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Integrin α2 and Akt in early hematopoiesis

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Abstract

Hematopoiesis is a tightly regulated process in which hematopoietic stem cells reside at the apex of the hierarchy, and produce all kinds of mature blood cells by differentiation to replenish the cell loss in homeostasis and acute injury. In past few decades, much effort has been made to purify hematopoietic stem cells (HSCs) and lineage-committed progenitor cells both in mouse and human, enabling further characterization of these cell populations not only in normal hematopoiesis, but also in various hematological malignancies. However, while the isolation of different cell populations in mouse hematopoietic system is achieved with a very high purity, purification of human hematopoietic stem and progenitor cells still far lags behind.

Integrins are heterodimeric transmembrane protein receptors regulating many important cellular processes including homing of HSCs by binding to neighboring cells or extracellular matrix proteins. First, we showed that integrin α2 is a novel marker improving the prospective isolation of human cord blood HSCs. We found integrin α2 receptor was preferentially expressed in cord blood-derived CD34+CD38-CD90+ *in vivo* long-term repopulating cells, demonstrated by 24-week limiting-dilution xenotransplantations using immunodeficient mice. Second, we revealed that integrin α2, which is a marker for megakaryoctyes and platelets, was not expressed in the immature CD34+CD38-CD45RA- bipotential megakaryocyte-erythrocyte progenitors in human bone marrow, providing a means for enriching this novel bipotent progenitor population for further studies on early megakaryocytic and erythroid lineage fate decisions. In addition, we demonstrated that hyperactivation of Akt, which is a key intrinsic factor regulating the homeostasis of HSCs, was incompatible with the survival and growth promoting ability of FMS-like tyrosine kinase 3-internal tandem duplication (FLT3-ITD) signaling in murine stem and progenitor cells.

Prospective isolation of more homogenous stem and progenitor cell populations in human and understanding the instrinsic regulation of HSC homeostasis will give important insights into the HSC maintenance and fate decisions in normal hematopoiesis, as well as the pathogenesis of various hematological disorders.

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Hematopoiesis, stem cell, bone marrow, extracellular matrix, integrin, Akt, megakaryocyte-erythrocyte progenitor

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Dedicated To

My Beloved Family

Contents

List of abbreviations

AGM	Aorta-gonad-mesonephros
AML	Acute myeloid leukemia
BFU-E	Burst-forming units-erythroid
BM	Bone marrow
BMP4	Bone morphogenetic protein 4
CAFC	Cobblestone area-forming cell
CAR	CXCL12-abundant reticular
CAS	Crk-associated substrate
CDK	Cyclin-dependent kinase
CFU-C	Colony-forming unit-progenitor
CFU-G	Colony-forming unit-granulocyte
CFU-GEMM	Colony-forming unit-granulocyte/ erythrocyte/ macrophage/ megakaryocyte
CFU-GM	Colony-forming unit-granulocyte/ macrophage
CFU-M	Colony-forming unit-monocyte/ macrophage
CFU-MK	Colony-forming unit-megakaryocyte
CFU-S	Colony-forming unit-spleen
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CXCL12	CXC chemokine ligand 12
CXCR4	CXC chemokine receptor 4
Cy	Cyclophosphamide
DNMT	DNA methyltransferase
E	Embryonic day
ECM	Extracellular matrix
EPO	Erythropoietin
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
FAK	Focal adhesion kinase
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FL	FMS-like tyrosine kinase 3 ligand
FLT3	FMS-like tyrosine kinase 3
FLT3-ITD	FMS-like tyrosine kinase 3-internal tandem duplication

FOXO	Forkhead box O
GMP	Granulocyte-monocyte progenitor
GM-CSF	Granulocyte-macrophage colony stimulating factor
GP	Glycoprotein
G-CSF	Granulocyte colony stimulating factor
HAT	Histone acetyltransferase
HBD	Hemoglobin delta
HDAC	Histone deacetylase
HES-1	Hairy enhancer of split-1
HIF	Hypoxia inducible factor
HLF	Hepatocyte leukemia factor
HOX	Homeobox
HPC	Hematopoietic progenitor cell
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cell
ICAM	Intercellular adhesion molecule
IL	Interleukin
ILK	Integrin-linked kinase
ITD	Internal tandem duplication
JNK	c-Jun NH2-terminal kinase
LFA	Lymphocyte function-associated antigen
LMPP	Lymphoid-primed multipotent progenitor
LSK	Lin-Sca1+Ckit+
LTC-IC	Long-term culture-initiating cell
MAPK	Mitogen-activated protein kinase
MEK	MAPK/ ERK kinase
MEKK	MAPK/ ERK kinase kinase
MEP	Megakaryocyte-erythrocyte progenitor
MLP	Multilymphoid progenitor
MPP	Multipotent progenitor
MSC	Mesenchymal stem cell
Myr	Myristylated
M-CSF	Macrophage colony stimulating factor
NK	Natural killer
PAK	P21-activated kinase
PI3K	Phosphatidylinositide 3-kinase
PIP3	Phosphatidylinositol-3, 4, 5-trisphosphate 3
PTEN	Phosphatase and tensin homolog
Rho123	Rhodamine 123
ROS	Reactive oxygen species
RUNX1	Runt-related transcription factor 1
SCF	Stem cell factor

SDF-1	Stromal cell-derived factor-1
SFK	Src-family kinase
SHC	Src homology 2 domain containing
SLAM	Signaling lymphocytic activation molecule
SP	Side population
SPAK	Stress-activated protein kinase
SRF	Serum response element
TGFβ	Transforming growth factor beta
TGFβ-R	Transforming growth factor beta receptor
TPO	Thrombopoietin
VASP	Vasodilator-stimulated phosphoprotein
VCAM	Vascular cell adhesion molecule
VLA	Very late antigen
WNT	Wingless-type MMTV integration site family
Wt	Wildtype

List of articles included in the thesis

This thesis is based on articles listed below, which are referred to in the text by their roman numerals (I-III)

I.   <u>Wan Man Wong</u>, Mikael Sigvardsson, Ingbritt Åstrand-Grundström, Donna Hogge, Jonas Larsson, Hong Qian, Marja Ekblom. Expression of integrin α2 receptor in human cord blood CD34+CD38-CD90+ stem cells engrafting long-term in NOD/SCID-IL2Rγcnull mice. Stem Cells, 2013; 31: 360-371.

II.   <u>Wan Man Wong</u>, Mikael Sigvardsson, Ingbritt Åstrand-Grundström, Hong Qian, Marja Ekblom. Identification of bipotential Lin-CD34+CD38- integrin α2- megakaryocyte-erythrocyte progenitors in human bone marrow. Manuscript 2013.

III. Yanjuan Tang, Camilla Halvarsson, Amanda Nordigården, Josefine Åhsberg, <u>Wan Man Wong</u>, and Jan-Ingvar Jönsson. Hyperactivated AKT is incompatible with survival when coexpressed with additional oncogenes and drives hematopoietic stem and progenitor cells to cell cycle inhibition and apoptosis. (2013). Submitted.

Overview of the thesis

Hematopoiesis is a tightly regulated process in which hematopoietic stem cells reside at the apex of the hierarchy, and produce all kinds of mature blood cells by differentiation to replenish the cell loss in homeostasis and acute injury. In past few decades, much effort has been made to purify hematopoietic stem cells (HSCs) and lineage-committed progenitor cells both in mouse and human, enabling further characterization of these cell populations not only in normal hematopoiesis, but also in various hematological malignancies. However, while the isolation of different cell populations in mouse hematopoietic system is achieved with a very high purity, purification of human hematopoietic stem and progenitor cells (HSPCs) still far lags behind.

Integrins are heterodimeric transmembrane protein receptors regulating many important cellular processes including homing of HSCs by binding to neighboring cells or extracellular matrix (ECM) proteins. First, we showed that integrin α2 is a novel marker improving the prospective isolation of human cord blood HSCs. We found integrin α2 receptor was preferentially expressed in cord blood-derived CD34+CD38-CD90+ *in vivo* long-term repopulating cells, demonstrated by 24-week limiting-dilution xenotransplantations using immunodeficient mice. Second, we revealed that integrin α2, which is a marker for megakaryocytes and platelets, was not expressed in the immature CD34+CD38-CD45RA- bipotential megakaryocyte-erythrocyte progenitors (MEPs) in human bone marrow (BM), providing a means for enriching this novel bipotent progenitor population for further studies on early megakaryocytic and erythroid lineage fate decisions. In addition, we demonstrated that hyperactivation of Akt, which is a key intrinsic factor regulating the homeostasis of HSCs, was incompatible with the survival and growth promoting ability of FMS-like tyrosine kinase 3-internal tandem duplication (FLT3-ITD) signaling in murine stem and progenitor cells.

Prospective isolation of more homogenous stem and progenitor cell populations in human and understanding the instrinsic regulation of HSC homeostasis will give important insights into the HSC maintenance and fate decisions in normal hematopoiesis, as well as the pathogenesis of various hematological disorders.

Introduction

Hematopoiesis

Mature blood cells are produced from the primitive HSCs, which reside at the apex of the hematopoietic hierarchy, and this highly orchestrated process is termed hematopoiesis. In adult human, approximately 10^{12} blood cells are generated in the BM every day to maintain the hematopoietic homeostasis (Ogawa, 1993). Hematopoiesis is a tightly regulated process. HSCs are characterized by their self-renewal ability and multi-lineage differentiation capacity. Differentiation from HSCs to intermediate lineage-committed progenitors, morphologically recognizable precursors and terminally differentiated mature blood cells is precisely controlled by the intrinsic factors of the cells and extrinsic factors in the BM niches. Understanding the regulation of HSC maintenance and fate decisions in normal hematopoiesis is not only critical for unraveling the pathogenesis in various hematological malignancies, but also important for the development of clinical transplantation methods.

Mammalian hematopoiesis is divided into two distinct phases, primitive and definitive phases (Figure 1). Both are initiated in the fetal life. Primitive hematopoiesis, which starts on embryonic day (E) 7.5 in mouse and 30 days post-conception in human, produces primitive erythrocytes extra-embryonically in the yolk sac before the onset of the circulation (Badillo and Flake, 2006; Lux, 2007; Ottersbach et al., 2010; Palis et al., 1999). The generation of HSCs, which have multi-lineage differentiation potential and are able to engraft the irradiated adult recipient mice long-term, is referred to definitive hematopoiesis and firstly seen in aorta-gonad-mesonephros (AGM) region at E10.5 in mouse and 4 weeks post-conception in human (Boisset et al., 2010; Cumano et al., 1996; Eilken et al., 2009). Definitive HSCs then migrate from AGM to seed placenta, liver, spleen and BM at different time points of development in mouse and human (Dzierzak and Speck, 2008). BM is the only site of hematopoiesis after birth in humans. However, extramedullary hematopoiesis is seen with some hematological disorders (Sohawon et al., 2012). By birth in mouse, BM is the predominant site of hematopoiesis but spleen also supports

production of the blood cells for several weeks after birth (Wolber et al., 2002).
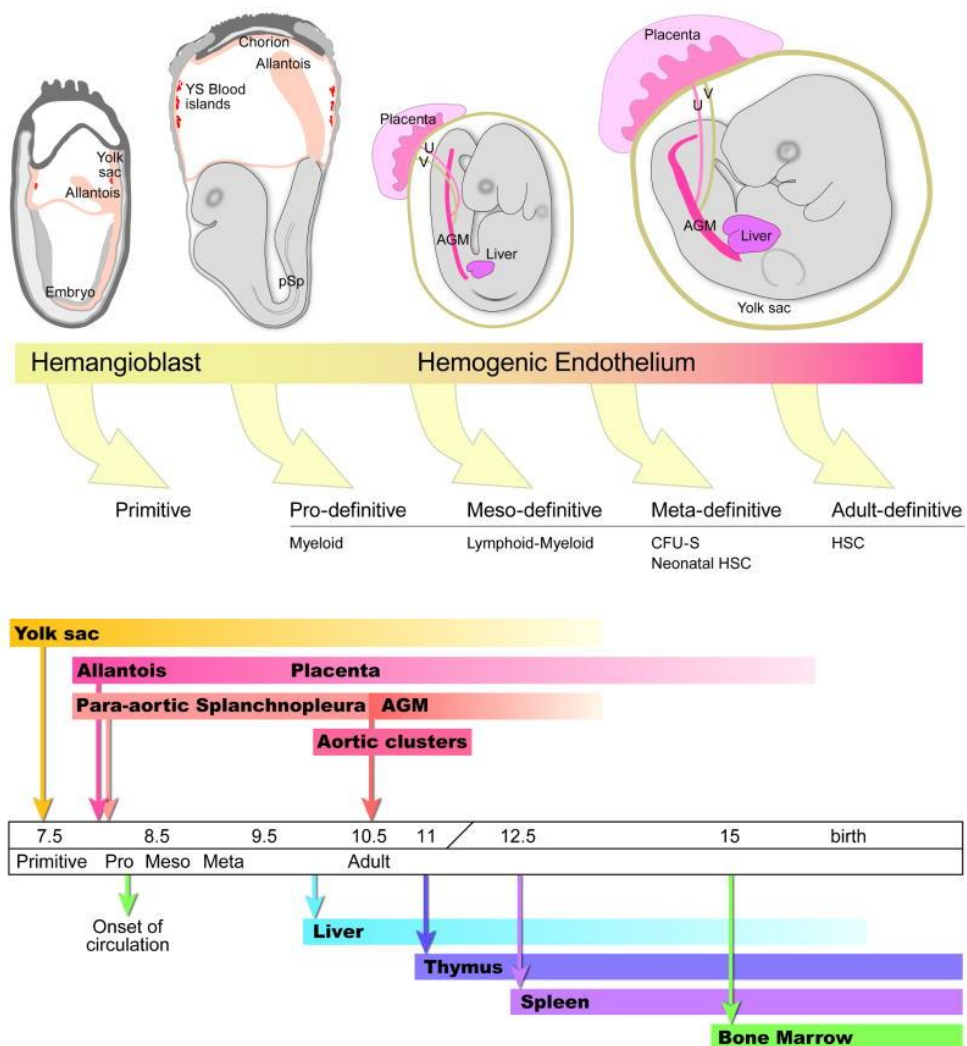
Figure 1. The development of murine hematopoietic stem cells. At least five classes of hematopoietic cells are generated at different time points of the development in mouse (Upper panel). The timeline indicates the onset of hematopoietic events during mouse embryogenesis. Arrows above and below show the organs where various hematopoietic events take place and the onset time of colonization of the secondary hematopoietic territories, respectively (Lower panel). CFU-S, colony-forming units in the spleen; V, vitelline arteries; U, umbilical arteries; AGM, aorta-gonad-mesonephros; HSC, hematopoietic stem cells. Reproduced with permission from the copyright owner (Dzierzak and Speck, 2008).

Hematopoietic hierarchy

Mature blood cells are categorized into two distinct lineages, myeloid and lymphoid lineages. Myeloid cells include granulocytes (neutrophils, eosinophils and basophils), monocytes, megakaryocytes and erythrocytes. Many of them are short-lived and have to be replenished continuously to maintain the homeostasis. Lymphoid lineage cells, which consist of T cells, B cells and natural killer (NK) cells, are relatively long-lived and play an important role in maintaining the adaptive and innate immunity in the body.

All mature hematopoietic cells are generated from the HSCs residing at the apex of the hematopoietic hierarchy. The differentiation roadmap of HSCs is highly complex and involves a lot of intrinsic regulations of the cells *per se* and extrinsic regulations from the microenvironment. A 'classical model' of HSC hierarchy has been widely adopted in both mouse and human hematopoiesis. The classical model demonstrates that the primitive HSCs lose self-renewal capacity but preserve the multipotency right after the first differentiation into the immediate progenitor cells, called multipotent progenitors (MPPs) (Adolfsson et al., 2001; Christensen and Weissman, 2001; Morrison et al., 1997; Morrison and Weissman, 1994; Osawa et al., 1996a) and the MPPs in turn give rise to two oligopotent progenitors, common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs) (Akashi et al., 2000; Kondo et al., 1997; Reya et al., 2001). CMPs produce granulocyte-monocyte progenitors (GMPs) and MEPs and hence differentiate into mature granulocytes/ monocytes and megakaryocytes/ erythrocytes respectively. CLPs give rise to B-, T- and NK- lineage precursor cells and in turn generate the mature lymphocytes to carry out immune responses.

However, this classical model of hematopoietic hierarchy has been challenged in both mouse and human by the identification of various progenitor cells which cannot be fit into the classical hierarchy. This is due to the recent advances in technologies such as fluorescence activated cell sorting (FACS) and in the production of monoclonal antibodies against cell-surface proteins, that have improved the prospective isolation and hence characterization of specific cell populations by functional stem cell transplantation assays. Furthermore, although human and mouse display a similar roadmap in hematopoiesis, there are still some discrepancies in lineage determination at the hematopoietic progenitor level.

Mouse hematopoietic hierarchy

Murine hematopoiesis has been extensively studied and the immunophenotypes of various hematopoietic cell populations are much better defined than in human. In adult mouse, all functional BM-derived HSCs reside in a population which does not express markers expressed on lineage-committed or differentiated hematopoietic cells (Lin), but expresses Sca-1 and high level of C-Kit. However, this Lin-Sca1+Ckit+ (LSK) fraction is still highly heterogeneous, and less than 10% of cells in this fraction possess long-term repopulation ability in transplantation assays (Okada et al., 1992; Osawa et al., 1996b). Long-term HSCs are the most primitive cells in the hierarchy, dormant in nature and contain life-long self-renewal capacity. They are defined as LSK FLT3-CD34-CD150+CD48- cells in the adult mouse BM (Christensen and Weissman, 2001; Kiel et al., 2005; Osawa et al., 1996a). HSPCs with transient repopulating capability reside in the LSK fraction but have CD34+, CD38-, CD150-, or Thy1.1- phenotypes (Kiel et al., 2005; Osawa et al., 1996a; Pronk et al., 2007; Randall et al., 1996). These cells are termed as short-term HSCs or MPPs. Different reports tried to discriminate the cells from these two categories; however, there is no general agreement in distinguishing between short-term HSCs and MPPs in adult murine hematopoiesis. Myeloid-lineage progenitor cells have been shown to reside in the LSK cell compartment (Pronk et al., 2007).

In the classical model of hematopoiesis, lineage commitment occurs at MPP level and results in the bifurcation of myeloid and lymphoid lineages. This idea has been challenged by the identification of LSK FLT3+ lymphoid-primed multipotent progenitor (LMPP) in adult mouse BM. LMPPs are committed to both myeloid and lymphoid differentiation but with limited megakaryocytic and erythrocytic potential, whereas MEPs can be isolated in LSK FLT3- fraction of adult BM cells (Adolfsson et al., 2005; Buza-Vidas et al., 2011). Kondo and coworkers proposed a similar idea that the MEPs can only be generated from LSK Thy1.1-FLT3-VCAM+ CMPs whereas LSK Thy1.1-FLT3+ LMPPs give rise to both lymphoid and myeloid progenies (Lai and Kondo, 2006). Nevertheless, the lineage commitment roadmap in adult mouse hematopoiesis at MPP level is still highly controversial (Forsberg et al., 2006) (Figure 2). In addition, in a study using long-term *in vivo* repopulation assays including secondary transplantations of single cells, differentiation roadmap of HSCs into myeloid progenitors by bypassing the MPP/ LMPP stage has been recently proposed (Yamamoto et al., 2013).
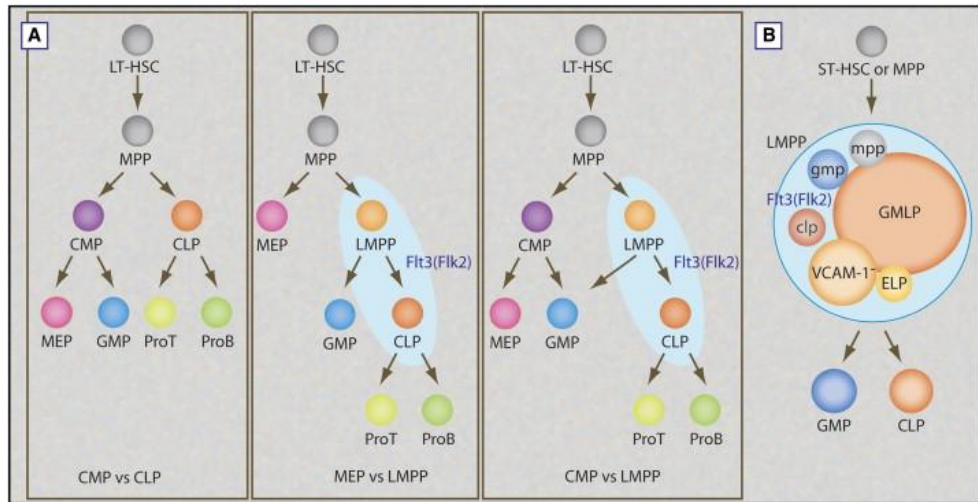
Figure 2. Different proposed developmental pathways in adult murine hematopoietic system, in which the lineage commitment at MPP level is still highly controversial. MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythrocyte progenitor; GMP, granulocyte-monocyte progenitor; LMPP, lymphoid-primed multipotent progenitor; and GMLP, granulocyte-monocyte-lymphoid progenitor. Reproduced with permission from the copyright owner (Iwasaki and Akashi, 2007).

Human hematopoietic hierarchy

Human HSCs can be isolated from fetal liver, fetal BM, cord blood, adult BM and peripheral blood after administration of stem-cell mobilizing agents. Cord blood is the main source for isolation and characterization of human HSCs for research purposes because it is relatively easier to obtain and the cord blood-derived HSCs have a superior *in vivo* repopulating capacity, assayed in experimental transplantations using immunodeficient mice, compared with HSCs from other sources (Holyoake et al., 1999; Noort et al., 2001; Ueda et al., 2001). The immunophenotypes of HSPCs are considerably different between human and mouse. Human HSCs are Lin-, express a transmembrane glycoprotein (GP) CD34 and have a low or no expression of an ectoenzyme CD38 (Bhatia et al., 1997; Civin et al., 1984; Hogan et al., 2002; Vogel et al., 2000). Nevertheless, CD34- human HSCs might exist in an extremely low frequency (Bhatia et al., 1998; Ishii et al., 2011). With the use of transplantation assays into immunodeficient SCID mouse model, human repopulating cells were shown to express a glycophosphatidylinositol-linked membrane glycoprotein CD90 (Baum et al., 1992). In agreement with this, Weissman and coworkers have recently showed that human cord blood-derived HSCs, reconstituting immunodeficient (NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ) NSG mice, are enriched in Lin-CD34+CD38-CD90+CD45RA- fraction whereas Lin-CD34+CD38-CD90-CD45RA- cell fraction contains fewer HSCs (Majeti et al., 2007). Furthermore, human HSCs can be preferentially isolated from the cord blood Lin-CD34+CD38-CD90+CD45RA- fraction expressing integrin α6 at a frequency of 1/10 (Notta et al., 2011).

Human cord blood MPPs were first suggested to reside in Lin-CD34+CD38-CD90-CD45RA- population, a heterogeneous fraction containing also more mature colony-forming unit progenitors (CFU-Cs) and, to a lesser extent, cells with repopulating potential (Majeti et al., 2007). Recently, the immunophenotype of MPP has been better refined as Lin-CD34+CD38-CD90-CD45RA- integrin α6-. The integrin α6-expressing cells in Lin-CD34+CD38-CD90-CD45RA- fraction possess long-term repopulating potential as good as Lin-CD34+CD38-CD90+CD45RA- integrin α6+ cells, although the frequency of long-term repopulating cells, assessed by limiting dilution transplantation, is lower in the CD90- than that in the CD90+ fraction (Notta et al., 2011).

Lin-CD34+CD38-CD90-CD45RA- multilymphoid progenitors (MLPs) have been recently identified in both cord blood and BM. This MLP population gives rise to all kinds of lymphoid-committed cells and possesses dendritic and monocytic potentials (Doulatov et al., 2010). Cord blood-derived MLPs lack *in vivo* repopulating capacity. The identification

18

of this immature lymphoid progenitor cell population has challenged the classical human hematopoietic hierarchy in which the CLP have long been suggested as the earliest lymphoid progenitor differentiated from MPP, but not MLP. (Galy et al., 1995; Hao et al., 2001; Hoebeke et al., 2007; Six et al., 2007). The hierarchical relationship between the newly identified and conventional progenitor cell populations remains poorly understood, mainly due to the heterogeneity of the phenotypically-defined cell populations comprising cells at highly variable developmental stages.

CMPs, GMPs and MEPs reside in a more mature Lin-CD34+CD38+ progenitor cell population, which does not contain any long-term *in vivo* reconstituting activity, and can be isolated from CD123+CD45RA-, CD123+CD45RA+ and CD123-CD45RA- fractions respectively (Manz et al., 2002). In addition, Edvardsson et al. suggested that CD34+CD19- CMPs, GMPs and MEPs could be more effectively purified from CD123lo/-CD110-CD45RA-, CD123loCD110-CD45RA+, CD123lo/- CD110+CD45RA- fractions respectively (Edvardsson et al., 2006). However, the CMP population is still highly heterogeneous. FLT3- expressing CMPs preferentially give rise to granulocytes and monocytes whereas CMPs lacking FLT3 expression are biased to megakaryocytic and erythrocytic lineage differentiation (Edvardsson et al., 2006; Kikushige et al., 2008). Furthermore, IL-5Rα+ CMPs exclusively generate eosinophil colonies (Mori et al., 2009). Refining the purification of CMP population is important for investigating the regulatory elements involved in granulocyte/ monocyte versus megakaryocyte/ erythrocyte lineage commitment process.

Hematopoietic stem cells

HSCs differentiate into intermediate transit-amplifying cells and then mature blood cells required for maintaining the homeostasis of the body. This process is balanced between the cues executing self-renewal and differentiation. Current model of hematopoiesis accepts that self-renewal and multipotency of HSC are compromised along the road of differentiation into terminally differentiated cells.

Differentiation

HSCs have long been regarded as a population with the equal ability of differentiation into myeloid and lymphoid progenitors. The development of recent technologies such as multicolor flow cytometry and the well-established cell-surface markers defining murine HSCs, have enabled the purification of murine HSCs to nearly homogeneity (Kiel et al., 2005; Osawa et al., 1996a). By transplanting a single murine HSC into the congenic recipient mouse, *in vivo* clonal output from single long-term repopulating cells can be examined.

Presence of murine HSC subsets with distinct self-renewal potentials and bias in producing lineage-restricted progenitor cells has been reported (Dykstra et al., 2007; Muller-Sieburg et al., 2012; Sieburg et al., 2006; Wilson et al., 2008). In these studies, these phenotypically homogeneous HSCs were basically categorized into 'Myeloid-biased (My-bi)'/ 'Lymphoid-deficient (α)', 'Balanced (Bala/ β)' and 'Lymphoid-biased (Ly-bi)'/ 'Myeloid-deficient (γ and δ)' subtypes, according to the predominant lineage output normalized to the total donor-derived mature cells or to the total of circulating mature cells in the recipient mouse. α and β HSCs defined by the Eaves laboratory could be serially transplanted and the pattern of the clonal output cells was stably maintained in the primary recipient mice. Several laboratories tried to identify markers expressed on the surface or based on the functional properties of various HSC subsets (Challen et al., 2010; Kent et al., 2009; Morita et al., 2010); however, at present the methods of prospectively isolating different functionally distinct HSC subsets are not sufficient for further characterization at molecular level. Recently, myeloid progenitors with long-term repopulating capacity have been identified and the study showed that these progenitors were differentiated from HSCs via asymmetric self-renewal division without passing through the MPP stage, suggesting more comprehensive criteria may be needed for defining HSCs in the future (Yamamoto et al., 2013).

Self-renewal

HSCs residing at the apex of the hematopoietic hierarchy are characterized by their life-long self-renewal ability and multipotency. Under the steady state of hematopoiesis, HSCs remain quiescent, and the number of HSCs in BM or circulation is tightly regulated and remains approximately the same (Jude et al., 2008). However, in the acute need of hematopoiesis, HSCs actively cycle, give rise to non self-renewing daughter progenitor cells and hence boost the production of various mature hematopoietic cells for satisfying the immediate needs. HSC pool must be maintained throughout the life time for continuously replenishing the daily loss of mature hematopoietic cells. The maintenance of the HSC pool is regulated by both self-renewal and differentiation cues. Loss of self-renewal potential of HSCs leads to HSC exhaustion and hence results in hematological disorders such as BM failure.

HSC self-renewal occurs in BM niche. With the use of various knockout mice, intrinsic regulators, extrinsic factors and epigenetic regulators controlling self-renewal have been identified in the past decades (Rossi et al., 2012). Identifying these major components for regulating HSC self-renewal capability at cellular and molecular levels is important for expanding HSCs *in vitro* and hence for transplantation in the patients with hematological disorders.

Bone marrow hematopoietic stem cell niche

HSCs are housed in the BM for maintaining the stem cell properties such as self-renewal, differentiation and quiescence (Scadden, 2006). The BM is a microenvironment providing cues from different components for regulating HSC behaviors by direct contact with neighboring cells, by secretion of growth factors and cytokines and by interactions with molecules composing the ECM. Identification of components in the BM niche in regulating HSC properties is important for *ex vivo* expansion of HSCs and hence for HSC-based therapeutic purposes. Furthermore, understanding the role of BM niche in the maintenance of malignant stem cells during the development of leukemia may provide an insight into the establishment of complementary therapies for stem cell-derived blood malignancies.

The identification of signaling lymphocytic activation molecule (SLAM) markers for the prospective purification of murine HSCs, showing that 50% of LSK CD150+CD48-CD41- cells have long-term repopulating ability, enabled the localization of HSCs in the mouse BM by fluorescence microscopy (Kiel et al., 2005). HSCs were found to reside in multiple locations: in the vicinity of vascular sinusoids, in the immediate proximity of endosteal surfaces or in a poorly defined position between two sinusoids (Figure 3). These findings suggested the presence of multiple niches for HSCs.
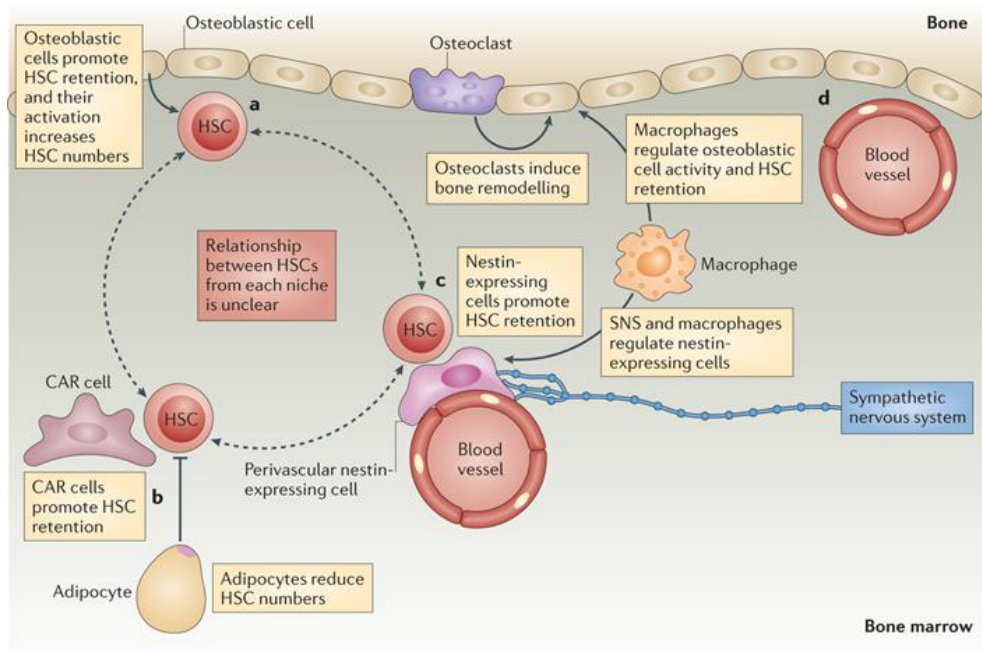
Figure 3. Hematopoietic stem cell niches. In the bone marrow, hematopoietic stem cells (HSCs) are (a) located in the immediate proximity of endosteal surfaces, (b) associated with the CXCL12-abundant reticular (CAR) cells or (c) connected with nestin-expressing cells and sinusoids. (d) Some sinusoids reside in the vicinity of the endosteal surfaces, but the interaction between these two parties remains poorly understood. SNS, sympathetic nervous system. Reproduced with permission from the copyright owner (Mercier et al., 2012).

Endosteal niche

Some HSCs were found to reside close to the bone-forming osteoblasts, which line on the bone surfaces at the endosteum, by confocal/ two-photon intravital imaging (Calvi et al., 2003; Nilsson et al., 2005; Zhang et al., 2003). Osteoblasts are generated from nestin-expressing mesenchymal stem cells (MSCs) and CXCL12-abundant reticular (CAR) cells have been recently suggested to possess osteogenic potential (Méndez-Ferrer et al., 2010; Omatsu et al., 2010; Pittenger et al., 1999). Osteoblasts secrete various growth factors and cytokines including membrane-bound stem cell factor (SCF), CXC chemokine ligand 12 (CXCL12), granulocyte colony stimulating factor (G-CSF), macrophage colony stimulating factor (M-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), interleukin (IL)-1, IL-6, IL-7, Notch ligands, wingless-type MMTV integration site family (WNT) ligands, thrombopoietin (TPO), and angiopoietin 1 that may be important for HSC maintenance (Arai et al., 2004; Calvi et al., 2003; Fleming et al., 2008; Qian et al., 2007a; Stier et al., 2002; Taichman, 2005; Yoshihara et al., 2007). In addition, osteoblasts express several cell adhesion molecules such as vascular cell adhesion molecule (VCAM) 1, intercellular adhesion molecule (ICAM) 1, CD164, CD44, N-cadherin and annexin II which may regulate HSC behaviors via cell-cell interactions (Jung et al., 2007). Some, but not all, studies demonstrated that changes in the number of osteoblastic cells and osteoclasts led to the corresponding changes in the number of HSCs in mouse, and mobilization of HSCs by G-CSF was hampered by depletion of osteoblasts in mouse BM (Calvi et al., 2003; Kiel et al., 2007; Lymperi et al., 2011; Miyamoto et al., 2011). Osteopontin, which is an ECM protein secreted by osteoblasts, modulates the HSC numbers and migration towards the endosteal surface in the BM (Nilsson et al., 2005; Stier et al., 2005). These studies suggested that osteolineage cells may be crucial for HSC maintenance and retention in the BM.

Most mouse HSCs reside in trabecular bone. HSCs purified from endosteal region possess superior reconstitution potential compared to those isolated from the center of the BM (Grassinger et al., 2010). Endosteal region is highly mineralized and a microenvironment with high concentration of calcium ion is created at the periendosteal surface. Calcium-rich endosteal region is recognized by the calcium-sensing receptor on HSCs and is important for the preservation of HSC engraftment potential (Adams et al., 2006; Chattopadhyay et al., 1997).

Vascular niche

HSCs have also been shown to reside at the perivascular region (Kiel et al., 2005). Similar to osteoblasts, endothelial cells surrounding sinusoids secrete multiple factors including GM-CSF, M-CSF, G-CSF, IL-6, SCF and FMS-like tyrosine kinase 3 ligand (FL) and express cell adhesion molecules such as VCAM-1, ICAM-1, E-selectin and P-selectin for regulating hematopoiesis (Mazo et al., 1998; Rafii et al., 1997).

Multipotent MSCs play a critical role in regulating HSCs. They surround the blood vessels in the BM and are able to differentiate into various cell types, such as osteoblasts, adipocytes and chondrocytes (Prockop, 1997). MSCs express CD146 in humans and nestin in mice. These cells directly contact with HSCs and also secret CXCL12, angiopoietin 1 and SCF for regulating the HSC maintenance. Depletion of nestin-expressing MSCs resulted in extramedullary hematopoiesis in spleen and 50% reduction of HSC numbers in the BM, suggesting MSCs are important for HSC retention in perivascular niche (Méndez-Ferrer et al., 2010; Sacchetti et al., 2007). Besides, MSCs modulate hematopoiesis indirectly by interacting with adrenergic nerve fibres of sympathetic nervous system, which regulates the egress of HSCs from the BM in mobilization and circadian oscillations (Katayama et al., 2006; Méndez-Ferrer et al., 2008).

Similar as MSCs, CAR cells interact tightly with HSCs in perivascular region of the BM, although some of them can also be found close to sinusoids near endosteum (Crisan et al., 2008). CAR cells express high levels of CXCL12 and SCF, which are critical factors in regulating HSC retention and maintenance respectively (Broxmeyer, 2008; Lapidot and Petit, 2002; Sugiyama et al., 2006). Depletion of CAR cells in mouse model resulted in a reduction in HSC numbers and increase in HSC quiescence (Omatsu et al., 2010). Since nestin-expressing MSCs are four times less abundant than CAR cells in the BM, and both MSCs and CAR cells express high level of CXCL12, MSCs have been proposed to represent a subpopulation of CAR cells in the BM niche (Ehninger and Trumpp, 2011; Mercier et al., 2012); however, more experiments are needed for further characterization of these two cell populations.

Recently, several studies have reported macrophages as a new component of the BM niche for regulating HSCs. Depletion of monocytes/ macrophages in the mouse BM resulted in the mobilization of HSCs into peripheral blood and spleen. In addition, reduction of 40% of CXCL12 was observed in BM extracellular fluid. Some but not all studies reported the expression of HSC retention factors such as CXCL12 was reduced in nestin-expressing MSCs in perivascular region and osteoblasts near

endosteal lining in the absence of phagocytes (Chow et al., 2011; Christopher et al., 2011; Winkler et al., 2010b). These findings suggested that monocytes/ macrophages may form part of both niches in the mouse BM.

Regulation of hematopoietic stem cells

Regulators of HSC functions such as differentiation, self-renewal and quiescence have been a primary interest and extensively investigated in the past decades. These studies were mainly conducted by gain or loss of gene function experiments using not only *in vitro* lentiviral overexpression or shRNA-based gene silencing systems into the primary cells but also transgenic or knockout mouse models for characterization. Properties of HSCs are controlled by various intrinsic regulators in the cells *per se* and external cues from the BM microenvironment. Recently, epigenetic modifications and reprogramming of HSCs have also been shown to play an important role in governing the corresponding self-renewal and multilineage potentials.

Extrinsic regulators of HSCs

The maintenance of self-renewal and the differentiation of HSCs require both intrinsic and extrinsic pathways. Extrinsic pathways are supported by stem cell microenvironment for the regulation of various HSC functions such as self-renewal and expansion, growth inhibition or quiescence, adhesion and migration. In general, different classes of signaling molecules either secreted from HSC niches or expressed on the osteoblasts and other niche components trigger the responses of HSCs through the corresponding receptors (Wang and Wagers, 2011).

Several ligand-receptor binding pairs play a major role in self-renewal and expansion control of HSCs and hence regulate the number of HSCs in the hematopoietic BM niches. This well-known ligand-receptor signaling includes fibroblast growth factor (FGF) and FGF receptor (FGFR), FL and FMS-like tyrosine kinase 3 (FLT3) receptor, SCF and C-Kit, as well as WNT and Frizzled receptor (Austin et al., 1997; Itkin et al., 2013; Lennartsson and Ronnstrand, 2012; Wodnar-Filipowicz, 2003). All the receptors are composed of an extracellular multi-transmembrane domain and a cytoplasmic signaling domain. Engagement of the receptors by corresponding ligands triggers a series of downstream signaling. For example, signaling generated from FGF/ FGFR, FL/ FLT3 and SCF/ C-Kit activates STAT3 and STAT5 proteins, resulting in the upregulation of Mucin 1 and Rac expression, and downregulation of C/EBP α and p19 expression (Itkin et al., 2013; Lennartsson and Ronnstrand, 2012; Wodnar-Filipowicz, 2003). For the WNT signaling, the engagement of Fizzled receptor by extracellular WNT protein triggers a series of downstream signaling and finally activates transcriptional factors Myc and β-catenin.

Myc then induces the expression of BMI-1 and reduces the expression of p21, while β-catenin induces the expression of both cyclin D1 and Myc (Austin et al., 1997; Cain and Manilay, 2013). Taken together, all the FGF/ FGFR, FL/ FLT3, SCF/ C-Kit and WNT/ Frizzled signaling tightly control the self-renewal and expansion of HSCs.

For the growth control of HSCs, transforming growth factor beta (TGFβ) / TGFβ-receptor (TGFβ-R) signaling plays a main role in negatively regulating the proliferation of HSCs (Blank and Karlsson, 2011; Isufi et al., 2007). Engagement of TGFβ-R by TGFβ secreted from the osteoblasts or stem cell niches activates SMAD signaling pathway. Activated SMAD complex induces the transcription of cyclin-dependent kinase (CDK) inhibitor 1B (p27) and CDK inhibitor 1C (p57), resulting in the inhibition of cell proliferation.

In addition, N-cadherin/ N-cadherin, VCAM-1/ very late antigen (VLA)-4, fibronectin/ VLA-5, ICAM-1/ lymphocyte function-associated antigen (LFA)-1 and ICAM-1/ Mucin 1 pairs play important roles in regulating the adhesion, migration and quiescence of HSCs. Stimulation of these adhesion molecules activates the GTPase Rac, which in turn regulates cytoskeletal organization and controls cell growth (Williams et al., 2008). In addition, engagement of Mucin 1 by ICAM-1 expressed on the osteoblasts prevents the release of Mucin 1-bound β-catenin, resulting in the β-catenin dependent transcription repression of cyclin D1 and Myc (Kirstetter et al., 2006; Scheller et al., 2006).

Instrinsic regulators of HSCs

Long-term HSCs are usually kept at quiescent state for their life-long maintenance in the BM to prevent exhaustion. Under homeostasis conditions, the fate of HSCs is determined by the molecular mechanisms regulating the balance between self-renewal and differentiation. Several transcription factors and cell cycle regulators have been identified for modulating these properties in the recent decades.

BMI-1 belongs to a polycomb group gene family and is a crucial regulator of adult HSC self-renewal and proliferation. It represses the expression of downstream CDK inhibitor p16 and tumor suppressor gene p19, resulting in cell cycle arrest, apoptosis, DNA repair and senescence (Vousden and Prives, 2009; Zilfou and Lowe, 2009). BMI-1-deficient HSPCs from mouse and human exhibited the loss of clonal potential, whereas overexpression of BMI-1 in human HSPCs enhanced the multilineage and self-renewal capabilities (Lessard and Sauvageau, 2003; Rizo et al., 2008; Rizo et al., 2009). In addition, upregulation of BMI-1 was found in leukemia and

28

various types of solid tumors (Gil et al., 2005; Schuringa and Vellenga, 2010).

Homeobox (HOX) -B4 is a transcription factor regulating self-renewal in HSPCs. Overexpression of HOX-B4 in murine HSCs by retroviral transduction significantly expanded HSC numbers (Antonchuk et al., 2002). However, overexpression of HOX-B4 in human HSPCs only showed limited degree of expansion of cells with stem cell characteristics (Amsellem et al., 2003; Buske et al., 2002). These findings suggested that the regulatory role of HOX-B4 in human and mouse HSPCs may be different.

Transcriptional factors hairy enhancer of split-1 (HES-1) and hepatocyte leukemia factor (HLF) were identified as human HSC regulators by comparing the global gene expression profiles of HSC-enriched to progenitor-enriched cell populations. Transducing human HSPCs with HES-1 or HLF expression vector led to enhanced *in vivo* repopulating potential (Shojaei et al., 2005). HES-1 is a Notch target. Activation of Notch pathway by extracellular modulator NOV results in the upregulation of HES-1, and increased long-term culture-initiating cell (LTC-IC) activity and engraftment potential of human HSPCs. Reduction or loss of NOV leads to decreased LTC-IC activity and repopulating capacity, which is accompanied with suppressed expression of HES-1 (Gupta et al., 2007; Sakamoto et al., 2002). These findings suggested that Notch signaling pathway may be involved in HES-1- mediated mechanism regulating HSC maintenance.

Phosphatidylinositide 3-kinase (PI3K) signaling pathway is important in HSC maintenance. PI3K pathway controls survival, proliferation and growth of the cells and it involves several serine/ threonine kinases and tumor suppressor proteins (Figure 4). PI3K activates AKT. AKT1 gene encodes a serine-threonine protein kinase and further activates mTOC1 which is a protein complex initiating the translation of proteins. Phosphatase and tensin homolog (PTEN) was originally identified as a tumor suppressor gene and encodes a phosphatidylinositol-3, 4, 5-trisphosphate 3 (PIP3)-phosphatse. It inhibits the activation of AKT by dephosphorylation of the PI3K substrate called PIP3 and thus impedes proliferation. Loss of PTEN gene in mice induces hyperactivation of AKT and drives the HSCs to proliferate. Therefore, PTEN are required for the maintenance of HSCs in the BM by inhibiting the AKT pathway (Chalhoub and Baker, 2009).

In addition to activating mTOC1, AKT inhibits the activation of forkhead box O (FOXO) family of transcription factors, which are the crucial modulators of oxidative stress. FOXO3a has been shown to be important

for regulating self-renewal of murine purified LSK CD34- HSCs (Miyamoto et al., 2007; Yamazaki et al., 2006). However, mice triply deficient of FOXO1, FOXO3a and FOXO4 have a more profound defect in hematopoiesis, suggesting different members in FOXO family may have redundancy between each other (Tothova et al., 2007).

Another study has demonstrated an important role of AKT in controlling proliferation and differenation of HSCs. HSCs isolated from the fetal liver of double knock-out mice deficient of AKT 1/2-/-, which are the isoforms most expressed in hematopoietic cells, produce fewer lineage-committed cells and are deficit in colony formation, demonstrated by both *in vivo* and *in vitro* experiments respectively (Juntilla et al., 2010).

Dormant HSCs are thought to primarily reside close to the endosteal lining of the bone, where the oxygen supply is relatively insufficient (Parmar et al., 2007; Winkler et al., 2010a). Recent reports demonstrated that hypoxia and the hypoxia inducible factors (HIFs) play important roles in HSC self-renewal and differentiation (Parmar et al., 2007; Takubo et al., 2010). In addition, loss of function of HIF-1α and HIF-2α severely affected the multilineage embryonic hematopoiesis and development of the embryonic vasculature (Adelman et al., 1999; Ramirez-Bergeron et al., 2006).
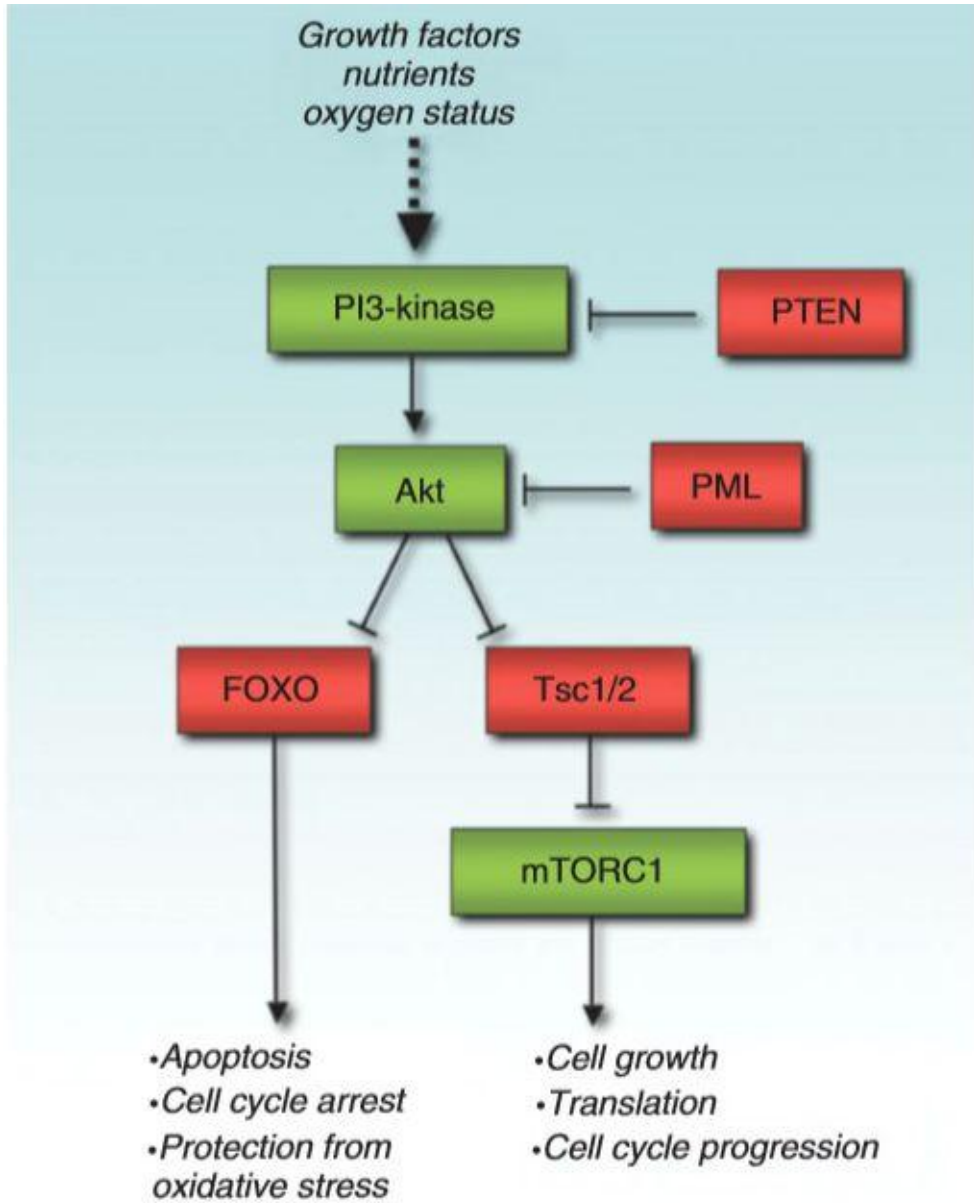
Figure 4. Phosphatidylinositide 3-kinase signaling pathway. Reproduced with permission from the copyright owner (Warr et al., 2011).

Epigenetic regulation of HSCs

In general, changes in gene expression can be regulated by transcription, translation, post-translational modification, small RNA regulation, DNA methylation and chromatin remodeling. The genome-wide analysis revealed that the differential gene expression between CD34+ HSPCs and CD34-differentiated cells is partially regulated by DNA methylation and chromatin remodeling (Oh and Humphries, 2012). In DNA methylation, a methyl group is added to the cytosine nucleotides in the promoter region and hence alters the gene expression. Methylation is regulated by DNA methyltransferase (DNMT) 1, DNMT 3a and DNMT 3b (Eberharter and Becker, 2002). Chromatin remodeling is the change of chromatin structure by the post-translational modification of histones such as acetylation, methylation, phosphorylation, sumoylation and ubiquitination. The histones in open chromatin structure are acetylated by histone acetyltransferases (HATs). The open chromatin structure can be switched to the closed chromatin structure after deacetylation by histone deacetylases (HDACs). Histones can also be modified by methylation on arginine and lysine residues. For example, the chromatin structure can be remodeled by polycomb group proteins such as BMI-1 through H3K27 methylation (Bracken et al., 2006).

A set of regulatory lineage-specific genes are expressed in HSCs and down regulated right after being exposed to differentiation cues. Changes of these lineage-affiliated genes have been shown to associate with the status of DNA methylation and histone modification at the promoter region (Bruno et al., 2004). Moreover, HSCs deficient of DNMT 1, DNMT 3a and/or DNMT 3b are defective in *in vivo* long-term repopulating capability, suggesting that methylation is important for the self-renewal of HSCs (Tadokoro et al., 2007; Trowbridge et al., 2009). In addition, loss of polycomb proteins BMI-1 and Mph1/ Rae28 results in the reduction of *in vivo* long-term repopulating ability of HSCs (Ohta et al., 2002; Park et al., 2003). Furthermore, chemical inhibition of DNA methylation and histone deacetylation, by 5-Aza-2'-deoxycytidine and trichostatin A respectively, enhances the self-renewal ability of transplanted HSCs in irradiated recipient mice (Chung et al., 2009). Taken together, epigenetic modifications facilitating the formation of open chromatin structure increase the self-renewal ability and maintain the undifferentiated status of HSCs.

Isolation and characterization of hematopoietic stem and progenitor cells

Understanding the roadmap of hematopoietic hierarchy and then prospectively isolating distinct cell populations along various differentiation stages are prerequisites for studying not only the properties of HSPCs, but also the regulatory mechanisms of hematopoiesis.

Functional identification of HSPCs

In the last twenty years, much effort has been made for identifying cell-surface markers on hematopoietic cells along various stages of differentiation. Recent antibody technology and FACS-based methods enable the simultaneous detection of a panel of stage-specific antigens and hence improve the homogeneity of FACS-purified cell populations for further characterization.

In general, murine hematopoietic system is much better defined compared to human hematopoiesis. In recent years, a panel of mouse hematopoietic stem cell markers identifies functionally defined HSCs in a purity of 50-96% (Matsuzaki et al., 2004; Yilmaz et al., 2006). However, the human HSC population is still highly heterogeneous and less than 10% of phenotypically defined HSCs possess functional HSC properties (Notta et al., 2011).

High drug efflux capacity

In addition to the expression pattern of various stage-specific stem cell markers, a population enriching HSCs can also be defined by the functional properties unique for the immature hematopoietic cell populations.

HSCs have a high drug efflux capability, thus resulting in dye-exclusion characteristic after being stained with fluorescent dyes compared to the more mature lineage-committed precursors. Various vital dyes, such as DNA-binding dye Hoescht 33342 and the mitochondrial-binding dye rhodamine 123 (Rho123), have been used for revealing this unique property of primitive hematopoietic cells. Hoescht 33342 is commonly used in a combination with other immunophenotypic markers in the purification of HSCs. It is excited at two wavelengths simultaneously by flow cytometry and HSCs form the signature 'tail' profile which is named as 'side population (SP)' (Goodell et al., 1996; Li and Johnson, 1992; McAlister et

al., 1990; Phillips et al., 1992; Wolf et al., 1993). Recently, a study suggested that murine HSCs biased to different lineage potentials can be purified based on the corresponding positions within SP (Challen et al., 2010).

Characterization of long-term HSCs: *In vivo* long-term repopulation assay

HSCs are defined by their life-long self-renewal capability and multipotency. *In vivo* long-term repopulation assay using mouse models is a golden standard for evaluating the stem-cell properties of the test cells. Functional HSCs are defined by their ability to long-term or serially reconstitute the whole blood system in the lethally irradiated or sublethally irradiated recipient mice. However, the stringency in designing the parameters measured in the experiment, including the length of transplantation assay, the level of engraftment and the type of mature donor-derived cells detected at the end of assay, is critical to avoid discrepancies in the results obtained.

In vivo long-term repopulation assay allows the discrimination of long-term HSCs from the remaining more mature hematopoietic cell populations, including short-term HSCs and multipotent progenitors. The multipotency of the repopulating cells is revealed by the presence of donor-derived mature hematopoietic cells from various lineages in the BM of recipient mice. The self-renewal potential of the test cells is often demonstrated by sustained multilineage repopulation after serial transplantation, or after a prolonged period time (>4 months) after primary transplantation (Benveniste et al., 2010; Schroeder, 2010). Some studies demonstrated that reconstitution from donor cells in the recipient mice fluctuated until 16 weeks or later after transplantation, suggesting that a minimum of 16 weeks is needed for evaluating the *in vivo* repopulating potential of long-term HSCs (Dykstra et al., 2007; Jordan and Lemischka, 1990), whereas the *in vivo* reconstitution from short-term HSCs or MPPs is assayed at earlier time point posttransplant due to their inability to sustain the repopulation long-term (Notta et al., 2011). Besides, the frequency of HSCs can be measured by limiting dilution transplantation assay based on the estimation of Poisson statistics (Szilvassy et al., 1990).

Murine *in vivo* repopulating cells are usually assayed in congenic mouse models which are with the equivalent genetic background but expressing different variants of a tyrosine phosphatase CD45 on all hematopoietic cells except erythrocytes and platelets (Spangrude et al., 1988). Competitive repopulation assay is the most common assay for examining the

34

repopulating potential of mouse HSCs from unknown source against a group of known numbers of HSCs, and provides qualitative and semi-quantitative measurements of HSCs in the test population (Purton and Scadden, 2007).

The functional potential of human HSCs can be evaluated by *in vivo* long-term repopulation analysis using immunodeficient mice in a non-competitive manner. The absolute frequency of human HSCs in an unknown cell population can be determined by either limiting-dilution or single-cell transplantation assays. A recent study comparing the human HSC engraftment between different immunocompromised mouse strains demonstrated that transplantation of human cord blood HSCs into NSG mice resulted in a higher level of chimerism in the BM compared with other mouse strains (McDermott et al., 2010). In addition, engraftment from a limiting dose of human HSCs is more efficient in female than male NSG mice (Notta et al., 2010).

Characterization of short-term HSCs: *In vivo* colony-forming unit-spleen (CFU-S) assay

The existence of mouse short-term HSCs can be revealed by the formation of macroscopic colonies in spleen of irradiated recipient mice at 1-3 weeks after injection. The CFU-Ss are early engrafting cells which provide a short-term radioprotection to the recipient mice. CFU-S assay was extensively used for evaluating the HSC activity in the early studies, but now is considered as an assay for measuring short-term HSCs or more mature progenitors (Purton and Scadden, 2007).

Characterization of primitive hematopoietic progenitor cells: *In vitro* long-term culture-initiating cell (LTC-IC) and cobblestone area-forming cell (CAFC) assays

Both LTC-IC and CAFC assays measure the existence of immature hematopoietic progenitor cells (HPCs) *in vitro*. These are coculture systems in which the test cells are cultured on the monolayer of irradiated stromal cells providing various regulatory factors in the culture. Fibroblasts or other cell lines, which can be engineered to express human cytokines (Hogge et al., 1996) are used as supporting cells for sustaining the growth and self-renewal of immature progenitor cells for 3-5 weeks. At readout, daughter cells produced from the immature progenitors can be detected by *in vitro* colony forming assay, whereas the presence of primitive progenitors in

CAFC assay can be revealed by the formation of 'cobblestone area' showing the integration of the immature cells to the adherent supportive cells in the culture (Coulombel, 2004; Van Os et al., 2004).

Characterization of lineage-committed HPCs: *In vitro* colony-forming cell assays

In vitro colony forming cell assays detect the existence and determine the frequency of lineage-committed precursor cells in a given population. These lineage committed progenitor cells include burst forming unit-erythroid (BFU-E), colony-forming unit-megakaryocyte (CFU-MK), colony-forming unit-granulocyte/ macrophage (CFU-GM), colony-forming unit-granulocyte (CFU-G) and colony-forming unit-monocyte/ macrophage (CFU-M). Immature multipotent progenitor cells colony-forming unit-granulocyte/ erythrocyte/ macrophage/ megakaryocyte (CFU-GEMM) can also be detected, but the generation of megakaryocytes from CFU-GEMM may be inhibited by the presence of serum in the culture medium. The tested cell population is cultured in a semi-solid medium, such as methylcellulose, agar or collagen-based medium supplemented with suitable cocktail of cytokines for 12 to 14 days. The presence of lymphoid progenitors cannot be evaluated in the conventional CFU-C assays, although Pre-B cells may be detected after optimization of the culture system (Coulombel, 2004; Doulatov et al., 2012; Purton and Scadden, 2007).

Early lymphoid development is difficult to be assessed because the generation of B and T cells is ineffective in the culture system supplemented only with cytokines. Therefore the coculture system utilizing specialized stromal cells, such as mouse BM stromal cell-lines OP9 and OP9-DL1, is often used for supporting the differentiation of lymphoid progenitors *in vitro* (Collins and Dorshkind, 1987; Itoh et al., 1989; La Motte-Mohs et al., 2005; Schmitt and Zúñiga-Pflücker, 2002).

Integrin receptors

Adhesive interactions of ECM and cell surface receptors provide a platform for the communication between cells and the surrounding environment. This interaction modulates many important cellular processes including cell death and survival, growth and differentiation, adhesion and migration (Schwartz and Ginsberg, 2002). In addition, this adhesive signaling integrates to other receptor signaling such as growth factor receptor-mediated signaling pathways for controlling various cellular signal transduction processes. Integrins are a key family of adhesion receptors expressed on most of the cell types, including hematopoietic cells. These adhesion receptors sense the immediate environment and response to the changes in ECM or neighboring cells.

Integrin subunits

Integrins are heterodimeric transmembrane protein receptors consisting of α and β subunits and 24 receptor complexes have been identified in mammalian system. Although a high structural similarity has been found within the family, most integrin receptors are specific on various biological signaling (Kumar, 1998).

α-integrins include α1 (CD49a), α2 (CD49b), α3 (CD49c), α4 (CD49d), α5 (CD49e), α6 (CD49f), α7 (CD49g), α8 (CD49h), α9 (CD49i), α10 (CD49j), α11 (CD49k), αD (CD11D), αE (CD103), αIIb (CD41), αL (CD11a), αM (CD11b), αV (CD51) and αX (CD11c). All these 18 subunits possess type I transmembrane domain and a small cytoplasmic domain for the activation of downstream signaling.

β-integrins include β1 (CD29), β2 (CD18; LFA-1), β3 (CD61; GPIIIa), β4 (CD104), β5 (ITGB5), β6 (ITGB6), β7 (ITGB7) and β8 (ITGB8). Similar to the α subunits, all 8 β-subunits consist of type I transmembrane domain, and also a small cytoplasmic domain with the except for the β4, which contains a huge cytoplasmic domain of 1088 amino acids.

α-integrins and β-integrins bind non-covalently to each other. Twenty four integrin receptor complexes have been studied for their functions in the mammalian system, these include α1β1, α2β1, α3β1, α4β1, α4β7, α5β1, α6β1, α6β4, α7β1, α8β1, α9β1, α10β1, α11β1, αDβ2, αXβ2, αMβ2, αLβ2, αEβ7, αIIbβ3, αVβ1, αVβ3, αVβ5, αVβ6, αVβ8 (Kumar, 1998; Margadant et al., 2011).

Integrin signaling

α- and β-integrins belong to type I transmembrane receptors with a large extracellular domain. The α subunit and β subunit form a non-covalently associated heterodimeric complex that possesses a N-terminal ligand-binding 'head' domain and a C-terminal 'leg' domain (Nermut et al., 1988). The association of the ligand-binding domain of the receptor complex and ECM generates signals to the short cytoplasmic tail through the 'leg' domain. These signals trigger different cellular responses and are integrated with the signals generated by the co-signaling receptors such as growth-factor receptors and G-protein-coupled receptors (Harburger and Calderwood, 2009). In addition to this conventional outside-in signaling, the binding affinity to the extracellular ligands is altered by conformational changes of the extracellular domain. This inside-out signaling can be modulated through the cytoplasmic tail of integrin receptors (Calderwood, 2004).

Integrin receptor signaling can be activated or inhibited through the binding to the cytoplasmic tail domain. For example, binding of talin to cytoplasmic tail of β-integrin (αIIbβ3) plays an important role in integrin activation for platelet aggregation (Petrich et al., 2007). Kindlin is another recently identified integrin activator and plays a similar role as talin in platelet and leukocyte adhesion (Ye and Petrich, 2011).

Downstream signaling molecules of integrins include focal adhesion kinase (FAK), Src-family kinases (SFKs), integrin-linked kinase (ILK), paxillin and vinculin. FAK acts as a phosphorylation-dependent scaffold and associates with the cytoplasmic tail of β3-integrins, which in turn regulate adhesion, Rho GTPase activity and migration of the cells (Mitra et al., 2005).

SFKs are Src-family protein kinases and activated upon binding to the cytoplasmic tail of the β-integrins. The activation of Src by SFKs further induces downstream kinases such as FAK, resulting in an alteration of integrin-dependent adhesion (Arias-Salgado et al., 2003). This can be mediated by the growth factor receptor bound protein complex (GRB2-SOS complex) which then activates Ras. Stimulation of Ras signaling sequentially activates PI3K and Raf.

Another Src substrate is Crk-associated substrate (CAS). Phosphorylated CAS binds to CrkII and DOCK180, resulting in the activation of Rac1. The association of activated Rac1 and activated CDC42 further activates MAPK/ ERK kinase kinases (MEKKs), p21-activated kinase (PAK), MAPK/ ERK kinases (MEKs), guanine nucleotide exchange factor Vav1, c-Jun NH2-terminal kinase (JNK) and stress-activated protein kinase

(SPAK), and hence regulates the gene expression and controls the cell proliferation. In addition, extracellular signal-regulated kinase (ERK) activates serum response element (SRF) and Myc and thus regulates cell growth and differentiation. Signal mediated by another protein Src homology 2 domain containing (SHC), together with that generated from the growth receptors, synergistically stimulates Ras/ mitogen-activated protein kinase (MAPK) signaling pathway and hence regulates the cell invasion and migration (Kumar, 1998).

ILK is a key player in the activation of integrin signaling. ILK associates with kindlin, PINCH1, PINCH2, paxillin and the cytoplasmic tail of β-integrins. Although the detailed mechanism of the activation remains to be elucidated, loss of ILK in mice showed defects in platelet aggregation, fibrinogen binding and thrombus formation, suggesting the importance of ILK in the control of platelet function and extracellular binding affinity of integrin receptors (Tucker et al., 2008).

There are other scaffold proteins, such as paxillin and vinculin, which are important for mediating integrin signaling. Paxillin is a scaffold protein for FAK, Src, ILK, vinculin, parvins, CrkII-DOCK180-ELMO complex and the C-terminal of α4-integrin, and enhances the cell migration (Kummer and Ginsberg, 2006). Although vinculin does not associate with the cytoplasmic tail domain of β-integrins, it interacts with F-actin, talin, α-actinin, vasodilator-stimulated phosphoprotein (VASP) and Arp2/3, and is important for the integrin-dependent cell adhesion (Ziegler et al., 2006).

The integrin signaling is terminated by disengaging the intracellular protein complex from the cytoplasmic tail domain of integrin receptor. The dissociation of the signaling complex is mediated by protein phosphorylation, proteolysis and external forces. For example, proteolysis of talin by the protease calpain leads to disengagement of other adhesion components, including paxillin and vinculin, from the signaling complex, resulting in the alteration of adhesion turnover (Franco et al., 2004).

Integrins in hematopoiesis

An important feature of HSCs is the homing ability. This ensures the HSCs staying in the proper niche for the maintenance of HSC homeostasis. Circulating HSCs first bind to the E-Selectin, P-Selectin and CD44 expressed on the endothelial cells in BM sinusoids. The extracellular stromal cell-derived factor-1 (SDF-1)/ CXCL12 activates integrin signaling in a dose dependent manner and enhances the binding of HSCs to the endothelial cells. HSCs then transmigrate through the endothelial layer and basal lamina which are rich in fibronectin, laminin and collagen, and lodge

in endosteal niche for maintenance. ECM proteins and cytokines in the BM microenvironment regulate the adhesion and migration of HSCs. The major players of integrin receptor complexes in this homing process are integrin α4β1 (CD49d/ CD29), α5β1 (CD49e/ CD29) and α6β1 (CD49f/ CD29) receptors (Sahin and Buitenhuis, 2012).

Integrins are critical for the retention of HSCs in the BM niche by regulating the adhesion and transendothelial migration. Blocking the functions of integrin α4β1, α5β1 and αLβ2 (CD11a/ CD18) receptors by antibodies attenuated the adhesion of HSCs to endothelial cells and inhibited the transendothelial migration (Craddock et al., 1997; Imai et al., 1999; Peled et al., 2000; Voermans et al., 2000). In addition, the SDF-1 induced migration of HSCs is mediated by integrin α4β1 and α5β1 receptors (Peled et al., 2000). Other transplantation assays demonstrated that treatment of function-blocking antibodies against integrin α4β1, α5β1, α4β7 receptors reduced the homing ability of HSCs with different extents (Katayama et al., 2004; Scott et al., 2003). Functional blocking of integrin α4 and α6 receptors impaired the BM homing of both short-term and long-term multilineage engrafting stem cells (Qian et al., 2006). However, different observations were reported that integrin α6 receptor is dispensable for the BM homing of cells from different sources, suggesting the contribution of integrin α6 receptor to HSC homing is cell type, specie and developmental-stage specific (Bonig et al., 2009; Qian et al., 2007b).

Integrin αLβ2 receptor may involve in the homing of HSCs. Loss of the integrin β2 subunit showed no difference in HSC homing ability compared to wildtype HSCs. However, treatment of function blocking antibodies against integrin α4β1 to integrin β2 knockout HSCs impaired the homing ability more severely compared to wildtype HSCs, suggesting the importance of integrin β2 subunit in the BM homing and the corresponding functional redundancy with integrin α4 subunit in regulating HSC homing (Papayannopoulou et al., 2001).

Integrin α2 receptor in early mouse hematopoiesis

Integrin α2 (CD49b) receptor is one of the stem cell markers in mouse hematopoietic system, identifying the Thy1.1lo LSK HSCs from a heterogeneous population of mouse BM cells. One study on murine HSCs demonstrated that after a treatment of cyclophosphamide (Cy)/ G-CSF, which is a mobilizing inducer of HSCs, the expression of integrin α2 was reduced whereas that of integrin α5 was elevated (Wagers et al., 2002). Functional studies on murine HSCs showed that integrin α2- HSCs were long-term reconstituting stem cells, while integrin α2+ HSCs only

reconstituted short-term in the recipient mice (Wagers and Weissman, 2006). Similar findings were observed from another study on the identification of long-term, intermediate-term and short-term mouse HSCs, demonstrating integrin α2- HSCs are more primitive than integrin α2+ HSCs in mouse hematopoietic system (Benveniste et al., 2010).

Integrin α2 receptor in platelets and megakaryopoiesis

In general, along the road of myeloid lineage commitment, HSC differentiates sequentially into MPP and CMP. MEP, which is one of the immediate progenitor cell populations generated from CMP, gives rise to erythrocytes and megakaryocytes. Megakaryocytes are giant precursor cells that generate numerous blood platelets. The production of blood platelets undergoes a series of cytoskeleton remodeling such as elongation of proplatelet by microtubules, bending and branching of proplatelet by actin and stabilization of proplatelet architecture by spectin (Italiano, 2013). Defects in these processes result in thrombocytopenia or low platelet level.

Integrin receptor complexes such as α2β1 and αIIbβ3 play crucial roles in platelet adhesion and thrombus formation. For example, activation of platelet triggers inside-out integrin signaling, resulting in conformational changes of integrin α2β1 receptor, and hence converts platelet from non-adherent to adherent state. This process can be reversible by changing the extracellular affinity of integrin receptor complexes. Mechanistically, association of talin and kindlin to the cytoplasmic domain of β1 subunit of integrin α2β1 receptor complex regulates the receptor binding affinity for the extracellular ligands (Calderwood, 2004).

In addition, integrin α2β1 receptor complementarily facilitates the contribution of GPVI, a receptor for collagen, in the platelet activation process, resulting in thrombus formation. Activation of GPVI co-stimulates integrin α2β1 receptor during collagen-induced thrombus formation process (Lecut et al., 2004) whereas the loss of integrin α2β1 receptor attenuates GPVI-induced responses (Kuijpers et al., 2003). Taken together, integrin α2β1 receptor plays an essential role in thrombus formation by complementing with GPVI.

Recent studies demonstrated that activation of integrin α2β1 and αIIbβ3 receptors on platelet was interrelated (Riederer et al., 2002; Van de Walle et al., 2007). Activation of integrin αIIbβ3 receptor by its ligand-mimetic peptide triggered the outside-in signaling, and simultaneously activated the integrin α2β1 receptor (Van de Walle et al., 2007). However, another study demonstrated that blocking the binding of fibrinogen to platelets inhibited the activation of integrin αIIbβ3 receptor but concurrently enhanced the

α2β1-dependent platelet adhesion (Riederer et al., 2002). A better understanding of the interplay between integrin α2β1 and αIIbβ3 signaling will provide important insights into the platelet biology.

A recent study demonstrated knocking out of integrin α2 receptor in megakaryocytes led to a decrease in mean platelet volume and defects in the collagen-induced platelet adhesion and aggregation (Habart et al., 2013). Other studies showed that during the megakaryocytic induction and differentiation, integrin α2 receptor was upregulated by runt-related transcription factor 1 (RUNX1) (Burger et al., 1992; Elagib et al., 2003; Zutter et al., 1992). Both integrin α2 receptor and GPVI are expressed in megakaryocytes. Integrin α2 receptor is crucial for the stress fiber formation process, whereas GPVI is involved in the activation of Erk1/2 pathway (Sabri et al., 2004). Taken together, integrin α2β1 receptor complex might play a role in megakaryopoiesis

Integrin β1 receptors in bone marrow niche

Integrin β1 subunit associates with various integrin α subunits forming the largest family of integrin receptor complexes, including integrin α1β1, α2β1, α3β1, α4β1, α5β1, α6β1, α7β1, α8β1, α9β1, α10β1, α11β1 and αVβ1 receptors (Hynes, 2002). Since HSCs are harbored in the BM microenvironment rich in ECM, the expression of integrin β1 receptors on HSCs suggests β1-integrins may play a role in modulating HSC proliferation, survival and differentiation (Legate et al., 2009). Since both growth factor signaling and cytokine signaling are essential for the HSC homeostasis, the BM niche may regulate HSC self-renewal and differentiation by integrating integrin signaling, growth factor signaling and cytokine signaling (Legate et al., 2009).

β1-integrins are essential for HSC homing to fetal liver, spleen and BM (Potocnik et al., 2000). They are also critical for the control of self-renewal and differentiation of HSCs (Legate et al., 2009). For example, β1-integrins activate the downstream signaling by the association to ECM such as VCAM-1 or fibronectin. In addition, SDF-1 and SCF activate CXC chemokine receptor 4 (CXCR4) and C-Kit, respectively (Driessen et al., 2003; Ma et al., 1999; Williams et al., 2008). Integrating the signals generated from SDF-1/ CXCR4, SCF/ C-Kit and β1-integrin activation controls the cytoskeleton remodeling, and regulates the proliferation and survival of HSCs. The GTPase Rac is one of the key modulators integrating these extrinsic signals (Cancelas et al., 2005).

β1-integrins are ubiquitously expressed in Thy1.1lo LSK HSCs and changes in the corresponding expression level in HSCs resulted in an

alternation of the homing ability (Wagers et al., 2002). Besides, murine HSCs lacking β1-integrins failed to reconstitute the irradiated recipient mice (Potocnik et al., 2000). In addition to acting as critical adhesion receptors mediating the homing and migration of HSCs, β1-integrins are also involved in the maintenance of niches for other tissue-specific stem cells such as MSCs and embryonic stem cells (Pruszak et al., 2009; Suh and Han, 2011; Veevers-Lowe et al., 2011). However, the role of integrin β1 receptor complexes in the HSC niche remains to be elucidated.

Integrin α9β1 and α4β1 receptors have been recently shown to play a regulatory role in the attraction, retention and release of HSCs from the stem cell niche through the binding to osteopontin (Grassinger et al., 2009). Impaired association of osteopontin with integrin α9β1 or α4β1 receptor resulted in the mobilization of HSCs into the peripheral circulation. In addition, bone morphogenetic protein 4 (BMP4) has recently been shown to maintain integrin α4 expression on cultured HSCs through p38 MAPK signaling, resulting in the enhanced homing ability of cultured HSCs (Khurana et al., 2013). Future studies on the functional role of β1-integrins in the HSC niche will provide more fundamental knowledge in HSC homeostasis.

Specific aims of the thesis

Aim 1 (article I):

Human cord blood HSCs reside in CD34+CD38- cell compartment and are enriched in the CD90-expressing subfraction (Majeti et al., 2007). However, this stem cell-enriched population is still highly heterogeneous, and the exact immunophenotype of the rare primitive stem cells remains poorly defined. Our preliminary data showed the heterogeneous expression of integrin α2 receptor in CD34+CD38- human cord blood cells, and that this receptor was expressed in the highest frequency of cells within the stem-cell enriched CD90+CD45RA- subfraction. The aim of this study was to examine the expression of integrin α2 receptor in long-term reconstituting stem cells and hence the possibility of using intergrin α2 receptor for the prospective isolation of primitive stem cells from human cord blood.

Aim 2 (article II):

Human CMPs, GMPs and MEPs have been characterized in the Lin-CD34+CD38+ progenitor cell enriched fraction by differential expression of CD123 and CD45RA markers both in adult BM and cord blood (Manz et al., 2002). However, progenitor cells with restricted lineage potential, including myeloid and erythroid progenitors, have been shown to reside also in the Lin-CD34+CD38- cell fraction (Debili et al., 1996; Doulatov et al., 2010; Majeti et al., 2007), and the phenotypical characteristic of these Lin-CD34+CD38- megakaryocyte-erythrocyte bipotent progenitor cell population is so far largely unknown. In this study, we investigated the expression of integrin α2 in adult human BM Lin-CD34+CD38- stem and progenitor cells, and examined the myeloid differentiation capacity of (Lin-) CD34+CD38- integrin α2+ and α2- cells, and the bipotential megakaryocyte-erythrocyte differentiation potential in Lin-CD34+CD38- integrin α2- cells in human adult BM.

Aim 3 (article III):

The PI3K-AKT signaling pathway plays an important role in cell growth and metabolism. Increased AKT activity is frequently seen in patients with acute myeloid leukemia (AML), providing leukemic cells with both growth-promoting and survival signals involved in the transformation process. The aim of this study was to investigate the effects of hyperactivated AKT on cell cycle arrest and apoptosis in murine HSPCs.

Results and discussion

Article I

Expression of integrin α2 receptor in human cord blood CD34+CD38-CD90+ stem cells engrafting long-term in NOD/SCID-IL2Rγcnull mice

<u>Wan Man Wong</u>, Mikael Sigvardsson, Ingbritt Åstrand-Grundström, Donna Hogge, Jonas Larsson, Hong Qian, Marja Ekblom. Stem Cells, 2013; 31: 360-371.

Recently, integrin α6 receptor has been identified as a novel stem cell marker (Notta et al., 2011). A subfraction of cells expressing integrin α6 in Lin-CD34+CD38-CD90+CD45RA- cord blood-derived cell population enriched stem cells reconstituting NSG mice long-term to a frequency of 1/10.5. Furthermore, reconstitution in 14-28% of mice was reported after single-cell transplantation of Lin-CD34+CD38-CD90+CD45RA-Rho123 lo integrin α6+ cells. However, while mouse stem cell markers enable the prospective isolation of long-term *in vivo* reconstituting stem cells in a purity of at least 50% (Wilson et al., 2007), additional markers are needed for the identification of human *in vivo* long-term reconstituting stem cells.

1.1   Expression of integrins in cord blood stem and progenitor cells

The human cord blood CD34+ hematopoietic cells were isolated by ficoll density gradient centrifugation followed by antigen-specific magnetic bead separation. The expression of integrin α1-α6 and β1 chains in cord blood CD34+CD38- stem and progenitor cells was analyzed by monoclonal antibodies and flow cytometry. While integrin α4, α5 and β1 chains were ubiquitously expressed, integrin α2, α3 and α6 chains were expressed only in a fraction of cells. Specifically, the integrin α2 receptor was expressed in 33.4 ± 7.2% (mean ± SD, n=6) of cord blood Lin-CD34+CD38- cells. The frequency of CD34+CD38- cells expressing integrin α2 was highest in the

stem-cell enriched CD90+CD45RA- cell fraction, compared to the CD90-CD45RA- cell population with lower frequency of *in vivo* repopulating cells and CD90-CD45RA+ cell fraction containing no *in vivo* repopulating activity (Doulatov et al., 2010; Majeti et al., 2007), suggesting that the integrin α2+ cells are upstream of the integrin α2- cells in the HSC developmental hierarchy.

1.2   Cord blood *in vivo* short-term reconstituting cells reside in both integrin α2+ and α2- fraction

We analyzed the expression of integrin α2 receptor in cord blood CD34+CD38- short-term repopulating cells by 8-12 week transplantation assay. CD34+CD38- integrin α2+ or α2- cord blood cells were sorted by FACS and intravenously transplanted into immunodeficient irradiated NSG mice (300-400 cell/ mouse). At 8-12 weeks after transplantation, the reconstitution of human CD45+ hematopoietic cells, CD34+ progenitor cells, CD19+ B-cells and CD15/CD33/CD66b+ myeloid cells in the recipient mouse BM were analyzed by flow cytometry. A similar frequency of mice were reconstituted from the CD34+CD38- integrin α2+ as the α2- cells (11/14 and 12/12 mice, respectively). In accordance with this, the levels of human CD45+, myeloid, B-lymphocytic, and CD34+ chimerism were similar in both groups, showing that the stem cells and progenitors engrafting NSG mice short-term are heterogeneous in the expression of integrin α2 receptor.

1.3   Cord blood *in vivo* long-term reconstituting stem cells are highly enriched in the integrin α2+ fraction

Besides short-term repopulating cells, the expression of integrin α2 receptor in the CD34+CD38- and CD34+CD38-CD90+ long-term repopulating cells was examined by transplantation analysis. At 16-18 weeks after intravenous transplantation of 300 or 650 CD34+CD38- integrin α2+ or α2- cord blood cells into each irradiated NSG mouse, the engraftment of human CD45+, myeloid, B-lymphocytic, and CD34+ cells was examined in the recipient mouse BM by flow cytometry. Cord blood long-term reconstituting stem cells within the CD34+CD38- population were significantly enriched in the integrin α2+ cell fraction, with 14/15 and 8/15 mice engrafted with integrin α2+ cells and integrin α2- cells, respectively. Accordingly, the levels of human CD45+ as well as myeloid and B-lymphoid chimerism were significantly higher in mice transplanted with CD34+CD38- integrin α2+ cells than with the integrin α2- cells. The difference in CD34+ cell

chimerism approached statistically significance (p= 0.064). These findings indicate that within the CD34+CD38- compartment the long-term engrafting stem cells are enriched in the integrin α2+ cell population.

The possible effect of the higher proportion of CD90+ cells in the CD34+CD38- integrin α2+ than α2- fraction for the differences in long-term *in vivo* reconstitution at 16-18 weeks and the frequency of integrin α2+ and α2- long-term reconstituting stem cells within the CD34+CD38-CD90+ cell fraction were examined by 24-week limiting dilution transplantation assay. At 24 weeks post transplantation of CD34+CD38-CD90+ cord blood cells by intrafemoral injection, the proportion of mice reconstituted with the integrin α2+ cells (11/15) was significantly higher than mice reconstituted with the integrin α2- cells (6/18), and the estimated frequency of long-term reconstituting stem cells in the integrin α2+ fraction (1/29) was significantly higher than in the integrin α2- subset (1/157). Accordingly, the levels of human myeloid and CD34+ cell chimerism after transplantation of 150 CD34+CD38-CD90+ cells were significantly higher in mice which received integrin α2+ than integrin α2- cells. The estimated frequencies of long-term repopulating cells, calculated based on the frequencies from the limiting dilution assay and FACS analyses of CD34+CD38-CD90+ cells, was 10.6±2.6 and 1.5±0.6/ 1000 CD34+CD38- cells for integrin α2+ and integrin α2- cells respectively (mean±SD). Therefore, approximately six of seven stem cells within CD34+CD38-CD90+ population reside in the integrin α2+ fraction. Robust lymphomyeloid engraftment was obtained from as few as 10 transplanted integrin α2+ cells. These results show that long-term engrafting stem cells within cord blood CD34+CD38-CD90+ cell population are highly enriched in the fraction expressing integrin α2 receptor and therefore integrin α2 receptor is a novel stem cell marker, which may improve prospective isolation of the primitive HSCs in cord blood.

Notably, the frequency of long-term reconstituting cells in the total cord blood CD34+CD38-CD90+ fraction (1/20), reported by Notta et al. (Notta et al., 2011) is higher than the frequency of long-term reconstituting cells in the integrin α2+ subset of the corresponding cell fraction (1/29) in our study. In this study, positive engraftment was defined as >0.1% human CD45+ cells (Notta et al., 2011). However, myeloid cell reconstitution, because of the short life span of granulocytes, is considered to be the best indicator of ongoing stem cell self-renewal (Uchida et al., 1997). Therefore, we have used the criteria of positive engraftment requiring 0.025% reconstitution of both lymphoid and myeloid cells (Christ et al., 2007), in addition to 0.1% human CD45+ reconstitution. Using these criteria, the number of reconstituted mice was lower than the numbers calculated using the criteria of Notta et al. (Notta et al., 2011) in both groups, since mice having

engraftment of human CD45+ lymphoid cells only were not considered as reconstituted from stem cells in our study.

The expression of integrin α2 in primitive human HSCs is in striking contrast to the findings in mouse HSCs. In mouse BM, integrin α2 was shown to be partially expressed in Thy.1.1lo LSK and Rho123lo CD34lo FLT3- LSK stem-cells. Transplantation of integrin α2hi or α2- cells from the latter cell fraction into congenic recipients resulted in long-term engraftment only from integrin α2- cells while the integrin α2hi cells could maintain multilineage lymphomyeloid reconstitution only for 4-8 weeks (Benveniste et al., 2010; Wagers and Weissman, 2006).

1.4 Integrin α2 receptor expression is maintained in all reconstituted Lin-CD34+CD38- CD90+CD45RA- cells after transplantation of CD34+CD38-CD90+ integrin α2+ cells, while differentiation into Lin-CD34+CD38-CD90-CD45RA- is accompanied by a loss of integrin α2 in a subset of cells

We examined the expression of integrin α2 receptor in the long-term reconstituted stem and progenitor cell fractions after 22-23 week intrafemoral transplantation of 50 CD34+CD38-CD90+ integrin α2+ cord blood cells by FACS. All reconstituted Lin-CD34+CD38-CD90+CD45RA- primitive cells expressed the integrin α2 receptor, further supporting the notion that integrin α2 is a stem cell marker. The engrafted downstream Lin-CD34+CD38-CD90-CD45RA- cell population contained both integrin α2+ and α2- cells, showing that the primitive integrin α2+ cells give rise to integrin α2- cells and reside upstream in the hematopoietic cell developmental hierarchy.

1.5 The integrin α2 receptor is not involved in the homing of cord blood stem and progenitor cells to mouse bone marrow

Experimental studies using function blocking antibodies and gene deletion have established a crucial role for β1-integrins in homing of HSPCs to the BM (Sixt et al., 2006). We therefore studied the role of integrin α2 and β1 receptors in homing of cord blood stem and progenitor cells into the BM of NSG mice using function blocking antibodies. Lineage-depleted cord blood cells were preincubated with the function blocking antibody against integrin α2 or β1 receptor or the corresponding isotype control and then injected intravenously into irradiated NSG mice. The frequency of human CD45+CD34+ cells homed to the recipient mouse BM at 3 hours after

injection was examined by FACS. 9-10% of cord blood CD34+ cells preincubated with isotype control antibodies were recovered in the BM. Pre-treatment with the function blocking antibody against integrin α2 chain did not perturb homing, whereas the anti-integrin β1 antibody significantly inhibited the homing of cord blood CD34+ progenitors. This finding, together with our results showing equal short-term reconstitution levels of CD34+CD38- integrin α2+ and α2- cells, indicates that the difference in long-term reconstitution experiments is due to the enrichment of primitive long-term engrafting cells in the integrin α2+ fractions of both CD34+CD38- and CD34+CD38-CD90+ cell populations.

1.6 Lin-CD34+CD38-CD90+CD45RA- integrin α2+ cells display a distinct gene expression pattern from the integrin α2- subsets and Lin-CD34+CD38-CD90-CD45RA- cell fraction

To further investigate the differences between integrin α2+ and α2- stem and progenitor cells, we analyzed global mRNA expression profiles from 3 separate FACS-sorted populations of Lin-CD34+CD38-CD90+CD45RA- integrin α2+ and α2- cells and compared these to the Lin-CD34+CD38-CD90-CD45RA- cell fraction reported to enrich for MPPs (Goardon et al., 2011; Majeti et al., 2007; Pang et al., 2011). Importantly, the heat map and principal component analysis show that the three individual integrin α2+ cell populations display a gene expression profile distinct from the corresponding integrin α2- cell populations, whereas the integrin α2- cell populations appear to be more closely related to the CD90-CD45RA- MPP cell population. These results are in agreement with our findings from *in vivo* functional assays showing that CD34+CD38-CD90+ integrin α2+ cell population is distinct from the integrin α2- subset and enriches for the primitive hematopoietic stem cells.

1.7 Expression of integrin α2 and α6 chains in human cord-blood derived hematopoietic stem and progenitor cells

The integrin α6 receptor was recently reported as a stem cell marker in human cord blood hematopoietic cells (Notta et al., 2011). Therefore we analyzed its coexpression with integrin α2 chain in Lin-CD34+CD38-CD90+CD45RA- and Lin-CD34+CD38-CD90-CD45RA- cells by flow cytometry. Notably, the expression of these receptors was only partially overlapping, indicating distinct regulation of the expression of these two receptors in primitive human hematopoietic cells. These findings identified integrin α2 receptor as a novel stem cell marker, and suggested the most

primitive human cord blood long-term reconstituting stem cells may be enriched in the cell fraction coexpressing integrin α2 and α6 receptors, which should be addressed in further experimental transplantation studies.

Article II

Identification of bipotential Lin-CD34+CD38- integrin α2-megakaryocyte-erythrocyte progenitors in human bone marrow

Wan Man Wong, Mikael Sigvardsson, Ingbritt Åstrand-Grundström, Hong Qian, Marja Ekblom. Manuscript in preparation.

Human CMPs, GMPs and MEPs have been characterized in the Lin-CD34+CD38+ progenitor cell enriched fraction by differential expression of CD123 and CD45RA markers both in adult BM and cord blood (Manz et al., 2002). However, progenitor cells with restricted lineage potential, including myeloid and erythroid progenitors, have been shown to reside also in the Lin-CD34+CD38- cell fraction (Debili et al., 1996; Doulatov et al., 2010; Majeti et al., 2007). In this article, our *in vitro* progenitor assays show that Lin-CD34+CD38- BFU-Es and CFU-MKs are enriched in the integrin α2- fraction, and that this fraction contains a high frequency of cells with bipotential megakaryocyte-erythrocyte differentiation capacity.

2.1 Expression of integrin receptors in human adult bone marrow hematopoietic stem and progenitor cells

The human adult BM CD34+ hematopoietic cells were isolated by ficoll density gradient centrifugation followed by antigen-specific magnetic bead separation. The expression of integrin α1-α6 and β1 chains in CD34+CD38- BM cells was examined by monoclonal antibodies and flow cytometry. The adult BM cells did not express integrin α1 chain, had a partial expression of integrin α3 and ubiquitous expression of integrin α4, α5 chains and the common β1 chain. Integrin α2 and α6 chains were expressed in most CD34+CD38- adult BM cells. Specifically, the integrin α2 receptor was expressed in 96.7±3.2% (mean±SD, n=25) of CD34+CD38- cells. Within the Lin-CD34+CD38- cell fraction in BM, integrin α2 was expressed in the vast majority of CD90+CD45RA- and CD90-CD45RA- cells, whereas among the CD90-CD45RA+ cells we were able to detect a somewhat larger fraction of cells lacking the expression of integrin α2.

2.2 CD34+CD38- BFU-Es and CFU-MKs are highly enriched in integrin α2- cell fraction

In order to investigate potential functional differences associated with the expression of integrin α2 in CD34+CD38- cells, we analyzed the distribution of multipotent and lineage-committed progenitors by *in vitro* differentiation cultures of sorted BM stem and progenitor cells in methylcellulose. 50 CD34+CD38- integrin α2+ or α2- adult BM cells were sorted by flow cytometer and cultured in the semi-solid methylcellulose medium supplemented with a panel of cytokines (SCF, G-CSF, GM-CSF, IL-3, IL-6 and erythropoietin (EPO)), which supports the myeloid differentiation of progenitor cells, for 14 days. Colonies were scored based on morphology. In addition, colonies with less typical morphology were transferred onto cytospin slides and analyzed after staining with May-Grünwald Giemsa. The frequency of total CFU-Cs and the minor population of CFU-GEMMs were equal in both fractions, whereas CFU-GMs was significantly enriched in the integrin α2+ fraction. In contrast, the integrin α2- fraction was highly enriched in erythroid BFU-E progenitors. The CFU-MK potential of CD34+CD38- integrin α2+ or α2- cells was analyzed in serum free collagen-based culture system, since serum components, including TGF-β, used in methylcellulose cultures are known to inhibit the growth of megakaryocytic colonies (Debili et al., 1996). 100-200 test cells were cultured in each well for 12-13 days and the megakaryocytes were then identified by staining for the megakaryocytic marker CD41 and Giemsa. The CFU-MK frequency was significantly higher in the integrin α2- than α2+ fraction, suggesting an enrichment of megakaryocyte-erythrocyte restricted progenitors in this fraction.

2.3 Lin-CD34+CD38- BFU-Es are highly enriched in integrin α2- cell fraction but not restricted in CD110+CD45RA- subfraction

We evaluated the cloning efficiency and the myeloerythroid potential of adult BM Lin-CD34+CD38- integrin α2+ and α2- cell populations more precisely by single-cell methylcellulose colony assay. Single cells were sorted by FACS-based automated cell deposition unit into a well containing methylcellulose medium supplemented with cytokines in the 96-well culture plate. As shown in the previous cultures, the primitive CFU-GEMM and myeloid CFU-GM progenitors resided in both fractions, while the integrin α2- fraction was highly enriched in erythroid progenitors. 22% of the single integrin α2- cells were found to give rise to erythroid BFU-E

colonies, in contrast to 0.7% of cells in integrin α2+ cell fraction. Besides, approximately 70% of the Lin-CD34+CD38- intergrin α2- colony forming unit possessed erythrocytic potential.

In addition to the enrichment of BFU-Es in Lin-CD34+CD38- integrin α2-fraction, some of CFU-GMs also resided in the integrin α2- cell fraction. We therefore tested whether additional markers could increase the purity of the integrin α2- erythroid progenitor population. In adult BM Lin-CD34+CD38+CD123lo/-CD45RA- cell fraction, the thrombopoietin receptor CD110 can further discriminate MEPs from CMPs (Edvardsson et al., 2006; Manz et al., 2002). We therefore subfractionated Lin-CD34+CD38- integrin α2- cells into CD110+CD45RA-, CD110-CD45RA- and CD110-CD45RA+ populations and assayed these by single-cell colony forming assay. The erythroid progenitors were enriched in but did not uniformly segregate into the CD110+ cell fraction, whereas CFU-GMs were partially depleted from the CD110+ cell fraction. The CD45RA+ cell population within the Lin-CD34+CD38-CD110- integrin α2- cells showed very low clonogenic potential, consistent with the previous report showing that Lin-CD34+CD38-CD45RA+ cells are multilymphoid progenitors and contain only a limited myeloerythroid differentiation capacity (Doulatov et al., 2010). Therefore, the CD45RA+ cell fraction was excluded from further assays.

However, the methylcellulose colony assay does not support megakaryocyte differentiation, and the commitment to erythroid or bipotential megakaryocyte-erythroid lineages cannot be distinguished. A single-cell culture supporting both megakaryocyte and erythroid differentiation has been used for characterizing the bipotency of Lin-CD34+CD38-CD45RA- integrin α2- human adult BM cells.

2.4 Single cell analysis revealed the existence of the bipotential megakaryocyte-erythrocyte progenitors in Lin-CD34+CD38-CD45RA- integrin α2- fraction

Bipotent megakaryocyte-erythrocyte progenitors have been previously by single cell colony assays and liquid cultures shown to exist in the BM CD34+CD38lo/- cell population (Debili et al., 1996). We performed single cell cultures of Lin-CD34+CD38-CD45RA- integrin α2- cells in mini-tray Terasaki plates and 96-well plates using serum free culture conditions and cytokines (SCF, TPO, IL3, EPO) which specifically promote differentiation into erythroid and megakaryocytic lineages (Debili et al., 1996). At day 12-14 of culture, the wells containing colonies were counted and the sizes of the colonies in the Terasaki plates were scored based on the cell numbers or

the percent coverage of the well surface. 30-40% of the wells contained clones, with a similar cloning efficiency in Terasaki plates or 96-well plates. The clone sizes, evaluated in Terasaki plates, showed a high proliferative capacity of the single cells, with one third of the colonies covering 50-100% of the well surface area. Some of the clones were picked for FACS analysis and stained with a panel of monoclonal antibodies against CD41, CD235ab, CD15/CD33/CD66b and CD49b for the presence of megakaryocytes (>0.1% CD41+CD235- cells), erythrocytes (>0.1% CD41-CD235+ cells), myeloid cells (>0.1% CD15/CD33/CD66b+ cells) and the expression of integrin α2. Other randomly picked clones were transferred onto cytospin slides and identified morphologically after May-Grünwald Giemsa staining.

In the flow cytometry assay, clones derived from bipotential progenitors contained both CD41+CD235- megakaryocytic and CD235+CD41-erythrocytic lineage-differentiated cells, whereas erythroid progenitors only produced CD235+CD41- cells. Importantly, as shown by FACS analysis, all 32 analyzed clones, derived from single cells, contained erythroid cells, whereas 22 of the clones contained both erythroid and megakaryocytic cells (using a criteria of >0.1% positive cells), showing a high degree of bipotential megakaryocyte-erythrocyte activity in the integrin α2-population. Minimal myeloid differentiation (CD15/CD33/CD66b+ cells 0.13%, 0.2% and 0.41%) was seen in the FACS analysis in 3 of 32 clones along with erythroid and megakaryocytic differentiation. During differentiation in the single cell cultures, the integrin α2 receptor was upregulated on 51.6±30.5% of CD41+ megakaryocytic cells (mean±SD; n=22), consistent with the notion that integrin α2 is a receptor on platelets with upregulation of expression during maturation of megakaryocytes (Nieswandt et al., 2001). In contrast, integrin α2 receptor was not expressed in CD235+ erythroid cells (0.1 ±0.2%; mean±SD; n=32).

For morphological analysis 83 clones derived from single cells after 12-14 days of liquid culture were individually transferred to cytospin slides and stained by May-Grünwald Giemsa. This allowed also evaluation of several of the clones with very limited cell numbers, which could not be analyzed by flow cytometry. These small colonies invariably consisted of megakaryocytic cells, indicating the corresponding low proliferative potential as demonstrated in previous report (Debili et al., 1996). Apart from these small megakaryocytic clones, bipotential clones containing both erythroid and megakaryocytic cells were identified by morphology in a similar proportion, approximately 2/3 of the clones as by FACS analysis.

In these single-cell liquid cultures of Lin-CD34+CD38-CD45RA- integrin α2- cells at a condition favoring both megakaryocytic and erythrocytic lineage differentiation, approximately half of the single cells were

differentiated along both erythroid and megakaryocytic lineages, while a minority of the cells showed unilineage either erythroid or megakaryocytic differentiation, confirmed by flow cytometry or morphological analysis. Since 40% of Lin-CD34+CD38-CD45RA- integrin α2- single cells gave rise to colonies in Terasaki wells, it can be estimated that 20% of these single sorted cells had a bipotential megakaryocytic-erythroid commitment, demonstrating a high frequency of bipotential MEP progenitors in adult BM Lin-CD34+CD38-CD45RA- integrin α2- cell fraction.

The liquid culture conditions and cytokines were chosen to favor megakaryocyte-erythroid differentiation. In these cultures, myeloid markers were found expressed, albeit in a low level (<0.5% of cells) in 3 of 32 clones analyzed by FACS, suggesting that some of the Lin-CD34+CD38-CD45RA- integrin α2- cells were more primitive progenitors with commitment to both granulocyte-macrophage and megakaryocyte-erythrocyte lineages. However, the single cell colony assays in methylcellulose with cytokine supplementation, also supporting the growth of multipotent myeloid, erythroid and granulocyte-macrophage progenitors, showed that only 1% of the cells in the Lin-CD34+CD38- integrin α2- cell fraction were multipotent CFU-GEMM progenitors. This result supports the notion that most of the CD34+CD38- integrin α2- cells giving rise to megakaryocytic and erythroid cells in the liquid culture system employed here are bipotent MEPs, whereas only few are multipotent myeloid progenitors or stem cells.

2.5 Lin-CD34+CD38- integrin α2+ fraction contains primitive stem and progenitors with LTC-IC activity

We attempted to analyze the expression of integrin α2 receptor in multipotent lymphoid-myeloid progenitors by intrafemoral transplantation into immunodeficient NSG mice and multilineage lympho-myeloid reconstitution analysis at 12 weeks after injection. However, the frequency of mice reconstituted with 300-500 CD34+CD38- integrin α2+ or α2- cells was very low (3/25 and 1/9 respectively), and the low engraftment potential of adult BM stem and progenitor cells observed in this study is accordance with a previous report (Park et al., 2008). Therefore, we applied the *in vitro* LTC-IC assay to examine the presence of primitive progenitors in these populations. 50 CD34+CD38- integrin α2+ or α2- cells were cultured on irradiated murine stromal cells lines SL/SL and M2-10B4, which were engineered to express human cytokines SCF, IL-3 and G-CSF for sustaining the growth and self-renewal of immature progenitor cells (Hogge et al., 1996). Daughter cells produced from the immature progenitors after 6

week of culture were then detected by *in vitro* colony forming assays. Notably, the LTC-IC progenitors were found to reside in the CD34+CD38-integrin α2+ cell fraction. In line with this, short-term liquid culture of Lin-CD34+CD38- integrin α2+ and Lin-CD34+CD38-CD45RA- integrin α2-cells with multiple cytokines promoting differentiation, showed that 97.2% of cells in the integrin α2+ fraction remained CD34+, whereas 27.7 % of the integrin α2- cells differentiated into CD34- cells, further supporting the notion that integrin α2+ cell fraction contains more primitive progenitors than the integrin α2- fraction.

We have recently by employing limiting dilution transplantation assay in NSG mice shown that cord blood long-term multilineage reconstituting stem cells are significantly enriched in the CD34+CD38-CD90+ integrin α2+ cell fraction (Wong et al., 2013). In agreement with this, our present findings show that adult BM LTC-ICs express integrin α2. However, while integrin α2 receptor is expressed in most adult CD34+CD38- cells, the frequency of cells expressing this receptor in the corresponding cell fraction is much lower, approximately 30%, in cord blood. Furthermore, essentially all adult BM CD34+CD38-CD90+ cells express integrin α2+, in contrast to approximately half of the corresponding cord blood cells. The cord blood LTC-ICs were significantly enriched in the integrin α2+ subfraction within the more primitive CD34+CD38-CD90+ cell fraction but equally distributed within the total CD34+CD38- cell fraction, whereas erythroid progenitors were significantly enriched in integrin α2- fraction within CD34+CD38- cell population but resided in an equal frequency in the CD90+ subpopulation. These findings show profound ontogeny-related differences in integrin α2 receptor expression in distinct human hematopoietic progenitor populations.

2.6 Gene expression profiling provides molecular evidence for enrichment of erythroid and megakaryocytic primed progenitors in the CD34+CD38- integrin α2- cell fraction

We examined and compared the gene expression profiles between CD34+CD38- integrin α2+ and α2- adult BM cells. The analysis showed increased expression of hemoglobin delta (HBD) in integrin α2- cells. Q-PCR analysis of the gene expression of the key regulators GATA-2 and non-erythroid transcription factor GATA-3 in the sorted cells from 4 donors showed the upregulation of GATA-2 and downregulation of GATA-3 in the integrin α2- cell fraction.

The increased expression of genes associated with erythroid and megakaryocytic lineages in the integrin α2- population is likely an

indication of so called lineage priming. The transcription of lineage restricted genes has been suggested to reflect epigenetic changes, making genes associated with a defined future lineage more easily accessible for the transcription factor machinery. The link between lineage priming and terminal commitment has been extensively studied in lineage tracing experiments in mice and even though it is apparent that priming is not absolute, the expression of Rag-1 or TdT in multipotent progenitors marks cells prone to development into lymphoid lineages, while expression of GATA-1 and GATA-2 identify cells destined to erythroid-megakaryocytic pathways (Dore and Crispino, 2011; Mancini et al., 2012). Therefore, we believe that the observation of the integrin α2- cell population expressing genes associated with erythroid and megakaryocytic lineages, strongly supports the concept that these cells represent a population primed towards the development of defined lineages.

Article III

Hyperactivated AKT is incompatible with survival when coexpressed with additional oncogenes and drives hematopoietic stem and progenitor cells to cell cycle inhibition and apoptosis

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The PI3K-AKT signaling pathway plays an important role in cell growth and metabolism. Increased AKT activity is frequently seen in patients with AML, providing leukemic cells with both growth-promoting and survival signals involved in the transformation process. In this study, we investigated the consequence of simultaneously expressing hyperactivated AKT and STAT5 to reconstruct FLT3-ITD signaling. We examined the effect of hyperactivated AKT in HSPCs coexpressing STAT5, FLT3-ITD, or apoptotic Bcl-2. We found that AKT was unable to relieve cytokine-dependence. Surprisingly, uncontrolled AKT activity was linked to accumulation of cells in the G0 stage of the cell cycle and increased number of cells undergoing apoptosis. Hyperactivated AKT was incompatible with STAT5-driven proliferation and triggered apoptosis. Taken together, the constitutive AKT activity is incompatible with the growth and survival promoting ability of FLT3-ITD and its downstream targets. In addition, cells expressing hyperactivated AKT displayed higher levels of reactive oxygen species (ROS) and treatment of antioxidant partially prevented apoptosis. This suggested that the detrimental effects by hyperactivated AKT is correlated to accumulation of increased levels of ROS.

3.1 Constitutively active AKT promotes only short-term survival, is incompatible with STAT5-driven proliferation and triggers apoptosis.

Mutation in the FLT3 gene is one of the most common genetic alternations found in patient with AML. Constitutively activated FLT3 occurs most often as internal tandem duplications (ITDs) within the juxtamembrane domain and/or the first kinase domain, and is observed in approximately 20-30% of AML patients (Kayser et al., 2009; Small, 2006). FLT3-ITD mutations are associated with activation of the PI3K/AKT pathway similar to wildtype FLT3 (Brandts et al., 2005; Jonsson et al., 2004). In contrast, STAT5 has been demonstrated to be activated solely by FLT3-ITD signaling, which is required for transformation *in vivo* (Choudhary et al.,

2007; Rocnik et al., 2006). To identify the role of activated AKT in FLT3-ITD signaling, we generated STAT5-expressing cells in the hematopoietic progenitor cell lines Ba/F3 and FDC-P1. A constitutively active form of STAT5 (ca-STAT5) was retrovirally introduced to Ba/F3 and the effects of proliferation was studied. STAT5 alone was able to sustain proliferation upon cytokine deprivation. In contrast, the consequence of simultaneously expressing STAT5 and AKT in Ba/F3 cells, using constitutively active form of AKT, led to apoptosis.

Next, we tested if functional AKT is necessary for survival in the progenitors by introducing a dominant negative form of AKT, K179M, in which the ATP-binding site is mutated and functions as a kinase dead protein (Bellacosa et al., 1998). The level of apoptosis was increased in FDC-P1 cells expressing K179M in the presence of SCF (23%) compared to cells expressing wildtype AKT (9%), indicating that functional AKT is crucial for survival of cytokine signaling.

3.2 Expression of activated AKT induces cell cycle exit and apoptosis of FLT3-ITD expressing progenitor cells of bone marrow

We overexpressed hyperactivated AKT in progenitor cells of FLT3-ITD transgenic mice. BM cells enriched for C-Kit by magnetic positive selection were infected with control GFP virus or virus expressing both GFP and hyperactivated AKT (or myristylated AKT, myrAKT) in the presence of cytokines known to support survival and growth of HSPCs (i.e., SCF+FL or SCF+FL+TPO). Two days after infection, GFP+ cells were FACS-sorted and re-cultured in media with or without cytokines. Cell cycle analysis (after 48 hours) and annexin V-staining for apoptosis (after 72 hours) were performed by flow cytometry. In the absence of cytokine, the proportion of myrAKT/ GFP+ progenitor cells of FLT3-ITD mice in G0 of the cell cycle increased significantly after 2 days in comparison to wildtype cells. Surprisingly, this was also seen when cells were cultured with cytokines. Thus, the numbers of C-Kit+ progenitors in G0 after stimulation with either SCF and FL or SCF, FL and TPO were significantly higher from FLT3-ITD mice coexpressing myrAKT compared to wildtype mice, but this was also evident for control progenitors expressing myrAKT. The numbers of apoptotic cells increased in cultures of FLT3-ITD expressing cells, indicating that hyperactivated AKT did not synergize with FLT3-ITD but is detrimental to the cells.

3.3 Activated AKT is incompatible with survival of anti-apoptotic BCL-2

Since anti-apoptotic BCL-2 family members are transcriptionally activated by FLT3-ITD signaling, in particular BCL-XL and MCL-1 (Bagrintseva et al., 2005; Kasper et al., 2012; Yoshimoto et al., 2009), we decided to analyze the consequence of overexpressing myrAKT in progenitor cells of BCL-2 transgenic mice. BM cells enriched for C-Kit expression were infected with control virus or virus containing myrAKT, and after sorting GFP+ cells we analyzed cell cycle and apoptosis during 3 days. Surprisingly, overexpression of myrAKT induced apoptosis but the expression of BCL-2 was not able to inhibit this apoptotic effect.

3.4 Engraftment of HSPCs in recipient mice is impaired by hyperactivated AKT

The inability of myrAKT to sustain survival and instead forcing cells into cell cycle block and cell death is evident from the above findings; however we wanted to corroborate this in transplantable HSPCs *in vivo*. BM-derived progenitor cells enriched for C-Kit expression of either wildtype (wt) mice or mice transgenic for BCL-2 were infected with control virus or myrAKT-virus. Forty-eight hours post-infection, 10,000 GFP+ FACS-sorted cells were injected to lethally irradiated mice by intravenous tail injections in a non-competitive setting. Peripheral blood was collected by lateral tail vein bleeding and analyzed at 4, 8, and 12 weeks post-transplant for GFP expression. Already after 4 weeks, reconstitution was negatively affected in mice receiving myrAKT-expressing BM cells. The level of reconstitution at 4 weeks was 17.1% for wt-myrAKT, 54.9% for wt-GFP, 25.2% for BCL2-myrAKT, and 67.2% for BCL2-GFP. At longer time points, chosen to represent transplantable HSPCs (8 and 12 weeks), reconstitution level remained high in both wildtype and BCL-2 mice transplanted with control cells but was severely impaired in mice receiving myrAKT-expressing cells. Engraftment was almost absent in the mice transplanted with myrAKT cells and several mice succumbed to hematopoietic failure during the first 8 weeks post-transplant. In this experiment, presence of BCL-2 expression did not make a difference, again demonstrating the ability of hyperactivated AKT to overcome survival-mediating effects elicited by other pathways.

3.5 Impaired engraftment of bone marrow cells expressing hyperactivated AKT does not correlate to disturbed homing in recipient mice

In the experiments above, BM cells expressing myrAKT was unable to reconstitute recipient mice even with cells expressing BCL-2. However, we could not distinguish whether this was due to apoptotic effects or the ability of AKT to affect homing and/or adhesion of the progenitor cells to the BM. Since previous studies have demonstrated that AKT influences the expression of adhesion molecules and homing receptors (Buitenhuis et al., 2010), we decided to repeat the transplantation experiments and in parallel to intravenous injections, also directly injected cells to the BM by intrafemoral injection. However, the engraftment ability was still impaired after 2 weeks post-transplant and myrAKT-overexpressing cells injected directly in the marrow were unable to reconstitute the hematopoietic system.

3.6. Antioxidant N-acetyl-lysine inhibits hyperactivated Akt-mediated increased ROS

Previous studies have shown that too much activation of AKT can lead to accumulated levels of intracellular ROS which can be detrimental to hematopoietic cells (van Gorp et al., 2006). To investigate whether this could be the cause of the pro-apoptotic effects mediated by activated AKT in cells expressing FLT3-ITD, STAT5, or BCL-2 as shown herein, we analyzed ROS levels in cells overexpressing myrAKT. To enable detection by DCF-DA staining, we used a puromycin-containing myrAKT virus in these experiments. In hematopoietic cells infected with myrAKT virus, the levels of ROS increased significantly. Treatment of infected GFP+ FACS-sorted cells with N-acetyl-lysine, a well-known antioxidant, prevented apoptosis partially, suggesting that the detrimental effects by hyperactivated AKT are linked to the increased levels of ROS.

The inability of BCL-2 to inhibit myrAKT-mediated cell death-inducing effects is surprising when one bears in mind previous findings that BCL-2 is involved as an anti-oxidant agent to overcome ROS effects (Rassool et al., 2007). Furthermore, BCL-2 functions in tilting the balance between pro-apoptotic and anti-apoptotic in favor of the latter (Happo et al., 2012). The myristylated version of AKT utilized herein has been used in many other studies to mimic AKT activation upon ligand-engaged receptor activation and been used to activate AKT in tumor models. In some studies where it showed harmful effects, myrAKT was expressed as a fusion protein to the estrogen receptor. In this case, AKT is inactive until tamoxifen treatment

where the fusion protein is translocated to the nucleus (van Gorp et al., 2006). In contrast, our version is primarily located in the cytoplasm and at the cell membrane. This raises the question to whether myrAKT executes different functions when nuclear localized versus targeted to the cell membrane, and whether BCL-2 can counteract only normal action of AKT. It remains to be demonstrated, however, whether nuclear AKT is a pre-requisite for its apoptotic function.

In summary, it appears that any defective activity such as AKT deletion or AKT hyperactivation will ultimately lead to improper balance of ROS which could be harmful to the cells. A key rationale to novel therapeutic ways to eradicate leukemic cells in patients is to identify the critical signaling components downstream of oncogenic receptors and associated signaling molecules. Inhibition of genes participating in such networks may turn out to be important targets for new ways of intervention. Since activating mutations of FLT3-ITD and other signaling proteins in leukemia lead to activation of the PI3K/AKT pathway, the results herein that hyperactivated AKT can induce a block in cell cycle and under some circumstances leads to apoptosis of FLT3-ITD as well as BCL-2 expressing cells are important findings that could lead to future drug development.

Conclusions

Article I

➢ Integrin α2 is a novel stem cell marker.

➢ Integrin α2 was preferentially expressed in human cord blood HSCs.

➢ A subfraction of Lin-CD34+CD38-CD90+CD45RA- cord blood cells with non-overlapping expression of integrin α2 and α6 was observed.

➢ CD34+CD38-CD90+ cord blood *in vivo* long-term reconstituting stem cells are highly enriched in the integrin α2+ fraction.

➢ Integrin α2 receptor expression was maintained in all reconstituted HSCs after transplantation of integrin α2+ HSCs.

➢ The integrin α2 receptor was not involved in the homing of cord blood stem and progenitor cells to mouse BM.

Article II

➢ Single cell analysis revealed the existence of the bipotential megakaryocyte-erythrocyte progenitors in Lin-CD34+CD38-CD45RA- integrin α2- fraction.

➢ CD34+CD38- BFU-Es and CFU-MKs were highly enriched in integrin α2- cell fraction.

➢ Lin-CD34+CD38- BFU-Es were highly enriched in integrin α2- cell fraction but not restricted in CD110+CD45RA- subfraction.

➢ Lin-CD34+CD38- integrin α2+ fraction contained primitive stem and progenitors with LTC-IC activity.

➢ Gene expression profiling provided molecular evidence for enrichment of erythroid and megakaryocytic primed progenitors in the CD34+CD38- integrin α2- cell fraction.

Article III

➢ Constitutively active AKT promoted only short-term survival, was incompatible with STAT5-driven proliferation and triggered apoptosis.

➢ Expression of activated AKT induced cell cycle exit and apoptosis of FLT3-ITD expressing progenitor cells of BM.

➢ Activated AKT was incompatible with survival by anti-apoptotic BCL-2.

➢ Engraftment of HSPCs in recipient mice was impaired by hyperactivated AKT.

➢ Impaired engraftment of BM cells expressing hyperactivated AKT did not correlate to disturbed homing in recipient mice.

➢ Antioxidant N-acetyl-lysine partially inhibited hyperactivated AKT-mediated increased ROS.

Sammanfattning på Svenska
(Summary in Swedish)

Blodbildning är en mycket noggrant reglerad process och i toppen av hierarkin finns de blodbildande stamcellerna som producerar alla typer av mogna blodceller. Detta sker för att kompensera normal fysiologisk förlust av blodceller samt ökade krav under patologiska tillstånd som blödning eller infektion.

Under de senaste decennierna har flera studier fokuserat på att bättre identifiera och isolera specifika populationer av primitiva blodbildande stamceller och mera mogna progenitorceller från både mus och human hematopoes. Syftet har varit att bättre karakterisera de molekylära mekanismer som styr dessa cellers utveckling och förändringar vid malign transformation. Primitiva blodbildande mus stam- och progenitorcellspopulationer är redan väl karakteriserade medan humana stam-och progenitorcellspopulationer fortfarande är mera ofullständigt identifierade.

Integrin celladhesionsreceptorer reglerar väsentliga funktioner i blodbildande celler såsom proliferation, differentiation, överlevnad och migration genom celladhesiva interaktioner med benmärgens stromaceller och bindvävsmolekyler. Vi har analyserat integriner i normala humana hematopoetiska celler och funnit att uttrycket av integrin $\alpha 2$ identifierar nya specifika primitiva cellpopulationer både i navelsträngsblodet och benmärgen.

I navelsträngsblodet har vi visat att de mest primitiva stamceller som kan funktionellt identifieras genom transplantation i immundeficienta möss, är signifikant anrikade i integrin $\alpha 2+$ cellpopulation inom CD34+CD38-CD90+ stam- och progenitorceller. Detta resultat tyder på att integrin $\alpha 2$ är en ny stamcellsmarkör i humant navelsträngsblod.

I human benmärg uttrycker en majoritet av primitiva CD34+CD38- stam - och progenitorceller integrin $\alpha 2$ receptorn och i likhet med resultat från analys av navelsträngsblod, uttrycker benmärgens mera omogna stam- och progenitorceller, analyserade med in vitro LTC-IC assay, integrin $\alpha 2$ receptorn.

66

Vi har vidare visat att benmärgens integrin α2- CD34+CD38- cellfraktion innehåller i en hög frekvens primitiva bipotenta megakaryocyt-erythrocyt progenitorceller. Detta är ett viktigt fynd som kan bidra till ny kunskap om de molekylära mekanismer som styr tidig differentiering till dessa cellinjer.

I en tredje studie har vi visat att aktiverad Akt signalering i mus stamceller är oförenlig med signalering av FLT-ITD signalering.

Identifiering av specifika populationer av primitiva blodbildande stam- och progenitorceller är en förutsättning för studier om reglering av dessa cellers olika funktioner såsom överlevnad, proliferation och differentiering under normal hematopoes. Det är också betydelsefullt för studier om de molekylära mekanismer som leder till malign transformation i blodbildande stam- och progenitorceller.

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References

Adams, G.B., Chabner, K.T., Alley, I.R., Olson, D.P., Szczepiorkowski, Z.M., Poznansky, M.C., Kos, C.H., Pollak, M.R., Brown, E.M., and Scadden, D.T. (2006). Stem cell engraftment at the endosteal niche is specified by the calcium-sensing receptor. Nature 439, 599-603.

Adelman, D.M., Maltepe, E., and Simon, M.C. (1999). Multilineage embryonic hematopoiesis requires hypoxic ARNT activity. Genes Dev 13, 2478-2483.

Adolfsson, J., Borge, O.J., Bryder, D., Theilgaard-Mönch, K., Åstrand-Grundström, I., Sitnicka, E., Sasaki, Y., and Jacobsen, S.E.W. (2001). Upregulation of Flt3 expression within the bone marrow Lin-Sca1+c-kit+ stem cell compartment is accompanied by loss of self-renewal capacity. Immunity 15, 659-669.

Adolfsson, J., Mansson, R., Buza-Vidas, N., Hultquist, A., Liuba, K., Jensen, C.T., Bryder, D., Yang, L., Borge, O.J., Thoren, L.A., et al. (2005). Identification of Flt3+ lympho-myeloid stem cells lacking erythro-megakaryocytic potential a revised road map for adult blood lineage commitment. Cell 121, 295-306.

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I.L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature 404, 193-197.

Amsellem, S., Pflumio, F., Bardinet, D., Izac, B., Charneau, P., Romeo, P.H., Dubart-Kupperschmitt, A., and Fichelson, S. (2003). Ex vivo expansion of human hematopoietic stem cells by direct delivery of the HOXB4 homeoprotein. Nature Medicine 9, 1423-1427.

Antonchuk, J., Sauvageau, G., and Humphries, R.K. (2002). HOXB4-induced expansion of adult hematopoietic stem cells ex vivo. Cell 109, 39-45.

Arai, F., Hirao, A., Ohmura, M., Sato, H., Matsuoka, S., Takubo, K., Ito, K., Koh, G.Y., and Suda, T. (2004). Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche. Cell 118, 149-161.

Arias-Salgado, E.G., Lizano, S., Sarkar, S., Brugge, J.S., Ginsberg, M.H., and Shattil, S.J. (2003). Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. Proc Natl Acad Sci U S A 100, 13298-13302.

Austin, T.W., Solar, G.P., Ziegler, F.C., Liem, L., and Matthews, W. (1997). A role for the Wnt gene family in hematopoiesis: expansion of multilineage progenitor cells. Blood 89, 3624-3635.

Badillo, A.T., and Flake, A.W. (2006). The regulatory role of stromal microenvironments in fetal hematopoietic ontogeny. Stem Cell Reviews 2, 241-246.

Bagrintseva, K., Geisenhof, S., Kern, R., Eichenlaub, S., Reindl, C., Ellwart, J.W., Hiddemann, W., and Spiekermann, K. (2005). FLT3-ITD-TKD dual mutants associated with AML confer resistance to FLT3 PTK inhibitors and cytotoxic agents by overexpression of Bcl-x(L). Blood 105, 3679-3685.

Baum, C.M., Weissman, I.L., Tsukamoto, A.S., Buckle, A.M., and Peault, B. (1992). Isolation of a candidate human hematopoietic stem-cell population. Proceedings of the National Academy of Sciences of the United States of America 89, 2804-2808.

Bellacosa, A., Chan, T.O., Ahmed, N.N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J., and Tsichlis, P. (1998). Akt activation by growth factors is a multiple-step process: the role of the PH domain. Oncogene 17, 313-325.

Benveniste, P., Frelin, C., Janmohamed, S., Barbara, M., Herrington, R., Hyam, D., and Iscove, N.N. (2010). Intermediate-Term Hematopoietic Stem Cells with Extended but Time-Limited Reconstitution Potential. Cell Stem Cell 6, 48-58.

Bhatia, M., Bonnet, D., Murdoch, B., Gan, O.I., and Dick, J.E. (1998). A newly discovered class of human hematopoietic cells with SCID- repopulating activity. Nature Medicine 4, 1038-1045.

Bhatia, M., Wang, J.C.Y., Kapp, U., Bonnet, D., and Dick, J.E. (1997). Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proceedings of the National Academy of Sciences of the United States of America 94, 5320-5325.

Blank, U., and Karlsson, S. (2011). The role of Smad signaling in hematopoiesis and translational hematology. Leukemia 25, 1379-1388.

Boisset, J.C., Van Cappellen, W., Andrieu-Soler, C., Galjart, N., Dzierzak, E., and Robin, C. (2010). In vivo imaging of haematopoietic cells emerging from the mouse aortic endothelium. Nature 464, 116-120.

Bonig, H., Priestley, G.V., Wohlfahrt, M., Kiem, H.P., and Papayannopoulou, T. (2009). Blockade of alpha6-integrin reveals diversity in homing patterns among human, baboon, and murine cells. Stem Cells Dev 18, 839-844.

Bracken, A.P., Dietrich, N., Pasini, D., Hansen, K.H., and Helin, K. (2006). Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. Genes Dev 20, 1123-1136.

Brandts, C.H., Sargin, B., Rode, M., Biermann, C., Lindtner, B., Schwable, J., Buerger, H., Muller-Tidow, C., Choudhary, C., McMahon, M., et al. (2005). Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation. Cancer Res 65, 9643-9650.

Broxmeyer, H.E. (2008). Chemokines in hematopoiesis. Current Opinion in Hematology 15, 49-58.

Bruno, L., Hoffmann, R., McBlane, F., Brown, J., Gupta, R., Joshi, C., Pearson, S., Seidl, T., Heyworth, C., and Enver, T. (2004). Molecular signatures of self-renewal, differentiation, and lineage choice in multipotential hemopoietic progenitor cells in vitro. Mol Cell Biol 24, 741-756.

Buitenhuis, M., van der Linden, E., Ulfman, L.H., Hofhuis, F.M., Bierings, M.B., and Coffer, P.J. (2010). Protein kinase B (PKB/c-akt) regulates homing of hematopoietic progenitors through modulation of their adhesive and migratory properties. Blood 116, 2373-2384.

Burger, S.R., Zutter, M.M., Sturgill-Koszycki, S., and Santoro, S.A. (1992). Induced cell surface expression of functional alpha 2 beta 1 integrin during megakaryocytic differentiation of K562 leukemic cells. Exp Cell Res 202, 28-35.

Buske, C., Feuring-Buske, M., Abramovich, C., Spiekermann, K., Eaves, C.J., Coulombel, L., Sauvageau, G., Hogge, D.E., and Humphries, R.K. (2002). Deregulated expression of HOXB4 enhances the primitive growth activity of human hematopoietic cells. Blood 100, 862-868.

Buza-Vidas, N., Woll, P., Hultquist, A., Duarte, S., Lutteropp, M., Bouriez-Jones, T., Ferry, H., Luc, S., and Jacobsen, S.E. (2011). FLT3 expression initiates in fully multipotent mouse hematopoietic progenitor cells. Blood 118, 1544-1548.

Cain, C.J., and Manilay, J.O. (2013). Hematopoietic stem cell fate decisions are regulated by Wnt antagonists: comparisons and current controversies. Exp Hematol 41, 3-16.

Calderwood, D.A. (2004). Integrin activation. J Cell Sci 117, 657-666.

Calvi, L.M., Adams, G.B., Weibrecht, K.W., Weber, J.M., Olson, D.P., Knight, M.C., Martin, R.P., Schipani, E., Divieti, P., Bringhurst, F.R., et al. (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425, 841-846.

Cancelas, J.A., Lee, A.W., Prabhakar, R., Stringer, K.F., Zheng, Y., and Williams, D.A. (2005). Rac GTPases differentially integrate signals regulating hematopoietic stem cell localization. Nat Med 11, 886-891.

Chalhoub, N., and Baker, S.J. (2009). PTEN and the PI3-kinase pathway in cancer. Annual Review of Pathology: Mechanisms of Disease 4, 127-150.

Challen, G.A., Boles, N.C., Chambers, S.M., and Goodell, M.A. (2010). Distinct Hematopoietic Stem Cell Subtypes Are Differentially Regulated by TGF-β1. Cell Stem Cell 6, 265-278.

Chattopadhyay, N., Vassilev, P.M., and Brown, E.M. (1997). Calcium-sensing receptor: Roles in and beyond systemic calcium homeostasis. Biological Chemistry 378, 759-768.

Choudhary, C., Brandts, C., Schwable, J., Tickenbrock, L., Sargin, B., Ueker, A., Bohmer, F.D., Berdel, W.E., Muller-Tidow, C., and Serve, H. (2007). Activation mechanisms of STAT5 by oncogenic Flt3-ITD. Blood 110, 370-374.

Chow, A., Lucas, D., Hidalgo, A., Méndez-Ferrer, S., Hashimoto, D., Scheiermann, C., Battista, M., Leboeuf, M., Prophete, C., Van Rooijen, N., et al. (2011). Bone marrow CD169+ macrophages promote the retention of hematopoietic stem and progenitor cells in the mesenchymal stem cell niche. Journal of Experimental Medicine 208, 761-771.

Christ, O., Lucke, K., Imren, S., Leung, K., Hamilton, M., Eaves, A., Smith, C., and Eaves, C. (2007). Improved purification of hematopoietic stem cells based on their elevated aldehyde dehydrogenase activity. Haematologica 92, 1165-1172.

Christensen, J.L., and Weissman, I.L. (2001). Flk-2 is a marker in hematopoietic stem cell differentiation: A simple method to isolate long-term stem cells. Proceedings of the National Academy of Sciences of the United States of America 98, 14541-14546.

Christopher, M.J., Rao, M., Liu, F., Woloszynek, J.R., and Link, D.C. (2011). Expression of the G-CSF receptor in monocytic cells is sufficient to mediate hematopoietic progenitor mobilization by G-CSF in mice. Journal of Experimental Medicine 208, 251-260.

Chung, Y.S., Kim, H.J., Kim, T.M., Hong, S.H., Kwon, K.R., An, S., Park, J.H., Lee, S., and Oh, I.H. (2009). Undifferentiated hematopoietic cells are characterized by a

genome-wide undermethylation dip around the transcription start site and a hierarchical epigenetic plasticity. Blood 114, 4968-4978.

Civin, C.I., Strauss, L.C., Brovall, C., Fackler, M.J., Schwartz, J.F., and Shaper, J.H. (1984). Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. Journal of Immunology 133, 157-165.

Collins, L.S., and Dorshkind, K. (1987). A stromal cell line from myeloid long-term bone marrow cultures can support myelopoiesis and B lymphopoiesis. Journal of Immunology 138, 1082-1087.

Coulombel, L. (2004). Identification of hematopoietic stem/progenitor cells: strength and drawbacks of functional assays. Oncogene 23, 7210-7222.

Craddock, C.F., Nakamoto, B., Andrews, R.G., Priestley, G.V., and Papayannopoulou, T. (1997). Antibodies to VLA4 integrin mobilize long-term repopulating cells and augment cytokine-induced mobilization in primates and mice. Blood 90, 4779-4788.

Crisan, M., Yap, S., Casteilla, L., Chen, C.W., Corselli, M., Park, T.S., Andriolo, G., Sun, B., Zheng, B., Zhang, L., et al. (2008). A Perivascular Origin for Mesenchymal Stem Cells in Multiple Human Organs. Cell Stem Cell 3, 301-313.

Cumano, A., Dieterlen-Lievre, F., and Godin, I. (1996). Lymphoid potential, probed before circulation in mouse, is restricted to caudal intraembryonic splanchnopleura. Cell 86, 907-916.

Debili, N., Coulombel, L., Croisille, L., Katz, A., Guichard, J., Breton-Gorius, J., and Vainchenker, W. (1996). Characterization of a bipotent erythro-megakaryocytic progenitor in human bone marrow. Blood 88, 1284-1296.

Dore, L.C., and Crispino, J.D. (2011). Transcription factor networks in erythroid cell and megakaryocyte development. Blood 118, 231-239.

Doulatov, S., Notta, F., Eppert, K., Nguyen, L.T., Ohashi, P.S., and Dick, J.E. (2010). Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. Nat Immunol 11, 585-593.

Doulatov, S., Notta, F., Laurenti, E., and Dick, J.E. (2012). Hematopoiesis: a human perspective. Cell Stem Cell 10, 120-136.

Driessen, R.L., Johnston, H.M., and Nilsson, S.K. (2003). Membrane-bound stem cell factor is a key regulator in the initial lodgment of stem cells within the endosteal marrow region. Exp Hematol 31, 1284-1291.

Dykstra, B., Kent, D., Bowie, M., McCaffrey, L., Hamilton, M., Lyons, K., Lee, S.J., Brinkman, R., and Eaves, C. (2007). Long-Term Propagation of Distinct Hematopoietic Differentiation Programs In Vivo. Cell Stem Cell 1, 218-229.

Dzierzak, E., and Speck, N.A. (2008). Of lineage and legacy: The development of mammalian hematopoietic stem cells. Nature Immunology 9, 129-136.

Eberharter, A., and Becker, P.B. (2002). Histone acetylation: a switch between repressive and permissive chromatin. Second in review series on chromatin dynamics. EMBO Rep 3, 224-229.

Edvardsson, L., Dykes, J., and Olofsson, T. (2006). Isolation and characterization of human myeloid progenitor populations--TpoR as discriminator between common myeloid and megakaryocyte/erythroid progenitors. Exp Hematol 34, 599-609.

Ehninger, A., and Trumpp, A. (2011). The bone marrow stem cell niche grows up: mesenchymal stem cells and macrophages move in. J Exp Med 208, 421-428.

Eilken, H.M., Nishikawa, S.I., and Schroeder, T. (2009). Continuous single-cell imaging of blood generation from haemogenic endothelium. Nature 457, 896-900.

Elagib, K.E., Racke, F.K., Mogass, M., Khetawat, R., Delehanty, L.L., and Goldfarb, A.N. (2003). RUNX1 and GATA-1 coexpression and cooperation in megakaryocytic differentiation. Blood 101, 4333-4341.

Fleming, H.E., Janzen, V., Lo Celso, C., Guo, J., Leahy, K.M., Kronenberg, H.M., and Scadden, D.T. (2008). Wnt Signaling in the Niche Enforces Hematopoietic Stem Cell Quiescence and Is Necessary to Preserve Self-Renewal In Vivo. Cell Stem Cell 2, 274-283.

Forsberg, E.C., Serwold, T., Kogan, S., Weissman, I.L., and Passegue, E. (2006). New evidence supporting megakaryocyte-erythrocyte potential of flk2/flt3+ multipotent hematopoietic progenitors. Cell 126, 415-426.

Franco, S.J., Rodgers, M.A., Perrin, B.J., Han, J., Bennin, D.A., Critchley, D.R., and Huttenlocher, A. (2004). Calpain-mediated proteolysis of talin regulates adhesion dynamics. Nat Cell Biol 6, 977-983.

Galy, A., Travis, M., Cen, D., and Chen, B. (1995). Human T, B, natural killer, and dendritic cells arise from a common bone marrow progenitor cell subset. Immunity 3, 459-473.

Gil, J., Bernard, D., and Peters, G. (2005). Role of polycomb group proteins in stem cell self-renewal and cancer. DNA and Cell Biology 24, 117-125.

Goardon, N., Marchi, E., Atzberger, A., Quek, L., Schuh, A., Soneji, S., Woll, P., Mead, A., Alford, K.A., Rout, R., et al. (2011). Coexistence of LMPP-like and GMP-like leukemia stem cells in acute myeloid leukemia. Cancer Cell 19, 138-152.

Goodell, M.A., Brose, K., Paradis, G., Conner, A.S., and Mulligan, R.C. (1996). Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. Journal of Experimental Medicine 183, 1797-1806.

Grassinger, J., Haylock, D.N., Storan, M.J., Haines, G.O., Williams, B., Whitty, G.A., Vinson, A.R., Be, C.L., Li, S., Sorensen, E.S., et al. (2009). Thrombin-cleaved osteopontin regulates hemopoietic stem and progenitor cell functions through interactions with alpha9beta1 and alpha4beta1 integrins. Blood 114, 49-59.

Grassinger, J., Haylock, D.N., Williams, B., Olsen, G.H., and Nilsson, S.K. (2010). Phenotypically identical hemopoietic stem cells isolated from different regions of bone marrow have different biologic potential. Blood 116, 3185-3196.

Gupta, R., Hong, D., Iborra, F., Sarno, S., and Enver, T. (2007). NOV (CCN3) functions as a regulator of human hematopoietic stem or progenitor cells. Science 316, 590-593.

Habart, D., Cheli, Y., Nugent, D.J., Ruggeri, Z.M., and Kunicki, T.J. (2013). Conditional knockout of integrin alpha2beta1 in murine megakaryocytes leads to reduced mean platelet volume. PLoS One 8, e55094.

Hao, Q.L., Zhu, J., Price, M.A., Payne, K.J., Barsky, L.W., and Crooks, G.M. (2001). Identification of a novel, human multilymphoid progenitor in cord blood. Blood 97, 3683-3690.

Happo, L., Strasser, A., and Cory, S. (2012). BH3-only proteins in apoptosis at a glance. J Cell Sci 125, 1081-1087.

74

Harburger, D.S., and Calderwood, D.A. (2009). Integrin signalling at a glance. J Cell Sci 122, 159-163.

Hoebeke, I., De Smedt, M., Stolz, F., Pike-Overzet, K., Staal, F.J.T., Plum, J., and Leclercq, G. (2007). T-, B- and NK-lymphoid, but not myeloid cells arise from human CD34+CD38-CD7+ common lymphoid progenitors expressing lymphoid-specific genes. Leukemia 21, 311-319.

Hogan, C.J., Shpall, E.J., and Keller, G. (2002). Differential long-term and multilineage engraftment potential from subfractions of human CD34+ cord blood cells transplanted into NOD/SCID mice. Proceedings of the National Academy of Sciences of the United States of America 99, 413-418.

Hogge, D.E., Lansdorp, P.M., Reid, D., Gerhard, B., and Eaves, C.J. (1996). Enhanced detection, maintenance, and differentiation of primitive human hematopoietic cells in cultures containing murine fibroblasts engineered to produce human steel factor, interleukin-3, and granulocyte colony-stimulating factor. Blood 88, 3765-3773.

Holyoake, T.L., Nicolini, F.E., and Eaves, C.J. (1999). Functional differences between transplantable human hematopoietic stem cells from fetal liver, cord blood, and adult marrow. Experimental Hematology 27, 1418-1427.

Hynes, R.O. (2002). Integrins: bidirectional, allosteric signaling machines. Cell 110, 673-687.

Imai, K., Kobayashi, M., Wang, J., Ohiro, Y., Hamada, J., Cho, Y., Imamura, M., Musashi, M., Kondo, T., Hosokawa, M., et al. (1999). Selective transendothelial migration of hematopoietic progenitor cells: a role in homing of progenitor cells. Blood 93, 149-156.

Ishii, M., Matsuoka, Y., Sasaki, Y., Nakatsuka, R., Takahashi, M., Nakamoto, T., Yasuda, K., Matsui, K., Asano, H., Uemura, Y., et al. (2011). Development of a high-resolution purification method for precise functional characterization of primitive human cord blood-derived CD34-negative SCID-repopulating cells. Experimental Hematology 39, 203-213.e201.

Isufi, I., Seetharam, M., Zhou, L., Sohal, D., Opalinska, J., Pahanish, P., and Verma, A. (2007). Transforming growth factor-beta signaling in normal and malignant hematopoiesis. Journal of interferon & cytokine research : the official journal of the International Society for Interferon and Cytokine Research 27, 543-552.

Italiano, J.E., Jr. (2013). Unraveling mechanisms that control platelet production. Seminars in thrombosis and hemostasis 39, 15-24.

Itkin, T., Kaufmann, K.B., Gur-Cohen, S., Ludin, A., and Lapidot, T. (2013). Fibroblast growth factor signaling promotes physiological bone remodeling and stem cell self-renewal. Curr Opin Hematol 20, 237-244.

Itoh, K., Tezuka, H., Sakoda, H., Konno, M., Nagata, K., Uchiyama, T., Uchino, H., and Mori, K.J. (1989). Reproducible establishment of hemopoietic supportive stromal cell lines from murine bone marrow. Experimental Hematology 17, 145-153.

Iwasaki, H., and Akashi, K. (2007). Myeloid Lineage Commitment from the Hematopoietic Stem Cell. Immunity 26, 726-740.

Jonsson, M., Engstrom, M., and Jonsson, J.I. (2004). FLT3 ligand regulates apoptosis through AKT-dependent inactivation of transcription factor FoxO3. Biochem Biophys Res Commun 318, 899-903.

Jordan, C.T., and Lemischka, I.R. (1990). Clonal and systemic analysis of long-term hematopoiesis in the mouse. Genes and Development 4, 220-232.

Jude, C.D., Gaudet, J.J., Speck, N.A., and Ernst, P. (2008). Leukemia and hematopoietic stem cells: Balancing proliferation and quiescence. Cell Cycle 7, 586-591.

Jung, Y., Wang, J., Song, J., Shiozawa, Y., Havens, A., Wang, Z., Sun, Y.X., Emerson, S.G., Krebsbach, P.H., and Taichman, R.S. (2007). Annexin II expressed by osteoblasts and endothelial cells regulates stem cell adhesion, homing, and engraftment following transplantation. Blood 110, 82-90.

Juntilla, M.M., Patil, V.D., Calamito, M., Joshi, R.P., Birnbaum, M.J., and Koretzky, G.A. (2010). AKT1 and AKT2 maintain hematopoietic stem cell function by regulating reactive oxygen species. Blood 115, 4030-4038.

Kasper, S., Breitenbuecher, F., Heidel, F., Hoffarth, S., Markova, B., Schuler, M., and Fischer, T. (2012). Targeting MCL-1 sensitizes FLT3-ITD-positive leukemias to cytotoxic therapies. Blood cancer journal 2, e60.

Katayama, Y., Battista, M., Kao, W.M., Hidalgo, A., Peired, A.J., Thomas, S.A., and Frenette, P.S. (2006). Signals from the sympathetic nervous system regulate hematopoietic stem cell egress from bone marrow. Cell 124, 407-421.

Katayama, Y., Hidalgo, A., Peired, A., and Frenette, P.S. (2004). Integrin alpha4beta7 and its counterreceptor MAdCAM-1 contribute to hematopoietic progenitor recruitment into bone marrow following transplantation. Blood 104, 2020-2026.

Kayser, S., Schlenk, R.F., Londono, M.C., Breitenbuecher, F., Wittke, K., Du, J., Groner, S., Spath, D., Krauter, J., Ganser, A., et al. (2009). Insertion of FLT3 internal tandem duplication in the tyrosine kinase domain-1 is associated with resistance to chemotherapy and inferior outcome. Blood 114, 2386-2392.

Kent, D.G., Copley, M.R., Benz, C., Wöhrer, S., Dykstra, B.J., Ma, E., Cheyne, J., Zhao, Y., Bowie, M.B., Gasparetto, M., et al. (2009). Prospective isolation and molecular characterization of hematopoietic stem cells with durable self-renewal potential. Blood 113, 6342-6350.

Khurana, S., Buckley, S., Schouteden, S., Ekker, S., Petryk, A., Delforge, M., Zwijsen, A., and Verfaillie, C.M. (2013). A novel role of BMP4 in adult hematopoietic stem and progenitor cell homing via Smad independent regulation of integrin-alpha4 expression. Blood 121, 781-790.

Kiel, M.J., Radice, G.L., and Morrison, S.J. (2007). Lack of Evidence that Hematopoietic Stem Cells Depend on N-Cadherin-Mediated Adhesion to Osteoblasts for Their Maintenance. Cell Stem Cell 1, 204-217.

Kiel, M.J., Yilmaz, O.H., Iwashita, T., Terhorst, C., and Morrison, S.J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 121, 1109-1121.

Kikushige, Y., Yoshimoto, G., Miyamoto, T., Iino, T., Mori, Y., Iwasaki, H., Niiro, H., Takenaka, K., Nagafuji, K., Harada, M., et al. (2008). Human Flt3 is expressed at the hematopoietic stem cell and the granulocyte/macrophage progenitor stages to maintain cell survival. J Immunol 180, 7358-7367.

Kirstetter, P., Anderson, K., Porse, B.T., Jacobsen, S.E., and Nerlov, C. (2006). Activation of the canonical Wnt pathway leads to loss of hematopoietic stem cell repopulation and multilineage differentiation block. Nat Immunol 7, 1048-1056.

Kondo, M., Weissman, I.L., and Akashi, K. (1997). Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell 91, 661-672.

Kuijpers, M.J., Schulte, V., Bergmeier, W., Lindhout, T., Brakebusch, C., Offermanns, S., Fassler, R., Heemskerk, J.W., and Nieswandt, B. (2003). Complementary roles of glycoprotein VI and alpha2beta1 integrin in collagen-induced thrombus formation in flowing whole blood ex vivo. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 17, 685-687.

Kumar, C.C. (1998). Signaling by integrin receptors. Oncogene 17, 1365-1373.

Kummer, C., and Ginsberg, M.H. (2006). New approaches to blockade of alpha4-integrins, proven therapeutic targets in chronic inflammation. Biochem Pharmacol 72, 1460-1468.

La Motte-Mohs, R.N., Herer, E., and Zúñiga-Pflücker, J.C. (2005). Induction of T-cell development from human cord blood hematopoietic stem cells by Delta-like 1 in vitro. Blood 105, 1431-1439.

Lai, A.Y., and Kondo, M. (2006). Asymmetrical lymphoid and myeloid lineage commitment in multipotent hematopoietic progenitors. Journal of Experimental Medicine 203, 1867-1873.

Lapidot, T., and Petit, I. (2002). Current understanding of stem cell mobilization: The roles of chemokines, proteolytic enzymes, adhesion molecules, cytokines, and stromal cells. Experimental Hematology 30, 973-981.

Lecut, C., Schoolmeester, A., Kuijpers, M.J., Broers, J.L., van Zandvoort, M.A., Vanhoorelbeke, K., Deckmyn, H., Jandrot-Perrus, M., and Heemskerk, J.W. (2004). Principal role of glycoprotein VI in alpha2beta1 and alphaIIbbeta3 activation during collagen-induced thrombus formation. Arterioscler Thromb Vasc Biol 24, 1727-1733.

Legate, K.R., Wickstrom, S.A., and Fassler, R. (2009). Genetic and cell biological analysis of integrin outside-in signaling. Genes Dev 23, 397-418.

Lennartsson, J., and Ronnstrand, L. (2012). Stem cell factor receptor/c-Kit: from basic science to clinical implications. Physiol Rev 92, 1619-1649.

Lessard, J., and Sauvageau, G. (2003). Bmi-1 determines the proliferative capacity of normal and leukaemic stem cells. Nature 423, 255-260.

Li, C.L., and Johnson, G.R. (1992). Rhodamine123 reveals heterogeneity within murine Lin-, Sca-1+ hemopoietic stem cells. Journal of Experimental Medicine 175, 1443-1447.

Lux, C.T. (2007). All primitive and definitive hematopoietic progenitor cells emerging prior to E10 in the mouse embryo are products of the yolk sac. Blood.

Lymperi, S., Ersek, A., Ferraro, F., Dazzi, F., and Horwood, N.J. (2011). Inhibition of osteoclast function reduces hematopoietic stem cell numbers in vivo. Blood 117, 1540-1549.

Méndez-Ferrer, S., Lucas, D., Battista, M., and Frenette, P.S. (2008). Haematopoietic stem cell release is regulated by circadian oscillations. Nature 452, 442-447.

Méndez-Ferrer, S., Michurina, T.V., Ferraro, F., Mazloom, A.R., MacArthur, B.D., Lira, S.A., Scadden, D.T., Mag'Ayan, A., Enikolopov, G.N., and Frenette, P.S. (2010). Mesenchymal and haematopoietic stem cells form a unique bone marrow niche. Nature 466, 829-834.

Ma, Q., Jones, D., and Springer, T.A. (1999). The chemokine receptor CXCR4 is required for the retention of B lineage and granulocytic precursors within the bone marrow microenvironment. Immunity 10, 463-471.

Majeti, R., Park, C.Y., and Weissman, I.L. (2007). Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell 1, 635-645.

Mancini, E., Sanjuan-Pla, A., Luciani, L., Moore, S., Grover, A., Zay, A., Rasmussen, K.D., Luc, S., Bilbao, D., O'Carroll, D., et al. (2012). FOG-1 and GATA-1 act sequentially to specify definitive megakaryocytic and erythroid progenitors. The EMBO journal 31, 351-365.

Manz, M.G., Miyamoto, T., Akashi, K., and Weissman, I.L. (2002). Prospective isolation of human clonogenic common myeloid progenitors. Proceedings of the National Academy of Sciences of the United States of America 99, 11872-11877.

Margadant, C., Monsuur, H.N., Norman, J.C., and Sonnenberg, A. (2011). Mechanisms of integrin activation and trafficking. Curr Opin Cell Biol 23, 607-614.

Matsuzaki, Y., Kinjo, K., Mulligan, R.C., and Okano, H. (2004). Unexpectedly Efficient Homing Capacity of Purified Murine Hematopoietic Stem Cells. Immunity 20, 87-93.

Mazo, I.B., Gutierrez-Ramos, J.C., Frenette, P.S., Hynes, R.O., Wagner, D.D., and Von Andrian, U.H. (1998). Hematopoietic progenitor cell rolling in bone marrow microvessels: Parallel contributions by endothelial selectins and vascular cell adhesion molecule 1. Journal of Experimental Medicine 188, 465-474.

McAlister, I., Wolf, N.S., Pietrzyk, M.E., Rabinovitch, P.S., Priestley, G., and Jaeger, B. (1990). Transplantation of hematopoietic stem cells obtained by a combined dye method fractionation of murine bone marrow. Blood 75, 1240-1246.

McDermott, S.P., Eppert, K., Lechman, E.R., Doedens, M., and Dick, J.E. (2010). Comparison of human cord blood engraftment between immunocompromised mouse strains. Blood 116, 193-200.

Mercier, F.E., Ragu, C., and Scadden, D.T. (2012). The bone marrow at the crossroads of blood and immunity. Nat Rev Immunol 12, 49-60.

Mitra, S.K., Hanson, D.A., and Schlaepfer, D.D. (2005). Focal adhesion kinase: in command and control of cell motility. Nature reviews Molecular cell biology 6, 56-68.

Miyamoto, K., Araki, K.Y., Naka, K., Arai, F., Takubo, K., Yamazaki, S., Matsuoka, S., Miyamoto, T., Ito, K., Ohmura, M., et al. (2007). Foxo3a Is Essential for Maintenance of the Hematopoietic Stem Cell Pool. Cell Stem Cell 1, 101-112.

Miyamoto, K., Yoshida, S., Kawasumi, M., Hashimoto, K., Kimura, T., Sato, Y., Kobayashi, T., Miyauchi, Y., Hoshi, H., Iwasaki, R., et al. (2011). Osteoclasts are dispensable for hematopoietic stem cell maintenance and mobilization. Journal of Experimental Medicine 208, 2175-2181.

Mori, Y., Iwasaki, H., Kohno, K., Yoshimoto, G., Kikushige, Y., Okeda, A., Uike, N., Niiro, H., Takenaka, K., Nagafuji, K., et al. (2009). Identification of the human eosinophil lineage-committed progenitor: revision of phenotypic definition of the human common myeloid progenitor. J Exp Med 206, 183-193.

Morita, Y., Ema, H., and Nakauchi, H. (2010). Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. Journal of Experimental Medicine 207, 1173-1182.

Morrison, S.J., Wandycz, A.M., Hemmati, H.D., Wright, D.E., and Weissman, I.L. (1997). Identification of a lineage of multipotent hematopoietic progenitors. Development 124, 1929-1939.

Morrison, S.J., and Weissman, I.L. (1994). The long-term repopulating subset of hematopoietic stem cells is deterministic and isolatable by phenotype. Immunity 1, 661-673.

Muller-Sieburg, C.E., Sieburg, H.B., Bernitz, J.M., and Cattarossi, G. (2012). Stem cell heterogeneity: Implications for aging and regenerative medicine. Blood 119, 3900-3907.

Nermut, M.V., Green, N.M., Eason, P., Yamada, S.S., and Yamada, K.M. (1988). Electron microscopy and structural model of human fibronectin receptor. The EMBO journal 7, 4093-4099.

Nieswandt, B., Brakebusch, C., Bergmeier, W., Schulte, V., Bouvard, D., Mokhtari-Nejad, R., Lindhout, T., Heemskerk, J.W.M., Zirngibl, H., and Fässler, R. (2001). Glycoprotein VI but not $\alpha 2 \beta 1$ integrin is essential for platelet interaction with collagen. EMBO Journal 20, 2120-2130.

Nilsson, S.K., Johnston, H.M., Whitty, G.A., Williams, B., Webb, R.J., Denhardt, D.T., Bertoncello, I., Bendall, L.J., Simmons, P.J., and Haylock, D.N. (2005). Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells. Blood 106, 1232-1239.

Noort, W.A., Wilpshaar, J., Hertogh, C.D., Rad, M., Lurvink, E.G., van Luxemburg-Heijs, S.A., Zwinderman, K., Verwey, R.A., Willemze, R., and Falkenburg, J.H. (2001). Similar myeloid recovery despite superior overall engraftment in NOD/SCID mice after transplantation of human CD34(+) cells from umbilical cord blood as compared to adult sources. Bone Marrow Transplant 28, 163-171.

Notta, F., Doulatov, S., and Dick, J.E. (2010). Engraftment of human hematopoietic stem cells is more efficient in female NOD/SCID/IL-2Rgc-null recipients. Blood 115, 3704-3707.

Notta, F., Doulatov, S., Laurenti, E., Poeppl, A., Jurisica, I., and Dick, J.E. (2011). Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science 333, 218-221.

Ogawa, M. (1993). Differentiation and proliferation of hematopoietic stem cells. Blood 81, 2844-2853.

Oh, I.H., and Humphries, R.K. (2012). Concise review: Multidimensional regulation of the hematopoietic stem cell state. Stem Cells 30, 82-88.

Ohta, H., Sawada, A., Kim, J.Y., Tokimasa, S., Nishiguchi, S., Keith Humphries, R., Hara, J., and Takihara, Y. (2002). Polycomb group gene rae28 is required for sustaining activity of hematopoietic stem cells. Journal of Experimental Medicine 195, 759-770.

Okada, S., Nakauchi, H., Nagayoshi, K., Nishikawa, S.I., Miura, Y., and Suda, T. (1992). In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. Blood 80, 3044-3050.

Omatsu, Y., Sugiyama, T., Kohara, H., Kondoh, G., Fujii, N., Kohno, K., and Nagasawa, T. (2010). The Essential Functions of Adipo-osteogenic Progenitors as the Hematopoietic Stem and Progenitor Cell Niche. Immunity 33, 387-399.

Osawa, M., Hanada, K.I., Hamada, H., and Nakauchi, H. (1996a). Long-term lymphohematopoietic reconstitution by a single CD34- low/negative hematopoietic stem cell. Science 273, 242-245.

Osawa, M., Nakamura, K., Nishi, N., Takahashi, N., Tokuomoto, Y., Inoue, H., and Nakauchi, H. (1996b). In vivo self-renewal of c-Kit+ Sca-1+ Linlow/- hemopoietic stem cells. Journal of Immunology 156, 3207-3214.

Ottersbach, K., Smith, A., Wood, A., and Göttgens, B. (2010). Ontogeny of haematopoiesis: Recent advances and open questions. British Journal of Haematology 148, 343-355.

Palis, J., Robertson, S., Kennedy, M., Wall, C., and Keller, G. (1999). Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. Development 126, 5073-5084.

Pang, W.W., Price, E.A., Sahoo, D., Beerman, I., Maloney, W.J., Rossi, D.J., Schrier, S.L., and Weissman, I.L. (2011). Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. Proc Natl Acad Sci U S A 108, 20012-20017.

Papayannopoulou, T., Priestley, G.V., Nakamoto, B., Zafiropoulos, V., and Scott, L.M. (2001). Molecular pathways in bone marrow homing: dominant role of alpha(4)beta(1) over beta(2)-integrins and selectins. Blood 98, 2403-2411.

Park, C.Y., Majeti, R., and Weissman, I.L. (2008). In vivo evaluation of human hematopoiesis through xenotransplantation of purified hematopoietic stem cells from umbilical cord blood. Nat Protoc 3, 1932-1940.

Park, I.K., Qian, D., Kiel, M., Becker, M.W., Pihalja, M., Weissman, I.L., Morrison, S.J., and Clarke, M.F. (2003). Bmi-1 is required for maintenance of adult self-renewing haematopoietic stem cells. Nature 423, 302-305.

Parmar, K., Mauch, P., Vergilio, J.A., Sackstein, R., and Down, J.D. (2007). Distribution of hematopoietic stem cells in the bone marrow according to regional hypoxia. Proc Natl Acad Sci U S A 104, 5431-5436.

Peled, A., Kollet, O., Ponomaryov, T., Petit, I., Franitza, S., Grabovsky, V., Slav, M.M., Nagler, A., Lider, O., Alon, R., et al. (2000). The chemokine SDF-1 activates the integrins LFA-1, VLA-4, and VLA-5 on immature human CD34(+) cells: role in transendothelial/stromal migration and engraftment of NOD/SCID mice. Blood 95, 3289-3296.

Petrich, B.G., Fogelstrand, P., Partridge, A.W., Yousefi, N., Ablooglu, A.J., Shattil, S.J., and Ginsberg, M.H. (2007). The antithrombotic potential of selective blockade of talin-dependent integrin alpha IIb beta 3 (platelet GPIIb-IIIa) activation. J Clin Invest 117, 2250-2259.

Phillips, R.L., Reinhart, A.J., and Van Zant, G. (1992). Genetic control of murine hematopoietic stem cell pool sizes and cycling kinetics. Proceedings of the National Academy of Sciences of the United States of America 89, 11607-11611.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science 284, 143-147.

Potocnik, A.J., Brakebusch, C., and Fassler, R. (2000). Fetal and adult hematopoietic stem cells require beta1 integrin function for colonizing fetal liver, spleen, and bone marrow. Immunity 12, 653-663.

Prockop, D.J. (1997). Marrow stromal cells as stem cells for nonhematopoietic tissues. Science 276, 71-74.

Pronk, C.J., Rossi, D.J., Mansson, R., Attema, J.L., Norddahl, G.L., Chan, C.K., Sigvardsson, M., Weissman, I.L., and Bryder, D. (2007). Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. Cell Stem Cell 1, 428-442.

Pruszak, J., Ludwig, W., Blak, A., Alavian, K., and Isacson, O. (2009). CD15, CD24, and CD29 define a surface biomarker code for neural lineage differentiation of stem cells. Stem Cells 27, 2928-2940.

Purton, L.E., and Scadden, D.T. (2007). Limiting Factors in Murine Hematopoietic Stem Cell Assays. Cell Stem Cell 1, 263-270.

Qian, H., Buza-Vidas, N., Hyland, C.D., Jensen, C.T., Antonchuk, J., Månsson, R., Thoren, L.A., Ekblom, M., Alexander, W.S., and Jacobsen, S.E.W. (2007a). Critical Role of Thrombopoietin in Maintaining Adult Quiescent Hematopoietic Stem Cells. Cell Stem Cell 1, 671-684.

Qian, H., Georges-Labouesse, E., Nystrom, A., Domogatskaya, A., Tryggvason, K., Jacobsen, S.E., and Ekblom, M. (2007b). Distinct roles of integrins alpha6 and alpha4 in homing of fetal liver hematopoietic stem and progenitor cells. Blood 110, 2399-2407.

Qian, H., Tryggvason, K., Jacobsen, S.E., and Ekblom, M. (2006). Contribution of alpha6 integrins to hematopoietic stem and progenitor cell homing to bone marrow and collaboration with alpha4 integrins. Blood 107, 3503-3510.

Rafii, S., Mohle, R., Shapiro, F., Frey, B.M., and Moore, M.A.S. (1997). Regulation of hematopoiesis by microvascular endothelium. Leukemia and Lymphoma 27, 375-386.

Ramirez-Bergeron, D.L., Runge, A., Adelman, D.M., Gohil, M., and Simon, M.C. (2006). HIF-dependent hematopoietic factors regulate the development of the embryonic vasculature. Dev Cell 11, 81-92.

Randall, T.D., Lund, F.E., Howard, M.C., and Weissman, I.L. (1996). Expression of murine CD38 defines a population of long-term reconstituting hematopoietic stem cells. Blood 87, 4057-4067.

Rassool, F.V., Gaymes, T.J., Omidvar, N., Brady, N., Beurlet, S., Pla, M., Reboul, M., Lea, N., Chomienne, C., Thomas, N.S., et al. (2007). Reactive oxygen species, DNA damage, and error-prone repair: a model for genomic instability with progression in myeloid leukemia? Cancer Res 67, 8762-8771.

Reya, T., Morrison, S.J., Clarke, M.F., and Weissman, I.L. (2001). Stem cells, cancer, and cancer stem cells. Nature 414, 105-111.

Riederer, M.A., Ginsberg, M.H., and Steiner, B. (2002). Blockade of platelet GPIIB-IIIA (Integrin alphaII(b)beta(3)) in flowing human blood leads to passivation of prothrombotic surfaces. Thromb Haemost 88, 858-864.

Rizo, A., Dontje, B., Vellenga, E., De Haan, G., and Sehuringa, J.J. (2008). Long-term maintenance of human hematopoietic stem/progenitor cells by expression of BMI1. Blood 111, 2621-2630.

Rizo, A., Olthof, S., Han, L., Vellenga, E., De Haan, G., and Schuringa, J.J. (2009). Repression of BMI1 in normal and leukemic human CD34+ cells impairs self-renewal and induces apoptosis. Blood 114, 1498-1505.

Rocnik, J.L., Okabe, R., Yu, J.C., Lee, B.H., Giese, N., Schenkein, D.P., and Gilliland, D.G. (2006). Roles of tyrosine 589 and 591 in STAT5 activation and transformation mediated by FLT3-ITD. Blood 108, 1339-1345.

Rossi, L., Lin, K.K., Boles, N.C., Yang, L., King, K.Y., Jeong, M., Mayle, A., and Goodell, M.A. (2012). Less is more: unveiling the functional core of hematopoietic stem cells through knockout mice. Cell Stem Cell 11, 302-317.

Sabri, S., Jandrot-Perrus, M., Bertoglio, J., Farndale, R.W., Mas, V.M., Debili, N., and Vainchenker, W. (2004). Differential regulation of actin stress fiber assembly and proplatelet formation by alpha2beta1 integrin and GPVI in human megakaryocytes. Blood 104, 3117-3125.

Sacchetti, B., Funari, A., Michienzi, S., Di Cesare, S., Piersanti, S., Saggio, I., Tagliafico, E., Ferrari, S., Robey, P.G., Riminucci, M., et al. (2007). Self-Renewing Osteoprogenitors in Bone Marrow Sinusoids Can Organize a Hematopoietic Microenvironment. Cell 131, 324-336.

Sahin, A.O., and Buitenhuis, M. (2012). Molecular mechanisms underlying adhesion and migration of hematopoietic stem cells. Cell adhesion & migration 6, 39-48.

Sakamoto, K., Yamaguchi, S., Ando, R., Miyawaki, A., Kabasawa, Y., Takagi, M., Li, C.L., Perbal, B., and Katsube, K.I. (2002). The nephroblastoma overexpressed gene (NOV/ccn3) protein associates with Notch1 extracellular domain and inhibits myoblast differentiation via Notch signaling pathway. Journal of Biological Chemistry 277, 29399-29405.

Scadden, D.T. (2006). The stem-cell niche as an entity of action. Nature 441, 1075-1079.

Scheller, M., Huelsken, J., Rosenbauer, F., Taketo, M.M., Birchmeier, W., Tenen, D.G., and Leutz, A. (2006). Hematopoietic stem cell and multilineage defects generated by constitutive beta-catenin activation. Nat Immunol 7, 1037-1047.

Schmitt, T.M., and Zúñiga-Pflücker, J.C. (2002). Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. Immunity 17, 749-756.

Schroeder, T. (2010). Hematopoietic Stem Cell Heterogeneity: Subtypes, Not Unpredictable Behavior. Cell Stem Cell 6, 203-207.

Schuringa, J.J., and Vellenga, E. (2010). Role of the polycomb group gene BMI1 in normal and leukemic hematopoietic stem and progenitor cells. Current Opinion in Hematology 17, 294-299.

Schwartz, M.A., and Ginsberg, M.H. (2002). Networks and crosstalk: Integrin signalling spreads. Nature Cell Biology 4, E65-E68.

Scott, L.M., Priestley, G.V., and Papayannopoulou, T. (2003). Deletion of alpha4 integrins from adult hematopoietic cells reveals roles in homeostasis, regeneration, and homing. Mol Cell Biol 23, 9349-9360.

Shojaei, F., Trowbridge, J., Gallacher, L., Yuefei, L., Goodale, D., Karanu, F., Levac, K., and Bhatia, M. (2005). Hierarchical and ontogenic positions serve to define the

molecular basis of human hematopoietic stem cell behavior. Developmental Cell 8, 651-663.

Sieburg, H.B., Cho, R.H., Dykstra, B., Uchida, N., Eaves, C.J., and Muller-Sieburg, C.E. (2006). The hematopoietic stem compartment consists of a limited number of discrete stem cell subsets. Blood 107, 2311-2316.

Six, E.M., Bonhomme, D., Monteiro, M., Beldjord, K., Jurkowska, M., Cordier-Garcia, C., Garrigue, A., Dal Cortivo, L., Rocha, B., Fischer, A., et al. (2007). A human postnatal lymphoid progenitor capable of circulating and seeding the thymus. Journal of Experimental Medicine 204, 3085-3093.

Sixt, M., Bauer, M., Lammermann, T., and Fassler, R. (2006). Beta1 integrins: zip codes and signaling relay for blood cells. Curr Opin Cell Biol 18, 482-490.

Small, D. (2006). FLT3 mutations: biology and treatment. Hematology / the Education Program of the American Society of Hematology American Society of Hematology Education Program, 178-184.

Sohawon, D., Lau, K.K., Lau, T., and Bowden, D.K. (2012). Extra-medullary haematopoiesis: a pictorial review of its typical and atypical locations. J Med Imaging Radiat Oncol 56, 538-544.

Spangrude, G.J., Heimfeld, S., and Weissman, I.L. (1988). Purification and characterization of mouse hematopoietic stem cells. Science 241, 58-62.

Stier, S., Cheng, T., Dombkowski, D., Carlesso, N., and Scadden, D.T. (2002). Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. Blood 99, 2369-2378.

Stier, S., Ko, Y., Forkert, R., Lutz, C., Neuhaus, T., Grünewald, E., Cheng, T., Dombkowski, D., Calvi, L.M., Rittling, S.R., et al. (2005). Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size. Journal of Experimental Medicine 201, 1781-1791.

Sugiyama, T., Kohara, H., Noda, M., and Nagasawa, T. (2006). Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. Immunity 25, 977-988.

Suh, H.N., and Han, H.J. (2011). Collagen I regulates the self-renewal of mouse embryonic stem cells through alpha2beta1 integrin- and DDR1-dependent Bmi-1. J Cell Physiol 226, 3422-3432.

Szilvassy, S.J., Humphries, R.K., Lansdorp, P.M., Eaves, A.C., and Eaves, C.J. (1990). Quantitative assay for totipotent reconstituting hematopoietic stem cells by a competitive repopulation strategy. Proceedings of the National Academy of Sciences of the United States of America 87, 8736-8740.

Tadokoro, Y., Ema, H., Okano, M., Li, E., and Nakauchi, H. (2007). De novo DNA methyltransferase is essential for self-renewal, but not for differentiation, in hematopoietic stem cells. J Exp Med 204, 715-722.

Taichman, R.S. (2005). Blood and bone: Two tissues whose fates are intertwined to create the hematopoietic stem-cell niche. Blood 105, 2631-2639.

Takubo, K., Goda, N., Yamada, W., Iriuchishima, H., Ikeda, E., Kubota, Y., Shima, H., Johnson, R.S., Hirao, A., Suematsu, M., et al. (2010). Regulation of the HIF-1alpha level is essential for hematopoietic stem cells. Cell Stem Cell 7, 391-402.

Tothova, Z., Kollipara, R., Huntly, B.J., Lee, B.H., Castrillon, D.H., Cullen, D.E., McDowell, E.P., Lazo-Kallanian, S., Williams, I.R., Sears, C., et al. (2007). FoxOs Are Critical Mediators of Hematopoietic Stem Cell Resistance to Physiologic Oxidative Stress. Cell 128, 325-339.

Trowbridge, J.J., Snow, J.W., Kim, J., and Orkin, S.H. (2009). DNA methyltransferase 1 is essential for and uniquely regulates hematopoietic stem and progenitor cells. Cell Stem Cell 5, 442-449.

Tucker, K.L., Sage, T., Stevens, J.M., Jordan, P.A., Jones, S., Barrett, N.E., St-Arnaud, R., Frampton, J., Dedhar, S., and Gibbins, J.M. (2008). A dual role for integrin-linked kinase in platelets: regulating integrin function and alpha-granule secretion. Blood 112, 4523-4531.

Uchida, N., Friera, A.M., He, D., Reitsma, M.J., Tsukamoto, A.S., and Weissman, I.L. (1997). Hydroxyurea can be used to increase mouse c-kit+Thy-1. 1(lo)Lin-/loSca-1(+) hematopoietic cell number and frequency in cell cycle in vivo. Blood 90, 4354-4362.

Ueda, T., Yoshida, M., Yoshino, H., Kobayashi, K., Kawahata, M., Ebihara, Y., Ito, M., Asano, S., Nakahata, T., and Tsuji, K. (2001). Hematopoietic capability of CD34+ cord blood cells: a comparison with CD34+ adult bone marrow cells. Int J Hematol 73, 457-462.

Van de Walle, G.R., Schoolmeester, A., Iserbyt, B.F., Cosemans, J.M., Heemskerk, J.W., Hoylaerts, M.F., Nurden, A., Vanhoorelbeke, K., and Deckmyn, H. (2007). Activation of alphaIIbbeta3 is a sufficient but also an imperative prerequisite for activation of alpha2beta1 on platelets. Blood 109, 595-602.

van Gorp, A.G., Pomeranz, K.M., Birkenkamp, K.U., Hui, R.C., Lam, E.W., and Coffer, P.J. (2006). Chronic protein kinase B (PKB/c-akt) activation leads to apoptosis induced by oxidative stress-mediated Foxo3a transcriptional up-regulation. Cancer Res 66, 10760-10769.

Van Os, R., Kamminga, L.M., and De Haan, G. (2004). Stem cell assays: Something old, something new, something borrowed. Stem Cells 22, 1181-1190.

Veevers-Lowe, J., Ball, S.G., Shuttleworth, A., and Kielty, C.M. (2011). Mesenchymal stem cell migration is regulated by fibronectin through alpha5beta1-integrin-mediated activation of PDGFR-beta and potentiation of growth factor signals. J Cell Sci 124, 1288-1300.

Voermans, C., Rood, P.M., Hordijk, P.L., Gerritsen, W.R., and van der Schoot, C.E. (2000). Adhesion molecules involved in transendothelial migration of human hematopoietic progenitor cells. Stem Cells 18, 435-443.

Vogel, W., Scheding, S., Kanz, L., and Brugger, W. (2000). Clinical applications of CD34+ peripheral blood progenitor cells (PBPC). Stem Cells 18, 87-92.

Vousden, K.H., and Prives, C. (2009). Blinded by the Light: The Growing Complexity of p53. Cell 137, 413-431.

Wagers, A.J., Allsopp, R.C., and Weissman, I.L. (2002). Changes in integrin expression are associated with altered homing properties of Lin(-/lo)Thy1.1(lo)Sca-1(+)c-kit(+) hematopoietic stem cells following mobilization by cyclophosphamide/granulocyte colony-stimulating factor. Exp Hematol 30, 176-185.

Wagers, A.J., and Weissman, I.L. (2006). Differential expression of alpha2 integrin separates long-term and short-term reconstituting Lin-/loThy1.1(lo)c-kit+ Sca-1+ hematopoietic stem cells. Stem Cells 24, 1087-1094.

Wang, L.D., and Wagers, A.J. (2011). Dynamic niches in the origination and differentiation of haematopoietic stem cells. Nature reviews Molecular cell biology 12, 643-655.

Warr, M.R., Pietras, E.M., and Passegue, E. (2011). Mechanisms controlling hematopoietic stem cell functions during normal hematopoiesis and hematological malignancies. Wiley interdisciplinary reviews Systems biology and medicine 3, 681-701.

Williams, D.A., Zheng, Y., and Cancelas, J.A. (2008). Rho GTPases and regulation of hematopoietic stem cell localization. Methods Enzymol 439, 365-393.

Wilson, A., Laurenti, E., Oser, G., van der Wath, R.C., Blanco-Bose, W., Jaworski, M., Offner, S., Dunant, C.F., Eshkind, L., Bockamp, E., et al. (2008). Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. Cell 135, 1118-1129.

Wilson, A., Oser, G.M., Jaworski, M., Blanco-Bose, W.E., Laurenti, E., Adolphe, C., Essers, M.A., Macdonald, H.R., and Trumpp, A. (2007). Dormant and self-renewing hematopoietic stem cells and their niches. Ann N Y Acad Sci 1106, 64-75.

Winkler, I.G., Barbier, V., Wadley, R., Zannettino, A.C., Williams, S., and Levesque, J.P. (2010a). Positioning of bone marrow hematopoietic and stromal cells relative to blood flow in vivo: serially reconstituting hematopoietic stem cells reside in distinct nonperfused niches. Blood 116, 375-385.

Winkler, I.G., Sims, N.A., Pettit, A.R., Barbier, V., Nowlan, B., Helwani, F., Poulton, I.J., Van Rooijen, N., Alexander, K.A., Raggatt, L.J., et al. (2010b). Bone marrow macrophages maintain hematopoietic stem cell (HSC) niches and their depletion mobilizes HSCs. Blood 116, 4815-4828.

Wodnar-Filipowicz, A. (2003). Flt3 ligand: role in control of hematopoietic and immune functions of the bone marrow. News in physiological sciences : an international journal of physiology produced jointly by the International Union of Physiological Sciences and the American Physiological Society 18, 247-251.

Wolber, F.M., Leonard, E., Michael, S., Orschell-Traycoff, C.M., Yoder, M.C., and Srour, E.F. (2002). Roles of spleen and liver in development of the murine hematopoietic system. Experimental Hematology 30, 1010-1019.

Wolf, N.S., Kone, A., Priestley, G.V., and Bartelmez, S.H. (1993). In vivo and in vitro characterization of long-term repopulating primitive hematopoietic cells isolated by sequential Hoechst 33342-rhodamine 123 FACS selection. Exp Hematol 21, 614-622.

Wong, W.M., Sigvardsson, M., Astrand-Grundstrom, I., Hogge, D., Larsson, J., Qian, H., and Ekblom, M. (2013). Expression of integrin alpha2 receptor in human cord blood CD34+CD38-CD90+ stem cells engrafting long-term in NOD/SCID-IL2Rgamma(c) null mice. Stem Cells 31, 360-371.

Yamamoto, R., Morita, Y., Ooehara, J., Hamanaka, S., Onodera, M., Rudolph, K.L., Ema, H., and Nakauchi, H. (2013). Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. Cell 154, 1112-1126.

Yamazaki, S., Iwama, A., Takayanagi, S.I., Morita, Y., Eto, K., Ema, H., and Nakauchi, H. (2006). Cytokine signals modulated via lipid rafts mimic niche signals and induce hibernation in hematopoietic stem cells. EMBO Journal 25, 3515-3523.

Ye, F., and Petrich, B.G. (2011). Kindlin: helper, co-activator, or booster of talin in integrin activation? Curr Opin Hematol 18, 356-360.

Yilmaz, Ö.H., Kiel, M.J., and Morrison, S.J. (2006). SLAM family markers are conserved among hematopoietic stem cells from old and reconstituted mice and markedly increase their purity. Blood 107, 924-930.

Yoshihara, H., Arai, F., Hosokawa, K., Hagiwara, T., Takubo, K., Nakamura, Y., Gomei, Y., Iwasaki, H., Matsuoka, S., Miyamoto, K., et al. (2007). Thrombopoietin/MPL Signaling Regulates Hematopoietic Stem Cell Quiescence and Interaction with the Osteoblastic Niche. Cell Stem Cell 1, 685-697.

Yoshimoto, G., Miyamoto, T., Jabbarzadeh-Tabrizi, S., Iino, T., Rocnik, J.L., Kikushige, Y., Mori, Y., Shima, T., Iwasaki, H., Takenaka, K., et al. (2009). FLT3-ITD up-regulates MCL-1 to promote survival of stem cells in acute myeloid leukemia via FLT3-ITD-specific STAT5 activation. Blood 114, 5034-5043.

Zhang, J., Niu, C., Ye, L., Huang, H., He, X., Tong, W.G., Ross, J., Haug, J., Johnson, T., Feng, J.Q., et al. (2003). Identification of the haematopoietic stem cell niche and control of the niche size. Nature 425, 836-841.

Ziegler, W.H., Liddington, R.C., and Critchley, D.R. (2006). The structure and regulation of vinculin. Trends Cell Biol 16, 453-460.

Zilfou, J.T., and Lowe, S.W. (2009). Tumor suppressive functions of p53. Cold Spring Harbor perspectives in biology 1.

Zutter, M.M., Fong, A.M., Krigman, H.R., and Santoro, S.A. (1992). Differential regulation of the alpha 2 beta 1 and alpha IIb beta 3 integrin genes during megakaryocytic differentiation of pluripotential K562 cells. J Biol Chem 267, 20233-20238.

Appendices (Article I-III)

Article I

STEM CELLS®

TISSUE-SPECIFIC STEM CELLS

Expression of Integrin α2 Receptor in Human Cord Blood CD34+CD38−CD90+ Stem Cells Engrafting Long-Term in NOD/SCID-IL2Rγcnull Mice

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ABSTRACT

Human hematopoietic stem cells reside in the CD34+CD38−CD90+ population in cord blood and bone marrow. However, this cell fraction is heterogeneous, and the phenotype of the rare primitive stem cells remains poorly defined. We here report that primitive cord blood CD34+CD38−CD90+ stem cells, with the ability to reconstitute NOD/SCID-IL2Rγcnull (NSG) mice long-term, at 24 weeks after transplantation, can be prospectively isolated at an increased purity by using integrin α2 receptor as an additional stem cell marker. Using a limiting dilution transplantation assay, we found a highly significant enrichment of multilineage reconstituting stem cells in the CD34+CD38−CD90+ cell fraction expressing the integrin α2 receptor, with a frequency of 1/29 cells, as compared to a frequency of 1/157 in the corresponding integrin α2−

cells. In line with this, long-term reconstituting stem cells within the cord blood CD34+CD38− cell population were significantly enriched in the integrin α2+ fraction, while stem cells and progenitors reconstituting short-term, at 8–12 weeks, were heterogeneous in integrin α2 expression. Global gene expression profiling revealed that the lineage-marker negative (Lin−) CD34+CD38−CD90+CD45RA− integrin α2+ cell population was molecularly distinct from the integrin α2− cell population and the more mature Lin−CD34+CD38−CD90−CD45RA− cell population. Our findings identify integrin α2 as a novel stem cell marker, which improves prospective isolation of the primitive human hematopoietic stem cells within the CD34+CD38−CD90+ cell population for experimental and therapeutic stem cell applications. STEM CELLS 2013;31:360–371

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Identification of cell surface markers expressed in primitive human hematopoietic stem and progenitor cell populations is instrumental for a better characterization of molecular mechanisms regulating their functions, as well as for studies on the dysregulated gene expression networks in leukemic stem cells, for development of targeted therapies against leukemias, and for improvement of clinical stem cell transplantation methods.

The xenogeneic transplantation into immunodeficient mice followed by analysis of multilineage reconstitution at various time points is currently the best available experimental

method to assess human hematopoietic stem and progenitor cell populations. Human cells repopulating immunodeficient mice can be phenotypically separated into subpopulations with distinct reconstitution characteristics over time. The CD34+CD38+ progenitors have been shown to repopulate rapidly but are able to maintain hematopoiesis for a maximum of 8–12 weeks [1–3], whereas the more primitive stem cells reside in the lineage marker negative (Lin−) CD34+CD38− fraction. Essentially two types of stem cells within this cell population give rise to multilineage reconstitution in immunodeficient mice. The short-term reconstituting stem cells display limited self-renewal potential. They engraft rapidly but produce lymphoid and myeloid progeny only within the first

12 weeks after transplantation, whereas the more primitive long-term reconstituting stem cells produce myeloid and lymphoid progeny for 5 months or longer [2–4].

In recent years, the non-obese diabetic (NOD)/severe combined immunodeficiency (SCID)-IL2Rγcnull (NSG) mouse strain has been reported as a superior model to assay human hematopoietic stem cell (HSC) engraftment, allowing functional analysis of limited numbers of cells in phenotypically defined fractions [5, 6]. Because of longer survival, the NSG mouse strain allows a better discrimination of long-term repopulating stem cells from short-term stem cells and progenitors than previously used mouse strains. By using this mouse strain, it was shown that human cord blood stem cells reconstituting newborn recipients are enriched in CD90+ fraction within the CD34+CD38− cell population, whereas higher numbers of CD90− cells are required for reconstitution [5, 7].

We have here characterized the expression of integrin α2 receptor chain in human cord blood stem and progenitor cells by in vivo transplantation into NSG mice. Integrins are heterodimeric cell surface receptors consisting of noncovalently associated α and β subunits, which regulate essential cell functions, including transcriptional activation, survival, proliferation, differentiation, adhesion, and migration in a cell- and tissue-specific manner [8]. The largest group is formed by β1 integrins, which contain 12 different α subunits (α1–α11, αv). Studies using gene-deleted mice or function-blocking antibodies have established the crucial role of the integrins containing β1 chain for homing of hematopoietic cells to fetal and postnatal hematopoietic tissues [9, 10], and the role of α4, α5, and α6 integrins for homing or development of hematopoietic cells [11–17].

Recently, integrin α6 receptor has been identified as a novel stem cell marker [7]. Expression of integrin α6 receptor in cord blood CD34+CD38−CD90+ cells enriched stem cells reconstituting NSG mice long-term to a frequency of 1/10.5. Furthermore, reconstitution in 14%–28% of mice was reported after single-cell transplantation of Lin−CD34+CD38−CD90+ CD45RA−Rhodamine 123 (Rho123)^lo integrin α6+ cells. However, while mouse stem cell markers enable prospective isolation of long-term in vivo reconstituting stem cells in a purity of at least 50% [18], additional markers are needed for the identification of human in vivo long-term reconstituting stem cells.

Herein, we have performed limiting dilution transplantation of cord blood CD34+CD38−CD90+ cells in NSG mice and show that integrin α2 is a novel hematopoietic stem cell marker, which is expressed in most primitive long-term multilineage reconstituting stem cells in human cord blood.

MATERIALS AND METHODS

Analysis and Isolation of Cord Blood Hematopoietic Stem and Progenitor Cells

Human cord blood cells were obtained from full-term normal deliveries, in accordance with the protocol approved by the Regional Ethics Committee, Lund (LU 2010/140) and with written informed consent. Functional assays were performed using freshly isolated cells. Mononuclear cells were isolated by Ficoll-Hypaque (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway) density gradient centrifugation. CD34+ and lineage-depleted cells were isolated using magnetic sorting (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com).

Flow Cytometric Analysis and Cell Sorting

Expression analysis of integrin subunits and sorting of cells for functional assays were performed by using the following

human specific monoclonal antibodies (mAb; BD Biosciences, San Jose, CA, http://www.bdbiosciences.com, unless otherwise specified), fluorescein isothiocyanate (FITC) or PerCP-Cy5.5 anti-CD34 (8G12), Allophycocyanin (APC) or PECy7 anti-CD38 (HB7), R-Phycoerythrin (PE) conjugated antibodies against integrin α1-6 chains, CD49a (SR84), CD49b (12F1-H6, AbD Serotec, Raleigh, NC, or 12F1, BD Biosciences), CD49c (17C6, AbD Serotec), CD49d (9F10), CD49e (IIA1), CD49f (GoH3) and integrin β1 chain, CD29 (4B7R, AbD Serotec), APC or FITC anti-CD90 (5E10; FITC anti-CD90: Biolegend, San Diego, CA), biotinylated anti-CD49f (GoH3, eBioscience, San Diego, CA) and streptavidin-APC Alexa Fluor 750 (Invitrogen, Carlsbad, CA, http://www.invitrogen.com). The cells expressing lineage markers were depleted by magnetic sorting or excluded after staining with PECy5 or Tri-color conjugated antibodies against CD3 (S4.1), CD4 (S3.5), CD7 (CD7-6B7), CD8 (3B5), CD14 (TüK4) (Invitrogen), CD19 (HIB19), CD20 (2H7), CD2 (RPA-2.10), CD10 (HI10a), CD11b (ICRF44), CD56 (B159), CD235a (GA-R2) (BD Biosciences). Pacific Blue anti-CD45RA (MEM-56) was from Invitrogen and eFluor450 anti-CD45RA (HI100) was from eBioscience. Dead cells were excluded by propidium iodide (Invitrogen) or 7-amino-actinomycin D (7AAD; Sigma, St Louis, MO) staining. Background staining was determined using appropriate isotype matched control antibodies. Cells were analyzed by LSRII Flow Cytometer System (BD Biosciences) and FlowJo software (TreeStar Inc., San Carlos, CA) or sorted by FACSAria or FACSDiVa Cell Sorter (BD Biosciences, San Jose, California, USA). Quadrant gates shown in fluorescence-activated cell sorting (FACS) plots were set according to fluorescence-minus-one (FMO) controls using corresponding isotype matched control antibodies in all analyses, except for CD34+CD38− cells, which were gated as the lowest 5%–10% CD38− cells out of total CD34+ cells [3].

Mouse Transplantation

NOD.Cg-Prkdcscid Il2rgtml Wjl/SzJ (NSG) mice (Jackson Laboratory, Bar Harbor, ME, http://www.jax.org) were bred and maintained in the germ-free isolators in the animal facility of Lund University. All animal experiments were approved by the animal ethics committee, Lund University (M 148-09).

NSG mice (8–12 weeks old) were sublethally irradiated 4–24 hours prior transplantation with 300 cGy using a ^{137}Cs-γ irradiator. Sex- and age-matched mice were used as recipients in all experimental groups. Cord blood cells were transplanted together with 1×10^6 irradiated (1,500 cGy) CD34− cells from the same donors. The sorted cells were injected intravenously into the tail vein or intrafemorally. For intrafemoral injection, an insulin syringe with 30-gauge needle (BD Biosciences, San Jose, California, USA) was used to drill a hole in the right femur, and 10 μl cell suspension was injected into bone marrow cavity using Hamilton Syringe with 31-gauge needle (Hamilton Bonaduz AG, Bonaduz, GR, Switzerland). One or two mice injected with phosphate-buffered saline (PBS) or irradiated (1,500 cGy) CD34− accessory cells were included in each experiment as controls. For serial transplantation, high numbers of cord blood CD34+CD38−CD90+ integrin α2+ or α2− cells (300 cells per mouse) were intravenously transplanted to primary recipients. After 19 weeks, bone marrow cells from one tibia and one femur of primary recipients with high level of human cell reconstitution were injected intravenously into secondary recipients.

Bone marrow cells were harvested from femurs and tibias of recipient mice at 8–24 weeks after transplantation and stained with human specific APC conjugated anti-CD45 mAb (HI30, BD Biosciences) for detecting human hematopoietic cells, FITC anti-CD15 (HI98), anti-CD33 (HIM3-4), and anti-CD66b

(G10F5) mAb (BD Biosciences) for detecting myeloid cells, PE anti-CD19 mAb (HIB19, BD Biosciences, San Jose, California, USA) for detecting lymphoid cells, and PerCP-Cy5.5 anti-CD34 mAb (8G12, BD Biosciences) for detecting progenitor cells. Gates in FACS plots were set according to FMO controls using corresponding isotype antibodies. Engraftment was determined as positive when a minimum of 0.1% human CD45+ cells, together with at least 0.025% human CD45+CD15/CD33/CD66b+ and 0.025% human CD45+CD19+ cells were detected, with a minimum of 100,000 viable cells collected for analysis [2]. Recently, female NSG mice were reported to be superior in engrafting human HSCs [6]. Therefore, the gender of recipient mice was shown in the results.

For analysis of integrin α2 expression in engrafted stem and progenitor cell populations, bone marrow cells from femurs, tibias, and iliac crests, obtained from three to four recipient mice at 22–23 weeks after transplantation of 50 cord blood CD34+CD38−CD90+ integrin α2+ cells, were pooled, depleted of mouse CD45-positive and human lineage-positive cells (Miltenyi Biotec), and treated with ammonium chloride (Stem Cell Technologies, Vancouver, BC, Canada, http://www.stemcell.com). Expression of human CD45, CD34, CD38, CD90, CD45RA, and CD49b was analyzed by FACS.

Homing Assay

4–24 hours prior injection of cells, NSG mice (8–12 weeks old) were sublethally irradiated with 300 cGy using a ^{137}Cs-γ irradiator. Sex- and age-matched mice were used as recipients in all experimental groups. Fresh lineage-depleted cord blood cells were incubated with azide-free function-blocking mAb against integrin α2 (P1E6) or β1 (P4C10) receptors (Millipore, Billerica, MA, http://www.millipore.com) or corresponding isotype matched control antibodies for 30 minutes on ice. 5 × 10^5 Lin− cord blood cells were injected intravenously into each mouse. Bone marrow cells from femurs were harvested at 3 hours after injection and stained with mouse-specific FITC anti-CD45.1 mAb (A20), human-specific AlexaFluor 700 anti-CD45 (HI30) and PerCP-Cy55 anti-CD34 mAb (BD Biosciences). The proportion of human stem and progenitor cells (mCD45.1-hCD45+CD34+) homed was calculated based on the assumption that mouse bone marrow cells from two femurs constitute 10% of total marrow cellularity [19].

Methylcellulose Colony Assay

The cells (50–100 cells per well) were resuspended in 100 μl Iscoves's modified Dulbecco's medium (PAA, Pasching, Austria, http://www.paa.at) containing 20% fetal bovine serum (FBS) (v/v, Sigma), 2 mM L-glutamine (PAA), 100 U/ml penicillin-streptomycin (PAA), and 10^{-4} M 2-mercaptoethanol (Sigma) and added into 900 μl Methocult H4435 (Stem Cell Technologies, Vancouver, BC, Canada). Cultures were plated in duplicates and maintained at 37°C and 5% CO_2 for 14 days. Colonies were scored based on morphology and the mean value of the duplicate colonies was calculated.

Long-Term Culture Initiating Cell (LTC-IC) Assay

Test cells (25–50 cells per well) were cultured in 200 μl Myelocult H5100 (Stem Cell Technologies), 10^{-6} M hydrocortisone (Sigma) in Collagen I 96-well microplates (BD Biosciences) on irradiated (8,000 cGy) murine stromal cell lines Sl/Sl and M2-10B4 (5,000 each/well), engineered to express human cytokines KL, IL-3 and G-CSF [20]. The cells were maintained at 37°C and 5% CO_2 for 6 weeks with weekly half-medium change and thereafter plated in Methocult H4435 in duplicates (20% and 80% of the cells from each well). The mean values of the duplicate colonies, assayed after 13–14 days, were calculated.

Microarray Analysis

RNA from Lin−CD34+CD38−CD90+CD45RA− integrin α2+, Lin−CD34+CD38− CD90+CD45RA− integrin α2−, and Lin−CD34+CD38−CD90−CD45RA− cells, each sorted from three independent pooled cord blood samples, was extracted as previously described [21], amplified by two rounds of linear in vitro transcription and labeled according to Affymetrix GeneChip Expression Analysis Technical Manual. Chips were scanned using GeneChipTM Scanner 3000. Human Genome U133 plus 2.0 Chips were normalized by robust multiarray average using invariant set normalization and probe level expression values were calculated using the PM-MM model provided by the DNA-Chip Analyzers (dCHIP) software (www.dchip.org). The cell populations were classified and the variations among these were determined using dCHIP built-in principal component analysis (PCA). The graph for the PCA was made in Microsoft Excel based on dimension values generated from dCHIP analysis.

Statistical Analysis

Statistical differences were evaluated by Mann–Whitney test unless stated otherwise. The difference of the proportion of reconstituted mice between groups was evaluated by Fisher's exact test. Based on the results from limiting dilution transplantation, the frequency of long-term repopulating cells was calculated using L-Calc software (Stem Cell Technologies, Vancouver, BC, Canada), using Poisson statistics and the method of maximum likelihood. The two-sided p-value was considered to be significant when $p < .05$.

RESULTS

Expression of Integrins in Cord Blood Stem and Progenitor Cells

The expression of integrin α1–α6 and β1 chains in cord blood CD34+CD38− stem and progenitor cells was analyzed by flow cytometry (Fig. 1 A). While integrin α4, α5, and β1 chains were ubiquitously expressed, integrin α2, α3, and α6 chains were expressed only in a fraction of cells. Specifically, the integrin α2 receptor was expressed in 33.4% ± 7.2% (mean ± SD, $n = 6$) of cord blood Lin−CD34+CD38− cells.

Lin−CD34+CD38− cells can be subdivided according to CD90 and CD45RA expression into three populations representing different stages in the developmental hierarchy [5]. The primitive stem cells, with the capacity for in vivo repopulation in NSG mice, are enriched in the CD90+CD45RA− fraction, whereas the CD90−CD45RA− cell fraction contains a lower frequency of in vivo repopulating cells. The CD90−CD45RA+ fraction contains no in vivo repopulating activity [5], but instead, multilymphoid progenitors and cells with myeloid and dendritic cell potential [22]. We analyzed integrin α2 expression in these CD90/CD45RA subpopulations of Lin−CD34+CD38− cells (Fig. 1B, 1C). The highest frequency of cells expressing integrin α2 was found in the cell populations enriched for the stem cells, suggesting that the integrin α2+ cells are upstream of the integrin α2− cells in the stem cell developmental hierarchy.

The integrin α6 receptor was recently reported as a stem cell marker in human cord blood hematopoietic cells [7]. Therefore, we analyzed its coexpression with integrin α2 chain in Lin−CD34+CD38−CD90+CD45RA− and Lin−CD34+CD38−CD90−CD45RA− cells by flow cytometry. Notably, the expression of these receptors was only partially overlapping (Fig. 1D, 1E), indicating distinct regulation
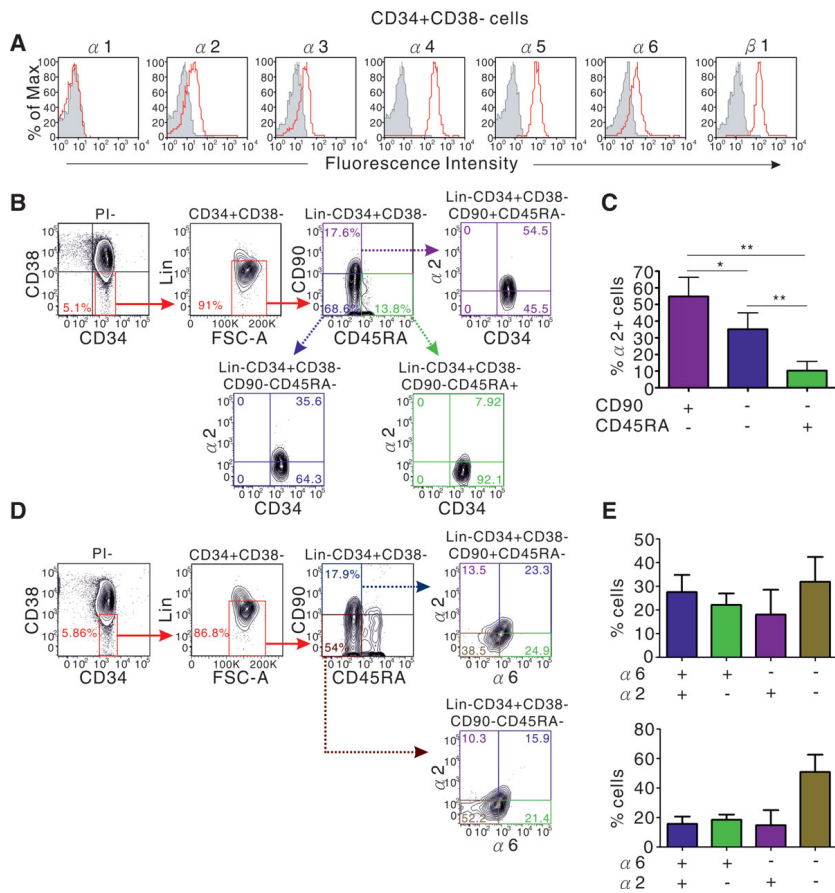
Figure 1. Expression of integrins in human cord blood stem and progenitor cells. **(A):** Representative fluorescence-activated cell sorting (FACS) analysis showing the expression of integrin α1–α6 and β1 chains in cord blood CD34+CD38− cells (open histograms). Shaded histograms show the corresponding isotype control stainings. **(B):** Representative FACS profiles showing viable (PI−) CD34+CD38− cells gated for lineage-marker negative cells (Lin−), CD90 and CD45RA. The expression of integrin α2 receptor is shown in the cells gated from the corresponding quadrants. **(C):** The percentage of cells expressing the integrin α2 receptor in CD90/CD45 quadrants of Lin−CD34+CD38− cells (mean ± SD; three independent analyses, each with two to four pooled cord blood samples). *, $p < .05$; **, $p < .01$. **(D):** Representative FACS profiles showing the expression of integrin α2 and α6 receptors in cord blood Lin−CD34+CD38−CD90+CD45RA− and Lin−CD34+CD38−CD90-CD45RA− cells gated from viable (PI−) cells. **(E):** The percentage of Lin−CD34+CD38−CD90+CD45RA− and Lin−CD34+CD38−CD90-CD45RA− cells expressing integrin α6, α2, both or neither receptor (mean ± SD; two independent experiments using one to three separate pooled cord blood samples). The numbers in each FACS plot show the percentage of cells in each gate or quadrant. Abbreviations: FSC, forward scatter; PI, propidium iodide.

of the expression of these two receptors in primitive human hematopoietic cells.

Cord Blood In Vivo Short-Term Reconstituting Cells Reside in Both Integrin α2+ and α2− Fractions

For analysis of integrin α2 expression in the functionally defined primitive cord blood stem and progenitor cells, CD34+CD38− cells with high or low integrin α2 expression

(α2+ and α2−, respectively) were transplanted into NSG mice (Fig. 2 A). Reconstitution was assayed by analysis of human CD45+ cells, myeloid and B-lymphoid lineages, and CD34+ progenitors in the bone marrow of the recipient mice. No human engraftment was seen in control mice injected only with PBS (Fig. 2B). At 8–12 weeks after i.v. transplantation of 300–400 cord blood CD34+CD38− cells, a similar frequency of mice were reconstituted from the CD34+CD38− integrin α2+ as the α2− cells (11/14 and 12/12 mice,

Figure 2. Integrin α2 expression in cord blood CD34+CD38− cells reconstituting NSG mice short term and long-term. **(A):** Fluorescence-activated cell sorting (FACS) profiles showing gating strategy for the isolation of cord blood CD34+CD38− integrin α2+ and α2− cells for in vivo transplantation into NOD/SCID-IL2Rγ_null (NSG) mice (red gates). Quadrant gates were set on the basis of fluorescence-minus-one (FMO) control staining profiles. **(B):** Representative FACS profiles showing analysis of human cell reconstitution in the bone marrow of NSG mice. The human CD45+ cell reconstitution is shown in the left panel. The middle panels show reconstitution of human myeloid (CD45+CD15/CD33/CD66b+), B-lymphoid (CD45+CD19+), and progenitor (CD45+CD34+) cells in the gated human CD45+ cell fraction. The right panel shows the analysis of the control mouse injected with PBS instead of human cells. The gates were set on the basis of FMO control staining profiles. 7AAD−, viable cells gated by exclusion of 7AAD+ dead cells. **(C):** Bone marrow chimerism of human CD45+, myeloid, B-lymphoid, and CD34+ cells in NSG mice at 8–12 weeks after i.v. transplantation of 300–400 cord blood CD34+CD38− integrin α2+ or α2− cells. **(D):** The number of reconstituted mice/total number of mice intravenously transplanted with indicated numbers of cells. The results are from two independent experiments with pooled cord blood samples. **(E):** Bone marrow chimerism of human CD45+, myeloid, B-lymphoid, and CD34+ cells in NSG mice at 16–18 weeks after i.v. transplantation of 300 CD34+CD38− integrin α2+ or α2− cells. *, $p < .05$. **(F):** The number of reconstituted mice/total number of mice intravenously transplanted with indicated numbers of CD34+CD38−integrin α2+ or α2− cells. The difference in the proportion of reconstituted mice between groups was evaluated by Fisher's exact test. *, $p < .05$. The results are from two independent experiments with pooled cord blood samples. The horizontal bars show median values. Red and blue dots represent individual female and male recipients, respectively. The numbers in each FACS plot show the percentage of cells in each gate or quadrant. Abbreviations: 7AAD, 7-aminoactinomycin D; CB, cord blood; FSC, forward scatter.

respectively). In accordance with this, the levels of human CD45+, myeloid, B-lymphocytic, and CD34+ cell chimerism were similar in both groups, showing that the stem cells and progenitors engrafting NSG mice short-term are heterogeneous in the expression of integrin α2 receptor (Fig. 2C, 2D).

Cord Blood In Vivo Long-Term Reconstituting Stem Cells Are Highly Enriched in the Integrin α2+ Fraction

In contrast to short-term engrafting stem cells and progenitors, cord blood long-term reconstituting stem cells within the CD34+CD38− population, assayed at 16–18 weeks after i.v. transplantation, were significantly enriched in the integrin

α2+ cell fraction (Fig. 2E, 2F), with 14/15 and 8/15 mice engrafted with integrin α2+ cells and α2− cells, respectively (*, $p < .05$; Fisher's exact test). Accordingly, the levels of human CD45+ as well as myeloid and B-lymphoid chimerism were significantly higher in mice transplanted with CD34+CD38− integrin α2+ cells than with the α2− cells. The difference in CD34+ cell chimerism approached statistical significance ($p = .064$). These findings indicate that within the CD34+CD38− compartment the long-term engrafting stem cells are enriched in the integrin α2+ cell population.

To address the possible effect of the higher proportion of CD90+ cells in the CD34+CD38− integrin α2+ than α2− fraction for the differences in long-term in vivo reconstitution at 16–18 weeks and to determine the frequencies of integrin
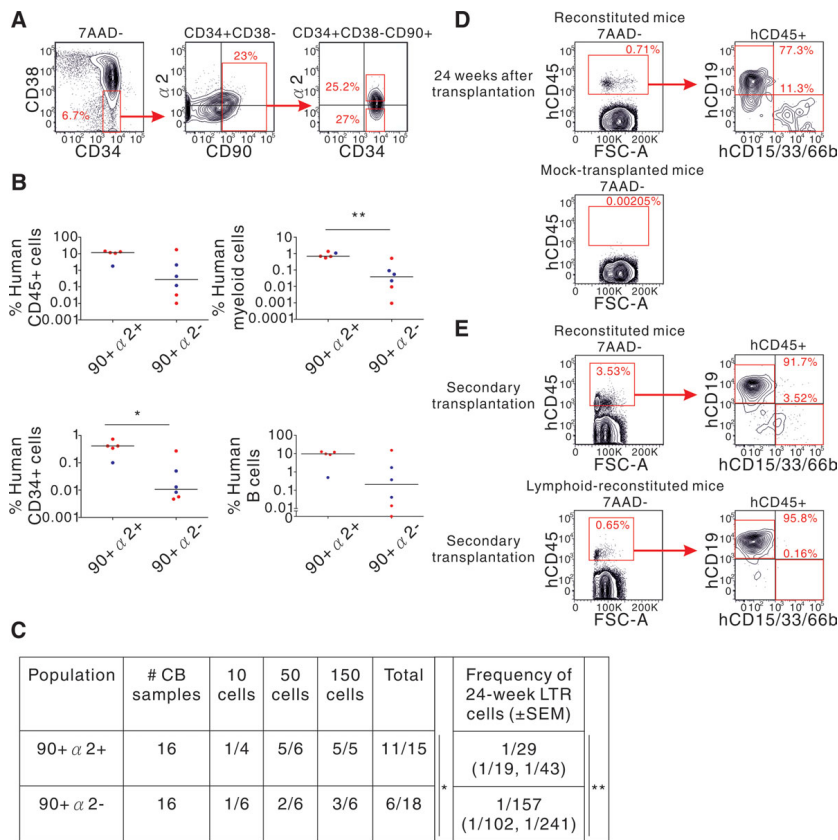
Figure 3. Human cord blood CD34+CD38−CD90+ cells reconstituting NOD/SCID-IL2Rγ_cnull (NSG) mice at 24 weeks after transplantation are enriched in the integrin α2+ fraction. **(A):** Fluorescence-activated cell sorting (FACS) profiles showing gating strategy for the isolation of cord blood viable (7AAD−) CD34+CD38−CD90+ integrin α2+ and α2− cells (red gates). Quadrant gates were set on the basis of fluorescence-minus-one control staining profiles. **(B):** Bone marrow chimerism of human CD45+, myeloid, B-lymphoid, and CD34+ cells in NSG mice at 24 weeks after intrafemoral transplantation of 150 CD34+CD38−CD90+ integrin α2+ or α2− cells. The horizontal bars show median values. *, $p < .05$; **, $p < .01$. Red and blue dots represent individual female and male recipients, respectively. **(C):** Limiting dilution transplantation of CD34+CD38−CD90+ integrin α2+ and α2− cells (10, 50, or 150 cells per mouse) showing the number of reconstituted mice/number of transplanted mice. The frequency of the long-term reconstituting stem cells (LTR; mean ± SEM) and the difference between groups were determined by L-Calc software (**, $p < .01$). The difference in the proportion of reconstituted mice between groups was evaluated by Fisher's exact test (*, $p < .05$). The results are from two independent experiments with pooled cord blood samples. **(D):** Representative FACS profiles showing reconstitution in a NSG mouse at 24 weeks after transplantation of 10 cord blood CD34+CD38−CD90+ integrin α2+ cells (upper panels). The lower panel shows FACS analysis of the bone marrow from a control mouse injected with PBS instead of human cells. **(E):** Representative FACS analysis showing reconstitution at 12 weeks after serial transplantation of cord blood CD34+CD38−CD90+ integrin α2+ cells (upper panels). The lower panels show bone marrow analysis of a transplanted mouse reconstituted with human CD45+ lymphoid cells but not with myeloid cells. The frequency of cells in the CD19−CD15/CD33/CD66b+ quadrant is 0.001% of the viable (7AAD−) cells in the mouse bone marrow. The numbers in each FACS plot show the percentage of cells in each gate or quadrant. Abbreviations: 7AAD, 7-amino-actinomycin D; CB, cord blood; FSC, forward scatter.

α2+ and α2− long-term reconstituting stem cells within the CD34+CD38−CD90+ cell fraction, we performed limiting dilution transplantation assay (Fig. 3A–3D). The CD90+CD45RA+ cell population was only 1.96% ± 2.2% (mean ± SD; $n = 6$) of Lin−CD34+CD38− cells and therefore was not excluded from the CD90+ population. Intrabone transplantation of human cells into immunodeficient mice has

been shown to result in a higher level of reconstitution than i.v. transplantation [23]. Therefore, the cells were transplanted by intrafemoral injection. At 24 weeks post-transplantation, the proportion of mice reconstituted with the integrin α2+ cells (11/15) was significantly higher than mice reconstituted with the integrin α2− cells (6/18), and the estimated frequency of long-term reconstituting stem cells in the integrin
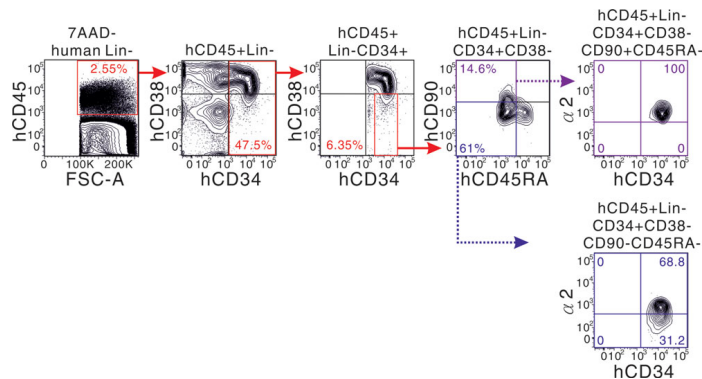
Figure 4. The integrin α2 receptor expression is maintained in all reconstituted Lin−CD34+CD38−CD90+CD45RA− cells after transplantation of CD34+CD38−CD90+ integrin α2+ cells, while differentiation into Lin−CD34+CD38−CD90−CD45RA− cells is accompanied by a loss of integrin α2. Fluorescence activated cell sorting (FACS) analysis of integrin α2 receptor expression in engrafted hematopoietic stem cells after transplantation of CD34+CD38−CD90+ integrin α2+ cells. Viable (propidium iodide −), mouse CD45-marker depleted and human lineage-marker depleted (Lin−) human CD45+Lin−CD34+CD38−CD90+CD45RA− cells (upper right panel) ubiquitously express integrin α2 receptor, while differentiation into the downstream Lin−CD34+CD38−CD90−CD45RA− cell population (lower right panel) is accompanied by a loss of integrin α2 expression in a subset of cells. Representative FACS plots shown are from one of two experiments of pooled bone marrow cells from mice reconstituted at 22–23 weeks after intrafemoral transplantation with 50 CD34+CD38−CD90+ integrin α2+ cells. The numbers in each FACS plot show the percentage of cells in each gate or quadrant. Abbreviations: 7AAD, 7-amino-actinomycin D; FSC, forward scatter.

α2+ fraction (1/29) was significantly higher than in the integrin α2− subset (1/157) (Fig. 3C). Accordingly, the levels of human myeloid and CD34+ cell chimerism after transplantation of 150 CD34+CD38−CD90+ cells were significantly higher in mice which received integrin α2+ than integrin α2− cells (Fig. 3B). The estimated frequencies of long-term repopulating cells, calculated based on the frequencies from the limiting dilution assay and FACS analyses of CD34+CD38−CD90+ cells, was 10.6 ± 2.6 and 1.5 ± 0.6/1,000 CD34+CD38− cells for integrin α2+ and α2− cells, respectively (mean ± SD). Therefore, approximately six seven stem cells within CD34+CD38−CD90+ population reside in the integrin α2+ fraction. Robust lymphomyeloid engraftment was obtained from as few as 10 transplanted integrin α2+ cells (Fig. 3D). These results show that long-term engrafting stem cells within cord blood CD34+CD38−CD90+ cell population are highly enriched in the fraction expressing integrin α2 receptor.

Serial transplantation was only performed from primary recipients with high level of reconstitution. After 12 weeks, reconstitution in secondary recipients was obtained from bone marrow cells one third and 2/2 of primary recipients transplanted with integrin α2+ or α2− cells, respectively (Fig. 3E, upper panel). A similar disparity in secondary transfer efficiency, with a higher frequency of mice reconstituted from CD90− cells than from the stem cell enriched CD90+ fraction of Lin−CD34+ CD38−CD45RA− cells, was shown by Notta et al. [7] but the basis for this remains unknown. With the reconstitution criteria used [2], mice having engraftment of human CD45+ lymphoid cells only were not considered as reconstituted from stem cells (Fig. 3E, lower panel). Taken together, our results show that long-term stem cells reside in both integrin α2+ and α2− cell fractions of CD34+CD38−CD90− cells, albeit, as shown by limiting dilution transplantation, in a significantly higher frequency in the α2+ cell fraction. Engraftment of human cells could be detected in blood, spleen, and thymus in some mice at 22–23 weeks after intrafemoral transplantation of 50 CD34+CD38−CD90+ integrin α2+ and α2− cells (supporting information Fig. S1), whereas no bone marrow

or blood reconstitution was observed in recipients which received only irradiated CD34- accessory cells (not shown).

The Integrin α2 Receptor Expression Is Maintained in All Reconstituted Lin−CD34+CD38−CD90+CD45RA− Cells After Transplantation of CD34+CD38−CD90+ Integrin α2+ Cells, While Differentiation into Lin−CD34+CD38−CD90−CD45RA− Is Accompanied by a Loss of Integrin α2 in a Subset of Cells

The expression of integrin α2 in the long-term reconstituted stem and progenitor cell fractions after intrafemoral transplantation of 50 CD34+CD38−CD90+ integrin α2+ cord blood cells was analyzed by FACS (Fig. 4). As shown previously [5], the transplanted CD34+CD38−CD90+ cells gave rise to the CD90+CD45RA−, as well as the more mature CD90−CD45RA− and CD90−CD45RA+ cell subpopulations of Lin−CD34+CD38− fraction. Importantly, all reconstituted Lin−CD34+CD38−CD90+CD45RA− primitive cells expressed the integrin α2 receptor, further supporting the notion that integrin α2 is a stem cell marker. The engrafted downstream Lin−CD34+CD38−CD90−CD45RA− cell population contained both integrin α2+ and α2− cells, showing that the primitive integrin α2+ cells give rise to integrin α2− stem and progenitor cells during maturation.

The Integrin α2 Receptor Is Not Involved in the Homing of Cord Blood Stem and Progenitor Cells to Mouse Bone Marrow

Experimental studies using function-blocking antibodies and gene deletion have established a crucial role for $\beta 1$ integrins in homing of hematopoietic stem and progenitor cells to bone marrow [8]. We therefore studied the role of integrin α2 and $\beta 1$ receptors in homing of cord blood stem and progenitor cells into bone marrow of NSG mice using function-blocking antibodies (Fig. 5). 9%–10% of cord blood CD34+ cells preincubated with isotype control antibodies were recovered in bone

marrow at 3 hours after i.v. injection. Pretreatment with a function-blocking antibody against integrin $\alpha2$ chain did not perturb homing, whereas the anti-integrin $\beta1$ antibody significantly ($p < .05$) inhibited the homing of cord blood CD34+ progenitors. This finding, together our results showing equal short-term reconstitution levels of CD34+CD38− integrin $\alpha2+$ and $\alpha2−$ cells, indicates that the difference in long-term reconstitution experiments is due to the enrichment of primitive long-term engrafting cells in the integrin $\alpha2+$ fractions of both CD34+CD38− and CD34+CD38−CD90+ cell populations.
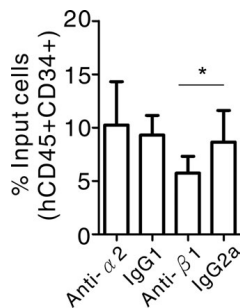

Figure 5. Integrin $\alpha2$ receptor is not involved in homing of cord blood CD34+ stem and progenitor cells into mouse bone marrow. Homing of human cord blood stem and progenitor cells to NOD/SCID-IL2R$\gamma_c$null mouse bone marrow was studied after preincubation of lineage-marker depleted cord blood cells with function-blocking antibodies against integrin $\alpha2$ or $\beta1$ receptors or isotype matched control antibodies (IgG1 and IgG2a, respectively). Treatment with the anti-integrin $\alpha2$ antibody does not inhibit homing, whereas preincubation with anti-integrin $\beta1$antibody significantly inhibits homing of cord blood CD34+ cells, as compared to the corresponding isotype control antibodies (*, $p < .05$). The results are from three independent experiments with pooled cord blood samples (7–9 mice per group).

CD34+CD38− and CD34+CD38−CD90+integrin $\alpha2+$ Cell Fractions Are Heterogeneous, Containing Long-Term Culture Initiating Cell and Committed Progenitors

Previous studies have shown that committed granulocyte-macrophage (CFU-GM), erythroid (BFU-E), mixed (CFU-GEMM), and total (CFU-C) progenitors reside in the CD34+CD38+ but also nearly at the same frequency (approximately 70%) in the CD34+CD38− cell fraction, when analyzed by single cell methylcellulose culture [24]. Furthermore, single cell cultures have shown a frequency of approximately 30% of CFU-Cs in both CD90+CD45RA− and CD90−CD45RA− fractions of CD34+CD38− cells, indicating that these cell populations are still highly heterogeneous and contain cells at variable stages of differentiation [5]. We therefore assessed the lineage potential of the integrin $\alpha2+$ and $\alpha2−$ cord blood cells by in vitro colony and long-term culture initiating cell (LTC-IC) assays. Cord blood CD34+CD38−CD90+ as well as CD34+CD38− LTC-ICs resided both in integrin $\alpha2+$ and $\alpha2−$ cell fractions (Fig. 6A, 6C), although significantly more colonies, the readout of LTC-IC assay, were derived from integrin $\alpha2+$ than $\alpha2−$ cells in the CD90+ fraction.

Within the cord blood CD34+CD38−CD90+ fraction, the frequency of progenitors with integrin $\alpha2+$ and $\alpha2−$ phenotypes was equal (Fig. 6B), while cord blood CD34+CD38− integrin $\alpha2−$ cells gave rise to more colonies than the $\alpha2+$ cells (Fig. 6D). Taken together, the results from both in vivo and in vitro functional assays show that the cord blood CD34+CD38−CD90+ integrin $\alpha2+$ population is still highly heterogeneous, consisting of cells with variable self-renewal and differentiation capacities.

Lin−CD34+CD38−CD90+CD45RA− Integrin $\alpha2+$ Cells Display a Distinct Gene Expression Pattern from the $\alpha2−$ Subsets and Lin−CD34+CD38−CD90−CD45RA− Cell Fraction

To further investigate the differences between integrin a2+ and $\alpha2−$ stem and progenitor cells, we analyzed global mRNA
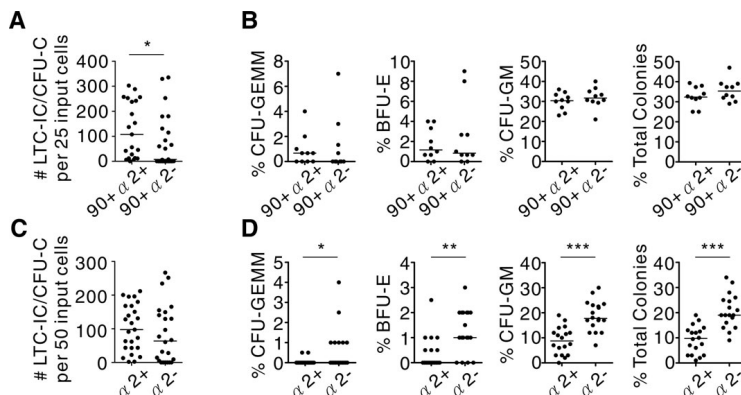
Figure 6. Presence of in vitro assayed LTC-ICs and lineage-committed progenitors in cord blood CD34+CD38− and CD34+CD38−CD90+ integrin $\alpha2+$ and $\alpha2−$ cell fractions. The number of colonies per well obtained in LTC-IC assay of **(A)** cord blood CD34+CD38−CD90+ and **(C)** CD34+CD38− integrin $\alpha2+$ and $\alpha2−$ cells. The frequencies of mixed (CFU-GEMM), erythroid (BFU-E), granulocyte-macrophage (CFU-GM), and total CFU-C progenitors in **(B)** cord blood CD34+CD38−CD90+ and **(D)** CD34+CD38− integrin $\alpha2+$ and $\alpha2−$ cells. The horizontal bars show median values. *, $p < .05$; **, $p < .01$; ***, $p < .001$. All results are from two independent experiments with pooled cord blood samples. Abbreviations: BFU-E, burst forming unit-erythrocyte; CFU-C, colony-forming unit in culture; CFU-GM, CFU-granulocyte and macrophage; CFU-GEMM, CFU-granulocyte, erythrocyte, monocyte and megakaryocyte; LTC-IC, long-term culture initiating cell.

expression profiles from three separate FACS-sorted populations of Lin−CD34+CD38−CD90+CD45RA− integrin α2+ and α2− cells and compared these to the Lin−CD34+CD38−CD90−CD45RA− cell fraction reported to be enriched in multipotent progenitors [5, 25, 26]. The list of twofold differentially expressed genes in each subset is shown in supporting information (Table S1). Importantly, the heat map and PCA (Fig. 7) show that the three individual integrin α2+ cell populations display a gene expression profile distinct from the corresponding integrin α2− cell populations, whereas the integrin α2− cell populations appear to be more closely related to the CD90−CD45RA− cell populations. These findings are in agreement with our findings from in vivo functional assays showing that CD34+CD38−CD90+ integrin α2+ cell population is distinct from the α2− subset and enriched in the primitive hematopoietic stem cells.

DISCUSSION

Early hematopoietic development proceeds along an organized hierarchical process, initiated by primitive long-term engrafting stem cells, which progressively differentiate into more mature stem cells with a restricted self-renewal potential, multipotent progenitors, and lineage committed progenitors. Transplantation into immunodeficient mice followed by multilineage repopulation analysis is currently the best experimental method to assay human hematopoietic stem and progenitor cells. However, studies from different laboratories have used different mouse strains, different routes of transplantation, and variable criteria for reconstitution, resulting in highly variable definitions of functional short-term and long-term stem cells [2, 27].

Markers previously reported to identify human hematopoietic stem cells include elevated aldehyde dehydrogenase activity among Lin−CD34+CD38− cells, by which hematopoietic stem cells capable of multilineage reconstitution in NOD/SCID or NOD/SCID-β2m$^{-/-}$ mice for 20 weeks or more could be isolated at a purity of one per 360 cells [2] and low Rho123 retention in Lin−CD34+CD38− cells, by which enrichment to a frequency of 1 per 30 was reported [28]. However, in the latter study, analysis was performed at 7–10 weeks after transplantation, and, therefore, included also short-term stem cells and progenitors. Recently, long-term reconstitution in NSG mice was obtained in three of four mice transplanted with 25 Lin−CD34+CD38−CD90+Rho 123^{high} cells, and five of eight mice transplanted with 10 corresponding Rho 123^{low} cells [7], and therefore, further studies are needed to address the role of Rho 123 as a human primitive stem cell marker.

Our present findings from in vivo engraftment analysis of transplanted cord blood CD34+CD38−CD90+ cells at 24 weeks after limiting dilution transplantation into NSG mice show a highly significant enrichment of long-term repopulating stem cells in a fraction expressing the integrin α2 receptor, with a frequency of 1/29, in contrast to the frequency of 1/157 in the corresponding integrin α2 negative cell fraction. In accordance with this, transplantation of cord blood CD34+CD38− cells followed by engraftment analysis at 16–18 weeks showed a significant enrichment of long-term repopulating cells in the integrin α2+ cell fraction. Furthermore, analysis of long-term engrafted stem and progenitor cells after transplantation of CD34+CD38−CD90+ integrin α2+ cord blood cells showed that the most primitive Lin−CD34+CD38−CD90+CD45RA− cell fraction uniformly retained the integrin α2 expression, supporting the notion that integrin α2 is a stem cell marker. In contrast, the more mature engrafted Lin−CD34+CD38−CD90−CD45RA−

cell population contained both integrin α2+ and integrin α2− cells, indicating that the integrin α2+ cells give rise to integrin α2− cells and reside upstream in the hematopoietic cell developmental hierarchy.

Recently, limiting dilution intrafemoral transplantation into female NSG mice showed long-term reconstituting stem cells among cord blood Lin−CD34+CD38−CD90+ CD45RA− cells in a frequency of 1/20, whereas the frequency in the corresponding CD90−CD45RA− cell fraction was 1/100 [7], showing in agreement with a previous study [5] that most but not all long-term reconstituting stem cells in cord blood reside in the Lin−CD34+CD38−CD90+ CD45RA− cell fraction. This recent study by Notta et al. reported a highly significant enrichment of the long-term repopulating cells in the integrin α6+ fraction of cord blood Lin−CD34+CD38−CD90+CD45RA− cell fraction with a frequency of 1/10.5, while the frequency in the corresponding integrin α6− fraction was 1/113 [7]. Importantly, we show by FACS analysis of cord blood Lin−CD34+CD38−CD90+ CD45RA− and Lin−CD34+CD38−CD90−CD45RA− cells that the integrin α2 and α6 chains in both cell populations are only partially coexpressed. Consequently, our findings identify integrin α2 receptor as a novel stem cell marker, which may improve prospective isolation of the primitive human hematopoietic stem cells.

Notably, the frequency of long-term reconstituting cells in the total cord blood CD34+ CD38−CD90+ fraction (1/20) reported by Notta et al. [7] is higher than the frequency of long-term reconstituting cells in the integrin α2+ subset of the corresponding cell fraction (1/29) in our study. In this study, positive engraftment was defined as >0.1% human CD45+ cells (supporting information Table S1) [7]. However, myeloid cell reconstitution, because of the short life span of granulocytes, is considered to be the best indicator of ongoing stem cell self-renewal [29]. Therefore, we have used the criteria of positive engraftment requiring 0.025% reconstitution of both lymphoid and myeloid cells [2], in addition to 0.1% human CD45+ reconstitution. Using these criteria, the number of reconstituted mice was lower than the numbers calculated using the criteria of Notta et al. [7] in both groups, since mice having engraftment of human CD45+ lymphoid cells only were not considered as reconstituted from stem cells in our study.

Gene expression profiling showed that some genes, previously reported to be downregulated (CDK6, CYCLIN C, and IL1RL) or upregulated (C4orf18 and CNKSR3) in cord blood Lin−CD34+CD38−CD90+/CD90−CD45RA− integrin α6+ cell fractions compared to the multipotent progenitor cell-enriched Lin−CD34+CD38−CD90−CD45RA− integrin α6− fraction [7], were also correspondingly regulated in the Lin−CD34+CD38−CD90+CD45RA− integrin α2+ cells compared to the Lin−CD34+CD38−CD90−CD45RA− cells in our study. Importantly, the PCA of the gene expression profiling revealed that within the Lin−CD34+CD38− fraction, CD90+CD45RA− integrin α2+ cells were distinct from the CD90+CD45RA− integrin α2− cells, whereas the latter cell population was more closely related to the CD90−CD45RA− multipotent progenitor-enriched cell population, in full agreement with the results from in vivo transplantation assays.

The expression of integrin α2 in primitive human hematopoietic stem cells is in striking contrast to the findings in mouse hematopoietic stem cells. In mouse bone marrow, integrin α2 was shown to be partially expressed in Lin$^{-/lo}$Thy.1.1^{lo}Sca-1+c-kit+ and Rho^{lo}Kit+Sca-1+CD34-FLT3- stem cells. Transplantation of integrin α2^{hi} or α2^{-} cells into congenic recipients resulted in long-term engraftment only from α2− cells while the integrin α2^{hi} cells could maintain
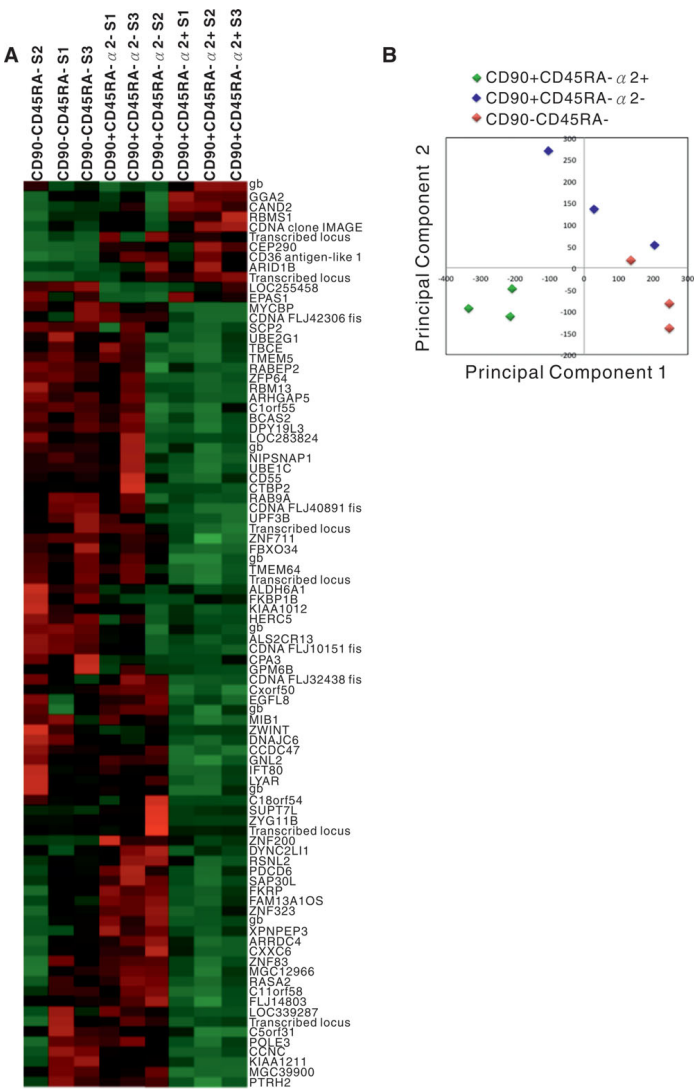
Figure 7. Microarray analysis reveals distinct gene expression pattern of the Lin−CD34+CD38−CD90+CD45RA− integrin α2+ cells from integrin α2− cells and the Lin−CD34+CD38−CD90−CD45RA− cells. **(A):** Heat-map of the expression levels of the 90 genes differentially expressed in Lin−CD34+CD38−CD90+CD45RA− integrin α2+, Lin−CD34+CD38−CD90+CD45RA− integrin α2− and Lin−CD34+CD38−CD90−CD45RA− cells. Clustering shows genes being upregulated or downregulated by twofold (using lower bound of 90% CI) in the CD90+CD45RA−integrin α2+ cells compared to the CD90+CD45RA− integrin α2− cells or the CD90−CD45RA− cells from Lin−CD34+CD38− fraction. Red and green represent upregulation and downregulation of genes, respectively. Data are from three independent FACS-sorted pooled cord blood samples. See supporting information Table S1 for details about the list of the genes. **(B):** Principal component analysis of the microarray data shows distinct gene expression pattern of the Lin−CD34+CD38−CD90+CD45RA− integrin α2+ cells from the corresponding integrin α2− cells and the Lin−CD34+CD38−CD90−CD45RA− cells. Each dot represents an independent FACS-sorted population of pooled cord blood cells.

multilineage lymphomyeloid reconstitution only for 4–8 weeks [30, 31].

   Integrins regulate multiple cellular processes including transcriptional activation, survival, proliferation, differentiation, and migration in a cell- and tissue-specific manner [8]. In this study, the integrin α2 receptor was not found to be involved in homing of human hematopoietic stem and progenitor cells to NSG mouse bone marrow. However, its expression in primitive hematopoietic stem cells suggests a role in regulating stem cell functions in the bone marrow niches by binding to its extracellular and cell surface ligands, including collagens, laminins, E-cadherin, and matrix metalloproteinase-1 [8, 32]. In human leukocytes, expression of integrin α2β1 receptor is associated with activation and migration during inflammation [32, 33]. In cancer, integrin α2β1 receptor acts as a metastasis suppressor, regulating tumor cell invasion and mobilization into circulation [34]. Recently, binding of α2β1 integrin in human T acute lymphoblastic leukemia cells to collagen I was identified as a survival mechanism promoting chemoresistance [35]. Further studies, including experimental inactivation of integrin α2 gene are needed to investigate its functional role in normal human hematopoietic stem and progenitor cells and leukemic cells.

CONCLUSION

Our present findings identify integrin α2 as a novel stem cell marker in human cord blood hematopoietic cells. Specifically, by using limiting dilution transplantation assay of CD34+CD38−CD90+ cells into NSG mice, we show a highly significant enrichment of long-term repopulating stem cells in a fraction expressing the integrin α2 receptor. Likewise, transplantation of cord blood CD34+CD38− cells showed a significant enrichment of long-term repopulating cells in the integrin α2+ cell fraction. In agreement with the functional in vivo transplantation assays, gene expression profiling of the Lin−CD34+CD38−CD90+CD45RA− integrin α2+ and α2− cell populations reveals that integrin α2+ cells are distinct from the integrin α2− cells, whereas the integrin α2− cell population appears to be more closely related to the CD90−CD45RA− multipotent progenitor enriched cell population.

   Recently, integrin α6 chain was reported to be a marker identifying the primitive human cord blood hematopoietic cells [7]. We here show that integrin α2 and α6 receptors are only partially coexpressed in cord blood Lin−CD34+CD38−CD90+CD45RA− and Lin−CD34+CD38−CD90−CD45RA− cells. Our results, together with the published results, suggest that the most primitive human cord blood long-term reconstituting stem cells may be enriched in the cell fraction coexpressing integrin α2 and α6 receptors, which should be addressed in further experimental transplantation studies.

DISCLOSURE OF POTENTIAL CONFLICT OF INTEREST

The authors indicate no potential conflicts of interest.

REFERENCES

1   Bhatia M, Wang JC, Kapp U et al. Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice. Proc Natl Acad Sci USA 1997;94:5320–5325.
2   Christ O, Lucke K, Imren S et al. Improved purification of hematopoietic stem cells based on their elevated aldehyde dehydrogenase activity. Haematologica 2007;92:1165–1172.
3   Hogan CJ, Shpall EJ, Keller G. Differential long-term and multilineage engraftment potential from subfractions of human CD34+ cord blood cells transplanted into NOD/SCID mice. Proc Natl Acad Sci USA 2002;99:413–418.
4   Glimm H, Eisterer W, Lee K et al. Previously undetected human hematopoietic cell populations with short-term repopulating activity selectively engraft NOD/SCID-beta2 microglobulin-null mice. J Clin Invest 2001;107:199–206.
5   Majeti R, Park CY, Weissman IL. Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood. Cell Stem Cell 2007;1:635–645.
6   Notta F, Doulatov S, Dick JE. Engraftment of human hematopoietic stem cells is more efficient in female NOD/SCID/IL-2Rgc-null recipients. Blood 2010;115:3704–3707.
7   Notta F, Doulatov S, Laurenti E et al. Isolation of single human hematopoietic stem cells capable of long-term multilineage engraftment. Science 2011;333:218–221.
8   Sixt M, Bauer M, Lammermann T et al. Beta1 integrins: Zip codes and signaling relay for blood cells. Curr Opin Cell Biol 2006;18:482–490.
9   Hirsch E, Iglesias A, Potocnik AJ et al. Impaired migration but not differentiation of haematopoietic stem cells in the absence of beta1 integrins. Nature 1996;380:171–175.
10 Potocnik AJ, Brakebusch C, Fassler R. Fetal and adult hematopoietic stem cells require beta1 integrin function for colonizing fetal liver, spleen, and bone marrow. Immunity 2000;12:653–663.
11 Arroyo AG, Yang JT, Rayburn H et al. Differential requirements for alpha4 integrins during fetal and adult hematopoiesis. Cell 1996;85:997–1008.
12 Arroyo AG, Yang JT, Rayburn H et al. Alpha4 integrins regulate the proliferation/differentiation balance of multilineage hematopoietic progenitors in vivo. Immunity 1999;11:555–566.
13 Lapidot T, Dar A, Kollet O. How do stem cells find their way home? Blood 2005;106:1901–1910.
14 Papayannopoulou T, Priestley GV, Nakamoto B et al. Molecular pathways in bone marrow homing: Dominant role of alpha(4)beta(1) over beta(2)-integrins and selectins. Blood 2001;98:2403–2411.
15 Priestley GV, Scott LM, Ulyanova T et al. Lack of alpha4 integrin expression in stem cells restricts competitive function and self-renewal activity. Blood 2006;107:2959–2967.
16 Qian H, Georges-Labouesse E, Nystrom A et al. Distinct roles of integrins alpha6 and alpha4 in homing of fetal liver hematopoietic stem and progenitor cells. Blood 2007;110:2399–2407.
17 Qian H, Tryggvason K, Jacobsen SE et al. Contribution of alpha6 integrins to hematopoietic stem and progenitor cell homing to bone marrow and collaboration with alpha4 integrins. Blood 2006;107:3503–3510.
18 Wilson A, Oser GM, Jaworski M et al. Dormant and self-renewing hematopoietic stem cells and their niches. Ann N Y Acad Sci 2007;1106:64–75.
19 Colvin GA, Lambert JF, Abedi M et al. Murine marrow cellularity and the concept of stem cell competition: Geographic and quantitative determinants in stem cell biology. Leukemia 2004;18:575–583.
20 Hogge DE, Lansdorp PM, Reid D et al. Enhanced detection, maintenance, and differentiation of primitive human hematopoietic cells in cultures containing murine fibroblasts engineered to produce human

steel factor, interleukin-3, and granulocyte colony-stimulating factor. Blood 1996;88:3765–3773.

21 Qian H, Le Blanc K, Sigvardsson M. Primary mesenchymal stem and progenitor cells from bone marrow lack expression of CD44 protein. J Biol Chem 2012;287:25795–25807.

22 Doulatov S, Notta F, Eppert K et al. Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development. Nat Immunol 2010;11: 585–593.

23 Yahata T, Ando K, Sato T et al. A highly sensitive strategy for SCID-repopulating cell assay by direct injection of primitive human hematopoietic cells into NOD/SCID mice bone marrow. Blood 2003;101: 2905–2913.

24 Manz MG, Miyamoto T, Akashi K et al. Prospective isolation of human clonogenic common myeloid progenitors. Proc Natl Acad Sci USA 2002;99:11872–11877.

25 Goardon N, Marchi E, Atzberger A et al. Coexistence of LMPP-like and GMP-like leukemia stem cells in acute myeloid leukemia. Cancer Cell 2011;19:138–152.

26 Pang WW, Price EA, Sahoo D et al. Human bone marrow hematopoietic stem cells are increased in frequency and myeloid-biased with age. Proc Natl Acad Sci USA 2011;108:20012–20017.

27 Park CY, Majeti R, Weissman IL. In vivo evaluation of human hematopoiesis through xenotransplantation of purified hematopoietic stem cells from umbilical cord blood. Nat Protoc 2008;3:1932–1940.

28 McKenzie JL, Takenaka K, Gan OI et al. Low rhodamine 123 retention identifies long-term human hematopoietic stem cells within the Lin−CD34+CD38− population. Blood 2007;109:543–545.

29 Uchida N, Friera AM, He D et al. Hydroxyurea can be used to increase mouse c-kit+Thy-1. 1(lo) Lin−/loSca-1(+) hematopoietic cell number and frequency in cell cycle in vivo. Blood 1997;90: 4354–4362.

30 Benveniste P, Frelin C, Janmohamed S et al. Intermediate-term hematopoietic stem cells with extended but time-limited reconstitution potential. Cell Stem Cell 2010;6:48–58.

31 Wagers AJ, Weissman IL. Differential expression of alpha2 integrin separates long-term and short-term reconstituting Lin−/loThy1.1(lo)c-kit+ Sca-1+ hematopoietic stem cells. Stem Cells 2006;24: 1087–1094.

32 Brakebusch C, Fassler R. beta 1 integrin function in vivo: Adhesion, migration and more. Cancer Metastasis Rev 2005;24:403–411.

33 Zutter MM, Edelson BT. The alpha2beta1 integrin: A novel collectin/C1q receptor. Immunobiology 2007;212:343–353.

34 Ramirez NE, Zhang Z, Madamanchi A et al. The alphabeta integrin is a metastasis suppressor in mouse models and human cancer. J Clin Invest 2011;121:226–237.

35 Naci D, El Azreq MA, Chetoui N et al. alpha2beta1 integrin promotes chemoresistance against doxorubicin in cancer cells through extracellular signal-regulated kinase (ERK). J Biol Chem 2012;287: 17065–17076.

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Article II

Identification of bipotential Lin-CD34+CD38- integrin α2- megakaryocyte-erythrocyte progenitors in human bone marrow

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Hong Qian, Marja Ekblom; equal contribution

ABSTRACT

Prospective isolation of stem cells and their downstream intermediate progenitors within the hematopoietic hierarchy is essential for the characterization of the molecular mechanisms regulating lineage commitment and differentiation and for understanding the aberrant molecular pathways in leukemias. Human common myeloid, granulocyte-macrophage and megakaryocyte-erythrocyte progenitors have been characterized in the Lin-CD34+CD38+ progenitor cell enriched fraction by differential expression of CD123 and CD45RA markers both in adult bone marrow and cord blood (Manz et al. 2002). However, progenitor cells with restricted lineage potential, including myeloid and erythroid progenitors, have been shown to reside also in the Lin-CD34+CD38- cell fractions (Debili et al. 1996; Majeti et al. 2007; Doulatov et al. 2010). Nevertheless, the phenotypical characteristics of these Lin-CD34+CD38- committed progenitor cell populations are so far largely unknown. We have here studied the expression of the integrin α2 receptor in adult human bone marrow Lin-CD34+CD38- stem and progenitor cells. We have, by employing single cell colony assays and liquid cultures, found that the integrin α2- cell fraction in the Lin-CD34+CD38- compartment contains in a high frequency bipotential megakaryocytic-erythroid progenitors. The identification of this novel bipotential megakaryocytic-erythroid cell population may provide a means for further analysis of early megakaryocytic and erythroid lineage fate decisions and may facilitate studies aiming to expand these lineages for clinical transplantation and transfusion trials.

INTRODUCTION

Haematopoiesis is a highly organized and complex developmental process where the hematopoietic stem cells lie at the apex of hierarchy and differentiate into all types of lineage-committed progenitors and mature blood cells. Identifying the distinct committed progenitors and gaining insights into the lineage commitment events are important for dissecting the developmental pathways of normal haematopoiesis, and for understanding the underlying mechanisms in leukemogenesis.

The technical advances of multicolour flow cytometry and development of monoclonal antibodies recognizing lineage and developmental stage specific cellular markers have provides a means to prospective isolate distinct stem and progenitor cell populations for functional *in vivo* and *in vitro* stem cell assays. While the immunophenotype of mouse primitive stem cells as well as intermediate progenitor stages are rather well defined (Iwasaki and Akashi 200; Pronk et al. 2007), the characterization of human hematopoietic stem cells and downstream committed intermediates remains largely elusive.

Transplantation into immunodeficient NSG mice followed by multilineage reconstitution analysis is the best method to characterize human primitive stem and progenitor cell populations. Such studies using cord blood cells have shown that the long-term *in vivo* reconstituting activity resides in the CD34+CD38- cell population, whereas the CD34+CD38+ cells are enriched in lineage committed progenitors and reconstitute only short-term *in vivo* (Bhatia et al. 1997;

Hogan et al. 2002; Christ et al. 2007). Within the CD34+CD38- fraction the most primitive stem cells reside in the CD90+CD45RA-, and in a lower frequency, in the CD90-CD45RA- fractions, whereas the CD45RA+ cell fraction contains lymphoid progenitors but does not have any *in vivo* long-term repopulating activity (Majeti et al. 2007; Doulatov et al. 2010).

In the Lin-CD34+CD38+ compartment of human bone marrow the megakaryocyte-erythrocyte progenitors (MEPs), assayed in methylcellulose culture with cytokines, have been identified by the lack of expression of IL3Rα receptor (CD123) and the CD45RA marker (Lin-CD34+CD38-CD123-CD45RA-), while common myeloid progenitors (CMPs) and granulocyte- macrophage progenitors (GMPs) have been defined as $CD123^{lo}CD45RA-$ and $CD123^{lo}CD45RA+$, respectively (Manz et al. 2002). However, within the CD123- MEP cell population the discrimination towards $CD123^{lo}$ CMP population is less distinct, and the MEP cell fraction can be further purified by expression of thrombopoietin receptor CD110 (Edvardsson et al. 2006). Within the CMP fraction, expression of FLT3 receptor has been shown to discriminate FLT3+ fraction highly enriched in GMPs and FLT3- fraction consisting only of MEPs, indicating heterogeneity in both phenotype and commitment into different lineages (Kikushige et al. 2008, Edvardsson et al. 2006).

Colony assays of Lin-CD34-CD38- CD45RA-stem cell-enriched cell fractions show that these also contain multipotent progenitors (GEMM), as well as lineage-committed progenitors (Majeti et al. 2007; Doulatov et al. 2010). However, the phenotypical markers to purify and isolate these different progenitor populations are yet to be identified.

In this study, we have applied *in vitro* progenitor assays, including single cell colony-forming unit in culture (CFU-C) and liquid culture megakaryocyte colony (CFU-MK) assays and show that Lin-CD34+CD38- erythroid colonies (BFU-E) and CFU-MK are enriched in the integrin α2- fraction, and that this fraction contains a high frequency of cells with bipotential megakaryocyte-erythrocyte differentiation capacity. In contrast, the long term culture initiating cell (LTC-IC) assay suggested that the integrin α2+ fraction in CD34+CD38- compartment contains the more primitive progenitors. This is in agreement with our previous findings on the enrichment of primitive long-term *in vivo* reconstituting stem cells in the integrin α2+ cell fraction in cord blood (Wong et al. 2013).

MATERIAL AND METHODS

Bone marrow aspiration and mononuclear cell isolation

Human bone marrow aspirates were collected from healthy adult volunteers in accordance with approved protocols (LU-195-00). All functional assays were performed using freshly isolated cells. Mononuclear cells (MNCs) were isolated by Ficoll-Hypaque (Lymphoprep, Axis-Shield PoC AS, Oslo, Norway) density gradient centrifugation. CD34+ and lineage-depleted cells were isolated using magnetic sorting (MACS; Miltenyi Biotech, Bergisch Gladbach, Germany), as described (Wong et al. 2013).

Flow cytometric analysis and isolation of hematopoietic stem and progenitor cells in human adult bone marrow

Expression analysis of integrin subunits and sorting of cell fractions for functional assays were performed using the following human specific monoclonal antibodies (mAb; BD Biosciences, San Jose, CA, USA, unless otherwise specified), FITC, APC or PerCP-Cy5.5 anti-CD34 (8G12), APC or PECy7 anti-CD38 (HB7), APC anti-CD90 (5E10), APC anti-CD110 (1.6.1), FITC anti-CD33 (HIM3-4), APC anti-CD135 (Biolegend, San Diego, CA, United States), PE conjugated antibodies against integrin α1-6 chains, CD49a (SR84), CD49b (12F1-H6, AbD Serotec, Raleigh, NC, USA or 12F1, BD Biosciences), CD49c (17C6, AbD Serotec), CD49d (9F10), CD49e (IIA1), CD49f (GoH3) and integrin β1 chain, CD29 (4B7R, AbD Serotec). The cells expressing lineage markers were depleted by magnetic sorting or excluded after staining with PECy5 or Tri-color conjugated antibodies against CD3 (S4.1), CD4 (S3.5), CD7 (CD7-6B7), CD8 (3B5), CD14 (TüK4) (Invitrogen, Carlsbad, CA, United States), CD19 (HIB19), CD20 (2H7), CD2 (RPA-2.10), CD10 (HI10a), CD11b (ICRF44), CD56 (B159), CD235a (GA-R2) (BD

Biosciences). FITC and Pacific Blue anti-CD45RA (MEM-56) were from Invitrogen while eFluor450 anti-CD45RA (HI100) was from eBioscience (San Diego, CA, USA). Dead cells were excluded by propidium iodide (PI; Invitrogen) or 7-Amino-actinomycin D (7AAD; Sigma, St Louis, MO, USA) staining. Cells were analysed by LSRII Flow Cytometer System (BD Biosciences) and FlowJo software (TreeStar Inc. San Carlos, CA, USA) or sorted by FACSAria, FACSAriaII or FACSAriaIII Cell Sorter (BD Biosciences). Quadrant gates shown in fluorescence-activated cell sorting (FACS) plots were set according to fluorescence-minus-one (FMO) controls in all analyses, except for CD34+CD38- cells, which were gated as the lowest 5-8% CD38- cells out of total CD34+ cells (Hogan et al. 2002).

Colony assays

To quantify colony-forming cells (CFU-C, CFU-GEMM, CFU-GM and BFU-E), 50 cells were resuspended in 100 μL Iscove's modified Dulbecco's medium (PAA, Pasching, Austria) containing 20% FBS (v/v, Sigma), 2 mM L-glutamine (PAA), 100 U/ mL penicillin-streptomycin (PAA) and 10^{-4} M 2-mercaptoethanol (Sigma) and added into 900 μL Methocult H4435 (Stem Cell Technologies, Vancouver, BC, Canada). Single-cell cultures were prepared by FACS single-cell deposition mode into 96-well plates in 100 μL Methocult H4435 per well. All cultures were maintained at 37 °C and 5% CO_2 for 14 days. Colonies were scored based on morphology. In addition, colonies with less typical morphology were transferred onto cytospin slides and analysed after staining with May-Grünwald Giemsa.

Megakaryocyte progenitors were quantified using Megacult-C kit (Stem Cell Technologies). One or two hundred cells were plated on each slide and the cultures were maintained at 37 °C and 5%

CO_2 for 12-13 days. The megakaryocytes were identified by staining for CD41 and Giemsa according to the manufacturer's instructions.

Long-term culture initiating cell (LTC-IC) assay

Test cells (50 cells/ well) were cultured in 200 μL Myelocult H5100 (Stem Cell Technologies), 10^{-6} M hydrocortisone (Sigma) in Collagen I 96-well microplates (BD Biosciences) on irradiated (8000 cGy) murine stromal cell lines Sl/Sl and M2-10B4 (5000 each/well), engineered to express human cytokines KL, IL-3 and G-CSF (Hogge et al. 1996). The cells were maintained at 37 °C and 5% CO_2 for 6 weeks with weekly half-medium change and thereafter plated in Methocult H4435 in duplicates (20% and 80% of the cells from each well). The mean values of the readout from the duplicate colony cultures, assayed after 13-14 days, were calculated.

Single cell liquid cultures

Single cells were deposited by FACS and cultured in StemSpan SFEM (Stem Cell Technologies) supplemented with 2 mM L-Glutamine, 100 U/ mL Penicillin and 0.1 mg/ mL Streptomycin, 40 μg/ mL human plasma-derived low density lipoprotein (Sigma) and human recombinant cytokines (50 ng/ mL SCF, 50 ng/ mL TPO, 10 ng/ mL IL-3 and 3 U/ mL EPO, all from R&D Systems; Minneapolis, MN, United States) either in mini-tray Terasaki plates (20 μL/ well; Thermo scientific, Waltham, MA, United States) or 96-well plates (40 μL/ well) for 12-14 days. The wells containing colonies were counted and the sizes of the colonies in the Terasaki plates were scored based on the cell numbers or the % coverage of the well surface. Some of the clones were picked for FACS analysis and stained by APC-Cy7 anti-CD41 (HIP8, Biolegend), PB anti-CD235ab (HIR2, Biolegend), FITC anti-CD15 (HI98), CD33 (HIM3-4) and CD66b (G10F5)

and PE anti-CD49b (12F1) (BD Biosciences) for the presence of megakaryocytes (>0.1% CD41+CD235- cells), erythrocytes (>0.1% CD41-CD235+ cells), myeloid cells (>0.1% CD15/CD33/CD66b+ cells) and the expression of integrin α2. Other randomly picked clones were transferred onto cytospin slides and identified morphologically after May-Grünwald Giemsa staining. The stained cells on the cytospin slides were analysed by a BX61 microscope (Olympus Corporation, Tokyo, Japan), and the images were acquired under 40X and 100X objectives using DP72 camera and cellSens Dimension software (Olympus). The colonies in Terasaki wells were analysed and photographed using an Eclipse TS100 microscope and DS-Fi2 camera (Nikon, Tokyo, Japan).

Short-term differentiation culture

500 Lin-CD34+CD38- integrin α2+ or Lin-CD34+CD38- CD45RA- integrin α2- cells or 1000-25000 Lin-CD34+CD38+ human bone marrow cells were plated in a well containing 1mL StemSpan SFEM supplemented with 2mM L-Glutamine, 100U/mL Penicillin and 0.1mg/mL Streptomycin, 40 μg/mL human plasma-derived low density lipoprotein and human recombinant cytokines (100ng/mL SCF, 100ng/mL FL, 50ng/mL TPO, 20ng/mL IL-3, 20ng/mL IL-6, 20ng/mL G-CSF, 20ng/mL GM-CSF and 3U/mL EPO, all from R&D Systems) in 24-well culture plate. Cells at each dose were plated in triplicates. After five days of culture, cells were harvested and stained with FITC anti-CD34 (8G12), PECy7 anti-CD38 (HB7), PE anti-CD235a (HIR2) (BD Biosciences) and APC-Cy7 anti-CD41 (HIP8, Biolegend) and analysed by FACS LSRII Flow Cytometer System (BD Biosciences) and FlowJo software (TreeStar Inc. San Carlos, CA, USA)

Microarray analysis

RNA from CD34+CD38- integrin α2+ and CD34+CD38- integrin α2- cells, each sorted from four independent bone marrow samples, was extracted as previously described (Qian et al. 2012), amplified by two rounds of linear *in vitro* transcription and labelled according to Affymetrix™ GeneChip Expression Analysis Technical Manual. Chips were scanned using GeneChipTM Scanner 3000. Human Genome U133 plus 2.0 Chips were normalized by Robust Multi-array Average (RMA) using invariant set normalization and probe level expression values were calculated using the PM-MM model provided by the DNA-Chip Analysers (dCHIP) software (www.dchip.org).

Quantitative RT-PCR

The CD34+CD38- integrin α2+ and CD34+CD38- integrin α2- cells were sorted directly into buffer-RLT (Qiagen, 2-mercaptoethanol 143mM (Sigma)) and frozen at –80°C. RNA extraction and DNase treatment was performed with the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Eluted RNA samples were reverse transcribed using SuperScript III and random primers (Invitrogen) according to protocol supplied by the manufacturer. Real-time quantitative PCR (Q-PCR) was performed by mixing 2 x TaqMan universal PCR master mix, 20 x TaqMan primer/probe mix, RNase-free water and 5 µl of cDNA for a final reaction volume of 20 µl. Assays-on-Demand probes were, GATA2: Hs00231119_m1, GATA3: Hs00231122_m1 and HPRT1: Hs99999909_m1.

Statistical Analysis

Statistical differences were evaluated by unpaired student t test (Q-PCR) or Mann-Whitney *t* test (other assays). The two-sided p-value was considered to be significant when p< 0.05.

RESULTS

Expression of integrin receptors in human adult bone marrow hematopoietic stem and progenitor cells

The expression of integrin α1-6 and β1 chains in bone marrow CD34+CD38- cells was examined by flow cytometry (Fig. 1A). In agreement with our previous findings from cord blood CD34-CD38- cells (Wong et al. 2013), the adult bone marrow cells did not express integrin α1 chain, had a partial expression of integrin α3 and a ubiquitous expression of integrin α4, α5 chains and the common β1 chain. Integrin α2 and integrin α6 chains were expressed in most adult bone marrow CD34+CD38- cells, in contrast to a more restricted expression in cord blood CD34-CD38- cell population. Specifically, the integrin α2 receptor was expressed in 96.7±3.2% (mean±SD, n=25) of CD34+CD38- cells but only an approximately half of the CD34+CD38+ cells (not shown) in adult bone marrow. Within the bone marrow Lin-CD34+CD38- cell fractions, integrin α2 was expressed in the vast majority of CD90+CD45RA- and CD90-CD45RA- cells, whereas among the CD90-CD45RA+ cells we were able to detect a somewhat larger fraction of cells lacking expression of integrin α2 (Fig.1B, C).

CD34+CD38- BFU-Es and CFU-MKs are highly enriched in integrin α2- cell fraction

In order to investigate potential functional differences associated with the expression of integrin α2 on CD34+CD38- cells we analyzed the distribution of multipotent and lineage-committed progenitors by *in vitro* differentiation cultures of sorted bone marrow progenitors in methylcellulose (Fig. 2A, B). The frequency of total colony-forming cells, CFU-C, and the minor population of mixed CFU-GEMM progenitors were equal in both fractions, whereas CFU-GM was significantly enriched in the integrin α2+ fraction. In contrast, the integrin α2- fraction

contained a higher frequency of erythroid BFU-E progenitors. The CFU-MK potential was analyzed in serum free collagen based culture system, since serum components, including TGF-β, used in methylcellulose cultures are known to inhibit megakaryocyte colony growth (Debili et al. 1996). Importantly, the CFU-MK frequency was significantly higher in integrin α2- than integrin α2+ fraction. (Fig. 2C), suggesting an enrichment of megakaryocyte-erythrocyte restricted progenitors in this fraction.

Lin-CD34+CD38- BFU-Es are highly enriched in integrin α2- cell fraction but not restricted in CD110+CD45RA- subfraction

In order to more precisely evaluate the cloning efficiency and the myeloerythroid potential of adult bone marrow Lin-CD34+CD38- integrin α2+ and the integrin α2- cell populations, we seeded single cells for methylcellulose colony assay (Fig. 3A). As shown in the previous cultures, the primitive CFU-GEMM and myeloid CFU-GM progenitors resided in both fractions, while the integrin α2- fraction was highly enriched in erythroid progenitors. 22% of the single integrin α2- cells were found to give rise to erythroid BFU-E colonies, in contrast to 0.7% of cells in integrin α2+ cell fraction (Fig. 3B).

In addition to the enrichment of BFU-Es in Lin-CD34+CD38- integrin α2- fraction, some of CFU-GM also resided in this fraction (Fig. 3B). We therefore tested whether additional markers could increase the purity of the erythroid progenitor population. In adult bone marrow Lin-CD34+CD38+CD123-CD45RA- cell fraction, the thrombopoietin receptor CD110 can further discriminate MEPs from CMPs (Manz et al. 2002; Edvardsson et al. 2006). We therefore

subfractionated Lin-CD34+CD38- integrin α2- cells into CD110+CD45RA-, CD110-CD45RA- and CD110-CD45RA+ populations and assayed these by single-cell colony forming assay (Fig. 3C). The erythroid progenitors were enriched in but did not uniformly segregate into the CD110+ cell fraction, whereas CFU-GM were partially depleted from the CD110+ cell fraction (Fig. 3D). The CD45RA+ cell population within the Lin-CD34+CD38- CD110- integrin α2- cells showed very low clonogenic potential, consistent with the previous report showing that Lin-CD34+CD38-CD45RA+ cells are multilymphoid progenitors and contain only limited myeloerythroid differentiation capacity (Doulatov et al. 2010). Therefore, the CD45RA+ cell fraction was excluded from further assays.

In an attempt to further discriminate the erythroid progenitor population, we analyzed the expression of CD33 and CD135 (FLT3) in the Lin-CD34+CD38-CD45RA- integrin α2- cell population. The expression of CD33, which is a pan-myeloid marker (van Lochem et al. 2004), in human adult bone marrow CD34+CD38- stem and progenitor cell compartment, is controversial (Sperr et al. 2005; Taussig et al. 2005; Kikushige et al. 2008; Herrmann et al. 2012), but low levels of expression has been reported in adult bone marrow CD34+CD38+CD123-CD45RA- MEPs (Manz et al. 2002). However, by FACS analysis, no CD33 expression was detected in the Lin-CD34+CD38-CD45RA- integrin α2- cell population (Fig. 3E).

The expression of the CD135 in bone marrow has been shown to segregate Lin-CD34+CD38+CD123^{lo}CD45RA- CMP cell fraction into two populations with diverging myelo-

erythroid differentiation potentials. CD135+ CMPs mainly contain CFU-GM whereas BFU-E are mainly reside in CD135- CMP fraction (Edvardsson et al. 2006; Kikushige et al. 2008). In agreement with this, CD34+CD38+CD123-CD45RA- MEPs are uniformly CD135- (Kikushige et al., 2008), suggesting that downregulation of CD135 marks commitment of progenitors into the megakaryocyte-erythrocyte lineage. CD135 is partially expressed in the CD34+CD38- cell compartment in adult bone marrow (Edvardsson et al. 2006; Kikushige et al. 2008; Hofmann et al. 2012). Our data show that the Lin-CD34+CD38- CD45RA- integrin α2- cells do not express CD135 (Fig. 3E) and thereby provides additional evidence for the downregulation of CD135 during early erythroid commitment.

Single cell analysis revealed the existence of the bipotential megakaryocyte-erythrocyte progenitors in Lin-CD34+CD38-CD45RA- integrin α2- fraction.

Bipotent megakaryocyte-erythrocyte progenitors have been previously by single cell colony assays and liquid cultures shown to exist in the bone marrow CD34+CD38$^{lo/-}$ cell population (Debili et al. 1996). Our results from colony assays show high frequencies of both the BFU-Es and CFU-MKs in the CD34+CD38- integrin α2- fraction, suggesting that the bipotent MEP progenitors reside in this cell fraction. Therefore, we performed single cell cultures of Lin-CD34+CD38-CD45RA- integrin α2- cells in mini-tray Terasaki plates and 96-well plates using serum free culture conditions and cytokines which specifically promote differentiation into erythroid and megakaryocytic lineages (Debili et al. 1996) (Fig. 4A). At day 12-14 of culture, 30-40% of the wells contained clones, with a similar cloning efficiency in Terasaki plates or 96-well plates (Fig. 4B). The clone sizes, evaluated in Terasaki plates, showed a high proliferative

capacity of the single cells, with one third of the colonies covering 50-100% of the well surface area (Fig. 4C).

The erythroid and megakaryocytic differentiation of the Lin-CD34+CD38-CD45RA- integrin α2- single cells cultured for 12-14 days was assessed by the immunophenotype and morphology (Fig. 5). In the flow cytometry assay, clones derived from bipotential progenitors contained both CD41+CD235- megakaryocytic and CD235+ CD41- erythrocytic lineage-differentiated cells (Fig.5A), whereas erythroid progenitors only produced CD235+CD41- cells (Fig.5B). Importantly, as shown by FACS analysis, all 32 analyzed clones, derived from single cells, contained erythroid cells, whereas 22 of the clones contained both erythroid and megakaryocytic cells (using a criteria of >0.1% positive cells), showing a high degree of bipotential megakaryocyte-erythrocyte activity in the integrin α2- population (Fig. 5C).

Previous report from single cell cultures have shown that megakaryocytic cells are a minority in clones derived from MEP progenitors, consisting of 2-30 cells among 500-1000 erythroblasts (Debili et al. 1996). In agreement with this, in our study the cells expressing the megakaryocyte marker CD41 were much fewer than cells expressing the erythroid CD235 marker (Fig. 5C), presumably indicating a much lower proliferative potential of megakaryocytic than erythroid progenitors and precursors. Minimal myeloid differentiation (CD15/CD33/CD66b+ cells 0.13%, 0.2% and 0.41%) was seen in the FACS analysis in 3 of 32 clones along with erythroid and megakaryocytic differentiation (not shown).

During differentiation in the single cell cultures, the integrin α2 receptor was upregulated on 51.6±30.5% of CD41+ megakaryocytic cells (mean±SD; n=22), consistent with the notion that integrin α2 is a collagen receptor on platelets with upregulation of expression during maturation of megakaryocytes (Nieswandt et al. 2001). In contrast, integrin α2 receptor was not expressed in CD235+ erythroid cells (0.1 ±0.2%; mean±SD; n=32).

For morphological analysis 83 clones derived from single cells after 12-14 days of liquid culture were individually transferred to cytospin slides and stained by May-Grünwald Giemsa (Fig. 5E). This allowed also evaluation of several of the clones with very limited cell numbers, which could not be analyzed by flow cytometry. These small colonies invariably consisted of megakaryocytic cells, again indicating the corresponding low proliferative potential (Fig. 5F). Apart from these small megakaryocytic clones, bipotential clones containing both erythroid and megakaryocytic cells were identified by morphology in a similar proportion, approximately 2/3 of the clones as by FACS analysis (Fig. 5D). Taken together, our data demonstrate a high frequency of bipotential MEP progenitors in adult bone marrow Lin-CD34+CD38-CD45RA- integrin α2- cell fraction.

Lin-CD34+CD38- integrin α2+ fraction contains primitive stem and progenitors with LTC-IC activity

To analyse the expression of integrin α2 receptor in multipotent lympho-myeloid progenitors, we performed transplantation of adult bone marrow CD34+CD38- integrin α2+ and integrin α2-

cells into immunodeficient NSG mice, followed by multilineage lympho-myeloid reconstitution analysis at 12 weeks. However, the frequency of mice reconstituted at 12 weeks after intrafemoral transplantation of 300-500 CD34+CD38- integrin α2+ and integrin α2- bone marrow cells was low, with 3/25 and 1/9 reconstituted mice, respectively (Suppl. Fig 1). This is in accordance with a previous report (Park et al. 2008), stating that adult human bone marrow stem and progenitor cells show a particularly low engraftment potential in immunodeficient mice, including the NSG strain. Therefore, we applied the *in vitro* LTC-IC assay to examine the presence of primitive progenitors in these populations. Notably, the LTC-IC progenitors were found to reside in the CD34+CD38- integrin α2+ cell fraction (Fig. 6A). In line with this, short-term liquid culture of Lin-CD34+CD38- integrin α2+ and Lin-CD34+CD38-CD45RA- integrin α2- cells with multiple cytokines promoting differentiation, showed that 97.2% of cells in the integrin α2+ fraction remained CD34+, whereas 27.7 % of the integrin α2- cells differentiated into CD34- cells, further supporting the notion that integrin α2+ cell fraction contains more primitive progenitors than the integrin α2- fraction (Fig. 6B, C). As reported previously, CD38 was not expressed in the cells after culture (not shown) (von Laer et al. 2000).

Gene expression profiling provides molecular evidence for enrichment of erythroid and megakaryocytic primed progenitors in the CD34+CD38- integrin α2- cell fraction.

Gene expression profiling of bone marrow CD34+CD38- cells showed increased expression of hemoglobin delta (HBD) in integrin α2- cells (Fig. 7A). In addition, we could see high expression of other erythroid and megakaryocytic lineage associated genes such as GATA2, CD36, and KLF in the integrin α2- cells from some donors (data not shown). Notably, Q-PCR

analysis of the gene expression of the key regulators GATA-2 and non-erythroid transcription factor GATA-3 in the sorted cells from 4 donors showed upregulation of GATA-2 and downregulation of GATA-3 in the integrin α2- cell fraction (Fig. 7B). These data support the findings from the *in vitro* cultures that integrin α2- cells are transcriptionally primed towards development erythroid and megakaryocytic lineages.

DISCUSSION

Studies focusing on the characterization of human hematopoietic stem cells and their downstream intermediates have shown that in the primitive Lin-CD34+CD38- cell fractions, the most primitive *in vivo* long-term repopulating stem cells reside in the CD90+CD45RA- and to a lesser extent, in the CD90-CD45RA- subpopulations, while the CD90-CD45RA+ subpopulation does not contain any stem cells but contains mainly lymphoid progenitors. However *in vivo* and *in vitro* stem and progenitor cell assays of these stem cell enriched fractions show that these are still very heterogeneous and contain progenitors of varying maturation and differentiation potentials, including granulocyte-macrophage, megakaryocyte-erythroid and multipotent-myeloid progenitors (Majeti et al. 2007; Doulatov al. 2010; Wong et al. 2013).

Human progenitor cells with both megakaryocyte and erythroid differentiation potential have been defined as the Lin-CD34+CD38+CD123-CD45RA- and in addition, CD110+ (Manz, Miyamoto et al. 2002; Edvardsson et al. 2006). However, previous colony assays as well as single cell liquid cultures have shown that cells with bipotential megakaryocyte-erythroid commitment also reside in the CD34+CD38- fraction in the adult bone marrow (Debili et al. 1996). So far, the phenotype of this MEP population has remained obscure. In this study, we show that the integrin α2 receptor is expressed on more than 90% of CD34+CD38- adult bone marrow cells, and that this cell fraction contains all primitive LTC-IC progenitors as well as most granulocyte-macrophage progenitors. In contrast, the integrin α2- fraction contains in a high frequency both erythroid and megakaryocytic progenitors. In single cell methylcellulose assay, approximately 70% of the Lin-CD34+CD38- integrin α2- colony forming unit had erythrocyte potential. However, this culture system does not support megakaryocyte differentiation and

commitment to erythroid or bipotential megakaryocyte-erythroid lineages cannot be distinguished. Therefore, we performed liquid cultures at single cell level in serum free culture medium supplemented with cytokines that favoured both megakaryocytic and erythrocytic lineage differentiation. These cell cultures showed a high proliferative capacity of the isolated single cells. All clones were shown to express markers for erythroid and/or megakaryocytic differentiation. Approximately half of the single cells were differentiated along both erythroid and megakaryocytic lineages, while a minority of the cells showed unilineage either erythroid or megakaryocytic differentiation, confirmed by flow cytometry or morphological analysis. Since 40% of Lin-CD34+CD38-CD45RA- integrin α2- single cells gave rise to colonies in Terasaki wells, it can be estimated that 20% of these single sorted cells had a bipotential megakaryocytic-erythroid commitment.

The liquid culture conditions and cytokines were chosen to favour megakaryocyte-erythroid differentiation. In these cultures, myeloid markers were found expressed, albeit in a low level (<0.5% of cells) in 3 of 32 clones analysed by FACS, suggesting that some of the Lin-CD34+CD38-CD45RA- integrin α2- cells were more primitive progenitors with commitment to both granulocyte-macrophage and megakaryocyte-erythrocyte lineages. However, the single cell colony assays in methylcellulose with cytokine supplementation, also supporting growth of multipotent myeloid, erythroid and granulocyte-macrophage progenitors, showed that only 1% of the cells in the Lin-CD34+CD38- integrin α2- cell fraction were multipotent CFU-GEMM progenitors. This result supports the notion that most of the CD34+CD38- integrin α2- cells

giving rise to megakaryocytic and erythroid cells in the liquid culture system employed here are bipotent MEPs, whereas only few are multipotent myeloid progenitors or stem cells.

The increased expression of genes associated with erythroid and megakaryocytic lineages in the integrin α2- population is likely an indication of so called lineage priming. This transcription of lineage restricted genes has been suggested to reflect epigenetic changes, making genes associated with a defined future lineage more easily accessible for the transcription factor machinery. The link between lineage priming and terminal commitment has been extensively studied in lineage tracing experiments in mice and even though it is apparent that priming is not absolute, the expression of Rag-1 or TdT in multipotent progenitors marks cells prone to development into lymphoid lineages, while expression of Gata-1 and Gata-2 identify cells destined to erythroid-megakaryocytic pathways (Mancini et al. 2012, Doré and Crispino 2011). Therefore, we believe that the observation that the integrin α2- cell population expressed genes associated with erythroid and megakaryocytic lineages, strongly supports the concept that these cells represent a population primed towards the development of defined lineages.

We have recently by employing limiting dilution transplantation assay in NSG mice shown that cord blood long-term multilineage reconstituting stem cells are significantly enriched in the CD34+CD38-CD90+ integrin α2+ cell fraction (Wong et al. 2013). In agreement with this, our present findings show that adult bone marrow LTC-ICs express integrin α2. However, while integrin α2 receptor is expressed in most adult CD34+CD38- cells, the frequency of cells expressing this receptor in the corresponding cell fraction is much lower, approximately 30%, in

cord blood. Furthermore, essentially all adult bone marrow CD34+CD38-CD90+ cells express integrin α2+, in contrast to approximately half of the corresponding cord blood cells. The cord blood LTC-ICs were significantly enriched in the integrin α2+ subfraction within the more primitive CD34+CD38- CD90+ cell fraction but equally distributed within the total CD34+CD38- cell fraction, whereas erythroid progenitors were significantly enriched in integrin α2- fraction within CD34+CD38- cell population but resided in an equal frequency in the CD90+ subpopulation. These finding show profound ontogeny-related differences in integrin α2 receptor expression in distinct human hematopoietic progenitor populations.

Knowledge on the molecular pathways regulating development of hematopoietic stem cells and their downstream progenitor stages is essential for elucidating the molecular mechanisms underlying normal and malignant haematopoiesis. This requires identification of novel lineage and differentiation stage specific cell surface markers which enable a better prospective isolation of distinct hematopoietic stem and progenitor cell populations in the Lin-CD34+CD38- compartments and validation of these markers by analysis of the isolated cell populations by *in vivo* and *in vitro* functional stem and progenitor cell assays. Here we describe a novel primitive megakaryocyte-erythrocyte bipotential Lin-CD34+CD38- integrin α2- progenitor in human adult bone marrow. The identification of this novel bipotential MEP population provides a means for further analysis on lineage fate decisions of primitive erythroid and megakaryocytic cells and may facilitate studies aiming to expand and differentiate these lineages for clinical transplantation and transfusion trials.

Figure 1.

Expression of integrin α1-α6 and β1 chains in human adult bone marrow stem and progenitor cells

(A) Representative FACS analysis showing the expression of integrin α-α6 and β1 chains in adult bone marrow CD34+CD38- cells (open histograms) and the corresponding isotype control stainings (shaded histograms).

(B) Representative FACS profiles showing viable (PI-) CD34+CD38- cells gated for lineage-marker negative cells (Lin-), CD90 and CD45RA. The expression of integrin α2 receptor is shown in the cells gated from the corresponding CD90/CD45RA quadrants. The quadrant gates were set on the basis of FMO control staining profiles.

(C) The percentage of cells expressing the integrin α2 receptor in CD90/CD45 quadrants of Lin-CD34+CD38- cells (mean±SD; 2 independent analyses, each with 2 separate human bone marrow samples). *p<0.05.

Figure 2.

Enrichment of the erythroid and megakaryocyte colony forming cells in human adult bone marrow CD34+CD38- integrin α2- cells

(A) FACS profiles showing gating strategy for the isolation of CD34+CD38- integrin α2+ and integrin α2- cells for *in vitro* colony forming assays. Quadrant gates in the FACS plots were set on the basis of FMO control staining profiles. The numbers in the plots represent the percentage of cells in the gates (red gates). 7AAD-, viable cells gated by exclusion of 7AAD+ dead cells.

(B) The frequencies of mixed (CFU-GEMM), granulocyte-macrophage (CFU-GM), erythroid (BFU-E) and total (CFU-C) progenitors in adult bone marrow CD34+CD38- integrin α2+ and integrin α2- cells assayed in the methylcellulose culture supplemented with cytokines.

(C) The frequency of megakaryocyte colonies (CFU-MK) in adult bone marrow CD34+CD38- integrin α2+ and integrin α2- cells assayed in collagen-based megakaryocyte colony forming assay.

The horizontal bars show median values.*$p<0.05$; **$p<0.01$; *** $p<0.001$. The results were obtained from 2 independent experiments with the use of 3-4 human samples.

Figure 3.

Single cell colony assay shows that adult bone marrow Lin-CD34+CD38- erythroid progenitors are highly enriched in integrin α2- population but not limited to CD110+ subfraction, whereas CD33 and CD135 are not expressed in the Lin-CD34+CD38-CD45RA- integrin α2- adult bone marrow cells

(A) FACS profiles showing gating strategy for the isolation of Lin-CD34+CD38- integrin α2+ and integrin α2- cells for *in vitro* single-cell colony forming assay (red gates)

(B) The frequencies of mixed (CFU-GEMM), granulocyte-macrophage (CFU-GM) and erythroid (BFU-E) progenitors in Lin-CD34+CD38- integrin α2+ and integrin α2- cell compartments. 460 Lin-CD34+CD38- integrin α2+ and 255 Lin-CD34+CD38- integrin α2- cells were plated. The result was obtained from 4 independent experiments with the use of 6 separate human samples in total.

(C) FACS profiles showing gating strategy for the isolation of CD110+CD45RA-, CD110-CD45RA- and CD110-CD45RA+ cells from Lin-CD34+CD38- integrin α2- population for *in vitro* single-cell colony forming assay (red gates).

(D) The frequencies of CFU-GEMM, CFU-GM and BFU-E progenitors in Lin-CD34+CD38-integrin α2-CD110+CD45RA-, CD110-CD45RA- and CD110-CD45RA+ cell compartments. 332 Lin-CD34+CD38- integrin α2-CD110+CD45RA- cells, 216 Lin-CD34+CD38- integrin α2-CD110-CD45RA- cells and 201 Lin-CD34+CD38- integrin α2-CD110-CD45RA+ cells were plated. The result was obtained from 2 independent experiments with the use of 4 separate human samples in total.

(E) Representative FACS profiles showing that CD33 and CD135 are not expressed in adult bone marrow Lin-CD34+CD38-CD45RA- integrin α2- cells. Shown are representative FACS plots of 2 separate experiments.

Quadrant gates in the FACS plots were set on the basis of FMO control staining profiles and the numbers in the plots represent the percentage of cells in the gates. 7AAD-, viable cells gated by exclusion of 7AAD+ dead cells. Lin-, lineage-depleted cells.

Figure 4.

Single cell liquid culture assay of adult bone marrow Lin-CD34+CD38-CD45RA- integrin α2- cells shows a high cloning efficiency after 12-14 days culture.

(A) FACS profiles showing gating strategy for the isolation of adult bone marrow Lin-CD34+CD38-CD45RA- integrin α2- cells for *in vitro* single-cell colony forming assay (red gates).

(B) Frequency of clonally expanded single Lin-CD34+CD38-CD45RA- integrin α2- adult bone marrow cells cultured in mini-tray Terasaki and 96-well plates.

(C) Sizes of clones derived from single Lin-CD34+CD38-CD45RA- integrin α2- cells. Terasaki plates were scored based on the cell numbers and the percent coverage of the culture-well surface. 151 clones were analyzed.

Figure 5.

Single cell clonogenic assay reveals the existence of bipotent megakaryocyte-erythrocyte progenitors in Lin-CD34+CD38-CD45RA- integrin α2- fraction in adult bone marrow

(A, B) Representative FACS profiles showing the expression of CD41, CD235, CD15/CD33/CD66b and integrin α2 in the cultured cells derived from a single Lin-CD34+CD38-CD45RA- integrin α2- cells with bipotential megakaryocytic-erythroid (A) and unipotential erythroid differentiation capacity (B). Quadrant gates in the FACS plots were set on the basis of fluorescence minus one (FMO) control staining profiles. The numbers in the plots represent the percentage of cells in the gates. 7AAD-, viable cells gated by exclusion of 7AAD+ dead cells. Lin-, lineage-depleted cells.

(C) The frequency of cells showing megakaryocyte (CD41+CD235-) or erythrocyte (CD235+ CD41-) differentiation in the FACS analysis of individual clones derived from single Lin-CD34+CD38-CD45RA- integrin α2- cells after 12-14 day of liquid cultures. 32 individual clones were analyzed by FACS.

(D) Lineage distribution of clones derived from a single Lin-CD34+CD38-CD45RA- integrin α2- adult bone marrow cells, evaluated based on immunophenotype (FACS analysis) or

morphology on cytospin slides after May-Grünwald-Giemsa staining of the single cell culture-derived cells.

(E) Morphology of the of the megakaryocyte (MK), erythroid (E) and mixed megakaryocyte-erythroid (MK/E) colonies obtained at 12-14 days after culture of single Lin-CD34+CD38-CD45RA- integrin α2- cells. Shown cells transferred onto cytospin slides and stained with May-Grünwald Giemsa.

(F) Morphology of the megakaryocyte (Mk), and mixed megakaryocyte-erythroid (Mk/E) colonies, obtained at 12-14 days after culture of single Lin-CD34+CD38-CD45RA- integrin α2- cells. The cells on Terasaki plates were photographed by an inverted light microscope. The morphology of both clones was confirmed after transferring onto cytospin slides and staining with May-Grünwald Giemsa.

The results were obtained from 5 independent experiments, each with one bone marrow sample.

Figure 6.

Human bone marrow CD34+CD38- integrin α2+ fraction contains more primitive progenitors than integrin α2- fraction

(A) The number of colonies per well obtained in the LTC-IC assay of bone marrow CD34+CD38- integrin α2+ and integrin α2- cells (plated 50 cells/well). The results were obtained from 2 independent experiments using 4 separate human samples in total.

(B) FACS profiles showing gating strategy for the isolation of adult bone marrow Lin-CD34+CD38- integrin α2+, Lin-CD34+CD38-CD45RA- integrin α2- and Lin-CD34+CD38+ cells for *in vitro* short-term liquid culture with multiple cytokines. The gates in the FACS plots were set on the basis of fluorescence minus one (FMO) control staining profiles. The numbers in

the plots represent the percentage of cells in the gates. 7AAD-, viable cells gated by exclusion of 7AAD+ dead cells.

(C) FACS plots showing expression of CD34 (red open histograms) after 5-day liquid culture of bone marrow Lin-CD34+CD38- integrin α2+, Lin-CD34+CD38-CD45RA- integrin α2- and Lin-CD34+CD38+ cells. The isotype control staining is shown by grey shaded histograms. The number in each plot shows the percentage of CD34+ cells.

Figure 7.

Microarray and Q-PCR analysis of Lin- CD34+CD38- integrin α2+ and integrin α2- cells

(A) Heatmap of the global gene expression in the Lin-CD34+CD38- integrin α2+ and Lin-CD34+CD38- integrin α2- cells. Clustering shows genes being up- or down-regulated by more than 1.5-fold in the integrin α2- cells compared to the integrin α2+ cells (using lower bound of 90% CI and P<0.05 for paired t test comparison). Red represents high and blue represents low expression. The data were normalized by RMA Express software and were from 4 independent sorting experiments and 8 microarray platforms.

(B) Q-PCR analysis showing the expression of GATA-2 and GATA-3 transcripts in the Lin-CD34+CD38- integrin α2+ and Lin-CD34+CD38- integrin α2- cells. The data were normalized to endogenous *HPRT* expression. The results (Mean±SEM) are from 4 independent bone marrow samples.

References

Bhatia, M., J. C. Wang, et al. (1997). "Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice." Proc Natl Acad Sci U S A 94(10): 5320-5325.

Christ, O., K. Lucke, et al. (2007). "Improved purification of hematopoietic stem cells based on their elevated aldehyde dehydrogenase activity." Haematologica 92(9): 1165-1172.

Debili, N., L. Coulombel, et al. (1996). "Characterization of a bipotent erythro-megakaryocytic progenitor in human bone marrow." Blood 88(4): 1284-1296.

Doré, L., Crispino, J.D. (2011). "Transcrption factor networks in erythroid cell and megakaryocyte development." Blood 118(2): 231-239.

Doulatov, S., F. Notta, et al. (2010). "Revised map of the human progenitor hierarchy shows the origin of macrophages and dendritic cells in early lymphoid development." Nat Immunol 11(7): 585-593.

Edvardsson, L., J. Dykes, et al. (2006). "Isolation and characterization of human myeloid progenitor populations--TpoR as discriminator between common myeloid and megakaryocyte/erythroid progenitors." Exp Hematol 34(5): 599-609.

Herrmann, H., S. Cerny-Reiterer, et al. (2012). "CD34(+)/CD38(-) stem cells in chronic myeloid leukemia express Siglec-3 (CD33) and are responsive to the CD33-targeting drug gemtuzumab/ozogamicin." Haematologica 97(2): 219-226.

Hofmann, M., L. Grosse-Hovest, et al. (2012). "Generation, selection and preclinical characterization of an Fc-optimized FLT3 antibody for the treatment of myeloid leukemia." Leukemia 26(6): 1228-1237.

Hogan, C. J., E. J. Shpall, et al. (2002). "Differential long-term and multilineage engraftment potential from subfractions of human CD34+ cord blood cells transplanted into NOD/SCID mice." Proc Natl Acad Sci U S A 99(1): 413-418.

Hogge, D. E., P. M. Lansdorp, et al. (1996). "Enhanced detection, maintenance, and differentiation of primitive human hematopoietic cells in cultures containing murine fibroblasts engineered to produce human steel factor, interleukin-3, and granulocyte colony-stimulating factor." Blood 88(10): 3765-3773.

Iwasaki, H. and K. Akashi (2007). "Hematopoietic developmental pathways: on cellular basis." Oncogene 26(47): 6687-6696.

Kikushige, Y., G. Yoshimoto, et al. (2008). "Human Flt3 is expressed at the hematopoietic stem cell and the granulocyte/macrophage progenitor stages to maintain cell survival." J Immunol 180(11): 7358-7367.

Majeti, R., C. Y. Park, et al. (2007). "Identification of a hierarchy of multipotent hematopoietic progenitors in human cord blood." Cell Stem Cell 1(6): 635-645.

Mancini, E., A. Sanjuan-Pla, et al. (2012). "FOG-1 and GATA-1 act sequentially to specify definitive megakaryocytic and erythroid progenitors." EMBO J 31(2): 351-365.

Manz, M. G., T. Miyamoto, et al. (2002). "Prospective isolation of human clonogenic common myeloid progenitors." Proc Natl Acad Sci U S A 99(18): 11872-11877.

Nieswandt, B., C. Brakebusch, et al. (2001). "Glycoprotein VI but not alpha2beta1 integrin is essential for platelet interaction with collagen." EMBO J 20(9): 2120-2130.

Park, C. Y., R. Majeti, et al. (2008). "In vivo evaluation of human hematopoiesis through xenotransplantation of purified hematopoietic stem cells from umbilical cord blood." Nat Protoc 3(12): 1932-1940.

Pronk, C. J., D. J. Rossi, et al. (2007). "Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy." Cell Stem Cell 1(4): 428-442.

Qian, H., K. Le Blanc, et al. (2012). "Primary Mesenchymal Stem and Progenitor Cells from Bone Marrow Lack Expression of CD44 Protein." J Biol Chem 287(31): 25795-25807.

Sperr, W. R., S. Florian, et al. (2005). "CD 33 as a target of therapy in acute myeloid leukemia: current status and future perspectives." Leuk Lymphoma 46(8): 1115-1120.

Taussig, D. C., D. J. Pearce, et al. (2005). "Hematopoietic stem cells express multiple myeloid markers: implications for the origin and targeted therapy of acute myeloid leukemia." Blood 106(13): 4086-4092.

van Lochem, E. G., V. H. van der Velden, et al. (2004). "Immunophenotypic differentiation patterns of normal hematopoiesis in human bone marrow: reference patterns for age-related changes and disease-induced shifts." Cytometry B Clin Cytom 60(1): 1-13.

von Laer, D., A. Corovic, et al. (2000). "Loss of CD38 antigen on CD34+CD38+ cells during short-term culture." Leukemia 14(5): 947-948.

Wong, W. M., M. Sigvardsson, et al. (2013). "Expression of integrin alpha2 receptor in human cord blood CD34+CD38-CD90+ stem cells engrafting long-term in NOD/SCID-IL2Rgamma(c) null mice." Stem Cells 31(2): 360-371.

Supplementary methods

Mouse Transplantation

NOD.Cg-Prkdcscid Il2rgtml Wjl/SzJ (NOD/SCID-IL2Rγ$_c$null; NSG) mice (Jackson Laboratory, Bar Harbor, ME, USA) were bred and maintained in the germ-free isolators in the animal facility of Lund University. All animal experiments were approved by the animal ethics committee, Lund University (M 148-09).

NSG mice (8-12 weeks old) were sublethally irradiated 4-24 hours prior transplantation with 300 cGy using a 137Cs-γ irradiator. Sex- and age-matched mice were used as recipients in all experimental groups. Human adult bone marrow bone marrow CD34+CD38- integrin α2+ or integrin α2- cells were transplanted together with 1 x 10^6 irradiated (1500 cGy) CD34- cells from the same donors. The sorted cells were injected intrafemorally. Briefly, an insulin syringe with 30-gauge needle (BD Biosciences) was used to drill a hole in the right femur, and 10 μL cell suspension was injected into bone marrow cavity using Hamilton Syringe with 31-gauge needle (Hamilton Bonaduz AG, Bonaduz, GR, Switzerland). One or two mice injected with PBS were included in each experiment as controls.

Bone marrow cells were harvested from femurs and tibias of recipient mice at 12 weeks after transplantation and stained with human specific APC conjugated anti-CD45 (HI30, BD Biosciences) for detecting human hematopoietic cells, FITC anti-CD15 (HI98), anti-CD33 (HIM3-4) and anti-CD66b (G10F5) (BD Biosciences) for detecting myeloid cells, PE anti-CD19 (HIB19, BD Biosciences) for detecting lymphoid cells and PerCP-Cy5.5 anti-CD34 (8G12, BD Biosciences) for detecting progenitor cells. Gates in FACS plots were set according to FMO

controls using corresponding isotype antibodies. Engraftment was determined as positive when a minimum of 0.1% human CD45+ cells, together with at least 0.025% human CD45+CD15/CD33/CD66b+ and 0.025% human CD45+CD19+ cells were detected, with a minimum of 100,000 viable cells collected for analysis (Wong et al. 2013). Recently, female NSG mice were reported to be superior in engrafting human HSCs (Notta et al. 2010). Therefore, the gender of recipient mice was indicated in the results.

Wong, W. M., M. Sigvardsson, et al. (2013). "Expression of integrin alpha2 receptor in human cord blood CD34+CD38-CD90+ stem cells engrafting long-term in NOD/SCID-IL2Rgamma(c) null mice." Stem Cells 31(2): 360-371.

Notta, F., S. Doulatov, et al. (2010). "Engraftment of human hematopoietic stem cells is more efficient in female NOD/SCID/IL-2Rgc-null recipients." Blood 115(18): 3704-3707.

Supplementary Figure 1

(A) Representative FACS profiles showing analysis of human cell reconstitution in the bone marrow of NSG mouse transplanted with CD34+CD38- integrin α2+ cells. Shown are CD45+ cell reconstitution (upper left panel), myeloid (CD45+CD15/CD33/CD66b+) and B-lymphoid (CD45+CD19+) (upper right panel), and progenitor (CD45+CD34+) cells (lower left panel) in the gated human CD45+ cell fraction. The lower right panel shows the analysis of the control mouse injected with PBS instead of human cells.

(B) Bone marrow chimerism of human CD45+, myeloid, B-lymphoid and CD34+ cells in

NSG mice at 12 weeks after intrafemoral transplantation of 300-500 adult bone marrow CD34+CD38- integrin α2+ or integrin α2- cells. The horizontal bars show median values. The red and blue dots represent reconstitution in individual female and male recipients, respectively.

(C) The number of reconstituted mice/ total number of mice transplanted with indicated numbers of cells.

The results are from 4 independent experiments with the use of 8 separate human samples in total.

Figure 1

A

CD34+CD38- cells

% of Max

α1 α2 α3 α4 α5 α6 β1

Fluorescence Intensity

B

C

Figure 2

A

B

C
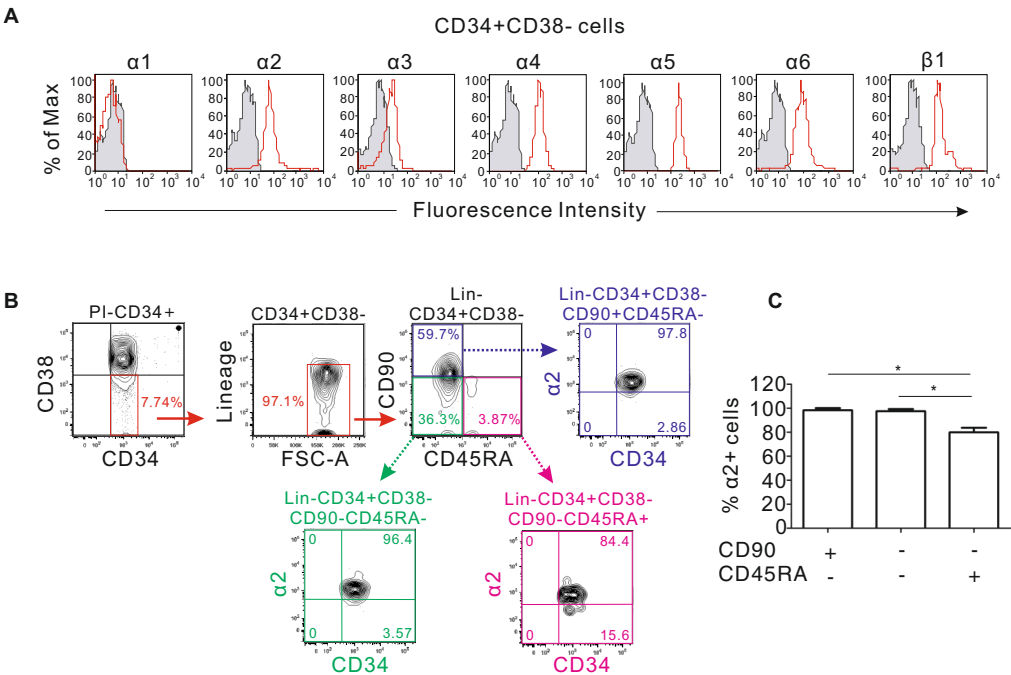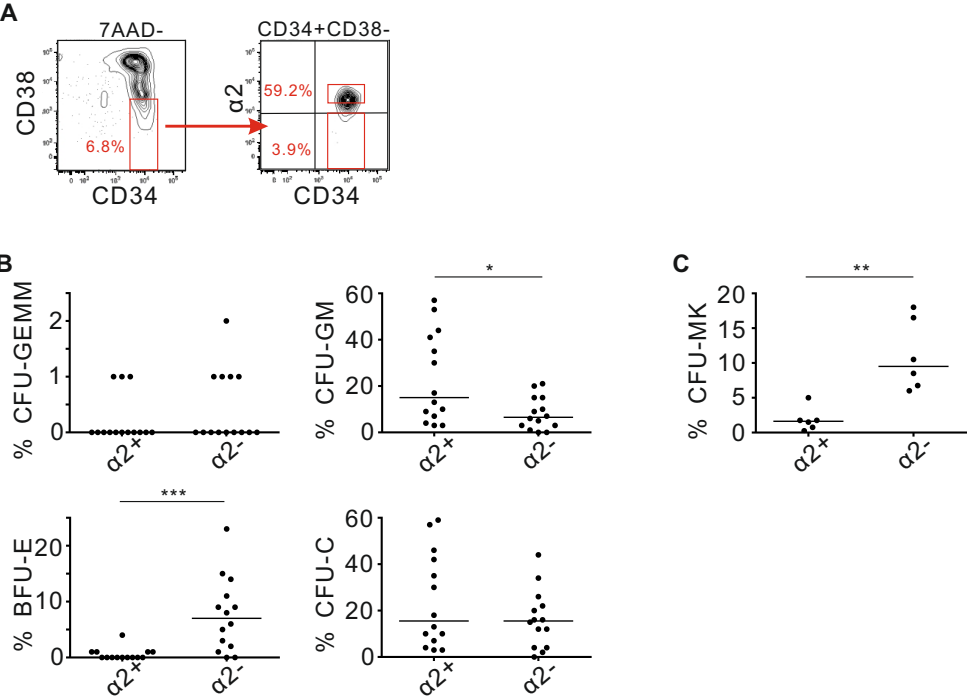
Figure 3

A
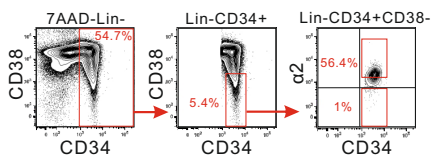
B

C

D

E

Figure 4

A

B

C

Figure 5

A Mk/E

7AAD-
CD41 / CD235 — 0.61% / 60%

7AAD-
α2 / CD41 — 0.03 / 0.48 / 99.4 / 0.12

7AAD-
α2 / CD235 — 0.51 / 0 / 42.6 / 56.9

7AAD-
α2 / CD15/33/66b — 0.51 / 0 / 99.5 / 0

B E

7AAD-
CD41 / CD235 — 0% / 83%

7AAD-
α2 / CD41 — 0.046 / 0 / 100 / 0

7AAD-
α2 / CD235 — 0.04 / 0.006 / 18.6 / 81.4

7AAD-
α2 / CD15/33/66b — 0.046 / 0 / 100 / 0

C

% of CD41+CD235- cells

% of CD235+CD41- cells

D

% of Clones — Immuno-phenotype

% of Clones — Morphology

☐ Mk
■ E
▦ Mk/E

E

Mk — 40μM / 20μM

E — 40μM / 20μM

Mk/E — 40μM / 20μM

F

Mk

Mk/E (Predominantly E)

Figure 6

A

B

C

Fig. 7 **A**

α2+   α2-   **Gene**   **Fold change**

Gene	Fold change
LOC221710	-2.9
IL12RB2	-3.8
LOC144997	-2.3
LRRC1	-2.1
MSTP020 (MST020)	-3.1
OPTN	-2.9
C12orf29	-2.9
FBXL17	-2.9
CDNA FLJ30906 fis, clone FEBRA2006055	-2.7
gb:AI741415 /DB_XREF	-3.0
gb:AI806174 /DB_XREF	-2.8
NDNL2	-2.6
gb:AI341383 /DB_XREF	-2.3
CDNA FLJ26539 fis, clone KDN09310	-2.1
CDNA FLJ34775 fis, clone NT2NE2003315	-3.0
Homo sapiens, clone IMAGE:4133122, mRNA	-2.0
DBNDD2	-2.4
gb:AI928513 /DB_XREF	-2.5
CTSO	-3.1
Transcribed locus	-2.1
Transcribed locus	-2.2
PLCL2	-2.3
GIPC2	-2.4
SNX7	-3.8
SLC2A11	-2.1
Transcribed locus	-2.9
LOC285949	2.1
ZNF395	1.8
CYP4F2	3.1
IGLL1	3.1
gb:AI890972 /DB_XREF	2.8
Full length insert cDNA clone YF47B02	2.9
SLC27A2	3.1
CSF2RB	7.6
HBD	6.5
gb:AL559474 /DB_XREF	2.5
TST	4.1
MRNA; cDNA DKFZp686D22106	4.3
STX7	2.2
BID	2.6
DBN1	3.0
PGDS	4.1
CDNA clone IMAGE:5285945	2.1
NPTX2	6.2
PRKAR2B	3.9
ZFHX1B	2.2
Clone FLB4246 PRO1102	2.7
MSL2L1	2.4
C20orf175	2.8

B

GATA2
P=0.036

GATA2 (Relative to HPRT)

α2+   α2-

GATA3
P=0.041

GATA3 (Relative to HPRT)

α2+   α2-

Article III

Hyperactivated AKT is incompatible with survival when coexpressed with additional oncogenes and drives hematopoietic stem and progenitor cells to cell cycle inhibition and apoptosis

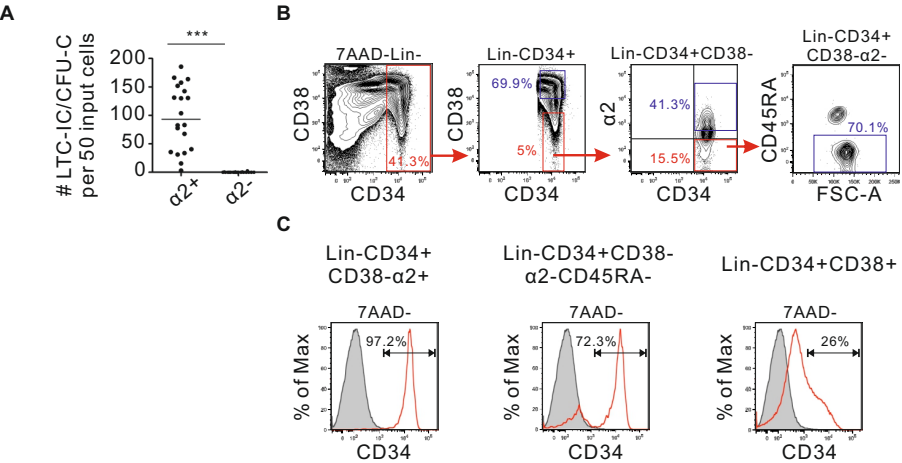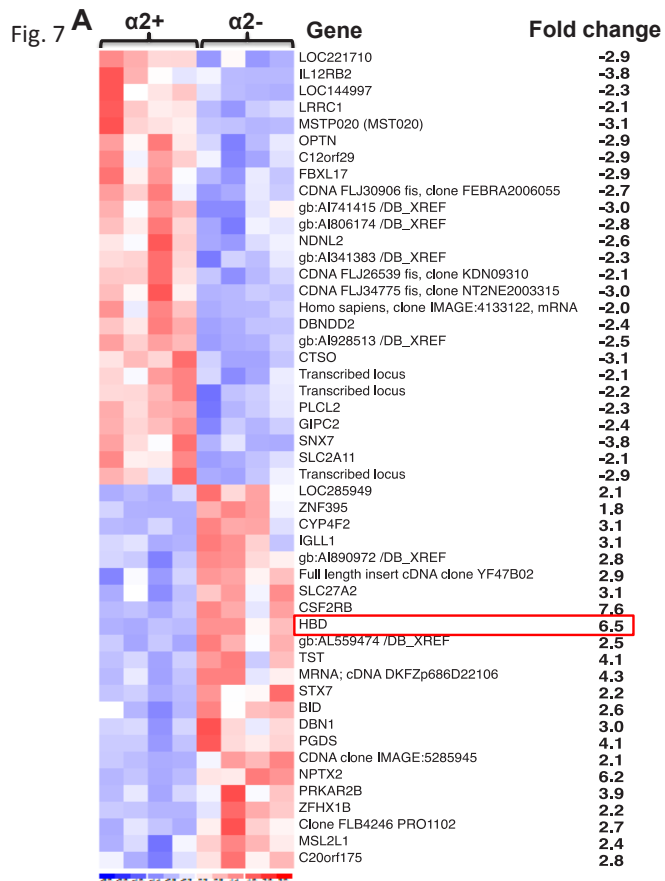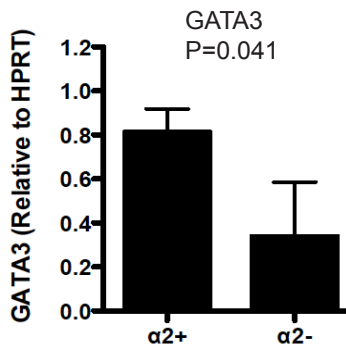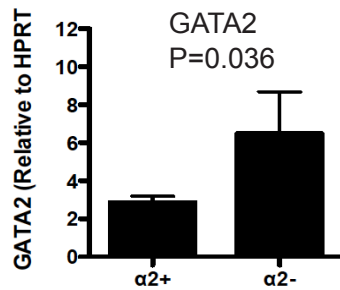Yanjuan Tang[1], Camilla Halvarsson[1,2], Amanda Nordigården[1], Josefine Åhsberg[1], Wan Man Wong[3], and Jan-Ingvar Jönsson[1,2]

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Running title: Pro-apoptoic effects by hyperactivated AKT in hematopoiesis

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CAPSULE (58 words)

Background: The PI3K-AKT signaling pathway is important in leukemia and associated to FLT3-ITD mediated transformation.

Results: Constitutively active AKT was incompatible with STAT5, FLT3-ITD, and Bcl-2 coexpression, inducing cell cycle block and apoptosis.

Conclusion: AKT is not only a single-function survival kinase but may facilitate rather than inhibit cell death.

Significance: These results could lead to future intervention of AKT in leukemia.

SUMMARY (240 words)

The PI3K-AKT signaling pathway plays an important role in cell growth and metabolism. Increased AKT activity is frequently seen in patients with acute myeloid leukemia (AML), providing leukemic cells with both growth-promoting and survival signals involved in the transformation process. In AML up to 30% of all patients carry activating mutations in the tyrosine kinase receptor FLT3, leading to activation of the PI3K/AKT pathway as well as STAT5. Here, we investigated the effect of hyperactivated AKT (myristylated AKT) by retroviral transfer to hematopoietic progenitor cells coexpressing STAT5, FLT3-ITD, or anti-apoptotic Bcl-2. AKT was unable to relieve cytokine-dependence. Surprisingly, uncontrolled AKT activity was linked to accumulation of cells in the G_0 stage of the cell cycle and increased cell numbers became apoptotic. Hyperactivated AKT was incompatible with STAT5-driven proliferation and triggered apoptosis. The same was true also in FLT3-ITD-expressing progenitor cells of transgenic mice. Transplantable hematopoietic stem cells of wildtype and Bcl-2 transgenic mice were impaired in their engraftment ability to recipient mice when expressing hyperactivated AKT. This was linked to AKT-mediated pro-apoptotic functions and not due to effects on homing or migration. Cells expressing hyperactivated AKT displayed higher levels of reactive oxygen species. However, the addition of the antioxidant N-acetyl-L-lysine significantly reduced apoptosis. Taken together, the results indicate that constitutive AKT activity is incompatible with the growth- and survival-promoting ability of FLT3-ITD and its downstream targets. These findings may provide a novel tool to intervene with AKT activity in leukemia.

INTRODUCTION

The phosphatidylinositol 3-kinase (PI3K)/AKT pathway is an integral component of signaling pathways involved in the development of myeloid leukemias, such as chronic myeloid leukemia (CML) and acute myeloid leukemia (AML). While the PI3KAKT pathway is frequently activated in AML (1-3), the mechanisms leading to AKT activation are not completely clear. Recent studies have shown that AKT is active by phosphorylation in the majority of cases of AML (1,2,4,5). Multiple mechanisms are likely to account for these findings, but while amplification and mutation of *PIK3CA* and loss of tumor suppressor PTEN (phosphatase and tensin homolog deleted from chromosome 10) contribute to constitutive activation of the PI3K/AKT pathway in many human cancers, such mutations are rare in myeloid leukemias (6,7).

Mutations in the Fms-like tyrosine kinase-3 (FLT3) gene is one of the most common genetic alterations found in patients with AML. Constitutively activated FLT3 occurs most often as internal tandem duplications (ITDs) within the juxtamembrane domain and/or the first kinase domain, and is observed in approximately 20-30% of AML patients (8,9). FLT3-ITD mutations are associated with activation of the PI3K/AKT pathway similar to wildtype FLT3 (10,11). In contrast, STAT5 has been demonstrated to be activated solely by FLT3-ITD signaling, which is required for transformation *in vivo* (12,13).

AKT overexpression by itself or constitutive active forms of AKT do not seem to induce oncogenic transformation of hematopoietic cells but may act in combination with other pathways to mediate transformation. For instance, AKT and Bcl-X$_L$ cooperate to promote leukemogenesis when coexpressed in a transplantation model (14). When activated AKT1 was expressed in a murine bone marrow transplantation model, recipient mice developed myeloproliferative disease, T-cell lymphoma, or AML (15). Analysis of hematopoietic stem cells (HSCs) in recipients revealed transient expansion and increased cycling as well as impaired engraftment, implying that AKT activation is an important mechanism of transformation in AML and that HSCs are sensitive to excess AKT signaling.
Here, we have investigated the consequence of overexpression of hyperactivated AKT in HSCs and progenitors from bone marrow of mice transgenic for FLT3-ITD as well as in hematopoietic progenitor cell lines expressing constitutively activated STAT5. In addition, we tested AKT function when coexpressed with Bcl-2 protein as an example of FLT3-ITD anti-apoptotic functions. Activation of AKT was accomplished by fusing the protein to the Src

myristoylation signal (myristylated AKT, myrAKT), targeting the protein to the cell membrane where it becomes constitutively activated (16). Hyperactivation of AKT surprisingly led to exit of cell cycle followed by an increase in apoptosis in all three cases. Furthermore, HSCs from both wildtype and Bcl-2 transgenic mice were impaired in their engraftment ability to recipient mice when myrAKT was overexpressed. These results suggest that constitutive AKT activity is incompatible with the growth- and survival-promoting ability of FLT3-ITD and its downstream targets.

EXPERIMENTAL PROCEDURES

Mice

C57BL6/J mice and C57BL6-vav-*Bcl2* mice (17) were housed and handled in the Animal Facility at Linköping University according to Swedish Legislation. The Animal ethics committee approved all mouse experiments. Bone marrow of *Flt3-ITD* knockin mice on C57BL/6 background (18) was provided by Ewa Sitnicka (Lund, Sweden).

Cell lines

Ba/F3 cells were cultured in complete RPMI 1640 medium (PAA laboratories, Les Mureaux, France) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA), 25 nM Hepes, 50 µM 2-mercaptoethanol (Sigma-Aldrich), whereas FDC-P1 cells were cultured in Iscove´s modified Dulbecco´s medium (IMDM; PAA) with 10% FCS and 2 mM L-glutamine. Both cell lines were also supplemented with 1 % penicillin-streptomycin and 5% IL-3-containing supernatant when routinely maintained. Cells stably expressing ca-STAT5 and/or myristylated AKT were done by spinoculation with retrovirus supernatants made by transfecting 293T cells with the corresponding retroviral cDNA constructs (pMX-puro-caSTAT5 (19) and pLZRS-myrAKT-EGFP (20), respectively) as previously described (21). Expression of STAT5 and AKT was confirmed by Western blot analysis.

Transfection and magnetic isolation with pMACS K^kII. cDNA corresponding to wt AKT or myrAKT were cloned into the expression vector pMACS K^kII (Miltenyi) and transfected into FDC-P1 cells using Amaxa nucleofection (Cologne, Germnay). Dead cells were removed by Ficoll centrifugation. $H2K^k$-positive cells were selected with antibodies coupled to magnetic beads (Miltenyi). $H2K^k$- expressing cells were analyzed for apoptosis by flow cytometry (FACS Calibur, BD Biosciences) using FITC-conjugated $H2K^k$ antibodies (Miltenyi) and Annexin V-PE (BD Biosciences) after 24 hours of stimulation with SCF (100 ng/mL).

Isolation and culture of c-kit^{+} cells

Mononuclear BM cells were harvested by crushing femurs and tibiae from 8-14 weeks old mice. MACS immunomagnetic cell separation using magnetically labeled anti-CD117 beads (Miltenyi) was used to isolate c-kit positive cells. After selection, cells were cultured overnight in IMDM supplemented with 20% FCS, 10 U/mL penicillin/streptomycin, 50ng/mL murine stem cell factor (mSCF), human IL6 (hIL6) and human thrombopoietin (hTPO) (all from Peprotech Inc., Rocky Hill, NJ) before retroviral infections.

Retroviral transduction of c-kit^{+} cells

The bicistronic retroviral vector pLZRS-eGFP, containing an internal ribosomal entry site followed by the gene encoding for eGFP, expressing myrAKT was kindly provided by Dr. Paul Coffer (Utrecht, the Netherlands). Retroviral supernatant was produced by transient transfection of 293T cell line using calcium phosphate co-precipitation with a total of 4µg of cDNA. Cells were plated in 3.5-cm dishes, and 2.5 mL IMDM medium was refreshed 18 hours after transfection. After an additional 24 and 48 hours, viral supernatants were collected, pooled, and filtered through a 0.45µm filter. Transduction was performed by spinoculation for 1,5 hours at 1,800g in 24-well plates in 0.5 mL of retroviral supernatant and 0.5 mL IMDM medium supplemented with 20% FCS, 50ng/mL mSCF, hIL6, hTPO, and 5µg/mL polybrene (American Bioanalytical, Natick, MA). When c-kit^{+} cells of FLT3-ITD mice were used, IL-6 was substituted by FL. Three days after infection, GFP^{+} cells were sorted on FACS Aria (BD Biosciences) and cultured in IMDM supplemented with 20% FCS in presence or absence of cytokines as described in the experiments.

Cell cycle analysis

Cell cycle was assessed by Ki67/propidium iodide (PI) staining and analyzed by flow cytometry. Cultured cells were collected and fixed with Cytofix/Cytoperm (BD Biosciences) for 30 minutes at 4˚C, followed by washing twice with Perm/Wash (BD Biosciences) buffer. After washing, the cells were stained with PE conjugated anti-Ki67 antibody (BD Biosciences) and DAPI (0.5μg/mL). Cell-cycle status was determined by simultaneous two-parameter analysis using DAPI versus Ki-67 on FACS Canto (BD Biosciences), and the amount of cells in G_0/G_1, S-phase, and G_2/M was calculated.

Apoptosis assays
For apoptosis measurements, cells were collected at the indicated time points, washed with cold PBS+5% FCS twice, re-suspended in Annexin V binding buffer, and incubated with Annexin V-APC and 7-aminoactinomycin (both from BD Biosciences) at room temperature for 15 minutes. Cells were analyzed on FACS Canto within an hour. Acquired data was analyzed using FlowJo software Version 7.6 for Macintosh.

Transplantations
Before transplantation, eight- to ten-week-old mice were lethally irradiated with 9Gy. For intravenous transplantation group, recipient mice received 10,000 sorted GFP^{+} cells via tail vein injections in a volume of 200 μl. For intra-femoral transplantations, after subcutaneous injection with Temgensic, a hole was drilled of the right femur by a 30G needle (BD Biosciences), after which 5,000 GFP^{+} sorted cells were injected directly in the BM cavity by a Hamilton syringe with a 31G needle (Hamilton Bonaduz AG, Bonaduz, GR, Switzerland) in a volume of 10 μl. After transplantation, mice were maintained under sterile conditions in microisolater cages and provided with autoclaved food and water containing 111mg/L ciprofloxacin (Ciproxin). Peripheral blood was collected by lateral tail vein bleeding and analyzed 2, 4, 8 and 12 weeks following transplantation by tracking GFP fluorescence expression on FACS Canto. Erythrocytes were lysed with ammonium chloride (Stem Cell Technologies), and the leukocytes suspended in PBS with 5% FCS. All data were analyzed using FlowJo software Version 7.6 for Macintosh.

Flow cytometry analysis of ROS by DCFDA staining
To measure intracellular levels of ROS, cells were stained with 10 μM 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy-H_2DCFDA) (Molecular Probes) for 30 minutes at 37°C and then washed with PBS. Dead cells were detected with 7AAD (0.5μg/mL). DCF fluorescence was detected by flow cytometry in the FL1 channel.

Statistical analysis
Statistical testing was performed using InStat software (Graphpad Software, San Diego, CA, USA). The experiments performed with sorted GFP^{+} cells were done at least in triplicate. Data were expressed as mean values and s.d. Statistical significance of differences observed between groups were determined as using Student's t-test. *, ** and *** means $p<0.05$, $p<0.001$ and $p<0.001$ respectively and 'ns' indicates non-significant P values.

RESULTS

Constitutively active AKT promotes only short-term survival and is incompatible with STAT5-driven proliferation and triggers apoptosis

This study was initiated to more exactly identify the role of activated AKT in FLT3-ITD signaling by stepwise reconstructing individual events. Since STAT5 is activated by FLT3-ITD receptor signaling and not wildtype FLT3, we generated STAT5-expressing cells in the hematopoietic progenitor cell lines Ba/F3 and FDC-P1. A constitutively active form of STAT5 (ca-STAT5) was retrovirally introduced to Ba/F3 and the effects of proliferation was studied. STAT5 alone was able to sustain proliferation upon cytokine deprivation as cells increased in numbers during the five days analyzed (Figure 1a). In contrast, the consequence of simultaneously expressing STAT5 and AKT in Ba/F3 cells, using constitutively active form of AKT, i.e., myristylated AKT (myrAKT) (16) (Figure 1b), led to a decline in numbers of viable cells by trypan blue exclusion (Figure 1a). Since AKT-expressing Ba/F3 were unable to survive cytokine deprivation (Figure 1a), we also tested overexpression of myrAKT in FDC-P1 cells. After 4 days in culture with IL-3, the numbers of cells expressing myrAKT had increased to 30% more in comparison with control cells. However, after cytokine deprivation cell numbers gradually decreased (Figure 1c), and the decline in cell numbers was similar between control FDC-P1 cells and cells containing myrAKT.

Finally, we tested if functional AKT is necessary for survival in the progenitors by introducing a dominant negative form of AKT, K179M, in which the ATP-binding site is mutated and functions as a kinase dead protein (22). The level of apoptosis was increased in FDC-P1 cells expressing K179M in the presence of SCF (23%) compared to cells expressing wildtype (wt) AKT (9%) (Figure 1d), indicating that functional AKT is crucial for survival of cytokine signaling.

Expression of activated AKT induces cell cycle exit and apoptosis of FLT3-ITD expressing progenitor cells of bone marrow from transgenic mice

We next decided to overexpress myristylated AKT in progenitor cells of FLT3-ITD transgenic mice. Bone marrow cells enriched for c-kit by magnetic positive selection were infected with control GFP virus or virus expressing both GFP and myrAKT in the presence of cytokines known to support survival and growth of hematopoietic stem cells and progenitors (i.e., SCF+FL or SCF+FL+Tpo). Two days after infection, GFP^{+} cells were FACS-sorted and re-cultured in media with or without cytokines. As indicated in Figure 2, cell cycle analysis

(after 48 hours) and annexin V-staining for apoptosis (after 72 hours) were performed by flow cytometry. In the absence of cytokine, the proportion of myrAKT/GFP$^+$ progenitor cells of FLT3-ITD mice in G_0 of the cell cycle increased significantly after 2 days in comparison to wildtype cells (Figure 2a). Surprisingly, this was also seen when cells were cultured with cytokines. Thus, the numbers of c-kit$^+$ progenitors in G_0 after stimulation with either SCF and FL or SCF, FL, and Tpo were significantly higher from FLT3-ITD mice coexpressing myrAKT compared to wildtype mice, but this was also evident for control progenitors expressing myrAKT. At the same time although with some delay, the numbers of apoptotic cells increased in cultures of FLT3-ITD expressing cells (Figure 2b), indicating that hyperactivated AKT does not synergize with FLT3-ITD but is detrimental to the cells.

Activated Akt is incompatible with survival of anti-apoptotic Bcl-2

Since anti-apoptotic Bcl-2 family members are transcriptionally activated by FLT3-ITD signaling, in particular Bcl-X$_L$ and Mcl-1 (23-25), we decided to analyze the consequence of overexpressing myrAKT in progenitor cells of Bcl-2 transgenic mice. Bone marrow cells enriched for c-kit expression were infected with control virus or virus containing myrAKT, and after sorting GFP$^+$ cells we analyzed cell cycle and apoptosis during 3 days. The results confirmed the apoptotic-inducing effects of myrAKT (Figure 3b), although we could not detect any significant difference in cell cycle distribution compared to control-infected progenitors (Figure 3a). However, surprisingly Bcl-2 expression was not able to inhibit this effect (Figure 3b), and after 24 hours c-kit$^+$ progenitor cells from Bcl-2 mice coexpressing myrAKT accumulated in G_0 of cell cycle (Figure 3a), and become apoptotic after 72 hours (Figure 3b).

Both long-term and short-term hematopoietic engraftment to recipient mice is impaired by hyperactivated AKT

The inability of myristylated AKT to sustain survival and instead forcing cells into cell cycle block and cell death is evident from the experiments presented above, however we wanted to corroborate this in transplantable hematopoietic stem cells *in vivo*. Bone marrow-derived progenitor cells enriched for c-kit expression of either wildtype mice or mice transgenic for Bcl-2 were infected with control virus or myrAKT-virus. Forty-eight hours post-infection, 10,000 GFP$^+$ FACS-sorted cells were injected to lethally irradiated mice by intravenous tail injections in a non-competitive setting. Peripheral blood was collected by lateral tail vein

bleeding and analyzed 4, 8, and 12 weeks post-transplant for GFP expression. Already after 4 weeks, reconstitution was negatively affected in mice receiving myrAKT-expressing bone marrow cells. The level of reconstitution at 4 weeks was 17.1% for B6-myrAKT, 54.9% for B6-GFP, 25.2% for Bcl2-myrAKT, and 67.2% for Bcl2-GFP (Figure 4a). At longer time points, chosen to represent transplantable long-term HSCs (8 and 12 weeks), reconstitution level remained high in both GFP control and Bcl-2 mice but was severly impaired in mice receiving myrAKT-expressing cells. Engraftment was almost absent in the mice transplanted with myrAKT cells (Figure 4a) and several mice succumb to hematopoietic failure during the first 8 weeks post-transplant (Figure 4b). In this experiment, presence of Bcl-2 expression did not make a difference, again demonstrating the ability of hyperactivated AKT to overcome survival-mediating effects elicited by other pathways.

Impaired engraftment of bone marrow cells expressing hyperactivated AKT does not correlate to disturbed homing in recipient mice

In the experiments above, bone marrow cells expressing myrAKT was unable to reconstitute recipient mice even with cells expressing Bcl-2. However, we could not distinguish whether this was due to apoptotic effects or the ability of AKT to affect homing and/or adhesion of the progenitor cells to the bone marrow. Since previous studies have demonstrated that AKT influences the expression of adhesion molecules and homing receptors (20), we decided to repeat the transplantation experiments and in parallel to intravenous injections also directly inject cells to the bone marrow by intrafemoral injections. However, the engraftment ability was still impaired after 2 weeks post-transplant and myrAKT-overexpressing cells injected directly in the marrow were unable to reconstitute the hematopoietic system (Figure 4c).

Antioxidant N-acetyl-lysine (NAC) inhibits hyperactivated Akt-mediated increased ROS production and apoptosis

Previous studies have shown that too much activation of AKT can lead to accumulated levels of intracellular reactive oxygen species (ROS) which can be detrimental to hematopoietic cells (26). To investigate whether this could be the cause of the pro-apoptotic effects mediated by activated AKT in cells expressing FLT3-ITD, STAT5, or Bcl-2 as shown herein, we analysed ROS levels in cells overexpressing myrAKT. To enable detection by DCF-DA staining, we used a puromycin-containing myrAKT virus in these experiments. In hematopoietic cells infected with myrAKT virus, the levels of ROS increased significantly 48

hours post- (Figure 5a). Treatment of infected GFP[+] FACS-sorted cells with N-acetyl-lysine (NAC), a well-known antioxidant, prevented apoptosis partially (Figure 5b), suggesting that the detrimental effects by hyperactivated AKT is linked to accumulation of increased levels of ROS.

DISCUSSION

In this study, we have investigated the consequence of simultaneously expressing constitutive active AKT and STAT5 to reconstruct FLT3-ITD signaling. As previously described, expression of myristylated AKT alone was insufficient in sustaining cell growth and survival of IL-3-dependent hematopoietic progenitor cell lines when cells were deprived of cytokine (26,27). However, hyperactivated AKT unexpectedly disabled STAT5-mediated proliferation of Ba/F3 by forcing the cells to exit the cell cycle followed by the onset of apoptosis. This result came as a surprise; multiple studies have demonstrated that FLT3-ITD mediated transformation is associated with activation of both AKT and STAT5 (28,29). While STAT5 appears as an indispensable downstream target in leukemia, as cells transformed by bcr-abl (30,31) or FLT3-ITD (13,24,32) is addicted to STAT5 for maintaining the leukemic state, the importance of AKT in hematological malignancies is not as clear. AKT is frequently activated in AML (2,33), and the majority of leukemic cells from AML patients have been shown to display elevated levels of phosphorylated AKT as evidence for its activated state (5,34). However, as mutations in AKT are absent in AML (35), the cause of its activation remains elusive although in some cases it is due to constitutive receptor activation (for example, FLT3-ITD) or mutations in *phosphatase and tensin* homolog (PTEN) (36). While the key residues involved in FLT3-ITD-mediated activation of STAT5 have been identified and involves tyrosine residues 589 and 591 (13,37), the mechanisms for AKT-mediated effects have been more difficult to decipher. It is therefore possible that myristylated AKT activates different downstream effector molecules than endogenous AKT activated via FLT3-ITD signaling.

Since the rationale of our study was to dissect individual contribution of signaling proteins downstream of FLT3-ITD, we also studied the consequence of overexpressing myrAKT in bone marrow-derived progenitors from FLT3-ITD transgenic mice. Although a cell cycle block was more evident when FLT3-ITD cells were cultured in the absence of cytokines, where most cells are likely to die by apoptosis, FLT3-ITD

progenitor cells cultured in stem cell factor, FLT3 ligand, and Tpo displayed significantly more cells in G_0 after 24 hours and were approximately 20% more apoptotic after 48 hours. This indicates that AKT-driven cell cycle exit and apoptosis is not inhibited by FLT3-ITD activity. Since AKT is a downstream target of FLT3-ITD we interpret the results as an incompatibility of FLT3-ITD and hyperactivated AKT, leading to overstimulation of the cells.

Recent studies have demonstrated that besides its major pro-survival role AKT is not a single-function kinase but may facilitate rather than inhibit cell death under certain conditions (38-40). This previously undetected function of AKT appears to be associated by its ability to increase reactive oxygen species (ROS) and to suppress the action of antioxidant enzymes (40,41). We could confirm that myrAKT-expressing hematopoietic progenitors did express higher levels of ROS. Interestingly, the harmful effect of myrAKT was counteracted by addition of NAC (N-acetyl-L-lysine), known to act as a scavenger of excess levels of ROS. This suggests that myrAKT-mediated apoptosis is indeed linked to increased generation of ROS. Recent studies have demonstrated that HSC function is impaired in double-deficient AKT1/AKT2. In these mice, HSCs were found to persist in the G_0 phase of the cell cycle and the intracellular content of ROS was decreased, implicating that AKT regulates ROS for proper HSC maintenance and differentiation (42). These results also imply that AKT hyperactivity may have the opposing effect, driving HSCs to excess cell cycle and harmful effects by an increase in ROS levels. Actually this has been observed in Ba/F3 cells as hyperactivated AKT led to short-lived survival upon growth factor-deprivation, only at later time points to induce cell death (26). Cells expressing hyperactivated AKT displayed an increase in FOXO3a activity as well as increased aerobic glycolysis and mitochondrial activity resulting in ROS overproduction.

Selective upregulation of the anti-apoptotic BCL-2 family member myeloid cell leukemia-1 (MCL-1) has been demonstrated in FLT3-ITD[+] cell lines and primary mononuclear cells from AML patients. This upregulation is dependent of FLT3-ITD since it is reversible upon pharmacological inhibition of FLT3 activity by tyrosine kinase inhibitors (23). Recently, it was observed that FLT3-ITD-dependent upregulation of MCL-1 is connected to STAT5 (24). Considering our results that myrAKT interferred with both STAT5- and FLT3-ITD-driven proliferation, an obvious experiment to perform was to express myrAKT simultaneously with an anti-apoptotic BCL-2 family member protein. We did this by expressing myrAKT in hematopoietic progenitor cells from Bcl-2 transgenic mice. Surprisingly, the negative effect of hyperactivated AKT on cell viability was corroborated. In

contrast to FLT3-ITD cells, however, Bcl-2 expressing progenitor cells from transgenic mice when coexpressing myrAKT did not display any significant survival difference *in vitro* compared to wildtype cells. However, Bcl-2 and myrAKT did lead to more cells accumulating in G_0.

Since coexpression of AKT and anti-apoptotic BCL-X_L protein has been reported to lead to a leukemic phenotype in mice (14), we tested the *in vivo* effects by transplanting infected bone marrow-derived progenitor cells with myrAKT to irradiated recipients. Both short-term (4 and 8 weeks posttransplant) and long-term (12 weeks posttransplant) HSCs were severely impaired and the reconstitution levels very low. Some animals even succumbed to hematopoietic failure. This was also seen with progenitor cells from wildtype mice transplantated with myrAKT. Thus, myrAKT appears to be detrimental for both HSCs and progenitors by inducing apoptosis. An alternative explanation to the findings that HSCs expressing myrAKT reconstitute poorly has been suggested in a recent report in which AKT regulated proper trafficking and homing of hematopoietic progenitors (20). By overexpressing myrAKT in human $CD34^+$ progenitor cells, and transplanting them to nonobese diabetic/severe combined immunodeficient (NOD/scid) mice, constitutively active AKT resulted in reduced migration to the bone marrow. However, this can be excluded as a plausible explanation in our study since intrafemoral injections of myrAKT/Bcl-2 expressing bone marrow cells reconstituted as poorly as intravenous distribution.

The inability of Bcl-2 to inhibit myrAKT-mediated cell death-inducing effects is surprising when one bears in mind previous findings that Bcl-2 is involved as an anti-oxidant agent to overcome ROS effects (43). Furthermore, Bcl-2 functions in tilting the balance between pro-apoptotic and anti-apoptotic in favour of the latter (44). The myristylated version of AKT utilized herein has been used in many other studies to mimic AKT activation upon ligand-engaged receptor activation and been used to activate AKT in tumor models. In some studies where it showed harmful effects, myrAKT was expressed as a fusion protein to the estrogen receptor (ER). In this case, AKT is inactive until tamoxifen treatment where the fusion protein is translocated to the nucleus (26). In contrast, our version is primarily located in the cytoplasm and at the cell membrane (data not shown). This raises the question to whether myrAKT executes different functions when nuclear localized versus targeted to the cell membrane, and whether Bcl-2 can counteract only normal action of AKT. It remains to be demonstrated, however, whether nuclear AKT is a pre-requisite for its apoptotic function.

In summary, it appears that any defective activity such as AKT deletion or AKT hyperactivation will ultimately lead to improper balance of ROS which could be harmful to the cells. A key rationale to novel therapeutic ways to eradicate leukemic cells in patients is to identify the critical signaling components downstream of oncogenic receptors and associated signaling molecules. Inhibition of genes participating in such networks may turn out to be important targets for new ways of intervention. Since activating mutations of FLT3-ITD and other signaling proteins in leukemia lead to activation of the PI3K/AKT pathway, the results herein that hyperactivated AKT can induce a block in cell cycle and under some circumstances leads to apoptosis of FLT3-ITD as well as Bcl-2 expressing cells are important findings that could lead to future drug development.

CONTRIBUTIONS

J.I.J: Conception and design, collection and assembly of data, data analysis, and drafted the manuscript; YJ.T: collection and assembly of data, data analysis, and drafted the manuscript; C.H., A.N., J.Å., M.K: collection and assembly of data, data analysis; W.M.W. performed intrafemoral injections. All authors have particpipated in evaluation of art work, and have participated in the final writing of the manuscript.

FIGURE LEGENDS

Figure 1. *Survival kinase AKT does not relieve cytokine-dependence of hematopoietic progenitor cells and is incompatible with STAT5-driven proliferation and triggers apoptosis.* (**a**) Ba/F3 cells were infected with retrovirus containing caSTAT5-puro and/or myrAKT-GFP (results from control virus not shown). After selection in puromycin (1.5 µg/ml) and/or FACS-sorting for GFP expression, cells were cytokine deprived and plated at 50.000 per well in triplicates on 96-well plates. After 2 and 5 days, respectively, the number of viable cells were determined by trypan blue exclusion. Data represent the average number of cells/ml and S.D. from three independent experiments. (**b**) Cell lysates were prepared from uninfected FDC-P1 cells or from FDC-P1 cells infected with retrovirus containing either wildtype AKT or myristylated AKT (myrAKT) stimulated for 0 or 15 minutes with SCF (100 ng/mL) and immunoblotted for pAKT (Ser ^{473}) and total AKT. The experiment was repeated once with similar results. (**c**) Unifected FDC-P1 cells or cells infected with myrAKT (50.000/well) was grown in triplicates on 96-well plates in IL-3 (1 ng/mL) for 4 days, after which the cells were washed and plated in cytokine-deprived medium. The number of viable cells was counted by trypan blue exclusion after 4, 6, 8, 10, and 12 days. Data represent average cell number from triplicates and S.D. from two independent experiments. (**d**) FDC-P1 cells were transfected with pMACS K^{k}II-plasmid containing wildtype (wt) AKTor K179M AKT. Apoptosis of H2-K^k positive cells were analyzed by flow cytometry using FITC-conjugated α-H-2K^k and Annexin V-biotin/streptavidin-PE. Percent indicates number of annexin V-positive cells. Data are from one of three independent experiments.

Figure 2. *Expression of myristylated AKT induces cell cycle exit and apoptosis in hematopoietic progenitor cells of bone marrow from FLT3-ITD transgenic mice.* c-kit$^+$ progenitor cells, isolated from bone marrow of C57BL6/J control or FLT3-ITD mice, were infected by spinoculation with supernatants containing either pLZRS-EGFP or pLZRS-myrAKT-EGFP retrovirus. Forty-eight hours later, GFP$^+$ cells were FACS-sorted and cultured in 96-well plates in duplicates without cytokines or with SCF+FL or SCF+Tpo+FL (50 ng/ml each). (**a**) After an additional 48 hours, the cell-cycle status was determined by two-parameter using PE-conjugated anti-Ki67 antibody and DAPI (0.5µg/ml), and the amount of cells in G_0/G_1, S-phase, and G_2/M was calculated. (**b**) The percent of apoptotic cells were determined after 72 hours after cell sorting using APC-conjugated Annexin V and 7-aminoactinomycin (7AAD). Data are from two independent experiments.

Figure 3. *Activated AKT induces cell cycle exit and increased apoptosis of bone marrow progenitors from Bcl-2 transgenic mice.* c-kit[+] bone marrow-progenitors from C57BL6 or Bcl-2 transgenic mice were infected by spinoculation with supernatants containing pLZRS-EGFP or pLZRS-myrAKT-EGFP retrovirus. Forty-eight hours later, GFP^{+} cells were FACS-sorted and cultured in 96-well plates in duplicates without cytokines (white bars) or with SCF+Tpo+ILK-6 (50 ng/mL each; black bars). (**a**) After 24 or 72 hours, the cell-cycle status was determined by two-parameter flow cytometry using PE-conjugated anti-Ki67 antibody and DAPI (0.5μg/ml), and the amount of cells in G_0/G_1, S-phase, and G_2/M was calculated. (**b**) The percent of apoptotic cells were determined after 72 hours after cell sorting using APC-conjugated Annexin V and 7-aminoactinomycin (7AAD). Data are mean value $\pm$ S.D. from three independent experiments.

Figure 4. *Short-term and long-term hematopoietic stem cells coexpressing activated AKT are impaired of engraftment potential even when expressing Bcl-2.* (**a**) Level of engraftment in recipient mice (n=5) transplanted with 1×10^4 c-kit[+] bone marrow cells of C57BL6 or Bcl-2 transgenic mice. Cells were infected for 2 days with control GFP or myrAKT-GFP virus in the presence of SCF, Tpo, and IL-6 (50 ng/mL), and then FACS-sorted for GFP expression before lateral tail injections. The level of reconstitution of donor cells in recipient mice was analysed for GFP expression after 4, 8, and 16 weeks by flow cytometry on peripheral blood (n.s. = not significant; ** and ***, $p<0.01$ and $p<0.001$, respectively). (**b**) Survival curves during 85 days post-transplant of mice (n=10) transplanted with c-kit[+] bone marrow cells of C57BL6 or Bcl-2 transgenic mice infected with control GFP och myrAKT-GFP virus. (**c**) Engraftment levels as measured two weeks post-transplant for GFP expression in peripheral blood of individual mice (n=5) transplanted with c-kit[+] bone marrow cells of C57BL6 or Bcl-2 transgenic mice infected with GFP or myrAKT/GFP virus injected either by intravenous tail injections (10,000 c-kit^{+} cells) or intrafemoral injections (5,000 c-kit^{+} cells (** and ***, $p<0.01$ and $p<0.001$, respectively).

Figure 5. *The levels of intracellular ROS increase by activated AKT, but antioxidant N-acetyl-L-cysteine (NAC) protects from activated AKT-induced apoptosis.* (**a**) c-kit[+] bone marrow progenitors cells of C57BL6 mice were infected with a puromycin-retrovirus containg myrAKT or the corresponding empty virus in the presence of SCF, Tpo, and IL-6

(50ng/mL each). After 3 days of puromycin selection (1.5 μg/mL), the level of ROS was measured by flow cytometry on carboxy-H$_2$DCFDA-stained (10 μM) cells. Results are presented as mean $\pm$ SEM for MFI (mean fluorescence intensity) of DCF-DA staining from two independent experiments. (**b**) c-kit[+] bone marrow progenitors cells of C57BL6 or Bcl-2 transgenic mice were infected for 2 days with GFP-retrovirus containg myrAKT or the corresponding empty virus in the presence of SCF, Tpo, and IL-6. After FACS-sorting for GFP^{+} cells, cells were re-cultured in the same cytokines with or without NAC (0.1, 1.0, and 2.0 nM) for 72 hours and the percent of apoptotic cells were determined using APC-conjugated Annexin V and 7-aminoactinomycin (7AAD). Data are mean value $\pm$ SEM from two independent experiments.

REFERENCES

1. Xu, Q., Simpson, S. E., Scialla, T. J., Bagg, A., and Carroll, M. (2003) Survival of acute myeloid leukemia cells requires PI3 kinase activation. *Blood* **102**, 972-980

2. Grandage, V. L., Gale, R. E., Linch, D. C., and Khwaja, A. (2005) PI3-kinase/Akt is constitutively active in primary acute myeloid leukaemia cells and regulates survival and chemoresistance via NF-kappaB, Mapkinase and p53 pathways. *Leukemia* **19**, 586-594

3. Park, S., Chapuis, N., Tamburini, J., Bardet, V., Cornillet-Lefebvre, P., Willems, L., Green, A., Mayeux, P., Lacombe, C., and Bouscary, D. (2010) Role of the PI3K/AKT and mTOR signaling pathways in acute myeloid leukemia. *Haematologica* **95**, 819-828

4. Min, Y. H., Cheong, J. W., Kim, J. Y., Eom, J. I., Lee, S. T., Hahn, J. S., Ko, Y. W., and Lee, M. H. (2004) Cytoplasmic mislocalization of p27Kip1 protein is associated with constitutive phosphorylation of Akt or protein kinase B and poor prognosis in acute myelogenous leukemia. *Cancer Res* **64**, 5225-5231

5. Tazzari, P. L., Cappellini, A., Grafone, T., Mantovani, I., Ricci, F., Billi, A. M., Ottaviani, E., Conte, R., Martinelli, G., and Martelli, A. M. (2004) Detection of serine 473 phosphorylated Akt in acute myeloid leukaemia blasts by flow cytometry. *Br J Haematol* **126**, 675-681

6. Hummerdal, P., Andersson, P., Willander, K., Linderholm, M., Soderkvist, P., and Jonsson, J. I. (2006) Absence of hot spot mutations of the PIK3CA gene in acute myeloid leukaemia. *Eur J Haematol* **77**, 86-87

7. Khwaja, A. (2010) PI3K as a target for therapy in haematological malignancies. *Curr Top Microbiol Immunol* **347**, 169-188

8. Small, D. (2006) FLT3 mutations: biology and treatment. *Hematology Am Soc Hematol Educ Program*, 178-184

9. Kayser, S., Schlenk, R. F., Londono, M. C., Breitenbuecher, F., Wittke, K., Du, J., Groner, S., Spath, D., Krauter, J., Ganser, A., Dohner, H., Fischer, T., and Dohner, K. (2009) Insertion of FLT3 internal tandem duplication in the tyrosine kinase domain-1 is associated with resistance to chemotherapy and inferior outcome. *Blood* **114**, 2386-2392

10. Brandts, C. H., Sargin, B., Rode, M., Biermann, C., Lindtner, B., Schwable, J., Buerger, H., Muller-Tidow, C., Choudhary, C., McMahon, M., Berdel, W. E., and Serve, H. (2005) Constitutive activation of Akt by Flt3 internal tandem duplications is necessary for increased survival, proliferation, and myeloid transformation. *Cancer Res* **65**, 9643-9650

11. Jönsson, M., Engstrom, M., and Jönsson, J. I. (2004) FLT3 ligand regulates apoptosis through AKT-dependent inactivation of transcription factor FoxO3. *Biochem Biophys Res Commun* **318**, 899-903

12. Choudhary, C., Brandts, C., Schwable, J., Tickenbrock, L., Sargin, B., Ueker, A., Bohmer, F. D., Berdel, W. E., Muller-Tidow, C., and Serve, H. (2007) Activation mechanisms of STAT5 by oncogenic Flt3-ITD. *Blood* **110**, 370-374

13. Rocnik, J. L., Okabe, R., Yu, J. C., Lee, B. H., Giese, N., Schenkein, D. P., and Gilliland, D. G. (2006) Roles of tyrosine 589 and 591 in STAT5 activation and transformation mediated by FLT3-ITD. *Blood* **108**, 1339-1345

14. Karnauskas, R., Niu, Q., Talapatra, S., Plas, D. R., Greene, M. E., Crispino, J. D., and Rudin, C. M. (2003) Bcl-x(L) and Akt cooperate to promote leukemogenesis in vivo. *Oncogene* **22**, 688-698

15. Kharas, M. G., Okabe, R., Ganis, J. J., Gozo, M., Khandan, T., Paktinat, M., Gilliland, D. G., and Gritsman, K. (2010) Constitutively active AKT depletes hematopoietic stem cells and induces leukemia in mice. *Blood* **115**, 1406-1415

16. Kohn, A. D., Takeuchi, F., and Roth, R. A. (1996) Akt, a pleckstrin homology domain containing kinase, is activated primarily by phosphorylation. *J Biol Chem* **271**, 21920-21926

17. Ogilvy, S., Metcalf, D., Print, C. G., Bath, M. L., Harris, A. W., and Adams, J. M. (1999) Constitutive Bcl-2 expression throughout the hematopoietic compartment affects multiple lineages and enhances progenitor cell survival. *Proc Natl Acad Sci U S A* **96**, 14943-14948

18. Lee, B. H., Tothova, Z., Levine, R. L., Anderson, K., Buza-Vidas, N., Cullen, D. E., McDowell, E. P., Adelsperger, J., Frohling, S., Huntly, B. J., Beran, M., Jacobsen, S. E., and

Gilliland, D. G. (2007) FLT3 mutations confer enhanced proliferation and survival properties to multipotent progenitors in a murine model of chronic myelomonocytic leukemia. *Cancer Cell* **12**, 367-380

19. Royer, Y., Menu, C., Liu, X., and Constantinescu, S. N. (2004) High-throughput gateway bicistronic retroviral vectors for stable expression in mammalian cells: exploring the biologic effects of STAT5 overexpression. *DNA Cell Biol* **23**, 355-365

20. Buitenhuis, M., van der Linden, E., Ulfman, L. H., Hofhuis, F. M., Bierings, M. B., and Coffer, P. J. (2010) Protein kinase B (PKB/c-akt) regulates homing of hematopoietic progenitors through modulation of their adhesive and migratory properties. *Blood* **116**, 2373-2384

21. Ahsberg, J., Tsapogas, P., Qian, H., Zetterblad, J., Zandi, S., Mansson, R., Jonsson, J. I., and Sigvardsson, M. (2010) Interleukin-7-induced Stat-5 acts in synergy with Flt-3 signaling to stimulate expansion of hematopoietic progenitor cells. *J Biol Chem* **285**, 36275-36284

22. Bellacosa, A., Chan, T. O., Ahmed, N. N., Datta, K., Malstrom, S., Stokoe, D., McCormick, F., Feng, J., and Tsichlis, P. (1998) Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene* **17**, 313-325

23. Kasper, S., Breitenbuecher, F., Heidel, F., Hoffarth, S., Markova, B., Schuler, M., and Fischer, T. (2012) Targeting MCL-1 sensitizes FLT3-ITD-positive leukemias to cytotoxic therapies. *Blood Cancer J* **2**, e60

24. Yoshimoto, G., Miyamoto, T., Jabbarzadeh-Tabrizi, S., Iino, T., Rocnik, J. L., Kikushige, Y., Mori, Y., Shima, T., Iwasaki, H., Takenaka, K., Nagafuji, K., Mizuno, S., Niiro, H., Gilliland, G. D., and Akashi, K. (2009) FLT3-ITD up-regulates MCL-1 to promote survival of stem cells in acute myeloid leukemia via FLT3-ITD-specific STAT5 activation. *Blood* **114**, 5034-5043

25. Bagrintseva, K., Geisenhof, S., Kern, R., Eichenlaub, S., Reindl, C., Ellwart, J. W., Hiddemann, W., and Spiekermann, K. (2005) FLT3-ITD-TKD dual mutants associated with AML confer resistance to FLT3 PTK inhibitors and cytotoxic agents by overexpression of Bcl-x(L). *Blood* **105**, 3679-3685

26. van Gorp, A. G., Pomeranz, K. M., Birkenkamp, K. U., Hui, R. C., Lam, E. W., and Coffer, P. J. (2006) Chronic protein kinase B (PKB/c-akt) activation leads to apoptosis induced by oxidative stress-mediated Foxo3a transcriptional up-regulation. *Cancer Res* **66**, 10760-10769

27. Shelton, J. G., Blalock, W. L., White, E. R., Steelman, L. S., and McCubrey, J. A. (2004) Ability of the activated PI3K/Akt oncoproteins to synergize with MEK1 and induce cell cycle progression and abrogate the cytokine-dependence of hematopoietic cells. *Cell Cycle* **3**, 503-512

28. Fernandes, M. S., Reddy, M. M., Croteau, N. J., Walz, C., Weisbach, H., Podar, K., Band, H., Carroll, M., Reiter, A., Larson, R. A., Salgia, R., Griffin, J. D., and Sattler, M. (2010) Novel oncogenic mutations of CBL in human acute myeloid leukemia that activate growth and survival pathways depend on increased metabolism. *J Biol Chem* **285**, 32596-32605

29. Masson, K., and Ronnstrand, L. (2009) Oncogenic signaling from the hematopoietic growth factor receptors c-Kit and Flt3. *Cell Signal* **21**, 1717-1726

30. Hoelbl, A., Schuster, C., Kovacic, B., Zhu, B., Wickre, M., Hoelzl, M. A., Fajmann, S., Grebien, F., Warsch, W., Stengl, G., Hennighausen, L., Poli, V., Beug, H., Moriggl, R., and Sexl, V. (2010) Stat5 is indispensable for the maintenance of bcr/abl-positive leukaemia. *EMBO Mol Med* **2**, 98-110

31. Warsch, W., Kollmann, K., Eckelhart, E., Fajmann, S., Cerny-Reiterer, S., Holbl, A., Gleixner, K. V., Dworzak, M., Mayerhofer, M., Hoermann, G., Herrmann, H., Sillaber, C., Egger, G., Valent, P., Moriggl, R., and Sexl, V. (2011) High STAT5 levels mediate imatinib resistance and indicate disease progression in chronic myeloid leukemia. *Blood* **117**, 3409-3420

32. Kiyoi, H., Shiotsu, Y., Ozeki, K., Yamaji, S., Kosugi, H., Umehara, H., Shimizu, M., Arai, H., Ishii, K., Akinaga, S., and Naoe, T. (2007) A novel FLT3 inhibitor FI-700 selectively suppresses the growth of leukemia cells with FLT3 mutations. *Clin Cancer Res* **13**, 4575-4582

33. Sujobert, P., Bardet, V., Cornillet-Lefebvre, P., Hayflick, J. S., Prie, N., Verdier, F., Vanhaesebroeck, B., Muller, O., Pesce, F., Ifrah, N., Hunault-Berger, M., Berthou, C., Villemagne, B., Jourdan, E., Audhuy, B., Solary, E., Witz, B., Harousseau, J. L., Himberlin, C., Lamy, T., Lioure, B., Cahn, J. Y., Dreyfus, F., Mayeux, P., Lacombe, C., and Bouscary, D. (2005) Essential role for the p110delta isoform in phosphoinositide 3-kinase activation and cell proliferation in acute myeloid leukemia. *Blood* **106**, 1063-1066

34. Gallay, N., Dos Santos, C., Cuzin, L., Bousquet, M., Simmonet Gouy, V., Chaussade, C., Attal, M., Payrastre, B., Demur, C., and Recher, C. (2009) The level of AKT phosphorylation on threonine 308 but not on serine 473 is associated with high-risk cytogenetics and predicts poor overall survival in acute myeloid leukaemia. *Leukemia* **23**, 1029-1038

35. Tibes, R., Kornblau, S. M., Qiu, Y., Mousses, S. M., Robbins, C., Moses, T., and Carpten, J. D. (2008) PI3K/AKT pathway activation in acute myeloid leukaemias is not associated with AKT1 pleckstrin homology domain mutation. *Br J Haematol* **140**, 344-347

36. Cheong, J. W., Eom, J. I., Maeng, H. Y., Lee, S. T., Hahn, J. S., Ko, Y. W., and Min, Y. H. (2003) Phosphatase and tensin homologue phosphorylation in the C-terminal regulatory domain is frequently observed in acute myeloid leukaemia and associated with poor clinical outcome. *Br J Haematol* **122**, 454-456

37. Vempati, S., Reindl, C., Wolf, U., Kern, R., Petropoulos, K., Naidu, V. M., Buske, C., Hiddemann, W., Kohl, T. M., and Spiekermann, K. (2008) Transformation by oncogenic mutants and ligand-dependent activation of FLT3 wild-type requires the tyrosine residues 589 and 591. *Clin Cancer Res* **14**, 4437-4445

38. Benbrook, D. M., and Masamha, C. P. (2011) The pro-survival function of Akt kinase can be overridden or altered to contribute to induction of apoptosis. *Curr Cancer Drug Targets* **11**, 586-599

39. Los, M., Maddika, S., Erb, B., and Schulze-Osthoff, K. (2009) Switching Akt: from survival signaling to deadly response. *Bioessays* **31**, 492-495

40. Nogueira, V., Park, Y., Chen, C. C., Xu, P. Z., Chen, M. L., Tonic, I., Unterman, T., and Hay, N. (2008) Akt determines replicative senescence and oxidative or oncogenic premature senescence and sensitizes cells to oxidative apoptosis. *Cancer Cell* **14**, 458-470

41. Esposito, F., Chirico, G., Montesano Gesualdi, N., Posadas, I., Ammendola, R., Russo, T., Cirino, G., and Cimino, F. (2003) Protein kinase B activation by reactive oxygen species is independent of tyrosine kinase receptor phosphorylation and requires SRC activity. *J Biol Chem* **278**, 20828-20834

42. Juntilla, M. M., Patil, V. D., Calamito, M., Joshi, R. P., Birnbaum, M. J., and Koretzky, G. A. (2010) AKT1 and AKT2 maintain hematopoietic stem cell function by regulating reactive oxygen species. *Blood* **115**, 4030-4038

43. Rassool, F. V., Gaymes, T. J., Omidvar, N., Brady, N., Beurlet, S., Pla, M., Reboul, M., Lea, N., Chomienne, C., Thomas, N. S., Mufti, G. J., and Padua, R. A. (2007) Reactive oxygen species, DNA damage, and error-prone repair: a model for genomic instability with progression in myeloid leukemia? *Cancer Res* **67**, 8762-8771

44. Happo, L., Strasser, A., and Cory, S. (2012) BH3-only proteins in apoptosis at a glance. *J Cell Sci* **125**, 1081-1087
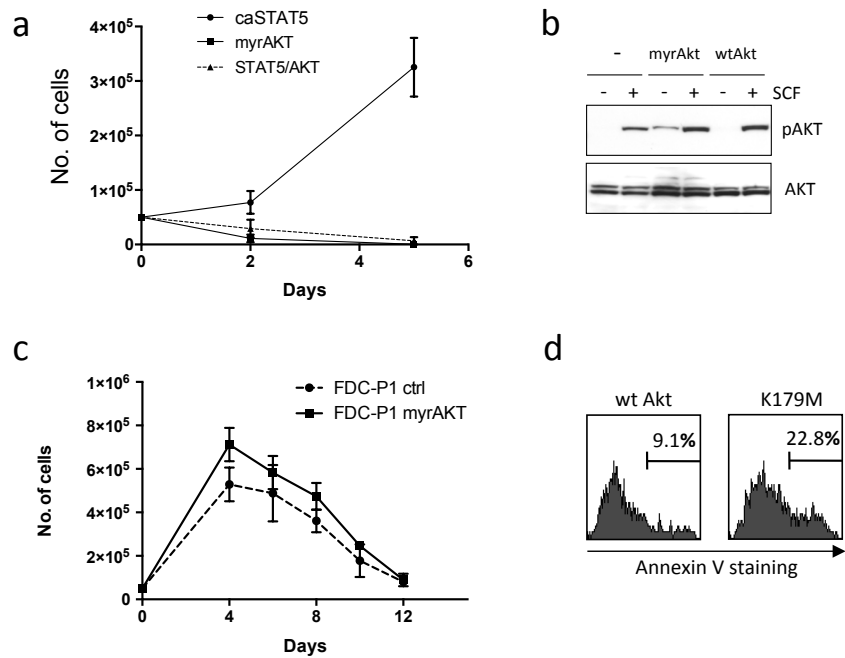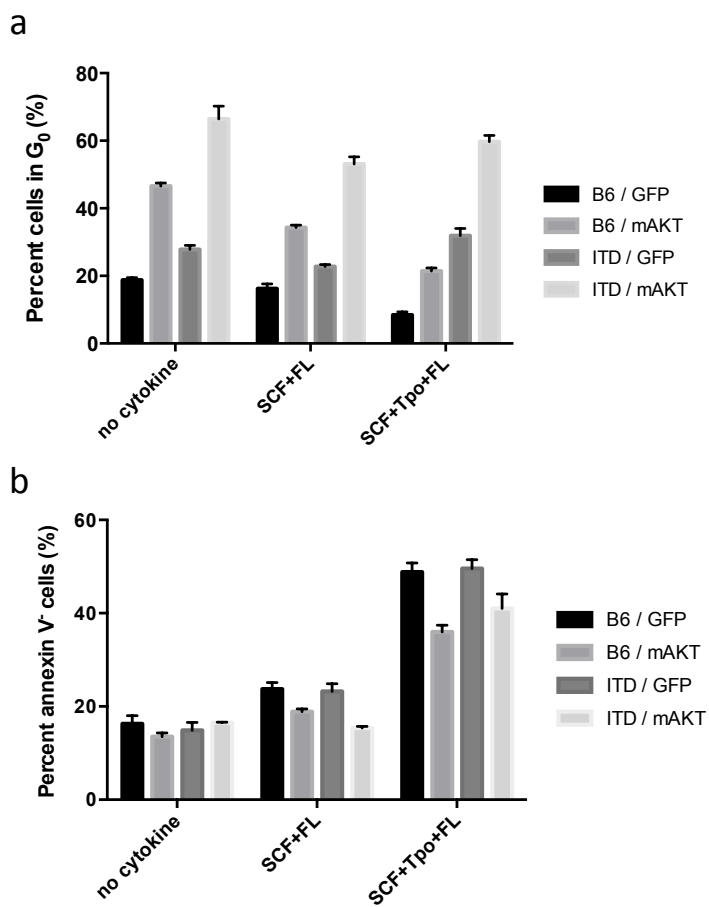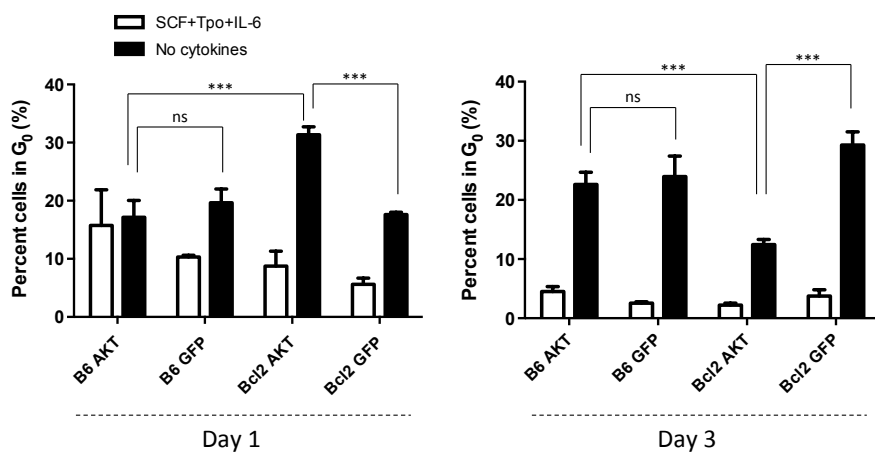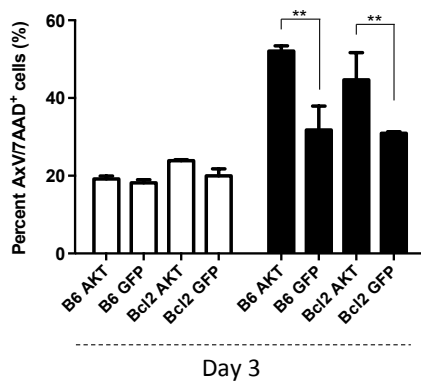
a

b

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Figure 1

a

b

Figure 2

Figure 3

Figure 4

a

b

Figure 5