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UNIVERZITA KARLOVA
1. lékařská fakulta

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# Úloha kmenových a progenitorových buněk v regeneraci krvetvorné tkáně

# The role of stem and progenitor cells in regeneration of hematopoietic tissue

Disertační práce

Ph.D. Thesis

Vedoucí závěrečné práce/školitel: Prof. MUDr. Emanuel Nečas, DrSc.

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# Content

# 1. List of Abbreviations

| | |
|---|---|
| ACK2 | c-Kit blocking antibody |
| AGM | aorta-gonad-mesonephros |
| ANOVA | analysis of variance |
| BFU-E | burst-forming unit-erythroid |
| BM | bone marrow |
| c-Kit | CD117, stem cell factor receptor |
| CFU-E | colony-forming unit-erythroid |
| CFU-GEMM | colony-forming unit-granulocyte-erythroid-macrophage-megakaryocyte |
| CFU-GM | colony-forming unit-granulocyte-macrophage |
| CFU-M | colony-forming unit-megakaryocyte |
| CLP | common lymphoid progenitor |
| CMP | common myeloid progenitor |
| CTRL | control |
| D | day after irradiation of mice |
| dHSC | definitive hematpoietic stem cell |
| E | embyronic day |
| EHT | endothelial-to-hematopoietic transition |
| EMP | erythro-myeloid progenitors |
| EPO | erythropoietin |
| EryP-CFC | primitive erythroid progenitors |
| FACS | fluorescence-activated cell sorting |
| FSC | forward scatter |
| FL | fetal liver |
| GFP | green fluorescein protein |
| GM-CSF | granulocyte-macrophage colony-stimulating factor |
| GMP | granulocyte-megakaryocyte progenitor |
| HCT | hematokcrit |
| HSC | hematopoietic stem cell |
| HSPC | hematopoietic stem and progenitor cell |

| | |
|---|---|
| LK | $Lin^{-}c\text{-}Kit^{+}$ |
| LSK | $Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{+}$ |
| LS-K | $Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{-}$ |
| LT-HSC | long term repopulating hematopoietic stem cell |
| Mac-CFC | primitive macrofage progenitors |
| MEP | megakaryocyte-erythrocyte progenitor |
| MPP | multipotent progenitor |
| M-CSF | monocyte colony-stimulating factor |
| PB | peripheral blood |
| Pre-HSC | precursor of hematopoietic stem cell |
| RBC | red blood cell |
| SC | side scatter |
| Sca-1 | stem cell antigen |
| SCF | stem cell factor |
| SP | side population |
| ST-HSC | short term repopulating hematopoietic stem cell |
| T | transfusion of red cells |
| TPO | trombopoetin |

# 2. Introduction

## 2.1. Steady state hematopoiesis

Steady-state hematopoiesis in adult mammals is highly studied using well-established methods like flow cytometry, cell sorting, clonal cultivation, transplantation, and gene expression analysis. These studies found a generally accepted developmental hierarchy of steady-state hematopoiesis complemented with specific transcriptomes of particular cell types in this cellular system (Gazit et al., 2013; Guo et al., 2013; Moignard et al., 2013; Klimmeck et al., 2014; Franziska Paul et al., 2015; Nestorowa et al., 2016; Giladi et al., 2018). At the top of the cell development hierarchy is rare hematopoietic stem cells (HSCs) characterized by the self-renewing ability allowing them to prevent their exhaustion when giving rise to more differentiated multipotent progenitor cells (MPPs). MPPs then give rise to lineage-committed progenitor cells further developing into the myeloid (CMPs) or lymphoid (CLPs) precursor cells (Weissman, 2000; Adolfsson et al., 2001; Na Nakorn et al., 2002; Kiel et al., 2005; Yang et al., 2005; Pronk et al., 2007; Wilson et al., 2007; Morita et al., 2010; Oguro et al., 2013) to finally accomplish the need for mature blood cells.

### 2.1.1. Hemtatopoietic stem cell

The bone marrow transplantation and transplantation of specified separated cells to syngeneic hosts with hematopoietic tissue damaged by ionizing radiation have become the key experiments for the final identification of pluripotent HSCs the middle of the last century (Ford et al., 1956). The main feature of the HSC has become its transplantation ability and restoration of destroyed hematopoiesis in the host. The rigorous definition of the HSC requires that the cell produces all types of blood cells, i.e., demonstrates its multipotent (pluripotent) developmental potential, and, at the same time, restores its population of transplantable stem cells, i.e., demonstrates the ability to self-renew. This fundamental attribute of hematopoietic stem cell is expressed by its functional designation “Long Term Repopulating Cell” (LTRC). Recent research has shown that not all HSCs are fully multipotent and that some long-term repopulating cells produce only specific blood cells (Carrelha et al., 2018).

The self-renewing ability of stem cells by cell division is thus their critical defining feature. The self-renewal capability assumes that after cell division, at least one daughter cell, or both, is/are entirely identical to the cell that has divided. After such cell division, the cell is regenerated (renewed) and is still present in the tissue in unchanged or increased numbers (Figure 1).

The concept of asymmetric and symmetric cell division is associated with the self-renewing ability of cells. Three possibilities can arise in connection with cell division and eventual differentiation: the result of cell division can be asymmetric self-renewing-differentiating, symmetrically self-renewing, or symmetrically differentiating, in the latter case, the divided cell has not been replaced by any of the two daughter cell arising from the cell division (Figure 1A, B, C).

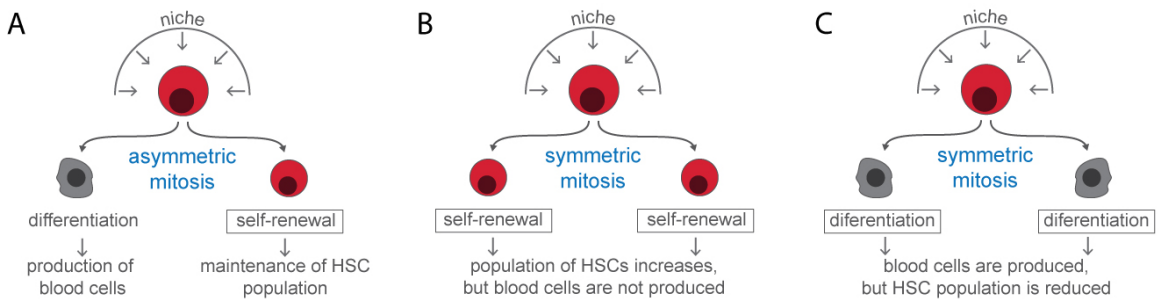


**Figure 1. Cell division associated with cell self-renewal or differentiation. A)** asymmetric self-renewal and differentiation division, **B)** symmetric self-renewal division, **C)** symmetric differentiation division

Evidence for the self-renewal ability of HSCs by the symmetrical self-renewal division has been provided by transplantation of a very small tanumber of stem cells (Brecher et al., 1993) and by transplantation of single cells (Ogawa, 1991; Morita et al., 2010), resulting in long-term monoclonal hematopoiesis, i.e., the full-fledged hematopoiesis derived from a single cell. Because bone marrow with monoclonal hematopoiesis can be successfully transplanted to several mice, the original transplanted cells had to generate several transplantable cells with the stem cell developmental potential.

The asymmetric result of cell division is based on the uneven distribution of cytoplasmic factors, which consequently lead to different gene expression in two daughter cells (Loeffler et al., 2019). This is an example of the internal asymmetric mitosis. Asymmetric mitosis can also be induced externally by different environments acting on daughter cells. These different external cues induce different gene expression through receptors and signaling pathways which are in both cells. Examples of such external cues are morphogens, e.g., Wnt, Hedgehog, BMP (Santoro et al., 2016). In the case of HSCs, there is no direct evidence for either of the two possibilities. However, indirect evidence for asymmetric outcome of the cell division in immature hematopoietic cells has been provided by Páral et al. (2018). This study indicated that approximately half of immature bone marrow cells carrying Sca-1 antigen lose the

antigen after their division while the second half of these cells maintain it when starting the next cell cycle.

### 2.1.2. Developmental hierarchy of hematopoietic cells

The introduction of flow cytometry into experimental studies of hematopoietic tissue, together with cell sorting and their transplantation, enabled the establishment of an immunophenotype-based developmental tree-like hierarchy model of immature hematopoietic cells. This hierarchy depends on, and is controlled, by precisely balanced/conducted key transcription factors and cytokines. It is considered a one-way developmental hierarchy in which the ability of self-renewal divisions progressively decreases in favor of the differentiation into more mature cell types. Various surface antigens discernible by monoclonal antibodies on murine hematopoietic cells are used to identify the individual hierarchical steps in this hierarchy by different research groups. In the classical model of transplantable stem and progenitor cells, two main subgroups are distinguished according to their capacity to reconstitute and maintain damaged hematopoiesis. The long-term (LT-) repopulating cells are identified with HSCs, also termed as LT-HSCs. They are rare quiescent cells with a full long-term (> 3-4 months) reconstitution capacity and the ability to restore their population of HSCs (Kondo et al., 1997; Akashi et al., 2000). These cells are usually identified with $CD201^{+}$ $CD150^{+}CD48^{-}Lin^{-}$ $c\text{-}Kit^{+}Sca\text{-}1^{+}$ or $CD34^{-}Flt3^{-}Lin^{-}c\text{-}Kit^{+}Sca\text{-}1^{+}$ cells. On the other hand, short-term (ST-) repopulating cells are CD34 positive can become a transient source of blood cells after transplantation for less than three months and mostly during the first month after their transplantation. They are also termed ST-HSCs. LT-HSC are thought to differentiate into ST-HSC and the later subsequently into multipotent progenitors (MPPs), which already lack a significant self-renewing capacity (Yang et al., 2005).

The hallmark of murine early hematopoietic cells, including stem and progenitor cells, is the c-Kit tyrosine kinase receptor (Ogawa et al., 1991; Okada et al., 1992). The c-Kit is activated after binding its ligand, the cytokine stem cell factor (SCF) which results in the formation of c-Kit homodimers. After activating of three internal signal cascades, those of PI3K/AKT, Ras-Erk and JAK/STAT, c-Kit homodimers with bound SCF are internalized and degraded (Lennartsson and Rönnstrand, 2012). Stem and progenitor cells thus depend on c-Kit mediated signals for their survival and proliferation. The expression of c-Kit is lost during differentiation into differentiated precursors of blood cells except for mast cells and dendritic cells. The hematopoietic cells expressing c-Kit receptor and lacking markers of lineage-restricted cells are designated as LK cells, ie. Lineage negative and c-Kit positive cells (Figure 2).

Another marker commonly used to characterize HSPCs in the most widely used mice strain C57Bl, is the Sca-1 antigen (Sca-1). LT-HSCs, ST-HSCs, and MPPs all express Sca-1. The loss of Sca-1 expression shifts LK cells into developmentally more advanced progenitor cells with restricted differentiation potential.

Two Sca-1-negative ($LS^{-}K$) cells populations and developmental lineages can be distinguished according to the expression of IL-7R: the myeloid lineage lacking IL-7R, starting with common myeloid progenitors (CMPs) and having the myeloid, erythroid and megakaryocytic developmental potential, and the IL-7R positive lymphoid lineage, starting with common lymphoid progenitors (CLPs) and having the developmental potential into lymphoid T, B, NK, and dendritic cells. The myeloid branch of hematopoiesis (which is the main object of our research interest) is in the classical hierarchical models bifurcated into the bipotent granulocyte-macrophage progenitors (GMPs) and the megakaryocyte-erythrocyte progenitors (MEPs) (Figure 2).

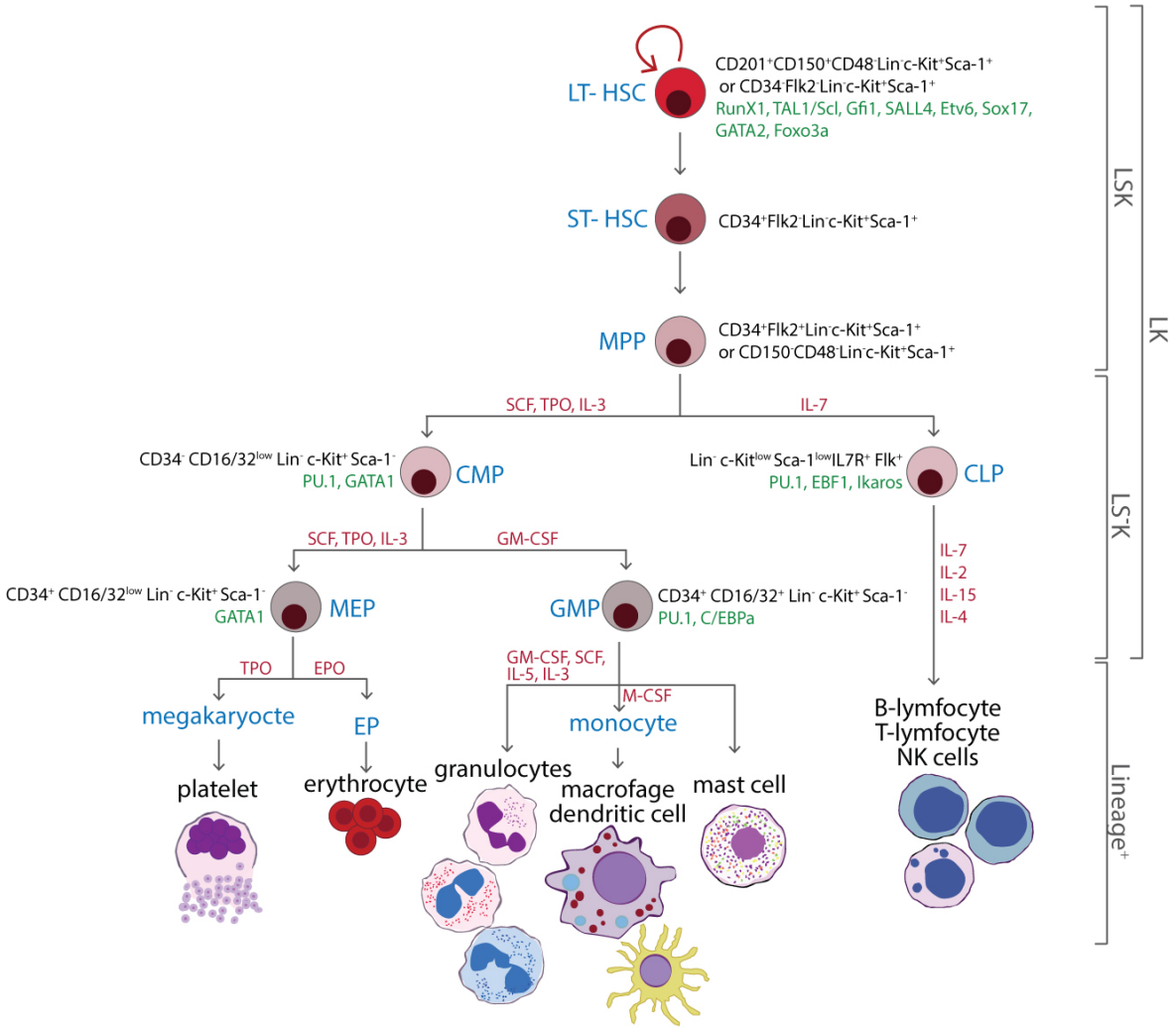


**Figure 2. The classical model of hematopoietic hierarchy.** The phenotype of the cells is written in black, upregulated genes in green, and cytokines to which cells are responsive in red letters. The picture is adjusted according to Cheng et al. (2020).

The classical model based mainly on surface markers and transplantation results using sorted bulks of cells helps understand the differentiation process in the hierarchy of the hematopoietic cell. The situation is much more complicated. This has been revealed over the past several years by applying new technologies as, for example, single-cell analysis and gene-targeted mouse models.

The classical model of the developmental hierarchy within immature hematopoietic cells suffers from the fact that the populations overlap and thus are not homogenous. There are many examples for this also at the level of HSCs. Some of the HSCs have equal potential to produce myeloid and lymphoid cells, others of them are myeloid- or lymphoid-biased HSCs regarding the ratio of myeloid to lymphoid cell output (Dykstra et al., 2007). A platelet-biased subset of myeloid-biased HSCs has been reported

(Sanjuan-Pla et al., 2013). Yamamoto et al. distinguished within the population of HSC, defined as LSK $CD34^{-}CD150^{+}$ cells, two fractions using the CD41 marker (Yamamoto et al., 2013, 2018). The CD41 negative fraction was enriched for functionally multipotent long/intermediate-term reconstituting HSCs after transplantation, whereas the CD41 positive fraction tended to show a more myeloid commitment. In addition, the proportion of these two fractions of HSCs changed with the age of the mouse. In aged mice, Yamamoto et al. showed an increased CD41 positive fraction of LSK cells

Cheng et al. (2020) reviewed the heterogeneity of HSCs and concluded that the cell fate predetermination occurs in HSCs before their differentiation towards progenitors.

Multipotent progenitors, the developmental category of cells directly derived from HSCs, are often divided into four subgroups according to their immunophenotype, developmental lineage bias, cell cycle status and bone marrow abundance (Wilson et al., 2008; Pietras et al., 2015; Boyer et al., 2019; Cheng et al., 2020). The subgroup MPP1 may have a multiple lineage reconstitution ability up to 4 months after transplantation demonstrating the self-renewal capacity. The MPP2, MPP3, and MPP4 subgroups lack the self-renewal potential and have the ability for a time-limited to 1 month. MPP2 cells are megakaryocyte-biased, MPP3 are rather myeloid-biased progenitors. Both these groups are functionally distinct from MPP4, which are considered lymphoid-primed MPPs.

Another new insight into the population of HSC was brought by Yamamoto et al. (2013), who described the myeloid-bypass pathway (Figure 3) directly branching from the most primitive HSCs and concluded that this bypass could be essential for the fast response to hematopoiesis ablation stress.

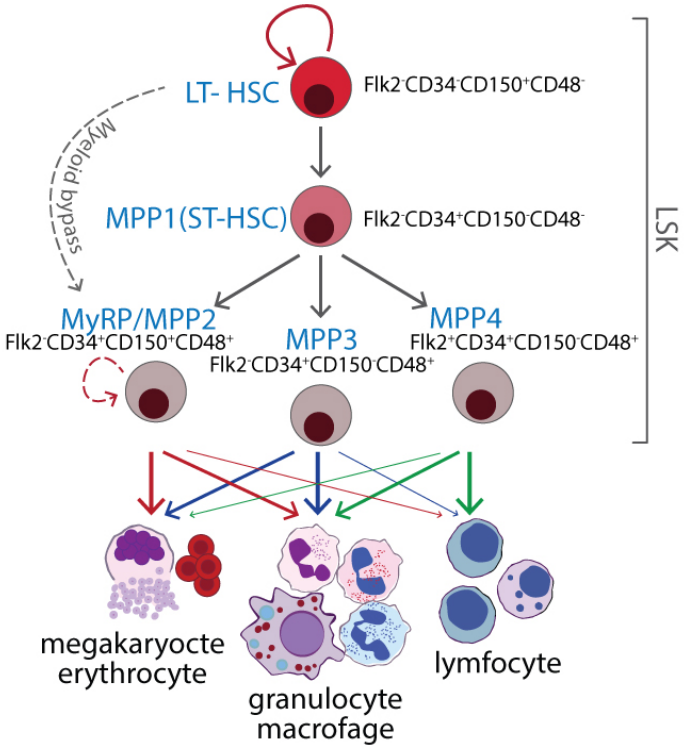


**Figure 3. The revised model for multipotent progenitors.** Adjusted according to Wilson et al., (2008), Yamamoto et al., (2013), Pietras et al., (2015) and Cheng et al. (2020).

CMPs, derived from MPPs, are defined as a homogenous population of $LS^{-}K$ $CD34^{+}CD16/32^{-}$ cells in the classical hierarchical model, but later studies revealed their heterogeneity in the lineage commitment. By employing additional cell membrane markers, CD150, CD105 (endoglin) and CD41, Pronk et al. (2007) distinguished within CMPs $CD41^{-}$ $CD150^{-}$ $CD16/32^{+}$ cells as GMPs and $CD41^{-}CD150^{-}$ $CD16/32^{-}$ as a mixed population of classical CMPs and MEPs (Figure 2). Relatively newly were defined subpopulations like Pre MegE, Pre GM, Pre CFU-E and CFU-E and MkPs (Pronk et al., 2007), and the process of early myeloid differentiation has thus been specified by describing novel groups of intermediate progenitors (Figure 4).

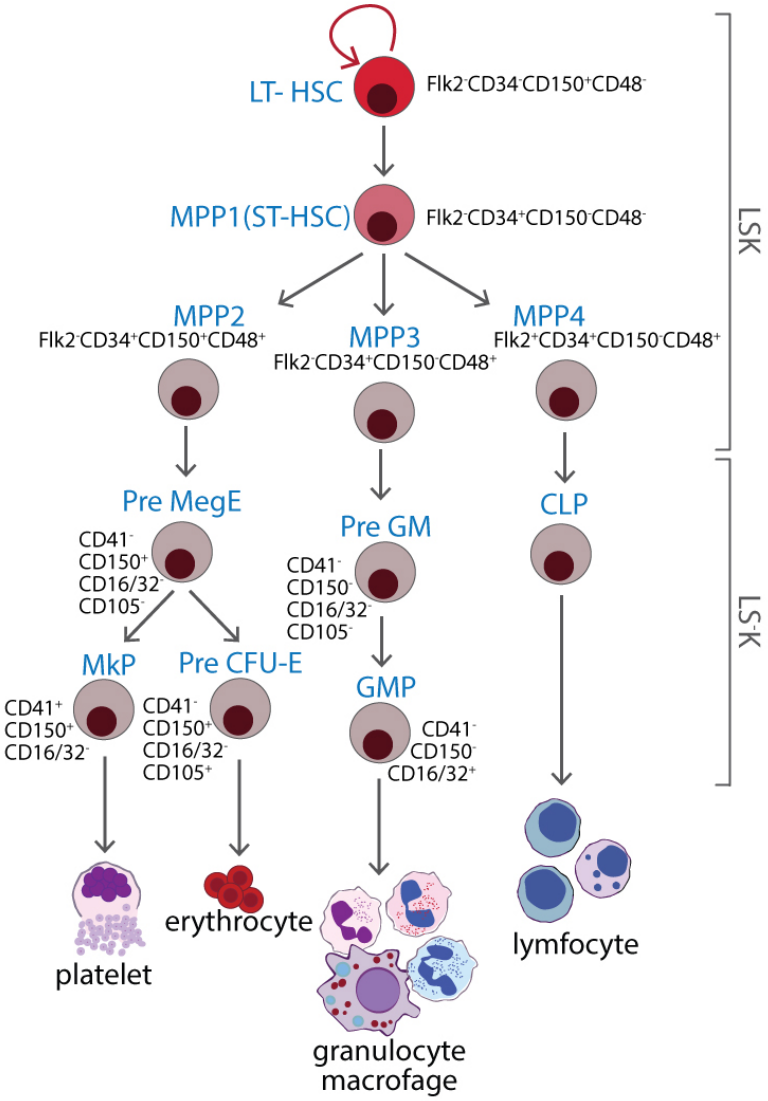


**Figure 4. The revised model for HSC differentiation.** The picture was adjusted according to Cheng et al. (2020) and Pronk et al. (2007).

Differentiation of HSCs into various types of progenitors does not occur in clearly different steps but occurs continusously by the stepwise and quantitatively graded acqiuisition/loss of specific cellular features. Therefore, efforts are made to replace the classical hierarchical model with a cell continuum model which is more flexible and accounts for the overlap of cell population. This cellular continuum is orchestrated by a highly coordinated transcriptional program, simultaneously suppressing cell proliferation-related genes and upregulating lineage-specific genes.

### 2.1.3. Stem cell niche in the hematopoietic tissue

Hematopoiesis occurs in specific tissues: bone marrow and lymphatic tissues. In rodents, the spleen retains hematopoietic function in adulthood. In embryonic development, the yolk sac and fetal liver

are vital hematopoietic organs. Thus, hematopoiesis must be supported by a specific tissue environment, a source of vital and controlling factors that largely determine the fate of hematopoietic cells. The tissue microenvironment that allows stem cells their function is named a "niche". It is assumed that the stimuli and information from the niche determine whether the stem cell would:

- remains resting, and therefore not active in hematopoiesis
- divide by symmetrical or asymmetrical self-renewal division
- differentiate or divide by symmetrical differentiating division
- leave the niche either by local migration or blood circulation
- undergo apoptotic death (Figure 5).

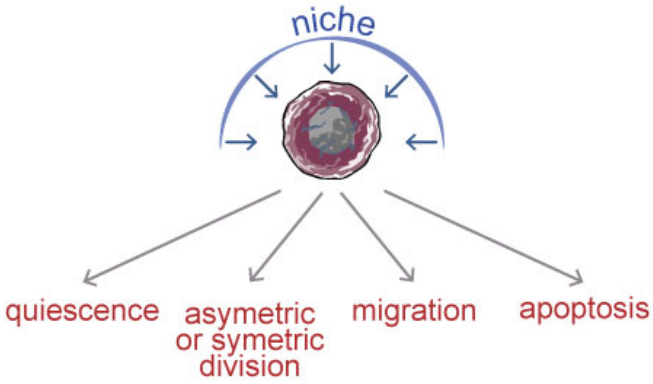


**Figure 5. The behavior and fate of a stem cell are determined by its internal state of gene expression and the cell environment - niche.**

Research the HSC niche is difficult because of its scarcity in hematopoietic tissues. Only a few thousand niches in whole bone marrow distributed to a large number of bones exists (Forgacova et al., 2013). Because HSCs are heterogeneous in terms of their developmental potential, it should be assumed that their niches are also specialized (Morrison and Scadden, 2014).

Initially, the HSC niche was associated with osteoblasts in the bone endosteum. However, later studies located HSCs near vascular sinusoids (perivascular) and near cells that produce the cytokine stem cell factor (SCF) and the chemokine CXCL12 (also SDF-1). The predominant source of these factors are mesenchymal stromal cells, partly also endothelium, and adipocytes. Osteoblasts do not produce these cytokines, and their role in the support and control of HSCs is probably indirect. The low oxygen partial pressure (hypoxia) as a significant feature of the niche has also not been confirmed (Morrison and Scadden, 2014). It can be thus concluded that the stromal cell support for hematopoiesis is essential but still poorly understood. This control underlies the shift of hematopoiesis from the

extraembryonic yolk sac and placenta to the embryo proper. Hematopoiesis then migrates within the emryo from the aorta-gonad-mesonephros region to the fetal liver and spleen and finally to the bone marrow (see further on in chapter Embryonal hematopoiesis).

### 2.1.4. Transplantability of hematopoietic stem cells

The ability to be transplanted and subsequently repair damaged hematopoietic tissue is a fundamental feature of the HSC. It is also the basis for the treatment by bone marrow transplantation and transplantation of hematopoietic cells obtained from blood (Cetkovský et al., 2016). However, the biological processes underlying HSC transplantation are still only partially understood. The procedure involves the introduction of bone marrow cells or separated hematopoietic cells into the bloodstream. The efficiency with which stem cells engraft in the host bone marrow is surprisingly high. Morita et al. (2010) reported that up to 50 % of single cells bearing the stem cell features and transplanted intravenously to more than 2000 mice become the source of blood cells. Forgacova et al. (2013) demonstrated the very high efficiency of bone marrow transplantation by competitive transplantation to submyeloablatively irradiated hosts. Virtually all stem cells contained in bone marrow transplanted to mice exposed to various sublethal doses of ionizing radiation began to produce blood cells and became the source of blood cells for six months of the follow-up period.

The nature of the navigation of intravenously administered stem cells into their niche in hematopoietic tissues is partially known. The secretion of the chemokine CXCL12 by niche cells and the presence of its CXCR4 receptor on stem cells is significat for the engraftment of transplanted cells to hematopoietic tissues (Vagima et al., 2011). Niche is also a source of the cytokine SCF, a ligand for the c-Kit receptor, present on the membrane of all immature hematopoietic cells. However, a significant reduction in the c-Kit receptor expression on transplanted cells did not reduce their transplantation efficiency (Chen et al., 2016).

### 2.1.5. Participation of hematopoietic stem cells in steady-state hematopoiesis

In recent years, the active participation of HSCs in the day-to-day production of blood cells has been questioned and investigated. Sun et al. (2014) reported that hematopoiesis in adult mice is maintained by cells that are hierarchically below stem cells, i.e., by multipotent progenitor cells. They concluded that the mouse bone marrow contains a significant amount of multipotent progenitors which are successively used for blood cell production and hematopoietic stem cells become activated only when hematopoietic tissues are damaged. This communication elicited a significant experimental response

with inconsistent results. The issue of "dormant" and “active” HSCs in undisturbed adult hematopoiesis remained unresolved and the relevant results were reviewed by McRae et al. (2019).

### 2.1.6. Amplification of cell production in blood cell formation

A small number of stem cells or multipotent progenitors give rise to a much larger number of blood cells in the process of hematopoiesis. This increase in cells produced is the result of repeated cell divisions in blood cell production. In our laboratory, we analyzed at which developmental stage of blood cells the most important amplification factor (Páral et al., 2018). By determining the cell cycle duration of different progenitor cells and their number in steady state murine hematopoiesis, we concluded the major amplification step is at the developmental level of myeloid progenitors that no longer express the Sca-1 antigen. This discovery demonstrated the enormous potential of these particular cells to rapidly answer to changing needs for blood cell production in the myeloid lineages covering erythropoiesis and production of granulocytes and macrophages.

## 3.1. Embryonal hematopoiesis

The stem cell-based hematopoiesis is established during embryonic development in mice around the tenth embryonic day (E10). However, the first blood cells are produced earlier in the yolk sac around the embryonic day seven (E7). Thus, hematopoiesis is functional before the first transplantable stem cells are formed (Miiller et al., 1994; Schaefer et al., 2001; Kumaravelu et al., 2002; Batsivari et al., 2017; Dzierzak and Bigas, 2018; Balounová et al., 2019). The development of embryonic hematopoiesis can be divided into three successive and overlapping waves (Figure 6). The pre-stem cell embryonic hematopoiesis demonstrates the ability of progenitor cells to produce functional blood cells independently from stem cells transiently.

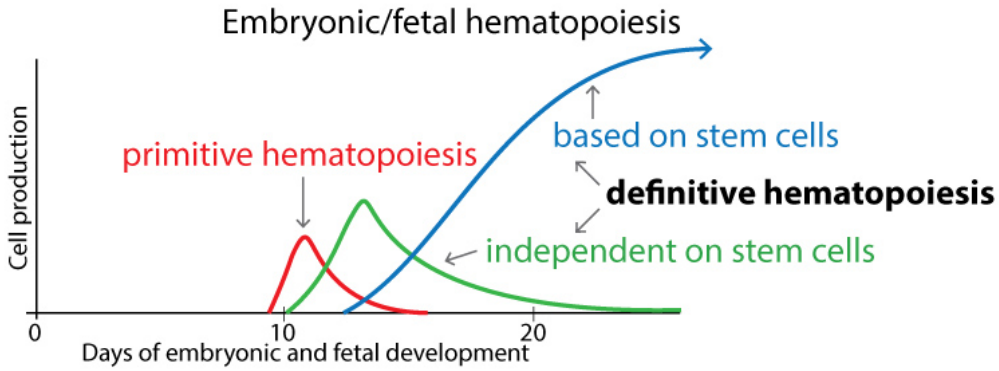


**Figure 6. Primitive and definitive hematopoiesis in the yolk sac, embryo, and fetus (delivery of mouse fetuses occurs on days 20-21 after oocyte fertilization).**

### 3.1.1. Primitive embryonic hematopoiesis

The first blood cells appear early after gastrulation around the seventh embryonic day (E7) in the blood islands within the yolk sac outside the embryo proper. They originate from hemangioblast precursors, giving rise to primitive erythroid, megakaryocyte, and macrophage progenitors (Palis et al., 1999; Huber et al., 2004).

The first primitive erythroid progenitors (EryP-CFC) have been detected at the primitive streak stage of the mouse embryo at the developmental stage E7.O in the proximal regions of the egg cylinder containing presumptive extraembryonic tissues. Palis (1999, 2014) described EryP-CFC cells as in vitro erythroid colony-forming cells that gave rise to colonies containing several hundred mature primitive erythroid cells after 5 days in an in vitro culture containing erythropoietin (EPO). These primitive hematopoietic progenitors lack a distinct c-Kit$^{high}$ population at E7.5 but expressed c-Kit and CD41 at a low level at E 8.5 (McGrath et al., 2015). In the next stage of embryo development, from the neural plate to the early somite stage, there is a dramatic expansion of the primitive erythroid progenitors when the number of EryP-CFC increases 7-fold. Subsequently, there is a sharp decline in their number and from E9.0 onwards, there is no more detectable either in the yolk sacor in the embryo proper (Palis et al., 1999). Between E9.5 and E12.5, EryP-CFC matures into erythroid precursors which undergo expansion through a limited set of symmetric cell divisions and simultaneously accumulate hemoglobin, decrease in cell size, nuclei undergo pyknosis, and the content of RNA decreases. Unlike in the definitive erythropoiesis, the transformation process from proerythrpblasts to primitive nucleated red blood cells is running in the circulation. Primitive red blood cells are distinguishable from the later definitive red blood produced in the fetal liver and bone marrow by their large size and embryonic globin expression (Palis, 2014). Mature primitive red blood cells persist in the blood for several days after birth (Kingsley et al., 2004; Fraser et al., 2007).

First primitive macrophage progenitors (Mac-CFC) were detected concomitantly with primitive erythroid progenitors (EryP-CFC) at E7.0 in the yolk sac (Vobořil et al., 2020). Until E9.0, Mac-CFC were also detectable in blood, but most of them resided in the yolk sac. At E10, Mac-CFC decreased in the yolk sac and increased in the blood (Palis et al., 1999).

### 3.1.2. Definitive hematopoiesis before hematopoietic stem cells

Primitive hematopoiesis is superseded by definitive hematopoiesis which is also transient but in contrast to the primitive hematopoiesis gives rise to red blood cells with fetal or adult hemoglobin. It

initially originates in the yolk sac (Palis et al., 1999; Chen et al., 2011; Palis, 2016) and colonizes the fetal liver earlier than HSCs emerge from the embryo proper. As the source of this definitive embryonic hematopoiesis, McGrath et al. (2015) identified erythro-myeloid progenitors (EMPs) with the erythroid and broad myeloid, but not lymphoid developing potential. The EMPs, emerging in the yolk sac at E9.5, were identified as c-Kit$^{high}$, CD41$^{+}$, 16/32$^{+}$, VEC$^{+/-}$,CD45$^{+/-}$, and Sca-1$^{-}$ cells. EMPs have a similar immunophenotype like the later emerging stem cells derived from hemogenic endothelial cells (see further on) with the significant difference in lacking Sca-1 and expressing CD16/32.

### 3.1.3. Hematopoietic stem cells emergence

Functional HSCs are first detected in hematopoietic clusters in the endothelial cell layer, lining the dorsal aorta wall in the aorta-gonad-mesonephros (AGM) region (Müller et al., 1994; de Bruijn et al., 2002; North et al., 2002). Only 2 cells from 700 cells in the cluster are functional HSCs defined by transplantation (Kumaravelu et al., 2002). The emergence of these HSCs is independent of the yolk sac and the foetal liver (Medvinsky and Dzierzak, 1996; Taoudi et al., 2005) and is known as the endothelial-to-hematopoietic transition (EHT). The cell type responsible for HSC emergence was determined as VEC$^{+}$ endothelial cells appearing around E10. Recently was specified that the cells could be identified by CD44 marker, a receptor for hyaluronan (Oatley et al., 2020).

The basis of EHT is the activation of the hematopoietic transcriptional program characterized by the upregulation of three key transcription factors, Runx1, Gata2 and Gfi1, in a subset of endothelial cells. The co-expression of these transcription factors indicates the affiliation of cells to the endothelium and the blood. The cells round up and break the tight junctions between neighbouring cells. Intra-aortic hematopoietic clusters are then formed and HSCs and are released into the blood-stream (Figure 7). Runx1 is an essential transcriptional factor for definitive-HSC-supported hematopoiesis (North et al., 1999, 2002; Rybtsov et al., 2014).

Rybtsov (2011) described several developmental steps in the emergence of fully comptent HSCs. Precursors of HSCs are the pre-HSCs that expand and mature in the AGM region and cannot yet repopulate hematopoiesis in adult irradiated recipients. Pre-HSCs multiply in the AGM region dramatically from E9.5 to E11.5 (Rybtsov et al., 2011) and develop into definitive HSCa (dHSCs), which already rescue damaged hematopoiesis in adult transplanted mice. Later, Rybtsov et al. (2016) identified as early embryonic ancestors of dHSCs pro-HSCs in E9.5 in the dorsal aorta. These cells are CD34 negative and have low expression of the c-Kit marker. Their progression into definitive HSCs is promoted by SCF (but not by IL-3) and represents the first step in the four-step developmental process resulting in dHSCs.

VEC$^{+}$CD41$^{+}$CD43$^{-}$CD45$^{-}$ pro-HSCs develop into the first type of pre-HSCs (preHSC-1), which are VEC$^{+}$CD41$^{low}$CD43$^{+}$CD45$^{-}$. The pre-HSCs of the second type (preHSC-2) are VEC$^{+}$CD41$^{low}$CD43$^{high}$CD45$^{+}$. Definitive HSCs have a similar expression pattern to preHSC-2 (VEC$^{+}$CD41$^{-/low}$CD43$^{high}$CD45$^{+}$) but are distinguished by transplantation into adult irradiated mice hosts they reconstitute adult hematopoiesis (Rybtsov et al., 2014, 2016). Very recently, the sc-RNA-seq analysis of the cells further specified the identification of all the main populations involved in the EHT in linkage to the expression of the CD44 marker (Oatley et al., 2020; Figure 7).

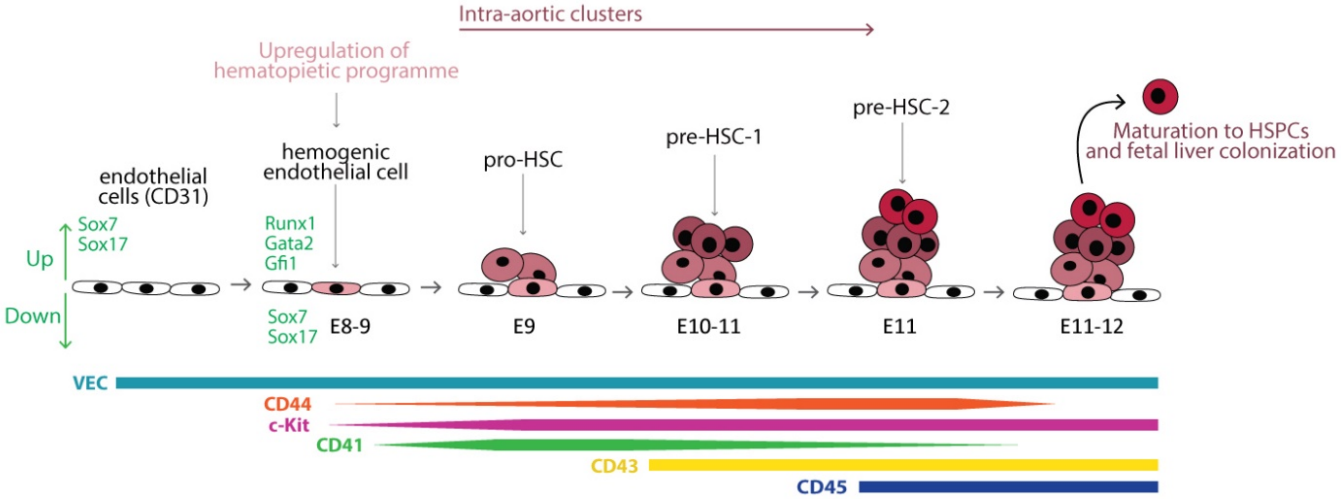


**Figure 7**. **Endothelial-to-hematopoietic transition.** Morphological events, regulation by transcription factors (in green), and phenotypes (lines below). Adjusted according to Rybtsov et al. (2014), Ottersbach (2019) a Oatley et al. (2020).

By E11.5, the number of pre-HSCs in the AGM region is sufficient to give rise to a large pool of dHSCs in the fetal liver through a quick maturation step which does not require any significant cell proliferation (Rybtsov et al., 2016). In the fetal liver, HSCs numbers increase exponentially from E12 to E15 (Ikuta and Weissman, 1992). As the critical chemokine in the process of fetal liver, HSC homing and retaining was identified stroma derived factor 1 (SDF-1/CXCL12) expressed by fetal liver stromal cells (Soares-da-Silva et al., 2020). Christensen et al. (2004) described the ensuing seeding of the fetal spleen and fetal bone marrow by HSCs from the fetal liver as a gradual process occurring over several days.

## 3.2. Regenerating hematopoiesis

A submyeloablative damage to hematopoietic tissues triggers tissue regeneration. In this specific situation, the adult hematopoiesis face very similar challenges as the embryonic and fetal hematopoiesis: hematopoiesis has to be reconstituted from a small number of founder cells initiating the early and vital production of functional blood cells. Any tissue regeneration is a complex process dedending on cells with the potential to restore tissue structure and on the controlling factors providing by the tissue microenvironment, the tissue stroma. After partial damage, the hematopoietic tissue regenerates efficiently. From the phylogenetic aspect, the tremendous regenerative capacity of hematopoiesis is not entirely clear. The pathogenic noxes that severely damage bone marrow are a very recent results of the human activity: exposure to ionizing radiation at effective doses and cytostatics. The high regenerative capacity of hematopoietic tissue is generally associated with stem cells and continuous cell production based on cell proliferation. Stem cells are generally considered the initial source of regeneration.

The reactions of hematopoietic tissues to increased needs of the organism for blood cells or to their damage by cytostatics or ionizing radiation are sometimes called stress hematopoiesis. However, there are significant differences in the responses of hematopoiesis to increased needs for blood cells. For example, acute stimulation of erythropoiesis by erythropoietin occurs at the level of late erythroid progenitors CFU-E (Tusi et al., 2018). However, long-term stimulation with erythropoietin activates hematopoiesis also at the level of stem cells. Stimulated „stress“ erythropoiesis can then be derived directly from stem cells and bypass the normal developmental hierarchy of hematopoiesis (Singh et al., 2018). However, only the „stres hematopoiesis“ after its partial damage correspond to the above-discussed embryonic and fetal hematopoiesis when increased production of functional blood cell is realized with concurrent expansion of populations of stem and progenitor cells.

### 3.3.1. Damage of hematopoietic tissue by ionizing irradiation

Exposure to ionizing radiation (IR) induces immediate and acute damage to all cellular subsets of hematopoiesis, including stem cells. Acute myelosuppression after IR is caused by induction of apoptosis, notably in rapidly proliferating hematopoietic progenitors and in relatively

quiescent HSCs. HSCs are more resistant than progenitor and differentiated precursors of blood cells (Meijne et al., 1991; Ploemacher et al., 1992; Down et al., 1995; Wagemaker, 1995) but far less than cells forming stroma of hematopoietic tissues. Ionizing radiation reduces pools of hematopoietic stem and progenitor cells in a dose-dependent manner (Forgacova et al., 2013). The whole body irradiation of mice with 6 Gy destroys about 99.9% of stem and hematopoietic cells, and in some mice, hematopoiesis is restored from only one cell in the long term (McCarthy, 1997).

Simonnet et al. (2009) studied the response of hematopoietic stem and progenitor cells to exposure of mice to 3 Gy or 6 Gy total body irradiation. An acute dose-dependent reduction in bone marrow cellularity and significant damage to rare cells characterized by very active expulsion of Hoechst 33342 dye, so-called SP (side population) cells highly enriched by stem cells, occured. Some SP cells persisted in the bone marrow of irradiated mice, but their phenotype changed significantly. The expression of the c-Kit receptor was decreased, and that of the Sca-1 antigen was increased. There was an increased expression of CD150 and CD105 markers in c-Kit$^{+/low}$ and Sca-1 $^{+/bright}$ SP cells. The proliferation rate of SP cells increased. The study concluded that SP cells are relatively resistant to ionizing radiation and are the source of early regeneration in hematopoiesis (Simonnet et al., 2009). Ten weeks after irradiation, the number of blood cells in the bone marrow and the expression of c-Kit and Sca-1 on the surface of SP cells were similar to those in untreated mice. However, the frequency of LSK cells in bone marrow was reduced while the proportion of SP cells in LSK cells fraction was increased, and the proportion of LSK cells with a specific phenotype was changed.

There is a limited knowledge about the response of hematopoietic stroma tissue to ionizing radiation. Its damage and recovery were studied by Dominici et al., 2010. The gene for c-Kit ligand SCF was shown significantly upregulated at the mRNA level in hematopoiesis damaged by irradiation (Limanni et al.; Sugimoto et al., 2001).

## 3.4. An overview of research methods in mouse hematopoiesis with emphasis on flow cytometry

Research efforts to understand the mechanisms of hematopoiesis have focused on the hematopoiesis of mice mainly due to the availability of syngeneic and congenic mouse strains

that allow transplantation of hematopoietic tissues and their cells and the monitoring of transplantation results. The research was bolstered by introducing flow cytometry and the availability of antibodies against membrane markers of various hematopoietic cells. Figure 8 is an overview of the developmental stages in hematopoiesis and the most apropriate methods for its experimental dissection. The most frequent analytical approaches are the listed in Table 1. The panhematopoietic marker CD45 generally identifies the relationship of cells to hematopoiesis. Table 2 presents the major membrane markers of murine hematopoietic cells detectable by specific monoclonal antibodies. Figure 9 gives an example of identifying primary subtypes of immature hematopoietic cells, stem cells, multipotent progenitors, developmentally restricted progenitors in the bone marrow of C57Bl mice. Table 3 and Figure 10 show the essential markers used to determine cells beloning to major developmental lineages of blood cells.

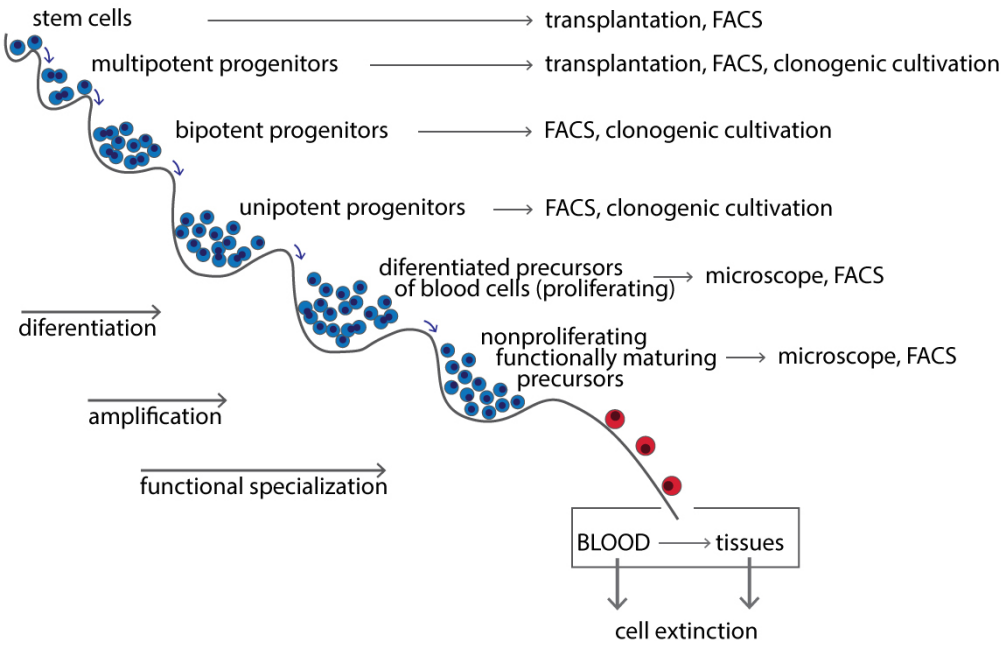


**Figure 8. Methods by which individual types of hematopoietic cells can be studied.**

| **Experimental research on hematopoietic tissue uses:** |
|---|
| bone marrow transplantation in syngeneic and congenic settings |
| transplantation of specific sorted cells |
| hematopoietic tissue damage and its spontaneous regeneration |
| cultivation of HSCs in vitro |
| flow cytometry (FC) and flow cytometry cell sorting (FACS) |
| cell labeling and monitoring of cell clones |
| cell labeling and monitoring of cell clones |
| analysis of natural mutations significantly affecting hematopoiesis |
| targeted gene manipulation |
| advanced microscopic analysis of bone marrow |
| study of the supporting and controlling tissue microenvironment (stroma, niche) |
| study of hematopoietic tissue embryogenesis |
| analogies of molecular mechanisms of hematopoiesis in Danio rerio, fish, and mammals |
| analysis of basic regulation of hematopoiesis by cytokines and systemically acting humoral factors (erythropoietin, thrombopoietin, granulocyte-macrophage growth factors) |
| spleen colony technique |

**Table 1**

| **Cytometric analysis of hematopoietic stem and progenitor cells** | |
|---|---|
| Basic markers: | |
| CD45 | tyrosine phosphatase is expressed on the surface of hematopoietic cells, except erythroid cells; this makes it possible to distinguish hematopoietic cells from stromal cells |
| "Lin" | markers of various differentiated blood cell precursors used usually as a cocktail of antibodies against the lineage features: B220, CD4, CD8, Gr-1, Mac-1, and Ter119 |
| c-Kit | cytokine stem cell factor (SCF) receptor |
| Sca-1 | Stem cell antigen-1 |
| Alternative markers: | |
| CD201 | endothelial receptor for protein C (EPCR) |
| CD105 | endoglin; constituent part of the receptor for the cytokine TGFβ |
| Subsidiary characters: | |
| CD150 | glycoprotein (signaling lymphocytic activation molecule 1; SLAMF1) |
| CD48 | glycoprotein (signaling lymphocytic activation molecule 2; SLAMF2) |
| CD41 | integrin α2b; this integrin has an established role in platelet function (Phillips et al., 1988) |
| CD34 | glycoprotein with presumed adhesive function |
| CD135 | Flt3 receptor (Flk2) for Flt-3 ligand |
| CD16/32 | III/II receptors for the Fc-fragment of immunoglobulins |
| CD127 | interleukin 7 receptor |

**Table 2**

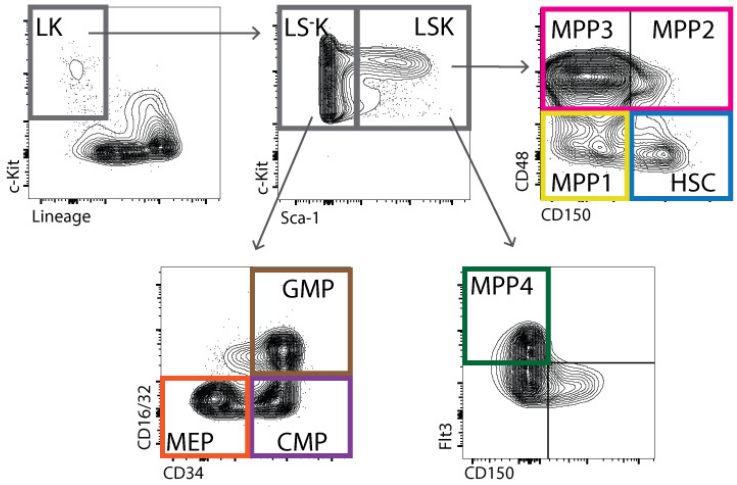


**Figure 9.** The figure shows an example of the resolution of immature hematopoietic cells, progenitors, and stem cells that do not yet have the characteristics of differentiated cells.

| **Cytometric analysis of differentiated precursors and blood cells** | |
|---|---|
| Basic antigenic features of differentiated blood cells: | |
| Ter119 | antigenic marker of erythroid cells from proerythroblasts to erythrocytes |
| CD71 | transferrin receptor 1 |
| CD44 | a glycoprotein, a receptor for hyaluronic acid and other substances in the extracellular mass (collagen, osteopontin) |
| CD41 | integrin α2b (megakaryocytes) |
| CD61 | integrin β3 (trombocytes) |
| Gr-1 | (Ly6C and Ly6G); Ly6G marker is expressed only on granulocytes, Ly6C is less specific, it is expressed on granulocytes and monocytes |
| Mac1 | integrin αMβ2; CR3 complement receptor; consists of CD11b (integrinαM) and CD18 (integrin β2); (monocytes) |
| CD16/32 | III/II receptors for Fc-fragment of immunoglobulins (granulocytes, monocytes) |
| B220 | (CD45R), a B-cell specific isoform of the CD45 marker |
| CD19 | the transmembrane glycoprotein of B-lymphocytes |
| CD11b | a marker expressed on monocytes, neutrophils and eosinophils, and granulocytes |
| CD11c | dendritic cells |
| F4/80 | macrophages |
| CD4 and CD8 | Th4 and Th8 lymphocytes |

**Table 3**

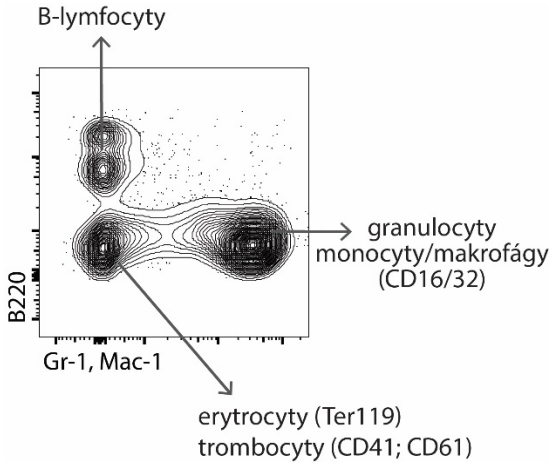


**Figure 10.** The figure shows the resolution of B cells (B220 positive), granulocyte-monocytes (Gr-1/Mac-1 positive), and cells negative for these markers. Among these "negative" cells, erythroblasts that express Ter119 marker predominate (not shown). Erythrocytes also carry the Ter119 marker, but are excluded from the analysis due to their small size. It is also possible to remove erythrocytes by lysis caused by exposure to a hypotonic alkaline environment.

# 4. Hypothesis

**Stem cells are not the exclusive carriers of regeneration of damaged hematopoiesis. Progenitor cells significantly participate in the recovery of blood cell production and early hematopoietic tissue reconstitution. It is assumed that a transient period in the regeneration of damaged hematopoiesis mimics the embryonic stage when production of blood cells is not derived from stem cells.**

# 5. Aims

1) Analysis of progenitor and stem cells in intensively regenerating hematopoiesis.

2) Determination of the role of the SCF/c-Kit receptor signaling and erythropoietin stimulation in early phase of hematopoiesis regeneration.

3) Comparison of the expanding adult hematopoiesis during its regeneration with the expanding hematopoiesis in the fetal liver.

# 6. Materials and methods

## 6.1 Mice

C57BL/6J (CD45.2) and B6.SJL-Ptprca Pepcb/BoyJ (CD45.1) mice were bred in a specific-pathogen free facility of the Center for Experimental Biomodels, First Faculty of Medicine, Charles University and housed in a clean conventional part of the facility (12:12 h light-dark cycle, 22 ± 1 °C, 60 ± 5 % humidity) during the experiments. Adult mice (mostly 8-12 weeks old) of both sexes were used in the experiments. Female mice in estrus were placed in cages with males (two females with one male) to gain mouse fetuses of the required developmental stage. The females were checked in the morning for copulation plugs, and positive ones were followed for pregnancy starting at the E0.5 day. All experiments were performed following national and international guidelines for laboratory animal care and approved by the Laboratory Animal Care and Use Committee of the First Faculty of Medicine, Charles University, and the Ministry of Education, Youth and Sports of the Czech Republic (MSMT-6316/2014-46 and MSMT-4502/2017-2).

## 6.2 Irradiation

Irradiation was performed in a plastic cage and a $^{60}$Co source (Chisobalt 2-B75, MEDIN, formerly Chirana, Czech Republic) from a distance of 123.5 cm with a dose rate of ~ 0.35 Gy per minute.

## 6.3 Spleen colonies (CFU-S)

Spleen colonies were determined in submyeloablatively irradiated mice (4 Gy or 6 Gy) 8-14 days after irradiation (endogenous spleen colonies). Spleens were fixed in Tellesniczky´s solution (18:1:1 volume parts of 70 % ethanol, glacial acetic acid and formalin), and spleen colonies were observed with the naked eye and counted.

## 6.4 Bone marrow collection

Bone marrow cells were obtained from the long bones (femurs and tibias, or femurs only) by flushing the bone cavity with PBS supplemented with 1% bovine serum albumin (BSA) through a hole in one end of the bone without clipping off the epiphyses. A single-cell suspension was obtained by repeated passage through the needle (25G) and kept on ice before further handling.

## 6.5 Flow cytometry and cell sorting

Cells were filtered through a 70µm nylon cell filter (BD Biosciences, San Jose, CA, USA) and stained with fluorochrome-labeled antibodies. Bone marrow cell populations were defined by immunophenotyping and forward (FSC) and side (SSC) scatter characteristics. Bone marrow cells were stained by fluorescently labeled antibodies for 20 minutes at 4$^{o}$ C in the dark with optimal dilutions of commercially prepared antibodies listed in Table 4.

Stained bone marrow cells were analyzed using a digital FACS Canto II flow cytometer, equipped with 405nm (60 mW), 488nm (20 mW), and 633nm (15 mW) lasers and the relevant configuration of optical filters and signal detectors (BD Biosciences). For data acquisition, BD FACSDiva software version 6.1.3 was used. CS&T beads (BD Biosciences) were used for the automated cytometer setup and the performance tracking procedure before measurements. The generated flow cytometry data were analyzed using FlowJo vX software (FlowJo, Tree Star, OR, USA). Debris, red blood cells, and dead cells were excluded from the analysis by gating the FSC-A/SSC-A dot plot. For cell doublet discrimination, a FSC-A/FSC-H dot plot was used. To correctly interpret flow cytometry data, Fluorescence-Minus-One (FMOs) controls were used for gating. Recordings containing less than 50 cells of a particular phenotype were excluded from further analysis.

Cells were sorted with a FACSAria IIu cell sorter (BD Biosciences) equipped with 489nm (50 mW), 561nm (100 mW), 638nm (140 mW), 404nm (100 Mw) and 355nm (20 mW) lasers. Cells were sorted with the use of either 70-micron or 85-micron integrated nozzles (with corresponding sheath pressure), under a "0-18-0" precision mode setup (yield mask 0, purity mask 18, phase mask 0). Cells were sorted into polypropylene microcentrifuge tubes (Eppendorf, Hamburg, Germany) containing 1x PBS supplied with 3-5% albumin fraction V, biotin-free (Carl Roth GmbH, Karlsruhe, Germany). Sterile 1x PBS was used as sheath fluid. BD FACS Diva software version 8.0.1 was used for data acquisition.

| **Antibody** | **Clone** | **Producer** | **Fluorochrome** |
|---|---|---|---|
| anti-Lineage cocktail of antibodies CD45R(B220)/CD3/Ly-6G(Ly-6C)/CD11b/Ter-119) | | http://www.biolegend.com/ | Alexa Fluor® 700 |
| CD117 (c-kit) | 2b8 | http://www.biolegend.com/ | Brilliant Violet 421™ |
| Ly-6A/E (Sca-1) | D7 | http://www.biolegend.com/ | PerCP |
| Ly-6A/E (Sca-1) | E13-161.7 | http://www.biolegend.com/ | PE/Cy7 |
| CD48 | HM48-1 | http://www.biolegend.com/ | PE |
| CD48 | HM48-1 | http://www.biolegend.com/ | FITC |
| CD150 (SLAM) | TC15-12F12.2 | http://www.biolegend.com/ | APC |
| CD150 (SLAM) | TC15-12F12.2 | http://www.biolegend.com/ | Brilliant Violet 605™ |
| CD71 | RI7217 | http://www.biolegend.com/ | FITC |
| CD71 | RI7217 | http://www.biolegend.com/ | PE |
| CD45.2 | 104 | http://www.biolegend.com/ | PE/Cy7 |
| CD41 | MWReg30 | http://www.biolegend.com/ | APC |
| CD41 | MWReg30 | http://www.biolegend.com/ | PE |
| CD45.1 | A20 | http://www.biolegend.com/ | APC |
| CD45R/B220 | RA3-6B2 | http://www.biolegend.com/ | FITC |
| CD4 | 53-6,7 | http://www.biolegend.com/ | PerCP |
| CD8 | GK1.5 | http://www.biolegend.com/ | PerCP |
| Ly-6G(Gr-1) | RB6-8C5 | http://www.biolegend.com/ | Brilliant Violet 421™ |
| CD11b | M1/70 | http://www.biolegend.com/ | Brilliant Violet 421™ |
| TER-119/Erythroid Cells | TER119 | http://www.biolegend.com/ | PerCP |
| CD 127 IL7 | 93 | http://www.biolegend.com/ | Brilliant Violet 785™ |
| CD 16/32 | A7R34 | http://www.biolegend.com/ | Brilliant Violet 510™ |

| | | | |
|---|---|---|---|
| CD 34 | RAM34 | http://www.ebioscience.com/ | Biotin |
| CD 135 | A2F10 | http://www.biolegend.com/ | APC |
| Streptavidin | | http://www.biolegend.com/ | PE/Cy7 |

**Table 4. Reagents used in immunophenotyping of hematopoietic cells**

## 6.6 Identification of immature lineage negative c-Kit positive (LK) cells and their subsets

LK cells and their subsets were identified by flow cytometry, as is shown in the result figures. LK cells were analyzed in their c-Kit$^{high}$ and c-Kit$^{low}$ fractions.

## 6.7 Red blood cell transfusion

Mice under deep anesthesia were exsanguinated from the retro-orbital venous sinus by heparinized capillaries and sacrificed by cervical dislocation. Red blood cells were washed with an excess of PBS, and a 75% suspension of red blood cells in PBS was prepared. The suspension was intravenously injected in a volume of 0.5-0.6 ml to mice via the retro-orbital route (29G needle).

## 6.8 Erythropoietin administration to normal mice

Male mice were injected with recombinant human erythropoietin (NeoRecormon epoetin beta; Roche, Basel, Switzerland) intraperitoneally for four consecutive days, thecumulative dose was 200 IU/mouse. Bone marrow was collected 24 hours after the last EPO injection.

## 6.9 A state of iron deficiency

Male mice were put on a low-Fe diet (C 1038; Altromin Spezialfutter GmbH & Co. KG, Hamburg, Germany) for 7 days and were bled 0.5-0.6 ml from the retro-orbital venous sinus 5 days and 1 day before the examination of peripheral blood and bone marrow.

## 6.10 ACK2 c-Kit blocking antibody administration

ACK2 In Vivo Ready$^{TM}$ Anti-Mouse CD117 (c-Kit) was used as the c-Kit blocking antibody. The reagent was from Tonbo Biosciences (U.S.A.). ACK2 antibody was administered to mice intravenously (0.5 mg in 0.25 ml per mouse).

## 6.11 Peripheral blood analysis

Peripheral blood was collected from the retro-orbital venous sinus of anesthetized mice using capillaries (75mm/60µl; KERAGLASS, Otovice, Czech Republic) containing a small volume of EDTA and was analyzed with a BC-5300Vet Auto Hemato Analyzer (Mindray Bio-Medical Electronics, Shenzhen, China) calibrated for mouse blood samples.

## 6.12 In vitro cultivation of bone marrow cells in semi-solid media

Bone marrow cells (pooled from 3 mice) of either untreated mice or mice irradiated (6 Gy) 14 days prior were cultured in duplicates on 30-mm Petri dishes in 1 ml of one of three types of MethoCult™ semi-solid media (STEMCELL Technologies, Vancouver, Canada). $3x10^4$ (GF M3434 medium), $1x10^5$ (SF M3436 medium), or $2x10^5$ (M3334 medium) cells of normal bone marrow were plated per dish. Sorted LSK and $LS^{neg}K$ cells were plated in 200-1000 umbers per dish. Single cells were sorted into 96-well plates for the analysis of cells with different CD71 expression levels. The cultures were conducted at 37° C in a humidified air atmosphere with 5 % $CO_2$ for 2 days in the M3334 medium (CFU-E clusters) and 8 – 12 days in the SF M3436 and GF M3434 media. Colonies were counted and analyzed by phase-contrast light microscopy and evaluated according to STEMCELL Technologies' Mouse Colony-Forming Unit (CFU) Assays Technical Manual (v 3.2.0; Document # 28405). Examples of the CFU-E clusters and BFU-E, CFU-GEMM, CFU-GM, CFU-G, CFU-M colonies are shown in Figure 11.

In three independent experiments, either LSK or $LS^-K$ were sorted from bone marrow pooled from three normal or three mice irradiated 14 days before bone marrow collection. 200 LSK cells or 500 $LS^-K$ cells sorted from the normal bone marrow and twice as many cells sorted from the regenerating bone marrow were cultured in GF M3434 medium per dish. Colonies were classified into CFU-GEMM, CFU-M, CFU-G, CFU-GM, or BFU-E.

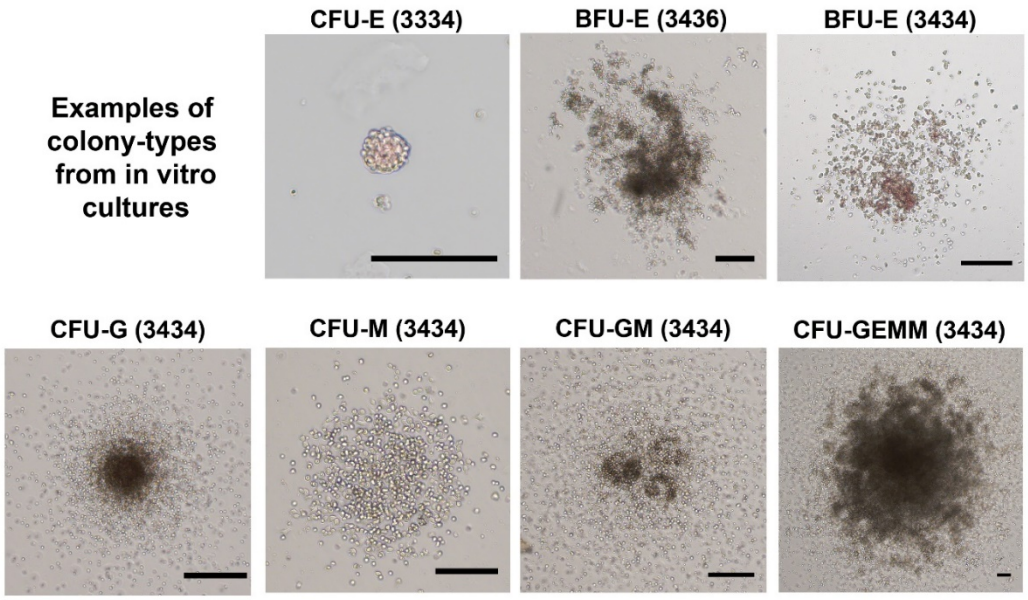


**Figure 11. Representative images of colony types in three semisolid media.** The images are from cultured whole bone marrow cells obtained from an untreated mouse. All media were from STEMCELL Technologies, Canada: M3334 medium for CFU-E clusters; SF M3436 medium for BFU-E colonies; GF M3434 medium for CFU-G, CFU-M, CFU-GM, BFU-E, and CFU-GEMM colonies. The images were acquired by Olympus microscope (Olympus IX71) with a color camera (Olympus DP74) and 4x UPlanFl (CFU-GEMM), 10x CPlanFl (all other) or 20x LCPlanFl (CFU-E) objectives. The scale bar is 100 µm for each image. The cells were cultured and the pictures were taken by Tomáš Heizer.

## 6.13 Bone marrow transplantation

A single-cell suspension of bone marrow cells was transplanted intravenously through the retro-orbital route. Recipient mice were dual CD45.1/CD45.2 (F1) mice irradiated at 8.5 Gy before transplantation. The recipients were transplanted with a mixture of bone marrow cells or sorted LSK $CD150^{+}CD48^{-}$ cells of regenerating (CD45.2) and untreated (CD45.1) mice (*vice versa* in one experiment; Table 6). Chimeric CD45.2/CD45.1 bone marrow from transplanted mice was retransplanted to secondary 8.5 Gy-irradiated F1 recipient mice. The experimental design of the transplantation assays is shown in Figure 22A.

## 6.14 Analysis of chimeric hematopoiesis in peripheral blood

The ratio of donor to host nucleated blood cells was determined in samples of peripheral blood drawn from the retro-orbital venous plexus of transplanted mice using capillaries containing 5µL of 0.5M EDTA. Approximately 50µL blood samples were stained with anti-CD45.1 and anti-CD45.2 antibodies for 30 minutes on ice in the dark and washed after. The samples were also stained for Gr-1/Mac-1,

B220, and CD3 or CD4 and CD8 markers. Only CD45.1 or CD45.2 single-positive cells were evaluated by flow cytometry, and double CD45.2/CD45.1-positive cells were excluded from the analysis.

## 6.15 Analysis of chimeric hematopoiesis in bone marrow

Femoral bone marrow cells were collected into PBS. Four million bone marrow cells were washed with PBS and centrifuged (4 °C, 400 g, 6 min). After removing the supernatant, the cells were stained for the CD45.2 and CD45.1 allotypes and Gr-1/Mac-1, B220 and CD3 or CD4 and CD8 markers to determine the ratio between CD45.2 cells (originating from regenerating bone marrow) and CD45.1 cells (originating from control bone marrow; *vice versa* in one experiment). Another set of samples were stained for CD45.2 and CD45.1 markers and with lineage cocktail, anti-c-Kit and anti-Sca-1 antibodies.

## 6.16 Statistical methods

Primarily multiple independent experiments were performed to verify the reproducibility of all experimental findings. Statistical analysis was done with GraphPad Prism version 5 (GraphPad Software, La Jolla, CA, USA). Two-tailed unpaired Student´s t-tests were used to determine statistical significance when the two groups were compared. One-way analysis of variance (ANOVA) with Dunnett´s post-test was used to compare each group to the control group when more than two groups were evaluated. Results are presented as means ± SEM.

# 7. Results

## 7.1 Immature hematopoietic cells expand their population rapidly and in parallel to increasing production of blood cells in regenerating hematopoiesis following significant damage

To define the experimental model of hematopoietic regeneration used in the present study, we estimated the extent of the initial damage inflicted on the hematopoietic tissue by irradiating mice with a dose of 6 Gy by determining the occurrence of endogenous spleen colonies arising from myeloid progenitor cells which survived irradiation. The colony numbers are shown in Table 5, and examples of spleens are shown in Figure 12A. There were only occasional spleen colonies in mice irradiated with a dose of 6 Gy. The rare colonies became large after 11 days, and from the kinetics of spleen colony development (Nečas and Znojil, 1989) it can be deduced that they originated from cells that had survived after irradiation and began their clonal expansion shortly after that. Numerous tiny colonies became visible on the spleens examined 12 – 14 days after irradiation. We hypothesize that they reflect the migration of progenitors from regenerating bone marrow to the spleen, as was reported by Peslak et al. (2012). Based on the spleen colony results and the results from our previous study (Forgacova et al., 2013) and the results reported by McCarthy (1997), we estimate that only very few cells with the hematopoietic tissue reconstituting capacity survived in the 6 Gy-irradiated mice.

Bone marrow cells are severely reduced by the damage caused by irradiation. In untreated mice, the bone marrow of one femur consists of 28.3 ± 5.3 x $10^6$ cells (Figure 12C). Ten days after irradiation at dose 6 Gy, bone marrow cells were reduced to one-quarter of its number (7.0 ± 2.7x $10^6$ cells), whereas the population of immature hematopoietic cells lacking lineage markers and expressing the c-Kit receptor (LK cells) was reduced to one-tenth (from 451,300 to only 4,400 LK cells/femur) at this time (not shown). The population of immature LK cells in the bone marrow rapidly expanded between the 14th and the 15th days after irradiation (Figure 12C). Intensive production of blood cells occurred 12 days and later after irradiation, as indicated by the increasing number of red blood cells in peripheral blood (Figure 12B). Altogether, these results suggest the similarity between the vigorously regenerating adult hematopoiesis and the physiologically expanding embryonic/fetal and early postnatal hematopoiesis in the concomitant increase of blood cell production and the expansion of populations of immature hematopoietic cells.

| Day | 8 | 11* | 12* | 12* | 14* |
|---|---|---|---|---|---|
| 4 Gy | 12.5±1.9 (4) | n.d. | n.d. | n.d. | n.d. |
| 6 Gy | 0.6±0.3 (12) | 0.4±0.2 (14) | 0.2±0.2 (#) (6) | 0.6±0.2 # (24) | 0.3±0.2 # (24) |

**Table 5. The number of spleen colonies in submyeloablatively irradiated mice.** Values are means ±SD; the number of mice is indicated in brackets; * - colonies > 2mm; # - a large number of tiny colonies

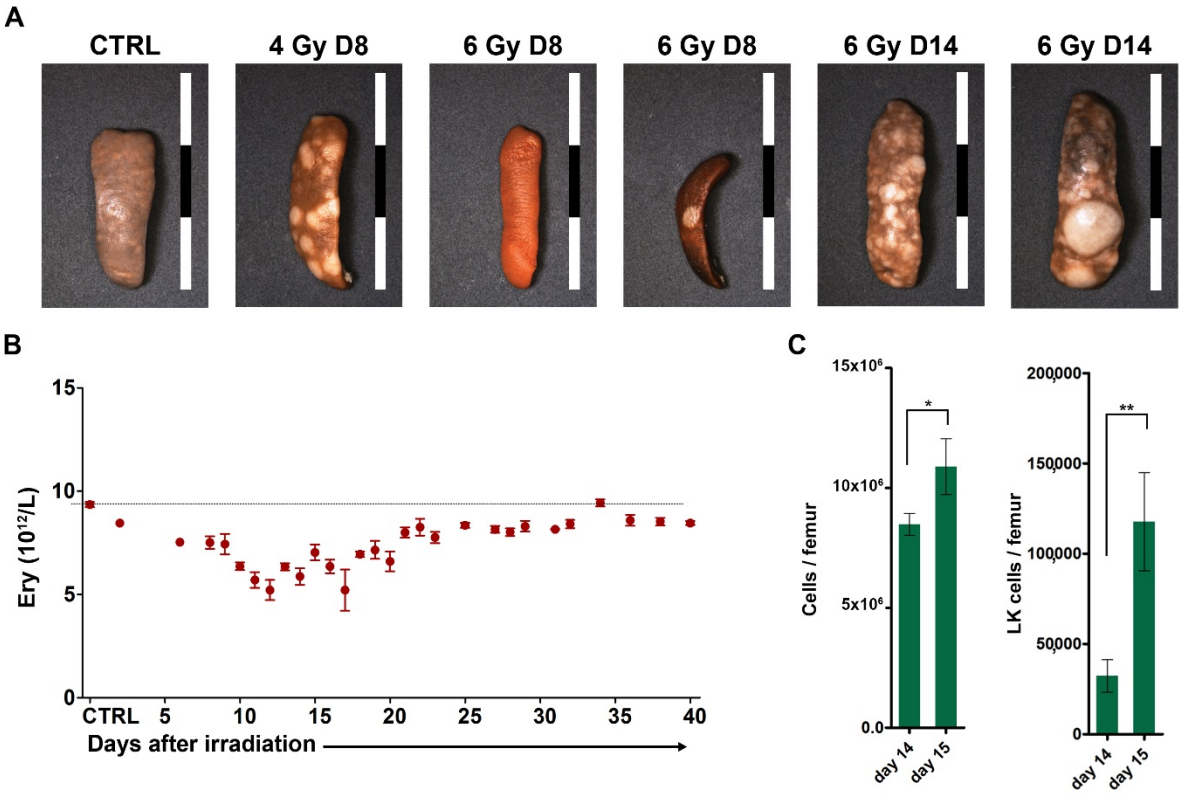


**Figure 12. Endogenous spleen colonies, red blood cell numbers, and expansion of hematopoiesis in bone marrow between days 14 and 15 after irradiation of mice with a dose of 6 Gy**. **A)** Representative spleens of untreated (CTRL) and submyeloablatively irradiated mice; D – day after irradiation; each segment of the scale bar equals 5 mm. Two spleens are shown after irradiation of mice at 6 Gy for spleen collected either after 8 or 14 days. **B)** Red blood cell (Ery) number achieves its nadir 12 days after irradiation of mice with 6 Gy. Data are from 52 untreated mice and 131 irradiated mice (both males). **C)** Bone marrow cellularity and the number of immature $Lin^{-}c\text{-}Kit^{+}$ (LK) cells significantly increased between days 14 (16 male mice) and 15 (12 male mice) after irradiation. There were 28.255 ± 5.386 million cells and 451,309 ± 153,982 LK cells in the femur of untreated mice (mean ± SD; 127 male mice).

## 7.2 The immunophenotype of LK cells is significantly altered in regenerating bone marrow

First, we analyzed the immunophenotype of the strongly reduced population of LK cells in intensively regenerating bone marrow by flow cytometry. We used the CD48 and CD150 markers according to Kiel et al. (2005) to visualize the subtypes of Sca-1 positive (LSK) cells with HSCs, MPP, and myeloid progenitor developmental potential. Figure 13A shows the representative sample wherein the immunophenotype of LK cells is shown in regenerating and normal bone marrow. The c-Kit expression level is significantly decreased (see Table 6), and the proportion of Sca-1 positive (LSK) cells is increased in the LK cells with low c-Kit expression level in regenerating bone marrow. The LSK cells are all CD48 positive, and the fraction of CD150 positive cells is increased.

Figure 13B compares LK cell numbers, and the ratio between Sca-1 negative ($LS^{-}K$) cells and LSK cells in untreated mice (CTRL) and mice examined 15 days after irradiation (6 Gy D15). In the regenerating bone marrow, the proportion of LSK cells was significantly increased, and consequently, the $LS^{-}K$/LSK ratio decreased. Figure 13B also shows the distribution of LSK cells in four CD48/CD150 subtypes in normal (CTRL) and regenerating (6 Gy D15) bone marrow. Because of the significantly altered c-Kit expression level of LK cells in regenerating bone marrow, we analyzed the CD48 and CD150 expression in LSK cells in the c-$Kit^{high}$ or c-$Kit^{low}$ gates separately so as not to exclude Sca-1 positive cells with decreased c-Kit expression from the analysis.

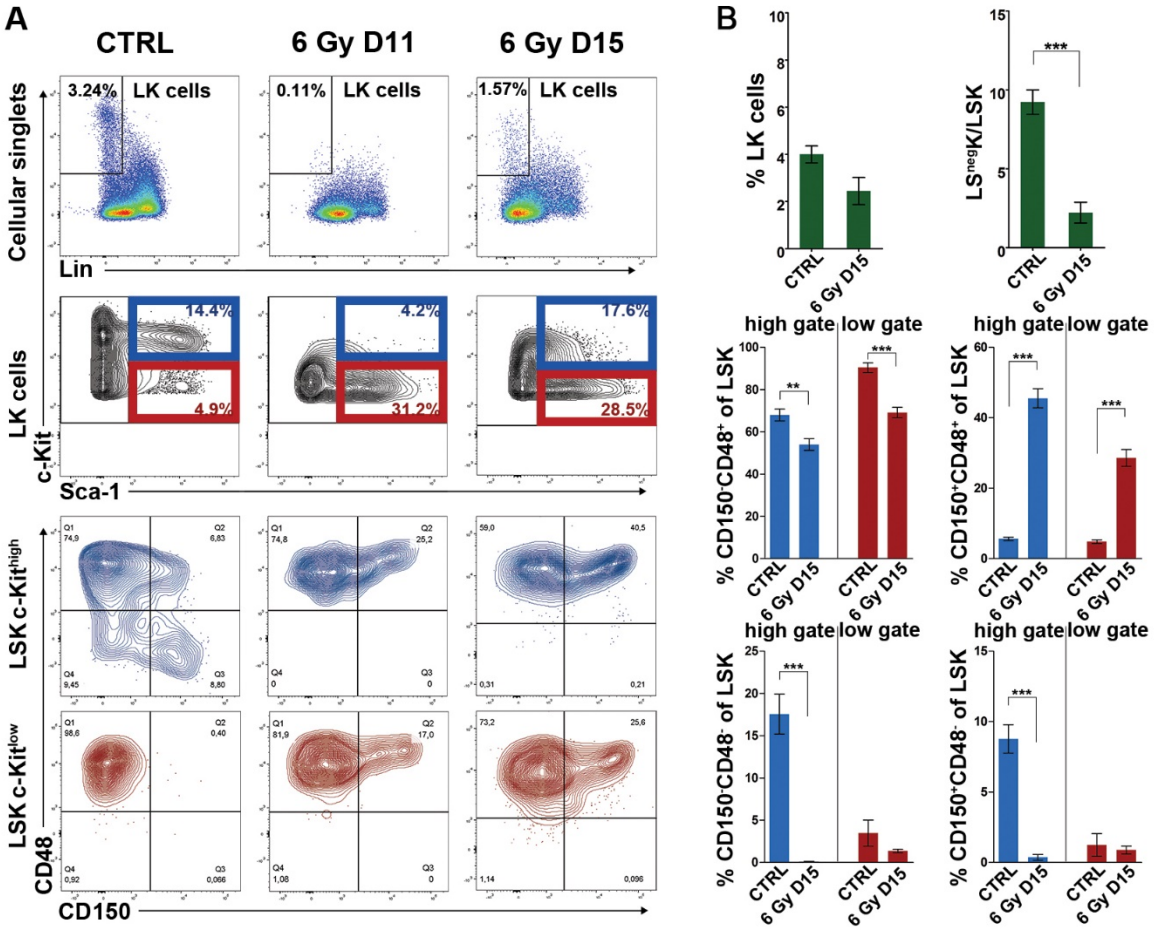


**Figure 13. LK cells are c-Kit$^{low}$ and are enriched for Sca-1$^{+}$ cells; LSK cells are CD48$^{+}$ and enriched for CD150$^{+}$ in regenerating bone marrow from irradiated mice. A)** Examples of immunophenotypes of LK cells present in normal bone marrow (CTRL) and bone marrow collected 11 (6 Gy D11) or 15 (6 Gy D15) days after irradiation of mice with 6 Gy. The CD150/CD48 expression profile was determined in c-Kit$^{high}$ and c-Kit$^{low}$ subsets of LSK cells. **B)** The percentage of LK cells and the ratio between LS$^{-}$K and LSK cells in normal (CTRL) and regenerating (6 Gy D15) bone marrow. All LK cells were analyzed as shown in Figure 13A by cell dotplots. However, the subsequent analysis of LSK cells regarding their distribution into the four CD150/CD48 expression profile subtypes was made separately for the LSK cells with "high" and "low" c-Kit expression levels distinguished by blue and dark red colors. Data were pooled from three independent experiments, including 5 untreated mice and 8 mice examined 15 days after irradiation. ** p<0.01, ***p<0. 001.

| | IRR | N | LS⁻K | LSK | LSK CD150⁻ CD48⁺ | LSK CD150⁻ CD48⁻ | LSK CD150⁺ CD48⁻ | LSK CD150⁺ CD48⁺ |
|---|---|---|---|---|---|---|---|---|
| ♂ CD45.2 | **CTRL** | 4 | 72.64 ±1.66 | 78.94 ±2.88 | 64.61 ±1.92 | 75.87 ±3.03 | 95.52 ±3.53 | 78.96 ±3.17 |
| | **Day 14** | 4 | 42.36 ±0.88 | 52.76 ±3.44 | 38.74 ±2.53 | 31.01 ±1.05 | 44.10 ±5.72 | 51.35 ±4.80 |
| | **t-test** | | **t=16.08; P<0.0001** | **t=5.827; P=0.0011** | **t=8.140; P=0.0002** | **t=14.02; P<0.0001** | **t=7.644; P=0.0003** | **t=4.803; P=0.0030** |
| ♂ CD45.1 | **CTRL** | 4 | 25.91 ±1.89 | 36.11 ±3.52 | 39.29 ±4.20 | 36.95 ±4.21 | 47.29 ±5.01 | 33.14 ±2.93 |
| | **Day 15** | 5 | 17.97 ±1.65 | 23.87 ±1.33 | 26.66 ±1.47 | Not enough cells | 27.39 ±2.29 | 24.31 ±1.00 |
| | **t-test** | | **t=3.170; P=0.0157** | **t=3.555; P=0.0093** | **t=3.120 P=0.0168** | **-** | **t=3.895 P=0.0059** | **t=3.140 P=0.0164** |

**Table 6. c-Kit fluorescence intensity (MFI) is decreased in various types of the Lin⁻Kit⁺ (LK) cells in regenerating bone marrow.** The mean fluorescence intensity (MFI) is in thousands of arbitrary units. All compared data were acquired in the same instrument run. Gating for LK cells was set to cover approximately 1.5-2.0 % of total bone marrow cells in normal mice. (Faltusová et al., 2020a; The results were obtained by Katarina Szikszai).

We added an anti-CD71 antibody to the antibody cocktail used to stain the analyzed bone marrow samples. The CD71/Sca-1 plot of LK cells is highly constant in the normal bone marrow and clearly distinguishes between LSK cells, which are uniformly CD71 negative, and LS⁻K cells that show variable expression CD71 ranging from CD71⁻ to CD71$^{high}$ expression (CTRL samples in Figure 14). Unexpectedly, there was a very high frequency of CD71 positive LSK cells in the regenerating bone marrow, which was in striking contrast to LSK cells in the normal bone marrow (Figure 14A). The CD71 marker corresponds to the transferrin receptor 1, which is highly expressed in erythroid cells and induced by a low iron body state. Therefore, we functionally tested the possibility that increased erythropoietin stimulation due to post-irradiation anemia (see Figure 12B) or a relative iron deficiency due to intensive erythropoiesis induced CD71 expression in LK cells in regenerating bone marrow. To differentiate these possibilities, we prevented the development of anemia by repeated transfusions of red blood cells. However, CD71 expression remained high in LSK cells in regenerating bone marrow and was equal in LSK and LS⁻K cells (Figure 14A). In non-irradiated mice, we tested, with a negative outcome, whether CD71 expression in LSK cells would respond to administration of erythropoietin

(EPO) or induction of a state of iron deficiency (Figure 14B). Furthermore, we functionally tested LK cells with different CD71 expression, sorted either from normal or regenerating bone marrow, by in vitro clonogenic assays. The capacity to form clones of hematopoietic cells declined with increasing CD71 expression, both in cells sorted from normal or regenerating bone marrow (Figure 15).

As the expression of CD71 in LSK cells was quite unusual in regenerating bone marrow, we plotted the CD71 expression level against that of Sca-1 in LK cells from normal and regenerating bone marrow. The Sca-1/CD71 expression profile of LK cells, highly conserved in normal bone marrow, was significantly altered in regenerating bone marrow and presented as irregular cell clusters (Figure 14C). The occurrence of the clusters was not affected by red blood cell transfusions and polycythemia (Figure 14C).

These results confirm the previously reported decreased c-Kit expression, increased Sca-1 expression, and increased CD150 expression in immature cells in post-irradiation bone marrow (Simonnet et al., 2009). They are novel in showing the virtual absence of CD48-negative LK cells, the expression of CD71 in LSK cells, and the previously unrecognized clusters of LK cells with highly variable Sca-1/CD71 expression profiles. They demonstrate that the altered immunophenotype of LK cells is not induced by erythropoietin stimulation.

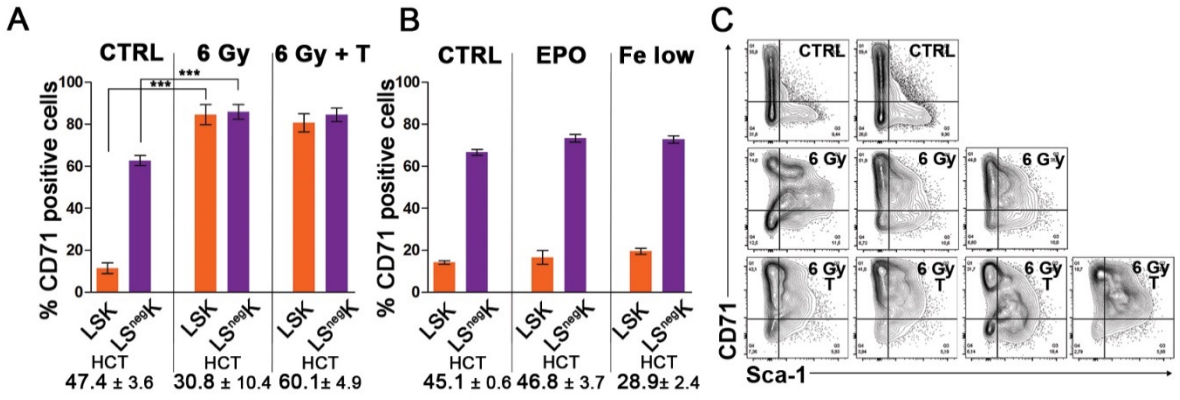


**Figure 14. Altered immunophenotype of LK cells is not induced by EPO stimulation. A)** Percentage of CD71 (transferrin receptor 1) positive LK cells increased in regenerating bone marrow, particularly in LSK cells. As defined in Figure 13A, all LK cells were divided into LS$^-$K and LSK cells and analyzed. In a part of the mice, transfusions of red blood cells were started five days after irradiation and were given in 3-4 day intervals to prevent post-irradiation anemia. Polycythemia induced in irradiated mice by repeated transfusions of red blood cells did not abolish the increase in CD71 expression. Data were pooled from 5 independent experiments, including a total of 6 untreated mice (CTRL), 13 irradiated mice (6 Gy), and 15 irradiated and transfused mice (6 Gy + T). Mice were examined on day 14 in 3 experiments and days 15 and 16 after irradiation in another 2 experiments. **B)** Administration of erythropoietin (EPO) to normal (non-irradiated) mice, or an iron-deficient diet combined with bleeding

(Fe low), did not influence the expression level of CD71 in LK cells. **C)** The Sca-1/CD71 expression profile of LK cells, highly conserved in normal bone marrow, became erratic in regenerating bone marrow and transfusions of red blood cells did not abrogate this. Examples of the Sca-1/CD71 immunophenotypes in LK cells from 2 untreated (CTRL), 3 irradiated (6 Gy) and 4 irradiated and transfused (6 Gy T) mice examined 15 days after irradiation are shown. HCT – hematocrit; T – transfusions of red blood cells.

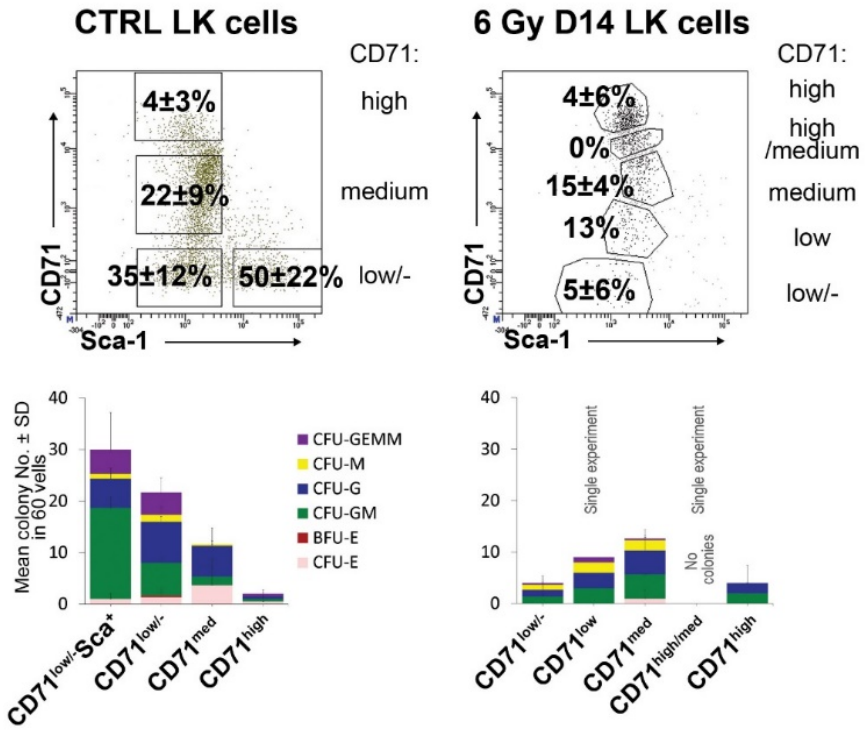


**Figure 15. Functional analysis of LK cells with various expression of CD71 in normal and in regenerating bone marrow.** Single LK cells were sorted into the GF M3434 culture medium (STEMCELL Technologies, Canada) using a 96-well plate. Sixty wells were used for cell culture, and the remaining wells on the plate edge were filled with water to prevent the desiccation of the medium. Positive wells containing hematopoietic cell colonies were determined after 10 days, and the colony types were established. The LK cells either from normal (CTRL) or regenerating bone marrow (6 Gy D14) were sorted based on Sca-1 and CD71 expression (see an example of the gates used). The plating efficiency is shown as the percentage of colony-positive wells (mean ± SD) for the cells from each gate. The number and the proportion of colony types are shown as the mean ± SD in the column graphs. Results are from three independent experiments, except $CD71^{low}$ and $CD71^{high/medium}$, which were examined in a single experiment. The cells were sorted and cultured by Martin Báječný and Tomáš Heizer.

## 7.3 LSK cells in regenerating bone marrow are similar to granulocyte-macrophage and erythroid progenitors

The unexpected finding of highly CD71 positive LSK cells in regenerating bone marrow prompted us to further explore LSK cells in regenerating bone marrow. We suspected that these cells could be developmentally restricted myeloid progenitors, originally Sca-1 negative, which had started reexpressing Sca-1. The myeloid Sca-1 negative progenitors can be further differentiated utilizing their CD34 and CD16/32 (FcγRIII/II) expression profiles into the common myeloid progenitors (CMPs; $CD34^{+}CD16/32^{-}$), granulocyte-macrophage progenitors (GMPs; $CD34^{+}CD16/32^{+}$), and megakaryocyte-erythroid progenitors (MEPs; $CD34^{-}CD16/32^{-}$) (Akashi et al., 2000). As we became suspicious that LSK cells in regenerating bone marrow are myeloid progenitor cells that reexpressed Sca-1, we applied the CD34 and CD16/32 marker analysis on all LK cells normal and regenerating bone marrow. We examined LK cells divided into four subsets: c-$Kit^{high}$ - c-$Kit^{low}$ and Sca-1 $^{negative}$ - Sca-1 $^{positive}$ (see Figure 16A, subgroups 1,2,3,4 of LK cells). Figure 16A shows examples of the CD34/CD16-32 expression profile in the four subsets of LK cells in normal (CTRL) and regenerating (6 Gy D13) bone marrow. Figure 16B shows the distribution of cells with the four CD34/CD16-32 immunophenotypes in female and male mice either normal (CTRL) or examined 13 days (6 Gy D13) or 14 days (6 Gy D14) after irradiation. The most striking difference between the LSK cells (subgroups 3 and 4 in Figure 16A) from normal and regenerating bone marrow is in the expression of CD16/32. LSK cells in the normal bone marrow are uniformly CD16/32 negative, while regenerating bone marrow LSK cells are mostly CD16/32 positive and became GMP-like (Figure 16C).

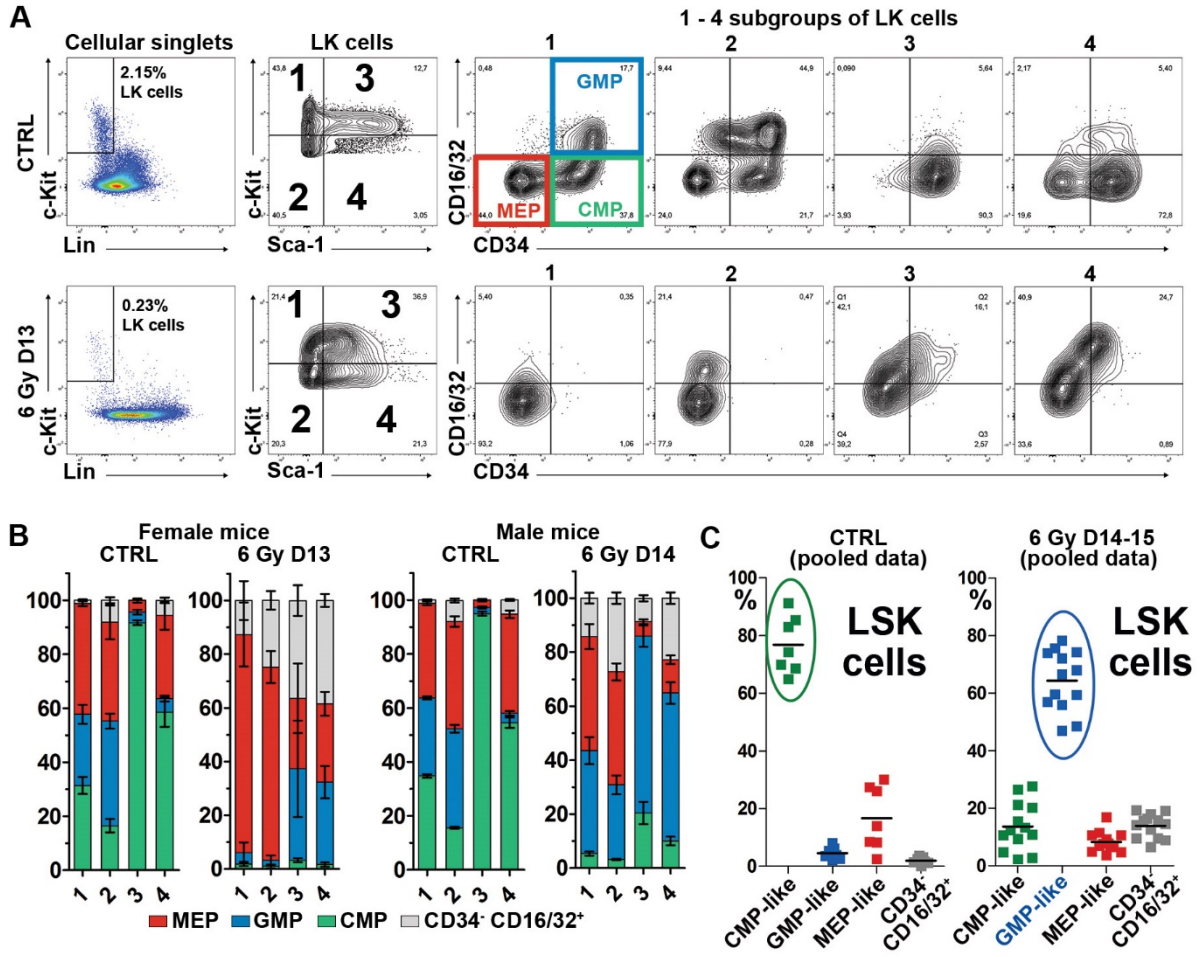


**Figure 16. The CD34$^{+}$CD16/32$^{-}$ cells are significantly reduced in regenerating bone marrow, and LSK cells express CD16/32 A)** Example of the CD34/CD16/32 expression profile in c-Kit$^{high}$ - c-Kit$^{low}$ and Sca-1$^{-}$ - Sca-1$^{+}$ (subgroups 1, 2, 3, 4) LK cells. CTRL – and untreated mouse; 6 Gy D13 – a mouse irradiated with 6 Gy before 13 days. The CD34/CD16/32 immunophenotypes characteristic for the common myeloid progenitors (CMP), granulocyte-macrophage progenitors (GMP), and megakaryocyte-erythroid progenitors (MEP) shown in subgroup 1 in the CTRL mouse were applied on cells in other subgroups of LK cells (2,3,4) and on the LK cells in the irradiated (6 Gy D13) mouse. **B)** Proportion of cells with the MEP, GMP, CMP, and CD34$^{-}$/CD16-32$^{+}$ immunophenotype profiles in 1, 2, 3, and 4 subgroups (see **A**) of LK cells in five untreated female mice (CTRL) and two female mice examined 13 days after irradiation (6 Gy D13), and four untreated male mice (CTRL) and six male mice examined 14 days after irradiation (6 Gy D14). **C)** LSK cells were analyzed for the CD34/CD16-32 immunophenotype in 7 untreated mice (CTRL; from five independent experiments) and 13 irradiated mice examined 14-15 days after irradiation (6 Gy D14-15; in four independent experiments). All mice were males. Cells immuphenotypically similar to CMP, GMP, or MEP cells have the suffix "-like" because of the Sca-1 positivity. The increased expression of CD16/32 in LSK cells in regenerating bone marrow changed their major immunophenotype from the CMP-like to the GMP-like.

We noticed that the GMPs and MEPs present in the bone marrow of untreated mice significantly differ from common myeloid progenitors (CMPs) and all LSK cells by their increased forward scatter (FSC; the

size of cells), and in GMPs, also by their higher side scatter (SSC; the granularity of cells) (Figure 17). Importantly, in the regenerating bone marrow, LSK cells had the FSC and SSC characteristics corresponding to of Sca-1$^-$ GMPs and MEPs in the bone marrow of untreated mice (Figure 17).

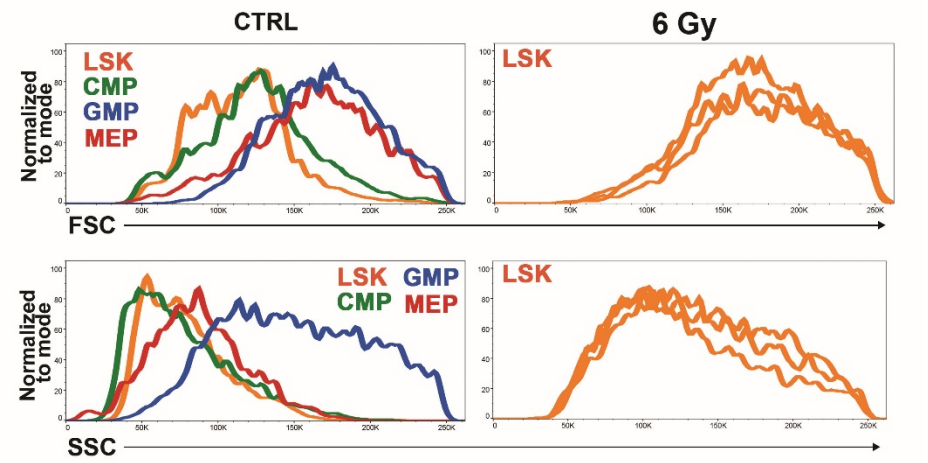


**Figure 17 Forward scatter (FSC) and side scatter (SSC) histograms** of LSK and CMP, GMP, and MEP cells in the bone marrow of one untreated mouse compared to the histograms of LSK cells of three mice irradiated with 6 Gy examined after 14 days.

We also compared the expression of Flt3 (CD135/Flk2), which marks lymphoid-biased MPPs (Buza-Vidas et al., 2011), in LK cells in normal and regenerating bone marrow. Approximately 40 % of the LSK cells in normal bone marrow expressed Flt3 while the LS$^-$K cells were homogenously Flt3$^{low}$ (Figure 18A). In regenerating bone marrow, the Flt3 expression became low in LSK cells and similar to its expression in LS$^-$K cells in normal bone marrow (Figure 18A).

The embryonic EMPs are mostly CD41 (integrin αIIb) positive (McGrath et al., 2015). Therefore, we determined CD41 expression in LK cells in normal and regenerating bone marrow. In the normal bone marrow, CD41 is highly expressed in a fraction of LS$^-$K cells (corresponding to CMPs; not shown). LK cells in regenerating bone marrow lack CD41$^{high}$ cells, and only a part of LSK cells express CD41 at a medium level (Figure 18B, C).

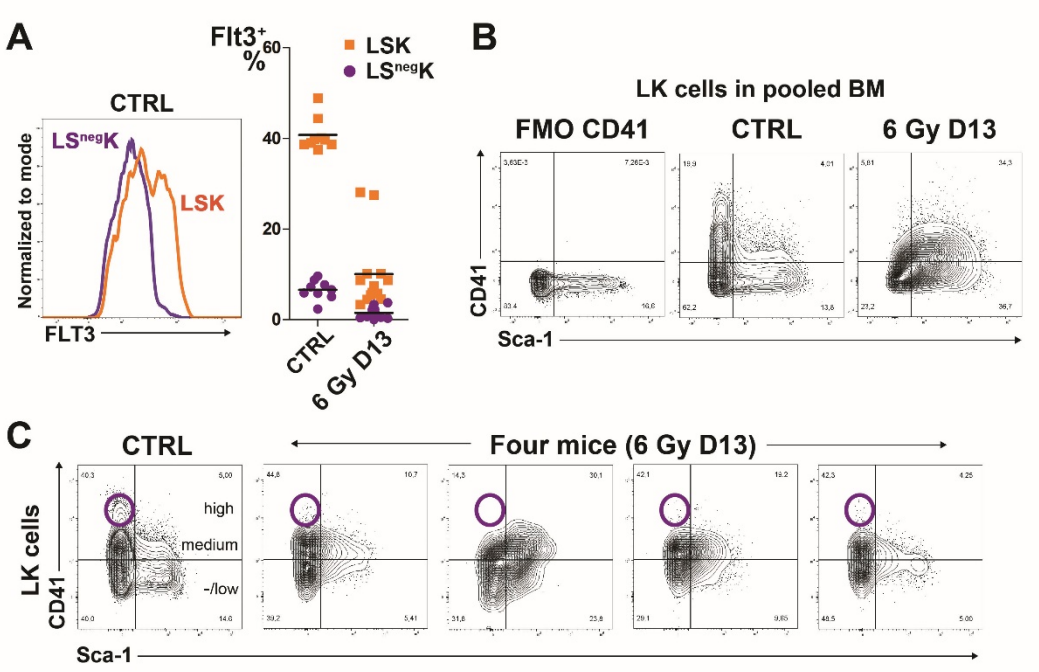


**Figure 18. LSK cells in regenerating bone marrow have diminished expression of Flt3 and altered expression of CD41 A)** Flt3 expression in LS$^-$K and LSK cells in normal bone marrow (CTRL), and the percentage of Flt3 positive cells in LS$^-$K and LSK cells in 9 untreated mice (CTRL; data pooled from four independent experiments) and 13 mice irradiated with 6 Gy (6 Gy D13; data pooled from three independent experiments). All mice were males. **B)** CD41 expression in LK cells compared to samples of bone marrow pooled from two normal (CTRL) or three irradiated (6 Gy D13) mice. FMO CD41 is an aliquot of normal bone marrow stained with the omission of anti-CD41 antibody. **C)** CD41 expression in LK cells of one normal (CTRL) mouse and four mice examined 13 days after irradiation (6 Gy D13).

We also determined the expression of CD201 (Endothelial protein C receptor – EPCR; expressed by LT-HSCs) and CD105 (endoglin - a proliferation-associated endothelial marker, which Simonnet et al. 2009 found increasing in SP cells after iradiation) in LK cells of normal and regenerating bone marrow. The expression of EPCR was decreased in regenerating bone marrow, while the expression of endoglin was increased and correlated with the expression of CD71 (Figure 19).

These results show that the LK cells in regenerating bone marrow are predominantly activated erythro-myeloid progenitors.

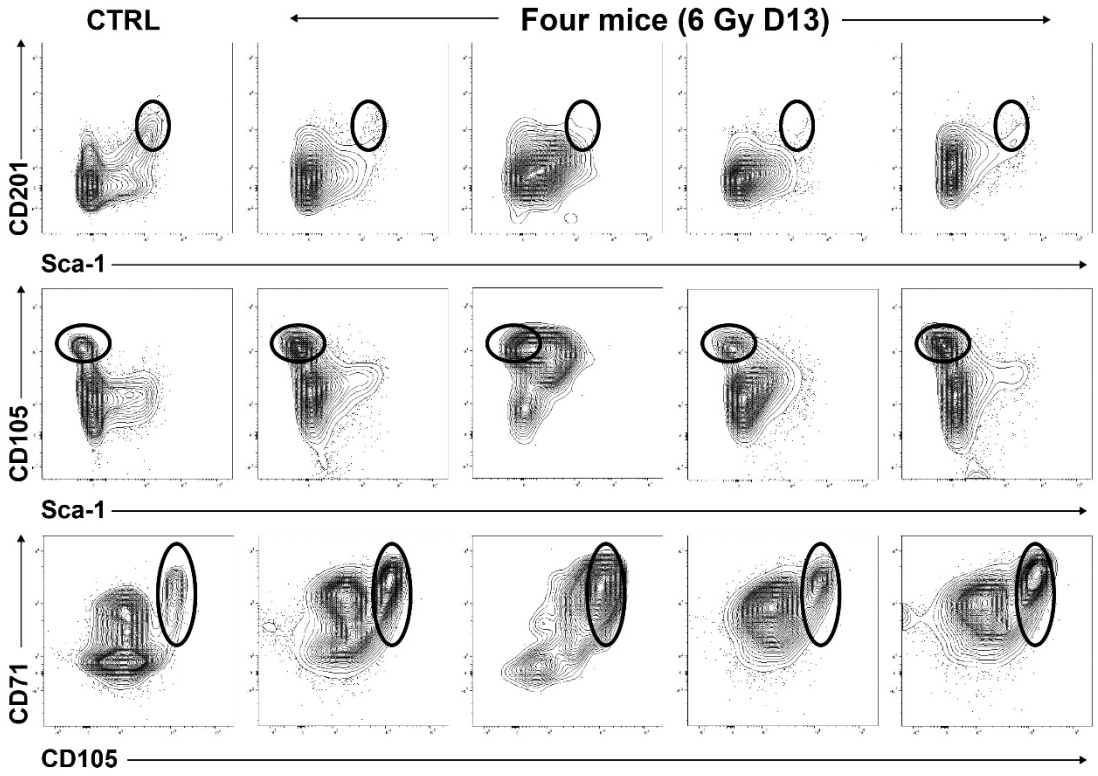


**Figure 19. The expression of CD201 (EPCR) and CD105 (endoglin) in normal and regenerating bone marrow.** The representative CD201/Sca-1, CD105/Sca-1, and CD105/CD71 expression profiles in LK cells of normal bone marrow are shown (CTRL). The same expression profiles are shown in bone marrow collected from four mice examined 13 days after irradiation (6 Gy D13).

## 7.4 LK cells in regenerating bone marrow are significantly different from the LK cells in expanding hematopoiesis in the fetal liver and early postnatal bone marrow

Regenerating bone marrow mimics hematopoiesis in the fetal liver and early postnatal bone marrow by rapidly expanding populations of immature cells accompanied by rapidly increasing production of mature blood cells. Therefore, we interrogated whether the vigorous expansion of regenerating bone marrow is similar in some aspects to the hematopoiesis in the fetal liver and early postnatal bone marrow. Immunophenotyping of LK cells from the normal adult bone marrow, fetal liver, and early postnatal bone marrow revealed significant differences from the LK cells in regenerating adult bone marrow (Figure 20B compared with Figure 13A). LK cells in the fetal liver and postnatal bone marrow were more frequent than in the adult and regenerating bone marrow (Figure 20C), exhibited high c-Kit expression (Figure 20B), and elevated LS$^-$K/LSK ratio (Figure 20C). The regenerating bone marrow

lacked the CD41high LK cells, which occur in the LS⁻K cells in the normal adult and postnatal bone marrow and the fetal liver (Figure 20D). CD41 is highly expressed in CMPs in normal bone marrow (not shown). The cells with the immunophenotype of CMPs are significantly suppressed in regenerating bone marrow (see Figure 16B), and the altered expression of CD41 thus correlates with this change in the composition of LK cells.

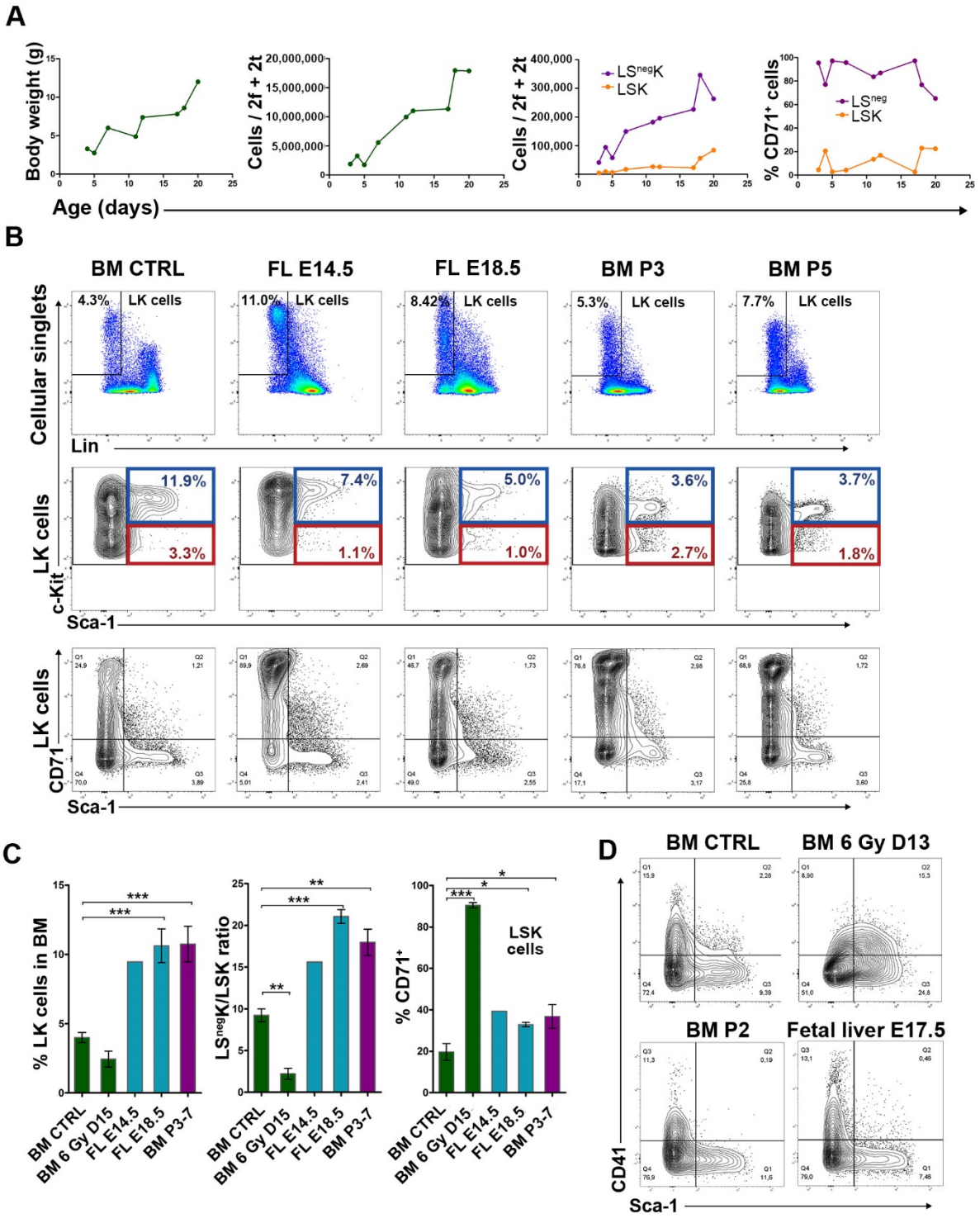

**Figure 20. Expanding hematopoiesis in fetal liver and postnatal bone marrow compared to normal adult and regenerating bone marrow. A)** Body weight, bone marrow cellularity, LSK, and LS⁻K cells, and CD71 expression in LSK and LS⁻K cells in postnatal mice. f – femur; t – the tibia. **B)** Examples of the immunophenotype of LK cells in adult bone marrow (Adult BM), fetal liver (FL-E14.5 or 18.5), and postnatal bone marrow (BM-P3 or P5). E – embryonic day; P – postnatal day. **C)** The percentage of LK cells, LS⁻K/LSK cell ratio, and percentage of CD71⁺ LSK cells in normal adult BM (BM – CTRL; 5 untreated male mice from three independent experiments); regenerating adult BM (6 Gy BM - D15; 8 adult male mice examined 15 days after the irradiation with 6 Gy from three independent experiments); in FL (FL – E14.5 and E18.5; 3 and 4 mice) and postnatal BM (BM – P3-7; 4 mice examined 3-7 days postpartum). *P<0.05, **P<0.01, ***P<0.001 **D)** Sca-1/CD41 expression profile in adult BM (BM CTRL), regenerating adult BM collected 13 days after irradiation (BM 6 Gy D13), BM collected on postnatal day 2 (BM P2) and fetal liver (FL - E17.5).

## 7.5 Regenerating bone marrow cells form in vitro colonies in semisolid cultures

The colony-forming potential of hematopoietic cells from regenerating bone marrow was determined in a series of in vitro experiments using three types of commercial culture media. Whole bone marrow cells or FACS-sorted LSK and LS⁻K cells were plated into semisolid media, which supported the growth of either erythroid progenitors (BFU-E and CFU-E) or a broad spectrum of myeloid progenitors marked here as HSPCs (Hematopoietic Stem and Progenitor Cells), which included CFU-GEMM, CFU-GM, CFU-G, CFU-M, and BFU-E progenitor cells. The results revealed various types of myeloid progenitor cells, both in whole normal and regenerating bone marrow and in sorted LS⁻K and LSK cells (Figure 21A, B, C; Figure 15). The capacity of LK cells from regenerating bone marrow to generate colonies of blood cell precursors was approximately half of that of the LK cells from normal bone marrow.

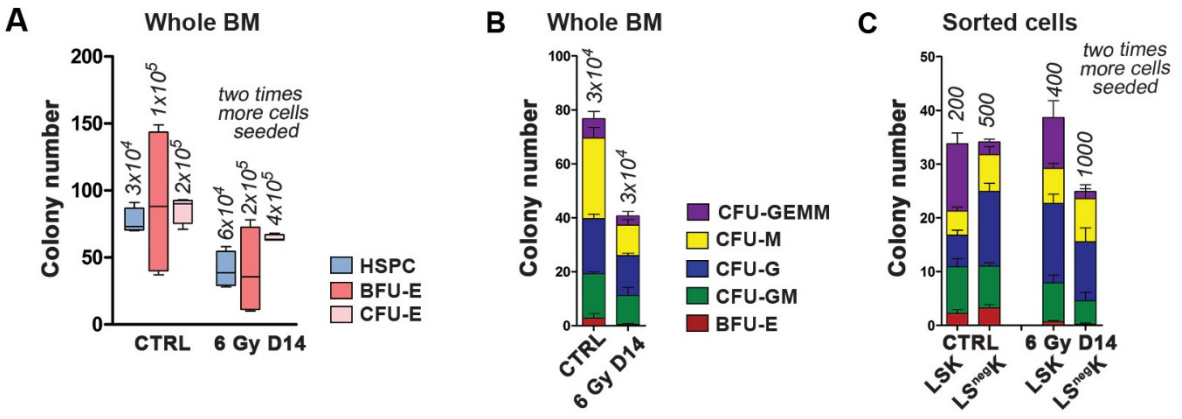


**Figure 21. Developmental potential of LK cells in regenerating bone marrow in cell cultures. The t**otal number of colonies of hematopoietic cells obtained in *in vitro* cultures of normal (CTRL) or regenerating (6 Gy D14) whole bone marrow or sorted LSK or LS⁻K cells. Results are pooled from three independent

experiments. **A**) Three types of culture media (from STEMCELL Technologies; Canada) were used. HSPC (Hematopoietic Stem and Progenitor Cells) is for the colonies cultured in the medium GF M3434 supporting the growth of CFU-GEMM, CFU-GM, CFU-M, CFU-G, and BFU-E cells; BFU-E is for the colonies cultured in the medium SF M3436; CFU-E is for cell clusters cultured in the medium M3334. Numbers of bone marrow cells seeded per dish are indicated in italics. **B**) The "HSPC" colonies cultured in the GF M3434 medium were differentiated into various colony types. **C**) LSK and LS¯K cells sorted from normal (CTRL) or regenerating (6 Gy D14) bone marrow were cultured in medium GF M3434.The numbers of cells seeded per dish are indicated in italics. Cell cultures were performed and the data were evaluated by Tomáš Heizer.

## 7.6 LK cells in regenerating bone marrow have a meager transplantation potential

Expanding hematopoiesis in the fetal liver is very potent in transplantation, and fetal liver cells outcompete bone marrow cells in co-transplantation assays (Rebel et al., 1996; Bowie et al., 2007; Copley et al., 2013). On the other hand, bone marrow collected 13 days after irradiation of mice with a dose of 5.5 Gy failed to produce blood cells when competitively transplanted with normal bone marrow (Harrison and Astle, 1982). This finding prompted us to perform a series of experiments that compared the transplantation power of the regenerating bone marrow to that of normal bone marrow. Preliminary experiments showed that regenerating bone marrow cells should be transplanted in a significant excess to the competing normal bone marrow cells to obtain similar blood cells in both branches of the resulting chimeric hematopoiesis. We then performed a series of experiments in which the transplantation and developmental potential of regenerating bone marrow were compared to that of normal bone marrow. The experimental design of these experiments is shown in Figure 22A.

In two experiments, we tested the short-term repopulating potential of co-transplanted regenerating and normal bone marrow. Cells from regenerating bone marrow (CD45.2) were given in 30-80 excess to the cells from normal bone marrow (CD45.1). The proportions of CD45.2 and CD45.1 cells were determined in blood and bone marrow 20 days after transplantation (Figure 22B, C). The capacity of regenerating bone marrow to produce bone marrow and mature blood cells was only ~ 2 % of the capacity of normal bone marrow. Regenerating bone marrow also produced fewer B-cells (B220) than granulocytes and macrophages (GM; Figure 22B, C).

We then compared the long-term reconstitution of damaged hematopoiesis in mice transplanted with a mixture of regenerating and normal bone marrow cells and employed three experimental settings in these experiments.

First, we co-transplanted bone marrow collected from mice 14 days after irradiation (CD45.2) with normal bone marrow (CD45.1) at an 80:1 ratio. The peripheral blood of the recipient mice was analyzed for the presence of CD45.2 nucleated blood cells for four months (Figure 22D). The 80-fold excess of transplanted regenerating bone marrow resulted in half of the blood cells of the regenerating bone marrow origin in three out of seven transplanted mice after 3 months (Figure 22D). More granulocytes and macrophages (GM cells) were produced by transplanted regenerating bone marrow than B-cells (B220) and T-cells (CD4+CD8) (Figure 22D). The bone marrow of the three mice was pooled, examined for the frequency of CD45.2 cells, and transplanted to secondary recipient mice to confirm the capacity of regenerating bone marrow to support hematopoiesis in the long term further. The transplanted chimeric bone marrow contained ~ 60 % of CD45.2 cells (Figure 22E, hatched column). The production of CD45.2 (regenerating) blood cells remained steady after the second transplantation for four months and was skewed for myeloid (GM) cells (Figure 22E). Four mice with the chimeric bone marrow containing approximately 25 % of CD45.2 cells (see Figure 22F, the hatched column) were sacrificed four months after co-transplantation of regenerating (CD45.2), and normal (CD45.1) bone marrow cells (see Figure 22D) and their bone marrow were transplanted to secondary recipient mice. The production of CD45.2 blood cells was then followed for 8 months (Figure 22F). The percentage of CD45.2 cells in peripheral blood steadily declined, but CD45.2 cells were still present in the peripheral blood of the secondary transplanted mice after 8 months (Figure 22F). These experiments revealed the occurrence of transplantable cells with the long-term repopulating capacity constituting ~ 1 % of their occurrence in the normal bone marrow. A hundred times more regenerating cells were needed to establish a 50: 50 chimeric hematopoiesis in the co-transplantation experiments.

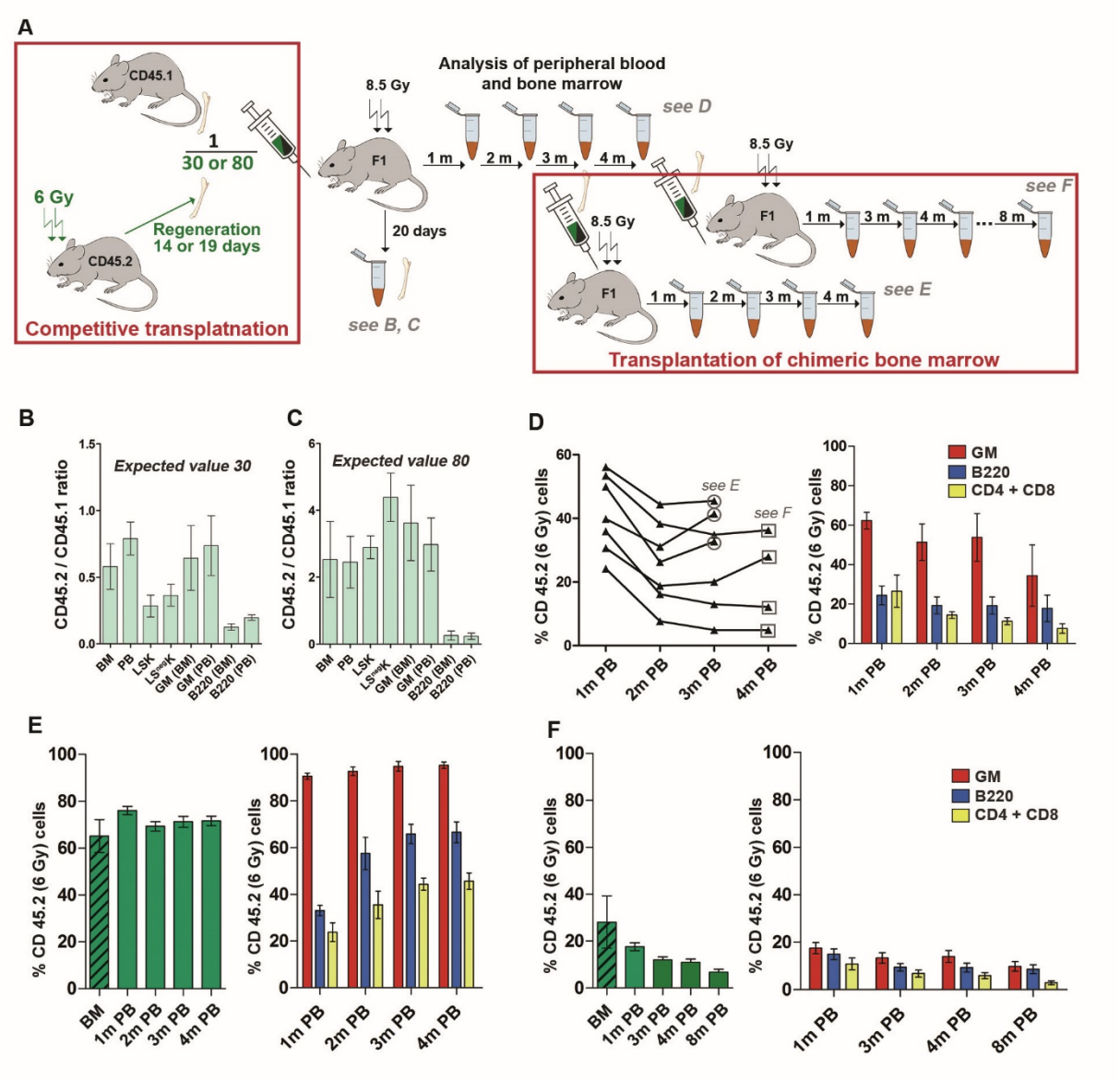


**Figure 22. Developmental potential of LK cells in regenerating bone marrow after transplantation.** **A)** Design of experiments studying the transplantability and developmental potential of regenerating bone marrow. **B), C)** Capacity of regenerating (CD45.2) and normal (CD45.1) bone marrow to restore blood cell production 20 days after transplantation (short-term hematopoiesis repopulation) was tested. 30 **(B)** or 80 **(C)** fold more CD45.2 bone marrow cells, collected 19 days in **B** and 14 days in **C** after irradiation, were mixed with CD45.1 normal bone marrow cells and co-transplanted to lethally irradiated dual CD45.1/CD45.2 F1 (F1) recipient mice. CD45.2 and CD45.1 cells were determined in various nucleated blood cells (PB) and bone marrow cells (BM; LSK, $LS^{-}K$) 20 days after transplantation. The CD45.2/CD451 ratio in various types of cells is shown. **D)** Regenerating (CD45.2; collected 14 days after irradiation of mice at 6 Gy) and normal (CD45.1) bone marrow was mixed in an 80: 1 ratio and transplanted (17.5 x $10^6$ per recipient mouse) to lethally (8.5 Gy) irradiated F1 mice. The percentage of CD45.2 nucleated blood cells originating from regenerating bone marrow was determined after 1, 2, and 3 months in seven individual mice and four mice after 4 months. The percentage of CD45.2 Gr-1/Mac-1(GM), B220, and $CD4^{+}CD8$ blood cells is shown in the column diagram. **E)** The CD45.2/CD45.1 chimeric bone marrow pooled from three mice sacrificed 3 months after transplantation (see D) was

analyzed for the presence of CD45.2 cells (hatched green column) and re-transplanted to secondary lethally irradiated F1 mice. Peripheral blood of the secondary transplanted mice was examined after 1, 2, 3, and 4 months similar to the first transplantation (see the empty green columns for all nucleated CD45.2 cells and the red, blue, and yellow columns for the GM, B220, and CD4+CD8 cells). **F)** The CD45.2/CD45.1 chimeric bone marrow pooled from four mice sacrificed 4 months after transplantation of the mixture of CD45.2 and CD45.1 bone marrow cells (see D) was similarly treated as that of the three mice sacrificed one month earlier (see E). Peripheral blood of the secondary transplanted mice was examined for the presence of CD45.2 cells for up to 8 months after transplantation.

Second, we compared the long-term reconstituting potential of LSK cells from regenerating and normal bone marrow. The regenerating bone marrow (CD45.2) was collected either 14 days or 23 days after irradiation. Normal bone marrow was from CD45.1 mice. To reduce pre-transplantation stress on LSK cells to be transplanted, we first determined the number of LSK cells in aliquots of CD45.2 and CD45.1 bone marrow samples kept at 4° C during the flow cytometry analysis of their aliquots. The regenerating and normal bone marrow cells were then mixed in the ratio wherein equal numbers of CD45.2 and CD45.1 LSK cells were present. The cell mixture was transplanted, and the percentage of CD45.2 and CD45.1 nucleated blood cells was determined in the peripheral blood of transplanted mice after 1 month and 6 months. Both these experiments revealed a meager capacity of the LSK cells from regenerating bone marrow to reconstitute and support hematopoiesis after transplantation (Table 7).

| Experiment | PB examined after | F1 (recipients) | normal BM (CD 45.1) | regenerating BM (CD 45.2) | 45.1/45.2 ratio |
|---|---|---|---|---|---|
| **6 Gy 14D** | **1 month** | 38.63 ± 6.33 | 60.63 ± 6.34 | 0.74 ± 0.09 | 83 |
| | **6 months** | 33.76 ± 6.62 | 66.1 ± 6.60 | 0.15 ± 0.05 | 478 |
| **6 Gy 23D** | **1 month** | 21.28 ± 3.82 | 77.55 ± 3.77 | 1.16 ± 0.29 | 70 |
| | **6 months** | 12.34 ± 4.39 | 87.41 ± 4.34 | 0.25 ± 0.12 | 516 |

**Table 7. Chimeric hematopoiesis resulting from co-transplantation of bone marrow cells from normal or irradiated mice containing equal number of LSK cells; two independent experiments (6 Gy 14D and 6 Gy 23D).** Five dual CD45.1/CD45.2 F1 (F1) hybrid mice were irradiated (5 Gy) and transplanted with a mixture of bone marrow (BM) cells from normal (CD45.1) and (CD45.2) irradiated mice. Mice were irradiated for either 14 days (14D) or 23 (23D) before BM collection. The number of LSK cells was determined in pooled samples of normal and regenerating BM cells by flow cytometry, and the two BM samples were mixed in such a ratio that 10,000 CD45.1 and 10,000 CD45.2 LSK cells were administered to F1 recipient mice. The transplanted cells competed with each other and against the repopulating cells, which survived in submyeloablatively irradiated F1 recipient mice. The contribution of the three types of repopulating cells to blood cell production was examined in the

peripheral blood (PB) of F1 transplant recipients after 1 and 6 months, and their proportion is shown as mean ± standard deviation. PB – peripheral blood; BM – bone marrow; D – day after irradiation of mice at 6 Gy

Third, we compared the transplantation potential of LSK CD150$^{+}$CD48$^{-}$ cells sorted from normal and regenerating bone marrow (for gating used, Figure 23). The regenerating bone marrow was collected from mice (CD45.1) irradiated before 20 days. An equal number of LSK CD150$^{+}$CD48$^{-}$ cells obtained from regenerating (CD45.1) and normal (CD45.2) bone marrow were co-transplanted. The resulting chimeric hematopoiesis was then examined in the peripheral blood of recipient mice after 1 and 4 months and in the bone marrow after 5 months (Table 8). The LSK CD150$^{+}$CD48$^{-}$ cells obtained from regenerating bone marrow had a significantly reduced transplantation potential.

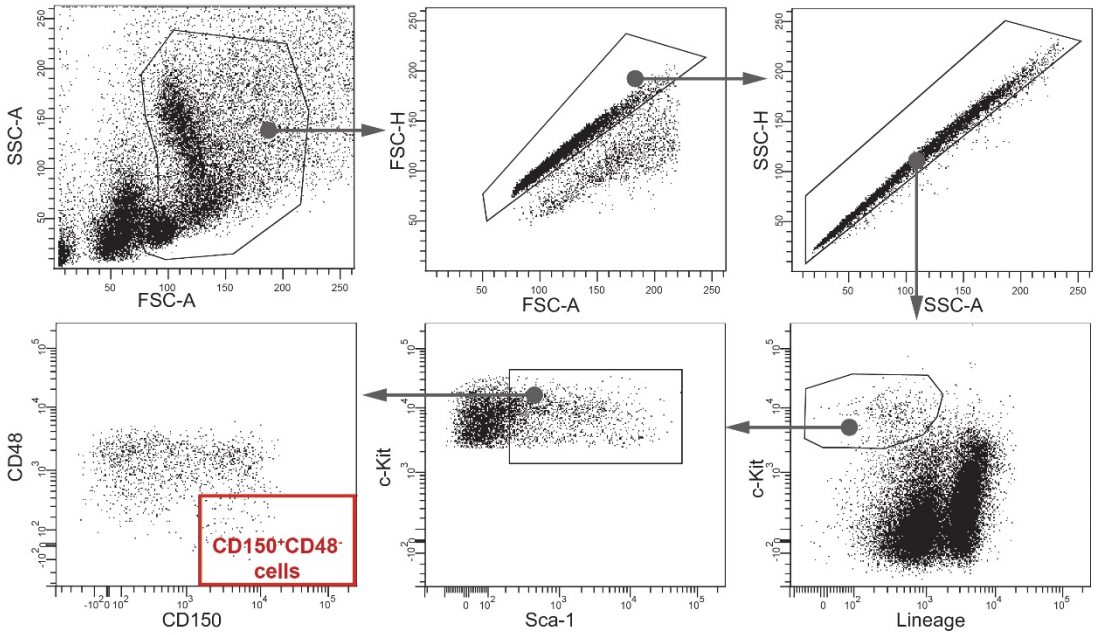


**Figure 23. The gating used to separate LSK CD150$^{+}$CD48$^{-}$ cells from either normal or regenerating bone marrow.** Cells were sorted by Filipp Savvulidi.

| Examined after | F1 recipients | LSK CD150$^{+}$48$^{-}$cells from normal BM (CD 45.2) | LSK CD150$^{+}$48$^{-}$ cells from regenerating BM (CD 45.1) | 45.2/45.1 ratio |
|---|---|---|---|---|
| **1 month PB** | 57.60 ± 7.31 | 33.59 ± 9.24 | 8.81 ± 2.95 | 3.8 |
| **4 months PB** | 25.58 ± 13.82 | 70.21 ± 11.65 | 2.21 ± 0.66 | 31.8 |
| **5 months BM** | 33.45 ± 19.10 | 66.01 ± 19.44 | 0.54 ± 0.47 | 123.2 |

**Table 8. Chimeric hematopoiesis derived from LSK CD150$^{+}$CD48$^{-}$ cells sorted from normal and regenerating bone marrow**. Five dual CD45.1/CD45.2 F1 (F1) hybrid mice were irradiated (7 Gy) and transplanted with 500 + 500 cells with the LSK CD150$^{+}$CD48$^{-}$ immunophenotype sorted either from normal (CD45.2) or regenerating (CD45.1) bone marrow (BM). The regenerating BM was collected 20 days after irradiation. The transplanted cells competed with each other and with the repopulating cells which survived in F1 recipient mice. The contribution of the three types of repopulating cells to blood cell production was examined in the peripheral blood (PB) of recipient F1 mice after 1 and 4 months and in BM after 5 months. The percentage of cells with the dual CD45.1/CD45.2 (recipient origin), CD45.2 (normal BM origin), and CD45.1 (regenerating BM origin) immunophenotypes in PB and BM is presented as the mean ± standard deviation. The gating used for sorting LSK CD150$^{+}$CD48$^{-}$ cells is shown in Figure 23. PB – peripheral blood; BM – bone marrow. The results were obtained by Katarína Forgáčová-Szikszai.

Thus, all these experiments demonstrated that expanding regenerating bone marrow contains a very low number (1 - 2 %) of cells that can reconstitute damaged hematopoiesis after transplantation compared to steady-state adult bone marrow.

## 7.7 The c-Kit receptor - stem cell factor interaction is essential for the expansion of myeloid progenitors in regenerating hematopoiesis

The low c-Kit expression level in LK cells in regenerating bone marrow was confusing since a high c-Kit expression level is a hallmark of hematopoietic stem and progenitor cells in the mouse (Okada et al., 1991; Osawa et al., 1996). We hypothesized that c-Kit might have been downregulated on the surface of LK cells by SCF, as we confirm in our laboratory in vitro (Chen et al., 2016). Therefore, we determined the expression of the mRNAs for the membrane-bound and soluble forms of SCF. Both mRNAs were significantly upregulated after irradiation (Figure 24A). To resolve the discrepancy between the downregulation of c-Kit by SCF and the inherently decreased production of c-Kit in LK cells in regenerating bone marrow, we determined the mRNA for c-Kit in normal and regenerating bone

marrow. The mRNA for c-Kit was low in regenerating bone marrow still 14 days after irradiation (Figure 24B), suggesting decreased production of c-Kit in LK cells.

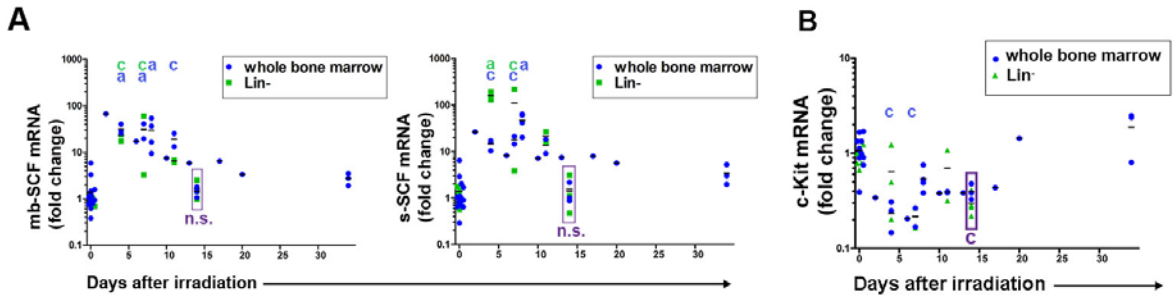


**Figure 24. A)** Stem cell factor (SCF) mRNA level was significantly increased in total bone marrow cells or magnetically separated Lin$^-$ cells of irradiated mice**.** mb - membrane-bound; s – soluble **B)** mRNA for c-Kit determined in all bone marrow cells and magnetically separated lineage negative fraction of bone marrow cells (Lin$^-$). The statistical significance between results from the bone marrow of irradiated mice and untreated mice is indicated by letters a: $p<0.05$, c: $p<0.005$. Petr Páral participated in obtaining these results.

Therefore, we functionally tested the role of c-Kit receptor-mediated signaling in developing myeloid progenitors in regenerating hematopoiesis by blocking c-Kit through the administration of a c-Kit-blocking antibody (ACK2) to mice. ACK2 effectively abrogated the c-Kit response to SCF for at least three days after a single dose of 0.5 mg (Figure 25B, C). ACK2's binding to c-Kit did not interfere with staining by fluorescently labeled anti-c-Kit antibodies (Figure 25A) used for flow cytometry.

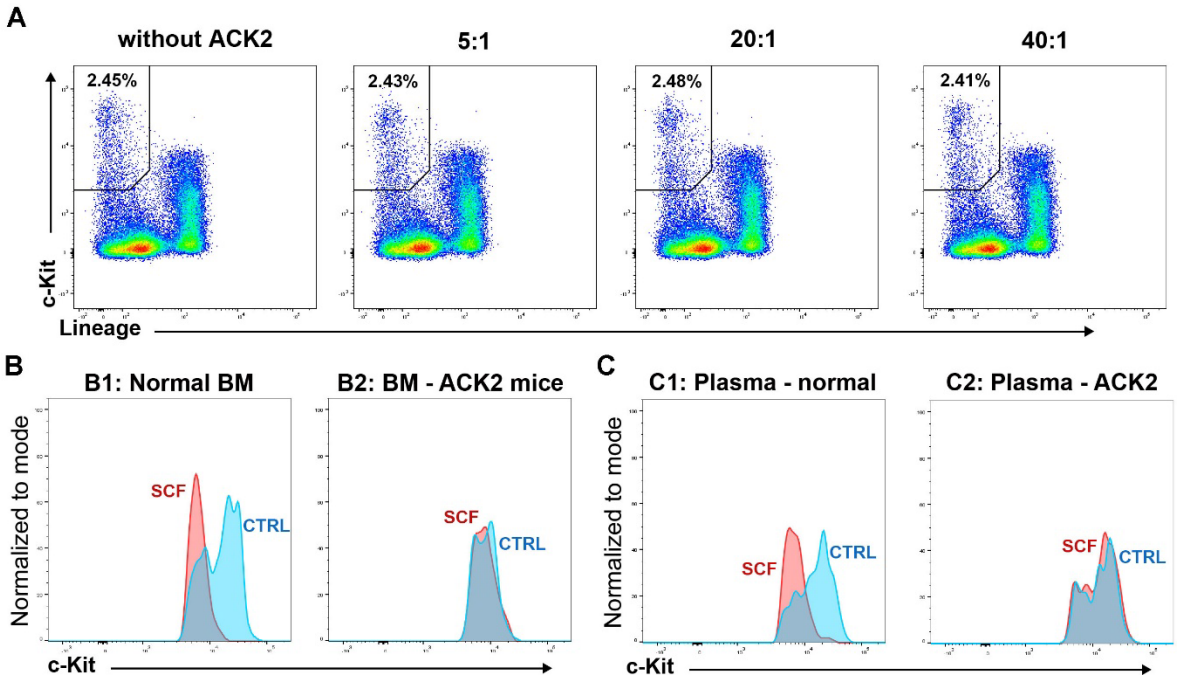

**Figure 25. ACK2 blocks c-Kit efficiently for at least 3 days and do not interfere with c-Kit detection. A)** Gating of LK cells (lineage negative c-Kit positive) for analysis of c-kit fluorescence intensity in control sample (without ACK2) and three samples with various mixtures of the c-Kit blocking antibody ACK2 and the anti-c-Kit fluorescently labeled antibody 2B8 used for measuring the c-Kit expression level. Aliquots of ten million normal bone marrow cells in 100 µl PBS were incubated at 4°C for 20 minutes in the presence of zero - 0.125 - 0.5 – 1.0 µl of ACK2 antibody (2 mg/ml). Without washing, fluorescent-labeled antibodies against lineage markers (cocktail LIN; A700), Sca-1 antigen (SCA1; Pe-Cy7), and c-Kit (2B8; BV421) were added, and the incubation continued for another 20 minutes. The ratio of ACK2/2B8 antibodies concentrations were 5:1, 20:1, and 40:1. Samples were then washed, and the percentage of $Lin^{-}c\text{-}Kit^{+}$ cells compared by flow cytometry. The pre-incubation of bone marrow cells with ACK2 antibody did not affect c-Kit detection. **B)** Bone marrow was collected into DMEM medium from normal mice (**B1**) and mice given 0.5 mg of ACK2 antibody three days previously (**B2**). The bone marrow cells were split into two aliquots of ten million cells, each in 1 ml of DMEM. One sample (SCF) was incubated with 200 ng of SCF, while the second sample (CTRL) was incubated without SCF (30 minutes at 37°C). After washing, cells were stained with fluorescent-labeled antibodies against lineage markers (cocktail LIN; A700), Sca-1 antigen (Sca1; Pe-Cy7), and c-Kit (2B8; BV421) and c-Kit fluorescence intensity was compared. Normal bone marrow cells down-regulated $c\text{-}Kit^{+}$ receptors when exposed to SCF (**B1**), but the cells from ACK2-treated mice did not respond to SCF (**B2**). **C)** Blood plasma was obtained from untreated mice and mice given 0.5 mg of ACK2 antibody three days before blood (plasma) collection. Normal bone marrow cells in 200 µl of PBS were pre-incubated for 20 min at 4°C with 300 µl of plasma from either untreated mice (**C1**) or ACK2-treated mice (**C2**). Both samples were then split into two aliquots and incubated for 30 minutes at 37°C in the presence (SCF; 300 ng per ml) or absence (CTRL) of SCF. After washing, cells were stained with fluorescently labeled antibodies against lineage markers (cocktail Lin A700), Sca-1 antigen (Sca1 Pe-Cy7), and c-Kit (2B8; BV421), and c-Kit fluorescence intensity was compared. Pre-incubation with plasma from ACK2-treated mice (**C2**) abolished the SCF-induced downregulation of c-Kit receptors, which occurred in the cells pre-incubated with the plasma of untreated mice and exposed to SCF (**C1**).

In a series of experiments, ACK2 was given to mice following their irradiation and untreated control mice. The mice were then examined 13 days after irradiation. Mice given ACK2 up to four days after irradiation did not survive, except for the one in a moribund condition (Table 9). The phenotype of LK cells in their $c\text{-}Kit^{high}$ and $c\text{-}Kit^{low}$ fractions in the mice given ACK2 1, 6, and 8 days after irradiation is shown in Figure 26A, B. The LK cells were predominantly Sca-1 and CD48 positive, CD150 negative, and lacked CD71 high cells.

**Females**

| | Days | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| 3 ○ | ACK2 | → | → | → | → | → | → | → | → | → | → | → | → | S |
| 3 ● | → | → | → | → | → | → | → | → | → | → | → | → | → | S |
| ● | ACK2 | → | → | → | → | → | → | → | → | → | Ø | | | |
| ● | → | ACK2 | → | → | → | → | → | → | → | → | → | Ø | | |
| ● | → | → | ACK2 | → | → | → | → | → | → | → | → | → | → | Ø |
| ● | → | → | → | ACK2 | → | → | → | → | → | → | → | → | Ø | |
| ● | → | → | → | → | → | → | ACK2 | → | → | → | → | → | → | S |
| ● | → | → | → | → | → | → | → | → | ACK2 | → | → | → | → | S |
| ● | → | → | → | → | → | → | → | → | → | → | ACK2 | → | → | S |

**Males**

| | Days | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| 2 ○ | ACK2 | → | → | → | → | → | → | → | → | → | → | → | → | S |
| 3 ● | → | → | → | → | → | → | → | → | → | → | → | → | → | S |
| ● | → | → | → | → | → | → | → | → | → | → | → | Ø | | |
| ● | ACK2 | → | → | → | → | → | → | → | → | → | → | → | Ø | |
| ● | → | ACK2 | → | → | → | → | → | → | → | → | → | → | → | (S) |
| ● | → | → | → | → | ACK2 | → | → | → | → | → | → | → | → | Ø |
| ● | → | → | → | → | → | → | ACK2 | → | → | → | → | → | → | S |
| ● | → | → | → | → | → | → | → | → | ACK2 | → | → | → | → | S |
| 8 ● | → | → | → | → | → | → | → | → | → | → | ACK2 | → | → | S |

**Table 9. ACK2 blocking antibody was given to mice following their irradiation and untreated mice.** c-Kit blocking ACK2 antibody was lethal if given during the first 4 days after irradiation (●), except in one mouse. Normal mice (o) and mice given ACK2 6, 8, and 10 days after irradiation survived for 13 days. Mouse death is indicated with a Ø.

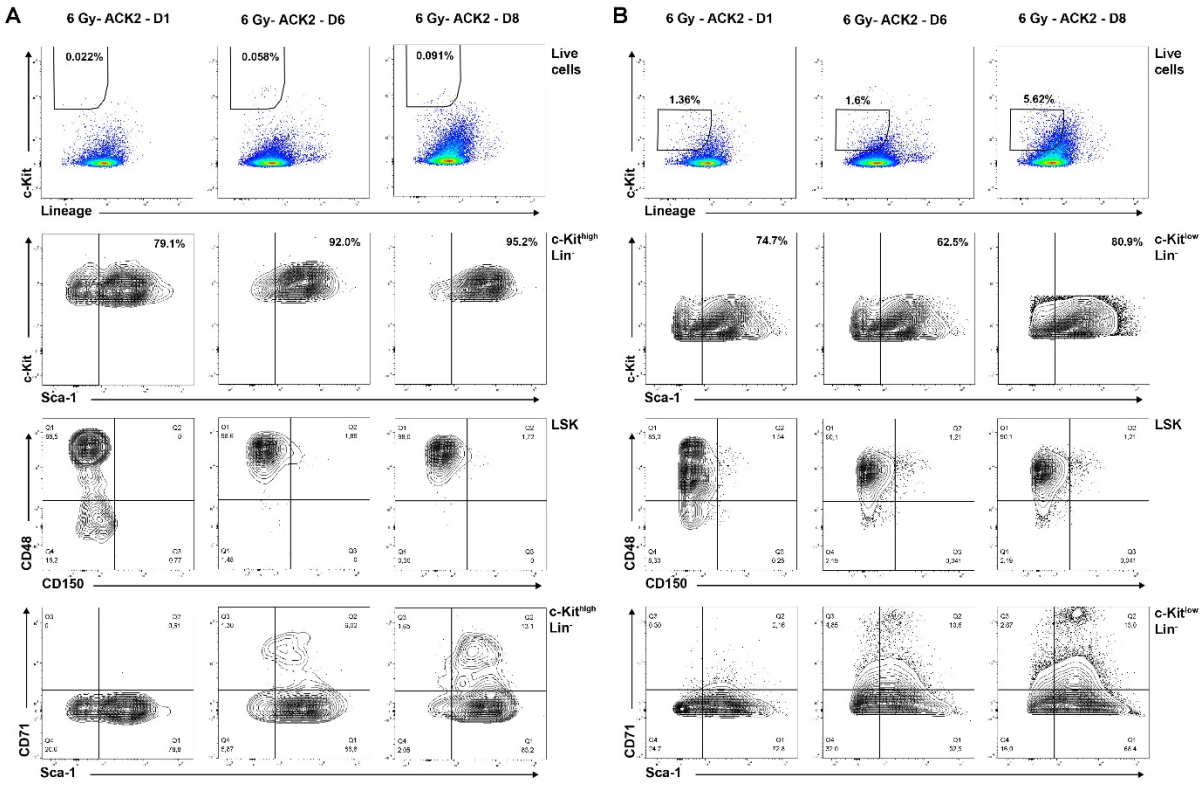


**Figure 26. Administration of ACK2 antibody to mice irradiated with 6 Gy suppressed the restoration of Lin$^-$c-Kit$^+$ (LK) cells and highlighted Sca-1$^+$ cells.** Bone marrow was analyzed in three mice that survived for 13 days after irradiation (6 Gy) and the administration of 0.5 mg of ACK2 antibody 1, 6, or 8 days (D1, D6, D8) after irradiation. The mouse receiving ACK2 1 day after irradiation was in a moribund condition (17.6 g b.w., 1.4x10$^6$ cells per femur, 0.022% LK cells which lacked CD71$^+$ cells). Sca-1$^+$ cells were highly abundant in all mice. **A)** c-Kit$^{high}$. **B)** c-Kit$^{low}$ LK cells. For normal bone marrow (CTRL), see Figures 13 and 14.

We then asked how much the reconstitution of damaged hematopoiesis still depended upon SCF/c-Kit signaling 10-13 days after irradiation, when the SCF mRNAs level started to decline (see Figure 24A) steeply, but the mRNA for c-Kit (Figure 24B) and c-Kit expression on LK cells (Table 6) was still low. All nine mice, eight males and one female, given ACK2 10 days after irradiation, survived until day 13 (Table 9). In these mice, ACK2 treatment significantly inhibited the recovery of LK cells (Figure 27D), and particularly their $LS^{-}K$ fraction (Figure 27A, B, D). LSK cells were also depressed after ACK2 administration (Figure 27D), but significantly less than $LS^{-}K$ cells, as shown by the $LS^{-}K$/LSK cell ratio, which was approximately 50-fold decreased in the ACK2-treated mice (Figure 27D). LK cells surviving ACK2 treatment in irradiated mice were almost exclusively positive for CD16/32 expression (Figure 27A, B). The differentiated precursors of red blood cells (Ter119) and granulocytes (Gr-1) were significantly suppressed in ACK2 treated mice, in contrast to B-cells (B220), which were not affected (Figure 27E).

These results reveal that the SCF/c-Kit signaling is essential for the development and population expansion of erythro-myeloid progenitors in regenerating bone marrow despite the decreased expression of the c-Kit receptor in these cells.

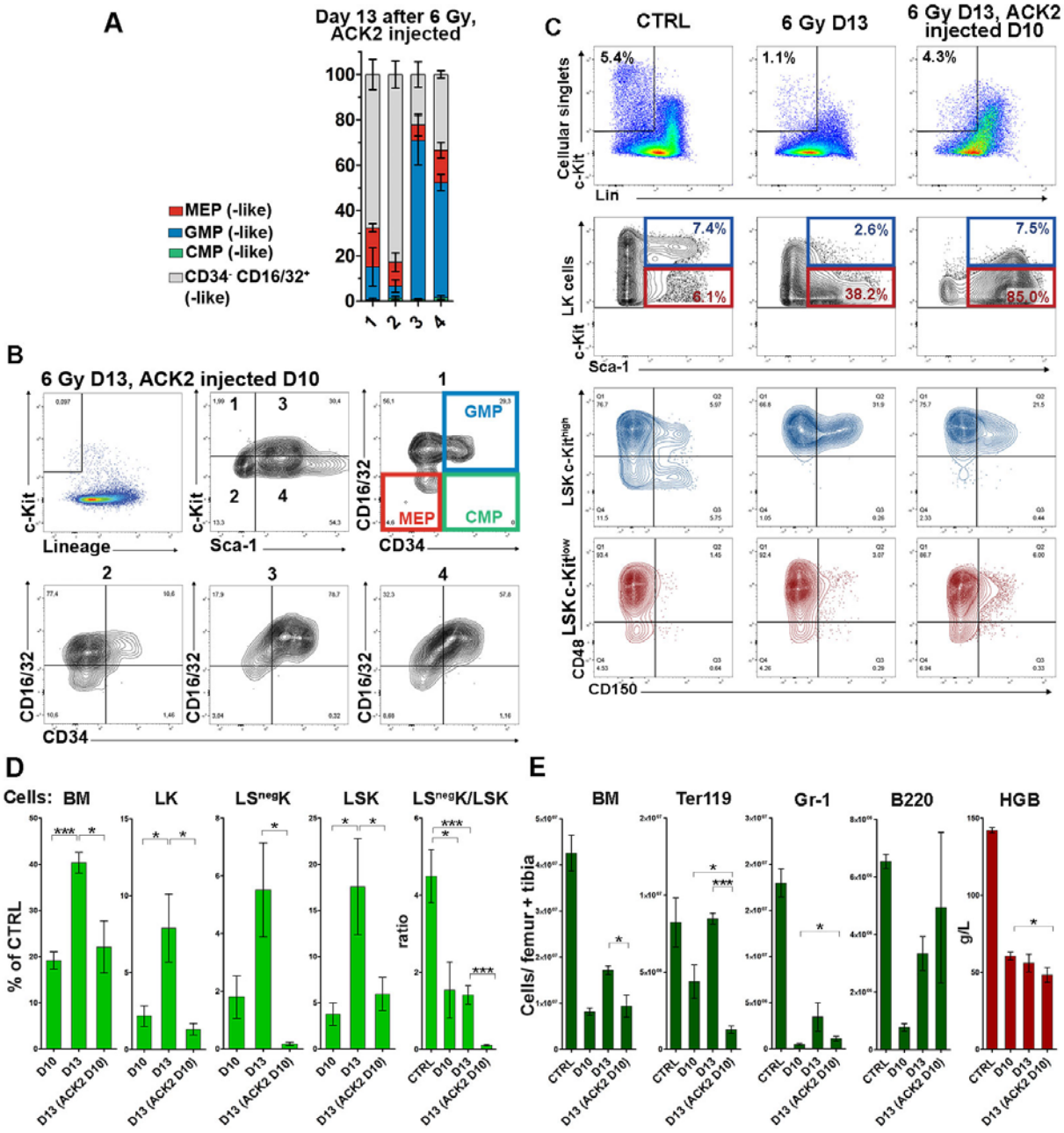


**Figure 27. Abrogation of c-Kit receptor signaling reveals its role in bone marrow regeneration. A,D**) The proportion of cells with the MEP-, GMP-, CMP-(like) and $CD34^{-}$/$CD16\text{-}32^{+}$ immunophenotypes in four subtypes of LK (see **B**) in mice given ACK2 10 days after irradiation and examined 13 days after irradiation. For the corresponding values in the bone marrow of untreated mice (CTRL) and those of only 6 Gy-irradiated mice, see Figure 16B. **B, C)** Representative immunophenotypes of $Lin^{-}c\text{-}Kit^{+}$ (LK) cells in the bone marrow of untreated (CTRL), irradiated (6 Gy D13) and irradiated and given ACK2 10 days after irradiation (6 Gy D13, ACK2 injected D10) mice examined 13 days after irradiation. **D)** Relative numbers of all bone marrow cells (BM) and LK, $LS^{-}K$, LSK cells in six irradiated mice examined after 10 days or after13 days, and five irradiated mice given ACK2 three days before examination on day 13. All mice were males, and the values from normal eight mice served as the reference 100% values. **E)** The absolute number of total BM cells, $Ter119^{+}$ cells, $Gr\text{-}1^{+}$ cells and $B220^{+}$ cells in these mice (**A**). Also, their hemoglobin concentration in the peripheral blood is shown. D10, D13 – days after irradiation with 6 Gy; HGB – hemoglobin concentration in blood. *P<0.05, **P<0.01, ***P<0.001

## 7.8 Transplanted bone marrow also regenerates by expanded committed myeloid progenitors partly masked by Sca-1 expression

We asked whether transplanted normal bone marrow regenerates similarly as spontaneously regenerating bone marrow, i.e., give rise to expanded populations of megakaryocyte-erythroid and granulocyte-macrophage progenitors. To answer this, we transplanted 1/100 (≈250.000 cells) of femoral bone marrow cells from untreated mice to lethally (myeloablative) irradiated syngeneic recipient mice. Fourteen days after transplantation, the Lin-c-Kit$^{+}$ (LK) cells in the bone marrow of recipient mice had features highly similar to the features of the LK cells in the spontaneously regenerating bone marrow of submyeloablatively irradiated mice (Figure 28A, B, compared to 13 A, 16 A). The fraction of LSK cells within LK cells was increased, and these cells were uniformly CD48 positive and similar in the c-Kit$^{high}$ and c-Kit$^{low}$ fractions (Figure 28A).

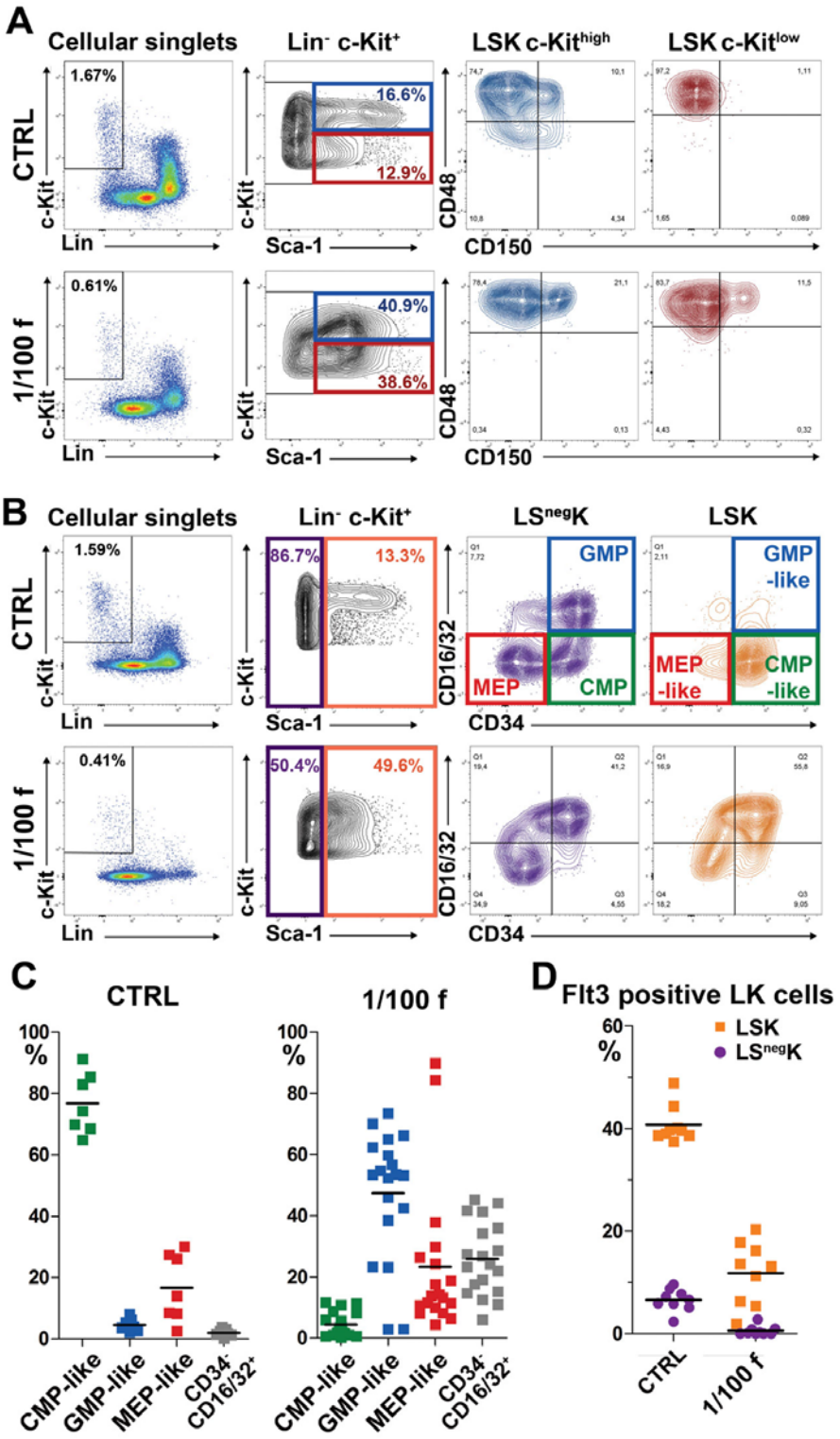


**Figure 28. Immature hematopoietic cells in the bone marrow regenerating from transplanted cells have the phenotype of granulocyte-macrophage and megakaryocyte-erythroid myeloid progenitors also when they express Sca-1 antigen. A)** Bone marrow pooled from 2 untreated mice (CTRL) and 3 mice irradiated with 9 Gy and transplanted with 1/100 of femoral bone marrow from a syngeneic donor (1/100 f) was analyzed 14 days after transplantation. Representative CD150/CD48 expression pattern in their c-Kit$^{high}$ (blue) and c-Kit$^{low}$ (red) portions of LSK cells. **B)** Representative CD34/CD16-32 expression patterns in LS$^{-}$K (violet) and LSK (orange) cells. **C)** LSK cells were analyzed for the

CD34/CD16-32 expression pattern (see b) in 7 untreated mice (from five independent experiments; CTRL) and 19 mice irradiated with 8 or 9 Gy and transplanted with 1/100 of syngeneic femoral bone marrow (1/100 f; examined after 14 days in four independent experiments). **D)** Flt3 positive cells in LSK and $LS^{-}K$ cells of 9 untreated male mice (from four independent experiments; CTRL) and 9 mice irradiated with 8 Gy and transplanted with 1/100 femoral bone marrow.

To further extend the characterization of LK cells present in bone marrow regenerating from transplanted cells, we compared the CD34/CD16-32 expression profile with the emphasis on LSK cells. Similarly, as in the spontaneously regenerating bone marrow (see Figure 16C), in bone marrow regenerating from transplanted cells, cells with the phenotype of CMPs (CMP-like) were suppressed, and those with the phenotype of GMPs and MEPs were significantly increased (Figure 28C).

Because in spontaneously regenerating bone marrow, the Flt3 (Flk2, marking the lymphoid-primed MPPs) expression became low (see Figure 18A), we explored whether the proportion of $Flt3^{+}$ cells in LSK cells present in bone marrow regenerating from transplanted cells is also similarly decreased. This analysis demonstrated the significantly diminished proportion of $Flt3^{+}$ within LSK cells in bone marrow regenerating from transplanted cells (Figure 28D) that corresponded to their low proportion in Sca-$1^{-}$ cells, similarly as in bone marrow regenerating spontaneously without transplantation.

In the regenerating bone marrow, LSK cells had the FSC and SSC characteristics corresponding to Sca-1 negative GMPs and MEPs in the bone marrow of untreated mice (Figure 17). The histograms in Figure 29 confirm that the situation is almost identical in bone marrow regenerating from transplanted cells.

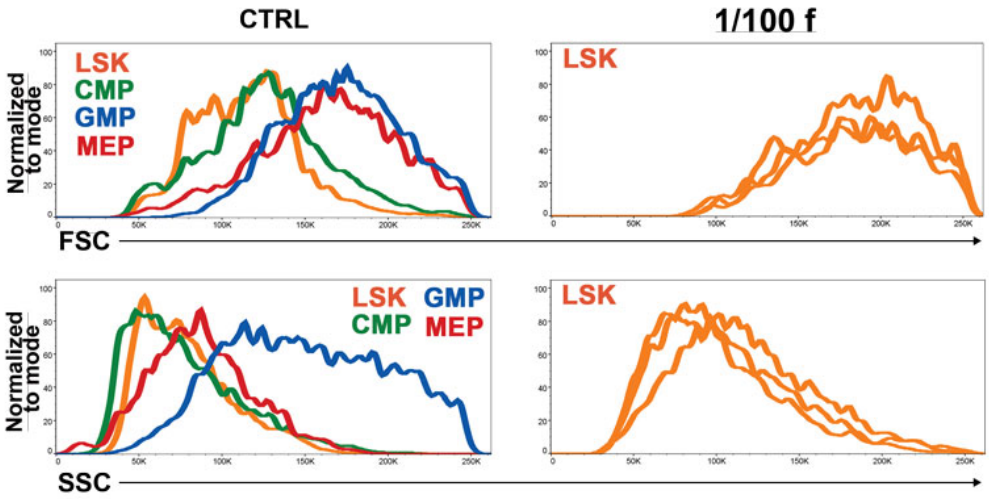


**Figure 29. LSK cells in BM regenerating from transplanted cells have the FSC and SSC characteristics corresponding to those of Sca-1 negative GMPs and MEPs in the bone marrow of untreated mice.** Forward scatter (FSC) and side scatter (SSC) histograms of LSK and CMP, GMP and MEP cells in the bone marrow of one untreated mouse (similar as in Figure 17) compared to the histograms of LSK cells of three mice irradiated with 8 Gy and transplanted with 1/100 of syngeneic femoral bone marrow examined after 14 days.

Thus, these data collectively confirm the similarity between hematopoiesis regenerating after submyeloablative irradiation and hematopoiesis regenerating after lethal irradiation from transplanted cells. In both, our analyzes revealed significantly expanded populations of MEPs and GMPs, partly presenting as LSK cells due to reexpression of Sca-1 antigen. On the other hand, LK cells with the immunophenotype of CMP, multipotent progenitors LSK $CD150^{-}CD48^{-}$, and HSCs are significantly suppressed.

## 7.9 The microenvironment of damaged bone marrow could activate reexpression of Sca-1 antigen in myeloid progenitors

Because LSK cells in regenerating hematopoiesis are similar to granulocyte-macrophage and erythroid progenitors, which are Sca-1 negative, we tested whether the microenvironment of damaged bone marrow would induce reexpression of Sca-1 in myeloid progenitor cells. We isolated $LS^{-}K$ cells from the bone marrow of UBC-GFP transgenic mice and transplanted them into congenic wild-type mice, either irradiated beforehand or untreated. Two days later, we analyzed GFP-positive cells in the bone marrow of recipient mice, focusing on their Sca-1 expression profile. In untreated recipient mice, transplanted $LS^{-}K$ cells remained

predominantly Sca-1$^{neg}$. However, a significant proportion of LS$^{-}$K cells transplanted into irradiated recipient mice expressed Sca-1 antigen and presented as LSK cells (Figure 30). Thus, these results directly demonstrate the activation of developmentally advanced myeloid progenitors by the stroma of damaged bone marrow.

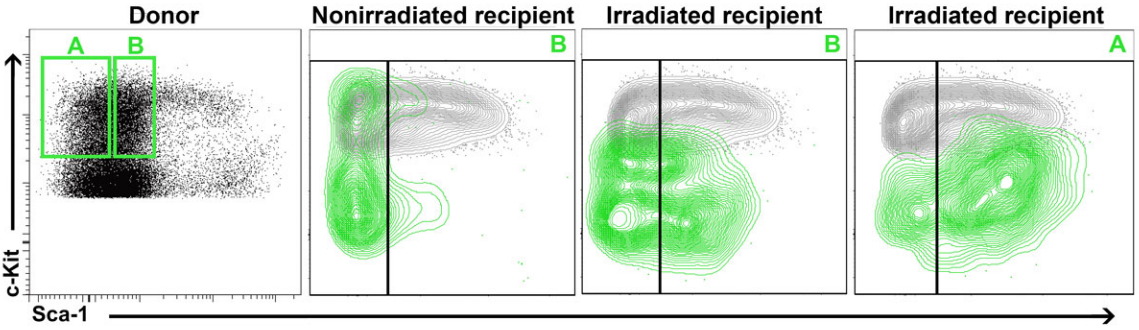


**Figure 30. Activation of developmentally advanced Sca-1$^{-}$ myeloid progenitors by the stroma of damaged bone marrow.** Lin$^{-}$Sca-1$^{-}$c-Kit+ cells were sorted from the bone marrow of untreated UBC-GFP mice (GFP-LS$^{-}$K cells) by BD FACS Aria IIu cell sorter into two fractions (A and B) according to the gating strategy shown here. 400 000-800 000 GFP-LS$^{-}$K cells were injected intravenously into C57Bl/6 mice, either untreated or irradiated two days prior with 7 Gy. Bone marrow from the femurs and tibias of the recipients was collected 48 hours after transplantation. Lin-c-Kit+ (LK) cells were sorted from the bone marrow of untreated C57Bl/6 mice and were mixed with bone marrow cells isolated from the transplant recipients for analysis. Red blood cells were lysed, and nuclear cells were stained with antibodies against lineage markers, the Sca-1 antigen, and the c-Kit receptor. The Sca-1 and c-Kit expression profiles were determined in lineage-negative cells, and the expression profiles of GFP-positive cells (green) were compared to those of the added untreated LK cells (grey). Representative results from three experiments are shown. Cells were sorted by Martin Báječný.

# 8. Discussion

This thesis provides a deep insight into the intensively regenerating bone marrow during its recovery from severe damage or after transplantation. It compares it to the physiologically expanding embryonic/fetal liver and early postnatal hematopoiesis which concomitantly execute two competing tasks: multiply their immature hematopoietic cells and increase production of mature blood cells.

In the embryo, the transient primitive hematopoiesis starts in the yolk sac and produces mainly large nucleated erythroblasts with fetal hemoglobin. This very early phase of hematopoiesis is followed by another transient hematopoiesis driven by erythro-myeloid progenitor cells, which originate in the yolk sac and migrate the newly established fetal liver. These two phases of embryonic hematopoiesis precede the appearance of transplantable HSCs (Frame et al., 2013; McGrath et al., 2015; reviewed in Palis, 2016 and Dzierzak and Bigas, 2018). HSCs are then generated separately in the embryo proper by differentiation from the hemogenic endothelium of large arteries in the aorta-gonad-mesonephros region and the vitelline arteries placenta in a stepwise process (Rybtsov et al., 2014, 2016). HSCs migrate into the fetal liver, where they initiate the production of all blood cells, including lymphocytes. The hierarchical structure of hematopoiesis with HSCs – MPPs and developmentally restricted progenitors is first established in the fetal liver. These HSCs are multipotent and have a high self-renewal and transplantation capacity (Copley et al., 2013). HSCs actively proliferate in the fetal liver and in bone marrow during the first three weeks of life in the mouse (Bowie et al., 2006). Afterward, many HSCs enter a dormant state but can still contribute to hematopoiesis (Sawai et al., 2016; Akinduro et al., 2018). The HSCs are induced to proliferate after bone marrow damage, infection, or sustained increased red blood cell production (Baldridge et al., 2010; Trumpp et al., 2010; Singh et al., 2018).

In this study, we targeted the brief period of bone marrow regeneration wherein the hematopoiesis, derived from a tiny number of founder cells, is challenged with similar tasks as in the embryonic hematopoiesis: to concurrently produce blood cells in large amounts, expand the pools of progenitors and to reconstruct the hierarchical structure of hematopoiesis. We focused on all immature cells lacking lineage markers and expressing the c-Kit receptor (LK cells). Thetargeted cell type of immature hematopoietic cells differs from the cells studied by Simonnet et al. (2009). These authors analyzed a subtype of LSK cells characterized by a high efflux of Hoechst 433342 dye (“side population” in flow cytometry; SP) in mice irradiated with either 3 or 6 Gy. They analyzed bone marrow in mice irradiated with 3 Gy after 2-14 days and after 10 weeks in mice irradiated with 3 or 6 Gy. These authors found decreased c-Kit expression and increased Sca-1 expression in SP-LSK cells during the first four days after the dose of 3 Gy and increased expression of CD150 marker up to 10 weeks-post irradiation. A

significant deficit in Flt3 (CD135) cells after ten weeks and SP-LSK CD150$^{+}$ cells showed reduced repopulating potential after transplantation. Our study thus principally differs from the study of Simonnet et al. (2009) in targeting all lineage-negative and c-Kit expressing cells (LK cells) in their c-Kit$^{low}$ and c-Kit$^{high}$ fractions, and also by targeting the transient regenerative phase characterized by the intensive production of mature blood cells and massive concurrent expansion of progenitor cells.

While studying Sca-1 expression in LK cells, we noticed Sca-1 expression in CD71-positive LSK cells in regenerating bone marrow, while the LSK cells in normal bone marrow were uniformly CD71 negative. CD71 is highly expressed in erythroid cells stimulated by erythropoietin, and its expression in LSK cells in regenerating bone marrow might signal their activation towards the erythroid commitment. The functional in vivo tests did not support this hypothesis. Therefore, we hypothesized that the normal Sca-1 negative and CD71 expressing early erythroid progenitors re-expressed the Sca-1 antigen in regenerating bone marrow as part of their activation.

These findings prompted us to focus on LSK cells in regenerating bone marrow and their comparison with LS$^{-}$K cells. Since we became suspected that LSK cells in regenerating bone marrow are, in fact, the myeloid progenitor cells which re-expressed Sca-1, we applied the CD34 and CD16/32 markers, traditionally used only for analysis of LS$^{-}$K cells, also on the analysis of LSK cells. This analysis revealed several similarities between the LSK cells and LS$^{-}$K cells in regenerating bone marrow. LSK cells in regenerating bone marrow had fewer Flt3 (CD135) positive cells: the lymphoid-primed multipotent progenitors with down-regulated megakaryocyte-erythroid potential (Buza-Vidas et al., 2011). LSK cells in regenerating bone marrow were mostly CD16/32 positive and expressed CD71 at a variable level. The expression of CD16/32 characterizes the granulocyte-macrophage progenitors. The CD71 expression is linked to the erythroid developmental lineage.

The flow cytometry analysis of regenerating bone marrow thus uncovered expanded populations of cells with phenotypic markers of the erythroid and myeloid (granulocyte-macrophage) progenitors masked by expression of Sca-1 in part of them and by the expression of CD16/32 by a majority of the cells. The CD16/32 expression makes these myeloid progenitors similar to the EMPs in the embryo (Frame et al., 2013; McGrath et al., 2015; Palis, 2016). However, there are significant differences between the EMPs and the regenerating myeloid progenitors since EMPs are c-Kit$^{high}$, uniformly Sca-1 and CD150 negative, and all express CD41. At the same time, LK cells in regenerating hematopoiesis are c-Kit low, partly Sca-1 and CD150 positive, and express CD41 only in a small fraction of LSK cells.

The gene expression analysis performed in connection with this thesis, revealed a strongly activated erythroid program in LS$^{-}$K and LSK cells in regenerating bone marrow(Faltusová et al., 2020a; the gene expression analyzis was performed by Chia-Ling Chen). Correspondingly, the number of erythrocytes

reached its nadir on day 12 after irradiation and then began to increase (Figure 12B). The blockade of the c-Kit receptor suppressed erythropoiesis in regenerating bone marrow significantly (Figure 27E). All these findings demonstrate significant erythroid activity in immature LK cells in intensively regenerating hematopoiesis. This intense erythroid activity was not fully reflected in in vitro clonogenic cultures of cells from regenerating bone marrow (Figure 21B, C, and Figure 15). We hypothesize that the culture conditions in GF M3434 medium did not fully substitute for the support and stimulation provided to erythro-myeloid progenitors by the microenvironment in regenerating bone marrow. The culture lacks the membrane-bound SCF and macrophages known to participate in the erythroblast differentiation and maturation. Thus, the conditions for the growth of erythroid cells are often in cultures suboptimal (Monette and Holden, 1982).

Analysis of the gene expression (Faltusová et al., 2020; not included in this thesis) in LSK and LS$^-$K cells separated from normal and regenerating bone marrow further supported the erythro-myeloid character of LK cells but revealed differences between their LSK and LS$^-$K subtypes. While LSK cells were both erythroid and granulocytic (myeloid) according to the enhanced gene expression linked to both these developmental lineages, the LS$^-$K cells had only the erythroid program enhanced (Faltusová et al., 2020). The suppression of erythropoietin stimulation by posttransfusion polycythemia did not inhibit the erythroid program in LK cells in regenerating hematopoiesis, nor did it suppress the enhanced expression of CD71. Peslak et al. (2012) showed that only Day-3 BFU-E and CFU-E erythroid progenitors were responsive to erythropoietin stimulation in regenerating bone marrow. Therefore, most LK cells in regenerating bone marrow have a less advanced erythroid status than Day-3 BFU-E. Contrary to the apparent erythropoietin independence, the expansion of LK cells in regenerating bone marrow, particularly of their LS$^-$K fraction, required stimulation by SCF mediated by the c-Kit receptor.

The LK cells in regenerating bone marrow differed markedly from the LK cells in the fetal liver and early postnatal bone marrow phenotypically and in their capacity to be transplanted. The expansion power of the fetal liver hematopoiesis can be transferred to adult mice with damaged hematopoiesis by transplantation and is markedly superior to adult bone marrow (Pawliuk et al., 1996; Harrison et al., 1997; Copley et al., 2013). Therefore, we explored the capacity of regenerating bone marrow to be similarly transplanted. This ultimate functional tests for HSCs revealed the most striking difference between the fetal liver hematopoietic cells and the regenerating bone marrow. The transplantation potential of regenerating bone marrow cells was severely reduced and deficient in producing of the lymphoid cells. In contrast, Harrison et al. ( 1997) demonstrated that 14-day fetal liver contains several times more long-term repopulating cells (corresponding to stem cells) relative to short-term repopulating cells (corresponding to progenitors) than adult marrow.

The functional testing of cells from the intensively regenerating bone marrow by in vitro clonogenic assays showed reduced capacity. We hypothesize that myeloid progenitors in regenerating bone marrow depend more on their direct interaction with the supporting tissue microenvironment, e.g. stimulation by the membrane-bound SCF, than normal progenitors. Another possibility to understand this paradoxical phenomenon would be increased sensitivity of clonogenic cells from regenerating bone marrow towards in vitro handling and adverse cultivation effects.

Because the population expansion of HSCs in the foetal liver is mainly independent of SCF induced signaling mediated by c-Kit receptor (Ikuta and Weissman, 1992; Thorén et al., 2008), we abrogated the c-Kit receptor signaling with the ACK2 antibody in irradiated mice to simulate the condition under which the fetal hematopoiesis rapidly expands. ACK2 administration to irradiated mice fully inhibited the expansion of Lin-c-Kit$^{+}$ cells and suppressed differentiated bone marrow cells excempt for B-cells. The expansion of MEPs appeared to be fully, and that of GMPs partly, dependent on uninterrupted SCF/c-Kit signalling. This difference in the SCF dependence further highlights the significant differences between the physiological expansion of hematopoiesis and its expansion during regeneration.

In the present study, we describe significantly expanded populations of altered erythro-myeloid progenitor cells in the Sca-1 positive and Sca-1 negative LK cells in regenerating bone marrow. The most significant feature of these cells, expressing c-Kit at a low level, is their highly restricted capacity for reconstitution of damaged hematopoiesis after transplantation which contrasts with their massive performance in the production of blood cells in situ and their concurrent population expansion. Randall and Weissman (1998) described cells present in murine bone marrow phenotypically similar to HSCs (Sca-1$^{high}$) lacking the c-Kit expression and the ability to reconstitute hematopoiesis in transplanted mice. Other reports found HSCs in c-Kit$^{low}$ immature cells (Doi et al., 1997; Lian et al., 1999; Yang et al., 2002; Thorén et al., 2008; Grinenko et al., 2014). However, these cells were identified as having a high capacity to reconstitute damaged hematopoiesis after their transplantation which contrasts with the meager capacity to transplant the erythro-myeloid progenitors from regenerating bone marrow we describe here. Our study could not identify the very few cells which were at the beginning of the bone marrow regeneration because of the significant initial damage to bone marrow.

However, and bearing the limitations of our study in mind, we provide compelling evidence that hematopoiesis in regenerating bone marrow contains expanded populations of activated erythro-myeloid progenitors which markedly outweigh the severely reduced populations of the short-term and long-term repopulating cells corresponding to MPPs and HSCs. We also find some previously unknown similarities between regenerating adult bone marrow and definitive embryonic hematopoiesis before the emergence of HSCs.

These novel experimental results shed light on how bone marrow regenerates after submyeloablative damage or after transplantation. They prompted us to develop a mechanistic model, inspired by Waddington´s landscape model of cell differentiation, describing the activation of developmentally very late progenitor cells governed by supporting stroma tissue (Figure 31). After that, we verified the model experimentally by analyzing of Sca-1-negative progenitor cells transplanted into either unconditioned or irradiated mice.

Using a two-dimensional model of the stroma and the hematopoietic stem and progenitor cells interactions, we present the significant differences between the steady-state hematopoiesis and the regenerating hematopoiesis in Figure 31. In regenerating bone marrow, the late myeloid progenitors (GMPs and MEPs) are significantly expanded, while the populations of HSCs, MPPs, and CMPs are diminished. A part of GMPs and MEPs reacquire Sca-1 expression. The Sca-1 expression in GMPs and MEPs may reflect their increased self-renewal capacity exceeding their differentiation and leading to their rapid population expansion. The model assumes that the transient suppression of the differentiation is initially induced in GMPs and MEPs by the external cues provided by the microenvironment of damaged bone marrow. Hypothetically, the highly upregulated gene for SCF in damaged bone marrow (Figure 24A) could provide one of the external cues inducing the early expansion of GMPs and MEPs.

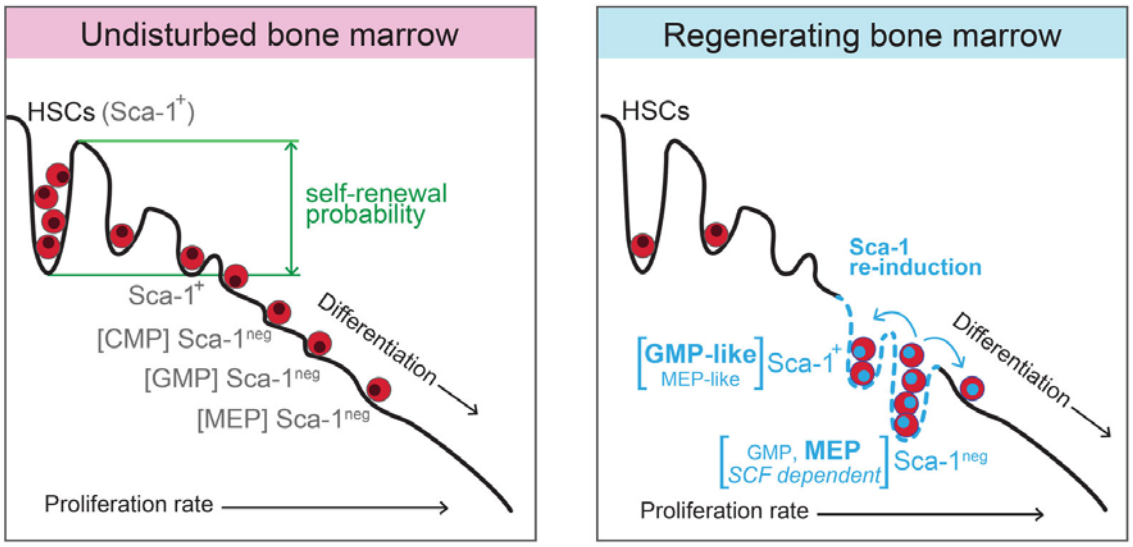


**Figure 31. The model of bone marrow regeneration with a robust response of the committed myeloid progenitors.** Sca-1 negative cells have most features of megakaryocyte-macrophage progenitors (MEPs) and are highly dependent on stimulation by SCF. Sca-1$^+$ cells are activated granulocyte-macrophage progenitors (GMPs) and MEPs which re-expressed Sca-1. MEPs and GMPs proliferate intensively. The model assumes that external cues emanating from injured bone marrow, e.g., SCF, transiently inhibit the differentiation of these cells, which leads to their early and rapid

population expansion. After the initial expansion, a portion of the cells resume differentiation and become the early source of myeloid blood cells.

In conclusion, our research unveils the unexpected extensive regenerative potential of the cells commonly attributed only to amplification function. Conceptually, it draws attention to the latent potential of developmentally advanced and committed progenitor cells to carry out the initial phase of tissue regeneration. Intensive proliferation and the proximity to mature blood cells predisposes these cells for a rapid and vigorous response to tissue damage. The conceptual advance demonstrates that bone marrow regeneration is a biologically markedly distinct process from the expansion of hematopoietic tissues occurring during the fetal and early postnatal development. Our results demonstrate, for the first time, that the function of stem cells and multipotent progenitors in blood cell production can be temporarily highly efficiently substituted by developmentally very distant progenitor cells.

# 9. Conclusions

The present study provides experimental evidence that stem cells are not the exclusive carriers of regeneration of damaged hematopoiesis. Expanded populations of developmentally advanced erythroid and myeloid progenitors with altered immunophenotype significantly participate in the recovery of blood cell production and early hematopoietic tissue reconstitution. These progenitors activate the erythroid developmental program independently from erythropoietin production but require effective stimulation by stem cell factor (SCF) for their expansion.

It was confirmed that a short period in the regeneration of damaged hematopoiesis mimics the early embryonic definitive stage of production of blood cells before the population of hematopoietic stem cells is established.

The data presnted in this study provide a novel insight into tissue regeneration by suggesting that cells other than stem cells and multipotent progenitors can be of fundamental importance for the rapid recovery of tissue function.

# 10. Summary

Tissue regeneration is a complex and highly orchestrated process dependent on cells with the potential to restore structures and functions and on controlling factors from the tissue microenvironment. Hematopoietic tissue has a high ability to regenerate, which is attributed to the presence of stem cells, but the regeneration of severely damaged adult tissue is still only partially understood. Hematopoietic tissue provides a unique opportunity to study tissue regeneration due to its well-established steady-state structure and function, easy accessibility, advanced research methods, and well-defined embryonic, fetal, and adult stages of development. Embryonic/fetal liver hematopoiesis and adult hematopoiesis recovering from damage share the need to expand populations of progenitors and stem cells in parallel with increasing production of mature blood cells.

We analyzed adult hematopoiesis in mice subjected to a submyeloablative dose (6 Gy) of gamma radiation, in which only a few cells with reconstituting capacity survived. We targeted the period of regeneration characterized by the renewed massive production of mature blood cells and the ongoing expansion of immature hematopoietic cells. Cells from the top of the hematopoietic hierarchy, hematopoietic stem cells, and multipotent progenitors are almost missing in this period of hematopoiesis regeneration. We uncovered significantly expanded populations of developmentally advanced erythroid and myeloid progenitors with significantly altered immunophenotype and with the ability for intensive proliferation. These immature hematopoietic cells differ from the progenitor cells present in normal bone marrow by the decreased expression level of the c-Kit receptor for stem cell factor, the expression of Sca-1 antigen also in the cells which express transferrin receptor 1 (CD71), by expression of CD16/32 in most of the cells, and by altered expression of CD41. These progenitors activated the erythroid developmental program independently from erythropoietin production. Despite decreased expression of the c-Kit receptor, progenitors require effective stimulation by stem cell factor (SCF) for their expansion.

Hematopoietic stem cells, defined by their ability to reconstitute destroyed hematopoiesis in the host, were reduced to 1 – 2 % of their normal number in the intensively regenerating hematopoiesis.

It was shown that the early reconstitution of hematopoiesis from transplanted cells that were not exposed to radiation gives rise to populations of altered progenitors, which are similar to those identified in the bone marrow regenerating from endogenous cells surviving exposure to ionizing radiation.

Regenerating hematopoiesis differs significantly from the expanding hematopoiesis in the fetal liver by the virtual lack of stem cells and different immunophenotypes of progenitor cells.

The data presented in this study provide a novel insight into tissue regeneration by suggesting that cells other than stem cells and multipotent progenitors can be of fundamental importance for the rapid recovery of tissue function, and the regenerating adult hematopoiesis shares some features with the embryonic hematopoiesis preceding the development of stem cells.

# 11. Souhrn

Regenerace tkáně je komplexní řízený proces závislý na buňkách s potenciálem obnovit buněčnou stavbu tkáně a její funkci, jakož i na řídících faktorech z tkáňového mikroprostředí. Krvetvorná tkáň má velkou schopnost regenerace, která je přičítána přítomnosti kmenových buněk. Regenerace silně poškozené dospělé krvetvorné tkáně je však prozkoumána jen částečně, a důraz na klíčovou úlohu kmenových buněk může zastírat poznání jiných významných mechanismů regenerace. Krvetvorná tkáň přitom poskytuje unikátní možnost poznání tkáňové regenerace díky její známé buněčné hierarchii, existenci pokročilých výzkumných metod včetně možnosti její transplantace, a také dobře definovaným embryonálním, fetálním a dospělým vývojovým fázím. Embryonální/fetální krvetvorba a regenerující dospělá krvetvorba spolu sdílejí potřebu zvyšování populací progenitorových a kmenových buněk při současné potřebě stálého zvyšování tvorby zralých krevních buněk.

Analyzovali jsme dospělou krvetvornou tkáň myší po jejím submyeloablativním poškození ionizujícím zářením (6 Gy). Použitou dávku celotělového záření přežije jen velmi málo buněk s kapacitou rekonstituce (regenerace) hematopoezy. Zaměřili jsme se na období intenzivní regenerace charakterizované obnovenou masivní produkcí zralých krevních buněk a souběžnou expanzí nezralých krvetvorných buněk progenitorových a kmenových. Zjistili jsme, že v této fázi regenerace kmenové buňky a multipotentní progenitorové buňky téměř chybí. Popsali jsme a definovali silně expandovanou populaci vývojové pokročilých erytroidních a myeloidních progenitorových buněk s významně změněným imunofenotypem. Od obdobných buněk přítomných v normální kostní dřeni se lišily sníženou expresí receptoru c-Kit pro cytokin stem cell factor (SCF), expresí Sca-1 antigenu na buňkách, které současně exprimovaly znak CD71 (transferinový receptor 1), zvýšenou expresí CD16/32 antigenu a změněnou expresí znaku CD41. Tyto fenotypové změny nebyly způsobeny stimulací erytropoetinem v důsledku anémie. Přestože buňky měly sníženou expresi c-Kit receptoru, byla jejich stimulace SCF nezbytná pro jejich početní expanzi.

Krvetvorné kmenové buňky definované schopností obnovit poškozenou krvetvorbu hostitele byly v intenzivně regenerující krvetvorbě redukovány na 1 – 2 % svého normálního počtu.

Regenerace krvetvorby se významně lišila od expandující krvetvorby ve fetálních játrech.

Regenerace krvetvorby vycházející z malého počtu z transplantovaných buněk, které nebyly vystaveny ionizujícímu záření, vykazovala shodné znaky s regenerací vycházející z endogenních buněk, které zůstaly v tkáni po působení ionizujícího záření.

Výzkum přináší nový pohled na regeneraci poškozené tkáně především tím, že odhaluje klíčovou úlohu vývojově pokročilých buněk progenitorových, které nahrazují funkci buněk kmenových a

multipotentních progenitorů. Poprvé je také upozorněno na podobnost regenerace krvetvorné tkáně se specifickou fází embryonální krvetvorby, která předchází vzniku krvetvorných kmenových buněk.

## 12. Key words

hematopoiesis, regeneration, transplantation, stem cells, progenitor cells, ionizing radiation

## 13. Klíčová slova

krvetvorba, regenerace, transplantace, kmenová buňka, progenitorová buňka, ionizující záření

# 14. References


Adolfsson, J., Borge, O. J., Bryder, D., Theilgaard-Mönch, K., Åstrand-Grundström, I., Sitnicka, E., et al. (2001). Upregulation of Flt3 expression within the bone marrow Lin-Sca1+c-kit+ stem cell compartment is accompanied by loss of self-renewal capacity. *Immunity* 15, 659–669. doi:10.1016/S1074-7613(01)00220-5.

Akashi, K., Traver, D., Miyamoto, T., and Weissman, I. L. (2000). A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. *Nature* 404, 193–7. doi:10.1038/35004599.

Akinduro, O., Weber, T. S., Ang, H., Haltalli, M. L. R., Ruivo, N., Duarte, D., et al. (2018). Proliferation dynamics of acute myeloid leukaemia and haematopoietic progenitors competing for bone marrow space. *Nat. Commun.* 9, 519. doi:10.1038/s41467-017-02376-5.

Baldridge, M. T., King, K. Y., Boles, N. C., Weksberg, D. C., and Goodell, M. A. (2010). Quiescent haematopoietic stem cells are activated by IFN-γ in response to chronic infection. *Nature* 465, 793–797. doi:10.1038/nature09135.

Balounová, J., Šplíchalová, I., Dobešová, M., Kolář, M., Fišer, K., Procházka, J., et al. (2019). Toll-like receptor 2 expression on c-kit+ cells tracks the emergence of embryonic definitive hematopoietic progenitors. *Nat. Commun.* 10. doi:10.1038/s41467-019-13150-0.

Batsivari, A., Rybtsov, S., Souilhol, C., Binagui-Casas, A., Hills, D., Zhao, S., et al. (2017). Understanding Hematopoietic Stem Cell Development through Functional Correlation of Their Proliferative Status with the Intra-aortic Cluster Architecture. *Stem cell reports* 8, 1549–1562. doi:10.1016/j.stemcr.2017.04.003.

Bowie, M. B., Kent, D. G., Copley, M. R., and Eaves, C. J. (2007). Steel factor responsiveness regulates the high self-renewal phenotype of fetal hematopoietic stem cells. *Blood* 109, 5043–8. doi:10.1182/blood-2006-08-037770.

Bowie, M. B., Mcknight, K. D., Kent, D. G., Mccaffrey, L., Hoodless, P. A., and Eaves, C. J. (2006). Hematopoietic stem cells proliferate until after birth and show a reversible phase-specific engraftment defect. 116. doi:10.1172/JCI28310.2808.

Boyer, S. W., Rajendiran, S., Beaudin, A. E., Smith-Berdan, S., Muthuswamy, P. K., Perez-Cunningham, J., et al. (2019). Clonal and Quantitative In Vivo Assessment of Hematopoietic Stem Cell Differentiation Reveals Strong Erythroid Potential of Multipotent Cells. *Stem Cell Reports* 12, 801–815. doi:10.1016/j.stemcr.2019.02.007.

Brecher, G., Bookstein, N., Redfearn, W., Necas, E., Pallavicini, M. G., and Cronkite, E. P. (1993). Self-renewal of the long-term repopulating stem cell. *Proc. Natl. Acad. Sci. U. S. A.* 90, 6028–31. doi:10.1073/pnas.90.13.6028.

Buza-Vidas, N., Woll, P., Hultquist, A., Duarte, S., Lutteropp, M., Bouriez-Jones, T., et al. (2011). FLT3 expression initiates in fully multipotent mouse hematopoietic progenitor cells. *Blood* 118, 1544–1548. doi:10.1182/blood-2010-10-316232.

Carrelha, J., Meng, Y., Kettyle, L. M., Luis, T. C., Norfo, R., Alcolea, V., et al. (2018). Hierarchically related lineage-restricted fates of multipotent haematopoietic stem cells. *Nature* 554, 106–111.

doi:10.1038/nature25455.

Cetkovský, P., Mayer, J., Starý, J., Hričinová, M., Adam, Z., Bartlová, R., et al. (2016). *Transplantace kostní dřeně a periferních hematopoetických buněk*. Galén.

Chen, C.-L., Faltusova, K., Molik, M., Savvulidi, F., Chang, K.-T., and Necas, E. (2016). Low c-Kit Expression Level Induced by Stem Cell Factor Does Not Compromise Transplantation of Hematopoietic Stem Cells. *Biol. Blood Marrow Transplant.* 22, 1167–1172. doi:10.1016/j.bbmt.2016.03.017.

Chen, M. J., Li, Y., De Obaldia, M. E., Yang, Q., Yzaguirre, A. D., Yamada-Inagawa, T., et al. (2011). Erythroid/myeloid progenitors and hematopoietic stem cells originate from distinct populations of endothelial cells. doi:10.1016/j.stem.2011.10.003.

Cheng, H., Zheng, Z., and Cheng, T. (2020). New paradigms on hematopoietic stem cell differentiation. *Protein Cell* 11, 34–44. doi:10.1007/s13238-019-0633-0.

Christensen, J. L., Wright, D. E., Wagers, A. J., and Weissman, I. L. (2004). Circulation and Chemotaxis of Fetal Hematopoietic Stem Cells. *PLoS Biol.* 2, e75. doi:10.1371/journal.pbio.0020075.

Copley, M. R., Babovic, S., Benz, C., Knapp, D. J. H. F., Beer, P. A., Kent, D. G., et al. (2013). The Lin28b–let-7–Hmga2 axis determines the higher self-renewal potential of fetal haematopoietic stem cells. *Nat. Cell Biol.* 15, 916–925. doi:10.1038/ncb2783.

de Bruijn, M. F. T. ., Ma, X., Robin, C., Ottersbach, K., Sanchez, M.-J., and Dzierzak, E. (2002). Hematopoietic Stem Cells Localize to the Endothelial Cell Layer in the Midgestation Mouse Aorta. *Immunity* 16, 673–683. doi:10.1016/S1074-7613(02)00313-8.

Doi, H., Inaba, M., Yamamoto, Y., Taketani, S., Mori, S.-I., Sugihara, A., et al. (1997). Pluripotent hemopoietic stem cells are c-kit. *Proc. Natl. Acad. Sci. U. S. A.* 94, 2513. doi:10.1073/PNAS.94.6.2513.

Down, J. D., Boudewijn, A., van Os, R., Thames, H. D., and Ploemacher, R. E. (1995). Variations in radiation sensitivity and repair among different hematopoietic stem cell subsets following fractionated irradiation. *Blood* 86, 122–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7795217 [Accessed July 23, 2019].

Dykstra, B., Kent, D., Bowie, M., McCaffrey, L., Hamilton, M., Lyons, K., et al. (2007). Long-Term Propagation of Distinct Hematopoietic Differentiation Programs In Vivo. *Cell Stem Cell* 1, 218–229. doi:10.1016/j.stem.2007.05.015.

Dzierzak, E., and Bigas, A. (2018). Blood Development: Hematopoietic Stem Cell Dependence and Independence. *Cell Stem Cell* 22, 639–651. doi:10.1016/j.stem.2018.04.015.

Faltusová, K., Chen, C.-L., Heizer, T., Báječný, M., Szikszai, K., Páral, P., et al. (2020). Altered erythro-myeloid progenitor cells are highly expanded in intensively regenerating hematopoiesis. *Front. Cell Dev. Biol.* 8, 98. doi:10.3389/FCELL.2020.00098.

FORD, C. E., HAMERTON, J. L., BARNES, D. W., and LOUTIT, J. F. (1956). Cytological identification of radiation-chimaeras. *Nature* 177, 452–4. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13309336 [Accessed October 17, 2017].

Forgacova, K., Savvulidi, F., Sefc, L., Linhartova, J., and Necas, E. (2013). All hematopoietic stem cells engraft in submyeloablatively irradiated mice. *Biol. Blood Marrow Transplant.* 19, 713–9. doi:10.1016/j.bbmt.2013.02.012.

Frame, J. M., McGrath, K. E., and Palis, J. (2013). Erythro-myeloid progenitors: "Definitive" hematopoiesis in the conceptus prior to the emergence of hematopoietic stem cells. *Blood Cells, Mol. Dis.* 51, 220–225. doi:10.1016/j.bcmd.2013.09.006.

Franziska Paul, A., Arkin, ara, Giladi, A., Torben Porse, B., Tanay, A., and Amit, I. (2015). Transcriptional Heterogeneity and Lineage Commitment in Myeloid Progenitors. *Cell* 163, 1663–1677. doi:10.1016/j.cell.2015.11.013.

Fraser, S. T., Isern, J., and Baron, M. H. (2007). Maturation and enucleation of primitive erythroblasts during mouse embryogenesis is accompanied by changes in cell-surface antigen expression. doi:10.1182/blood-2006-03-006569.

Gazit, R., Garrison, B. S., Rao, T. N., Shay, T., Costello, J., Ericson, J., et al. (2013). Transcriptome Analysis Identifies Regulators of Hematopoietic Stem and Progenitor Cells. *Stem Cell Reports* 1, 266–280. doi:10.1016/j.stemcr.2013.07.004.

Giladi, A., Paul, F., Herzog, Y., Lubling, Y., Weiner, A., Yofe, I., et al. (2018). Single-cell characterization of haematopoietic progenitors and their trajectories in homeostasis and perturbed haematopoiesis. *Nat. Cell Biol.* 20, 836–846. doi:10.1038/s41556-018-0121-4.

Grinenko, T., Arndt, K., Portz, M., Mende, N., Günther, M., Cosgun, K. N., et al. (2014). Clonal expansion capacity defines two consecutive developmental stages of long-term hematopoietic stem cells. *J. Exp. Med.* 211, 209–15. doi:10.1084/jem.20131115.

Guo, G., Luc, S., Marco, E., Lin, T.-W., Peng, C., Kerenyi, M. A., et al. (2013). Mapping Cellular Hierarchy by Single-Cell Analysis of the Cell Surface Repertoire. *Cell Stem Cell* 13, 492–505. doi:10.1016/j.stem.2013.07.017.

Harrison, D. E., and Astle, C. M. (1982). Loss of stem cell repopulating ability upon transplantation. Effects of donor age, cell number, and transplantation procedure. *J. Exp. Med.* 156, 1767–79. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2186863&tool=pmcentrez&rendertype=abstract [Accessed February 3, 2016].

Harrison, D. E., Zhong, R. K., Jordan, C. T., Lemischka, I. R., and Astle, C. M. (1997). Relative to adult marrow, fetal liver repopulates nearly five times more effectively long-term than short-term. *Exp. Hematol.* 25, 293–297. Available at: https://pubmed.ncbi.nlm.nih.gov/9131003/ [Accessed September 2, 2020].

Huber, T. L., Kouskoff, V., Joerg Fehling, H., Palis, J., and Keller, G. (2004). Haemangioblast commitment is initiated in the primitive streak of the mouse embryo. *Nature* 432, 625–630. doi:10.1038/nature03122.

Ikuta, K., and Weissman, I. L. (1992). Evidence that hematopoietic stem cells express mouse c-kit but do not depend on steel factor for their generation. *Proc. Natl. Acad. Sci. U. S. A.* 89, 1502–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1371359 [Accessed June 21, 2017].

Kiel, M. J., Yilmaz, O. H., Iwashita, T., Yilmaz, O. H., Terhorst, C., and Morrison, S. J. (2005). SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* 121, 1109–21. doi:10.1016/j.cell.2005.05.026.

Kingsley, P. D., Malik, J., Fantauzzo, K. A., and Palis, J. (2004). Yolk sac-derived primitive erythroblasts enucleate during mammalian embryogenesis. doi:10.1182/blood-2003.

Klimmeck, D., Cabezas-Wallscheid, N., Reyes, A., von Paleske, L., Renders, S., Hansson, J., et al. (2014). Transcriptome-wide Profiling and Posttranscriptional Analysis of Hematopoietic Stem/Progenitor Cell Differentiation toward Myeloid Commitment. *Stem Cell Reports* 3, 858–875. doi:10.1016/j.stemcr.2014.08.012.

Kondo, M., Weissman, I. L., and Akashi, K. (1997). Identification of Clonogenic Common Lymphoid Progenitors in Mouse Bone Marrow. *Cell* 91, 661–672.

Kumaravelu, P., Hook, L., Morrison, A. M., Ure, J., Zhao, S., Zuyev, S., et al. (2002). Quantitative developmental anatomy of definitive haematopoietic stem cells/long-term repopulating units (HSC/RUs): role of the aorta-gonad-mesonephros (AGM) region and the yolk sac in colonisation of the mouse embryonic liver. *Development* 129, 4891–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12397098 [Accessed May 23, 2019].

Lennartsson, J., and Rönnstrand, L. (2012). Stem cell factor receptor/c-Kit: from basic science to clinical implications. *Physiol. Rev.* 92, 1619–49. doi:10.1152/physrev.00046.2011.

Lian, Z., Feng, B., Sugiura, K., Inaba, M., Yu, C., Jin, T., et al. (1999). c‐kit $^{\<low}$ Pluripotent Hemopoietic Stem Cells Form CFU‐S on Day 16. *Stem Cells* 17, 39–44. doi:10.1002/stem.170039.

Limanni, A., Baker, W. H., Chang, C. M., Seemann, R., Williams, D. E., and Patchen, M. L. c-kit Ligand Gene Expression in Normal and Sublethally Irradiated Mice.

Loeffler, D., Wehling, A., Schneiter, F., Zhang, Y., Müller-Bötticher, N., Hoppe, P. S., et al. (2019). Asymmetric lysosome inheritance predicts activation of haematopoietic stem cells. *Nature* 573, 426–429. doi:10.1038/s41586-019-1531-6.

McCarthy, K. F. (1997). Population size and radiosensitivity of murine hematopoietic endogenous long-term repopulating cells. *Blood* 89, 834–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9028314 [Accessed July 13, 2016].

MCCULLOCH, E. A., and TILL, J. E. (1960). The radiation sensitivity of normal mouse bone marrow cells, determined by quantitative marrow transplantation into irradiated mice. *Radiat. Res.* 13, 115–25. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13858509 [Accessed July 23, 2019].

McGrath, K. E., Frame, J. M., Fegan, K. H., Bowen, J. R., Conway, S. J., Catherman, S. C., et al. (2015). Distinct Sources of Hematopoietic Progenitors Emerge before HSCs and Provide Functional Blood Cells in the Mammalian Embryo. *Cell Rep.* 11, 1892–1904. doi:10.1016/j.celrep.2015.05.036.

McRae, H. M., Voss, A. K., and Thomas, T. (2019). Are transplantable stem cells required for adult hematopoiesis? *Exp. Hematol.* 75, 1–10. doi:10.1016/j.exphem.2019.05.007.

Medvinsky, A., and Dzierzak, E. (1996). Definitive hematopoiesis is autonomously initiated by the AGM region. *Cell* 86, 897–906. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8808625 [Accessed June 11, 2019].

Meijne, E. I., van der Winden-van Groenewegen, R. J., Ploemacher, R. E., Vos, O., David, J. A., and Huiskamp, R. (1991). The effects of x-irradiation on hematopoietic stem cell compartments in the mouse. *Exp. Hematol.* 19, 617–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1893947 [Accessed July 23, 2019].

Miiller, A. M., Medvinsky, A., Strouboulıqt, J., Grosveld, F., and Dzierzakt, E. (1994). Development of Hematopoietic Stem Cell Activity in the Mouse Embryo. Available at: https://pdf.sciencedirectassets.com/272197/1-s2.0-S1074761300X00945/1-s2.0-1074761394900817/main.pdf?x-amz-security-token=AgoJb3JpZ2luX2VjELT%2F%2F%2F%2F%2F%2F%2F%2F%2F%2FwEaCXVzLWVhc3QtMSJIMEYCIQDIZ8PLkdTo2VNbSCJO1KQmzGQJ%2FdJu1xZiftrF0ilXJQIhAJeiGPPzpDn [Accessed May 23, 2019].

Moignard, V., Woodhouse, S., Fisher, J., and Göttgens, B. (2013). Transcriptional hierarchies regulating early blood cell development. *Blood Cells, Mol. Dis.* 51, 239–247. doi:10.1016/j.bcmd.2013.07.007.

Monette, F. C., and Holden, S. A. (1982). Hemin enhances the in vitro growth of primitive erythroid progenitor cells. *Blood* 60, 527–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7093531 [Accessed February 13, 2020].

Morita, Y., Ema, H., and Nakauchi, H. (2010). Heterogeneity and hierarchy within the most primitive hematopoietic stem cell compartment. *J. Exp. Med.* 207, 1173–1182. doi:10.1084/jem.20091318.

Morrison, S. J., and Scadden, D. T. (2014). The bone marrow niche for haematopoietic stem cells. *Nature* 505, 327–334. doi:10.1038/nature12984.

Müller, A. M., Medvinsky, A., Strouboulis, J., Grosveld, F., and Dzierzak, E. (1994). Development of hematopoietic stem cell activity in the mouse embryo. *Immunity* 1, 291–301. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7889417 [Accessed June 11, 2019].

Na Nakorn, T., Traver, D., Weissman, I. L., and Akashi, K. (2002). Myeloerythroid-restricted progenitors are sufficient to confer radioprotection and provide the majority of day 8 CFU-S. *J. Clin. Invest.* 109, 1579–85. doi:10.1172/JCI15272.

Nečas, E., and Znojil, V. (1989). A comparison of stem cell assays using early or late spleen colonies. *Cell Prolif.* 22, 111–121. doi:10.1111/j.1365-2184.1989.tb00204.x.

Nestorowa, S., Hamey, F. K., Pijuan Sala, B., Diamanti, E., Shepherd, M., Laurenti, E., et al. (2016). A single-cell resolution map of mouse hematopoietic stem and progenitor cell differentiation. *Blood* 128, e20–e31. doi:10.1182/blood-2016-05-716480.

North, T. E., de Bruijn, M. F. T. ., Stacy, T., Talebian, L., Lind, E., Robin, C., et al. (2002). Runx1 Expression Marks Long-Term Repopulating Hematopoietic Stem Cells in the Midgestation Mouse Embryo. *Immunity* 16, 661–672. doi:10.1016/S1074-7613(02)00296-0.

North, T., Gu, T. L., Stacy, T., Wang, Q., Howard, L., Binder, M., et al. (1999). Cbfa2 is required for the formation of intra-aortic hematopoietic clusters. *Development* 126, 2563–75. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10226014 [Accessed July 17, 2019].

Oatley, M., Bölükbası, Ö. V., Svensson, V., Shvartsman, M., Ganter, K., Zirngibl, K., et al. (2020). Single-cell transcriptomics identifies CD44 as a marker and regulator of endothelial to haematopoietic transition. *Nat. Commun.* 11. doi:10.1038/s41467-019-14171-5.

Ogawa, M. (1991). Expression and function of c-kit in hemopoietic progenitor cells. *J. Exp. Med.* 174, 63–71. doi:10.1084/jem.174.1.63.

Ogawa, M., Matsuzaki, Y., Nishikawa, S., Hayashi, S., Kunisada, T., Sudo, T., et al. (1991). Expression and function of c-kit in hemopoietic progenitor cells. *J. Exp. Med.* 174, 63–71. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2118893&tool=pmcentrez&rendertype=abstract [Accessed April 24, 2015].

Oguro, H., Ding, L., and Morrison, S. J. (2013). SLAM family markers resolve functionally distinct subpopulations of hematopoietic stem cells and multipotent progenitors. *Cell Stem Cell* 13, 102–16. doi:10.1016/j.stem.2013.05.014.

Okada, S., Nakauchi, H., Nagayoshi, K., Nishikawa, S., Miura, Y., and Suda, T. (1992). In vivo and in vitro stem cell function of c-kit- and Sca-1-positive murine hematopoietic cells. *Blood* 80, 3044–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1281687 [Accessed April 24, 2015].

Okada, S., Nakauchi, H., Nagayoshi, K., Nishikawa, S., Nishikawa, S., Miura, Y., et al. (1991). Enrichment and characterization of murine hematopoietic stem cells that express c-kit molecule. *Blood* 78, 1706–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1717068 [Accessed December 12, 2016].

Osawa, M., Nakamura, K., Nishi, N., Takahasi, N., Tokuomoto, Y., Inoue, H., et al. (1996). In vivo self-renewal of c-Kit+ Sca-1+ Lin(low/-) hemopoietic stem cells. *J. Immunol.* 156, 3207–3214. Available at: https://www.jimmunol.org/content/156/9/3207.long [Accessed September 6, 2019].

Ottersbach, K. (2019). Endothelial-to-haematopoietic transition: An update on the process of making blood. *Biochem. Soc. Trans.* 47, 591–601. doi:10.1042/BST20180320.

Palis, J. (2014). Primitive and definitive erythropoiesis in mammals. *Front. Physiol.* 5, 3. doi:10.3389/fphys.2014.00003.

Palis, J. (2016). Hematopoietic stem cell-independent hematopoiesis: emergence of erythroid, megakaryocyte, and myeloid potential in the mammalian embryo. *FEBS Lett.* 590, 3965–3974. doi:10.1002/1873-3468.12459.

Palis, J., Robertson, S., Kennedy, M., Wall, C., and Keller, G. (1999). Development of erythroid and myeloid progenitors in the yolk sac and embryo proper of the mouse. *Development* 126, 5073–84. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10529424 [Accessed May 23, 2019].

Páral, P., Faltusová, K., Molík, M., Renešová, N., Šefc, L., and Nečas, E. (2018). Cell cycle and differentiation of Sca-1$^{+}$ and Sca-1$^{-}$ hematopoietic stem and progenitor cells. *Cell Cycle* 17. doi:10.1080/15384101.2018.1502573.

Pawliuk, R., Eaves, C., and Humphries, R. K. (1996). Evidence of both ontogeny and transplant dose-regulated expansion of hematopoietic stem cells in vivo. *Blood* 88, 2852–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8874181 [Accessed May 3, 2019].

Peslak, S. a, Wenger, J., Bemis, J. C., Kingsley, P. D., Koniski, A. D., McGrath, K. E., et al. (2012). EPO-mediated expansion of late-stage erythroid progenitors in the bone marrow initiates recovery from sublethal radiation stress. *Blood* 120, 2501–11. doi:10.1182/blood-2011-11-394304.

Phillips, D., Charo, I., Parise, L., and Fitzgerald, L. (1988). The platelet membrane glycoprotein IIb-IIIa complex. *Blood* 71. Available at: http://www.bloodjournal.org/content/71/4/831?ijkey=d86254d4ba75d2278bbcc0bbcc978080af1344cb&keytype2=tf_ipsecsha [Accessed June 3, 2019].

Pietras, E. M., Reynaud, D., Kang, Y.-A., Carlin, D., Calero-Nieto, F. J., Leavitt, A. D., et al. (2015). Functionally Distinct Subsets of Lineage-Biased Multipotent Progenitors Control Blood Production in Normal and Regenerative Conditions. *Cell Stem Cell* 17, 35–46. doi:10.1016/j.stem.2015.05.003.

Ploemacher, R. E., van Os, R., van Beurden, C. A., and Down, J. D. (1992). Murine haemopoietic stem cells with long-term engraftment and marrow repopulating ability are more resistant to gamma-radiation than are spleen colony forming cells. *Int. J. Radiat. Biol.* 61, 489–99. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1349331 [Accessed July 13, 2016].

Pronk, C. J. H., Rossi, D. J., Månsson, R., Attema, J. L., Norddahl, G. L., Chan, C. K. F., et al. (2007). Elucidation of the phenotypic, functional, and molecular topography of a myeloerythroid progenitor cell hierarchy. *Cell Stem Cell* 1, 428–42. doi:10.1016/j.stem.2007.07.005.

Randall, T. D., and Weissman, I. L. (1998). Characterization of a population of cells in the bone marrow that phenotypically mimics hematopoietic stem cells: resting stem cells or mystery population? *Stem Cells* 16, 38–48. doi:10.1002/stem.160038.

Rebel, V. I., Miller, C. L., Eaves, C. J., and Lansdorp, P. M. (1996). The repopulation potential of fetal liver hematopoietic stem cells in mice exceeds that of their liver adult bone marrow counterparts. *Blood* 87, 3500–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8605370 [Accessed May 3, 2019].

Rybtsov, S., Batsivari, A., Bilotkach, K., Paruzina, D., Senserrich, J., Nerushev, O., et al. (2014). Tracing the Origin of the HSC Hierarchy Reveals an SCF-Dependent, IL-3-Independent CD43− Embryonic Precursor. *Stem Cell Reports* 3, 489–501. doi:10.1016/J.STEMCR.2014.07.009.

Rybtsov, S., Ivanovs, A., Zhao, S., and Medvinsky, A. (2016). Concealed expansion of immature precursors underpins acute burst of adult HSC activity in foetal liver. *Development* 143, 1284–9. doi:10.1242/dev.131193.

Rybtsov, S., Sobiesiak, M., Taoudi, S., Souilhol, C., Senserrich, J., Liakhovitskaia, A., et al. (2011). Hierarchical organization and early hematopoietic specification of the developing HSC lineage in the AGM region. *J. Exp. Med.* 208, 1305–15. doi:10.1084/jem.20102419.

Sanjuan-Pla, A., Macaulay, I. C., Jensen, C. T., Woll, P. S., Luis, T. C., Mead, A., et al. (2013). Platelet-biased stem cells reside at the apex of the haematopoietic stem-cell hierarchy. *Nature* 502.

doi:10.1038/nature12495.

Santoro, A., Vlachou, T., Carminati, M., Pelicci, P. G., and Mapelli, M. (2016). Molecular mechanisms of asymmetric divisions in mammary stem cells. *EMBO Rep.* 17, 1700–1720. doi:10.15252/embr.201643021.

Sawai, C. M., Babovic, S., Upadhaya, S., Knapp, D. J. H. F., Lavin, Y., Lau, C. M., et al. (2016). Hematopoietic Stem Cells Are the Major Source of Multilineage Hematopoiesis in Adult Animals. *Immunity* 45, 597–609. doi:10.1016/j.immuni.2016.08.007.

Schaefer, B. C., Schaefer, M. L., Kappler, J. W., Marrack, P., and Kedl, R. M. (2001). Observation of antigen-dependent CD8+ T-cell/ dendritic cell interactions in vivo. *Cell. Immunol.* 214, 110–22. doi:10.1006/cimm.2001.1895.

Simonnet, A. J., Nehmé, J., Vaigot, P., Barroca, V., Leboulch, P., and Tronik-Le Roux, D. (2009). Phenotypic and functional changes induced in hematopoietic stem/progenitor cells after gamma-ray radiation exposure. *Stem Cells* 27, 1400–9. doi:10.1002/stem.66.

Singh, R. P., Grinenko, T., Ramasz, B., Franke, K., Lesche, M., Dahl, A., et al. (2018). Hematopoietic Stem Cells but Not Multipotent Progenitors Drive Erythropoiesis during Chronic Erythroid Stress in EPO Transgenic Mice. *Stem cell reports* 10, 1908–1919. doi:10.1016/j.stemcr.2018.04.012.

Soares-da-Silva, F., Peixoto, M., Cumano, A., and Pinto-do-Ó, P. (2020). Crosstalk Between the Hepatic and Hematopoietic Systems During Embryonic Development. *Front. Cell Dev. Biol.* 8, 612. doi:10.3389/fcell.2020.00612.

Sugimoto, K., Adachi, Y., Moriyama, K., Qiong, W., Nakayama, A., Hosono, M., et al. (2001). Induction of the expression of SCF in mouse by lethal irradiation. *Growth Factors* 19, 219–231. doi:10.3109/08977190109001088.

Sun, J., Ramos, A., Chapman, B., Johnnidis, J. B., Le, L., Ho, Y.-J., et al. (2014). Clonal dynamics of native haematopoiesis. *Nature* 514, 322–7. doi:10.1038/nature13824.

Taoudi, S., Morrison, A. M., Inoue, H., Gribi, R., Ure, J., and Medvinsky, A. (2005). Progressive divergence of definitive haematopoietic stem cells from the endothelial compartment does not depend on contact with the foetal liver. *Development* 132, 4179–91. doi:10.1242/dev.01974.

Thorén, L. a, Liuba, K., Bryder, D., Nygren, J. M., Jensen, C. T., Qian, H., et al. (2008). Kit regulates maintenance of quiescent hematopoietic stem cells. *J. Immunol.* 180, 2045–53. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18250409.

Trumpp, A., Essers, M., and Wilson, A. (2010). Awakening dormant haematopoietic stem cells. *Nat. Rev. Immunol.* 10, 201–209. doi:10.1038/nri2726.

Tusi, B. K., Wolock, S. L., Weinreb, C., Hwang, Y., Hidalgo, D., Zilionis, R., et al. (2018). Population snapshots predict early haematopoietic and erythroid hierarchies. *Nature* 555, 54–60. doi:10.1038/nature25741.

Vagima, Y., Lapid, K., Kollet, O., Goichberg, P., Alon, R., and Lapidot, T. (2011). Pathways implicated in stem cell migration: the SDF-1/CXCR4 axis. *Methods Mol. Biol.* 750, 277–89. doi:10.1007/978-1-61779-145-1_19.

Vobořil, M., Brabec, T., Dobeš, J., Šplíchalová, I., Březina, J., Čepková, A., et al. (2020). Toll-like receptor signaling in thymic epithelium controls monocyte-derived dendritic cell recruitment and Treg generation. *Nat. Commun.* 11. doi:10.1038/s41467-020-16081-3.

Wagemaker, G. (1995). Chapter 6: Cell and cell system responses: Heterogeneity of radiation sensitivity of hemopoietic stem cell subsets. *Stem Cells* 13, 257–260. doi:10.1002/stem.5530130731.

Weissman, I. L. (2000). Stem Cells: Units of Development, Review Units of Regeneration, and Units in Evolution. *Cell* 100, 157–168. Available at: http://www.cell.com/cell/pdf/S0092-8674(00)81692-X.pdf [Accessed April 13, 2018].

Wilson, A., Laurenti, E., Oser, G., van der Wath, R. C., Blanco-Bose, W., Jaworski, M., et al. (2008). Hematopoietic Stem Cells Reversibly Switch from Dormancy to Self-Renewal during Homeostasis and Repair. *Cell* 135, 1118–1129. doi:10.1016/j.cell.2008.10.048.

Wilson, A., Oser, G. M., Jaworski, M., Blanco-Bose, W. E., Laurenti, E., Adolphe, C., et al. (2007). Dormant and self-renewing hematopoietic stem cells and their niches. in *Annals of the New York Academy of Sciences* (Blackwell Publishing Inc), 64–75. doi:10.1196/annals.1392.021.

Yamamoto, R., Morita, Y., Ooehara, J., Hamanaka, S., Onodera, M., Rudolph, K. L., et al. (2013). Clonal analysis unveils self-renewing lineage-restricted progenitors generated directly from hematopoietic stem cells. *Cell* 154, 1112–1126. doi:10.1016/j.cell.2013.08.007.

Yamamoto, R., Wilkinson, A. C., Ooehara, J., Lan, X., Lai, C. Y., Nakauchi, Y., et al. (2018). Large-Scale Clonal Analysis Resolves Aging of the Mouse Hematopoietic Stem Cell Compartment. *Cell Stem Cell* 22, 600-607.e4. doi:10.1016/j.stem.2018.03.013.

Yang, G., Hisha, H., Cui, Y., Fan, T., Jin, T., Li, Q., et al. (2002). A New Assay Method for Late CFU-S Formation and Long-Term Reconstituting Activity Using a Small Number of Pluripotent Hemopoietic Stem Cells. *Stem Cells* 20, 241–248. doi:10.1634/stemcells.20-3-241.

Yang, L., Bryder, D., Adolfsson, J., Nygren, J., Månsson, R., Sigvardsson, M., et al. (2005). Identification of Lin-Sca1+kit+CD34 +Flt3- short-term hematopoietic stem cells capable of rapidly reconstituting and rescuing myeloablated transplant recipients. *Blood* 105, 2717–2723. doi:10.1182/blood-2004-06-2159.

# 15. List of publications

**Publications with a direct relation to the thesis:**

**Faltusová, K.**, Chen, C.L., Heizer, T., Báječný, M., Szikszai, K., Páral,P., Savvulidi, F., Renešová,N., and Necas, E. (2020). Altered Erythro-Myeloid Progenitor Cells Are Highly Expanded in Intensively Regenerating Hematopoiesis. *Frontiers in Cell and Developmental Biology* 8:98. doi: 10.3389/fcell.2020.00098. IF=5.186 (2019).

Nečas, E. and **K. Faltusová**. (2020). Regenerující krvetvorba se podobá embryonální krvetvorbě nezávislé na kmenových buňkách. *Transfuze a Hematologie Dnes* 26(3):157–66.

Nečas, E., and **K. Faltusová**. (2019). Současný pohled na krvetvornou tkáň. *Československá fyziologie* 68(2):57–67.

Chen, C. L., **K. Faltusova**, M. Molik, F. Savvulidi, K. T. Chang, and E. Necas. (2016). Low C-Kit Expression Level Induced by Stem Cell Factor Does Not Compromise Transplantation of Hematopoietic Stem Cells. *Biology of Blood and Marrow Transplantation* 22(7):1167-1172. doi: 10.1016/j.bbmt.2016.03.017. IF=3.980 (2015)

**Other publications related to the research field:**

**Faltusová, K.**, Báječný, M., Heizer, T., Páral, P., and Nečas, E. (2020). T-lymphopoiesis is Severely Compromised in Ubiquitin-Green Fluorescent Protein Transgenic Mice. *Folia Biol. (Praha)* 66:47–59. IF=0.691 (2019)

Páral, P., **Faltusová, K.**, Molík, M., Renešová, N., Šefc, L., and Nečas, E. (2018). Cell cycle and differentiation of Sca-1 $^{+}$ and Sca-1 $^{-}$ hematopoietic stem and progenitor cells. *Cell Cycle* 17:1979–1991. doi:10.1080/15384101.2018.1502573. IF=3.304 (2017)

**Faltusová, K.**, Szikszai, K., Molík, M., Linhartová, J., Páral, P., Šefc, L., et al. (2018). Stem Cell Defect in Ubiquitin-Green Fluorescent Protein Mice Facilitates Engraftment of Lymphoid-Primed Hematopoietic Stem Cells. *Stem Cells*. doi:10.1002/stem.2828.

**Faltusová, K**., Szikszai, K., Molík, M., Linhartová, J., Páral, P., Šefc, L., et al. (2018). Stem Cell Defect in Ubiquitin-Green Fluorescent Protein Mice Facilitates Engraftment of Lymphoid-Primed Hematopoietic Stem Cells. *Stem Cells* 36(8):1237-1248. doi:10.1002/stem.2828. IF=5.587 (2017)

Michalova, J., Savvulidi, F., Sefc, L., **Faltusova, K.**, Forgacova, K., and Necas, E. (2011). Hematopoietic stem cells survive circulation arrest and reconstitute hematopoiesis in myeloablated mice. *Biol. Blood Marrow Transplant.* 17:1273–81. doi:10.1016/j.bbmt.2011.07.007. IF=3.762 (2011)

# 16. Appendices

Apendix 1

Faltusová, K., Chen, C.L., Heizer, T., Báječný, M., Szikszai, K., Páral,P., Savvulidi, F., Renešová,N., and Necas, E. (2020). "Altered Erythro-Myeloid Progenitor Cells Are Highly Expanded in Intensively Regenerating Hematopoiesis." *Frontiers in Cell and Developmental Biology* 8:98.

Apendix 2

Nečas, E. and K. Faltusová. (2020). "Regenerující krvetvorba se podobá embryonální krvetvorbě nezávislé na kmenových buňkách." *Transfuze a Hematologie Dnes* 26(3):157–66.

Apendix 3

Nečas, E., and K. Faltusová. (2019). "Současný pohled na krvetvornou tkáň." *Československá fyziologie* 68(2):57–67.

Apendix 4

Chen, C. L., K. Faltusova, M. Molik, F. Savvulidi, K. T. Chang, and E. Necas. (2016). "Low C-Kit Expression Level Induced by Stem Cell Factor Does Not Compromise Transplantation of Hematopoietic Stem Cells." *Biology of Blood and Marrow Transplantation* 22(7) :1167-1172.