lineage development, and transcriptionally regulates Hes1, Deltex1 and macrophage related genes.; 45th Annual Meeting of ASH, Dec 2003; San Diego, CA. (oral presentation)
12. **Crcareva A,** et al.; Time-to-relapse period shorter than 12 months is an extremely unfavourable prognostic sign in patients with Hodgkin's disease. 7th Congress of EHA; June 2002; Florence, Italy. (poster presentation)
13. **Crcareva A,** et al; Validation of the IPI concept on a single-centre study. 3rd International Symposium on Hodgkin Lymphoma; Sept 2001; Cologne, Germany. (poster presentation)

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