Requirement of the Transcription Factor and Onco-protein *Gfi1* for the Development and Function of Hematopoietic Stem Cells and Progenitor Cells

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Abbreviations

4-HC	4-hydroperoxy-cyclophosphamide
5-FU	5-fluorouracil
AGM	aorta-gonad-mesonephros
APC	allophycocyanin
BFU-E	burst-forming unit-erythroid
BrdU	bromodeoxyuridine
BM	bone marrow
BMP	bone morphogenetic protein
CAFC	cobblestone area-forming cell
CDK	cyclin dependent kinase
CFC	colony forming cell
CFU	colony forming unit
CFU-S	spleen colony-forming unit
CFU-S_8	day 8 CFU-S
CFU-S_{12}	day 12 CFU-S
CMPs	common myeloid progenitor
CSF	colony-stimulating factor
FACS	fluorescence-activated cell sorting
FITC	fluorescein isothiocyanate
FL	flt3 ligand
flt3	fms-like tyrosine kinase-3
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte-macrophage colony-stimulating factor
Gfi1	growth factor independence 1
GFP	green fluorescent protein
GMP	granulocyte/monocyte-restricted progenitor
HSC	hematopoietic stem cells
Id	inhibitor of DNA binding
KL	c-kit ligand
LKLF	lung Krüppel-like factor
IL	interleukin
LSK	$lin^-c\text{-}kit^+Sca\text{-}1^+$
LTC-IC	long-term culture-initiating cell
LT-HSC	long-term HSC
MEP	megakarytic/erythroid progenitor
MHC	major histocompatibility complex
MKP	monopotent megakaryocyte-committed progenitor
MPP	multipotent progenitor
NK	natural killer
PcG	Polycomb group
PE	phycoerythrin
PIAS	protein inhibitor of activated STAT
Rb	retinoblastoma
SCF	stem cell factor
SDF	stromal cell-derived factor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel

Shh	sonic hedgehog
STAT	signal transducers and activators of transcription
ST-HSC	short-term-HSC
TCR	T cell receptor
TPO	thrombopoietin
VLA	very-late antigen
WT	wild-type
YS	yolk sac

1. Introduction

1.1 Hematopoiesis

The healthy human individual daily produces an enormous number of differentiated blood cells to replace cells lost due to normal turnover as well as to illness or trauma. A variety of homeostatic mechanisms allow blood cells to respond quickly to bleeding, infection or other stress situations and to return to normal levels when the stress is resolved. This orchestrated, highly dynamic and developmental process of blood production and homeostasis is termed hematopoiesis.

The hematopoietic system is derived from the mesodermal germ layer early in embryogenesis (Muller et al., 1994). The hemangioblast, a common progenitor, gives rise to vascular endothelium and hematopoietic cells (Choi et al., 1998). The development of blood cells occurs in two waves during mouse embryogenesis. The first and less well-characterized wave, called primitive hematopoiesis, takes place in the visceral yolk sac (YS) on the seventh day of gestation (Moore et al., 1970). A second wave, termed definitive hematopoiesis, occurs in the aorta-gonad-mesonephros (AGM) region in intraembryonic areas, then shifts to fetal liver which is the main source of hematopoietic cells in fetal life, and finally resides in bone marrow (BM) around birth time (Johnson and Moore, 1975; Muller et al., 1994; Dzierzak and Medvinsky, 1995; Medvinsky and Dzierzak, 1996; de Bruijn MF, et al, 2000). A recent study reported the generation of definitive hematopoietic stem cells (HSCs) from both YS and AGM region (Matsuoka et al., 2001b). Once established, the hematopoietic system contributes to lifelong hematopoiesis in a highly regulated manner.

1.2 Hematopoietic cells

The hematopoietic system can be envisioned as a series of functional compartments: stem cells, progenitor cells, precursor cells and mature cells (Quesenberry et al., 2001). As the half-life of mature hematopoietic cells varies from several hours to years, a continuous

production of end-stage cells from HSCs is required throughout the life span of the organism. In general, models of stem cell regulation are hierarchical (Quesenberry et al., 2001) in that a primitive multipotential stem cell gives rise to a proliferating progenitor pool, which in turn produces to recognizable differentiated cells. During the transition from stem cells to differentiated cells, the proliferative potential is lost, while specific differentiated features are acquired. Terminally differentiated cells are incapable of further development. Thus, mature blood cell formation occurs as a result of a series of maturation cell divisions.

1.2.1 Hematopoietic stem cells

HSCs are defined as cells that have the ability to perpetuate themselves through self-renewal and to be responsible for the generation of the blood-forming and immune (hematolymphoid) systems through differentiation. The hematopoietic stem cell compartment is made up of rare primitive cells that are multipotential (maintain the capacity to give rise to all lineages of blood cells) and have a high self-renewal capacity (give rise to "identical" daughter stem cells to maintain its original pool). The total number of stem cells is strictly regulated via both extrinsic and intrinsic mechanisms, resulting in the stability of a stable stem cell pool (Domen et al., 2000; Weissman, 2000a; Calvi et al., 2003; Lemischka and Moore, 2003; Zhang et al., 2003).

In vivo limiting dilution analysis of sorted HSCs allowed the isolation of subsets of multipotent cells: long-term HSCs (LT-HSCs) and short-term-HSCs (ST-HSCs) (Harrison and Astle, 1997; Adolfsson et al., 2001; Christensen and Weissman, 2001; Guenechea, et al., 2001;). The long-term subset self-renews for the entire life of the host, while the short-time subset retains self-renewal capacity for a short period of time (approximately 8 weeks). The highly self-renewing LT-HSCs can transit into ST-HSCs that possess limited self-renewal activity. ST-HSCs subsequently generate multipotent progenitors (MPPs) that give rise to a successive series of intermediate committed progenitors to further generate the multiple hematopoietic lineages.

HSCs have an impressive regenerative potential, as demonstrated by transplantation experiments using limited numbers of cells (Weissman, 2000b). The regeneration of the hematolymphoid system following a lethal dose of whole-body irradiation or chemotherapy became the basis for the use of bone marrow transplantation (Thomas, 1991). As the best-characterized stem cell population, HSCs have been isolated from mice and humans, used extensively in therapeutic settings as the vital elements in bone-marrow transplantation as well as in gene therapy (Kurre and Kiem 2000). When a bone marrow or blood stem cell transplantation is performed, it appears that the progenitors contribute to the engraftment for only a short period of time, while long-term blood production is realized by HSCs. HSCs can rapidly home to bone marrow (Hendrikx, et al 1996), and settle in a bone marrow niche (Nilsson et al., 2001). The enormous potential of HSCs is demonstrated by the fact that very few HSCs or even single HSCs are capable of repopulating the entire hematopoietic system of a lethally irradiated recipient (Lemishka et al., 1986).

1.2.2 Hematopoietic progenitor cells

HSCs generate mature cells through a series of binary decisions during which progressively restricted progenitors commit to alternative cell fates. In the process of commitment from HSCs to progenitors, cells exquisite some growth factor receptors, lose others, and become mitotically active. The primary function of these hematopoietic progenitor cells is to increase the number of mature cells. Accompanied by excessive proliferation, the progenitor cells undergo sequential differentiation with a decrease of self-renewal capacity (Quesenberry et al., 2001).

Each stage of differentiation of multipotent cells involves functionally maturation steps. The progenitor cell compartment is comprised of oligopotential, bipotential and monopotential progenitor cells, which have been characterized as exclusively committed to the production of restricted progeny. These progenitor cells are generally defined functionally by the capacity of the cells to form colonies *in vitro*. The progeny of mouse HSCs includes two kinds of oligolineage-restricted cells: common lymphoid progenitors

(CLPs) which are the progenitors for T lymphocytes, B lymphocytes, and natural killer (NK) cells (Kondo et al., 1997), and common myeloid progenitors (CMPs), which generate myeloerythroid lineage and further give rise to granulocyte/monocyte-restricted progenitors (GMPs) and megakarytic/erythroid progenitors (MEPs) (Akashi et al., 2000). Downstream of GMPS and MEPs are more mature progenitors, which are further restricted in the number and type of lineages they can generate (Figure 1).

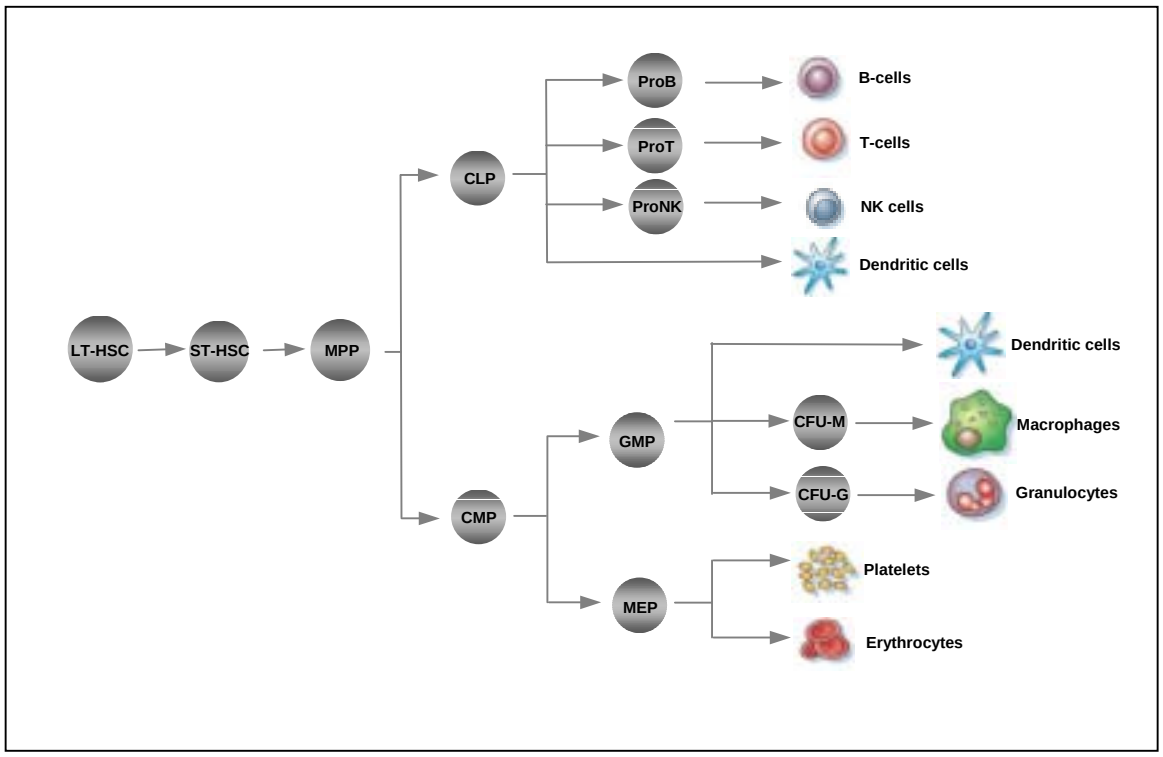

Figure 1. Proposed model of murine hematopoiesis based on prospectively isolatable bone marrow populations

HSCs are subdivided into long-term self-renewing HSCs (LT-HSCs), short-term self-renewing HSCs (ST-HSCs), and multipotent progenitors (MPPs). They give rise to common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs). CMPs give rise to granulocyte/macrophage progenitors (GMPs) and megakaryocyte/erythrocyte progenitors (MEPs). One of the most important issues in stem cell biology is to understand the mechanisms that regulate HSC self-renewal and commitment to differentiation. (Scheme according to Reya et al., 2001)

1.3 Identification, enrichment and isolation of HSCs and progenitors

1.3.1 Functional HSC assays

Much progress has been made on the development of reagents and practical and quantitative assays to characterize HSCs. However, functional assays are still the only reliable ways to identify HSCs.

The existence of HSCs and oligopotent progenitors within the hematopoietic system was initially shown by *in vivo* clonogenic assays. Till and McCulloch discovered that mouse bone marrow contains highly proliferative progenitors capable of giving rise to colonies of hematopoietic cells within the spleens of lethally irradiated hosts (Till and McCulloch, 1961). Thus, spleen colony-forming unit (CFU-S) assays became the cornerstone for much of our subsequent understanding of hematopoiesis. However, the colonies generated at day 10 after transplantation are composed of myeloerythroid cells, and only a fraction of the cells from these colonies have self-renewal potential and are capable of long-term multilineage reconstitution when re-injected into mice (Siminovitch et al., 1963; Lepault et al., 1993). Recent studies demonstrated that the vast majority of day 8 CFU-S ($CFU\text{-}S_8$) are derived from MEPs (Nakorn et al, 2002), while about half of day 12 CFU-S ($CFU\text{-}S_{12}$) are derived from the HSC/MPP compartment and the other half are derived from the MEP/CMP populations (Spangrude et al., 1988; Morrison and Weissman, 1994; Nakorn et al., 2002). Thus, the spleen-colony forming assays are only valuable to assay primitive progenitors.

Although HSCs undergo continuous self-renewal and differentiation to provide a continuous supply of hematopoietic cells throughout the organism lifespan (Cheshier et al., 1999), serial transplantation studies have suggested that HSCs can only be transplanted 5–7 times in mice (Harrison et al., 1978; Harrison and Astle, 1982; Harrison et al., 1990), indicating that the self-renewal capacity of HSCs may be intrinsically limited or at least subjected to exhaustion (Harrison and Astle, 1982; Harrison et al., 1990). Therefore, serial transplantation can be used for the evaluation of self-renewal ability of HSCs.

Another approach to quantitatively assess LT-HSC is competitive transplantation. The number and function of HSCs in each donor of a particular genotype can be tested by mixing donor bone marrow cells with a constant number of competitive bone marrow cells with a distinguishable marker (such as CD45.1, CD45.2, GFP), and measuring the relative ability of the donor cells to repopulate in stem-cell-depleted recipients (Harrison, 1980; Szilvassy et al., 1990).

In addition to *in vivo* assays, on the basis of long-term bone marrow culture, the "long-term culture-initiating cells" (LTC-IC) assay (Sutherland et al., 1989) and the "cobblestone area-forming cell" (CAFC) assay (Ploemacher et al., 1991; Neben et al., 1993) were developed as *in vitro* assays to determine the frequency of primitive hematopoietic cells. In the LTC-IC assay, LTC-IC-derived colony-forming cells (CFC) in semi-solid cultures are scored as readout. Alternatively, CAFC assay is visually assessed by the appearance of the cobblestone areas (tightly knit group of phase-dark, angular cells in the stroma) in phase contrast microscopy.

1.3.2 Functional assays for hematopoietic progenitors

The colony-forming cell assays were developed in the 1960s (Bradley and Metcalf, 1966; Pluznik and Sachs, 1965). Since then, many variations of the basic technique have been developed to allow quantification of progenitor cells that possess the ability to proliferate, differentiate and develop into phenotypically and functionally mature cells. In this test, cells are grown *in vitro* in a semi-solid matrix (tissue culture medium containing agar as a gelling agent or other highly viscous media, containing methylcellulose, plasma gel or fibrin clots). These semi-solid media reduce cell movement and allow individual progenitor cells to develop into cell clones that are identified as single colonies. These cells capable of generating colonies are called colony forming cells (CFC) or colony forming units (CFU).

The generation of hematopoietic colonies in a colony formation assay depends on the continuous presence of so-called colony-stimulating factors (CSF). In early experiments, colony-stimulating factors were provided by feeder cells or conditioned medium. The

diversity of hematopoietic colony stimulating factors in early culture systems caused ambiguous results. This problem was solved by the use of purified, recombinant form of hematopoietic colony-stimulating factors. The development of *in vitro* clonogenic assays has defined subsets of oligopotent progenitors for all myeloid lineage cells (mixed colony-forming cells, CFU-Mix) (Johnson and Metcalf, 1977), for granulocytes and macrophages (CFU-GM) (Ichikawa et al., 1966), and for megakaryocytes and erythrocytes (McLeod et al., 1976). Monopotent CFUs for granulocytes (CFU-G), macrophages (CFU-M), erythrocytes (CFU-E) (Stephenson et al., 1971), or megakaryocytes (CFU-Mk) (Metcalf et al., 1975) were also reported.

In retrospect, the nature of particular colony stimulating factors can be inferred sometimes after staining of these colonies and close examination of the morphology of the cells that have developed. Colony formation assays, therefore, allow the study of the influences of given growth factors or cytokines on the determination of the lineage along which colony forming cells differentiate.

1.3.3 Enrichment and isolation of HSCs and progenitors

HSCs and progenitors are very rare in both bone marrow cell populations and peripheral blood cell populations. The low frequency of HSCs and progenitors prevented the detailed understanding of their properties and application for clinical therapy. Whereas functional experiments provided evidence that stem cells exist, they did not allow the isolation of HSCs. However, on the basis of these functional HSC assays, different ways and markers have been developed over the years to phenotypically define, prospectively isolate and enrich candidate stem cell as well as progenitor cell populations using a high-speed cell sorter based on the presence or the absence of surface markers (usually detected by monoclonal antibodies), or biochemical markers (lectins, vital dyes such as Rhodamine 123 and Hoechst 33342).

The majority of HSC enrichment protocols rely on fluorescence-activated cell sorting (FACS), which allows cells to be positively selected based on the expression of a set of cell surface proteins. Murine stem cells have been defined based on their undifferentiated

characteristics (i.e., absence of lineage markers), as well as specific positive markers, such as Sca-1 and c-Kit. Most protocols use antibodies against lineage markers to exclude cells expressing proteins characteristic of a mature hematopoietic cell. This enrichment protocol permits the isolation of stem cell populations in which more than 80% of the cells have the potential to reconstitute the hematopoiesis (Lagasse et al., 2001). Combined staining with 4 to 5 markers (such as $Lin^{-/low}Sca\text{-}1^{high}c\text{-}Kit^{high}flt3^{-}$ and $Lin^{-/low}Sca\text{-}1^{high}c\text{-}Kit^{high}CD34^{-}$) allows an up to 2000-fold enrichment of to the HSC population (Uchida and Weissman, 1992; Morrison and Weissman, 1994; Zhao et al., 2000; Adolfsson et al., 2001; Christensen et al., 2001). Meanwhile, bone marrow progenitors including CLPs (Kondo et al., 1997), CMPs, GMPs, MEPs (Akashi et al., 2000) and monopotent megakaryocyte-committed progenitors (MKP) (Nakorn et al., 2003) have been prospectively isolated from mouse bone marrow.

Another approach for isolation of HSCs is based on the relative quiescence of stem cells and used the DNA binding dye Hoechst 33342 and the mitochondrial binding dye rhodamine 123. The lineage negative cells are isolation using magnetic beads, followed by Hoechst and Rhoda mine staining (Lemishka et al., 1986; Wolf et al., 1993) and enrichment of $Hoechst^{low}$ and $Rhodamine^{low}$ cells by FACS. Recently, Goodell and colleagues developed a method to isolate HSCs relying on the ability of stem cells to pump out the Hoechst dye (Goodell et al., 1996 and 1997). The murine bone marrow cells were stained with Hoechst 33342, and then the intensity of Hoechst fluorescence was analyzed simultaneously at two emission wavelengths. An extremely small and homogeneous population of cells revealed, and termed as “Hoechst-stained side population (SP)”. These cells have phenotypic markers of multipotential HSCs, and enable to contribute to both lymphoid and myeloid lineages in transplanted hosts.

The number of HSCs that can be obtained from animals is still very small, and it is very difficult to expand HSCs *in vitro*. Hence, most experiments need to be performed with a very low number of cells. Another problem is that the markers used to identify HSC may not directly correlate with their potential as stem cells, because none of the stem cell markers is known to be HSC-specific, and most of these markers are not known to be essential to stem cell function. Moreover, these markers can differ depending on alleles,

strains, developmental stages and activation stages (Randall and Weissman 1997; Sato et al., 1999; Matsuoka et al., 2001a; Henckaerts et al., 2002). For example, CD34 expression on LT-HSC has been found to be reversible and dependent not only on the activation state of the cells but also the developmental stage of the donor (Matsuoka et al., 2001a; Sato et al., 1999). The difference between negative and low expression of many of the markers used in such isolation protocols can be subtle. Thus, even the most rigorous isolation protocols currently available result in only relatively homogeneous HSC populations or highly enriched HSC populations, in which some of the cells fail to demonstrate pluripotency and/or long-term reconstituting ability (Morrison and Weissman, 1994). New markers and methods are required to allow the prospective isolation of stem and progenitor cells.

1.4 Proliferation of HSCs

1.4.1 The cell cycle status of hematopoietic stem cells

Regulated expansion of a small stem cell subset is necessary both to sustain a steady state level of mature blood cells and to compensate hematological stress. The hematopoietic tissues of an adult mouse must replace approximately 2.4×10^8 red blood cells and 4×10^6 nonlymphoid peripheral blood cells each day. Therefore, a subset of HSCs must undergo a massive expansion to produce mature blood cells. Such demands necessitate strict control over proliferation of hematopoietic stem cells and progenitor cells. On one side, maintenance of cell production requires a highly cytokine-responsive progenitor cell pool with prodigious proliferative capacity and a smaller population of stem cells intermittently feeding daughter cells into the proliferative compartment. On the other side, the stem cell pool itself is relatively quiescent and cytokine resistant, a state that seems to be necessary for the prevention of premature depletion during times of stress. It has been shown that most primitive hematopoietic stem cells are in G_0 stage of cell cycle; only 5% of LT-HSCs are in $S/G_2/M$ phases (Cheshier et al., 1999). The explanation might be either only a few hematopoietic stem cells are in cell cycle and contributing to blood cell production, or alternatively HSCs are constantly undergoing cell division, but these cells have prolonged active cell cycle or repeatedly entering and leaving the cell cycle.

The clonal succession model proposes that most of primitive hematopoietic stem cells are dormant or quiescent, and are thus protected from depletion or exhaustion. The production of blood cells is maintained sequentially by one or just a few hematopoietic stem cells at any time. The quiescent HSCs do not contribute to hematopoiesis until the proliferative capacity of the active HSCs clone is exhausted (Hodgson and Bradley, 1979; Lerner and Harrison, 1990). Significant proportions of immunophenotypically isolated stem cell candidates are in G_0 phase of the cell cycle, which are defined by 2N DNA content on Hoechst staining and by low metabolic rates (pyronin^{low}, or Rhodamine^{low}) (Morrison and Weissman, 1994). This is consistent with the finding that functionally selected HSCs are resistant to cell cycle-specific killing drugs such as 5-fluorouracil (5-FU) or 4-hydroperoxy-cyclophosphamide (4-HC) (Berardi et al., 1995).

Although cell cycle analysis revealed that HSCs are relatively quiescent at any one point in time, this does not reflect the proliferation history of HSCs. Studies with cell cycle-specific killing drugs did not distinguish between cells that are truly dormant and cells that are either in prolonged cell cycle or intermittently entering and exiting cell cycle at a slow rate. To overcome this obstacle, the proliferation history of HSCs was investigated with *in vivo* bromodeoxyuridine (BrdU) labeling to determine the rate of HSCs entering the cell cycle over time. BrdU can be incorporated into DNA during DNA synthesis and label the nuclei of dividing cells which have transited S phase while progressing through the cell cycle. Cheshier and coworkers immunophenotypically purified the LT-HSC subsets and found that about 50% of LT-HSCs incorporated BrdU by 6 days and more than 90% incorporated BrdU by 30 days; 99% of LT-HSCs had incorporated BrdU by 6 months. On average, 99% of lineage-negative HSCs with long-term self-renewing capabilities divided every 57 days, and approximately 8% of LT-HSCs asynchronously entered the cell cycle daily (Cheshier et al., 1999). These data demonstrate that although most of LT-HSCs are quiescent in G_0 at any given time, all HSCs are recruited into cycle regularly.

These results were confirmed by several groups using different species (Mahmud et al., 2001), different mouse strains (Bradford et al, 1997), and different stem cell separation

methods (Bradford et al, 1997; Abkowitz et al. 2000). Furthermore, HSC cycling appears to increase with age (Morrison et al., 1996). Thus, long-term *in vivo* BrdU incorporation indicated that hematopoietic stem cell are slowly proceeding through cell cycle and constantly undergo cell division.

1.4.2 Changing engraftable multipotent stem cell phenotype with cell cycle transit

A number of investigators observed a functional heterogeneity associated with cell cycle status of both murine and human HSCs. When immunophenotypically purified murine HSCs were fractionated into subsets from G_0/G_1 phase or $S/G_2/M$ phase, it could be observed that cells in G_0/G_1 phase have long-term engraftment capacity, while cells in $S/G_2/M$ phase show a decreased long-term engraftment capacity (Fleming et al, 1993a; Orschell-Traycoff et al, 2000). The ability for long-term *in vivo* engraftment is more pronounced in cells in the G_0 phase than cells in other cell cycle phases (Szilvassy et al., 2000; Huttman et al., 2001). Similarly, studies with human HSCs which were purified from bone marrow, cord blood or peripheral blood showed that their ability as transplantable stem cells in NOD/SCID mice was restricted to the G_0 or G_0/G_1 fraction, cells in S/G2/ or M phase had minimal engraftment potential (Gothot et al., 1997, 1998; Glimm et al., 2000; Summers et al., 2001). Furthermore, many studies showed that cytokine stimulation can induce HSCs to enter the cell cycle (Nilsson, et al., 1997; Reddy et al., 1997), which resulted in a loss of engraftment capacity of bone marrow cells (Peters et al., 1996; Habibian et al., 1998), supporting a reversible fluctuating engraftment phenotype associated with cell cycle phase position.

1.4.3 Cell cycle-related shifts in the engraftment phenotype is associated with an altered gene expression profile of HSCs

Early studies showed that the cell cycle dependent engraftment fluctuations may be due to a homing defect (Yamaguchi et al., 1998). Different adhesive characteristics and very-late antigen 4 (VLA-4) expression levels were observed in $CD34^+$ human progenitors in the G_0/G_1 and $S/G_2/M$ phases. Recently, it has been shown that gene expression patterns

shift in HSCs through the cell cycle in correlation with the cell cycle related alterations of stem cell phenotype, and that a wide variety of genes were found to be modulated in their expression with cell cycle progression (Lambert et al., 2003). These include genes involved in cytokine receptor expression, DNA damage and repair, RNA splicing, intracellular signaling, energy metabolism, cell cycle regulation, cytoskeleton, apoptosis regulation, and chromatin modification, indicating that a major shift of gene expression takes place between two specific cell cycle states. Non-cycling HSC selectively express mainly transcription regulators and protein synthesis factors, while they are fully capable of repopulating a myeloablated transplant recipient. In contrast, cells in S/G_2 have turned down most of the originally active genes, and express cell cycle related as well as chromatin remodeling genes (Lambert et al., 2003).

1.5 Regulation of stem cell self-renewal

One of the most important issues in stem cell biology is to understand the mechanisms that regulate self-renewal. In the past 10 years, based on gain-of-function and loss-of-function mouse models, some genes and signaling pathways have been found to play an important role in regulating self-renewal of HSCs.

1.5.1 Cyclin dependent kinase (CDK) inhibitors

The stochastic progression into the cell cycle depends on a matrix of expression levels of diverse cell cycle regulators. Slow cycling of HSCs necessitates the presence of appropriate cell cycle machinery to effect passage into and through G_1 phase. It has been shown that the intrinsic mitotic clocks regulate the cell cycle differently in stem cells than in more committed progenitors.

As CDKs regulate the cell cycle at different checkpoints, their different inhibitors are natural candidates for proteins that oppose cell cycle progression. Mice lacking $p21^{cip1/waf1}$, a G_1 specific CDK inhibitor, have a larger HSC pool, an increase in stem cell cycling accompanied by an increased susceptibility of the stem cell compartment to 5-FU-induced cell death and to rapid stem cell exhaustion upon serial transplantation

(Cheng et al., 2000a). This suggests that $p21^{cip1/waf1}$ normally acts to limit cycling of hematopoietic stem cells, and is required for stem cell quiescence. In contrast, $p27^{kip1}$, another G_1 specific inhibitor, does not affect stem cell number, cell cycling, or self-renewal, (Cheng et al., 2000b), and $p27^{kip1}$-deficient mice exhibited normal stem cell numbers as measured by CAFC assays, and serial bone marrow transplantation (Cheng et al., 2000b). However, deficiency of $p27^{kip1}$ markedly exhibited increased hematopoietic progenitor cell activity (Fero et al., 1996; Cheng et al., 2000b). The progenitor cell pool cycles more actively in these mice, as determined by colony formation by progenitor cells and 5-FU susceptibility of progenitor cells (Cheng et al., 2000b). These data support the notion that distinct members of the CDK inhibitor family have differentiation stage-specific roles in the stem cell and the progenitor cell compartments.

1.5.2 Signaling pathways

Accumulated evidence showed that many signaling pathways associated with oncogenesis, such as the Notch, Sonic hedgehog (Shh) and Wnt signaling pathways, may also regulate stem cell self-renewal.

1.5.2.1 Notch

Notch receptors are transmembrane proteins, which interact with a family of proteins containing a highly conserved Delta-Serrate-Lag-2 (DSL) domain in the extracellular region that serve as ligands (Milner and Bigas, 1999; Artavanis-Tsakonas, et al., 1999). In general, Notch-mediated cellular interactions have been shown to play a central role in regulating cell-fate decisions of various multipotent precursors (Milner and Bigas, 1999; Artavanis-Tsakonas, et al., 1999). The Notch ligand, Jagged-1, was found to be a potent activator of the Notch signaling pathway in a variety of cell types, mediating signals via cellular interactions with adjacent Notch-expressing cells. A role for Jagged-Notch signaling pathway in hematopoiesis was suggested since Notch was found on the hematopoietic cells and Notch ligands was found on bone marrow stromal cells (Karanu et al., 2001). An effect of Notch signaling on hematopoietic precursors has been suggested since numbers of murine primitive precursors increased upon incubation with

exogenously presented Notch ligands (Jones et al., 1998; Varnum-Finney et al., 1998). Recently, it has been shown that human Jagged-1 can maintain and expand primitive hematopoietic cells capable of multilineage reconstitution *in vivo* (Karanu et al., 2000). Notch1 activation increases hematopoietic stem cell self-renewal *in vivo* and favors lymphoid over myeloid lineage outcome (Stier et al., 2002) Constitutive Notch1 signaling in hematopoietic cells established immortalized, cytokine-dependent cell lines that generated progeny with either lymphoid or myeloid characteristics both *in vitro* and *in vivo* (Varnum-Finney et al., 2000). These data are consistent with the concept that Notch activation promotes HSC self-renewal, or at least the maintenance of their multipotentiality.

1.5.2.2 Wnt

Wnts are secreted glycoproteins that mediate cell-to-cell communication during development. Activation of Wnt-Wnt receptor complexes results in stabilization and accumulation of a protein called β-catenin which can translocate to the nucleus and bind and activate transcription factors of the TCF/LEF family thereby initiating transcription of TCF/LEF target genes. The Wnt signaling cascade has been shown to control early lymphopoiesis (van de Wetering et al., 2002) and gain of function approaches suggested that purified Wnt3A could act as stem cell growth factors and promote the self-renewal of HSCs (Reya et al., 2003; Willert et al., 2003; Murdoch et al., 2003). Overexpression of a dominant active form of β-catenin (lacking the NH_2-terminal phosphorylation domain) in long-term cultures of HSCs expands the pool of transplantable HSCs determined by both phenotype ($Thy1.1^{low}Lin^{-/low}Sca1^{+}c\text{-}kit^{+}$) and function (ability to reconstitute the hematopoietic system *in vivo*). In contrast, ectopic expression of Axin, which negatively regulates β-catenin by enhancing its degradation, leads to inhibition of HSC proliferation, increased death of HSCs *in vitro*, and reduced reconstitution *in vivo*. These data suggest that ß-catenin–mediated Wnt signaling is critical for a normal homeostasis of HSCs.

In contrast to earlier reports, inactivation of the β-catenin gene in bone marrow progenitors does not impair their ability for self-renewal and to reconstitute all hematopoietic lineages (myeloid, erythroid, and lymphoid), even in competitive mixed

chimeras (Cobas et al., 2004), suggesting that β-catenin-independent pathways may exist to mediate Wnt signaling in the hematopoietic compartment.

1.5.3 Transcription factors

1.5.3.1 Ikaros

Ikaros is a member of a family of zinc finger transcription factors, and is expressed in primitive and definitive hematopoietic precursors that reside within early hematopoietic sites, the yolk sac and fetal liver (Georgopoulos et al., 1992). It is also present in a rare bone marrow population that is enriched for HSCs ($Lin^{-}Sca1^{+}c\text{-}Kit^{+}$) (Kelley et al., 1998; Klug et al., 1998). Ikaros expression is downregulated during differentiation along the monocyte/macrophage and erythroid pathways but is maintained throughout granulocyte maturation (Klug et al., 1998). Ikaros is essential for specification of cells in lymphoid lineages (Georgopoulos et al., 1994; Wang et al., 1996)

Mice homozygous for an *Ikaros* null mutation display a more than 30-fold reduction in long-term repopulation units, whereas mice homozygous for an Ikaros dominant negative mutation have no measurable activity. A progressive reduction in multipotent CFU-S_{14} progenitors and the earliest erythroid-restricted precursors (burst-forming unit-erythroid, BFU-E) is also detected in the Ikaros mutant strains consistent with the reduction in HSCs. (Nichogiannopoulou et al., 1999)

1.5.3.2 Bmi1

Bmi1 is a member of the PcG (Polycomb group) family of transcriptional repressors that control development by the regulation of genes associated cell growth and differentiation. Studies of Bmi1-deficient mice revealed that the absence of Bmi1 results in progressive loss of all hematopoietic stem cells (van der Lugt et al., 1994). It has recently been shown that Bmi1 is necessary for efficient self-renewing cell divisions of adult HSCs (Park et al., 2003). In addition, lack of Bmi1 compromises the proliferative potential of leukaemic stem cells and progenitor cells, leading to transplant failure of the leukemia (Lessard and Sauvageau, 2003). Taken together with the detection of high levels of Bmi1 in human

AML stem cells (Lessard and Sauvageau, 2003), these results suggest that Bmi1 is also required for the self-renewal of leukemic stem cells. Similar to Bmi1, another member of PcG genes, the rae28 gene, has been shown to have a crucial role in sustaining the activity of HSCs to maintain hematopoiesis (Ohta et al., 2002).

The molecular mechanism by which Bmi1 affects the generation of HSCs has been revealed (Park et al., 2003). Bmi1 modulates HSC self-renewal through the regulation of genes important for stem cell fate decisions, as well as survival genes, antiproliferative genes, and stem cell-associated genes. Microarray analysis showed that Bmi1 target genes such as $p16^{Ink4a}$ and $p19^{Arf}$ (Jacobs et al., 1999) were elevated in bone marrow cells of the $Bmi1^{-/-}$ mice (Park et al., 2003). Enforced expression of $p16^{Ink4a}$ and $p19^{Arf}$ in HSCs leads to senescence and apoptosis of normal HSC, respectively (Park et al., 2003). In neural stem cells, $p16^{Ink4a}$ deficiency partially restored the ability of Bmi1-deficient stem cells for self-renewal (Molofsky, et al., 2003).

1.5.3.3 Homeobox (Hox) genes

Multiple Hox family members are expressed in the most primitive hematopoietic stem cell enriched populations, (Sauvageau et al., 1994). Engineered overexpression of several different members of the clustered Hox gene family has been shown to have major effects on the proliferation and differentiation of both murine and human HSCs and early hematopoietic progenitor cells both *in vivo* and *in vitro* (Sauvageau et al., 1995; Thorsteinsdottir, et al., 1997 and 1999; Antonchuk et al., 2002). Conversely, deficiency of both HoxB3 and HoxB4 impaired *in vitro* proliferative capacity of murine $Lin^{-}Sca\text{-}1^{+}$ $c\text{-}Kit^{+}$, and resulted in lower repopulating capability compared to normal littermates (Björnsson et al., 2002).

As in murine cells, retrovirally mediated expression of HoxB4 rapidly triggers an increase in the number of human HSCs and progenitor cells both measured by *in vitro* and *in vivo* assays (Buske et al., 2002; Amelia et al., 2003; Krosl et al., 2003a). This growth enhancement extended across primitive myeloid-erythroid and B-lymphoid progenitors

but did not lead to alterations in the balance of lymphomyeloid reconstitution *in vivo*, suggesting that HoxB4 does not affect control of end-cell output (Buske et al., 2002).

1.5.3.4 E2F

E2F family members are transcription factors involved in regulating S-phase progression of cell cycle. E2F protein complexes are defined by their ability to bind to a sequence element that was identified originally in the adenovirus E2 promoter (Kovesdi et al., 1987). The transcriptional activity of E2F is composed of a variety of heterodimers formed by the association of one of at least six E2F family members (E2F1 to E2F6) (Dyson, N. 1998). The E2F family of proteins can be aligned into three distinct groups, based on sequence/structural similarity as well as functional roles. E2F1, E2F2, and E2F3 are structurally similar, potent transcriptional activators (DeGregori et al., 1997). Overexpression of each of these E2F proteins is sufficient to drive quiescent cells to re-enter the cell cycle. In contrast, a second group of E2Fs, which includes E2F4 and E2F5, as well as a recently described, alternate version of E2F3 termed E2F3b (Leone et al., 2000), are thought to primarily function in the active repression of E2F target genes in quiescent cells by recruiting the retinoblastoma (Rb) family members. The third group is defined by the recently described E2F6 protein, which functions as a transcriptional repressor through a mechanism independent of pRB family members (Ogawa et al., 2002; Trimarchi et al., 2001). E2F family members play critical roles in cell cycle progression by regulating the expression of genes involved in nucleotide synthesis, DNA replication, and cell cycle control (Trimarchi and Lees, 2002).

The precise functional roles of individual E2F proteins in hematopoiesis are only poorly understood. The combined loss of E2F1 and E2F2 results in decreased hematopoietic cellularity of all examined hematopoietic compartments including bone marrow, thymus, lymph nodes and spleen, as well as significant reductions in the numbers of red blood cells, lymphocytes, and monocytes in the peripheral blood. Moreover, E2F1/E2F2 double-knockout bone marrow precursors competed poorly with wild-type (WT) precursors during hematopoiesis (Li et al., 2003). E2F4 deficient mice showed a deficiency of various mature hematopoietic cell types together with an increased number of immature

cells in several lineages (Rempel et al., 2000), suggesting a critical role for E2F4 activity in controlling the maturation of cells. E2F6 associates with numerous known PcG proteins *in vivo*, including the onco-protein Bmi1 (Trimarchi et al., 2001). This suggests that the transcriptionally repressive properties of E2F6 are mediated through its ability to recruit the PcG complex.

1.5.4 Interaction among regulators of stem cell self-renewal

HSC self-renewal is a complicated process, the knowledge of which is still limited. Many studies have shown links between several regulatory proteins and signaling pathways. For instance, sonic hedgehog protein (Shh) induces expression of bone morphogenetic protein (BMP) 4, HoxD11 and HoxD13, while HoxD12 regulates Shh expression in a positive feedback loop (Knezevic et al., 1997; Roberts et al., 1995). BMP4 has been shown to regulate HoxC8 expression (Shi, et al., 1999). In addition, expression of the HoxA9 gene is also affected in Bmi1-deficient hematopoietic tissues and neurospheres (Park et al., 2003; Molofsky, et al., 2003). Other factors important for maintenance of hematopoietic activity include molecules such as Pbx1 (Krosl et al., 2003b) and Rae28 (Ohta et al., 2002), both have connections to Hox proteins. It has also been shown that β-catenin could upregulates HoxB4 and Notch1 in HSCs (Reya et al., 2003).

Many studies have demonstrated that cytokines can regulate the expression of $p21^{cip1/waf1}$ and $p27^{kip1}$. The high level of $p21^{cip1/waf1}$ expression in stem cells could result in part from autologous production of TGF-β1 (Eaves et al., 1991; Hatzfeld et al., 1991) which is anti-proliferative. In addition, it has been reported that the edd gene and the c-fos gene exert their antiproliferative effects through $p21^{cip1/waf1}$ (Lessard et al., 1999; Okada et al., 1999) and $p21^{cip1/waf1}$ has been suggested to be a transcriptional target of HoxA10 (Bromleigh et al., 2000).

1.6 The transcription factor Gfi1 and hematopoiesis

1.6.1 The transcription factor Gfi1

The Growth factor independence 1 (Gfi1) gene was originally identified as a proto-oncogene during the analysis of proviral integration sites and their associated target genes in the NB2 rat lymphoma cell line after retroviral infection with the non-acute transforming Moloney murine leukemia virus (MoMuLV) (Gilks et al., 1993). Later, the Gfi1 locus in the human and mouse genomes was mapped to chromosomes 1p22 and 5 respectively (Bell et al., 1995; Roberts and Cowell, 1997). The Gfi1 gene encodes a 55-kD nuclear zinc finger transcription factor, which is a member of a protein family that includes Gfi1b (Fuchs et al., 1997; Rödel et al., 1998; Tong et al., 1998) as well as the murine proteins Snail and Slug (Grimes et al., 1996; Zweidler-McKay et al., 1996). All proteins of the Gfi1 family share six carboxy-terminal C_2-H_2 zinc finger domains and a characteristic N-terminal 20 amino acid stretch that was termed "SNAG" domain (Zweidler-McKay et al., 1996; Grimes et al., 1996; Tong et al., 1998) since it is well conserved between Gfi1 and the proteins Snail and Slug which bear similar zinc finger domains (Figure 2).

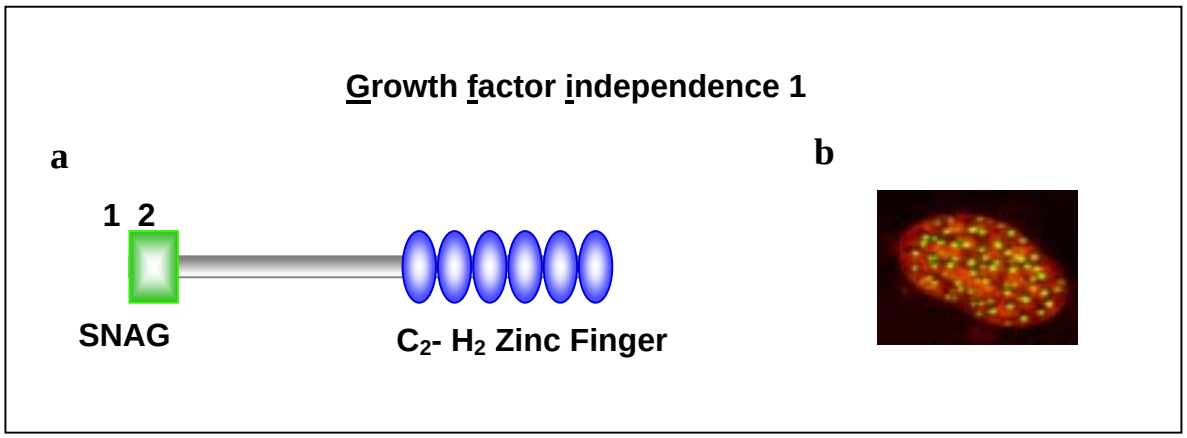

Figure 2. Zinc-finger transcription factor Gfi1

a. Gfi1 is a zinc-finger transcription factor which contains a SNAG domain and 6 Zinc-finger domains.
b. Expression of Gfi1 in nuclear dots was detected in NIH 3T3 cells by anti-Flag antibody after transfection with Flag epitope-tagged Gfi1 constructs (Rödel et al., 2000).

Gfi1 and Gfi1b are 97% homologous in the zinc finger region, and both proteins bind to virtually identical DNA consensus sequences. Experiments with reporter genes driven by synthetic promoters containing Gfi1 binding sites suggested a transcriptional repressor activity of Gfi1 (Grimes et al., 1996; Zweidler-McKay et al., 1996). Gfi1 binds to a specific DNA target sequences and this ability depends on the presence of some but not all of its zinc finger domains. Further mutational studies clearly delineated that this activity depends on the DNA binding activity of Gfi1 and on an intact N-terminal SNAG domain. The SNAG domain is responsible for nuclear localization and transcriptional repression (Grimes et al., 1996).

An alternative activity of Gfi1 has been discovered through its interaction with PIAS (protein inhibitor of activated STAT) 3, which is an inhibitor of signal transducers and activators of transcription (STAT) 3 (Rödel et al., 2000). PIAS3 can bind to activated, tyrosine phosphorylated STAT3 dimers and is able to down-regulate the activity of STAT3 as a transcriptional transactivator (Chung et al., 1997). By virtue of its interaction with PIAS3, Gfi1 is able to relieve STAT3 from PIAS3-mediated inhibition with the consequence of an enhanced STAT3 response (Rödel et al., 2000). This suggested a role of Gfi1 in a set of specific cytokine-signaling pathways because STAT3 is activated in response to a number of cytokines, among them Interleukin (IL) -2, IL-6, IL-10, or G-CSF.

1.6.2 Gfi1 in lymphomagenesis

Several studies with cultured cells indicated that a constitutive Gfi1 expression can relieve peripheral mature T cells from a requirement of IL-2 to overcome a G1 arrest (Grimes et al., 1996) or could help sustain cell proliferation of IL-2-dependent cells in the absence of the cytokine (Zörnig et al., 1996), indicating a role of Gfi1 in IL-2-dependent cell cycle progression of T cells. Similarly, the Gfi1 gene is a frequent target of proviral insection, and is transcriptionally activated by retroviral insertion in T cell lymphomas that arise in mice induced by infection with MoMuLV (Gilks et al., 1993; Zörnig et al, 1996; Schmidt et al., 1996, 1998a and 1998b; Scheijen et al., 1997). The constitutive expression of Gfi1 targeted to T cells by virtue of the proximal *lck* promoter predisposed mice to the

development of T cell lymphoma at low frequency (Schmidt et al., 1998a), indicating Gfi1 has a low oncogenic potential. Gfi1 acts as a dominant oncogene when overexpressed, and cooperates strongly with Pim (a cytoplasmic serine/threonine kinase) and Myc (an HLH-LZ transcription factor) in accelerating progression of T cell lymphomagenesis in the respective transgenic mice or in MoMuLV-infected mice (Schmidt et al., 1996, 1998a and 1998b; Zörnig et al., 1996; Scheijen et al., 1997).

1.6.3 Gfi1 in lymphopoiesis

Gfi1 is expressed at very high levels in thymic lymphoid cells compared with other organs and cell lineages (Gilks et al., 1993; Grimes et al., 1996; Schmidt et al., 1998a; Karsunky et al., 2002a and 2002b). Protein and RNA analyses showed that Gfi1 is expressed during T cell development beginning in the early stages of pre-T cell selection until the point where T cells express both CD4 and CD8 surface markers (Schmidt et al., 1998b), which suggested a role of Gfi1 in early T cell development. In mature CD4 or CD8 single positive (SP) T cells, Gfi1 expression is low or at least not readily detected but can be rapidly upregulated following T cell receptor (TCR) stimulation (Rödel et al., 2000). It has been demonstrated that Gfi1 inhibits activation-induced T cell death by overriding a G_1 cell-cycle checkpoint (Karsunky et al., 2002a). Gfi1 regulates IL-4/STAT6-dependent Th2 cell proliferation (Zhu et al., 2002) and IL-6/STAT3-mediated proliferative responses to antigenic stimulation (Rödel et al., 2000). Induction of Gfi1 by IL-4 increases Th2 cell expansion by promoting proliferation and preventing apoptosis (Zhu et al. 2002).

Overexpression of Gfi1 in transgenic mice results in a reduction of peripheral $CD4^+$ and $CD8^+$ cells by inhibiting “beta-selection”, a process that allows the preferential expansion of cells with a functional TCR beta-chain (Schmidt et al., 1998b). These results demonstrated that Gfi1 participates in the regulation of beta-selection-associated pre-T cell differentiation, and is important for disposal of those T cell precursors unsuccessful in T cell receptor rearrangement. In a loss-of-function model, mice lacking Gfi1 show reduced thymic cellularity (about 10% that of wild-type mice) due to an increased cell death rate, lack of proliferation, and a differentiation block in the very early uncommitted

$CD4^-/CD8^-/c\text{-}Kit^+$ cytokine-dependent T cell progenitors that have not yet initiated VDJ recombination (Yücel et al., 2003). In addition, Gfi1-deficient mice show increased major histocompatibility complex (MHC) class I-restricted positive selection, and a significant bias toward the production or selection of $CD8^+$ cells, suggesting a requirement of Gfi1 for a correct CD4/CD8 lineage decision (Yücel et al., 2003). This demonstrates that Gfi1 is generally required for a normal production of T cell progenitors and their differentiation.

1.6.4 Gfi1 in myelopoiesis

Gene targeting has recently revealed that Gfi1-deficient mice are unexpectedly neutropenic (Karsunky et al., 2002a), demonstrating Gfi1 contributions to myelopoiesis. Mature neutrophils are absent in $Gfi1^{-/-}$ mice, while atypical immature mono-myeloid cells accumulate in the bone marrow and peripheral blood, suggesting that the differentiation from $Gfi1^{-/-}$ precursors is severely skewed toward the monocyte/ macrophage lineage as a result of either a block of the granulocyte lineage or enhanced differentiation towards the monocyte/macrophage lineage. It has been recently found that heritable Gfi1 mutations cause human neutropenia and fail to repress neutrophil elastase (ELA2) (Person et al., 2003), encoding neutrophil elastase, mutations of which are the major cause of inherited human neutropenia syndromes (Horwitz et al., 1999; Dale et al. 2000). Two heterozygous mutations were found. The 1412A to G transition causes N382S amino acid mutation in the fifth zinc finger of Gfi1, subsequently abolishing the DNA binding activity of Gfi1. The second mutation is a 1475A to G transition in the sixth zinc finger of Gfi1, causing the coding sequence substitution K403R. The K403R mutation does not affect DNA binding but could perturb protein-protein interaction. Both mutations act in a dominant negative manner when expressed competitively with the wild-type protein. In both mice and humans with Gfi1 mutations, myeloid progenitor cells fail to differentiate to mature neutrophils, causing the accumulation of monocytes and abnormal cells that blend features of monocytes and granulocytes, thus highlighting essential contributions of Gfi1 to delineating the two cell types.

1.6.5 Target genes of Gfi1

A number of potential target genes have been proposed to be mediators of downstream effectors of Gfi1 function. In the lymphoid system, the transcription regulator lung Krüppel-like factor (LKLF), and the helix loop helix proteins inhibitor of DNA binding (Id) 1 and Id2 are supposed to be potential target genes of Gfi1 (Yücel et al., 2003).

In myeloid cells, Duan and Horwitz investigated 34 genes as potential Gfi1 targets, based on functional contributions to myelopoiesis, and found 16 of the tested genes to be direct Gfi1 targets. These genes include cell-cycle regulators ($p21^{cip1/waf1}$, E2F5, E2F6, and c-Myc), transcription factors (Ets2, C/EBPα, C/EBPε), growth factors and their receptors (IL2, Jak3, IL-6R, IL8) and neutrophil elastase (ELA2) (Duan and Horwitz, 2003). Cluster analysis of expression patterns and chromatin immunoprecipitation data revealed that Gfi1 targets a subset of genes affecting the differentiating hematopoietic lineages and therefore plays a relatively superior role in the hierarchy of factors governing stem cell differentiation.

1.7 The aim of the work

It has been shown that Gfi1 is expressed in the hematopoietic and immune systems, and lack of Gfi1 leads to defects in both myeloid and lymphoid hematopoiesis (Karsunky et al., 2002; Yücel et al., 2003). These results have been confirmed by the data from Gfi1-deficient mice generated by another group (Hock et al., 2003), and by the dominant negative mutations of Gfi1 found in neutropenic patients (Person et al., 2003).

However, the knowledge about Gfi1 is limited in relatively mature compartments of the hematopoietic system. Since self-renewal is required for HSCs to persist for the lifetime of the animal to maintain the stem cell function, to understand the mechanisms that regulate self-renewal is one of the most important issues in stem cell biology. The expression pattern and the function of Gfi1 in HSC compartment and in subsets of progenitor compartment are still unclear. Does Gfi1 contribute to development of HSCs and progenitors? Does loss of Gfi1 result in alteration of numbers of HSCs and progenitors in adult mouse bone marrow at steady stage? Does Gfi1 affect the functions

of HSCs, such as self-renewal, proliferation, and differentiation? Does Gfi1 play a role in regulation of hematopoiesis at stress stage? What are the mechanisms by which Gfi1 regulate hematopoiesis? The aim of the present study is to answer above questions using generated Gfi1 mutant mice, flow cytometry analysis, functional assays for HSCs and progenitors as well as other molecular cell biology techniques.

2. Materials and Methods

2.1. Materials

Chemicals

The chemicals used in the present study were from Fluka, Neu-Ulm; Invitrogen, Karlsuhe; Merck, Darmstadt; Roche, Mannheim; Roth, Karlsuhe; Serva, Heidelberg and Sigma, Deisenhofen.

Reagents for CFC-assay

Fetal bovine serum (FBS)	PAA Laboratories, Austria
Iscove's modified DMEM (IMDM)	Invitrogen Corporation
Dulbecco's PBS (DPBS)	Invitrogen Corporation
Penicillin-streptomycin	Invitrogen Corporation
L-Glutamine	Invitrogen Corporation
2.3% methylcellulose	GIBCO/BRL, Germany
Recombinant murine GM-CSF	PeproTech, USA
Recombinant murine IL-3	PeproTech, USA

Antibodies for flow cytomertric analysis

Specificity	Clone	Conjugation	Source
BrdU	3D4	FITC	PharMingen, USA
CD3	145-2C11	APC	PharMingen
CD4	RM4-5	PE, biotin	PharMingen
CD8	53-6.7	biotin	PharMingen
CD16/CD32 (Fcγ receptor III/II)	2.4G2	PE, biotin	PharMingen
CD11b (Mac1)	M1/70	PE, PerCP-Cy5.5	PharMingen

CD19	6D5	FITC	Caltag, USA
CD34	RAM34	FITC, PE	PharMingen
CD45.1	A20	PE	PharMingen
CD45.2	104	biotin	PharMingen
CD45R/B220	RA3-6B2	APC	PharMingen
CD127 (IL-7Rα chain)	B12-1	biotin	PharMingen
CD127 (IL-7Rα chain)	SB/14	PE	PharMingen
c-Kit (CD117)	2b8	FITC, APC	PharMingen
Flt3 (CD135, Flk2)	AF10.1	PE	PharMingen
Gr-1 (Ly6G)	RB6-8C5	FITC, PE	PharMingen
Sca-1 (Ly6A/E)	E13-161.7	FITC, PE, biotin	PharMingen
Streptavidin		PE, PerCP-Cy5.5	PharMingen
Ter119 (Ly-76)	Ter119	biotin	PharMingen
CXCR4	2b11	PE	PharMingen
VLA4 (CD49d)	9C10	PE	PharMingen
VLA5 (CD49e)	5H10-27	PE	PharMingen

Biotinylated lineage cocktail

CD11b#	100 μl
Gr-1#	100 μl
CD3#	100 μl
CD45R/B200#	100 μl
Ter119#	100 μl
CD4	10 μl
CD8	10 μl

Antibodies from BD PharMingen™ Biotin-conjugated Mouse Lineage Panel Kit

Antibody for Western blot

Specificity	Clone	Origin	Source
$p21^{waf1/cip1}$	Ab5	rabbit polyclonal	Oncogene, USA
$p27^{kip1}$	Ab2	mouse monoclonal	CALBIOCHEM, Bad Soden
E2F5	E-19	rabbit polyclonal	Santa Cruz, USA
E2F6	anti-sera	rabbit polyclonal	Dr. Stefan Gaubatz, Marburg

Kits

Ammonium chloride lysing reagent	PharMingen
Cytofix/Cytoperm Kit	PharMingen
Enhanced chemiluminescence plus (ECL) detection system	
Super Signal West Dura	PIERCE, Bonn

Methylcellulose medium

30 ml	FBS
40 ml	2.3% methylcellulose
1 ml	2-mercaptoethanol (7µl in 10 ml H_2O)
1 ml	Penicillin-streptomycin
1 ml	L-Glutamine
10 ml	10% bovine serum albumin
17 ml	IMDM

The media were mixed and aliquoted into Falcon tubes (# 2059) for a final volume of 3.6 ml each tube, which is sufficient material to generate triplicate plates. Store at -20°C. Individual tubes can be thawed at 37°C and used as needed.

Tellesniczky's solution:

375 ml	70 % ethanol
18.75 ml	glacial acetate acid
37 ml	37% formaldehyde solution
add water to 500 ml	

Staining buffer for FACS analysis

PBS supplemented with 10% FBS

Hoechst buffer

100 ml	Hanks' balanced salt solution
20 mM	HEPES
0.1 %(w/v)	glucose
10 ml	FBS

Whole Cell Extract (WCE) Buffer

50 mM	Tris-HCl, pH7.8
250mM	NaCl
0.2 mM	EDTA
1 mM	DTT
10% (w/v)	glycine
0.5% (v/v)	NP-40

Transfer Buffer

5.82 g	Tris base
2.93 g	glycine
3.75 ml	10% SDS
200 ml	methanol
add water to 1000 ml	

4×protein loading buffer

80mM	Tris-HCl, pH 6.8
3.2% (w/v)	SDS
8% (v/v)	β-mercaptoethanol
0.002% (v/v)	bromphenolblue
1.6% (v/v)	glycerin

10× TBS (Tris-buffered saline)

24.2g	Tris base
80g	NaCl
adjust pH to 7.6	

Wash Buffer (TBST)

1× TBS, 0.1% Tween-20

2.2 Mice

Gfi1-deficient mice were generated by homologous recombination in R1 embryonic stem cells and have been previously described (Karsunky et al., 2002a). Wild type and Gfi1$^{-/-}$ mice were bred and maintained under specific pathogen free conditions at the animal facility of the Institut für Zellbiologie, Universitätsklinikum Essen in individually ventilated cages. Mice that were used for analyses were healthy 4-8-week-old animals from a more than 20 generation backcross with C57BL/6 mice. Gfi1:GFP knock-in mice were generated by Raif Yücel in a similar way as the Gfi1$^{-/-}$ animals with the exception that a GFP open reading frame was inserted immediately downstream of the Gfi1 translation initiation codon and that the neo casette was flanked by loxP sites which allowed its germline deletion upon expression of a Cre recombinase. Gfi1 heterozygotes did not show any distinct phenotype and were used along with WT mice as controls. All animal experiments were carried out according to the German animal protection law and were done under a license granted by the Bezirksregierung Düsseldorf/NRW, Germany (Nr.:G004/98Z).

2.3 Flow cytometry analysis and sorting

2.3.1 Preparation of single cell suspension

All phenotypic analysis was performed in parallel using cells from age-matched Gfi1$^{-/-}$ and WT control mice (4 to 8 weeks old). The femurs were dissected after the mice were

sacrificed by CO_2 inhalation. The bone marrow cells were harvested by flushing the femurs with PBS. The bone marrow cells were passed through a 23 G needle to obtain a single cell suspension, and, if necessary, were passed again through a nylon mesh after ammonium chloride (PharMingen) lysis of the red blood cells. The bone marrow cells were washed with PBS supplemented with 10% FBS, then the total nucleated cell numbers were calculated using a CASY-1 cell counter (Schärfe System). Subsequently, the cells were resuspended in PBS (for bone marrow transplantation) or in staining buffer (for antibody Staining).

2.3.2 Flow cytometry analysis and sorting of HSC and progenitors

To define HSCs and progenitors, bone marrow cells from one mouse were suspended in 450 µl staining buffer. 150 µl of the cell suspension were transferred to a 5-ml tube for a single reaction. The cells were incubated with biotinylated lineage antibody cocktail (B220, Gr-1, CD11b, CD3, CD4, CD8, and TER-119) together with other antibodies (table 1) for 15 minutes at 4°C. After washing with 4 ml of staining buffer, the cells were resuspended in 150 µl of staining buffer and incubated with 1 µl of PerCP-Cy5.5-conjugated Streptavidin for 15 minutes at 4°C. Stained cells were analyzed with a FACSCalibur (Becton Dickinson) using CellQuest software as described before (Kondo et al., 1997; Akashi et al., 2000; Adolfsson et al., 2001; Christensen and Weissman, 2001).

To analyze the GFP expression in $Gfi1^{GFP/+}$ HSCs, the bone marrow cells were stained with biotinylated lineage cocktail and PE-conjugated-anti-Sca-1 and APC-conjugated-anti-c-Kit antibodies, followed by PerCP-Cy5.5-conjugated Streptavidin staining.

To analyze the GFP expression in CLPs, the bone marrow cells were stained with biotinylated lineage cocktail followed by staining with PerCP-Cy5.5-conjugated Streptavidin. The Lin^- cells were sorted on a FACSDiVa (Becton Dickinson), centrifuged and resuspended in 150 µl of staining buffer. The sorted cells were stained with antibodies against Sca-1 (PE), c-Kit (APC) and IL-7Rα (biotin) for 15 minutes at 4°C, followed by staining with PerCP-Cy5.5-conjugated Streptavidin for 15 minutes at 4°C.

Table 1. Antibodies for staining of HSCs and progenitors

Antibodies	HSC	CLP	CMP/GMP/MEP
biotinylated lineage cocktail	15 μl	15 μl	15 μl
Sca-1-FITC	1 μl	1 μl	
c-Kit-APC	1 μl	1 μl	1 μl
Flt3-PE (or CD34-PE)	1 μl		
IL-7R α chain-PE		1μl	
CD34-FITC			1 μl
CD16/CD32-PE			1 μl
Sca-1-biotin			1 μl
IL-7R α chain-biotin			1 μl

For CMPs/GMPs/MEPs, the bone marrow cells were first stained with biotinylated lineage cocktail together with biotinylated antibodies against Sca-1 and IL-7Rα , and then stained with PerCP-Cy5.5-conjugated Streptavidin. The $Lin^-Sca\text{-}1^-IL\text{-}7R\alpha^-$ cells were sorted on a FACSDiVa, and stained with antibodies against CD34 (PE), c-Kit (APC) and Fcγ receptors (biotin) for 15 minutes at 4°C. After washing with 4 ml of staining buffer, the cells were resuspended in 150 μl of staining buffer and stained with PerCP-Cy5.5-conjugated Streptavidin for 15 minutes at 4°C.

2.4 Expression analysis of Gfi1 by RT-PCR

This experiment was kindly performed by Dr. Karsunky (Stanford University, USA). Bone marrow cells were harvested from C57BL/6, Thy1.1 mice and sorted according to the following criteria: HSC: $Lin\text{-}c\text{-}Kit^+Sca\text{-}1^+Thy1.1^{low}$, CMP: Lin^- $c\text{-}Kit^+$ $Sca\text{-}1^-CD34^+$ $CD16/32^{int}$, GMP: $Lin^-c\text{-}Kit^+Sca\text{-}1^-CD34^+CD16/32^{low}$, MEP: $Lin^-c\text{-}Kit^+Sca1^-CD34^-$ $CD16/32^{low}$, CLP: $Lin^-c\text{-}Kit^+$ $Sca\text{-}1^+Thy1.1^-IL\text{-}7R\alpha^+$. Total RNA from 2000 double sorted cells was isolated using TRIzol Reagent (Invitrogen) according to manufactures protocol and 10 μg/ml linear acrylamide (Ambion) was used as a carrier. All RNA samples were treated with DNase1 to avoid genomic DNA contamination and reversed-transcribed into

cDNA using the SuperScript First Strand Synthesis System with random hexamers according to manufacture's protocol. Per PCR 1 μl of cDNA (equivalent of cDNA from 100 cells) and the following primer were used for amplification:

Gfi1: 5' CTG CTA CAA GAG GAG GCA TCA-3'

5'-GAA GCA CAG AAC ACA GGC TCT-3'

β-actin: 5'-ACG AGG CCC AGA GCA AGA GAG G-3',

5'-AGC CAC CGA TCC ACA CAG AGT A-3'

2.5 Bone marrow transplantation

The mice used as recipients were 8–12 week old. The lethal preconditioning regimen for HSC and bone marrow reconstitution was 9.6 Gy total body irradiation, given in two doses with a 4-hour interval using a ^{137}Cs radiation source at Institut für Medizinische Strahlenbiologie, Universitätsklinikums Essen with the help of Prof. Müller. After irradiation mice were given antibiotic water (1.1 g/L neomycin sulfate and 10^6 U/L polymyxin B sulfate) for at least 12 weeks to reduce the chance of infection from opportunistic pathogens.

Donor bone marrow cells were prepared as described above. The cell suspensions were adjusted to an appreciated concentration for injection. In all experiments, 200 μl of cells were injected into lethally irradiated recipient mice.

To confirm that Gfi1 deficient HSCs have an intrinsic defect and to exclude influences of a potentially defective bone marrow microenvironment in $Gfi1^{-/-}$ mice, $4x10^6$ WT or $Gfi1^{-/-}$ bone marrow cells were injected into lethally irradiated CD45.1 mice. 4 months after transplantation, the frequencies of HSCs and early progenitors derived from donor bone marrow cells ($CD45.2^+$) in recipients' bone marrow were measured by flow cytometry and described in above. To identify the donor derived cells, biotin-conjugated anti-CD45.1 antibodies were added to the biotinylated lineage cocktail. Reconstitution of donor myeloid cells was monitored by staining bone marrow cells with antibodies against CD45.2 (PE), CD11b (PerCP-Cy5.5), and Gr-1 (FITC).

Vice versa, $3x10^6$ CD45.1 WT bone marrow cells were injected into lethally irradiated WT or $Gfi1^{-/-}$ mice (both $CD45.2^+$). 4 months after transplantation, the frequencies of HSCs and early progenitors derived from donor bone marrow ($CD45.1^+$) were measured by flow cytometry and described above. To identify the donor-derived cells, biotin-conjugated anti-CD45.2 antibody was added to the biotinylated lineage antibody cocktail. Reconstitution of donor myeloid cells was monitored by staining bone marrow cells with antibodies against CD45.2 (PE), CD11b (PerCP-Cy5.5), and Gr-1 (FITC).

For competitive transplantation, WT or $Gfi1^{-/-}$ bone marrow cells (both $CD45.2^+$) were mixed with competitor $CD45.1^+$ bone marrow cells at a ratio of 1:1 (2×10^5 CD45.2 versus 2×10^5 CD45.1 cells per mouse) or 10:1 (2×10^6 CD45.2 versus 2×10^5 CD45.1 cells per mouse), and injected into lethally irradiated CD45.1 recipient mice.

The same experiments were performed using 500-sorted WT or $Gfi1^{-/-}$ LT-HSCs mixed with $2x10^5$ competitor CD45.1 bone marrow cells. 4000-sorted WT or $Gfi1^{-/-}$ $Lin^-Sca\text{-}1^+$ $c\text{-}Kit^+Flt3^-$ cells were sorted into 5-ml tubes then mixed with $1.6x10^6$ competitor CD45.1 bone marrow cells. The mixed cells were centrifuged, and resuspended in 1.6 ml of PBS. 200 μl of the mixed cells were injected into lethally irradiated CD45.1 recipient mice.

Donor-derived cells were distinguished from endogenous host cells by the expression of different CD45 antigens (CD45.1 vs. CD45.2). The peripheral blood was collected from retroorbital venous sinus of anesthetized mice into EDTA-containing tubes at different time points (3, 10 and 22 weeks). After ammonium chloride (PharMingen) lysis of the red blood cells, cells were washed, and resuspended in 150 μl of staining buffer. Antibodies against CD45.2 (PE), CD11b (PerCP-Cy5.5), and Gr-1 (FITC) were used to measure reconstitution of donor derived myeloid cells, while reconstitution of donor derived lymphoid cells was measured by staining with antibodies against CD45.2 (PE), CD3 (APC), and CD19 (FITC).

In some experiments, bone marrow cells were harvested at the end of experiments. Percentage of donor $Lin^-Sca\text{-}1^+c\text{-}Kit^+$ cells was detected by staining with the biotinylated lineage cocktail together with antibodies against CD45.2 (PE), c-Kit (APC), and Sca-1

(FITC) as described above.

2.6 Short-term radioprotection assay

Cell suspensions containing various amounts of WT or Gfi1$^{-/-}$ bone marrow cells were injected into lethally irradiated wild-type recipient mice. The animal survival frequency was plotted for each group over a time period of 35 days.

2.7 Spleen colony-forming assays

5x10^4 WT or Gfi1$^{-/-}$ bone marrow cells were injected into lethally irradiated (9.6Gy) wild-type recipient mice via the lateral tail vein (10 mice each group). Mice were killed 8 days or 12 days after the injection, and their spleens were fixed in Tellesniczky's fixative solution for at least 7 days for macroscopic examination. Irradiated mice injected with PBS were included as control in all experiments.

2.8 CFC assays

Single-cell suspensions of bone marrow were prepared as described in 2.3.1. Cell concentration was adjusted to 1×10^6/ml. 90 μl of single cell suspensions were added to methylcellulose media (2.5×10^4 cells/ml). Purified recombinant murine IL-3 and murine GM-CSF were used at the concentrations indicated in the results. After mixed thoroughly with 3 ml syringe, the mixture was plated in triplicate in 1 ml of methylcellulose medium in 35-mm petri dishes and incubated for 7 days at 37°C and 5% CO_2. Individual colonies (defined by >50 cells) were scored at day 8 post-plating.

2.9 Analysis of adhesion molecule expression on HSCs

Bone marrow cells were stained with antibodies for surface markers (lineage antibody cocktail, anti-c-Kit-APC, anti-Sca-1-FITC), and anti-CXCR-4-PE, or anti-VLA-4-PE, or anti-VLA-5-PE, respectively. Lin$^-$ Sca-1$^+$c-Kit$^+$ population was gated for analysis of adhesion molecule expression.

2.10 BrdU incorporation

Mice were initially injected intraperitoneally with 1.8 mg of BrdU in 200 μl of PBS and then were continuously given BrdU at 1 mg/ml in the drinking water. At different time points (1,2,5 and 10 days), bone marrow cells were harvested and were first stained for surface markers (lineage antibody cocktail, c-Kit, Sca-1). After washing with PBS supplemented with 10% FBS, the cells were resuspended with 100 μl of Cytofix/Cytoperm buffer (from Cytofix/Cytoperm Kit, PharMingen) and incubated for 1 hour at room temperature. The fixed and permeabilized cells were washed once with 1 ml of Perm/Wash buffer (from Cytofix/Cytoperm Kit, PharMingen), and incubated with 100 μl of diluted Dnase I (300 μg/ml in DPBS) for 1 hour at 37°C. After washing with 1 ml of Perm/Wash buffer, the cells were incubated with FITC-conjugated anti-BrdU antibody in Perm/Wash buffer for 20 minutes at room temperature. The cells were washed with 1 ml of Perm/Wash buffer, and resuspended in PBS supplemented with 10% FBS for flow cytomertric analysis. Bone marrow cells from mice without BrdU-injection were stained with antibody against surface marker and BrdU, and used as negative control.

2.11 Cell cycle analysis of HSCs

Bone marrow cells were stained with antibodies for surface markers (biotinylated lineage cocktail, c-Kit-APC, Sca-1-FITC) as described above, and resuspended in 1 ml of Hoechst buffer. Cells were incubated with Hoechst 33342 for 45 minutes at 10μg/ml at 37°C. Pyronin Y was then added to a final concentration of 1 μg/ml, and cells were incubated for another 15 minute prior to analyzed with a FACSDiVa to determine the cell cycle profile of $Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{+}$ cells

2.12 Western blot

The bone marrow cells were lysed with WCE buffer plus protease inhibitors, incubated on ice for 20 minutes, then centrifuged at 14,000 rpm for 10 minutes at 4°C. The supernatant (whole-cell extract) was transferred to a fresh Eppendorf tube and stored at -80°C.

Denaturing sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) were prepared with 12% separating gels and 5% stacking gels. 50 μg of protein extracts were added in 4×loading buffer, denatured at 95°C for 5 minutes, loaded onto the gels and electrophoresed in running buffer at 120 V for about 90 to 120 minutes. The samples were transferred from the gel to a Hybond^{TM}-C Extra membrane (Amersham Bioscience) using Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). The membrane was stained with Ponceau S solution to ensure equal protein loading and transfer. The membrane was washed with TBST, blocked with 5% non-fat dry milk powder in TBST for 60 minutes at room temperature, and incubated with primary antibody overnight at 4°C. After being washed with TBST, the membrane was incubated with Horseradish peroxidase-conjugated secondary antibody in TBST for 60 minutes at room temperature. The membrane was washed in TBST, and immunoreative proteins were visualized with the ECL detection system, followed by exposure to X-ray film.

3. Results

3.1 Generation and functional testing of the Gfi1:GFP knock-in mice

To investigate the function of Gfi1, Gfi1 deficient mice have been generated by homologous recombination in embryonic stem cells, replacing part of the coding sequence with a *neo*^{R} cassette (Karsunky et al., 2002a). Intercrossing of Gfi1$^{+/-}$ mice produced Gfi1 null mice (Gfi1$^{-/-}$) that completely lacked expression of Gfi1 protein. Gfi1 heterozygotes did not show any distinct phenotype, while mice lacking Gfi1 showed multilineage defects in hematopoiesis such as reduced numbers of lymphoid cells, monocytosis and severe neutropenia (Karsunky et al., 2002a; Yücel et al., 2003; Hock et al., 2003).

To further understand the role of Gfi1 in modulation of hematopoiesis, it is essential to precisely delineate the magnitude and compartmentalization of Gfi1 expression in different hematopoietic lineages at different differentiation stages during hematopoiesis. Since the green fluorescent protein (GFP) has been proven remarkably useful in tracking intracellular protein localization *in vitro* and recently for localizing expression to cellular subsets *in vivo* (Monroe et al., 1999), Raif Yücel used gene targeting to generate a knock-in mouse mutant in which the Gfi1 coding region was replaced by the gene encoding GFP (Yücel et al., submitted). The GFP gene was inserted in-frame with the ATG translation initiation codon of Gfi1, thereby placing it under the transcriptional control of the Gfi1 regulatory elements (Figure 3). The targeting construct was designed to replace exon 3 to 5 of Gfi1 by GFP and included a selectable marker gene (Neo) flanked by loxP sites (Figure 3). All endogenous Gfi1 regulatory sequences were maintained after homologous recombination. Therefore, GFP is expressed in place of the endogenous Gfi1 allele under the transcriptional control of the Gfi1 regulatory elements. The Gfi1:GFP heterozygous knock-in mice (Gfi1$^{GFP/+}$) derived from the targeted ES cells were mated with CMV-Cre transgenic mice, which express Cre recombinase transiently in the early blastocyst, to remove the neomycin resistance gene at the germline level. To minimize genetic background effects on the analysis of the role of Gfi1 in hematopoiesis, Gfi1$^{GFP/+}$ mice

without the neomycin gene were backcrossed into C57BL/6 mice. Intercrossing of Gfi1$^{GFP/+}$ mice produced Gfi1$^{GFP/GFP}$ mice that completely lacked expression of Gfi1 protein.

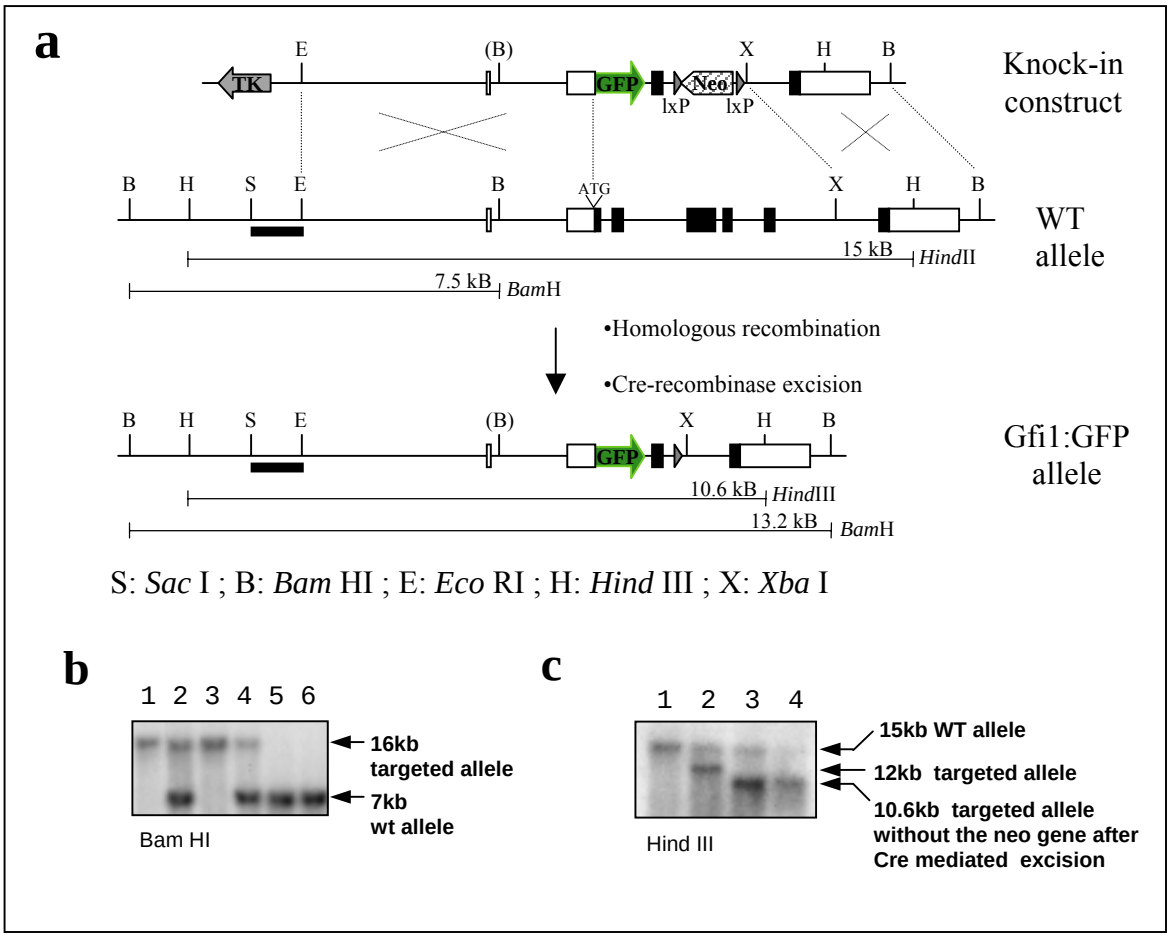

Figure 3. Generation of the Gfi1:GFP knock-in mutant (This figure was kindly provided by R. Yücel, Institut für Zellbiologie, Universitätsklinikum Essen)

a. Schematic representation of the targeting strategy to generate a Gfi1:GFP knock-in mutant. The neo cassette contains flox sites and was removed by crossing a Gfi1:GFP knock-in mouse with a general deleter strain carrying a CMV-Cre transgene. (TK: thymidin kinase)

b. Diagnostic Southern analysis for genotyping with the indicated 5' probe: lane 1: Gfi1 $^{GFP/GFP}$, lane 2: Gfi1$^{GFP/+}$, lane 3: Gfi1$^{GFP/GFP}$, lane 4: Gfi1$^{GFP/+}$, lanes 5 and 6: Gfi1$^{+/+}$.

c. Detection of the neo deletion after Cre expression. Lane 1: Gfi1$^{+/+}$, lane 2: Gfi1$^{GFPneo/+}$, lane 3 and 4: Gfi1$^{GFP/+}$.
(Gfi1$^{GFPneo/+}$: GFP allele with neo cassette)

As expected from experiments with previously described mice heterozygous for the Gfi1 allele (Karsunky et al., 2002a; Yücel et al., 2003), $Gfi1^{GFP/+}$ mice were indistinguishable from their WT littermates and from the previously described animals that carry a neo resistance marker gene in the Gfi1 locus disrupting one Gfi1 allele ($Gfi1^{+/-}$) (Karsunky et al., 2002a; Yücel et al., 2003; Yücel et al., submitted). Hematopoietic parameters of $Gfi1^{GFP/+}$ mice appeared normal, both with respect to developing populations as defined by surface marker expression and with respect to total thymocyte and bone marrow cell numbers. Thus, the results from $Gfi1^{GFP/+}$ mice reflected the situation under physiological conditions.

3.2 Gfi1 expression in adult mouse bone marrow hematopoietic cells

3.2.1 Gfi1 is expressed in myeloid cells but is absent in erythroid cells

To evaluate the expression of Gfi1 in various hematopoietic cell populations in adult mice, cells were harvested from several hematopoietic tissues in $Gfi1^{GFP/+}$ heterozygous animals and analyzed for GFP expression by flow cytometry. FACS analysis showed a single peak representing GFP-expressing cells in the thymus, lymph nodes and spleen, suggesting that most of the cells in these tissues express Gfi1 albeit at different levels. In contrast, in the bone marrow of adult mice there were consistently two populations of nucleated cells that expressed different levels of GFP, ranging from absent/low levels to high levels of expression compared to WT control (Figure 4). This indicated that Gfi1 gene is not expressed in all hematopoietic cell populations in adult mouse bone marrow.

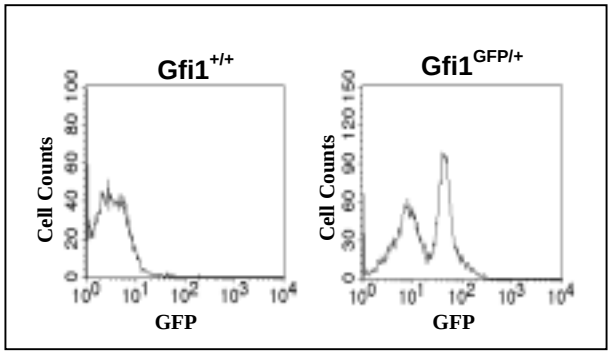

Figure 4. Gfi1 is heterogeneously expressed in adult mouse bone marrow hematopoietic cells

FACS analysis was performed on total nucleated bone marrow cells isolated from $Gfi1^{+/+}$ and $Gfi1^{GFP/+}$ mice. A representative FACS plot is shown for GFP expression in total nucleated bone marrow cells. Note that two peaks of GFP-expressing cells are present in $Gfi1^{GFP/+}$ mice.

To more closely examine the significance of the different GFP-expressing populations in the bone marrow, we analyzed Gfi1-GFP expression in various lineages by flow cytometry. As expected, GFP expression was not observed in $Ter119^{+}$ nucleated erythroid cells. Analysis of GFP expression in myeloid cells ($CD11b^{+}$) of bone marrow (Figure 5) revealed two subsets of GFP-expressing cells in immature mono-myeloid population ($CD11b^{+}Gr\text{-}1^{-/low}$), while a single positive subset of GFP-expressing cells existed in mature granulocyte population ($CD11b^{+}Gr\text{-}1^{high}$). These data were consistent with the results from previous experiments (Karsunky et al., 2002a; Hock et al., 2003), suggesting that Gfi1 is present in myeloid but absent in erythroid lineage cells.

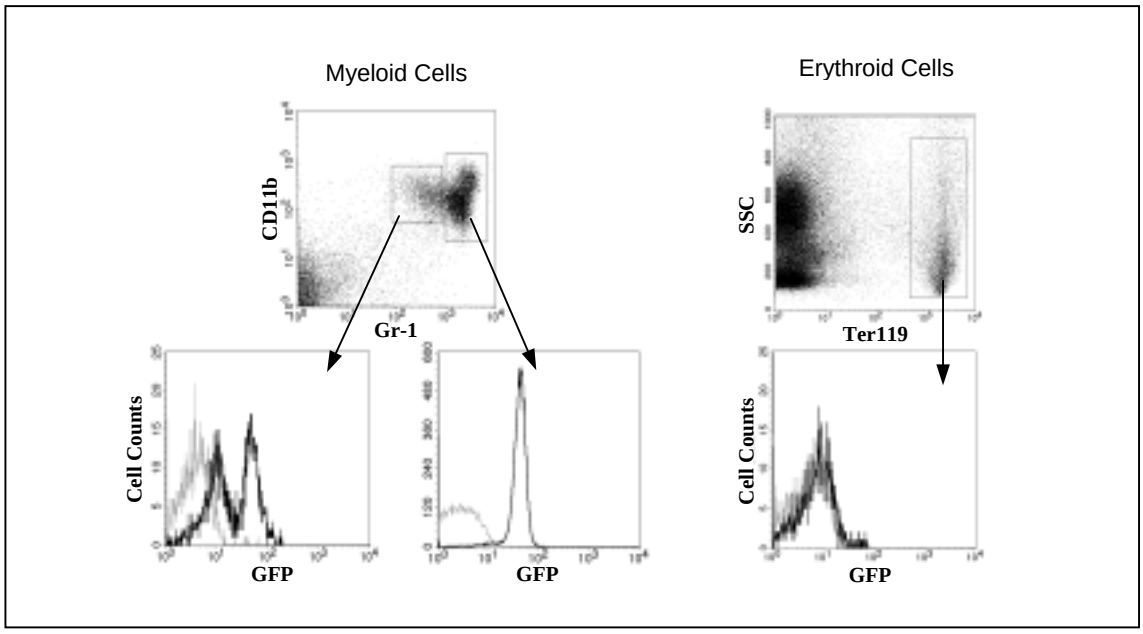

Figure 5. **Expression of Gfi1 in myeloid and erythroid cells**

Bone marrow cells were stained for the myeloid cell surface markers (CD11b and Gr-1) and erythroid cell surface marker (Ter119). Myeloid and erythroid subsets were gated respectively and analyzed for green fluorescence. $Gfi1^{+/+}$ histograms (gray line) were overlaid with $Gfi1^{GFP/+}$ histograms (black lines). GFP was highly expressed in granulocytes ($CD11b^{+}Gr\text{-}1^{high}$ cells) but absent in $Ter119^{+}$ nucleated erythroid cells, while two peaks of GFP-expressing cells was found in immature mono-myeloid population ($CD11b^{+}Gr\text{-}1^{-/low}$ cells).

3.2.2 Expression of Gfi1 in HSCs and subsets of hematopoietic progenitor cells

Gfi1 expression in hematopoietic stem cell and early progenitor compartments was analyzed by measuring the fluorescence of GFP in the $Gfi1^{GFP/+}$ mice. Using multiparameter flow cytometry and cell sorting, cells were isolated with surface marker expression patterns that discriminate among HSCs, CLPs, CMPs and more lineage-restricted GMPs and MEPs (Adolfsson et al., 2001; Christensen et al., 2001; Kondo et al., 1997; Akashi et al., 2000). A high intensity of green fluorescence was found in bone marrow $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) population which contains LT-HSCs, ST-HSCs and

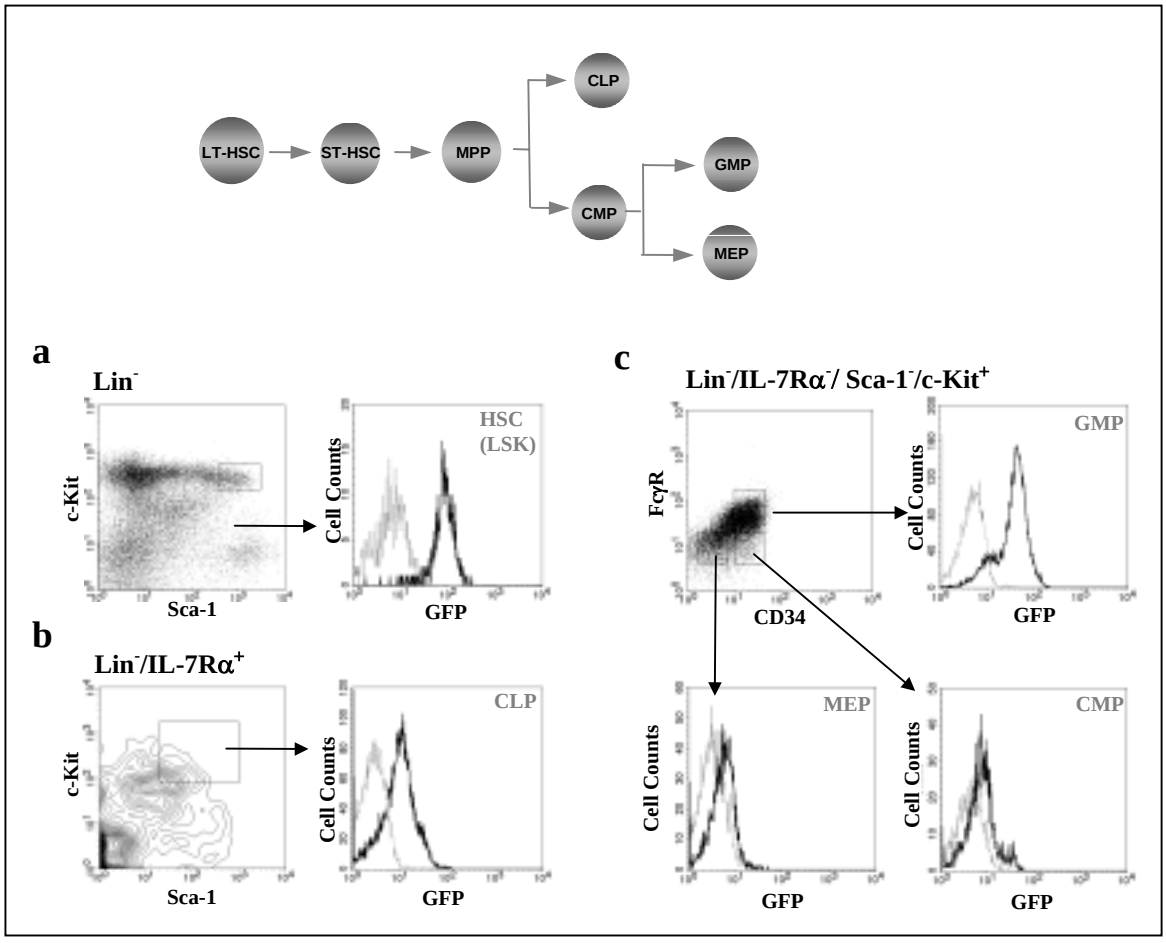

Figure 6. Expression of Gfi1 in hematopoietic stem cells and progenitors in adult mouse bone marrow

Electronically gated HSC and progenitor cell populations in $Gfi1^{GFP/+}$ mice were analyzed by measuring green fluorescence. $Gfi1^{+/+}$ histograms (gray line) were overlaid with $Gfi1^{GFP/+}$ histograms (black lines). a-c: Expression of GFP in HSCs (a), CLPs (b), CMPs, GMPs and MEPs (c) in $Gfi1^{GFP/+}$ mice.

MPPs (Adolfsson et al., 2001; Christensen et al., 2001), indicating that the Gfi1 gene is expressed in the entire HSC compartment (Figure 6a). Similarly, significant GFP expression was observed in CLPs ($Lin^-IL7R\alpha^+Sca\text{-}1^{low}c\text{-}Kit^{low}$ cells) (Figure 6b) and in GMPs ($Lin^-Sca\text{-}1^-IL\text{-}7R\alpha^-c\text{-}Kit^+CD34^+Fc\gamma R^{high}$) (Figure 6c) but not in MEPs, ($Lin^-Sca\text{-}1^-IL\text{-}7R\alpha^-c\text{-}Kit^+CD34^-Fc\gamma R^{low}$ cells) and CMPs ($Lin^-Sca\text{-}1^-IL\text{-}7R\alpha^-c\text{-}Kit^+CD34^+Fc\gamma R^{low}$ cells) (Figure 6c).

To confirm that GFP expression in these cells truly reflected Gfi1 gene expression, HSC and progenitor fractions were sorted from bone marrow of C57BL/6, Thy1.1 mice. 2000 cells for each population were used for RT-PCR analyses. The expression pattern of Gfi1 in HSCs and progenitors was completely concordant with GFP fluorescence in $Gfi1^{GFP/+}$ mice (Figure 7. This experiment was kindly performed by Dr. Karsunky, Standford University, USA).

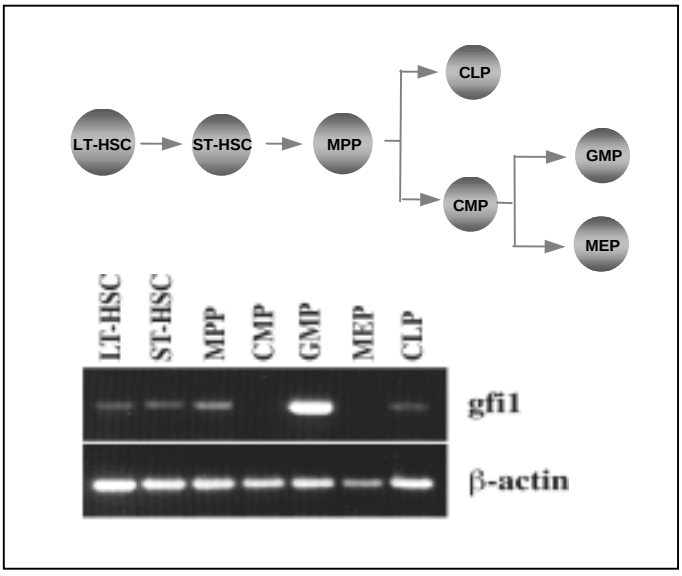

Figure 7. Expression analysis of Gfi1 in HSCs and progenitors by RT-PCR reaction

2000 HSCs, CMPs, GMPs, MEPs and CLPs were sorted from bone marrow of C57BL/6, Thy1.1 mice and used for RT-PCR analysis of Gfi1 expression. This experiment was kindly performed by Dr. H. Karsunky at Stanford University, USA.

3.3 Effect of Gfi1 deletion on the frequencies of HSCs and progenitors

3.3.1 Absence of Gfi1 results in a decrease of HSCs

To continuously produce end-stage hematopoietic cells, HSCs must be capable of self-renewal to maintain the HSC pool and their more mature progeny. Since expression of Gfi1 was found in bone marrow HSCs and progenitors, Gfi1 might play a role to maintain the number of HSCs and their downstream progenitors. To test whether the lack of Gfi1 resulted in a decrease in stem cell number in the basal state, the frequencies of HSCs and hematopoietic progenitors were analyzed in $Gfi1^{-/-}$ mice. Hematopoietic cells that lack expression of mature lineage markers (Lin^-) and that coexpress Sca-1 and the tyrosine kinase receptor c-Kit on their cell surfaces ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$, LSK) are highly enriched in HSC activity in normal murine bone marrow in steady-state hematopoiesis. Strikingly, $Gfi1^{-/-}$ mice showed a depletion of this LSK population in adult bone marrow (Figure 8, left panels and Figure 9a). Further analysis revealed a five-fold reduction of frequencies of HSCs with LSK phenotype in total $Gfi1^{-/-}$ bone marrow when compared to age matched WT littermates (0.1032±0.0139% and 0.0215±0.0052% for WT and $Gfi1^{-/-}$ respectively, n=8, $p<0.001$, Figure 9a).

Next, further studies were performed to analyze the LT-HSC population and ST-HSC/MPP population using additional markers such as CD34 and Flt3. LSK cells lacking CD34 or Flt3 expression are thought to represent a virtually pure HSC population in the bone marrow of adult mice (Adolfsson et al., 2001; Christensen et al., 2001; Goodell et al., 1997; Zhao et al., 2000). The fractions of LT-HSCs ($Lin^-Sca1^+c\text{-}Kit^+CD34^-$ or $Lin^-Sca1^+c\text{-}Kit^+Flt3^-$) and ST-HSCs/MPPs ($Lin^-Sca1^+c\text{-}Kit^+Flt3^+$) were dramatically reduced 2-4 fold and 10-fold respectively (Figure 9a), and $Lin^-Sca1^+c\text{-}Kit^+Flt3^{high}$ cells were almost completely depleted in $Gfi1^{-/-}$ bone marrow (Figure 8, right panels and Figure 9a), indicating that $Gfi1^{-/-}$ LT-HSCs may have a defect in self-renewal and/or in the generation of downstream primitive progenitors.

The absolute numbers of phenotypically defined HSCs were also clearly reduced in Gfi1 deficient mice, since the percentages of HSCs were reduced in $Gfi1^{-/-}$ mice while bone marrow cellularity was only slightly reduced in $Gfi1^{-/-}$ mice (Figure 9b).

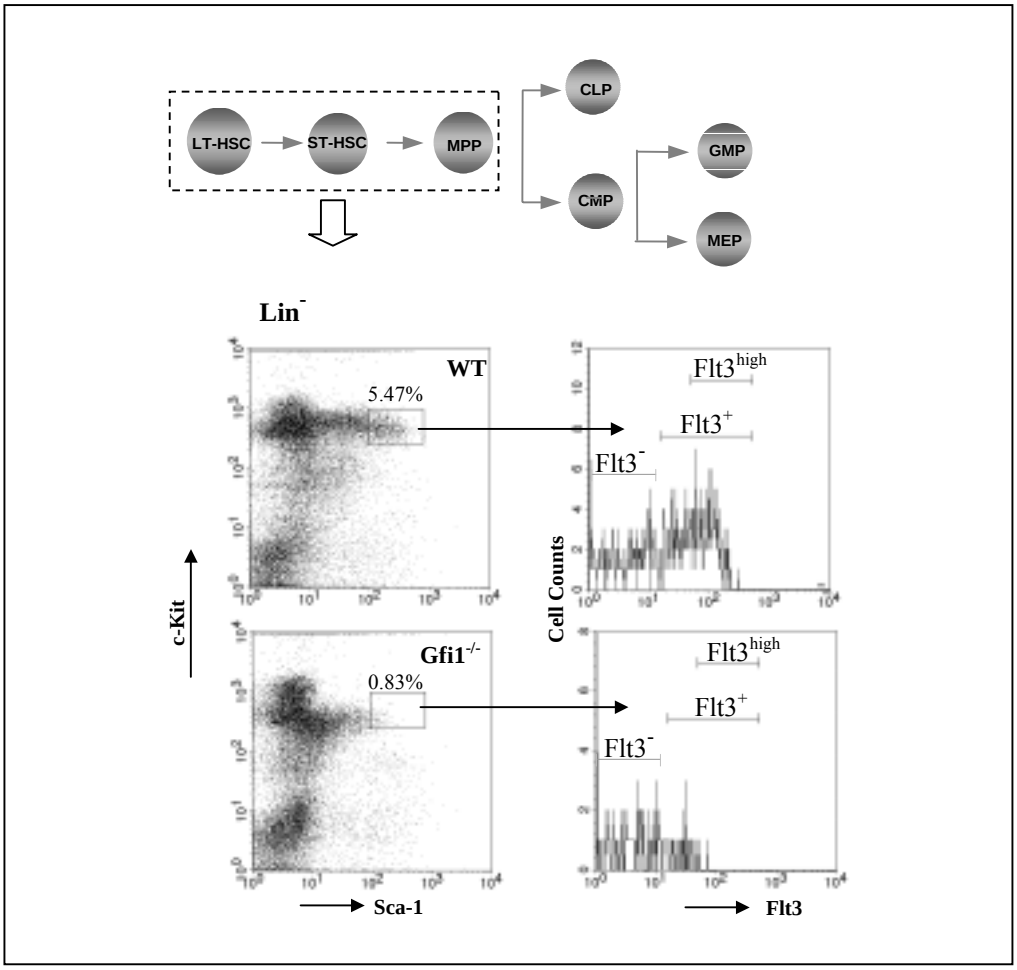

Figure 8. Flow cytometry analysis of HSC population in WT and Gfi1$^{-/-}$ mouse bone marrow

Bone marrow cells from WT and Gfi1$^{-/-}$ mice were stained with lineage antibody cocktail together with antibodies against Sca-1, c-Kit and Flt3. Representative FACS plots are shown for the comparison of frequencies of HSCs with Lin^{-}Sca1^{+}c-Kit^{+} (LSK) phenotype in total bone marrow between Gfi1$^{-/-}$ mice and WT littermates (left panels). In addition to a decrease of LSK population in Gfi1$^{-/-}$ bone marrow, the frequencies of Flt3^{+} in LSK cells were decreased. Lin^{-}Sca1^{+}c-Kit^{+}Flt3^{high} population was almost completely depleted in Gfi1$^{-/-}$ bone marrow (right panels).

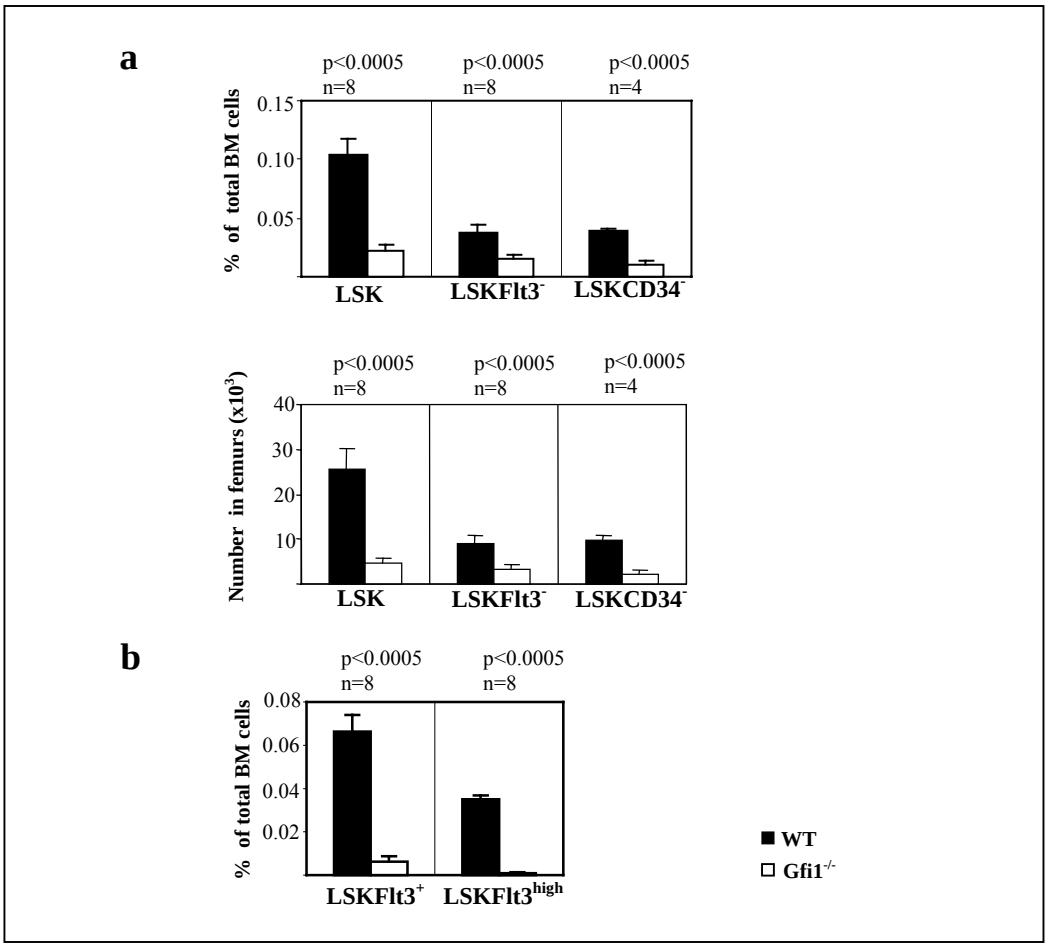

Figure 9. Numbers of HSCs are diminished in Gfi1$^{-/-}$ mouse bone marrow

a. Frequencies and absolute numbers of phenotypically defined HSC (LSK) and LT-HSC (LSKFlt3^{-}or LSKCD34^{-}) were calculated based on flow cytometry analysis and number of total bone marrow cells in femurs. Both HSC and LT-HSC population were reduced in Gfi1$^{-/-}$ bone marrow.

b. Frequencies of ST-HSC/MPP cells (LSKFlt3^{+}) and LSKFlt3^{high} population were dramatically decreased in Gfi1$^{-/-}$ bone marrow.

n: number of mice analyzed. P values were calculated using the Student's *t* test.

3.3.2 Alteration of the frequencies of hematopoietic progenitors

Since Gfi1$^{-/-}$ mice show defects in both lymphoid and myeloid lineages (Karsunky et al., 2002a; Yücel et al., 2003, Hock et al., 2003), it was of interest to investigate whether these defects were related to an alteration of development of relevant hematopoietic progenitors. As far as it is known to date, the segregation into CLP and CMP represents the earliest known HSC commitment step, and the CLP and CMP represent the earliest

stage of lymphoid and myeloid restricted development, respectively. A significant decrease of both CLP and CMP frequencies was observed in Gfi1$^{-/-}$ mouse bone marrow compared to the respective compartments in WT mice (Figure 10 and Figure 11). Strikingly, the over 40-fold reduction of CLPs (which express Gfi1) in Gfi1$^{-/-}$ mice was much more dramatic than the 2-fold reduction of CMPs (which do not express Gfi1) (Figure 10 and Figure 11).

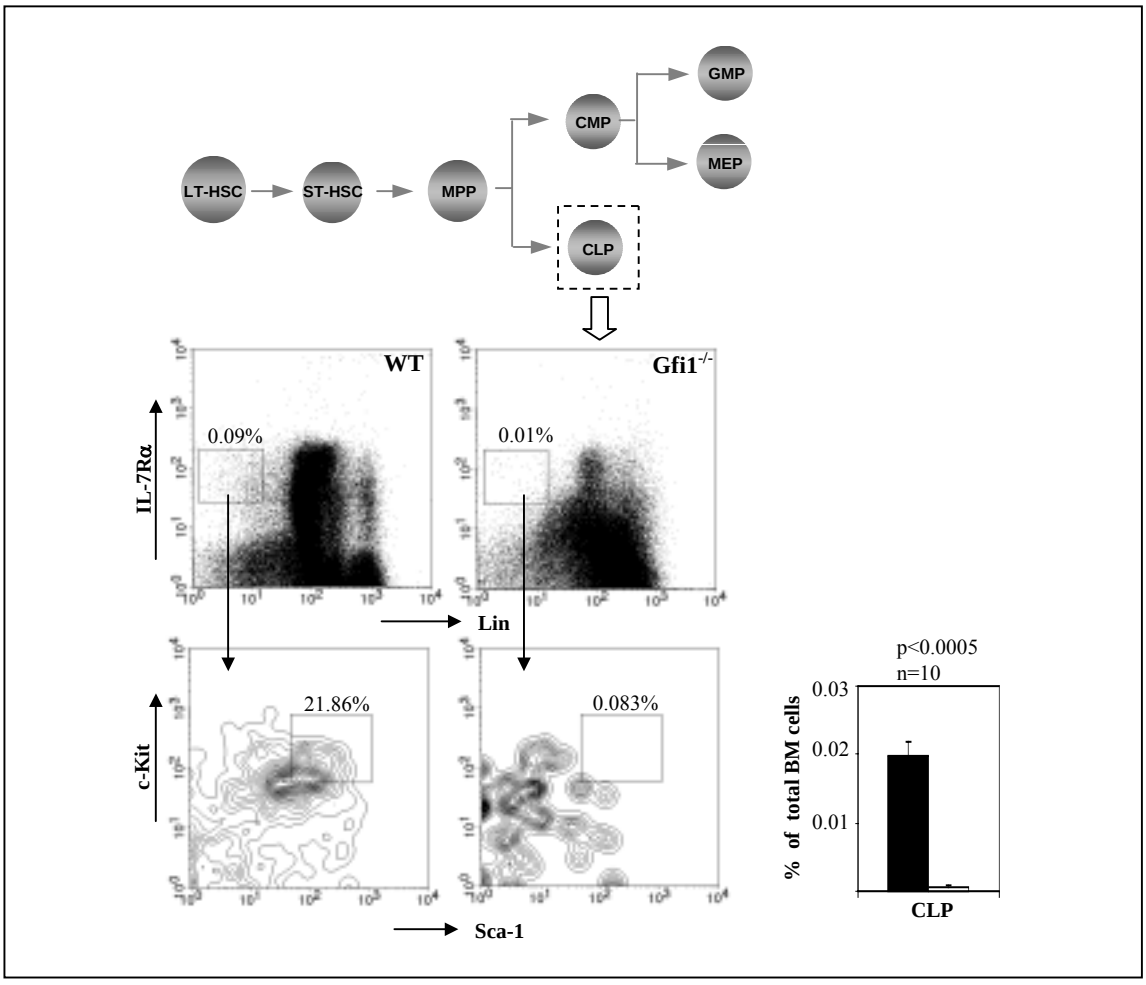

Figure 10. Flow cytometry analysis of CLP population in WT and Gfi1$^{-/-}$ mouse bone marrow

Bone marrow cells from WT control and Gfi1$^{-/-}$ mice were stained with antilineage cocktail together with antibodies against Sca-1, c-Kit and IL-7Rα. Representative FACS plots are shown for the comparison of frequencies of CLPs (defined by the Lin^{-}IL-7Rα^{+}Sca-1^{low}c-Kit^{low} phenotype) in total bone marrow cells between Gfi1$^{-/-}$ mice and WT littermates.
n: number of mice analyzed. P values were calculated using the Student's *t* test.

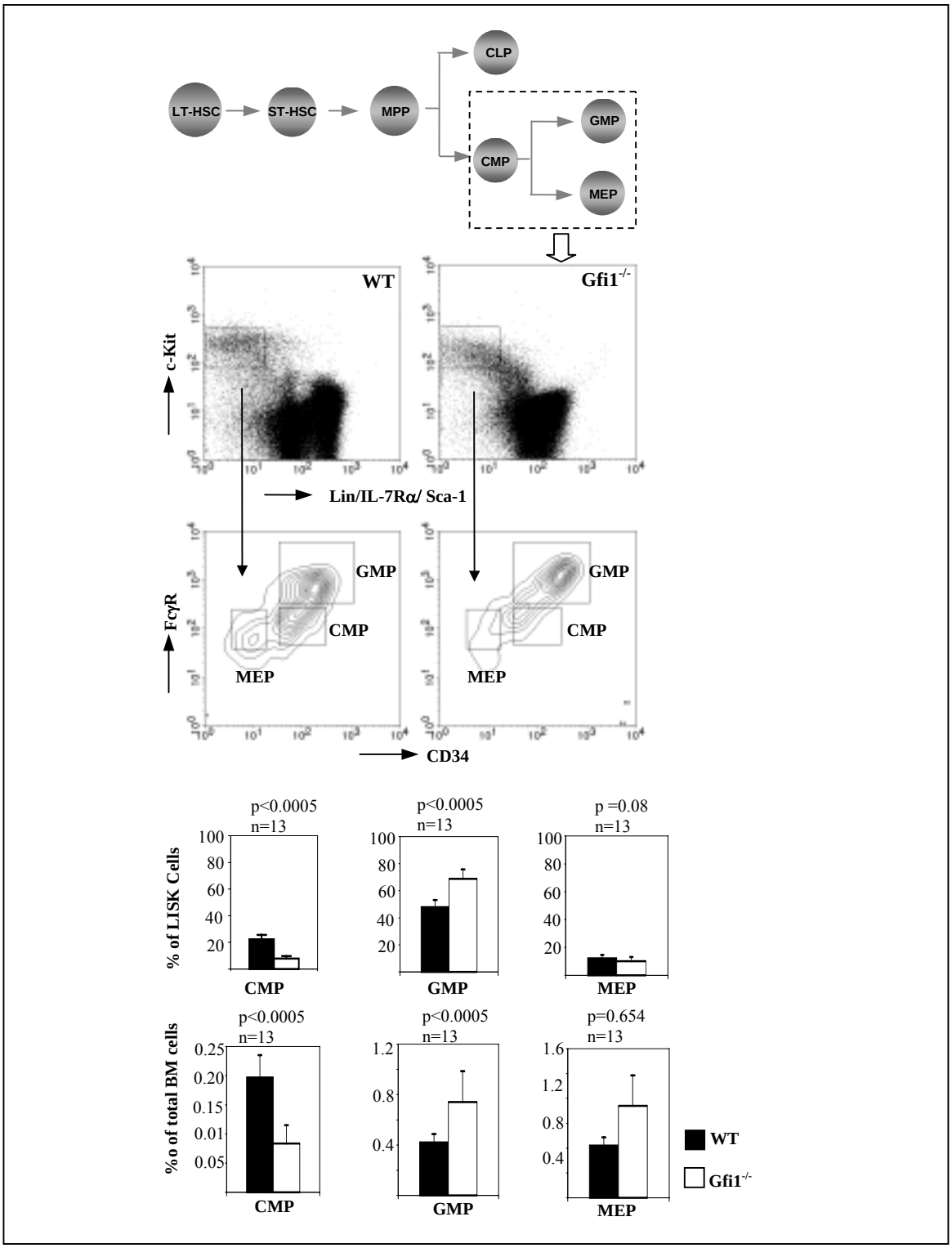

Figure 11. CMP and GMP but not MEP cell numbers are altered in Gfi1 deficient mice

Frequencies of CMP, GMP and MEP in WT and Gfi1$^{-/-}$ mouse bone marrow population were analyzed by flow cytometry by staining with antilineage cocktail together with antibodies against Sca-1, c-Kit, IL-7Rα, CD34 and Fcγ receptor. Gfi1 deficient mice show a decrease of the CMP population but an increase of the GMP population.
n: number of mice analyzed. P values were calculated using the Student's *t* test.

In contrast to reduced frequencies of HSCs, CMPs and CLPs, the frequency of GMPs was not reduced in Gfi1 deficient mice, but rather showed a significant increase with regard to the $Lin^{-}Sca\text{-}1^{-}IL\text{-}7R\alpha^{-}c\text{-}Kit^{+}$ population and also with respect to total bone marrow cell numbers compared to the respective compartments in WT mice. As expected, the frequency of MEPs (which do not express Gfi1) was not altered in Gfi1 deficient mice compared to WT littermates (Figure 11).

3.4 The homozygous $Gfi1^{GFP/GFP}$ mice are functional Gfi1 knock-outs

In contrast to $Gfi1^{GFP/+}$ mice which do not show any detectable hematopoietic defects, homozygous $Gfi1^{GFP/GFP}$ mice which were unable to produce the Gfi1 protein (Yücel et al., submitted) showed the same typical phenotypes of Gfi1 deficient animals such as monocytosis and neutropenia (Karsunky et al., 2002a; Hock et al., 2003) (Figure 12 a and b), loss of thymocytes and developmental defects during T cell differentiation (data not shown, Yücel et al., 2003 and submitted), and also a depletion of the LSK population in bone marrow (Figure 12c).

3.5 Defects of $Gfi1^{-/-}$ HSCs and progenitors are cell autonomous

To confirm that Gfi1 deficient HSCs have an intrinsic defect and to exclude influences of a potentially defective bone marrow microenvironment in $Gfi1^{-/-}$ mice, an excess of Gfi1 deficient bone marrow cells ($CD45.2^{+}$) were transplanted into lethally irradiated WT hosts ($CD45.1^{+}$). The phenotype of Gfi1 deficient mice with regard to frequencies of stem cells and progenitors, or previously reported hallmarks (neutropenia, accumulation of monocytic cells and reduced numbers of thymocytes (Karsunky et al., 2002a; Yücel et al., 2003; Hock et al., 2003) could be exactly reproduced in the transplanted hosts (Figure 13), suggesting that the hematopoietic defects in $Gfi1^{-/-}$ mice should be cell autonomous.

Vice versa, $CD45.1^{+}$ WT bone marrow cells were transplanted into lethally irradiated Gfi1 deficient mice ($CD45.2^{+}$) to test whether Gfi1 deficient mice have intact bone marrow microenvironment. Normal numbers of HSCs, progenitors, neutrophils and

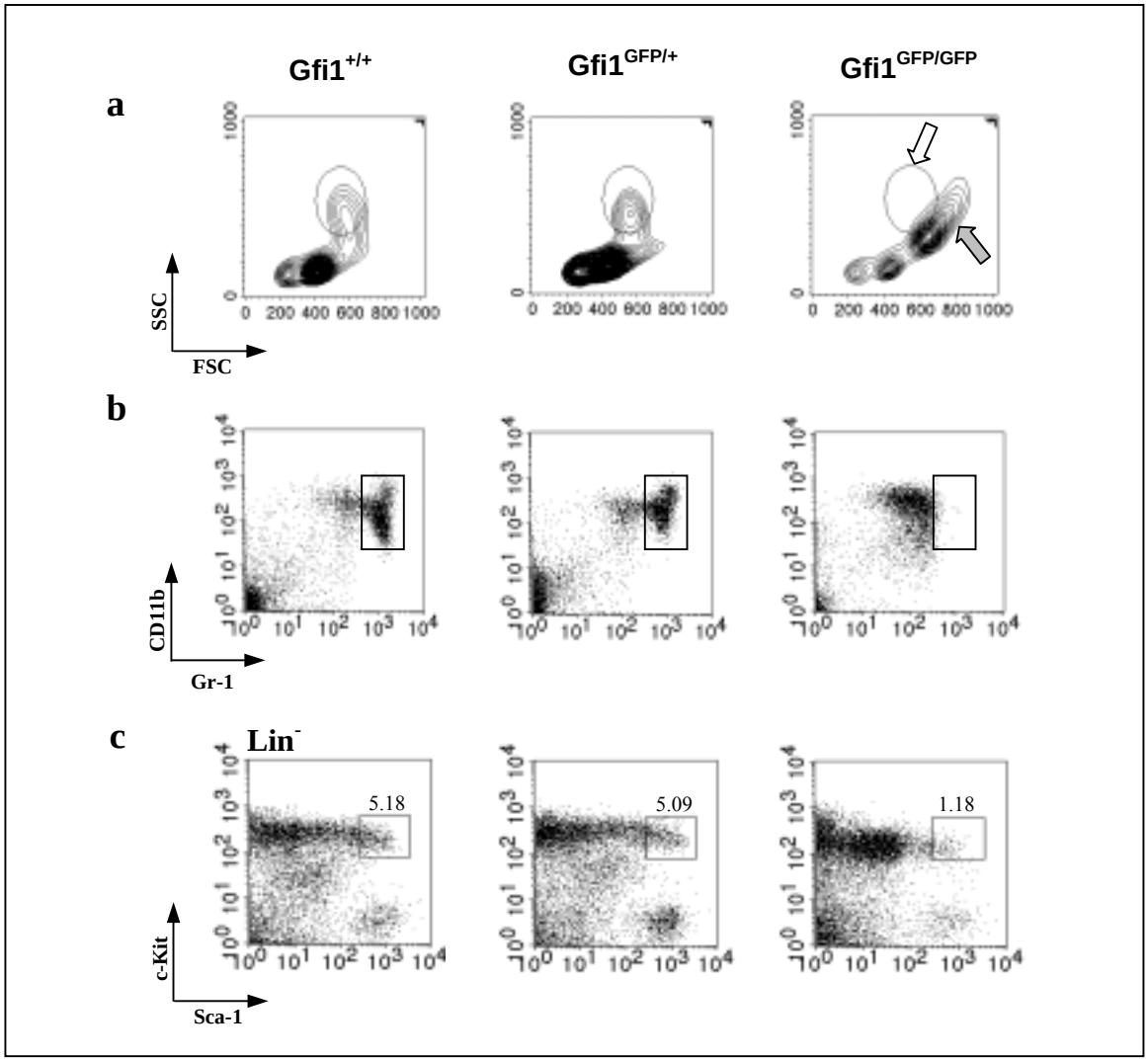

Figure 12. The homozygous Gfi1$^{GFP/GFP}$ mice showed the typical phenotype of Gfi1$^{-/-}$ animals

- **a.** Forward (FSC) and side (SSC) light-scatter of bone marrow cells showed absence of a cell population with granulocyte characteristic (open arrow) and presence of an abnormal myelo-monocytic population (filled arrow) in Gfi1$^{GFP/GFP}$ mice compared to control mice.
- **b.** Flow cytometric analysis of bone marrow confirmed that mature granulocytes, characterized by high levels of the surface markers CD11b and Gr-1 were barely detectable in Gfi1-deficient mice. In contrast, the number of apparently immature myelo-monocytic cells (CD11b^{+}Gr-1$^{-/low}$) was increased in Gfi1$^{GFP/GFP}$ bone marrow.
- **c.** A depletion of LSK population was detected in Gfi1$^{GFP/GFP}$ bone marrow.

normal numbers of thymocytes were found in the host 4 months after transplantation (Figure 14). This indicated that stroma cells and the environment in Gfi1 deficient bone marrow are functional and lack any detectable defects, and confirmed that the defect of Gfi1$^{-/-}$ HSCs and progenitors must be cell autonomous.

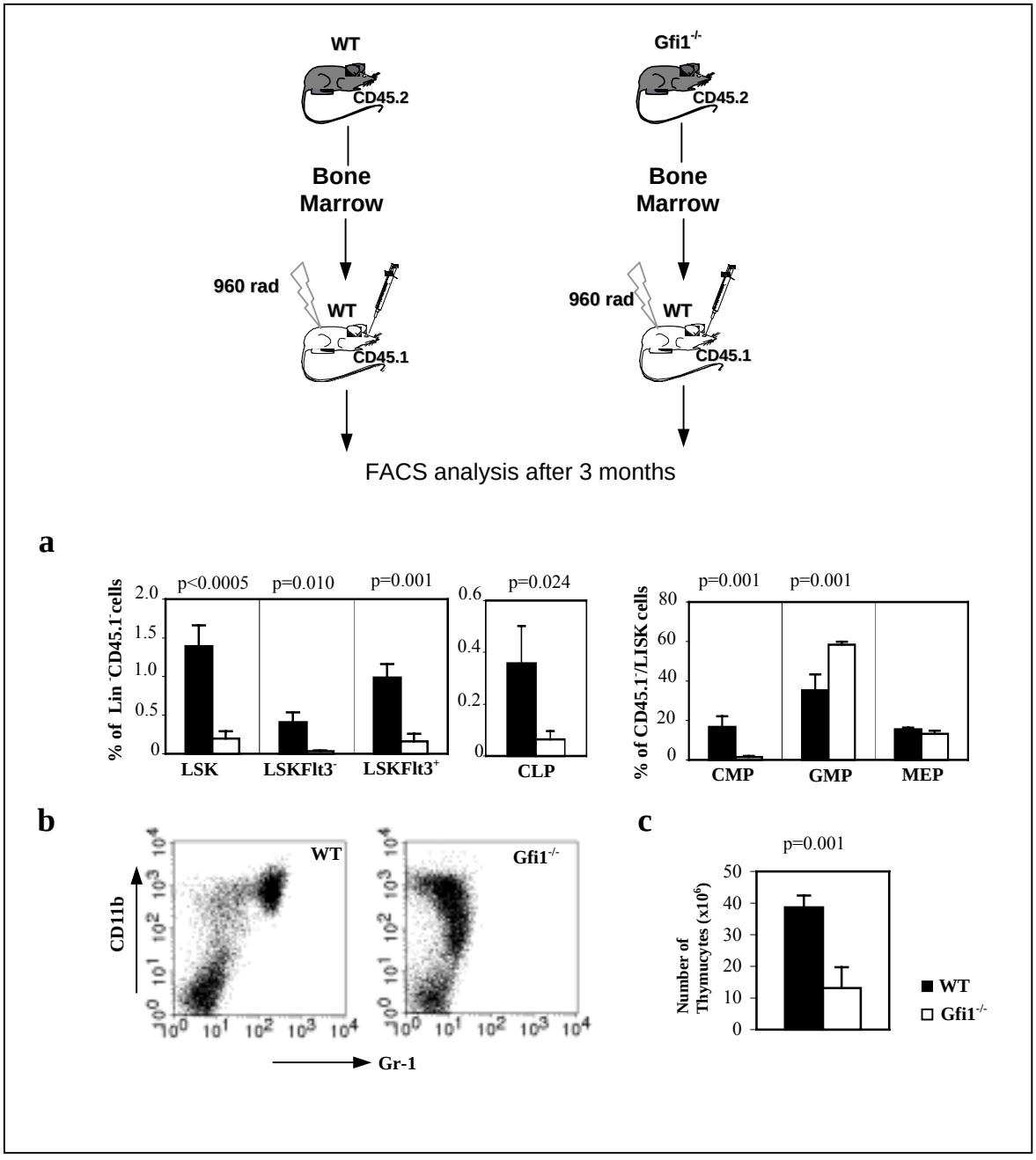

Figure 13. Defects in Gfi1$^{-/-}$ mice are cell autonomous

4x10^6 WT or Gfi1$^{-/-}$ bone marrow cells were injected into lethally irradiated CD45.1 mice (n=4 for each group). 4 months after transplantation, the frequencies of HSCs and early progenitors in recipients' bone marrow derived from donor bone marrow cells (CD45.2$^+$) were measured by flow cytometry. The phenotype of Gfi1 deficient mice with regard to frequencies of stem cells and progenitors (a), neutropenia (b) and reduced numbers of thymocytes (c) could be exactly reproduced in the transplanted hosts.
(LISK: Lin$^-$Sca-1$^-$IL-7Rα^-c-Kit$^+$).

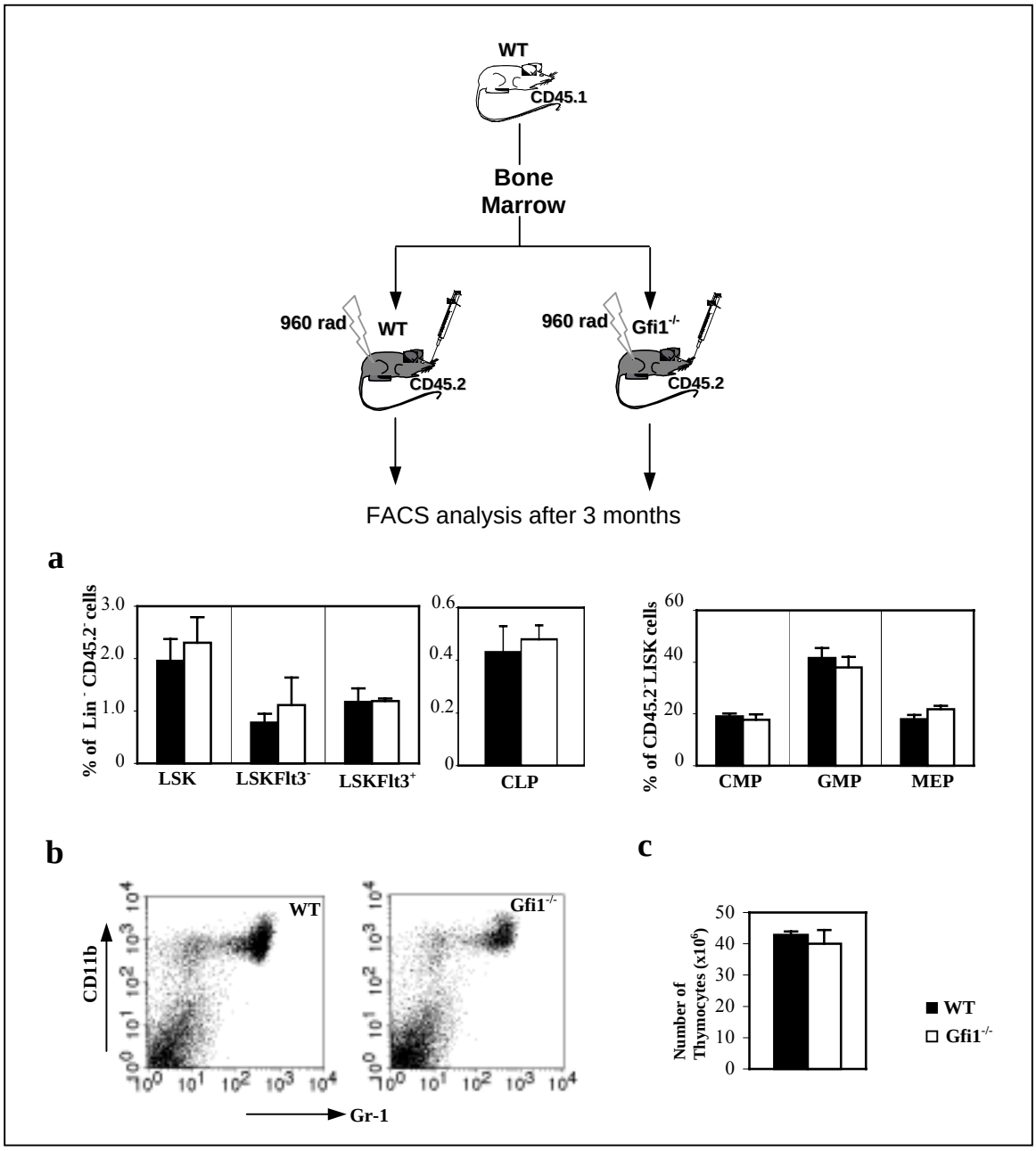

Figure 14. No defects were detected in Gfi1$^{-/-}$ bone marrow stromal environment

3x10^6 CD45.1 WT bone marrow cells were injected into lethally irradiated WT or Gfi1$^{-/-}$ mice (both CD45.2, n=3 to 4 for each group). 4 months after transplantation, the frequencies of HSCs and early progenitors derived from donor bone marrow (CD45.1$^+$) were measured. The frequency of HSCs and progenitors (a), the development of granulocytes (b) and the number of thymocytes (c) were normal, indicating that the microenvironment of Gfi1$^{-/-}$ bone marrow lacks detectable defects.
(LISK: Lin$^-$Sca-1$^-$IL-7Rα^-c-Kit$^+$).

3.6 Alteration of Gfi1$^{-/-}$ hematopoietic progenitor frequencies

3.6.1 Reduction in numbers of day 8 CFU-S but not day 12 CFU-S in Gfi1$^{-/-}$ mice

To confirm the above described findings from the phenotypic analysis of HSCs and progenitors, and to assess the function of HSCs and more committed myeloid progenitors of Gfi1$^{-/-}$ mice, CFU-S assays were performed. As shown in Figure 15 and 16, both the numbers and the size of CFU-S_{12} (which are derived from both the HSC/MPP compartment and from the CMP/MEP populations) were significantly reduced in Gfi1$^{-/-}$ bone marrow transplanted hosts (204$\pm$50.7 and 82$\pm$25.6 per 10^6 bone marrow cells respectively) (Figure 15 and 16). In contrast, CFU-S_8 which are mainly derived from

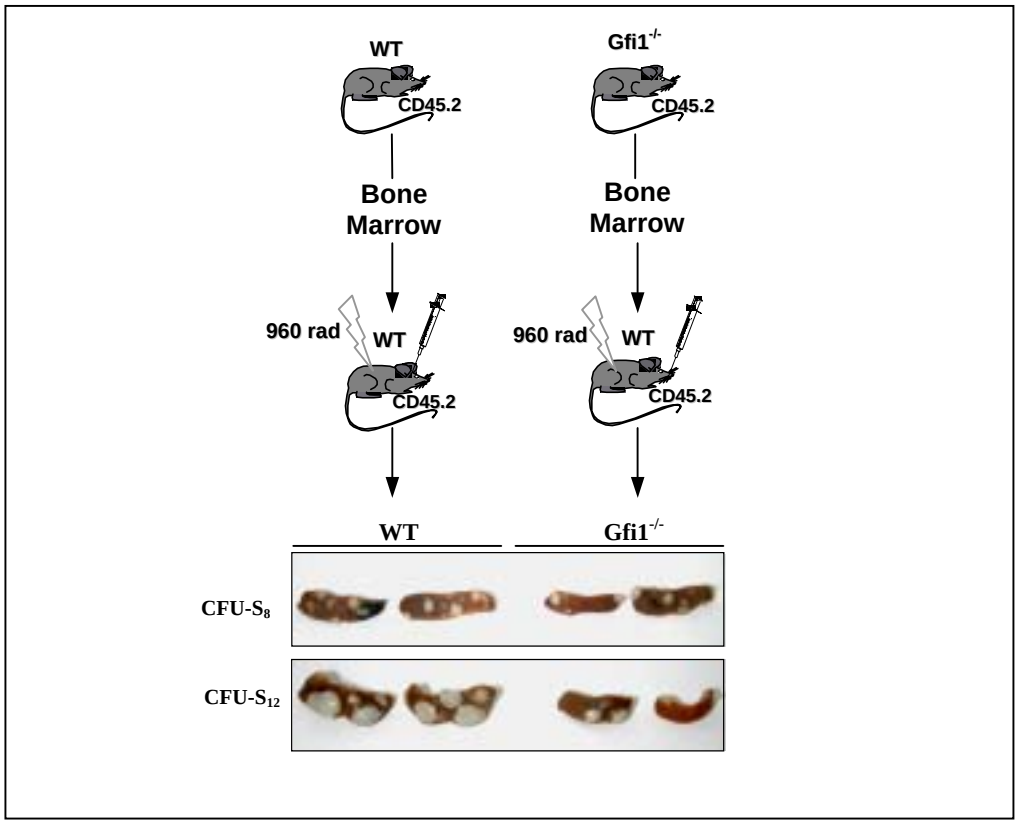

Figure 15. Measures the frequency and functional property of primitive progenitors with *in vivo* spleen colony forming unit (CFU-S) assays

CFU-S assay is an *in vivo* clonogenic method that measures the frequency and functional property of primitive progenitors. 5x10^4 WT or Gfi1$^{-/-}$ bone marrow cells were injected into lethally irradiated (9.6Gy) wild-type recipient mice (10 mice each group). Mice were sacrificed 8 days or 12 days after the injection, and their spleens were fixed for macroscopic examination. Spleen colonies are shown. Note that the size of day 12 CFU-S was significantly reduced in Gfi1$^{-/-}$ bone marrow transplanted hosts.

MEPs, remained unaltered with regard to numbers and size in irradiated hosts after transplantation of WT or Gfi1$^{-/-}$ bone marrow (174±28.2 and 137.3±15.5 per 10^6 bone marrow cells, respectively) (Figure 15 and 16). These results were consistent with phenotypically defined results of ST-HSCs, MPPs, CMPs and MEPs. Thus, the lack of Gfi1 affected the production of primitive hematopoietic progenitors but not the formation of megakaryocyte/erythrocyte progenitors.

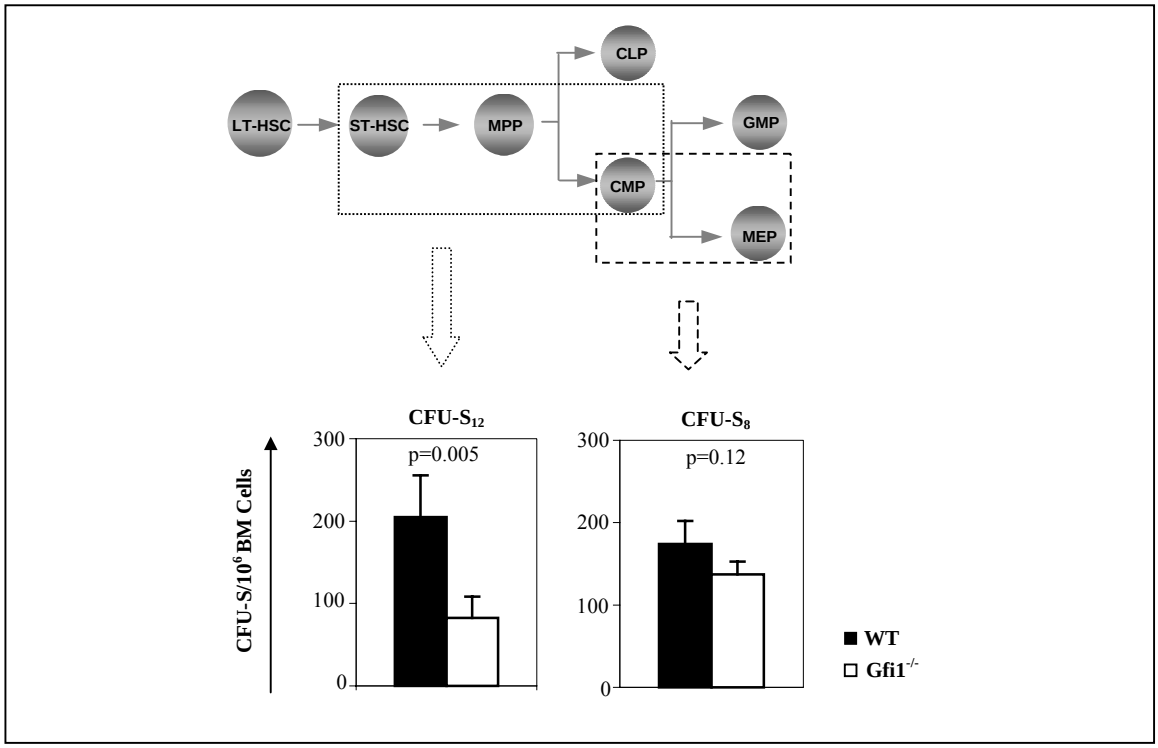

Figure 16. Reduction in CFU-S_{12} but not in CFU-S_8 in Gfi1 deficient mice

CFU-S_{12} are derived from primitive progenitors and that CFU-S_8 are mainly derived from relatively late myeloerythroid-committed progenitors. Numbers of spleen colonies generated upon injection of WT or Gfi1$^{-/-}$ bone marrow cells are shown. The data represent the mean of CFU-S numbers wit a SD determined from 3 independent experiments (n=10 for each group).

3.6.2 The short-term radioprotection capabilities of Gfi1$^{-/-}$ bone marrow

Consistent with this decrease in CFU-S_{12}, the short-term radioprotection capacity of Gfi1$^{-/-}$ bone marrow cells was also compromised. While the transplantation of 2 x 10^5 WT bone marrow cells led to a complete rescue of lethally irradiated recipients (Figure 17), the same number of Gfi1$^{-/-}$ bone marrow cells protected less well and about 1/3 of the

animals died from the consequences of irradiation within 35 days. However, transplantation of 1 x 10^6 Gfi1$^{-/-}$ bone marrow cells could provide almost full protection against irradiation (Figure 17), suggesting that the defect in the short-term radioprotection capacity of Gfi1$^{-/-}$ bone marrow cells was mainly due to the depletion of ST-HSC, MPP and CMP populations.

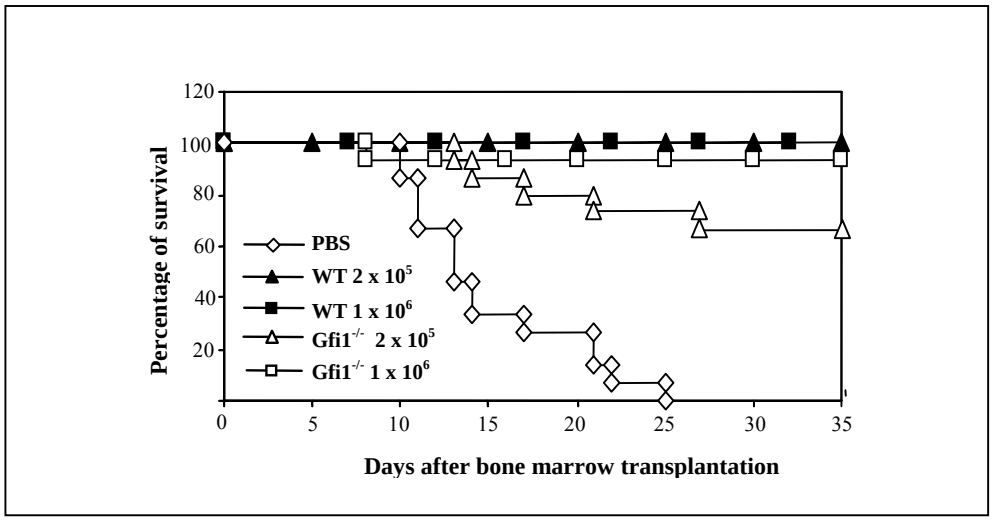

Figure 17. Short-term radioprotection capacity of Gfi1$^{-/-}$ bone marrow cells is moderately compromised

Survival graph of lethally irradiated mice reconstituted with various amount of WT or Gfi1$^{-/-}$ bone marrow cells, or PBS as control (15 mice per group). Short-term radioprotection capacity of Gfi1$^{-/-}$ bone marrow cells is moderately compromised.

3.6.3 Colony assays of granulocyte-monocyte progenitors

The defect of GMP in Gfi1 deficient mice cannot be assessed by the CFU-S assay and radioprotection assay. To test whether elevated frequency of phenotypically defined GMP was associated with an elevated frequency of granulocyte-monocyte colony forming cells, the colony-forming ability of Gfi1$^{-/-}$ hematopoietic progenitor cells was examined in semisolid medium. Treatment with IL-3 and GM-CSF elicited a significantly higher number of colonies from Gfi1$^{-/-}$ bone marrow cells than from WT bone marrow cells, even at reduced cytokine concentration (Figure 18, upper panels). This indicated that increased numbers of early myeloid progenitor cells are present in Gfi1$^{-/-}$ mice.

The specific cytokine sensitivity of myeloid precursor cells can be derived from dose-response curves obtained by plotting the number of colonies against the concentrations of the factor under examination. Dose-response curves for colony formation stimulated by IL-3 and GM-CSF were almost identical for Gfi1$^{-/-}$mice and WT controls (Figure 18, lower panels), suggesting that the increased numbers of colonies observed with Gfi1$^{-/-}$ cells are not the result from altered cytokine sensitivity of Gfi1$^{-/-}$ myeloid precursor cells.

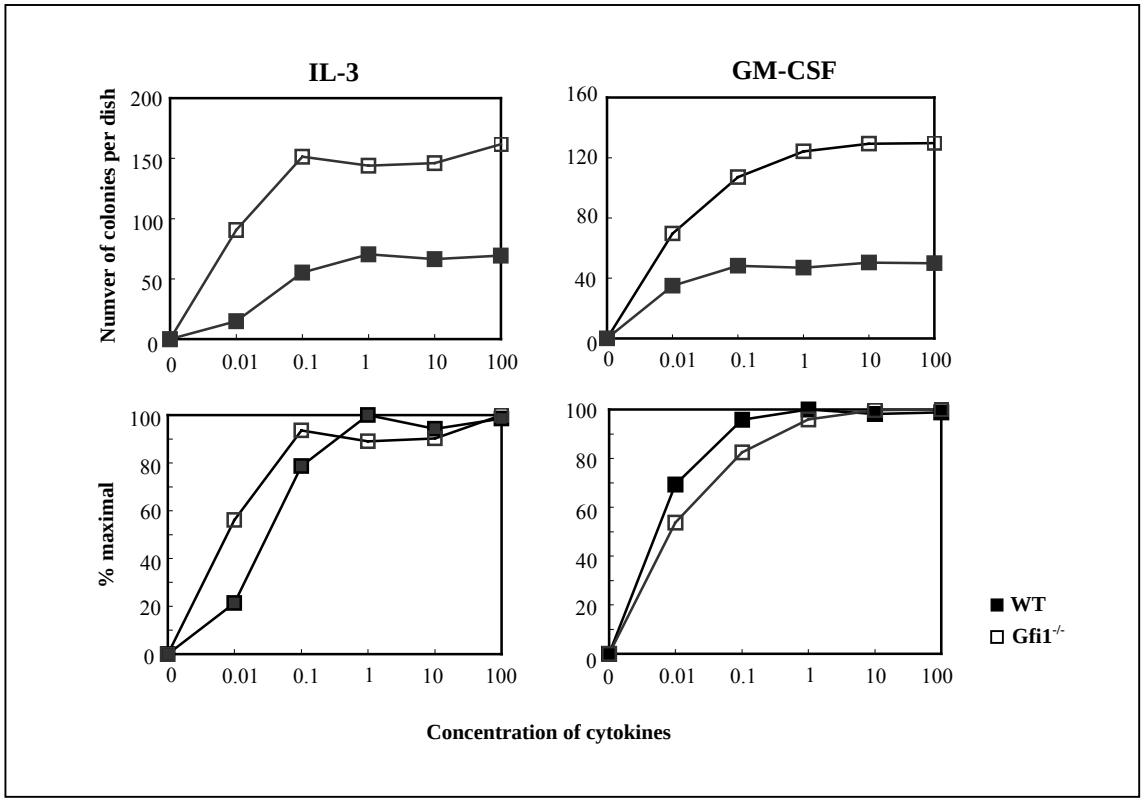

Figure 18. Analysis of myeloid progenitors of Gfi1-deficient mice

Single-cell suspensions from Gfi1$^{-/-}$ bone marrow (closed squares) or from WT bone marrow (open squares) were plated in methylcellulose in the presence of the indicated amounts of IL-3 and GM-CSF. Upper panels show numbers of colonies per dish in response to increasing cytokine concentration. Lower panels show dose–response curves of maximal colony numbers reached per dish at a given cytokine concentration. The results are representative for at least five independent experiments each with individual animals. Cytokine concentration is in ng/ml.

3.7 Decreased long-term reconstitution capacity of Gfi1$^{-/-}$ HSCs

The capability for self-renewal over the entire lifetime of an organism, the fostering of multilineage hematopoiesis and the potency to reconstitute multilineage hematopoiesis upon transplantation in a foreign host are the most important features of HSCs. Although our phenotypic analysis suggested that the HSC pool size is significantly reduced in Gfi1deficient mice, the depletion of stem cells under normal homeostatic conditions may not reflect a capacity to self-renew under conditions of stress. Thus, the function of HSCs can only be assessed by *in vivo* reconstitution assays such as bone marrow transplantation.

3.7.1 Inadequate self-renewal of Gfi1$^{-/-}$ HSCs

Since the Gfi1$^{-/-}$ bone marrow cells exhibited moderate decrease of radioprotection ability in the primary recipients, it was of interest to determine whether these findings would be more predominant upon further proliferative stress after transplantation to secondary recipients. 1×10^6 bone marrow cells from WT or Gfi1$^{-/-}$ mice were transplanted into lethally irradiated WT mice. 3 months later, secondary transplantations were performed with 1 x 10^6 bone marrow cells from primary recipients which had previously received either 1 x 10^6 WT or Gfi1$^{-/-}$ bone marrow cells. Within six months, four of ten secondary recipients that received bone marrow from primary recipients transplanted with Gfi1$^{-/-}$ cells died. In contrast, all secondary recipients injected with cells from primary recipients previously transplanted with WT bone marrow survived this six-month period. This suggested that a lack of Gfi1 is associated with a defect of the repopulation ability of HSCs.

3.7.2 Decreased competitive reconstitution capacity of Gfi1$^{-/-}$ HSCs

To further determine the long-term reconstituting abilities of Gfi1 deficient bone marrow cells, WT or Gfi1$^{-/-}$ bone marrow cells (both CD45.2$^+$) were mixed at a 1:1 ratio with competitor CD45.1$^+$ bone marrow cells. The mixtures were transplanted into irradiated CD45.1$^+$ recipients and myeloid and lymphoid reconstitution was measured by FACS analysis of peripheral blood over a period of 22 weeks. WT bone marrow cells

successfully reconstituted both myeloid and lymphoid lineages in irradiated recipients, whereas Gfi1$^{-/-}$ bone marrow cells showed a dramatically decreased capacity to reconstitute myeloid or lymphoid hematopoiesis (Figure 19, upper panels). The possibility could be excluded that the above defect is simply due to the reduction of phenotypically defined HSCs in Gfi1$^{-/-}$ bone marrow, because a 10:1 mixture of Gfi1$^{-/-}$ bone marrow with CD45.1 competitor cells which provided a large excess of phenotypically defined Gfi1$^{-/-}$ HSCs also failed to compete with CD45.1 competitor cells to reconstitute hematopoiesis in recipient mice in both myeloid and lymphoid lineages (Figure 19, lower panels).

To exclude the influences of the altered lineage profiles (monocytosis, neutropenia and lymphocytopenia) or a simple dilution effect in Gfi1$^{-/-}$ bone marrow, 500 sorted WT or Gfi1$^{-/-}$ LT-HSC cells (CD45.2^{+}Lin^{-}Sca1^{+}kit^{+}Flt3^{-}) were mixed with 2x10^{5} of competitor CD45.1^{+} bone marrow cells for competitive transplantation. Gfi1$^{-/-}$ HSCs were unable to foster the outgrowth of significant numbers of myeloid or lymphoid cells in irradiated hosts up to 22 weeks after transplantation (Figure 20a). Moreover, sorted Gfi1$^{-/-}$ LT-HSCs were also unable to give rise to any LSK cells upon transplantation, whereas 500 sorted WT cells could indeed generate LSK cells in a transplanted host (Figure 20b). Such a direct determination of HSC ratios in reconstituted animals showed that the competitive disadvantage starts at the level of HSC.

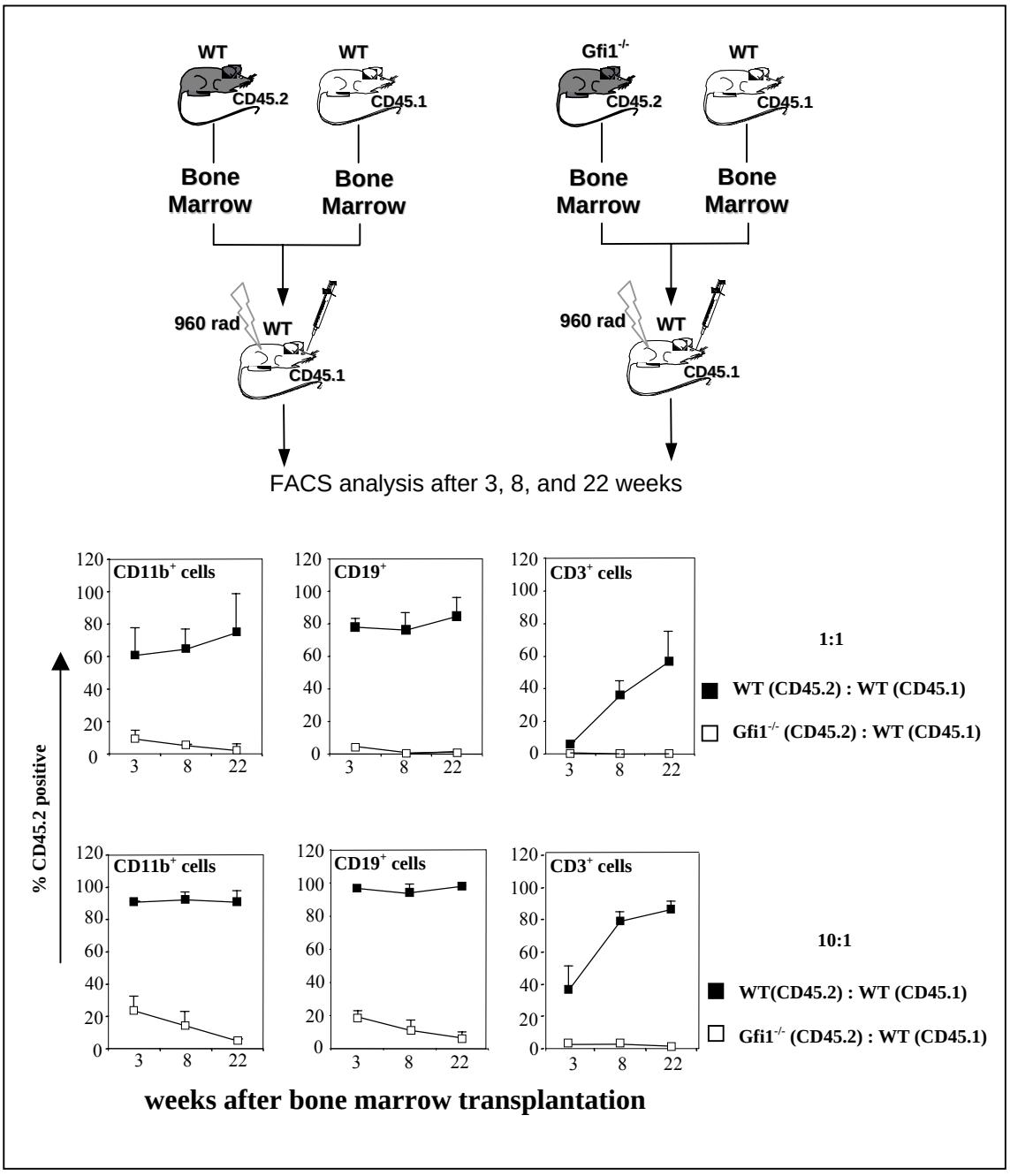

Figure 19. Competitive repopulation assay with WT and Gfi1$^{-/-}$ bone marrow cells

2x10^5 WT or Gfi1$^{-/-}$ bone marrow cells (CD45.2$^+$) were mixed with the same number of competitor CD45.1$^+$ bone marrow cells and were injected into lethally irradiated CD45.1$^+$ mice (n=4). Peripheral blood was analyzed at various times after reconstitution for WT or Gfi1$^{-/-}$ bone marrow cells (CD45.2$^+$)-derived myeloid, B-lymphoid and T-lymphoid cells (upper panels marked 1:1). The same experiments were performed using 2x10^6 WT or Gfi1$^{-/-}$ bone marrow cells (CD45.2$^+$) mixed with 2x10^5 of competitor CD45.1$^+$ bone marrow cells. After injection into lethally irradiated CD 45.1$^+$ mice (n=4) peripheral blood was analyzed as indicated above (lower panels marked 10:1).

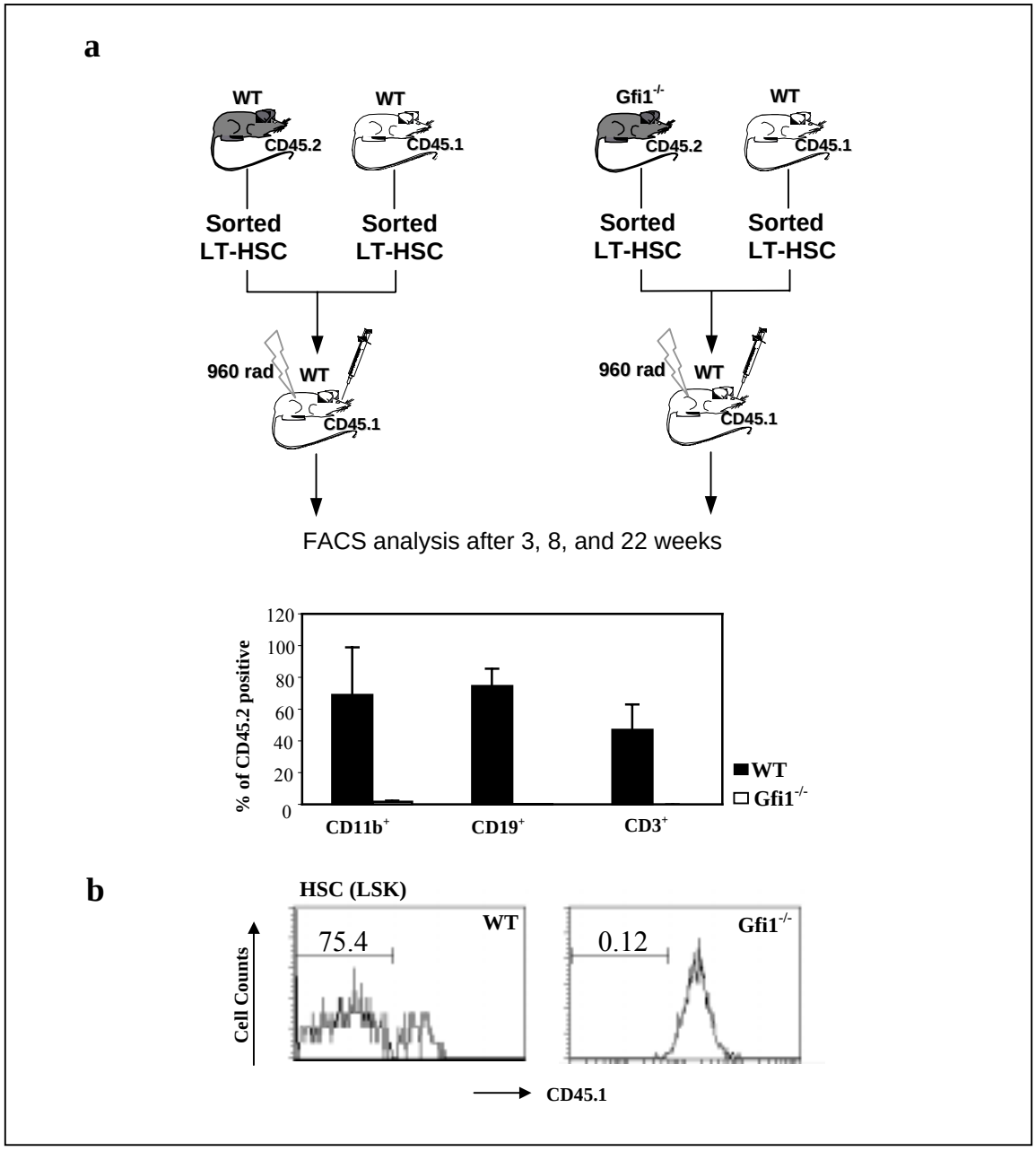

Figure 20. Competitive repopulation assay with sorted WT and Gfi1$^{-/-}$ LT-HSCs

500 sorted WT or Gfi1$^{-/-}$ LT-HSC cells (CD45.2^{+}) were mixed with 2x10^{5} of competitor CD45.1^{+} bone marrow cells and injected into lethally irradiated CD 45.1^{+} mice (n=4).

a. Peripheral blood was analyzed 22 weeks after reconstitution for WT or Gfi1$^{-/-}$ bone marrow cells (CD45.1^{-}) -derived myeloid, B-lymphoid and T-lymphoid cells.

b. Bone marrow cells were analyzed 6 months after reconstitution to measure the percentage of LSK cells derived from WT or Gfi1$^{-/-}$ LT-HSCs. Representative diagrams show the contribution of donor derived HSCs (CD45.1^{-} LSK) in WT and Gfi1$^{-/-}$ LT-HSCs transplanted recipients.

Although influences of a potentially defective bone marrow microenvironment in Gfi1$^{-/-}$ mice have been excluded (see chapter 3.5), the defect in HSCs in the bone marrow of Gfi1$^{-/-}$ mice could result from an impaired generation of self-renewing HSCs or defective stem cell homing. CXCR4 (SDF-1 receptor), VLA-4 and VLA-5 have been shown to be important for HSC homing (Peled et al., 1999; Wright et al., 2002; Papayannopoulou and Nakamoto, 1993; Craddock et al., 1997; Vermeulen et al., 1998; Scott et al. 2003), Flow cytometric analysis revealed no difference between WT and Gfi1$^{-/-}$ LSK cells (Figure 21), indicating that loss of Gfi1 does not cause changes in the expression of these receptors. In addition, the early engraftment of Gfi1 that was seen at three weeks after transplantation was followed by a gradual decrease of Gfi1$^{-/-}$ progeny (Figure 19). Therefore, a homing defect of Gfi1 deficient cells appeared as an unlikely explanation for the loss of competitive activity of Gfi1$^{-/-}$ HSCs.

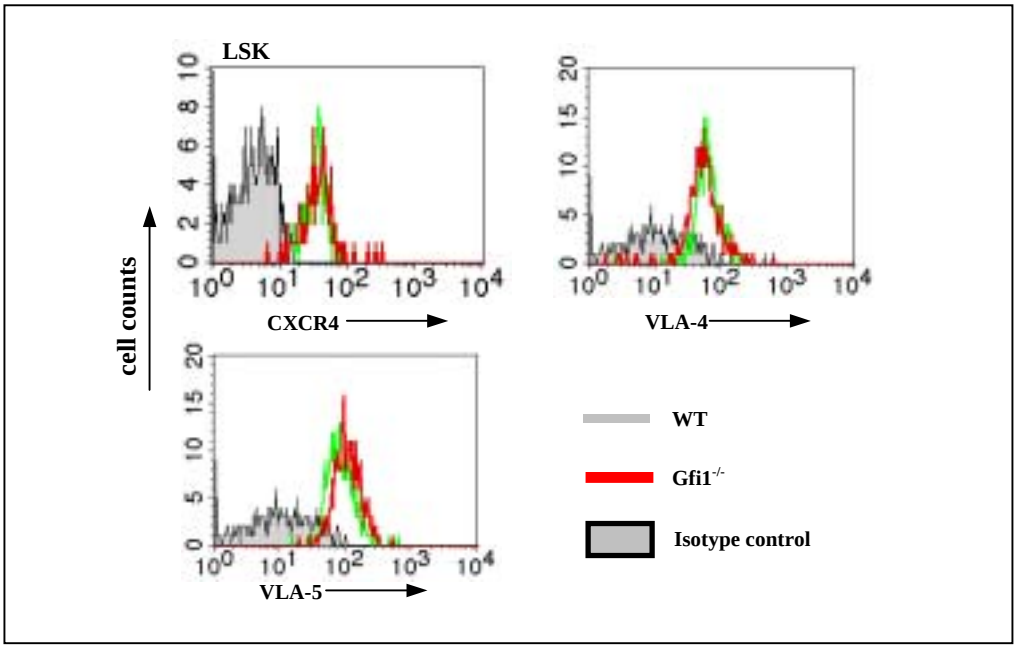

Figure 21. Expression levels of CXCR4 (SDF-1 receptor), VLA-4 or VLA-5 are expressed at identical levels on LSK cells from WT or Gfi1$^{-/-}$ mice

3.8 Altered *in vivo* proliferation kinetics of HSCs in the absence of Gfi1

It has been shown that LT-HSCs are asynchronously dividing, repeatedly entering and leaving the cell cycle with a constant fraction in G_0 phase under steady-state

hematopoietic conditions (Bradford et al., 1997; Cheshier et al., 1999). Obviously, the factors impeding HSC proliferation capacity could lead to a decreased repopulating ability of HSCs (Park et al., 2003; Björnsson et al., 2003). However, the enhancement of proliferation of HSCs does not result in a subsequent expansion of self-renewing HSCs, but results in an exhaustion of HSCs (Cheng et al., 2000a) and a dramatically decreased long-term engraftment capacity (Fleming et al, 1993a; Orschell-Traycoff et al, 2000; Szilvassy et al., 2000; Huttman et al., 2001). To analyze whether there was a defective cell cycle progression in Gfi1$^{-/-}$ HSCs, WT and Gfi1$^{-/-}$ mice were initially injected intraperitoneally with 1.8 mg/200 µl BrdU in saline and then continuously given BrdU at 1 mg/ml in the drinking water. The proliferative history of HSCs was determined by flow cytometry at different time points. As shown in Figure 22, the percentage of cells entering the cell cycle (BrdU positive) was significantly higher in the Gfi1$^{-/-}$ HSC compartment than in WT HSCs, indicating that absence of Gfi1 alters the *in vivo* proliferation kinetics of HSCs.

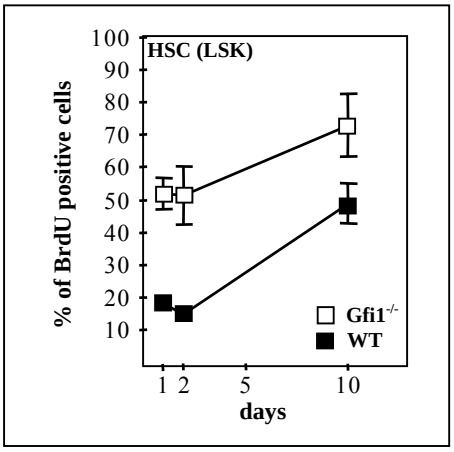

Figure 22. *In vivo* BrdU incorporation experiments revealed alteration of proliferation kinetics of Gfi1$^{-/-}$ HSCs

LSK cells were *in vivo* labelled with BrdU over the indicated time period. Gfi1$^{-/-}$ LSK cells show a significantly higher incorporation of BrdU than LSK cells from WT mice.

3.9 Drastic loss of HSCs in G_0 phase in Gfi1$^{-/-}$ bone marrow

To determine whether enhanced BrdU incorporation in Gfi1$^{-/-}$ HSCs is related to an increase of the proportion of HSC in cell cycle, the cell cycling status of stem cells was determined using the RNA dye pyronin Y (PY, as a measure of quiescence) and DNA dye Hoechst 33342. During cell cycle progression, there is a constant increase of cellular RNA, mainly because of the increased production of ribosomal RNA (Darzynkiewicz, et al., 1979 and 1988). Quiescent cells have, on average, 10-20% of the RNA as their

cycling counterparts (Johnson et al., 1975). Simultaneous staining of viable cells with the RNA-specific fluorochrome PY and the DNA-specific fluorochrome Hoechst has been widely used to determine the cell cycle status of many different cell types (Shapiro, 1981). By using this technique, the fraction of LSK in $G_1/S/G_2/M$ versus G_0 can be determined, revealing the fraction of actively proliferating cells (growth fraction) *in vivo* (Cheshier et al., 1999). As seen in Figure 23, the absence of Gfi1 correlated with a drastic loss of HSCs in G_0 phase and a significantly higher percentage of Gfi1$^{-/-}$ LSK cells in G_2/S/M phases compared to WT LSK cells. These findings demonstrated that during steady-state hematopoiesis, the ratio of Gfi1$^{-/-}$ cells in active cycle versus G_0/G_1 is affected compared to WT controls, indicating that the decrease of Gfi1$^{-/-}$ HSC long-term reconstituting capacity is not caused by a lower proportion of proliferating stem cells during endogenous hematopoiesis of adult mice, but might be due to a shift of HSCs from G_0 into the cycle which leads to exhaustion of the stem cell pool and also lowers the percentage of cells with high self-renewal potential.

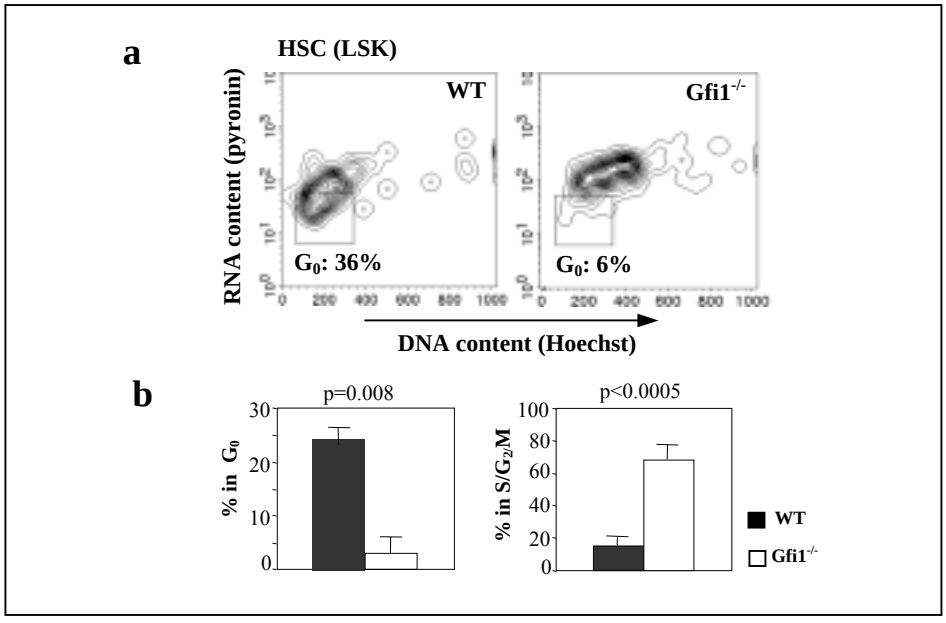

Figure 23. Absence of Gfi1 is correlated with a drastic loss of HSCs in G_0 phase and an increase of the proportion of HSC in cell cycle

a. A combination of DNA and RNA staining of bone marrow cells showed that the absence of Gfi1 correlated with a drastic loss of HSCs (LSK cells) in G_0 phase.

b. Percentages of HSCs (LSK cells) in different cell cycle phases. Given are average values with standard deviations from four WT and Gfi1$^{-/-}$ animals, respectively.

3.10 Expression of specific Gfi1 transcriptional target genes was altered in Gfi1$^{-/-}$ bone marrow cells

Recently, it has been reported that the genes encoding the cell cycle regulators E2F5, E2F6 and also p21$^{cip1/waf1}$ are putative Gfi1 targets or at least can be considered as downstream effectors of Gfi1 (Duan and Horwitz, 2002). Given the established roles for Gfi1 in regulation of cell cycle progression, protein expression levels of cell cycle regulators were determined by Western blot. As shown in Figure 24, whole bone marrow cells from Gfi1 deficient mice expressed significantly higher levels of E2F5 and E2F6 than WT cells and almost completely lacked p21$^{cip1/waf1}$ whereas another G_1 specific negative cell cycle regulator p27^{kip1} was expressed at comparable levels in both WT and Gfi1 null cells. This result suggested that Gfi1 regulates cell cycle progression of HSCs through up-regulating or down-regulating the expression of its target cell cycle regulators.

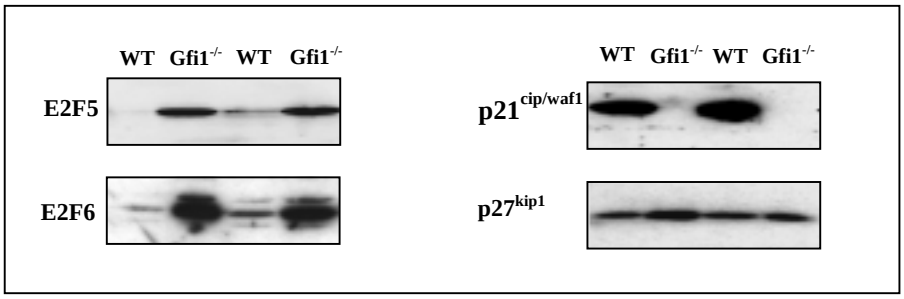

Figure 24. Expression of specific Gfi1 transcriptional target genes is altered in Gfi1$^{-/-}$ bone marrow cells

Protein expression levels of E2F5, E2F6, p21$^{cip/waf1}$ and p27^{kip1} in WT or Gfi1$^{-/-}$ bone marrow in two independent sets of mice (total number of animals analyzed, n = 4).

4. Discussion

4.1 Using mouse model systems to study the expression pattern and the function of Gfi1 during the process of hematopoietic cell development

In adult mammals, blood cells are generated in the bone marrow through a process named hematopoiesis by which the host continuously maintains adequate numbers of terminally differentiated cells of different lineages. It is well accepted that hematopoiesis is arranged as a hierarchy whereby the mature elements of the hematopoietic system are derived in a clonal fashion from HSCs. The bone marrow has the ability to markedly increase the production of blood cells to compensate for hematological stresses such as blood loss and infection. Thus, the need to continuously produce different types of hematopoietic cells necessitates strict control over HSC fate decisions between self-renewal and commitment toward lineage-restricted development.

Although the molecular mechanisms responsible for the control of self-renewal and differentiation outcomes of HSC divisions remain largely unknown, accumulating evidence suggests that self-renewal and commitment of hematopoietic stem cells to a distinct lineage is governed by complex external signals that modulate gene expression patterns through activation of specific transcription factors (Metcalf, 1998). A number of studies have implicated a variety of transcription factors as key regulatory components of these processes (Cross and Enver, 1997; Zhu and Emerson, 2002).

The role of transcription factors in regulating hematopoiesis used to be studied in cell lines. Since most of these cell lines have unknown and variable mutational histories, those *in vitro* data may not correctly reflect the *in vivo* function of transcription factors under physiological and pathological conditions. To overcome this obstacle, the mouse recently has become an irreplaceable model system to study the molecular mechanisms of hematopoiesis, because the mouse shares striking physiological, anatomical and genomic similarities with humans. Recently, the rapid development of a series of novel techniques for manipulating the murine genome has allowed the *in vivo* modification of virtually any

genomic region in a time and tissue specific manner. Such important technological developments allowed the generation of ideal mouse model systems that can provide more insights into the mechanisms underlying the hematopoietic process under physiological conditions (Adams and Cory, 1991; Adams et al., 1999; Bertoncello and Williams, 2001; Bernardi et al., 2002).

On the basis of gene-expression studies of the hematopoietic cell population, genes of interest have been selected and characterized mostly by using enforced gene expression and gene knockout strategies. In particular, production of mutant alleles by gene targeting in mouse embryonic stem cells has greatly accelerated genetic dissection of the hematolymphoid system. These studies have provided information on positive and negative regulators for HSC self-renewal and lineage commitment, differentiation, cycling and apoptosis (Adams, et al., 1999; Bertoncello and Williams, 2001; Bernardi et al., 2002). The cooperativity between different genes can be tested by cross breeding of two gene-mutant strains. A large number of mouse mutants were already generated to mimic human hematopoietic disease, to revealed molecular mechanisms underlying pathological conditions as well as to determine *in vivo* the consequences of aberrant gene function *in vivo* (Bernardi et al., 2002).

4.2 Expression pattern of Gfi1 in adult bone marrow hematopoietic cells

The importance of Gfi1 in hematopoiesis was revealed by the studies of Gfi1 deficient mice and prompted us to investigate the detailed expression pattern of Gfi1 in hematopoietic stem and progenitor cells. Since $Gfi1^{-/-}$ mice show multilineage defects (Karsunky et al., 2002a; Yücel et al., 2003; Hock et al., 2003), it was of interest to know whether Gfi1 acts on a wide spectrum of hematopoietic cells, or, on a limited subset of stem cells or progenitor cells and certain lineages.

To understand the role of Gfi1 in modulation of hematopoiesis, it is essential to precisely delineate the magnitude and cell type specificity of Gfi1 expression in different lineages in different development stages. By taking advantages of a Gfi-1:GFP knock-in mice and

characterizing HSCs and progenitors by FACS, the expression pattern Gfi1 in hematopoietic cells was determined as shown in Figure 25.

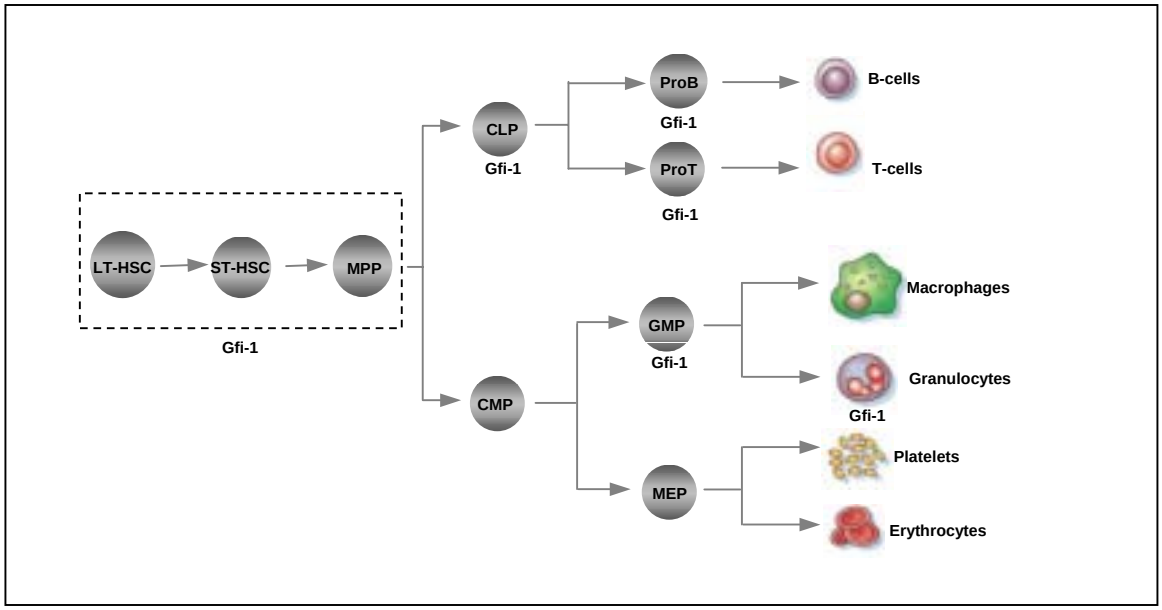

Figure 25. Gfi1 expression in adult mouse hematopoietic cells

It cannot be ruled out that a difference in the half-life between Gfi1 and GFP proteins exists, which may influence interpretation of our FACS results. However, the comparable expression pattern of Gfi1 in bone marrow cells estimated by FACS and RT-PCR, suggested that flow cytometric analysis of GFP provides an accurate reflection of endogenous Gfi1 gene expression. Since $Gfi1^{GFP/+}$ heterozygous knock-in mice are indistinguishable from their wild-type littermates and from the previously described animals that carry a neo resistance marker gene in the Gfi1 locus disrupting one Gfi1 allele ($Gfi1^{+/-}$), it can be inferred that GFP developmental expression patterns precisely mimic those of endogenous Gfi1 under physiological conditions.

Since the GFP marker can be used as a faithful indicator of Gfi1 expression, the Gfi1:GFP knock-in mice allowed Gfi1 expression to be assayed in hematopoietic populations through simple FACS analyses and electronic gating, and obviate more cumbersome and often less informative approaches such as extensive cell sorting coupled with RNA and protein analyses. Moreover, this novel model permitted more quantitative

judgments with respect to cell numbers and relative expression levels in given populations.

4.3 Expression pattern of Gfi1 in hematopoietic compartment is correlated with function of Gfi1 revealed in Gfi1 deficient mice

The analysis of hematopoietic defects in Gfi1$^{-/-}$ mice showed that expression pattern of Gfi1 in hematopoietic compartments is correlated with function of Gfi1 as revealed in Gfi1 deficient mice. For example, Gfi1 exhibits a biphasic expression in immature myeloid cells (CD11b^{+}Gr-1^{low}), and is highly homogenously expressed in mature granulocytes (CD11b^{+}Gr-1^{high}), while Gfi1 deficient mice show severe neutropenia and monocytosis. In contrast, Gfi1 is absent in the MEP and enucleated red cells, and no obvious defects were found in development of erythrocytes and megakaryocytes in Gfi1$^{-/-}$ mice. Collectively, these studies suggest that Gfi1 is required for myeloid differentiation and maturation but not for terminal differentiation of erythroid and megakaryocytic lineage. Importantly, the correlation between hematopoietic defects and Gfi1 expression pattern was further demonstrated by phenotypical and functional analysis of HSCs and progenitors in Gfi1$^{-/-}$ mice in the present study (see below). Together, the results suggest that Gfi1 is required for multilineage hematopoiesis from early stem cells to late committed cells.

4.4 Gfi1 is required for the maintenance of homeostasis of stem cell and early progenitor populations

HSCs are capable of self-renewal and have a tremendous differentiation potential, a single to a few cells being capable of repopulating the entire hematopoietic system of a lethally irradiated recipient. The two major goals to study HSCs are to understand these capacities at the molecular level and to clarify the mechanisms regulating self-renewal and lineage commitment. Several studies have indicated that, in steady-state hematopoiesis, the proliferation of HSC is tightly controlled. In mice, HSC numbers remain relatively constant throughout most of their adult life, although in very old mice

(older than 2 years) their numbers appears to increase, possibly due to accumulation of genetic lesions (Morrison et al., 1996). Recent data indicate that a variety of regulatory molecules active in early development may also play a role in the maintenance of hematopoietic stem cells with repopulating activity. Lack-of-function mouse models have generated important insight into the role of various transcription factors in hematopoiesis (such as GATA-2, SCL/tal-1, Rbtn2/Lmo2, AML1, PU.1/Spi1, Ikaros, HoxB6, and HoxA9, reviewed by Tenen et al., 1997 and Orkin, 2000).

Since Gfi1 expression has been observed in HSC, CLP and GMP compartments, the possibility existed that Gfi1 might also play an important role in early stage of hematopoiesis. As shown in the present study, loss of Gfi1 results in alteration of the numbers of HSC and certain progenitor populations including CMPs, CLP and GMP population.

The depletion of HSC population in $Gfi1^{-/-}$ mice affects the whole HSC compartment (the LSK population). Strikingly, $Flt3^{+}$ fraction in the LSK population was dramatically reduced, pointing out severe defects in number of $Flt3^{+}$ HSCs. Recently, two groups have demonstrated that upregulation of Flt3 expression within the bone marrow LSK compartment is accompanied by loss of self-renewal capacity (Christensen and Weissman, 2001; Adolfsson et al., 2001). Christensen and Weissman reported that in LSK compartment, the $Thy1.1^{low}Flt3^{+}$ subset contains predominantly ST-HSCs and $Thy1.1^{-}Flt3^{+}$ subset contains predominantly MPPs. Although Adolfsson and colleagues found that $LSKFlt3^{+}$ cells only reconstitute B and T lymphopoiesis but not myelopoiesis *in vivo* and further concluded that $LSKFlt3^{+}$ cells are progenitors for CLPs, they found $LSKFlt3^{+}$ cells have myeloid differentiation potential *in vitro*. Moreover, both groups showed that $LSKFlt3^{+}$ cells are generated from $LSKFlt3^{-}$ cells. Taken together, $LSKFlt3^{+}$ compartment was considered to represent progenitors downstream to LT-HSCs and upstream to CLPs. The severe depletion of LSK $Flt3^{+}$ and $LSKFlt3^{high}$ cells shown in the present study might be attributed to the impairment of the ability of $Gfi1^{-/-}$ LT-HSCs to generate direct downstream progenitors.

Downstream to HSCs, the segregation into CLP and CMP represents the earliest known HSC commitment step. Although the frequencies of both CMPs and CLPs were decreased in Gfi1$^{-/-}$ bone marrow, the over 40-fold reduction of CLPs (which express Gfi1) in Gfi1$^{-/-}$ mice was more dramatic than the 2-fold reduction of CMP (which do not express Gfi1). The moderate depletion of Gfi1$^{-/-}$ CMP might be a subsequent result from the loss of Gfi1$^{-/-}$ HSC compartment, which may also affect the number of their downstream lineage-committed progenitors. In contrast to the decrease of HSC, CLP and CMP frequencies, the frequency of GMPs (which express Gfi1) showed a significant increase in Gfi1 deficient mice, while the frequency of MEPs (which do not express Gfi1) was not altered in Gfi1 deficient mice compared to WT littermates. The fact that not all progenitor compartments were compromised suggests that Gfi1 performs complex and specific functions in regulating the development of certain, distinct myeloid and lymphoid progenitor cell populations.

4.5 The alteration of the number of progenitors was confirmed by *in vivo* and *in vitro* functional assays

Although the findings described above correlate very well with the previously reported reduction of thymocytes and peripheral T cells and B cells (Karsunky, et al., 2002a; Yücel et al., 2003; Hock et al., 2003), and normal counts of red blood cells and platelets in Gfi1$^{-/-}$ mice (Karsunky et al., 2002a; Hock et al., 2003), the functional-identified progenitors could only be investigated by *in vivo* and *in vitro* functional assays.

First, *in vitro* colony-forming assays in semisolid media confirmed the role of Gfi1 in lineage-committed precursors. Consistent with elevated percentages of GMPs in Gfi1$^{-/-}$ bone marrow, progenitors that respond to IL-3 and GM-CSF were all significantly increased Gfi1$^{-/-}$ bone marrow.

Second, *in vivo* spleen colony-forming assays were performed to analyze the role of Gfi1 in primitive progenitors and myelo-erythroid lineage committed progenitors. The unaltered formation of CFU-S_8 and the impaired formation of CFU-S_{12} in Gfi1$^{-/-}$ bone marrow transplanted hosts were consistent with unaltered percentages of MEPs, moderate

decreased CMP population and a dramatically decreased ST-HSC/MPP population in Gfi1$^{-/-}$ bone marrow,

In addition to the decrease in CFU-S_{12}, the short-term radioprotection capacity of Gfi1$^{-/-}$ bone marrow cells was also compromised in a dose-dependent manner. This fact suggested that the defect in the short-term radioprotection capacity of Gfi1$^{-/-}$ bone marrow cells is mainly due to the depletion of ST-HSC, MPP and CMP populations.

The *in vivo* and *in vitro* colony-forming assays also revealed that loss of Gfi1 affects the functions of early progenitors. The decrease of size of CFU-S_{12} suggests that Gfi1$^{-/-}$ primitive progenitors fail to generate substantial number of progeny. Although IL-3 and GM-CSF both of which can induce differentiation from myeloid precursors into the granulocyte and the monocyte/macrophage lineage elicited a significantly higher number of colonies from Gfi1$^{-/-}$ bone marrow cells than from WT bone marrow cells, these Gfi1$^{-/-}$ colonies contain immature atypical myelo-monocytes but few mature granulocytes (Karsunky, et al., 2002a; Hock et al., 2003). Thus, the differentiation from Gfi1$^{-/-}$ myeloid progenitors is severely skewed toward the monocyte/macrophage lineage as a result of either a block of the granulocyte lineage or enhanced differentiation towards the monocyte/macrophage lineage.

4.6 Gfi1 is required for the maintenance of long-term reconstitution capacity of HSCs

The initial results showed that lack of Gfi1 leads to a decrease of HSC number in adult mouse bone marrow, suggesting that Gfi1 could regulate the normal processes that control the HSC population size. However, two questions remain open. First, since the data from FACS analysis only reflect the level of phenotypically defined HSCs, the level of functionally defined HSCs which can only be reliably determined through *in vivo* long-term reconstitution studies had to be clarified. Second, the abnormalities in HSCs in steady state do not always reflect abnormalities under stress conditions. Despite the decrease in HSC frequency in the Gfi1$^{-/-}$ bone marrow, the bone marrow cellularity was relatively conserved in Gfi1$^{-/-}$ mice, probably because the reduction of HSCs can be

compensated under steady-state conditions. Therefore, whether Gfi1 is required for HSC maintenance under stress stage was further addressed.

The dramatic reduction in long-term radioprotection capacity of $Gfi1^{-/-}$ bone marrow cells in secondary bone marrow transplantation was the first evidence which prompted that Gfi1 plays an important role in HSC self-renewal, at least in response to hematological stress caused by lethal irradiation and serial transplantation. When measured the reconstitution capacity against wild-type bone marrow cells in a competitive assay, a severe depletion in both short-term and long-term repopulating activities is revealed. The peripheral blood cells derived from $Gfi1^{-/-}$ bone marrow cells were reduced more remarkably after 22 weeks than after 3 weeks post-transplantation, suggesting that defects in reconstitution is more prominent in the upper level of the hematopoietic hierarchy in $Gfi1^{-/-}$ mice. Importantly, the impaired competitive capacity could not be relieved by increasing the number of transplanted $Gfi1^{-/-}$ bone marrow cells. Even at a 10:1 ratio to WT bone marrow, $Gfi1^{-/-}$ bone marrow cells failed to compete with WT bone marrow cells to reconstitute hematopoiesis in recipient mice in both myeloid and lymphoid lineage, only limited hemopoietic contribution is observed from $Gfi1^{-/-}$ bone marrow. Thus, despite 2-4 fold decrease in phenotypically defined LT-HSC in adults mouse bone marrow, there is a quantitative reduction estimated to be more than 30-40-fold in the number of functionally-defined HSCs in $Gfi1^{-/-}$ mice. This observation suggested that the failure of competetivivity of $Gfi1^{-/-}$ HSCs is not caused only by a decrease of phenotypically defined HSCs, but rather seems to be caused by defects in the functions (self-renewal, proliferation, differentiation, homing) of HSCs.

In addition to these *in vivo* experiments, the long-term culture initiating cell (LTC-IC) assay was performed by our collaborators (Y, Li in Prof. Dr. U. Duehrsen's Lab) to quantitate primitive hematopoietic progenitors in a co-culture with stroma cells. Consistent with the results from *in vivo* competitive transplantation experiments, a 60-fold reduction of LTC-IC numbers were observed in cultures started with $Gfi1^{-/-}$ bone marrow compared to WT bone marrow. Since the number of LTC-IC correlates with the number of HSCs with *in vivo* repopulating potential, this result supports the notion that

the lack of Gfi1 confers a defect to HSCs for their long-term reconstituting capacity and the hypothesis that Gfi1 is required to maintain the self-renewal abilities of HSCs.

4.7 The defect in HSCs in Gfi1$^{-/-}$ bone marrow could result from the impaired generation of self-renewing HSCs

Many factors such as hormones and cytokines influence hematopoietic efficiency, and these factors are derived from both hematopoietic and nonhematopoietic cells. The defects in bone marrow Gfi1$^{-/-}$ HSCs could result from an impaired generation of self-renewing adult HSCs, defective stem cell homing, or a defective bone marrow microenvironment.

Bone marrow transplantation experiments can determine whether hematopoietic defects observed for Gfi1 deficient mice are autonomous to the hematopoietic system by examining the capacity of Gfi1 deficient bone marrow to reconstitute hematopoiesis in lethally irradiated recipient mice that provide an otherwise normal environment. If the defects in the Gfi1$^{-/-}$ mice were secondary to an abnormal microenvironment, then Gfi1$^{-/-}$ HSCs would be able to engraft the bone marrow of WT mice efficiently, while WT HSCs would show defects in engraftment and/or hematopoietic development in Gfi1$^{-/-}$ mice. The phenotype of Gfi1 deficient mice with regard to frequencies of stem cells and progenitors, or previously reported hallmarks (neutropenia, accumulation of monocytic cells and reduced numbers of thymocytes (Karsunky, et al., 2002a; Yücel et al., 2003; Hock et al., 2003) could be exactly reproduced in the transplanted hosts which provide otherwise normal environment. Vice versa, WT bone marrow transplanted into irradiated Gfi1 deficient mice enable to generate normal numbers of HSCs, progenitors, neutrophils and normal numbers of thymocytes. Both experiments indicate that the microenvironment in the bone marrow of Gfi1 deficient mice is functional without any detectable defects, and confirm that the hematopoietic defects in Gfi1$^{-/-}$ mice are cell autonomous.

The difference in engraftment between Gfi1$^{-/-}$ and WT HSCs in competitive repopulations showed a competitive disadvantage of Gfi1$^{-/-}$ stem cells. A homing defect of Gfi1 deficient cells could be excluded or at least seems unlikely. First, loss of Gfi1 did not

alter the expression of adhesion molecules important for HSC homing such as CXCR4, VLA-4 and VLA-5 in Gfi1$^{-/-}$ LSK populations. Second, the early engraftment of Gfi1$^{-/-}$ bone marrow cells that was seen at three weeks after transplantation was followed by a gradual decrease of Gfi1$^{-/-}$ progeny, suggesting that the severe impairment of long-term reconstituting abilities of Gfi1$^{-/-}$ HSCs is not a result of a homing defect. In addition to *in vivo* results, reduction of LTC-IC numbers generated from Gfi1$^{-/-}$ bone marrow cells also supports the notion, otherwise a reduction in LTC-IC numbers would not be expected.

Alternatively, since loss of Gfi1 leads to neutropenia and decreased B and T cell number in Gfi1$^{-/-}$ mice, the failure of generating peripheral blood of Gfi1$^{-/-}$ bone marrow cells seen in reconstituted animals might start at the level of HSC or at more differentiated cells. The reduction of Gfi1$^{-/-}$ HSCs with long-term repopulating capacity might be caused by alterations in lineage commitment of hematopoietic cells, or by failure of generating mature progenies, or failure of releasing of Gfi1$^{-/-}$ mature hematopoietic cells from bone marrow to peripheral blood.

However, Gfi1$^{-/-}$ HSCs can generate both myeloid and lymphoid hematopoiesis in the recipients when they were transplanted without competitive cells, indicating that Gfi1$^{-/-}$ HSCs do not lose multilineage differentiation potential. Furthermore, similar to the results from analysis of peripheral blood, flow cytometry analysis revealed that bone marrow cells from WT mice efficiently reconstituted myeloid cells and lymphoid cells, whereas the mice reconstituted with Gfi1$^{-/-}$ marrow lost nearly all donor-derived mature hematopoietic cells in bone marrow. Importantly, when competitive transplantation experiments were performed with sorted 500 LT-HSCs, a restoration of LSK cells was found in mice reconstituted with WT HSCs, whereas mice reconstituted with Gfi1$^{-/-}$ HSCs lost nearly all Gfi1$^{-/-}$ LSK population. These results indicate that the defect in Gfi1 deficient mice is not likely due to alteration of lineage-commitment. The defects should be at a more primitive level, within the stem cell compartment. Taken together, the data presented here suggest that Gfi1 is required for self-renewal and maintenance of adult HSCs.

4.8 Gfi1 is required to maintain hematopoietic stem cell quiescence

High levels of production of mature blood cells are needed to replace their rapid turnover. Since the vast majority of HSC population are resistant to cytotoxic agents such as 5-FU or hydroxyurea (Harrison DE, 1991; Lerner C and Harrison DE. 1990), it has therefore been hypothesized that in the hematopoietic tissue stem cells are relatively quiescent or slowly cycling, but their more differentiated offspring have extremely robust proliferative potential. Further experiments indicated that LT-HSCs are indeed slowly cycling, asynchronously dividing, repeatedly entering and leaving the cell cycle with a constant fraction in G_0 phase under steady hematopoietic conditions, with a turnover time of approximately 30 days (Bradford et al., 1997; Cheshier et al., 1999). Thus, it is conceivable the proliferative activity of HSCs is highly restricted in order to prevent susceptibility to myelotoxic insult or consumption of the regenerative cell pool.

Regulating cell cycling of the stem cell pool involves a very complex mixture of internal and external signals. A number of other molecules have also been reported to have an important role in this scheme. Deficiency of Bmi1, HoxB4 and HoxB3 can influence the repopulating ability of HSCs by causing a decreased proliferation capacity (Park et al., 2003; Björnsson et al., 2003). These findings intriguingly point to the possibility that regulatory molecules known for their growth-promoting roles in early developmental processes may also affect HSC activity.

Interestingly, in addition to the molecules which are needed for maintaining HSC proliferation, $p21^{cip1/waf1}$, a cyclin-dependent kinase inhibitor was shown to be necessary for HSC self-renewal by different mechanisms. $p21^{cip1/waf1}$ plays a role in at least some cell types in the transition out of the cell cycle and maintenance in G_0 phase. Hematopoietic stem cell proliferation and absolute number were increased under normal homeostatic conditions in $p21^{-/-}$ mice bone marrow, suggesting $p21^{cip1/waf1}$ impede stem cell cycling (Cheng et al., 2000a). In the absence of $p21^{cip1/waf1}$, the inhibition is alleviated, leading to an expansion of the primitive cell pool under resting conditions. However, under stress conditions, loss of $p21^{cip1/waf1}$ enhances 5-FU killing of primitive cells, leads to a reduced serial transplantation ability due to hematopoietic cell depletion (Cheng et al.,

2000a). Thus, deficiency of $p21^{cip1/waf1}$, which is necessary for maintaining stem cells in a quiescent state, and leads to stem cell exhaustion accompanied by impaired self-renewal.

In addition to the results from $p21^{-/-}$ mice, transplantation studies revealed that the ability of HSCs for engraftment in foreign hosts correlates with their cell cycle phase position. High long-term engraftment capacity is restricted to HSCs in G_0/G_1 phases, while HSCs in $S/G_2/M$ show a dramatically decreased long-term engraftment capacity. More recently, it has become clear that the transit of HSCs through different phases of the cell cycle is accompagnied by a significant shift in gene expression that not only affects homing but also other properties of HSCs (Lambert et al., 2003). It is therefore crucial for an organism to maintain a pool of HSCs in G_0 to assure a potent, self-renewable source of cells that can support lifelong multilineage hematopoiesis.

Several studies with cultured cells indicated that constitutive Gfi1 expression can relieve peripheral mature T cells from a requirement of IL-2 to overcome a G_1 arrest (Grimes et al., 1996) or could help to sustain cell proliferation of IL-2-dependent cells in the absence of the cytokine (Zörnig et al., 1996), which suggested a role for Gfi1 in IL-2-dependent cell cycle progression of T cells. Constitutive expression of Gfi1 accelerates entry of resting T cells into S phase and decreases apoptosis (Karsunky et al., 2002a). Induction of Gfi1 by IL-4 increases Th2 cell expansion by promoting proliferation and preventing apoptosis (Zhu et al., 2002). It has been shown that T cells of mice over expressing Gfi1 enter S-phase more quickly than WT cells after antigenic stimulation (Karsunky et al., 2002a). In the Gfi1 deficient mice, T cells are slow in responding to antigenic stimulation (Möröy et al., unpublished findings).

Above results are in agreement with a role of Gfi1 as a positive mediator to promote T cell proliferation. However, the *in vivo* BrdU labelling experiments described here revealed a significant increase of the percentage of cells entering the cell cycle in the $Gfi1^{-/-}$ HSC compartment compared to in WT HSCs. Meanwhile, combination of DNA and RNA staining of live LSK cells revealed that the absence of Gfi1 correlated with a drastic loss of HSCs in G_0 phase and a significantly higher percentage of $Gfi1^{-/-}$ LSK cells in $G_2/S/M$ phases. Thus, both experiments, demonstrated that lacking Gfi1

expression has heightened HSC cycling, indicating requirement of Gfi1 to maintain hematopoietic stem cell quiescence.

The results from T cells and HSCs showed entirely different roles of Gfi1 in cell cycle regulation, suggesting that Gfi1 may function in a tissue- or differentiation-specific manner. The different cell cycle distribution in lymphoid cells and HSCs can be explained by taking into consideration that Gfi1 very likely regulates different target genes in different cell types. The helix loop helix proteins Id1 and Id2 and the LKLF transcription regulator were found to be associated with T-cell quiescence. (Yücel et al., 2003), while C/EBPα, E2F family members were found to be Gfi1 target genes in myeloid cells (Duan and Horwitz, 2003). In addition, antigenic stimulation in T-cells triggers other pathways than those are active in stem cells or progenitors.

It has been shown that Gfi1 cooperates with the cell-cycle regulators Pim-1 and c-Myc (Schmidt et al., 1998a and 1998b). Recently, Gfi1 target gene analysis also pointed out a role of Gfi1 in cell cycle regulation. It was reported that the genes encoding the cell cycle regulators E2F5, E2F6 and also $p21^{cip1/waf1}$ which are involved in the regulation of G_1 phase progression and S-phase entry are putative Gfi1 targets or at least can be considered as downstream effectors of Gfi1 (Duan and Horwitz, 2003).

Consistent with a negative regulatory role for Gfi1 of HSC cycling, loss of Gfi1 resulted in an alteration of expression of Gfi1 downstream cycling regulators (such as $p21^{cip1/waf1}$, E2F5 and E2F6). Strikingly, $p21^{cip1/waf1}$ is almost completely lacked in $Gfi1^{-/-}$ bone marrow cells. Since Gfi1b may act either as an activator or repressor (Jegalian and Wu 2002), it is conceivable that Gfi1 could also function as a transcriptional activator or repressor, depending on the promoter, the cofactors recruited and developmental context. Recently, there is one indirect report of its potential as an activator (Sharina et al., 2003). It is also possible that Gfi1 represses the production of an intermediate factor that itself is a repressor leading to the activation of its target genes. An alternative possibility is that Gfi1 acts as an activator of STAT3 target genes through a mechanism that has been described before (Rödel et al., 2000).

It has been documented that a loss of $p21^{cip1/waf1}$ causes increased cell cycling and resulting in stem cell exhaustion (Cheng et al., 2000). However, absence of $p21^{cip1/waf1}$ leads to an elevated level of number of HSCs, while a decrease of HSC pool was found in $Gfi1^{-/-}$ bone marrow under steady-state conditions. The distinction in stem cell generation between these different mutants may have several potential explanations. It is most likely that as a transcription factor, Gfi1 affects a series of its targets genes, which not only affect cell cycle regulation, but also are required for HSC self-renewal. In turn, alteration of signalling pathways and yet unidentified effectors of Gfi1 may account for the more severe phenotypes manifested in Gfi1 deficient mice.

In contrast to a decrease of $p21^{cip1/waf1}$ expression, Gfi1 deficient mice expressed significantly higher levels of E2F5 and E2F6 than WT cells. The E2F family of transcription factors also plays a critical role in cell-cycle progression through its ability to regulate the expression of target genes, including cyclins and cyclin-dependent kinases (Dyson, 1998). Whereas the role of E2F5 in cell cycle regulation seems less clear, it is known that ectopic expression of E2F6 leads to accumulation of cells in S-phase (Trimarchi and Lees, 2002). Moreover, E2F6 is able to form complexes with the onco-protein Bmi1, which is a member of the polycomb group (Trimarchi et al., 2001) and an essential factor for the self-renewal of HSCs and leukemic stem cells (Park et al., 2003; Lessard et al., 2003). These findings suggest that Gfi1 acts upstream of E2F5, E2F6 and $p21^{cip1/waf1}$ and that a loss of Gfi1 causes a shift of HSCs from G_0 into the cycle which leads to exhaustion of the stem cell pool and also lowers the percentage of cells with high self-renewal potential. It is thus likely that Gfi1 controls cardinal features of HSCs such as self-renewal by regulating their cycle exit or entry through the maintenance of a constant proportion of HSCs in G_0 (Figure 26).

The data presented here support the notion that deficiency of Gfi1 positively affects HSCs cycling and proliferation. Furthermore, it appears that Gfi1 is mainly important to control the proliferation response and is dispensable for both normal, steady-state hematopoiesis and stress-state hematopoiesis. These findings are important for understanding the regulatory mechanisms that control fate, particularly self-renewal, of HSCs.

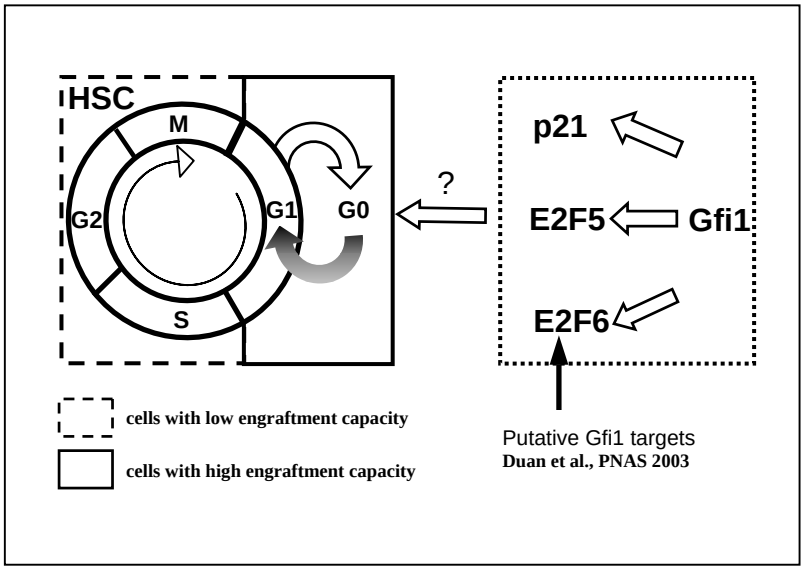

Figure 26. Proposed model for the role of Gfi1 in maintenance of long-term engraftment capacity

Long-term engraftment capacity is attributed to HSCs in G_0/G_1 phase but not to those in $S/G_2/M$ phase. The absence of Gfi1 results in a drastic loss of HSCs in G_0 phase. Therefore, Gfi1 may be one of the responsible factors that maintain or control cell cycle entry or exit of HSCs or the proportion of them remaining in G_0.

4.9 The defects in HSCs may not be attributable to deficient HSC surface receptors, but may be attributable to deficient cell signaling

Although detailed analysis of premature hematopoietic cells including HSCs in $Gfi1^{-/-}$ mice reported here reveals a crucial role for Gfi1 in sustaining HSC in G_0 phase, other mechanisms by which Gfi1 affects HSCs self-renewal cannot be excluded. The present knowledge of known Gfi1 target genes is based on myeloblasts (KG-1, HL-60), monnoblasts (U937) and T lymphocytes (Jurkat), but not on stem cells. The mechanism of the striking decrease in function of HSCs remains to be elucidated.

Most cytokines and their receptors appear to have no or redundant roles in regulating the HSC pool (Metcalf, 1998). However, thrombopoietin (TPO) and c-Kit ligand (KL) and their corresponding receptors c-mpl and c-Kit have been demonstrated through gene targeting studies to be important for regulating HSC numbers and/or function (Kimura et

al., 1998; Solar et al., 1998; Alexander et al., 1996; Miller et al., 1996; Geissler and Russell, 1983).

The *in vivo* roles of c-Kit and its ligand (stem cell factor, SCF) are well understood because of the existence of mutant mice. Mutations in the c-Kit receptor and SCF had the same complex phenotype that affects pigmentation, germ cells, and hematopoiesis (Witte, 1990; Fleischman et al., 1993; Broudy, 1997). Although the primary function of SCF in early hematopoiesis might be to induce the growth of quiescent progenitor/stem cells through synergistic interactions with other early-acting cytokines, ample evidence indicates that SCF, in the absence of other cytokines, selectively promotes viability rather than proliferation of primitive murine progenitor cells (Fleming et al, 1993b). Many reports have shown that the partial loss of function-*W* alleles ensues impaired HSC competitiveness and CFU-S activity, decreased CFU-C formation and mild anemia, as well as mast cell deficiencies, and mild thrombocytopenia (Lyman and Jacobsen, 1998). Null c-Kit mutations lead to embryonic lethality owing to severe anemia caused by decreased stem cells and progenitors (Lyman and Jacobsen, 1998).

It has been noted that the SCF/c-Kit signaling pathway specifically induces the expression of Slug gene, a zinc finger transcriptional repressor of the highly conserved Slug/Snail family of transcription factors with stretche of homology to Gfi1 (Nieto et al., 1994), in natural and artificially engineered c-Kit^{+} cells. Like c-Kit and SCF-defective mice, Slug$^{-/-}$ mice have a complex phenotype including pigmentation, gonadal defects, and hematopoietic defects (Pérez-Losada et al., 2002), identifying Slug as a molecular target that contributes to the biologic specificity of the SCF/c-Kit signaling pathway. However, there is no evidence to indicate that Gfi1 regulates c-Kit expression or to suggest that Gfi1 is downstream of the SCF/c-Kit signaling pathway. Although Gfi1 deficient mice present similar hematopoietic defects to mice deficient in the SCF/c-Kit signaling pathway mice, no pigmentation or gonadal defects were found. Moreover, a dramatic increase of the GMP population (which are c-Kit^{+}) was found in Gfi1$^{-/-}$ mice. Therefore, an impaired c-Kit/SCF signaling pathway could not be an explanation for the defects in Gfi1$^{-/-}$ bone marrow.

Like c-Kit, fms-like tyrosine kinase-3 (flt3) is selectively expressed and functional in early stages of mouse and human hematopoiesis (Lyman and Jacobsen, 1998). Flt3 was originally identified as a tyrosine kinase receptor expressed in fetal liver populations enriched for HSCs (Matthews, et al., 1991). The flt3 ligand (FL) has emerged as a key stimulator of growth of candidate murine and human HSCs through its ability to promote *ex vivo* expansion and onco-retroviral transduction of primitive human hematopoietic progenitors (Lyman and Jacobsen, 1998). As a consequence, FL has been frequently used to promote HSC *ex vivo* expansion and retroviral mediated gene transfer. Mice deficient in expression of Flt3 or FL have reductions in B and T lymphopoiesis, with a preferential reduction in the earliest proB cell progenitors (Mackarehtschian et al., 1995; McKenna et al., 2000). Furthermore, bone marrow HSC from mice deficient in Flt3 expression reveal reduced reconstitution potential *in vivo* (Mackarehtschian et al., 1995). Surface marker analysis of LSK hematopoietic populations in Gfi1 deficient mice revealed a severe reduction in expression of Flt3 in the LSK population. However, recent studies from two groups revealed that mouse adult long-term reconstituting HSCs do not express Flt3 during steady-state hematopoiesis, and the upregulation of flt3 expression within the HSC compartment is accompanied by loss of self-renewal capacity (Adolfsson, et al., 2001; Christensen and Weissman, 2001). Moreover, mice deficient in the expression of FL show deficient lymphopoiesis (Mackarehtschian et al., 1995; McKenna et al., 2000). Recently, Sitnicka and colleagues reported that $FL^{-/-}$ mice have normal frequencies of phenotypically ($Lin^{-}Sca1^{+}c\text{-}Kit^{+}CD34^{-}$) and functionally defined long-term HSC in adult bone marrow. $FL^{-/-}$ HSC have normal functional capacities when transplanted into lethally irradiated WT recipients, both with regard to reconstitution and self-renewal capacity (as determined through serial transplantation). In contrast, FL deficient mice have severely (10-fold) reduced levels of CLPs, accompanied by reductions in the earliest identifiable B and T cell progenitors, whereas CMP and other early myeloid progenitors are unaffected (Sitnicka et al., 2002). Therefore, loss of Flt-3 expression in $Gfi1^{-/-}$ HSC population can explain the decrease of CLP in $Gfi1^{-/-}$ mouse bone marrow, but cannot explain strikingly decreased of self-renewal capacity of $Gfi1^{-/-}$ HSCs.

A role of Gfi1 in cytokine or growth factor signaling had already been suggested earlier because the Gfi1 gene was found to be activated by proviral insertion in NB2 rat lymphoma cell clones selected for IL-2-independent growth after infection with Moloney murine leukemia virus (Gilks et al., 1993). Cytokines such as IL-2 or IL-6 mediate their signals after binding the respective membrane-bound cytokine receptors through the activation of several cytoplasmic proteins. In particular, signal transducers and activators of transcription (STAT) proteins are critical constituents of cytokine-mediated signal transduction (for a review see Ihle, 1996; Heinrich et al., 1998; Shuai, 1999). All seven STAT proteins identified so far are located in the cytoplasm as latent transcription factors. They are recruited via their SH2 domains to phosphotyrosine motifs of activated receptors and subsequently become tyrosine phosphorylated by Janus kinases (JAKs) (Schindler and Darnell, 1995; Darnell, 1997; Heinrich et al., 1998; Shuai, 1999). Phosphorylated STAT proteins dimerize and translocate to the nucleus where they act as transcriptional activators of specific target genes. STAT5 and STAT3 for instance can relay signals from the IL-2 receptor and STAT3 from the IL-6 receptor.

The cytokine response and the activation of STATs can also be negatively regulated. Among the known antagonists for STAT function are the suppressor of cytokine signaling (SOCS) proteins and PIAS (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997; Chung et al., 1997; Liu et al., 1998). While SOCS proteins interact with JAKs and very probably reduce their tyrosine kinase activity, PIAS proteins bind to activated STAT dimers and block their DNA binding activity (Chung *et al*., 1997; Liu *et al*., 1998).

STAT3 is a key downstream signalling intermediate of gp130, a receptor previously shown to activate HSC self-renewal divisions. Retrovirus-mediated over-expression of dominant negative form of STAT3 in HSCs markedly and permanently reduced *in vivo* reconstituting ability (Oh and Eaves, 2002). Similar to STAT3, competitive bone marrow transplantation studies *in vivo* revealed a profound impairment of repopulation potential of $STAT5^{-/-}$ HSCs (Bradley et al., 2002; Snow et al., 2002). These abnormalities were associated with heightened proliferation activity in the HSC fraction (Snow et al., 2002).

It has been demonstrated that Gfi1 interacts with PIAS3 and affects its function, as it is able to relieve the inhibitory effect of PIAS3 on STAT3 activity (Rödel et al., 2000). Cells infected with Gfi-1 retrovirus showed striking enhancement of IL-2-induced Stat5 phosphorylation (Zhu et al, 2002). Therefore, impaired HSC activity in Gfi1 deficient mice might be associated with a decrease of STAT3 and/or STAT5 activity.

4.10 Future directions

The inability to expand hematopoietic stem cells (HSCs) *ex vivo* imposes major limitations on the current use of HSC transplantation and gene therapy in patients. This is especially true in cases where the number of available stem cells is limiting (e.g. cord blood-derived stem cells for transplantation into adults). Current methods for expanding the number of stem cells often involve the use of recombinant cytokines. However, these molecules have pro-differentiative as well as proliferative effects, and expansion often occurs at the expense of stem cell differentiation and loss of pluripotent regenerative capacity (Bhatia et al., 1997). While studies have shown that self-renewal is clearly possible *in vitro* (Ema et al., 2000; Glimm and Eaves, 1999), most culture conditions nonetheless result in net HSC losses, indicating that differentiation is favoured over expansion. Conditions suitable for expanding reconstituting cells that do not induce differentiation and loss of stem cell function have yet to be found (Weissman, 2000b). Using single or a combination of cytokines does not seem to be sufficient to induce clinically significant HSC expansions. It appears they rather serve a role in HSC survival and proliferation than directing self-renewal. Hence, efficient means of selective expansion of HSCs, either *in vitro* prior to transplantation or *in vivo*, is of key importance for future progress.

Recent attention has focused on cell intrinsic pathways, whose activation has caused HSC expansion *ex vivo*. A few candidate genes with the ability to expand stem cells have been reported. Overexpression of the P glycoprotein pump genes MDR1 or ABCG2 led to the expansion of side population cells with retained repopulation ability (Sorrentino et al., 1992; Bunting et al., 1998 and 2000). Activation of retinoic acid receptor signaling by

addition of exogenous all-trans retinoic acid resulted in retention of long-term repopulating activity after 14 days in culture (Purton et al., 2000). Constitutive Notch activation in $lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{+}$ bone marrow cells led to immortalization of blast-like cells that retained lympho-myeloid differentiation and long-term repopulating ability (Varnum-Finney et al., 2000). Addition of soluble Sonic Hedgehog protein (Shh) to liquid cultures of human bone marrow cells led to at least 3-fold expansion of SCID-repopulating cells via modulation of BMP4 levels (Bhardwaj et al., 2001). Over-expression of the human HoxB4 gene in mouse bone marrow cells enables over 40-fold expansion of HSCs *in vitro* with enhanced stem cell repopulating capacity *in vivo* and maintenance of pluripotentiality. (Thorsteinsdottir et al., 1999; Antonchuk et al., 2002 Buske et al., 2002; Amelia et al., 2003; Krosl et al., 2003)

The present findings from loss-of-function models intriguingly point to the possibility that Gfi1 plays a positive functional role in HSC self-renewal. It is of interest to use gain-of-function models to further define the role of Gfi1 in hematopoiesis. Therefore, further studies are required to elucidate fully the mechanism of Gfi1 action in HSCs in order to determine whether enforced expression of Gfi1 can be used safely to generate or expand stem cells *ex vivo* for cell or gene therapy.

Gfi1 has low oncogenic potential, however, it could act as a dominant oncogene when overexpressed in T cells, and cooperates strongly with Pim1 and Myc in accelerating progression of T cell lymphomagenesis in MoMuLV-infected mice (Schmidt et al., 1996, 1998a and 1998b; Zörnig et al., 1996; Scheijen et al., 1997). Although accumulation of blastoid monocytic cells occurs in Gfi1 deficient mice, overt leukemic transformation was not seen (Karsunky et al., 2002a; Hock et al., 2003). Furthermore, Bmi1, an onco-protein which belongs to the same complementation group as Gfi1 during lymphoid transformation, has been shown to be essential for maintenance of self-renewal of both normal adult HSCs and leukemic HSCs (Park et al., 2003; Lessard and Sauvageau, 2003). Since Gfi1 is required for the self-renewal of normal HSCs, it is of interest to know weather Gfi1 is needed for regulating the self-renewal and proliferative activity of leukaemic HSCs.

Similarly, Bmi1 is required for the self-renewal of stem cells in the peripheral and central nervous systems (Molofsky et al., 2003). Given its tissue distribution (Wallis et al., 2003), it will be therefore important to know whether Gfi1 serves similar regulatory pathways in non-hematopoietic developmental systems, and to determine whether our findings reported here might also extend to other types of stem cells.

5. Summary

Multilineage hematopoiesis is maintained by a pool of stem cells, which ensures the formation of all blood cells and a functional immune system. To this aim, HSCs must self-renew and regulate the relative balance between self-renewal and differentiation. Gfi1 is a zinc-finger transcription factor and onco-protein, which is differentially expressed in cells the hematopoietic and immune compartment and plays important roles in development of myeloid cells and lymphoid cells. The expression pattern and the function of Gfi1 in development of adult mouse HSCs and progenitors were investigated by using Gfi1 deficient mice ($Gfi1^{-/-}$), and Gfi1:GFP knock-in mice in which the Gfi1 coding region is replaced by the GFP gene.

Gfi1 expression was followed by measuring green fluorescence, and it was found that Gfi1 is expressed HSCs, in CLPs and GMPs, but not in CMPs and MEPs. Consistent with the Gfi1 expression pattern, Gfi1 deficient mice show reduced frequencies of HSCs, CMPs and CLPs and an increase in the GMP population, suggesting that Gfi1 is essential for the maintenance of homeostasis of stem cell and early progenitor populations. The alteration of the number of progenitors was confirmed by *in vivo* (CFU-S, radioprotection) and *in vitro* functional assays (CFC). A reduction of the numbers and the size of $CFU\text{-}S_{12}$ and moderately compromised short-term radioprotection capacity were found in $Gfi1^{-/-}$ bone marrow transplanted hosts. Bone marrow transplantation experiments confirm that the hematopoietic defects in $Gfi1^{-/-}$ mice are cell autonomous.

Furthermore, when measured the reconstitution capacity of $Gfi1^{-/-}$ bone marrow cells against wild-type bone marrow cells in a competitive transplantation assay, we observed a severe impairment in long-term repopulating activities. This defect is not a result of a homing defect or a differentiation defect, but is correlated with an unusually high proportion of actively cycling HSCs. A large proportion of $Gfi1^{-/-}$ HSCs leave G_0 phase to enter the cell cycle. We present evidence that a deregulation of the Gfi1 downstream effectors and cell cycle regulators $p21^{cip1/waf1}$, E2F5 and E2F6 is responsible for these

defects, and suggest that Gfi1 controls self-renewal and engraftment abilities of HSCs by regulating their cell cycle exit or entry and by maintaining a constant proportion of HSCs in G_0 phase.

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Publications

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2. **Zeng H**, Jin L, Richard RE, et al. Receptor specificity in the self-renewal and differentiation of primary multipotential hemopoietic cells. Blood, 98:328, 2001.
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1. Jin L, **Zeng H**, Otto K. Emery DW, Blau CA. In vivo selection of genetically modified primary hemopoietic cells using a cell growth switch. 28th Annual Meeting of the International Society of Experimental Hematology, Monte Carlo, Monaco, July 10-15,1999.
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3. **Zeng H**, Zhao X, Ma Y, et al. Functional expression of Fas in HL-60 cells induced by retinoic acid . 4th China-Japan Symposium on Hematology, Kyoto, Japan, October 1996.

Erklärung:

Hiermit erkläre ich, gem. §6 Abs.2, Nr.7 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich das Arbeitsgebiet, dem das Thema „Requirement of the Transcription Factor and Onco-Protein Gfi1 for the Development and Function of Hematopoietic Stem Cells and Progenitor Cells“ zuzuordnen ist, in Forschung und Lehre vertrete und den Antrag von Herrn Hui Zeng befürworte.

Essen, den 9.2.2004

Prof. Dr. T. Möröy
(Betreuer der Arbeit)

Erklärung:

Hiermit erkläre ich, gem. §6 Abs.2, Nr.6 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich die vorliegende Dissertation selbstständig verfasst und mich keiner anderen als der angegebenen Hilfsmittel bedient habe.

Essen, den 9.2.2004

Hui Zeng
(Doktorand)

Erklärung:

Hiermit erkläre ich, gem. §6 Abs.2, Nr.8 der Promotionsordnung der Fachbereiche 6 bis 9 zur Erlangung des Dr. rer. nat., dass ich keine anderen Promotionen bzw. Promotionsversuche in der Vergangenheit durchgeführt habe und dass diese Arbeit von keiner Fakultät abgelehnt worden ist.

Essen, den 9.2.2004

Hui Zeng
(Doktorand)