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Cadaveric bone marrow transplantation: Effects of hypoxia and metabolic starvation on mouse hematopoietic stem cells

Ph.D. Thesis

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Declaration

I hereby declare that I have written this thesis on my own under the supervision of RNDr. Luděk Šefc, CSc. and that I have mentioned all sources used. This work has not been submitted for any other degree or award in any other university or educational establishment.

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Co-author statement

I hereby declare that Jana Michalová has substantially (20-90%) contributed to the publications, which are attached to this thesis. She has carried out the experiments and has significantly participated in their planning, in the interpretation of results and writing of the publications.

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Abstract

Objectives: Hematopoietic stem cell transplantation (HSCT) is a widely used method for treatment of hematological disorders and some other diseases. However, sometimes a suitable donor of hematopoietic stem cells (HSC) is not found for a patient. Because HSC have been described as cells with low proliferative and metabolic activity, their tolerance to the lack of oxygen or metabolic substrates may be assumed. In this study, we explored cadaveric bone marrow as an alternative source of HSC for HSCT, using a mouse experimental model. In addition, the effect of *in vitro* metabolic inhibition and short-term *in vitro* storage (1 - 4 days) on functional properties of mouse HSC was investigated.

Methods: C57Bl/6 mice (wild-type or $p53^{-/-}$) were used in the experiments. To explore cadaveric HSC, bone marrow (BM) was left in intact femurs at 37°C, 20°C and 4°C under the conditions of ischemia. The bone marrow cells were harvested after defined time periods ranging 0 – 48 hours. For metabolic inhibition, the electron transport chain inhibitor potassium cyanide (KCN) and inhibitor of glycolysis 2-deoxy-D-glucose (2-DG) were used *in vitro*. To determine the impact of ischemia, metabolic inhibition, or *in vitro* storage on transplantability of HSC, the competitive repopulation assay using Ly5.1/Ly5.2 congenic model was used. Besides, live/apoptotic/dead cells ratio in BM subpopulations was measured, and frequencies of LSK SLAM ($Lin^{low}Sca\text{-}1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}$) and LSK SP (side population) cells (highly enriched in HSC) were detected by flow cytometry.

Results: Chimerism arisen from the transplanted cadaveric donor bone marrow cells (followed up in recipient's peripheral blood (PB) for 6 months after transplantation) revealed that the long-term repopulating ability of HSC is fully preserved for at least 2 hours, 6 hours and 12 hours of ischemia at 37°C, 20°C and 4°C, respectively. Number of LSK SLAM and LSK SP cells decreased in compliance with the transplantability. Furthermore, the LSK subpopulation (enriched in HSC) contained less apoptotic and dead cells as compared to more differentiated subpopulations of the BM exposed to ischemia at 37°C. Cadaveric $p53^{-/-}$ HSC did not differ from wild-type HSC in their survival or transplantability. The incubations with inhibitors showed the LSK cells as more resistant to KCN in comparison with other populations tested; however the 2-DG inhibition was lethal for all bone marrow cells. Two-day or four-day *in vitro* storage of bone marrow cells at 37°C or 4°C, respectively, did not influence the transplantability of HSC.

Conclusions: Our findings suggest that HSC survive in cadaveric bone marrow for considerable time, without loss of their repopulating ability. They represent the most resistant population of BM to oxygen and metabolic starvation. The HSC survival is significantly extended during *in vitro* storage even without growth factors, thus bone marrow cells should be harvested as soon as possible.

Abstrakt (Czech)

Východisko: Transplantace krvetvorných kmenových buněk slouží k léčbě poruch krvetvorby, autoimunitních chorob i některých nehematologických onemocnění. Ne pro všechny pacienty se však podaří nalézt vhodného dárce krvetvorných kmenových buněk (HSC). Protože HSC vykazují nízkou proliferační a metabolickou aktivitu, lze u nich přepokládat odolnost vůči nedostatku kyslíku a metabolických substrátů, který nastává při ischemii, např. v kadaverózní kostní dřeni. Cílem této práce bylo pomocí experimentálního myšího modelu ověřit možnost odběru HSC z kadaverózní kostní dřeně. Navíc byla testována tolerance HSC k inhibitorům metabolismu a k uchovávání *in vitro.*

Metody: V experimentech byl použit modelový organismus mus musculus C57Bl/6 („wild-type“ a p53 knock-out). Kostní dřeň byla po určitou dobu (0 až 48 hod) ponechána v uzavřených femurech dárců při teplotě 37°C, 20°C nebo 4°C v podmínkách ischemie, nebo byla ihned odebrána a buňky byly vystaveny inhibitoru dýchacího řetězce kyanidu draselnému či inhibitoru glykolýzy 2-deoxy-D-glukose, příp. pouze skladovány *in vitro*. Vliv ischemie, metabolických inhibitorů a skladování *in vitro* na transplantabilitu HSC byl studován na myším kongenním modelu Ly5.1/Ly5.2 metodou kompetitivní repopulace. Poměry živých, apoptotických a mrtvých buněk v subpopulacích kostní dřeně, a zastoupení LSK SLAM ($Lin^{low}Sca\text{-}1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}$) a LSK SP (side population) buněk, reprezentujících HSC, byly měřeny pomocí průtokové cytometrie.

Výsledky: Na základě chimerismu buněk dárcovského původu sledovaného v periferní krvi příjemců po dobu 6 měsíců od transplantace bylo zjištěno, že dlouhodobá repopulační schopnost HSC vystavených ischemii zůstává při 37°C, 20°C resp. 4°C zachována po dobu minimálně 2, 6 resp. 12 hodin. S následným poklesem transplantability klesalo i zastoupení LSK SLAM a LSK SP buněk v kostní dřeni (BM). Ischemie způsobila také zvýšení počtu apoptotických a mrtvých buněk v BM, přičemž v LSK populaci (bohaté na HSC) byl pozorován menší nárůst než v populacích diferencovaných prekurzorů krevních buněk. U HSC odebraných z kadaverózní dřeně $p53^{-/-}$ myší nebyly v porovnání s „wild-type“ HSC pozorovány významné rozdíly v přežívání ani v transplantabilitě. Po inkubacích s inhibitory byla zjištěna vyšší odolnost LSK buněk k inhibici KCN; k inhibici 2-DG byly citlivé všechny buňky kostní dřeně. Dvoudenní skladování buněk při 37°C ani čtyřdenní skladování při 4° C transplantabilitu HSC neovlivnilo.

Závěr: Výsledky naší studie prokázaly, že HSC přežívají v kadaverózní kostní dřeni po významnou dobu, aniž by ztrácely svou repopulační schopnost. Ukázalo se, že představují populaci kostní dřeně nejodolnější k nedostatku kyslíku a metabolických substrátů. Přežívání HSC bylo významně prodlouženo při skladování *in vitro,* proto by buňky měly být vždy odebrány co nejdříve.

Contents

LIST OF ABBREVIATIONS

2-DG	2-deoxy-D-glucose
A	absorbance
ADP	adenosine diphosphate
ANOVA	analysis of variance
APC	allophycocyanin
APC-Cy7	APC cyanine 7 dyes conjugate
APO	apoptotic
ATP	adenosine triphosphate
BM	bone marrow
BSA	bovine serum albumin
CFU-S	colony-forming units - spleen
CTRL	control
CD	cluster of differentiation
c-Kit	CD117, stem cell factor receptor
DMEM	Dulbecco’s modified Eagle's medium
FACS	fluorescence-activated cell sorting
FIH-1	factor inhibiting hypoxia inducible factor 1
FITC	fluorescein isothiocyanate
FBS	fetal bovine serum
FSC	forward-scatter
g	gravitational acceleration
G	Gauge; unit of needle thickness
Gy	Gray; unit of ionizing radiation
glc	D-glucose
HIF-1	hypoxia inducible factor 1
HLA	human leukocyte antigen
HSC	hematopoietic stem cells
HSCT	hematopoietic stem cells transplantation
IMDM	Iscove’s Modified Dulbecco’s Medium
i.v.	intravenously
Lin	lineage

LSK	$Lin^-Sca\text{-}1^+c\text{-}Kit^+$
LSK SLAM	$Lin^-Sca\text{-}1^+c\text{-}Kit^+CD150^+CD48^-$
LSK SP	$Lin^-Sca\text{-}1^+c\text{-}Kit^+$ side population, Hoechst 33342 negative
LTRC	long-term repopulating cells
Ly5.1	CD45.1
Ly5.2	CD45.2
MHC	major histocompatibility complex
mM	$mmol/dm^3$
µM	$\mu mol/dm^3$
P	p-value
PBS	phosphate buffer saline
PBS/BSA	phosphate buffer saline supplemented with 0.5 % (w/v) BSA
PDH	pyruvate dehydrogenase
PE	phycoerythrin
PHD	proline hydroxylase
PDK1	PDH kinase
PI	propidium iodide
pVHL	von Hippel-Lindau protein
ROS	reactive oxygen species
SCF	stem cell factor
SDF-1	stromal cell derived factor 1 (CXCL12)
SEM	standard error of mean
SLAM	signaling lymphocyte activating molecule
SP	side population
SSC	side-scatter
STRC	short-term repopulating cells
λ_{em}	emission wavelength
λ_{ex}	excitation wavelength

1 INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) is a widely used therapeutic method for treatment of hematological disorders, solid tumors or autoimmunity diseases. During the transplantation procedure, hematopoietic stem (and progenitor) cells (HSC) obtained from bone marrow of living donors are transplanted to (sub)myeloablated patients.

For a successful HSCT, a suitable HLA-matched donor must be found [1]. Most often, the donor is a relative of the patient, or a volunteer from bone marrow donor register [2]. Unfortunately, sometimes these options fail and a question arises of whether an additional way of obtaining HSC exists, e.g. by harvesting of bone marrow from cadaveric organ donors. However, an impact of ischemia occurring in the cadaveric tissue must be considered.

Under physiological conditions, the HSC do not proliferate continuously but are in so called “quiescent” state [3]. This means that they are in G0 phase of the cell cycle for most of the time and divide approximately once per a month to maintain production of blood cells. Because of that, primitive HSC are often termed quiescent or dormant cells and their arrest in the G0 phase is called “quiescence”.

The quiescence of HSC is strictly regulated by both intrinsic and extrinsic regulators, mainly produced by stromal cells [4-8]. The stromal cells are non-hemopoietic cells in bone marrow (BM), including osteoblasts, fibroblasts or reticular cells, which form a special microenvironment for HSC, termed “niche”. Thus it can be said that the HSC quiescence is maintained by the niche. Because niches are located in hypoxic regions of BM, the quiescent HSC differ from mature cells in some respects [9, 10]. They cannot use the electron transport chain due to the low oxygen levels; hence they prefer cytoplasmic glycolysis to gain energy in the form of ATP [10]. Moreover, since HSC are slow-cycling cells, they probably do not have as high energy demands as actively proliferating cells.

Based on this knowledge, it is possible to assume that hematopoietic stem cells should be resistant to a metabolic stress as well as to oxygen shortage, which occur in the body after circulatory arrest. Therefore, a research focused on HSC harvested from cadaveric bone marrow remains a challenging task.

1.1 Hematopoietic stem cells

1.1.1 Hematopoiesis

Hematopoiesis is a strictly regulated process of blood cells formation. It occurs in hematopoietic tissues: bone marrow in human and BM and spleen in mice. Cornerstones of hematopoiesis are primitive hematopoietic stem cells, which have the potential to self-renew and to differentiate to any kind of blood cell (multipotency) [11].

The ability of **self-renewal** enables to maintain a pool of HSC in bone marrow and provides the repopulation ability of these cells. The **multilineage differentiation** is necessary for correct functioning of immune system and oxygen transportation. The whole differentiation process is orchestrated by a rank of interleukins and growth factors, as shown in Figure 1.

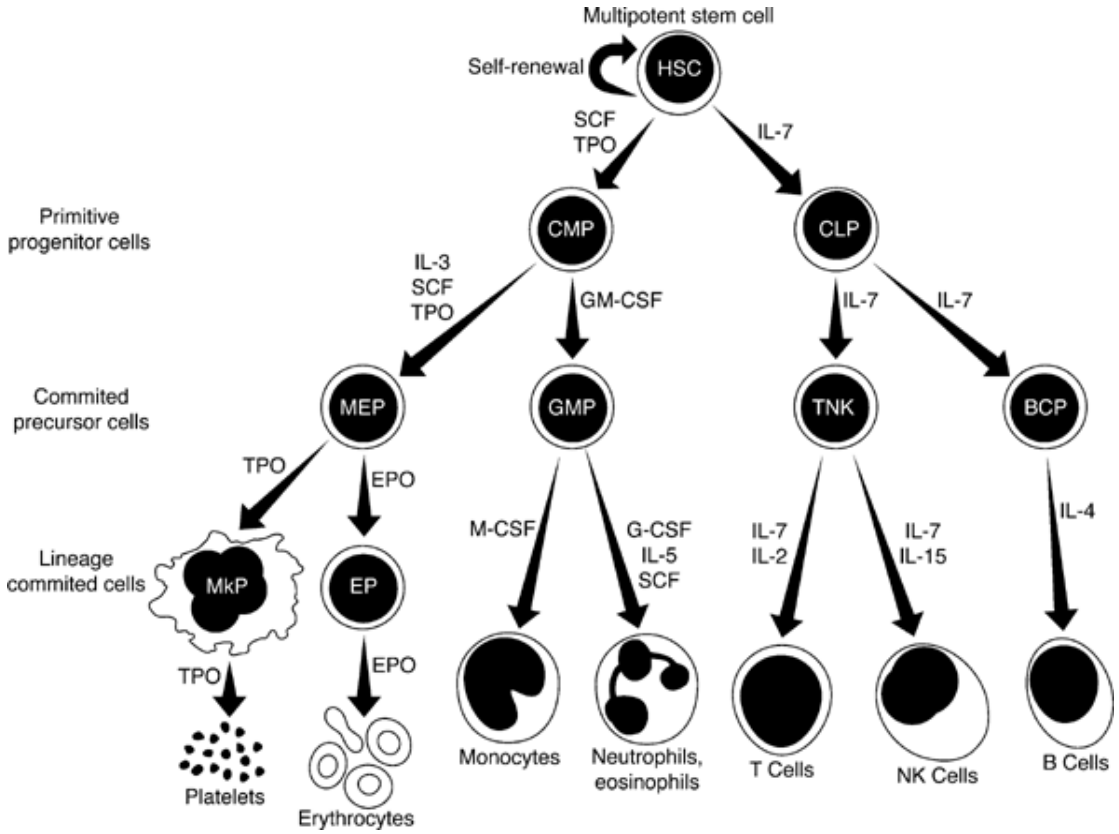

Figure 1 – Hematopoiesis and the role of cytokines. HSC – hematopoietic stem cell, CMP – common myeloid progenitor, CLP – common lymphoid progenitor, MEP – megakaryocyte-erythroid progenitor, GMP - granulocyte–macrophage progenitor, TNK - T-cell natural killer cell progenitor, BCP - B-cell progenitor, MkP - megakaryocyte progenitor, EP – erythroid progenitor, SCF – stem cell factor, TPO – thrombopoietin, IL-7 – interleukin 7, IL-3 – interleukin 3, EPO – erythropoietin, M-CSF – macrophage colony-stimulating factor, G-CSF – granulocyte colony stimulating factor, IL-5 – interleukin 5, IL-2 – interleukin 2, IL-15 – interleukin 15, IL-4 – interleukin 4 [11].

It starts from a hematopoietic stem cell, which is stimulated by stem cell factor (SCF), thrombopoietin (TPO), and interleukin 7 (IL-7) and gives rise to common myeloid progenitor (CMP), common lymphoid progenitor (CLP), or a new hematopoietic stem cell. The progenitor cells lose the ability of self-renewal, but they are still able to give rise to all blood cells of either lymphoid or myeloid lineage. More differentiated lineage restricted progenitors (committed precursor cells) then give rise to one or more blood cell types [11].

Once the cell has matured, it enters the circulation and starts to play its role in blood and/or immune system. However, a portion of differentiated cells is still present in the bone marrow. Among them, B-lymphopoietic population, granulopoietic population, monopoietic population, and erythropoietic population are the most represented [12].

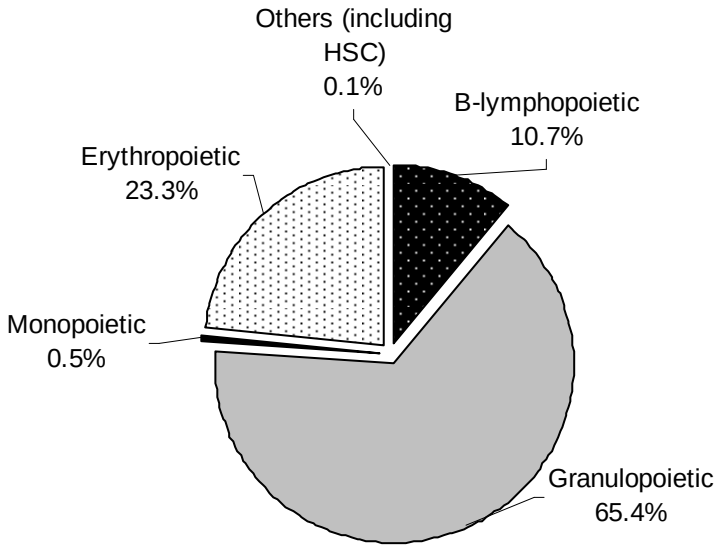

Figure 2 – Representation of various cell types in bone marrow according to [12].

1.1.1.1 Role of apoptosis in regulation of hematopoietic stem cells

Apoptosis, i.e. programmed cell death, plays an important role in normal development and homeostasis of various organs and tissues [13]. In hematopoietic compartment, apoptosis regulates the number of hematopoietic stem cells as well as of more differentiated blood precursors to prevent their overproduction and to eliminate damaged and non-functional cells. However, the mechanism of apoptotic regulation in hematopoiesis has not yet been completely understood.

Anti-apoptotic members of Bcl-2 family, Bcl-2 and Mcl-1, and a pro-apoptotic factor Bim have been described as the essential apoptosis regulators of HSC so far [14-16]. It has

been demonstrated that overexpression of anti-apoptotic regulator *Bcl2* in transgenic mice results in an increase number of HSC and their repopulating capacity, and in resistance of HSC to ionizing radiation and cytotoxic agents [14, 17, 18]. Ablation of another anti-apoptotic factor *Mcl1* led to loss of early bone marrow progenitor populations, including HSC [15]. On the contrary, deletion of pro-apoptotic factor *Bim* caused accumulation of lymphoid and myeloid cells and impairment of T-cell development[16].

1.1.2 Functional and phenotypic characterization of hematopoietic stem cells

The reconstitution capacity of HSC was first described in 1956 by Ford et al. [19]. Only five years later, Till and McCulloch developed a colony-forming unit – spleen (CFU-S) assay, which enabled them to study the repopulating cells *in vivo*: bone marrow cells are transplanted to lethally irradiated recipients and their repopulating ability is determined by the number of spleen colonies formed by the donor-derived cells [20]. It was believed for a long time that the CFU-S are the true HSC. Only with the arrival of flow cytometry, the CFU-S were defined as partially differentiated hematopoietic progenitors with restricted repopulating ability [21]. Flow cytometry also made it possible to measure chimerism (representation) of donor derived cells in the peripheral blood (or in other tissues, e.g. bone marrow or spleen) of the recipient. Detected chimerism reflects the percentage of donor HSC, which have engrafted into the recipient's bone marrow [22]. Besides, *in vitro* assays such as long-term culture initiating cell (LTC-IC) assay, cobble-stone area forming cell (CAFC) assay or colony-forming unit in culture (CFU-C) assay are used for measuring of hematopoietic stem and progenitor cells frequencies [23].

Phenotypic characterization of HSC is mainly based on combination of surface markers. Mouse HSC were first characterized as c-Kit^{+}Thy1^{low}Lin^{-}Sca-1^{high} population [24]. Because HSC are undifferentiated cells, they should belong to the population of cells negative for lineage markers (Lin^{-}). However, since representation of Lin^{-} fraction is dependent on the amount of used antibody, it is better to use Lin^{low} population, together with highly specific HSC markers. Protein markers highly expressed on mouse HSC are for example Sca-1 [24, 25], c-Kit (CD117) [24, 26], CD38 [27], CD43 [28] or CD150 [29]. Apart from these, also markers with low expression on HSC, such as CD34, CD90 (Thy1) [24, 25], CD48 or CD244 [29] are used for the detection.

Usually, mouse HSC are distinguished in the $Lin^{low/-}Sca\text{-}1^{+}c\text{-}Kit^{+}$ (LSK) population by means of other specific markers. Nowadays, combination of SLAM markers, according to Kiel et al. [29], is mostly preferred. SLAM (signaling lymphocyte activation molecule) family is a group of surface receptors, including CD150, CD48 and CD244, which are differentially expressed among functionally distinct hematopoietic progenitors. Primitive HSC are then characterized as $CD150^{+}CD48^{-}CD244^{-}$, multipotent progenitor cells as $CD150^{-}CD48^{-}CD244^{+}$, and lineage-restricted progenitors as $CD150^{-}CD48^{+}CD244^{+}$ [29]. Another possibility for HSC detection within the LSK population is staining with a vital dye Hoechst 33342 [30]. HSC over-express the ABCG2 transporter, which efficiently effluxes Hoechst 33342 [31]. After long-lasting (ninety-minute) staining with Hoechst 33342, a small fraction of bone marrow cells remains Hoechst 33342 negative. This fraction is called side population (SP) and it is highly enriched in HSC [30].

The most common marker for detection and isolation of human HSC is CD34. Contrary to mouse HSC, human HSC were identified as CD34-positive cells. CD34 is usually used together with additional markers, such as CD90 (Thy1) or CD133 [32-34].

1.1.3 Hematopoietic stem cell niche

In bone marrow, HSC are located in a special microenvironment, generally known as the niche. The concept of the hematopoietic stem cell niche was first formulated in 1978 by Raymond Schofield [35]. Based on experimental data demonstrating gradual loss of the stem cell phenotype as a result of repeated transplantations, Dr. Schofield hypothesized that a cellular environment exists in the BM, which conserves the stem cell phenotype, and he used the term “niche” for it.

Current concept of the niche for hematopoietic stem cells distinguishes between the endosteal (osteoblastic) and endothelial (vascular) niche. These two types of niches differ in their localization in BM, cellular composition, and function [29, 36, 37]. It is generally accepted that the **endosteal niche** is situated in a close proximity of inner bone surface (in endosteum), consists of osteoblasts, fibroblasts, and CXCL12-abundant reticular cells (CAR cells), and contains primitive multipotent HSC. The **endothelial niche** is located more centrally in the bone cavity, next to the sinusoids. It consists of endothelial cells and CAR cells, and contains dividing multipotent progenitors (Figure 3, p. 16) [38, 39].

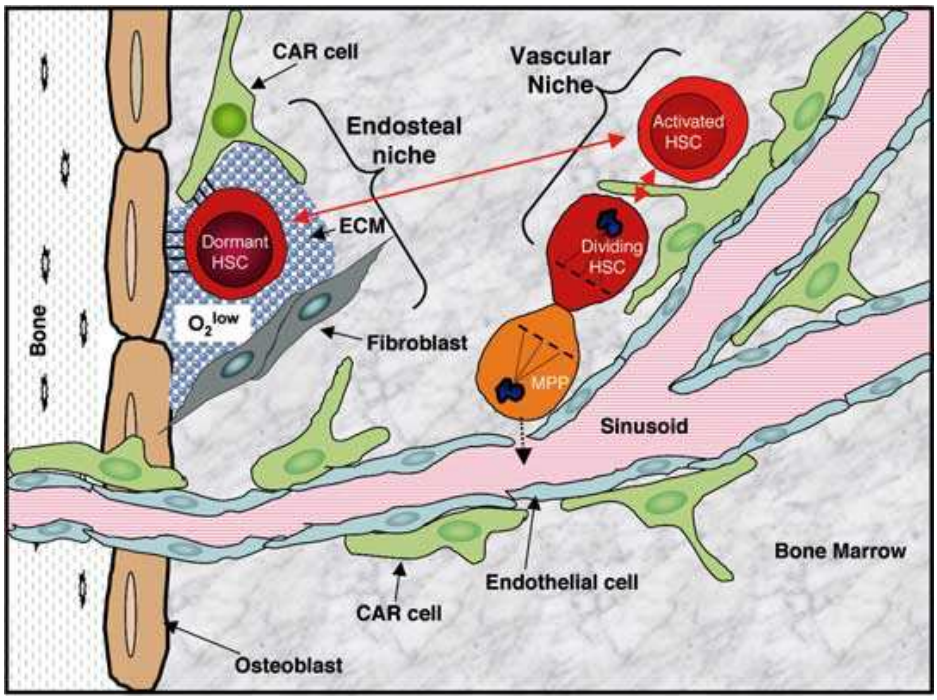

Figure 3 – Hematopoietic stem cell niches in bone marrow. Primitive hematopoietic stem cell (dormant HSC) reside in endosteal niche, situated near to the bone surface and formed by extracellular matrix (ECM), osteoblasts, CAR cells and fibroblasts. Activated HSC translocates to vascular niche, where it divides, self-renews or differentiates to a multipotent progenitor (MPP). MPP gives rise to a maturated blood cell, which enters the circulation [38].

However, Lo Celso et al. [40] demonstrated, using a high resolution confocal microscopy and two-photon video-imaging of single hematopoietic stem cell, that osteoblasts are also co-localized with microvessels and the osteoblastic niche is perivascular as well. The same conclusion was published by Xie et al. [41] by demonstrating vasculature in close proximity of endosteum in irradiated mice transplanted with HSC. On the other hand, numerous studies described a hypoxic character of the osteoblastic niche [9, 42-44]. It has been demonstrated, that bone marrow regions enriched in HSC overlap with those, which are the most positive for the hypoxic marker pimonidazole [9, 45]. Kiel et al. [46] attempted to resolve this obvious discrepancy by suggesting that the bone marrow sinusoids are functionally specialized blood vessels designed to allow cells to enter and exit circulation rather than to deliver oxygen to the tissue. Clearly, the oxygen tension can achieve different values in the microvasculature depending on its organization and intensity of the blood flow.

Although much remains unknown regarding the anatomy of the hematopoietic stem cell niche, its function has been described better. The osteoblastic niche maintains a pool of quiescent HSC, which are regulated by cytokines and adhesive molecules [4, 47-51], as is

described in more detail further on. The niche can also trigger HSC proliferation which provides daughter cells either preserving their phenotype or moving to the vascular niche and giving rise to actively proliferating progenitors [38].

1.1.4 Hematopoietic stem cell quiescence

Since the late nineties, many studies have been published that focused on hematopoietic stem cell quiescence and its regulation. Cheshier et al. [3] analyzed the proliferation kinetics of mouse HSC (characterized as $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}Thy1^{low}$) *in vivo* and cell cycle of the HSC *in vitro.* According to their findings, more than 50 %, more than 90 %, and more than 99 % of the HSC divide by 6 days, by 30 days, and by 6 months, respectively. Furthermore, just 20 % of the HSC are in G1-phase of the cell cycle and 5 % of the HSC are in S/G2/M phases of the cell cycle at one moment. Remaining 75 % of the HSC are in G0 phase of the cell cycle, which is defined as a quiescent one. Based on these results, it was calculated that 99 % of HSC divide on average every 57 days and that maximally 8 % of the HSC asynchronously proliferate every day (Figure 4). Although the number of HSC in the phases of cell cycle can be strain-specific, this study clearly demonstrated their restricted proliferation rate [3].

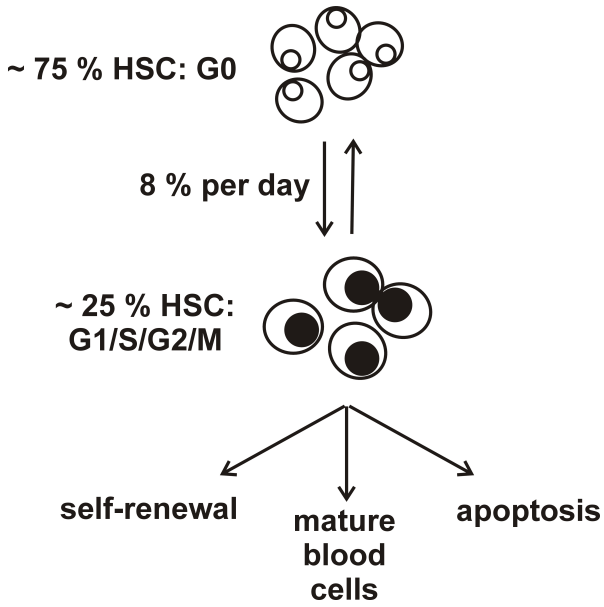

Figure 4 – Model of HSC proliferation kinetics. Under physiological conditions, approximately 75 % of HSC are in G0 phase of the cell cycle, whereas 25 % of HSC are in G1, S, G2, or in M phase. About 8 % of HSC enter the cell cycle every day. Proliferation of hematopoietic stem cell then leads to self-renewal, differentiation, or apoptosis. Adapted from [3].

Quiescence is a crucial property of HSC. It helps to maintain the unique HSC features – self-renewal capacity and ability to differentiate into multiple lineages. Because of that, regulatory mechanisms controlling the quiescence have been widely studied in the last decade [4-8, 52, 53]. The regulation of quiescence occurs mainly in the osteoblastic niche. Extrinsic regulators (cytokines, signaling glycoproteins and adhesion molecules) secreted mainly by the stromal cells in the niche, bind to their counterparts on the surface of HSC. The interactions then trigger multiple signaling pathways in the cell, some of which include intrinsic regulators of the quiescence. Finally, the signal is transduced to the nucleus and through the changes in expression of target genes, it causes the G0 arrest.

As **extrinsic** regulators of the HSC quiescence have been described for example: a key cytokine of stem cell homing - stromal cell-derived factor 1 (SDF-1, CXCL12) and its receptor CXCR4 [6, 54, 55], essential regulator of platelet production - thrombopoietin (TPO) and its receptor MPL [4, 56, 57], vascular specific growth factor angiopoietin 1 (Ang-1) and its receptor – protein kinase Tie2 [49], or Wnt ligands which bind to Frizzled receptor (Fzd) [5, 58].

Conditional knock-outs of genes encoding the listed proteins [6, 50, 55-57], as well as their blocking by a neutralizing antibody or by an antagonist [4, 5], caused exhaustion of primitive HSC as a consequence of their escape from G0 phase of the cell cycle. It resulted in the increased susceptibility to myelosuppressive agent 5-fluorouracyl (5-FU), in the reduced ability to form colonies *in vitro,* and in the decreased or even abolished repopulating ability of long-term repopulating HSC. On the contrary, stimulation of HSC with Ang-1 both *in vivo* and *in vitro* led to increased number of cells with SP phenotype and cells in G0 phase of the cells cycle, to resistance of HSC to 5-FU and to increased repopulating ability [49].

Among **intrinsic** regulators of the HSC quiescence belong inhibitors of mTOR serine threonine kinase – TSC1 and PTEN [8, 59, 60], tumor suppressor protein p53 [61-64] together with its target genes growth-factor independent-1 (Gfi-1) and transcriptional repressor Necdin [65, 66], FoxO subfamily of transcription factors [67, 68], serine/threonine kinase Lkb1 [69, 70] and others [71-73]. Conditional knock-outs of genes encoding these proteins led again to the loss of HSC “stemness” due to their escape from quiescence.

Apart from protein regulators mentioned above, the quiescence of HSC is probably controlled also by the hypoxic character of the stem cell niche [43]. It has been

demonstrated that hypoxia (0.1 % -1.5 % O_2) has a positive effect on maintenance of the primitive character of HSC [74-78]. For example, HSC cultured in 1 % O_2 expanded more slowly than their counterparts cultured under normoxic conditions (20 % O_2) and most of them were in G0 phase of the cell cycle. Furthermore, cyclin-dependent kinase inhibitor $p21^{Cip1}$ was upregulated in primitive HSC incubated in hypoxia, and expression of other CDK inhibitors $p57^{Kip1}$ and $p57^{Kip2}$ was shown to be dependent on hypoxia inducible factor 1α (HIF-1α). Moreover, competitive repopulation assay revealed an increase fraction of long-term repopulating HSC within "hypoxic" LSK cells, as compared to LSK cells cultured in normoxia [78].

1.1.4.1 p53 regulation of HSC quiescence

p53 is a tumor suppressor protein that participates in regulation of many processes in the cell. It is stabilized and activated in response to stress, to protect the cell against damage, or to induce apoptosis. Regarding HSC, p53 has been shown to regulate their quiescence and self-renewal. However, activity of p53 in HSC is much lower than its apoptosis-promoting activity [79].

It has been reported that deletion of *p53* in mice causes an increase in the frequency of HSC (LSK, LSK $CD34^-$ and LSK SLAM cells) [61-63]. The increase was reflected in both *in vitro* and *in vivo* functional assays [61, 62]. Increased numbers of colony forming units-spleen (CFU-S), cobblestone area-forming cells (CAFC), and long-term culture initiating cells (LTC-IC) were observed when $p53^{-/-}$ bone marrow cells were compared to control [61, 62, 64]. TeKippe et al. [61] also demonstrated higher engraftment of $p53^{-/-}$ HSC in a competitive repopulation assay. However, sorted $p53^{-/-}$ HSC were shown to be incapable of long-term repopulation [63]. *In vitro* as well as *in vivo* cell cycle analysis of $p53^{-/-}$ bone marrow cells revealed more cycling HSC in comparison to the wild-type control [62].

Together, these results indicate that *p53* deletion causes proliferation and expansion of HSC. Liu et al. [62] studied the *p53* deletion also on $Mef^{-/-}$ background. MEF/ELF4 is a transcription factor, regulating HSC self-renewal and quiescence [80]. Loss of *Mef* alone increases the number of quiescent HSC. When *p53* was also deleted, the effect of *Mef* deletion was abrogated, which indicates an important role of p53 in the regulation of HSC quiescence (Figure 5, p. 20) [62]. Two target genes of p53 participating of the quiescence regulation have been identified – growth factor independent-1 (*Gfi-1*) and *Necdin*. Gfi-1 is

a transcriptional repressor, which restricts proliferation of HSC [65, 66]. Growth suppressor Necdin functions as a negative cell cycle regulator in postmitotic neurons [81] and its overexpression in HSC results in increased quiescence [62]. Suppression of both *Gfi-1* and *Necdin* in *Mef*$^{-/-}$ mice led to the same result as deletion of *p53* – decline in HSC quiescence [62]. Therefore, p53 preserves HSC quiescence through these two factors (Figure 5).

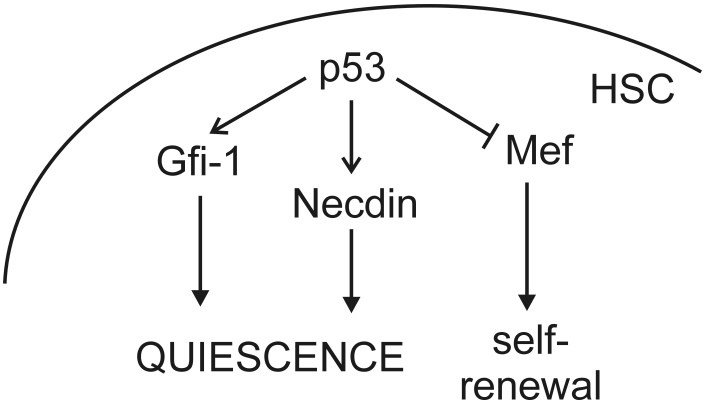

Figure 5 – p53 regulation of hematopoietic stem cell quiescence. p53 transcription factor triggers expression of growth factor independent (*Gfi-1*) and *Necdin*, and suppress regulator of self-renewal – myeloid elf-1-like factor (*Mef*) to support quiescence.

1.1.5 Hematopoietic stem cell metabolism

Because of the hypoxic character of hematopoietic stem cell niche, the energy metabolism of HSC differs from that of differentiated cells [10].

In the presence of oxygen, an animal cell gains energy by means of aerobic respiration. Glycolysis produces 4 molecules of ATP, 2 molecules of reducing equivalents $NADH+H^+$, and 2 molecules of pyruvate. Pyruvate is converted to acetyl-coenzyme A (Ac-CoA) through the oxidative decarboxylation, while reducing equivalents $NADH+H^+$ and carbon dioxide are released. Ac-CoA enters the tricarboxylic acid cycle (TCA), and is oxidized to CO_2 in a series of chemical reactions. During each TCA, 1 GTP (lately converted to ATP), 3 $NADH+H^+$ and 1 $FADH_2$ are produced. The reducing equivalents are then used in the electron transport chain to establish a proton gradient across the inner mitochondrial membrane. Proton gradient is used for phosphorylation of ADP to ATP, while oxygen is reduced to water (Figure 6, p. 21) [82].

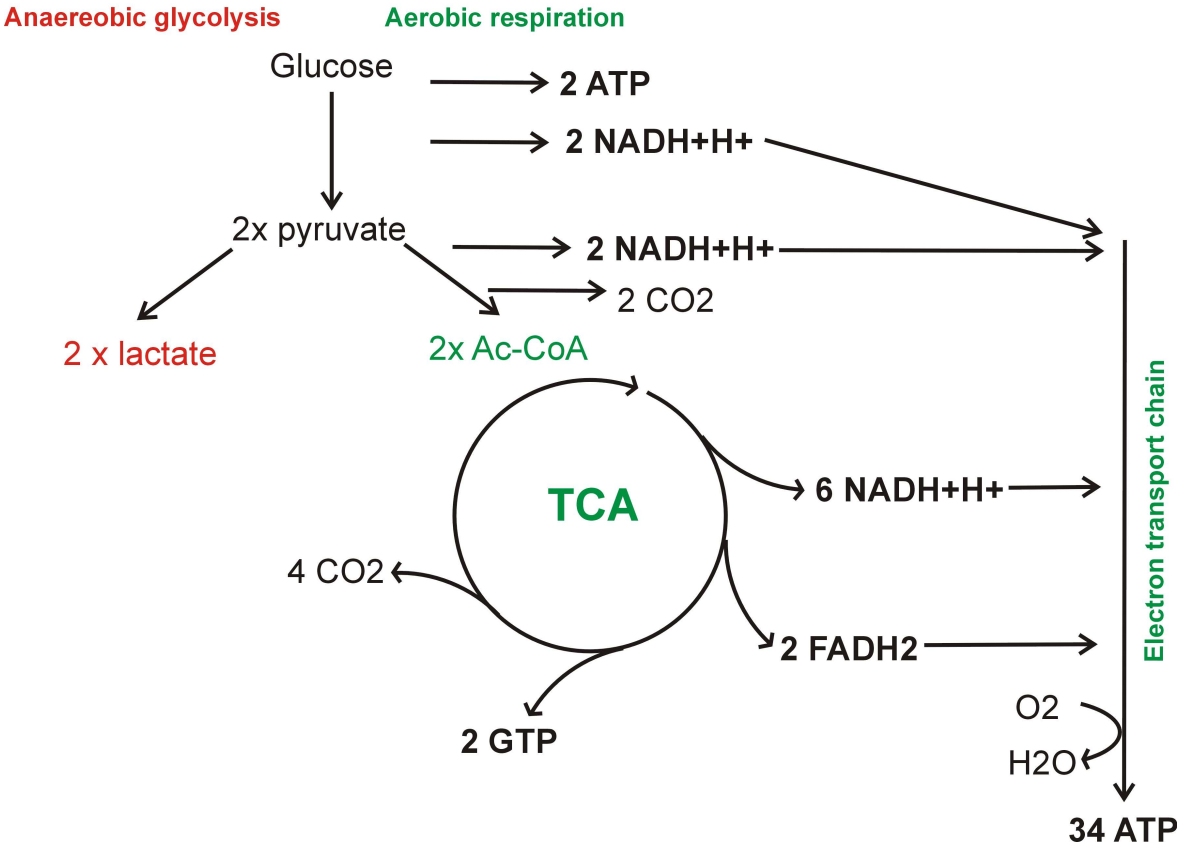

Figure 6 – Cellular respiration under aerobic and anaerobic conditions. Under aerobic conditions, product of glycolysis pyruvate is decarboxylated and provides acetyl – coenzyme A (Ac-CoA). Ac-CoA enters the tricarboxylic acid cycle (TCA), which provides reducing equivalents (NADH+H+ and FADH2) for electron transport chain (ETC). During the ETC, ATP is produced and oxygen is reduced to water. Under anaerobic conditions, pyruvate is reduced to lactate and ETC does not occur.

Each molecule of $NADH+H^+$ provides 3 molecules of ATP, and each molecule of $FADH_2$ provides 2 molecules of ATP in the electron transport chain. Because 2 molecules of ATP are used during glycolysis (phosphorylation of glucose to glucose-6-phosphate and phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate), the yield of ATP from cellular respiration is 38 molecules (8 (glycolysis) + 6 (oxidative phosphorylation of pyruvate) + 24 (TCA)).

When oxygen is not present, glycolysis can occur anaerobically. Pyruvate is reduced to lactate, and the yield is 2 molecules of ATP (Figure 6) [82].

Under conditions of **hypoxia** or anoxia, an animal cell is forced to use oxygen-independent pathways to survive. The switch to anaerobic metabolism is initiated by hypoxia inducible factor 1 (HIF-1) [83, 84]. HIF-1 is a transcription factor activated just under hypoxic conditions, which regulates both glycolysis and mitochondrial respiration

[85-89]. HIF-1 consists of two subunits, HIF-1α and HIF-1β [90]. Both of them are constitutively expressed in the cell, but in normoxia HIF-1α subunit is degraded rapidly [91]. HIF-1 regulation is shown in Figure 7.

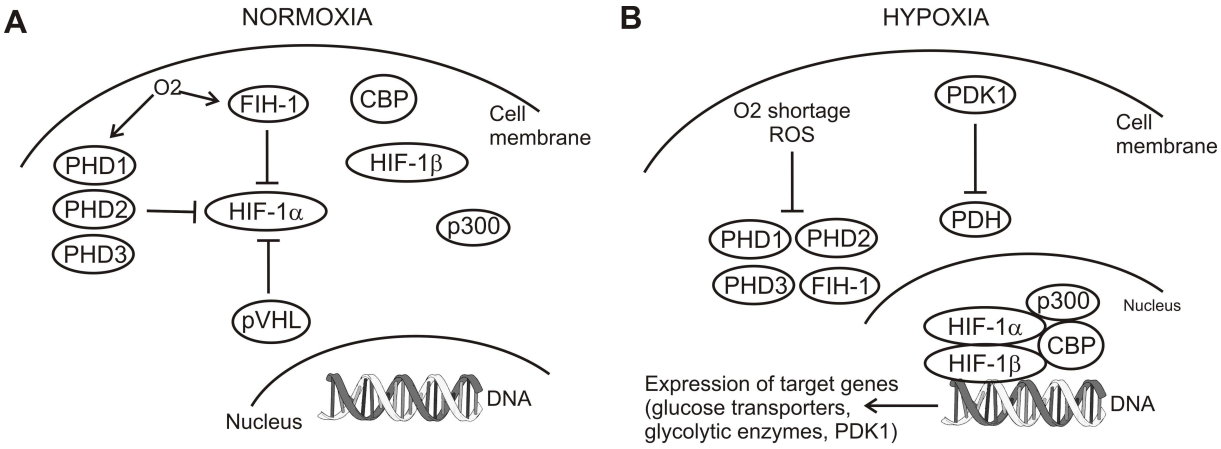

Figure 7 – Hypoxia inducible factor 1 regulation and function. In normoxia, HIF-1α subunit is inhibited by prolyl-hydroxylases (PHD1, PHD2 and PHD3). Hydroxylated form of HIF-1α is recognized by von Hippel-Lindau protein (pVHL), ubiquitinated and degraded. FIH-1 hydroxylates HIF-1α on an asparagine residue and prevents binding of p300 and CBP co-activators **(A)**. In hypoxia, PHD1, PHD2, PHD3 and FIH-1 are inhibited by substrate shortage and by reactive oxygen species (ROS). HIF-1 subunits interact, enter the nucleus and trigger the expression of target genes. PDH-kinase (PDK1, HIF-1 target gene) inhibits a tricarboxylic acid cycle enzyme pyruvate dehydrogenase (PDH) and prevents ROS production **(B)**.

At first, two proline residues of the HIF-1α subunit are hydroxylated by oxygen-dependent prolyl-4-hydroxylase enzymes PHD1, PHD2 and PHD3 [92-94]. During this process, one oxygen atom is used for proline hydroxylation whereas the other serves for conversion of tricarboxylic acid cycle intermediate 2-oxoglutarate to succinate [95, 96]. Hydroxylated proline residues are recognized by pVHL (von Hippel-Lindau protein) ubiquitin-ligase which marks HIF-1α with ubiquitin, and the molecule is subsequently degraded by proteasome [92, 97-99]. Under hypoxic conditions, activity of PHD enzymes is inhibited by the lack of substrates (oxygen and 2-oxoglutarate) and/or by oxidation of Fe(II) in their active sites by reactive oxygen species (ROS) generated by mitochondrial electron transport complex III. Thus HIF-1α can interact with HIF-1β creating active heterodimer HIF-1, which enters the nucleus, binds to DNA, and triggers transcription of target genes [95, 96].

Besides degradation, HIF-1 is regulated also by a factor inhibiting HIF-1 (FIH-1). FIH-1 adds hydroxyl groups onto an asparagine residue near the C-terminus of HIF-1α and prevents the co-activators p300 and CBP from binding to the HIF-1 [100, 101]. Because

FIH-1 needs oxygen as a substrate, its activity is also inhibited in hypoxia by limited O_2 level and by ROS [102].

HIF-1 binding to DNA under hypoxic conditions upregulates expression of many genes including those, which encode glucose transporters and all of the glycolytic enzymes [85-87]. Thus, glycolysis occurs anaerobically but faster than in normoxia and produces sufficient amount of ATP for a cell in steady-state such as the hematopoietic stem cell. Moreover, HIF-1 regulates also the pyruvate dehydrogenase (PDH) reaction, which provides acetyl coenzyme A as a product of aerobic glycolysis. In hypoxia, PDH is phosphorylated by PDH kinase (PDK1) which expression is induced by HIF-1 [103, 104]. Repression of PDH reaction causes cessation of tricarboxylic acid cycle producing reduction equivalents for respiratory chain, and prevents the generation of toxic levels of ROS under hypoxic conditions.

Metabolic patterns of HSC have been recently described by Simsek et al. [10]. HSC were shown to have low mitochondrial potential as well as low ATP levels and to utilize cytoplasmic glycolysis instead of mitochondrial oxidative phosphorylation to cover their energy demands. It has been also demonstrated that the HIF-1α subunit is overexpressed in HSC fraction, as compared to more differentiated cells [45]. On the contrary, expression of PHD2, an enzyme included in HIF-1α degradation, was lower in long-term HSC than in hematopoietic progenitors. Moreover, conditional knock-out of HIF-1α resulted in impaired repopulating ability of long-term repopulating HSC, as a consequence of their escape from quiescence [45].

1.2 Hematopoietic stem and progenitor cells transplantation

Multipotent HSC are able to reconstitute damaged or even destroyed hematopoiesis. This is used clinically for treatment of patients with various hematological disorders by means of hematopoietic stem cell transplantation. The treatment occurs in several stages. First, HSC for the HSCT have to be obtained from a suitable donor. Subsequently, the patient's (recipient's) own hematopoiesis is suppressed and the graft is injected intravenously. The graft injection is followed by homing, seeding (lodgment) and engraftment of the donor HSC, and if the engraftment occurred successfully, by long-term reconstitution of the patient's hematopoiesis [105].

The main criterion of the donor choice is HLA matching between the donor and patient. HLA (human leukocyte antigen) is a name for major histocompatibility complex (MHC) in humans. MHC is a part of immune system, which serves to distinguish non-self cells from self cells of the organism. The higher the HLA-match the lower the risk of graft-versus-host disease (GvHD) and graft rejection. This problem is eliminated in case of autologous HSCT, when the patient's own cells are harvested, frozen, and later used for the transplantation. However, the autologous HSCT can only be used for some hematological diseases and is associated with an increased likelihood of disease relapse.

In case of allogenic HSCT, HLA-matched donor is usually found among the relatives of the patient, most commonly a sibling. Another possibility is finding a donor in a registry of volunteers or using HSC from umbilical cord blood, obtained during childbirth and preserved in a cord blood bank [1, 2].

1.2.1 Sources of hematopoietic stem and progenitor cells

Nowadays, HSC for clinical transplantation can be obtained from bone marrow, peripheral blood or umbilical cord blood. From bone marrow, the cells are harvested by a hipbone puncture. Because the puncture is relatively exhausting for the donor, harvesting of HSC from peripheral blood is usually preferred [106-108]. Under steady-state conditions, most of the HSC reside in bone marrow. They have to be mobilized into circulation using mobilizing agents such as granulocyte-colony stimulating factor (G-CSF), cyclophosphamide, AMD3100 (CXCR4 antagonist) [109], and others. Mobilized cells are sorted on the basis of CD34 expression and a mixture of HSC, hematopoietic progenitors and white blood cells is obtained.

Umbilical cord blood has been used as a source of HSC since the nineties [110]. Blood from the placenta and umbilical cord is harvested during childbirth, frozen, and stored in a cord blood bank. Later, it can be used for autologous or allogenic HSCT.

1.2.2 Storage of hematopoietic stem and progenitor cells

HSC are harvested either directly from BM or more frequently from peripheral blood of a donor after previous mobilization by mobilizing agents (G-CSF, AMD3100, cyclophosphamide etc). Although the cells should be used for HSCT as soon as possible, sometimes the liquid storage lasts for several hours or even days [111]. This can be due to

harvesting being done via consecutive leukapheresis to collect a sufficient amount of cells, or because the cells need to be transported to a patient, e.g. to another country. The most commonly used temperature for the storage of bone marrow cells or peripheral blood progenitor cells in suspension is 4 – 8°C with the recommendation to not exceed 20°C and to transplant the cells within 72 hours after their initial collection.

1.2.3 Mechanism of transplantation

Prior to HSCT, the patient has to undergo conditioning, which suppresses their own malignant hematopoiesis. The most effective method of the suppression is irradiation, acting also on the non-cycling HSC. Because the irradiation is very harmful for the organism, it is commonly used in a submyeloablative dose in combination with chemotherapy [112].

After the conditioning, hematopoietic stem cell niches in the recipient's BM are prepared to receive donor cells ("open"). The graft is transplanted intravenously and homing, lodgment and engraftment of HSC follows.

Homing is a rapid process, by which the HSC find their way to the BM. It lasts a few hours in mouse and 1 - 2 days in human. As a crucial molecule of the homing process the stromal cell derived factor 1 (SDF-1, CXCL12) has been described [113-115]. SDF-1 is secreted by the stromal cells and endothelial cells of hematopoietic stem cell niche. Because HSC have SDF-1 receptor CXCR4 on their surface, a gradient of SDF-1 directs HSC from the circulation to the bone marrow. Once the HSC reach the bone marrow sinusoidal vessel, they adhere to the endothelial cells by means of cell-cell interactions. Role of E- and P-selectins in adhesion of HSC to endothelial cells has been demonstrated using mice deficient in both of them [116]. Also the interaction of $\alpha 4\beta 1$ integrin (very-late antigen, VLA-4) on the HSC surface with vascular cell adhesion molecule (VCAM-1), and interaction of CD44 with hyaluronic acid, have been shown to be involved in the homing process [116-118].

After the adhesion, HSC follow the SDF-1 gradient to the endosteal niches, where they lodge by means of SCF/c-Kit interaction [119], SDF-1/CXCR4 interaction, VLA-4/VCAM-1 interaction, and others. A schematic overview of the complex process is shown in Figure 8, p. 26).

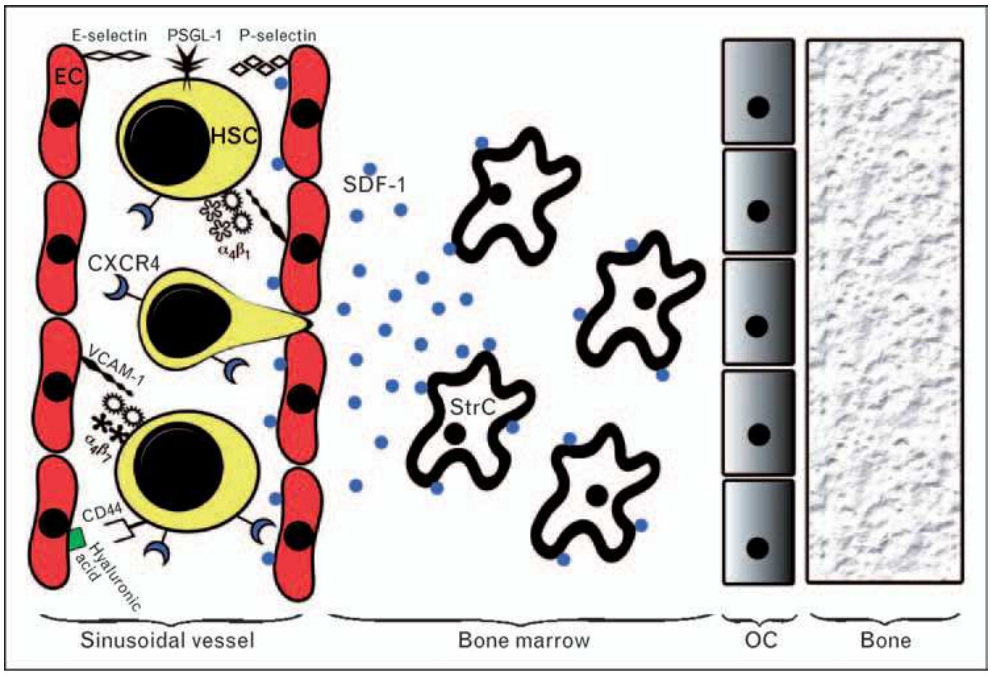

Figure 8 – Homing of hematopoietic stem cells. HSC adhere to endothelial cells (EC) through cell-cell interactions: α4β1 integrin (α4β1, very-late antigen 4)/ vascular cell adhesion molecule 1 (VCAM) interaction, CD44/hyaluronic acid interaction, P-selectin glycoprotein ligand (PSGL-1)/P-selectin interaction and E-selectins interaction. Stromal cells (StrC) in BM produce SDF-1 cytokine, which interacts with SDF-1 receptor (CXCR4) on the HSC membrane. Gradient of SDF-1 brings the HSC to the osteoblastic cells (OC) on the bone surface [120].

The **engraftment** is, contrary to the homing, a long-lasting process. At first, hematopoietic progenitors provide a short-term engraftment. The progenitors are not quiescent any more, and therefore their proliferation and differentiation to the blood cells can start in a short time after the HSCT. However, the progenitors lack the ability of self-renewal and in the course of time (weeks in mouse, months in human), they are exhausted. Around the time of exhaustion of progenitors, the HSC begin to proliferate and provide the long-term engraftment, which lasts years, or ideally a lifetime [120, 121]. Because of the short- and long-term lasting engraftment, sometimes the progenitors and the HSC are called the "short-term repopulating cells" (STRC) and the "long-term repopulating cells (LTRC), respectively.

1.3 Cadaveric organ donors

At present, hematopoietic stem and progenitor cells for transplantation are harvested exclusively from living donors or their source is cord blood. Nevertheless, Soderdahl et al. [122] explored the bone marrow from cadaveric organ donors and based on cell viability and CFU numbers, they assumed that the cells could be procured with a high degree of

engraftment potential. In addition, the CD34-positive cell fraction was not affected by storage for up to three days in a heparinized RPMI 1640 medium at room temperature (20°C) or in a refrigerator (4°C – 8°C) [122]. Furthermore, bone marrow cells harvested from cadaveric organ donors and stored at 4°C for seven days did not display a significant increase in apoptosis [123].

Nowadays, organs for transplantation are routinely harvested from non-heart-beating donors (NHBD) [124-126]. Four categories of NHBD exist: I (dead on arrival) and II (unsuccessful resuscitation) are uncontrolled donors; III (withdrawal of life-supporting therapy) and IV (cardiac arrest in a brain-dead donor) are controlled donors [127]. Transplantability of solid organs from NHBD and the tolerance of the organs to warm ischemia (ischemia at 37°C) has been widely studied over the last two decades [124-126, 128-130]. Nevertheless, practically no attention has been paid to the bone marrow of NHBD (cadaveric bone marrow), despite the fact that hematopoietic stem cells are quiescent cells with restricted aerobic metabolism, which could favor their survival after the cessation of circulation.

2 AIMS OF THE STUDY

The aim of the presented study was to explore cadaveric bone marrow as an alternative source of hematopoietic stem cells (HSC) for hematopoietic stem cell transplantation (HSCT), using mouse as a model organism. In order to address this task, several specific objectives were established:

- To investigate the repopulating ability of HSC, which have been exposed to ischemia at 37°C, 20°C, and 4°C for defined time periods, after transplantation to sublethally irradiated congenic recipients.

- To investigate the competitive repopulating ability of HSC, which have been exposed to ischemia at 37°C, 20°C, and 4°C for defined time periods, after co-transplantation with fresh bone marrow cells (1:1 ratio) to lethally irradiated congenic recipients.

- To determine the frequencies of HSC in bone marrow exposed to ischemia at 37°C, 20°C, and 4°C for defined time periods. To compare apoptosis and necrosis in population enriched in HSC ($Lin^{low}Sca\text{-}1^{+}c\text{-}Kit^{+}$) with differentiated subpopulations of bone marrow.

- To explore the impact of ischemia at 37°C on repopulating ability of $p53^{-/-}$ HSC, their frequencies, and apoptosis and necrosis in bone marrow.

- To examine an effect of metabolic inhibition by potassium cyanide and 2-deoxy-D-glucose *in vitro* on survival of bone marrow subpopulations, and on the repopulating ability and survival of HSC.

- To confirm the repopulating ability of hematopoietic stem cells after short-term *in vitro* storage (up to 4 days) in suspension without specific hematopoietic growth factors.

3 MATERIALS AND METHODS

3.1 Materials

Chemicals

Carl Roth GmbH

Bovine serum albumin – Fraction V, bovine serum albumin – Fraction V, biotin free

Honeywell Riedel-de Haën, Germany

Hoechst 33342

IPL, Czech Republic

Ammonium chloride (NH_4Cl), disodium phosphate ($Na_2HPO_4.12H_2O$), monosodium phosphate ($NaH_2PO_4.2H_2O$), sodium chloride (NaCl)

Lachema, Czech Republic

D-glucose

Lonza, Switzerland

Iscove's Modified Dulbecco's Medium with HEPES and L-glutamine

Penta, Czech Republic

Türck solution

Sigma-Aldrich, USA:

2-deoxy-D-glucose, Dulbecco's Modified Eagle's Medium - high glucose (with 4500 mg/dm^3 glc, 110 mg/dm^3 sodium pyruvate and L-glutamine), Dulbecco's Modified Eagle's Medium – low glucose (with 1000 mg/dm^3 glc, L-glutamine, and sodium bicarbonate), ethylendiaminetetraacetic acid (EDTA), fetal bovine serum, penicillin – streptomycin (with 10.000 units penicillin and 10 mg/cm^3 streptomycin), potassium cyanide, propidium iodide

Zentiva, Czech Republic

Heparin

Antibodies

BioLegend, USA

Anti-B220-Alexa700, anti-CD150-PE, anti-CD48-FITC, anti-c-Kit-APC-Cy7, anti-Gr-1-APC, anti-Lin-FITC, anti-Mac-1-APC, anti-Sca-1-APC, anti-Ter119-FITC, streptavidin-PE-Cy7

Miltenyi Biotec, Germany

Lineage cocktail - biotin

Buffers

Phosphate buffered saline (PBS): 16 mmol/dm^3 $Na_2HPO_4.12H_2O$, 4 mmol/dm^3 $NaH_2PO_4.2H_2O$, 0.15 mol/dm^3 NaCl, pH 7.4

Ammonium chloride lysis solution: 0.15 mol/dm^3 NH_4Cl, 0.1 mmol/dm^3 EDTA, 0.032 mol/dm^3 NaCl

Media

DMEM without drugs: DMEM high glucose, 20 % (v/v) FBS, 1 % (v/v) penicillin-streptomycin

DMEM with KCN: DMEM high glucose, 20 % (v/v) FBS, 1 % (v/v) penicillin-streptomycin, 1 mmol/dm^3 KCN or 10 mmol/dm^3 KCN

DMEM with 2-deoxy-D-glucose: DMEM low glucose, 20 % (v/v) FBS, 1 % (v/v) penicillin-streptomycin, 50 mmol/dm^3 2-DG

DMEM with D-glucose: DMEM low glucose, 20 % (v/v) FBS, 1 % (v/v) penicillin-streptomycin, 50 mmol/dm^3 D-glucose

IMDM: IMDM with HEPES and L-glutamine, 10 % (v/v) FBS, 1 % (v/v) penicillin-streptomycin

3.2 Instruments

Automatic micropipettes: Eppendorf, Germany

Cellometer AUTO T4: Nexelom Bioscience, USA

Centrifuges: 5804R and 5810R centrifuges, Eppendorf, Germany

CO_2 incubator: IGO 150 Cell life, Jouan GmbH, Germany

Flow box: Holten LaminAir, Model 1.2, Thermo-Scientific Inc., USA

Flow cytometers: BD FACS Aria IIu equipped with UV-laser, BD FACS Canto II, Becton Dickinson, USA

Irradiator: ^{60}Co Chisobalt, Chirana, Czech Republic

Other: AccuBlock^{TM} Digital Dry Bath, Labnet international, Inc., USA; analytical balance AB 104, Mettler Toledo, Czech Republic; pH meter 3305 Jenway, UK; vortex mixer VELP Scientifica, p-lab, Czech Republic; water bath, Heto lab equipment, Denmark, orbital incubator SI50, Stuart Scientific, UK

3.3 Animals

C57BL/6 (Ly5.2 and Ly5.1) and C57Bl/6J *Trp53*^{tm1Tyj} (p53$^{-/-}$ Ly5.2) mice were bred in the specific pathogen free facility of The Center of Experimental Biomodels, First Faculty of Medicine, Charles University in Prague, maintained in a clean conventional animal facility with a light-dark cycle of 12 hours during experiments, and fed ad libitum. Two to six-months-old mice, 20 – 25 g of body weight, were used in the experiments. All experiments were approved by the Laboratory Animal Care and Use Committee of the First Faculty of Medicine, Charles University, and were performed in accordance with national and international guidelines for laboratory animal care.

3.4 Harvesting of bone marrow cells

Donor mice were sacrificed by cervical dislocation. Femurs were removed from the body and carefully rid of muscles. Bone marrow cells were flushed with 1-milliliter insulin syringe (**21 G needle**) into 1 ml PBS supplemented with 0.5 % BSA (PBS/BSA) solution and kept on ice, or into 1 ml of a medium, depending on the experiment. A single cell suspension was created with **25 G needle**. Cellularity of the suspension was counted by Cellometer AUTO T4, using Türck solution for white blood cell count.

3.5 Preparation of cadaveric bone marrow

After sacrificing of C57Bl/6 Ly5.1 or C57Bl/6J Ly5.2 p53$^{-/-}$ donor mice, intact femurs were removed from the body, deprived of muscles and inserted in pre-tempered PBS solution. Afterwards, the femurs were maintained at the temperature of 37°C (in IGO 150 Cell life incubator), 20°C (in AccuBlock™ Digital Dry Bath), or 4°C (in a cold room) respectively, for various time periods as shown in Table 1.

Temperature	Period of ischemia	Genotype
37°C	0, 1, 2, 3, 4, 5 hours	wild-type
	0, 2, 4, 6 hours	p53-/-
20°C	0, 3, 4, 6, 12, 20 hours	wild-type
4°C	0, 8, 12, 16, 24, 48 hours	wild-type

Table 1 – Periods of ischemia used at 37°C, 20°C, and 4°C.

Subsequently, bone marrow cells were harvested from the femurs as described in section 3.4. The cells were used for bone marrow transplantation to congenic recipients, to analysis of apoptosis and necrosis, or to phenotypic analysis of HSC or lineage-restricted cells by flow cytometry.

In addition, the experimental design described above was compared to the situation when the whole body was exposed to ischemia. At the same time, femurs from sacrificed C57Bl/6 Ly5.1 mice were either removed from the body and exposed to ischemia as described above, or left in the body exposed to ischemia, and removed afterwards. Three-hour period of ischemia at 37°C was used for the comparison. These cells were used for phenotypic analysis of HSC populations and for bone marrow transplantation to congenic recipients.

3.6 Metabolic inhibition by KCN and 2-deoxy-D-glucose

Following combinations of media/chemicals were used:

- DMEM high glucose
- DMEM high glucose + 1 mM KCN
- DMEM high glucose + 10 mM KCN
- DMEM low glucose + 50 mM D-glucose
- DMEM low glucose + 50 mM 2-deoxy-D-glucose
- DMEM low glucose + 10 mM KCN + 50 mM 2-deoxy-D-glucose

Bone marrow cells from one femur of C57Bl/6 Ly5.1 mice were aseptically harvested into 1 ml DMEM high glucose or DMEM low glucose with/without chemicals, as listed above. The cells were transferred in 30-millimeter wells of a 6-well plate and incubated for 2 hours or 20 hours at 37°C in 5 % CO_2 atmosphere (CO_2 incubator IGO 150 Cell life). Afterwards, the cells were transferred in 5-ml plastic tubes and centrifuged at 400 g at 4°C for 5 minutes. Supernatants were discarded; the cells were resuspended in PBS/BSA and used for bone marrow transplantation, determination of apoptosis and necrosis in bone marrow, or phenotypic analysis by flow cytometry.

3.7 *In vitro* storage of bone marrow cells

Bone marrow cells were aseptically harvested from the femurs of sacrificed C57Bl/6 Ly 5.1 donors. Afterwards, the cells were washed and resuspended in IMDM medium with 10 % FBS at a concentration of 1 x 10^7 cells/ml. Cells were maintained in the medium for 1 and 2 days at 37°C (in IGO 150 Cell life incubator) or for 2 and 4 days at 4°C (in a cold room), respectively. Subsequently, the cells were transplanted to sublethally irradiated congenic recipients and chimerism of donor derived cells in peripheral blood was followed up for 6 months.

3.8 Total body irradiation

Before bone marrow transplantation, recipient mice were sublethally or lethally irradiated with the ^{60}Co source with a dose rate 0.5 Gy/min. The dose of 6 Gy and 9 Gy were used for sublethal and lethal irradiation, respectively.

3.9 Bone marrow transplantation

Ly5.1/Ly5.2 mouse congenic model were used in transplantation experiments. C57Bl/6 Ly5.1 and C57Bl/6 Ly5.2 are two inbred mouse strains that differ in the surface antigen CD45. They express isoforms Ly5.1 (CD45.1) and Ly5.2 (CD45.2), which are functionally identical but distinguishable by means of specific antibodies. Because CD45 is expressed by all nucleated blood cells, Ly5.1/Ly5.2 ratio in peripheral blood reflects the percentage of transplanted HSC which have engrafted to recipient's bone marrow (chimerism of donor cells).

Following transplantation experiments were performed:

- Cadaveric bone marrow cells from C57Bl/6 Ly5.1 donors were transplanted to sublethally or lethally irradiated C57Bl/6 Ly5.2 recipient mice.
- Cadaveric bone marrow cells from C57Bl/6J Ly5.2 $p53^{-/-}$ donors were transplanted to sublethally irradiated C57Bl/6 Ly5.1 recipient mice.
- C57Bl/6 Ly5.1 bone marrow cells after metabolic inhibition were transplanted to sublethally irradiated C57Bl/6 Ly5.2 recipient mice.
- C57Bl/6 Ly5.1 bone marrow cells after *in vitro* storage were transplanted to sublethally irradiated C57Bl/6J Ly5.2 recipient mice.

Fresh bone marrow cells were used as a control. Sublethally irradiated recipients were transplanted with cadaveric bone marrow cells (or with cells after metabolic inhibition or *in vitro* storage) from half of the femur (approximately 1 x 10^7 cells) (Figure 9A).

Lethally irradiated recipients were transplanted with a 1:1 ratio mixture of cadaveric congenic (Ly5.1) and fresh syngenic (Ly5.2) bone marrow cells from half of the femur of each donor (2 x 0.5 femur, Figure 9B). Expected chimerism for fresh bone marrow was 50 %.

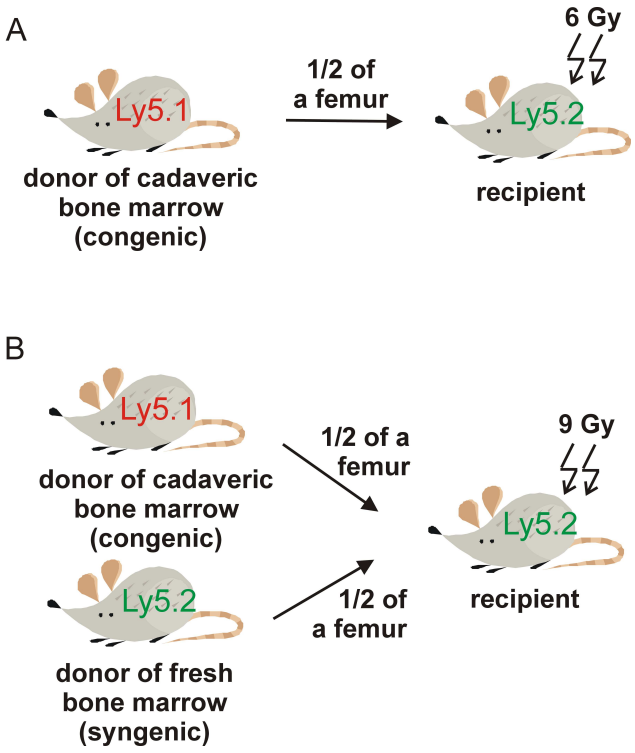

Figure 9 – Schematic overview of bone marrow transplantation experiments. Half of the congenic ischemic femur was transplanted to sublethally (6 Gy) irradiated recipient **(A)**. Half of the congenic ischemic femur was co-transplanted to lethally (9 Gy) irradiated recipients with a half of the untreated syngenic femur **(B)**.

A single-cell suspension of bone marrow cells was administered intravenously through the retrobulbar plexus in a volume of 0.5 ml PBS/BSA. At least four mice were used per group.

The engraftment of donor cells was determined in the peripheral blood of recipients two weeks after transplantation and then at monthly intervals up to 3, 5 or 6 months. The chimerism up to 1 month was considered to be caused by the short-term repopulating cells (STRC). Long-term repopulating cells (LTRC) were responsible for chimerism after 3 or more months. Blood samples of approximately 100 µL were collected through the

retrobulbar plexus puncture using heparinized glass capillary and the samples were prepared for the chimerism analysis as it is described below, in section 3.10.1.

3.10 Flow cytometry

Flow cytometry is a biological method, which serves to measure properties of individual cells (or other particles) in very short time [131, 132]. Along with optical parameters of the cell (size and granularity), the presence of various antigens in the cell can be visualized. The cells are labeled with fluorescent dye conjugated antibodies against specific antigens on their surface or in cytoplasm/nucleus. During the surface labeling, monoclonal antibodies conjugated with fluorochromes specifically bind to the antigens on the cell membrane. During the "inner" labeling, antibodies pass through the permeabilized cell membrane and bind to the proteins in cytoplasm or in nucleus (DNA complexes). Simple low-molecular dyes (propidium iodide, Hoechst stains etc.) can pass the cell membrane and stain DNA or other cellular structures. The fluorescent labeling allows us to distinguish the cells according to the phenotype, or to measure cell processes such as apoptosis or proliferation. In this study, surface labeling was used to detect populations of bone marrow or blood cells, and inner labeling was used to measure apoptosis or to detect side-population (SP) in bone marrow. Fluorescent dyes used in the study are listed in Table 2.

Fluorochrome	λ_{ex} [nm]	λ_{em} [nm]
Alexa-700	696	719
Allophycocyanin (APC)	650	767
APC-Cy7	650, 755	767
Fluorescein isothiocyanate (FITC)	495	519
Phycoerythrin (PE)	480, 565	578
PE-Cy7	480, 565, 743	767
Hoechst 33342	343	483
Propidium iodide (PI)	536	617

Table 2 – List of fluorochromes used in the study. λ_{ex} – excitation wavelength, λ_{em} – emission wavelength.

During the cytometric analysis, the cells are hydrodynamically focused in a core stream encased within a sheath fluid and one by one go through the laser beams (Figure 10).

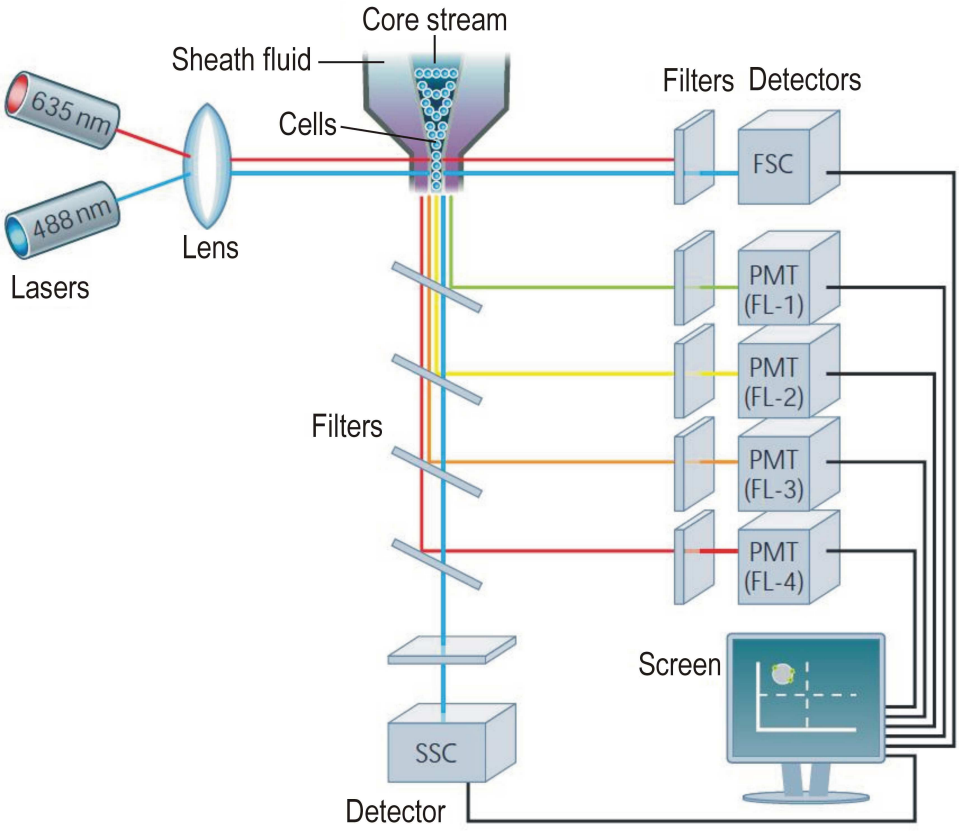

Figure 10 – Schematic overview of a typical flow cytometer setup. The cells, carried by the sheath fluid, go through the laser beams; diffracted light is detected as forward-scatter (FCS), refracted and reflected light as side-scatter (SSC). Fluorochromes excited by lasers emit light of defined wavelength. Emitted light goes through filters and photomultipliers (PMT) and is detected as intensity of fluorescence (FL-1, FL-2, FL-3, FL – 4) [132].

Lasers excite the fluorescent dyes, which subsequently emit light of defined wavelength. The light passes through a system of filters and photomultipliers and finally reaches the detectors. Analog signal from the detectors is transformed to a digital one and intensity of fluorescence is displayed on a screen [131, 132]. Moreover, forward-scatter (FCS) and side-scatter (SSC) of the laser beam going through the cell are measured. When a laser beam goes through the cell, certain amount of the light is diffracted on the cell surface and subsequently detected in the forward direction by a photodiode as FSC; another amount of the light is refracted by the cell organelles and detected at approximately 90 degrees to the laser beam as SSC. Therefore, FSC and SSC correspond to relative size and granularity of the cells, respectively [131].

3.10.1 Determination of chimerism in peripheral blood

The engraftment of donor cells after transplantation was determined from chimerism of donor-derived cells in recipient's peripheral blood two weeks after transplantation and then at monthly intervals up to 6 months to distinguish the short-term repopulating cells (STRC) and long-term repopulating cells (LTRC).

Fifty microliters of peripheral blood were added to 3 ml of preheated ammonium chloride lysis buffer and incubated for 15 minutes at 37°C and 150 rpm (orbital incubator SI50) to lyse erythrocytes. Afterwards, the samples were centrifuged for 5 minutes at 4°C, 400 g (centrifuge 5804R) and supernatants were discarded. The cells were washed with 3 ml PBS/BSA and centrifuged as previously. Pellets were stained with phycoerythrin (PE) conjugated anti-Ly5.1 and fluorescein isothiocyanate (FITC) conjugated anti-Ly5.2 antibodies – 0.5 µl of each antibody per sample – for 20 minutes on ice and in the dark. Samples were then washed with 3 ml PBS/BSA and centrifuged for 5 minutes at 4°C and 400 g. Pellets were resuspended in 250 µl PBS/BSA. Chimerism (percentage of donor derived cells) was determined by flow cytometry (BD FACS Canto II). Doublets and debris were excluded and populations of Ly5.1 and Ly5.2 positive cells were gated as shown in Figure S1, Appendix 1, p. I.

3.10.2 Analysis of LSK SLAM and LSK SP cells

Staining for $Lin^{low}Sca\text{-}1^{+}c\text{-}Kit^{+}$ $CD150^{+}CD48^{-}$ (LSK SLAM) markers according to Kiel et al. [29] together with detection of LSK side-population (LSK SP) according to Goodell et al. [30] were used to identify hematopoietic stem cells within bone marrow.

At first, fresh and cadaveric bone marrow cells or bone marrow cells after metabolic inhibition were stained for SP population. Briefly, approximately 2×10^7 cells were resuspended in preheated IMDM medium ($1 – 2 \times 10^6$ cells per 1 ml of IMDM) in fifty-milliliter plastic tubes. Hoechst 33342 dye was added to a final concentration of 5 µg/ml, and the suspension was incubated for exactly 90 minutes at 37°C in a water bath. Afterwards, the cells were centrifuged for 10 minutes at 4°C and 400 g (centrifuge 5810R) and washed with 3 ml of ice-cold PBS/BSA. Supernatants were removed and the cells were stained in the pellet (approximate volume of 50 µl) for specific surface markers LSK and SLAM with fluorochrome-conjugated monoclonal antibodies (20 min on ice and in the dark): lineage cocktail – biotin – 3.5 µl per sample, anti-Sca-1-APC, anti-cKit-APC-Cy7,

anti-CD150-PE and anti-CD48-FITC – 1,5 µl of each antibody per sample. After washing with 3 ml of PBS/BSA, the cells were incubated with streptavidin-PE-Cy7 antibody for 15 min on ice and in the dark, and washed again. Finally, the cells were resuspended in 400 µL PBS/BSA and analyzed by flow cytometry (BD FACS Aria IIu equipped with UV-laser). Doublets and debris were excluded and LSK SP and LSK SLAM populations were gated (Figure S2, Appendix 1, p. II).

3.10.3 Detection of apoptotic and dead cells in bone marrow subpopulations

Frequencies of live, apoptotic and dead cells in fresh and cadaveric bone marrow or in bone marrow after metabolic inhibition were detected in LSK population enriched in HSC, lymphopoietic population ($B220^+$), granulo-monopoietic population ($Gr\text{-}1^+Mac\text{-}1^+$) and erythropoietic population ($Ter119^+$). Specific monoclonal antibodies, Hoechst 33342 dye, and propidium iodide (PI) were used for staining. Hoechst 33342 permeates into cells and binds to the DNA. If the staining is short (7 minutes), just the apoptotic and dead cells are stained. PI functions in the same way; however it stains just the dead cells.

Approximately 2×10^6 and 5×10^5 bone marrow cells were used for detection of apoptosis in LSK population and in lineage restricted populations, respectively. The cells were washed with 3 ml PBS/BSA and centrifuged for 5 minutes at 4°C and 400 g (centrifuge 5804R). Supernatants were discarded and the cells in 50 µl PBS/BSA were stained with fluorochrome-conjugated monoclonal antibodies (20 min on ice and in the dark): anti-lineage-FITC - 2 µl per sample, anti-Sca-1-APC, anti-c-Kit-APC-Cy7, anti-B220-Alexa700, anti-Gr-1-APC, anti-Mac-1-APC and anti-Ter119-FITC – 0.6 µl of each antibody per sample. After subsequent washing with 3 ml PBS/BSA and removal of supernatants, the cells were stained with Hoechst 33342 and propidium iodide to detect apoptotic and dead fractions according to Ormerod et al. [133] and Schmid et al. [134]. Briefly, Hoechst 33342 was added to the pellet in a final concentration of 20 µg/ml. The samples were incubated for 7 minutes at 37°C (AccuBlock™ Digital Dry Bath). Subsequently, the cells were diluted with PSB/BSA to the volume of 250 µl. Immediately before analysis, 1 µl of PI stock solution (1 mg/ml) was added. The samples were analyzed by flow cytometry (BD FACS Aria IIu equipped with UV-laser). Doublets and debris were excluded and live (Hoechst 33342 negative, PI negative), apoptotic (Hoechst 33342 positive, PI negative) and dead (Hoechst 33342 positive, PI positive) cells were gated

within the population of nucleated bone marrow cells and within the bone marrow subpopulations. (Figure S3, Appendix 1, p. III, Figure S4, Appendix 1, p. IV)

3.11 Statistical analysis

Statistical analysis was performed with GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego, USA, www.graphpad.com). Data are presented as the mean ± SEM. To compare multiple groups to the control, one-way ANOVA using Dunnett's post test was applied. Student's two-tailed t-test was used for comparison of two groups to each other. P-values ***$P < 0.005$, **$P < 0.01$ and *$P < 0.05$ were considered statistically significant. In some figures, error bars are not presented because of better readability of the graphs. In this case, supplementary tables containing SEM values are presented in Appendix 2.

4 RESULTS

4.1 Effects of ischemia on bone marrow

Bone marrow in intact femurs (cadaveric bone marrow) was maintained for defined time periods at 37°C, 20°C and 4°C, respectively. Repopulating ability of the hematopoietic stem cells, frequencies of LSK SLAM and LSK SP cells, and viability of bone marrow cells were determined.

4.1.1 Transplantability of hematopoietic stem cells after ischemia

After exposition to ischemia at 37°C, 20°C, or 4°C, the bone marrow cells were transplanted to sublethally or to lethally (in 1:1 ratio with fresh congenic bone marrow cells) irradiated recipients. Donor chimerism was followed up in peripheral blood of recipients for 6 months after the transplantation.

4.1.1.1 Engraftment of hematopoietic stem cells after ischemia in sublethally irradiated recipients

Percentage of donor-derived cells in the peripheral blood of recipients is presented in Figure 11, p. 41 and in Tables S1-S3, Appendix 2, p. V-VI. At 37°C (Figure 11A; Table S1, Appendix 2, p. V), the transplantability was not decreased after 2 hours or 3 hours of ischemia for STRC or LTRC, respectively. At 20°C (Figure 11B; Table S2, Appendix 2, p. V), the engraftment was comparable to the control for up to six-hour ischemia. Twenty-hour ischemia at 20°C resulted in almost complete loss of repopulating ability. At 4°C (Figure 11C; Table S3, Appendix 2, p. VI), the engraftment was not significantly decreased up to 12 hours of ischemia, and a reduced engraftment was still present after 24 hours of ischemia.

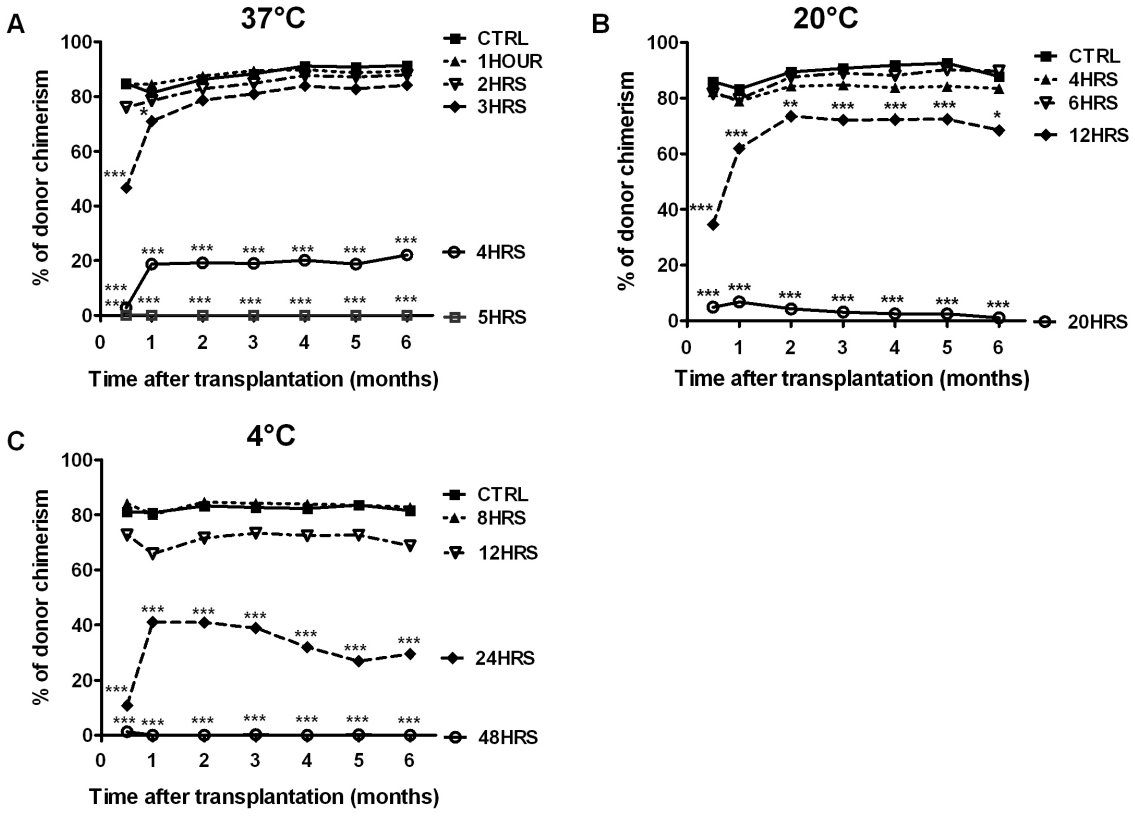

Figure 11 - Engraftment of ischemic HSC in sublethally irradiated recipients. Donor cells (Ly5.1) were exposed to ischemia for up to 5 hours at 37°C **(A)**, for up to 20 hours at 20°C **(B)**, or for up to 48 hours at 4°C **(C)**. Ly5.2 recipients (n=4-10) were irradiated with 6 Gy and transplanted with half of the donor femur. The engraftment is presented as chimerism of Ly5.1+ cells (mean) detected in peripheral blood of Ly5.2 recipients. Significance of difference from the controls at individual time points: ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.1.1.2 Engraftment of hematopoietic stem cells after ischemia in lethally irradiated recipients

In lethally irradiated recipients, ischemic Ly5.1 HSC competed for repopulation with simultaneously transplanted fresh Ly5.2 HSC. Chimerism of Ly5.1 cells in the peripheral blood of recipients is shown in Figure 12, p. 42 and in Tables S4-S6, Appendix 2, p. VI-VII. At 37°C (Figure 12A; Table S4, Appendix 2, p. VI), the engraftment of cells exposed to ischemia for 2 hours was comparable to that of their non-affected counterparts. At 20°C (Figure 12B; Table S5, Appendix 2, p. VII), the cells exposed to ischemia for 3 hours successfully competed with fresh ones in the short- and long-term repopulation, and those exposed to ischemia for 12 hours in the long-term repopulation. At 4°C (Figure 12C; Table S6, Appendix 2, p. VII), there was still a significant engraftment (chimerism about 30 %) even when the ischemia lasted for 48 hours.

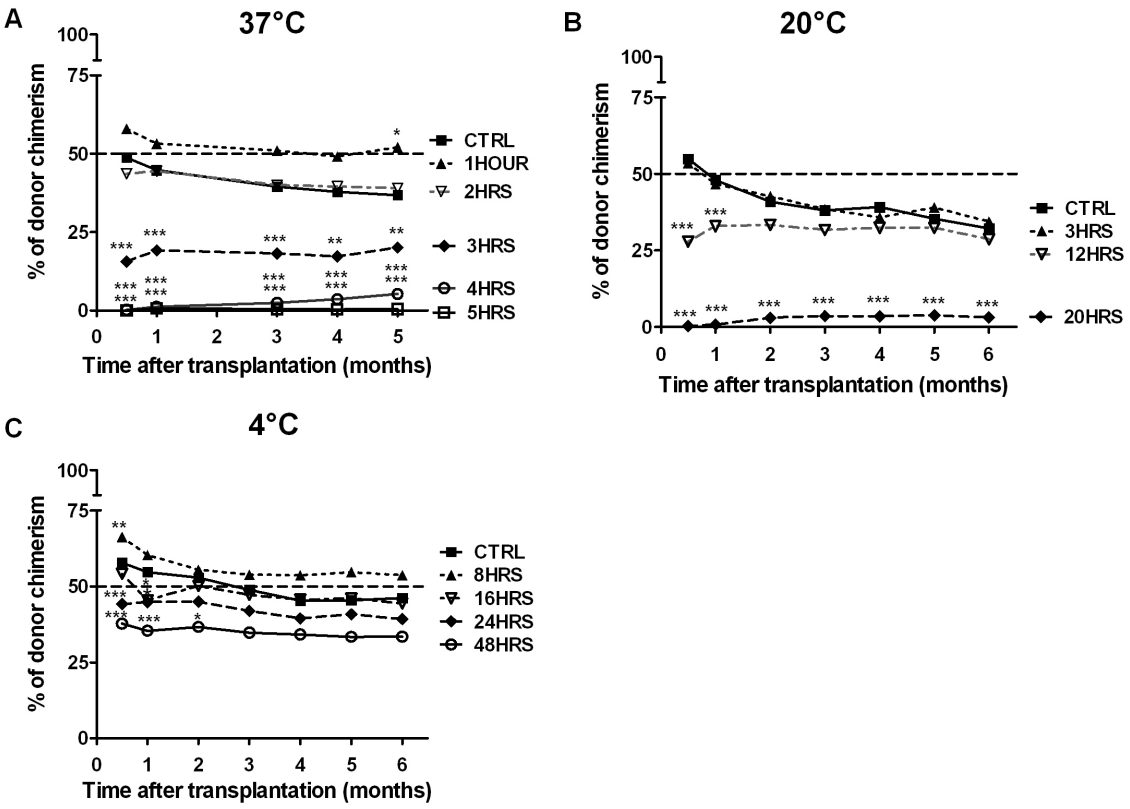

Figure 12 - Engraftment of ischemic HSC in lethally irradiated recipients (competitive transplantation). Donor cells (Ly5.1) were exposed to ischemia for up to 5 hours at 37°C **(A)**, for up to 20 hours at 20°C **(B)**, or for up to 48 hours at 4°C **(C)**. Ly5.2 recipients (n=4-17) were irradiated with 9 Gy and transplanted with a 1:1 mixture of cadaveric and fresh congenic donor bone marrow cells. The engraftment is presented as chimerism of Ly5.1+ cells (mean) detected in peripheral blood of Ly5.2 recipients. Significance of difference from the controls at individual time points: ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.1.2 Representation of LSK SLAM and LSK SP cells in ischemic bone marrow

Percentage of LSK SLAM ($Lin^{low}Sca\text{-}1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}$) and LSK SP (side population, Hoechst 33342 negative) cells in the BM after various periods of ischemia at 37°C, 20°C and 4°C is shown in Figure 13, p. 43. At 37°C (Figure 13A), the LSK SP percentage was significantly decreased already after 1 hour of ischemia. After 3 hours of ischemia at 37°C, the side-population was no longer detectable, whereas the LSK SLAM number was not significantly decreased. At 20°C (Figure 13B) and at 4°C (Figure 13C), the LSK SP and LSK SLAM populations were lost after 12 hours and 48 hours of ischemia, respectively.

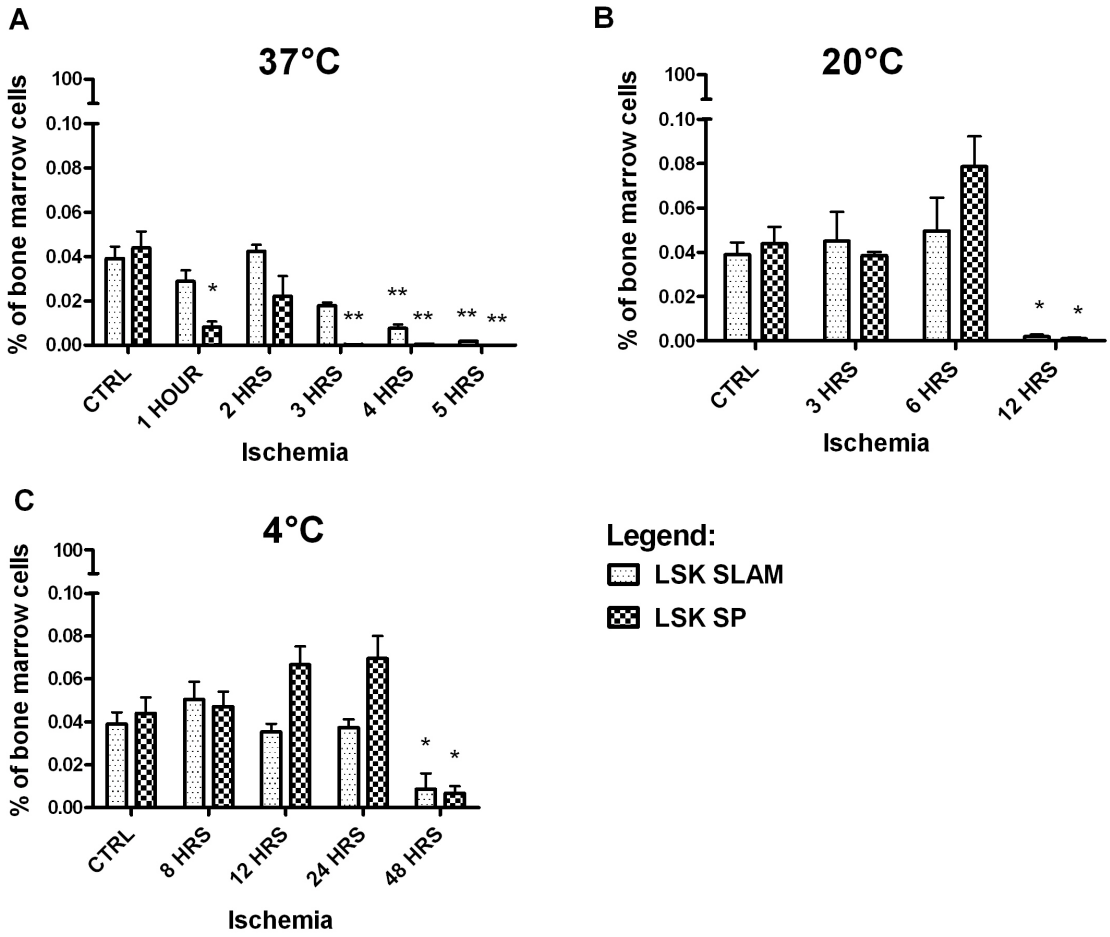

Figure 13 – Representation of LSK SLAM and LSK SP cells in ischemic bone marrow. LSK SLAM and LSK SP cells were detected in BM exposed to ischemia for up to 5 hours at 37°C **(A)**, for up to 20 hours at 20°C **(B)**, or for up to 48 hours at 4°C **(C)**. Data are presented as mean ± SEM (n=3). Significance of difference from the controls: ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.1.3 Viability of bone marrow cells during ischemia

The cellularity of BM (number of nucleated cells) was (18.5 ± 0.69) x 10^6 (mean ± SEM) cells per femur and did not significantly decrease during the periods of ischemia (Figure 14A, C, E, p. 44), as compared to the control. The percentage of live, apoptotic and dead cells after ischemia is shown in Figure 14B, D, F. After 2 hours of ischemia at 37°C, about 50 % of bone marrow cells were detected as apoptotic and dead cells and this number increased to 90 % within 5 hours (Figure 14B). At 20°C, the same increase in apoptotic and dead fractions was observed after 6 hours and 20 hours of ischemia, respectively (Figure 14D). After 48 hours of ischemia at 4°C, live cells still constituted more than 10 % of cells in the bone marrow (Figure 14F).

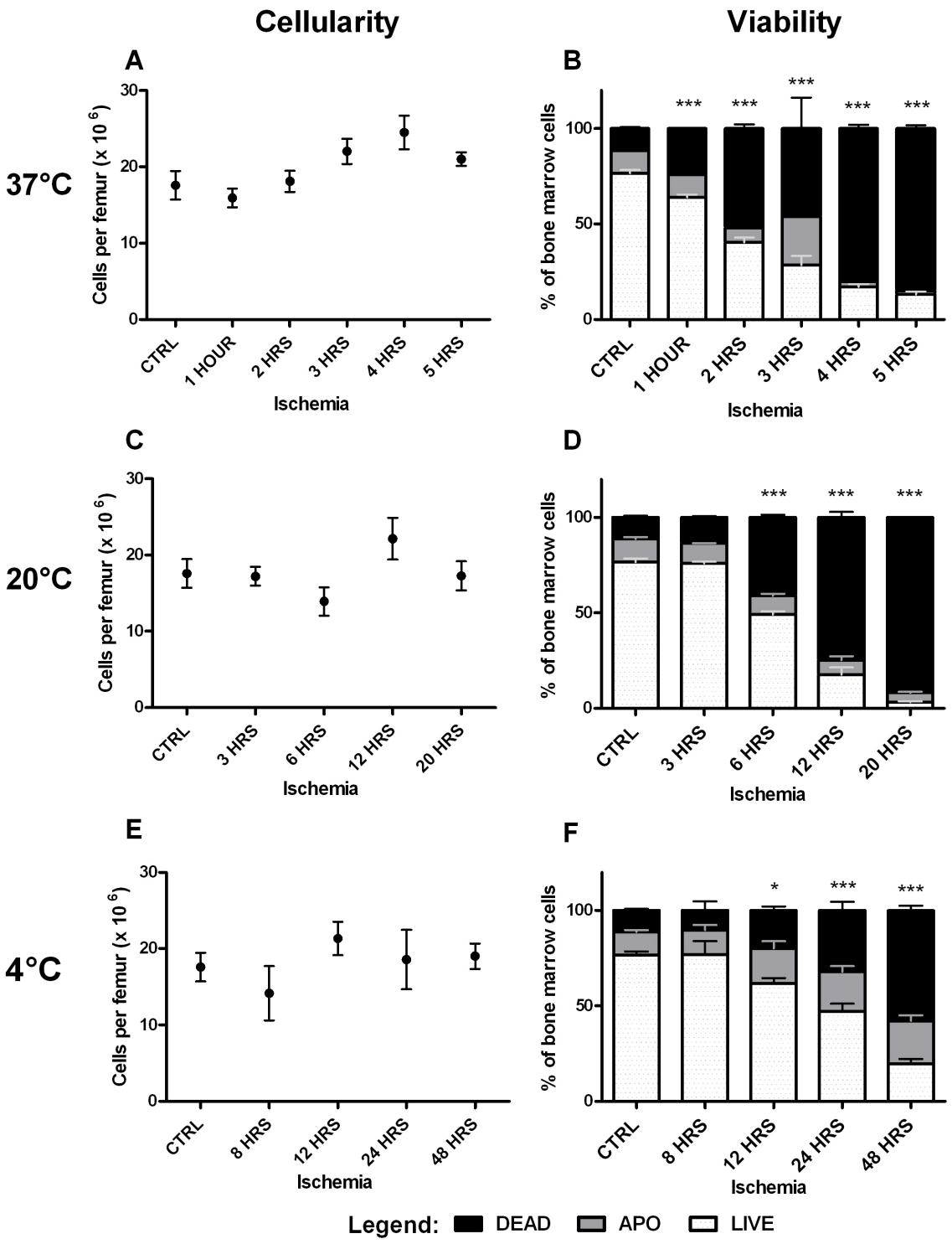

Figure 14 – **Cellularity and viability of bone marrow during ischemia.** Cellularity of the femur and ratio of live, apoptotic and dead cells was determined in BM exposed to ischemia for up to 5 hours at 37°C **(A, B)**, for up to 20 hours at 20°C **(C, D)**, or for up to 48 hours at 4°C **(E, F)**. Data are presented as mean ± SEM (n=3). Significance of difference from the controls (for live cell fraction): ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.1.3.1 Viability of bone marrow subpopulations during ischemia at 37°C

Representation of $Lin^{low}Sca\text{-}1^{+}c\text{-}Kit^{+}$ population (LSK, enriched in hematopoietic stem and progenitor cells), $B220^{+}$ population, $Ter119^{+}$ population, and $Gr\text{-}1^{+}Mac\text{-}1^{+}$ population in the bone marrow after ischemia at 37°C, and the amount of live, apoptotic and dead cells within these populations are shown in Figure 15. A noticeable decline in the amount of live cells was detected in the LSK population after 3 hours of ischemia (Figure 15A), in the $B220^{+}$ population after 1 hour of ischemia (Figure 15B), and in the $Ter119^{+}$ and $Gr\text{-}1^{+}Mac\text{-}1^{+}$ populations after 2 hours of ischemia (Figure 15C, D).

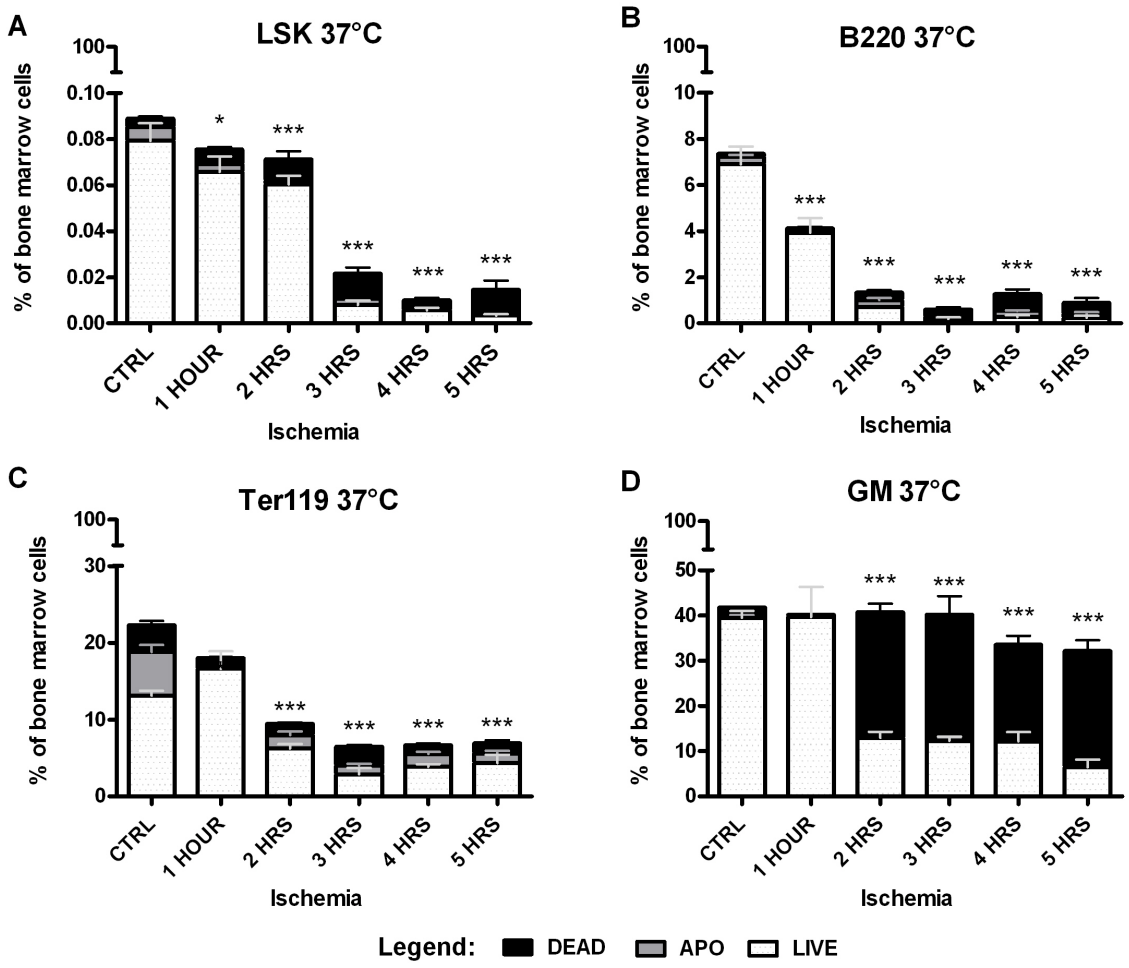

Figure 15 – Viability of bone marrow subpopulations during ischemia at 37°C. BM was exposed to ischemia for up to 5 hours at 37°C. Ratio of live, apoptotic and dead cells was determined in LSK population **(A)**, in B220+ (B220) population **(B)**, in Ter119+ (Ter119) population **(C)**, and in Gr-1+Mac-1+ (GM) population **(D)**. Data are presented as mean ± SEM (n=3). Significance of difference from the controls (for live cell fraction): ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.1.3.2 Viability of bone marrow subpopulations during ischemia at 20°C

Representation of LSK population, B220$^+$ population, Ter119$^+$ population and Gr-1$^+$Mac-1$^+$ population after ischemia at 20°C, and the ratio of live, apoptotic and dead cells within these populations are shown in Figure 16. A significant decrease in frequency of live cells was detected after 6 hours of ischemia in the LSK population (Figure 16A), in the B220$^+$ population (Figure 16B) and in the Gr-1$^+$Mac-1$^+$ population (Figure 16D), and after 12 hours in the Ter119$^+$ population (Figure 16C). After 20 hours of ischemia, the B220$^+$, Ter119$^+$ and Gr-1$^+$Mac-1$^+$ cells were not detectable according to the phenotype anymore.

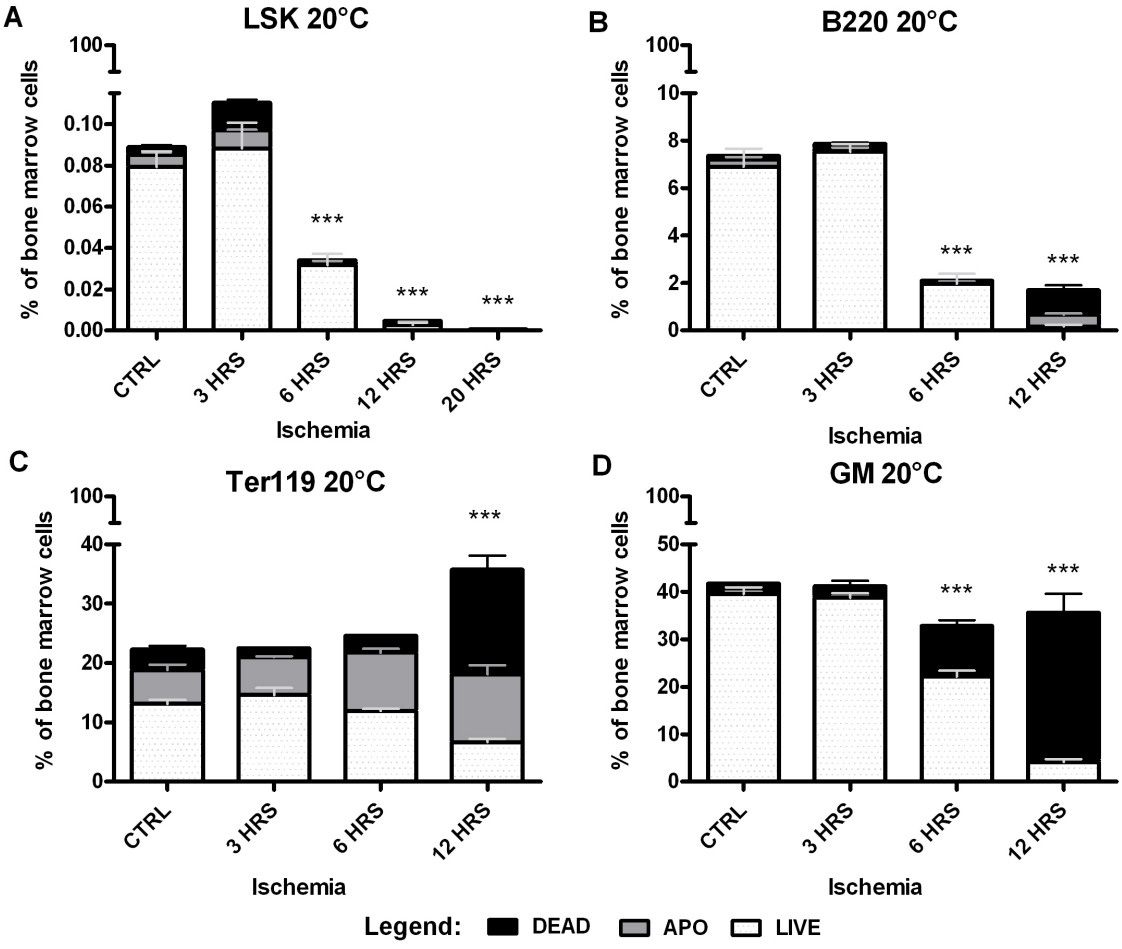

Figure 16 - Viability of bone marrow subpopulations during ischemia at 20°C. BM was exposed to ischemia for up to 20 hours at 20°C. Ratio of live, apoptotic and dead cells was determined in LSK population **(A)**, in B220+ (B220) population **(B)**, in Ter119+ (Ter119) population **(C)**, and in Gr-1+Mac-1+ (GM) population **(D)**. Data are presented as mean ± SEM (n=3). Significance of difference from the controls (for live cell fraction): ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.1.3.3 Viability of bone marrow subpopulations during ischemia at 4°C

Frequencies of LSK population, $B220^+$ population, $Ter119^+$ population and $Gr\text{-}1^+Mac\text{-}1^+$ population in the bone marrow after various periods (0 – 48 hours) of ischemia at 4°C, and the amount of live, apoptotic and dead cells within these populations are presented in Figure 17. A decline in the amount of live cells was detected after 12 hours in the LSK and $Gr\text{-}1^+Mac\text{-}1^+$ populations (Figure 17A, D) and after 24 hours in the $B220^+$ population (Figure 17B). No decline was observed in the $Ter119^+$ population (Figure 17C).

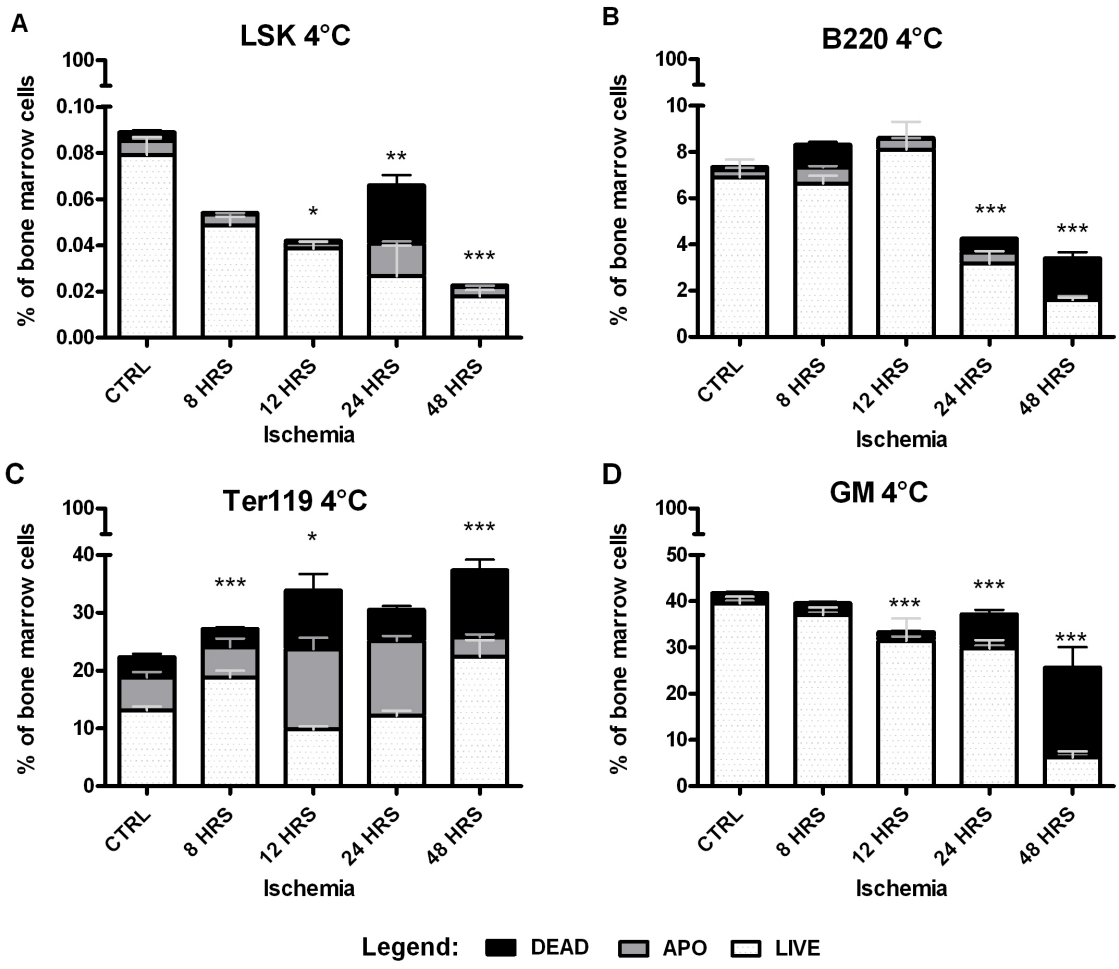

Figure 17 - Viability of bone marrow subpopulations during ischemia at 4°C. BM was exposed to ischemia for up to 48 hours at 4°C. Ratio of live, apoptotic and dead cells was determined in LSK population **(A)**, in B220+ (B220) population **(B)**, in Ter119+ (Ter119) population **(C)**, and in Gr-1+Mac-1+ (GM) population **(D)**. Data are presented as mean ± SEM (n=3). Significance of difference from the controls (for live cell fraction): ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.1.4 Influence of femur manipulation on the effects of ischemia

To exclude any impact of the femur manipulation on our results, we compared the effects of ischemia on bone marrow cells harvested 1) from intact femurs removed from the body and exposed to ischemia or 2) from femurs left in the body exposed to ischemia, and removed afterwards. Three-hour period of ischemia at 37°C was used for the comparison. Declines in frequencies of LSK SLAM and LSK SP populations (Figure 18A) as well as the repopulating ability of ischemic HSC (Figure 18B; Table S7, Appendix 2, p. VIII) were almost identical in both samples.

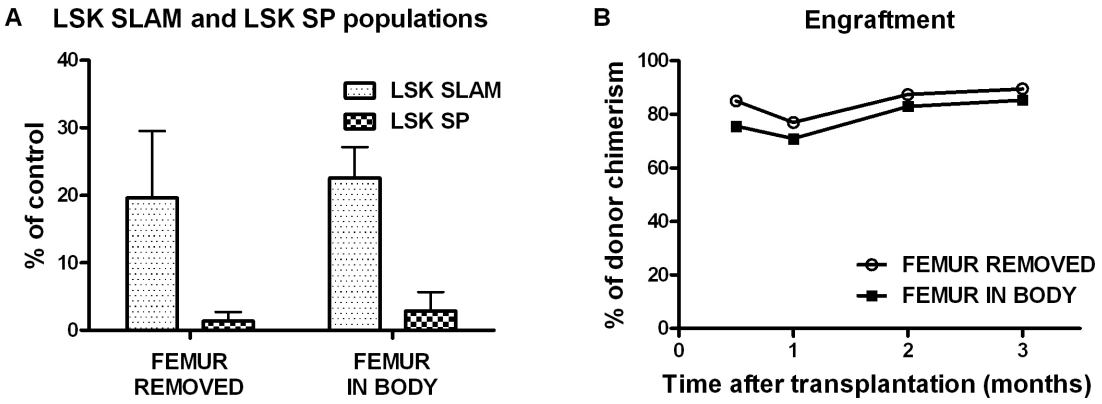

Figure 18 - Influence of femur manipulation on hematopoietic stem cells exposed to ischemia at 37°C for 3 hours. Decline in frequencies of LSK SLAM and LSK SP cells in bone marrow, shown as a percentage of control **(A)**. Engraftment of HSC in sublethally irradiated recipients **(B)**. **A**: Data are presented as mean ± SEM (n=3). Significance of difference from the controls: ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$. **B**: Donor cells (Ly5.1) were exposed to ischemia at 37°C in femurs removed from the body (femur removed) or in femurs left in the body (femur in body). Ly5.2 recipients (n=5) were irradiated with 6 Gy and transplanted with half of the donor femur. The engraftment is presented as chimerism of Ly5.1+ cells (mean) detected in peripheral blood of Ly5.2 recipients. Significance of difference from the controls at individual time points: ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$. There were no significant differences between the samples.

Average cellularity of femurs was (24.1 ± 1.59) x 10^6 (mean ± SEM). No differences between the experimental conditions were observed in the cellularity of bone marrow or in the fraction of dead cells in the BM and in the LSK population (Figure 19).

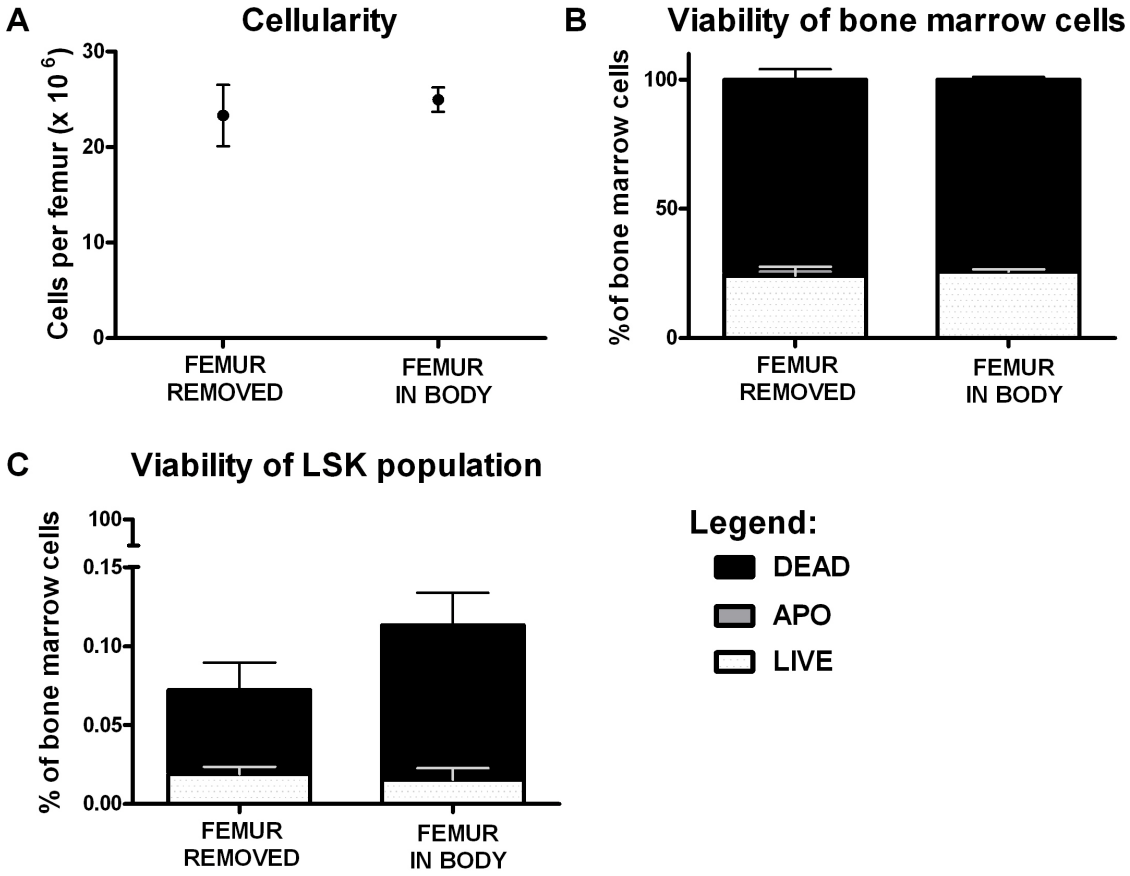

Figure 19 – Influence of femur manipulation on viability of bone marrow cells. Cellularity of bone marrow after 3 hours of ischemia at 37°C in femur removed from the body (femur removed) and in femur left in the body during ischemia (femur in body) **(A)**, percentage of live, apoptotic and dead cells in BM after 3 hours of ischemia at 37°C in femur removed from the body and in femur left in the body during ischemia **(B)**, frequency of LSK population in BM, and ratio of live, apoptotic and dead cells in LSK population after 3 hours of ischemia at 37°C in femur removed from the body and in femur left in the body during ischemia **(C)**. Data are presented as mean ± SEM (n=3). Significance of difference from the controls (for live cell fraction): ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$. There were no significant differences between the samples.

4.2 Effects of ischemia on p53$^{-/-}$ bone marrow

Intact femurs removed from sacrificed C57Bl/6J p53$^{-/-}$ mice were maintained for 0, 2, 4 or 6 hours at 37°C. Afterwards, bone marrow cells were harvested and tested for repopulating ability, frequency of HSC, and viability of bone marrow subpopulations.

4.2.1 Transplantability of p53$^{-/-}$ hematopoietic stem cells after ischemia at 37°C

The p53$^{-/-}$ bone marrow cells after various periods of ischemia were transplanted to sublethally (6 Gy) irradiated congenic recipients. Engraftment of the donor HSC was followed up in the peripheral blood of the recipients for 3 months. After 2 hours of ischemia, the repopulating ability of long-term repopulating cells was not significantly changed (Figure 20; Table S8, Appendix 2, p. VIII). After 4 hours or 6 hours of ischemia, the p53$^{-/-}$ HSC completely lost their short-term repopulating ability, whereas the long-term repopulating ability was partially preserved after the four-hour period.

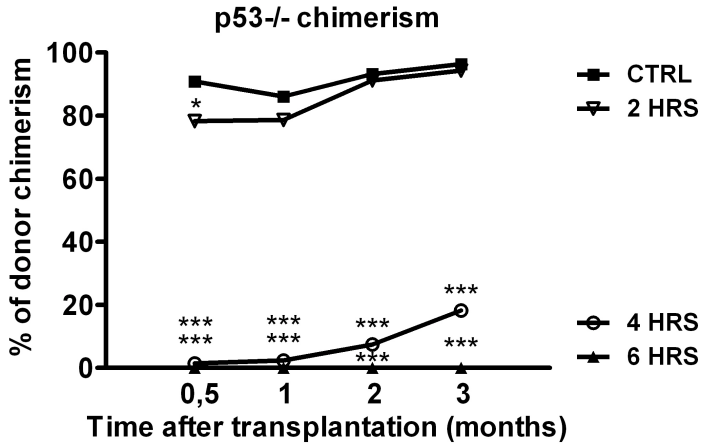

Figure 20 - Engraftment of ischemic p53-/- HSC in sublethally irradiated recipients. Donor cells (Ly5.2, p53-/-) were exposed to ischemia at 37°C for up to 6 hours. Ly5.1 recipients (n=4-5) were irradiated with 6 Gy and transplanted with half of the donor femur. Representation of donor-derived cells is presented as chimerism of p53-/- Ly5.2+ cells (mean) detected in peripheral blood of Ly5.1 recipients. Significance of difference from the controls at individual time points: ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.2.2 Representation of LSK SLAM and LSK SP cells in p53-/- bone marrow during ischemia at 37°C

Representation of the HSC defined as LSK SLAM ($Lin^{low}Sca\text{-}1^{+}c\text{-}Kit^{+}CD150^{+}CD48^{-}$) and LSK SP (side population, Hoechst 33342 negative) cells in the $p53^{-/-}$ bone marrow after 0, 2, 4 and 6 hours of ischemia at 37°C is presented in Figure 21. Frequency of the LSK SP population decreased after 2 hours. After 4 hours, LSK SLAM frequency also declined and the LSK SP cells were no more detectable. After 6 hours of ischemia, both the LSK SLAM and the LSK SP cells completely lost their phenotypes.

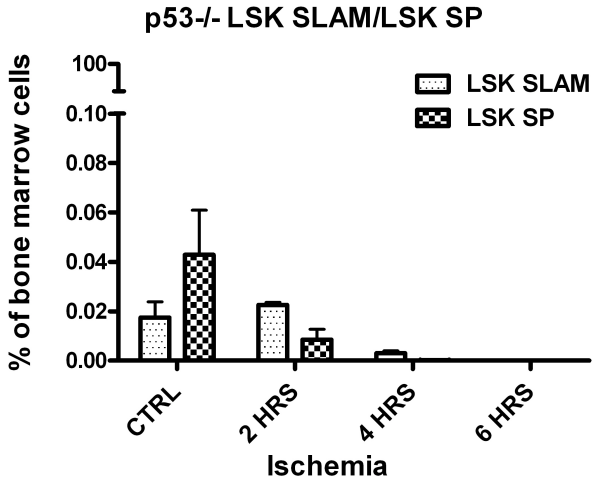

Figure 21 – Representation of LSK SLAM and LSK SP cells in p53-/- ischemic bone marrow. LSK SLAM and LSK SP cells were detected in p53-/- BM exposed to ischemia for up to 6 hours at 37°C. Data are presented as mean ± SEM (n=3). Significance of difference from the controls: ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.2.3 Viability of p53$^{-/-}$ bone marrow cells during ischemia at 37°C

Cellularity of p53$^{-/-}$ bone marrow after various periods of ischemia at 37°C is presented in Figure 22, together with the ratio of live, apoptotic and dead cells in p53$^{-/-}$ bone marrow as a whole. Average cellularity of the femur was (27.4 ± 1.12) x 10^6 (mean ± SEM) and there was no decrease caused by ischemia in the number of cells during the studied time periods (**Figure 22**A). After 2 hours of ischemia, fraction of apoptotic and dead cells was about 50 % in bone marrow, and increased to about 85 % after 4 hours and 6 hours of ischemia (**Figure 22**B).

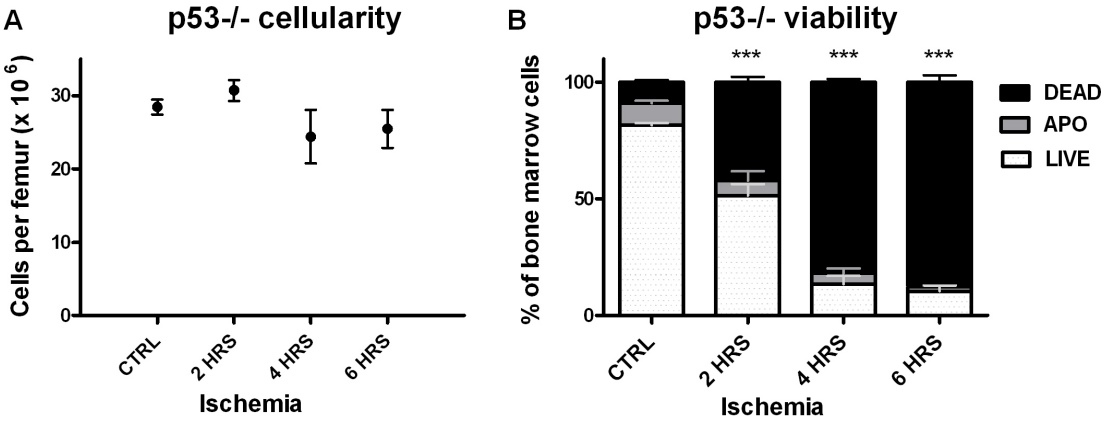

Figure 22 – Cellularity and viability of p53-/- bone marrow during ischemia at 37°C. Cellularity **(A)**, and ratio of live, apoptotic and dead cells **(B)** were determined in p53-/- BM exposed to ischemia at 37°C for up to 6 hours. Data are presented as mean ± SEM (n=3). Significance of difference from the controls (for the live cell fraction): ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.2.3.1 Viability of p53$^{-/-}$ bone marrow subpopulations during ischemia at 37°C

Frequencies of LSK population, B220$^+$ population, Ter119$^+$ population and Gr-1$^+$Mac-1$^+$ population in the p53$^{-/-}$ bone marrow as well as the ratio of live, apoptotic and dead cells after ischemia at 37°C are shown in Figure 23, p. 53. The p53$^{-/-}$ LSK cells remained alive for 2 hours of ischemia, became all dead after 4 hours of ischemia, and were completely depleted after 6 hours of ischemia at 37°C (Figure 23A). Frequency of the p53$^{-/-}$ B220$^+$ population declined as the period of ischemia prolonged. After 4 hours, there were no live cells in the B220$^+$ population and after 6 hours, B220$^+$ phenotype was barely detectable (Figure 23B). The p53$^{-/-}$ Ter119$^+$ population did not change significantly in frequency or in the ratio of live, apoptotic, and dead cells up to 6 hours of ischemia (Figure 23C). In the p53$^{-/-}$ Gr-1$^+$Mac-1$^+$ population, a third of the cells became dead after 2 hours of ischemia

at 37°C. After 4 hours and 6 hours of ischemia, dead cells covered almost the whole Gr-1^{+}Mac-1^{+} population (Figure 23D).

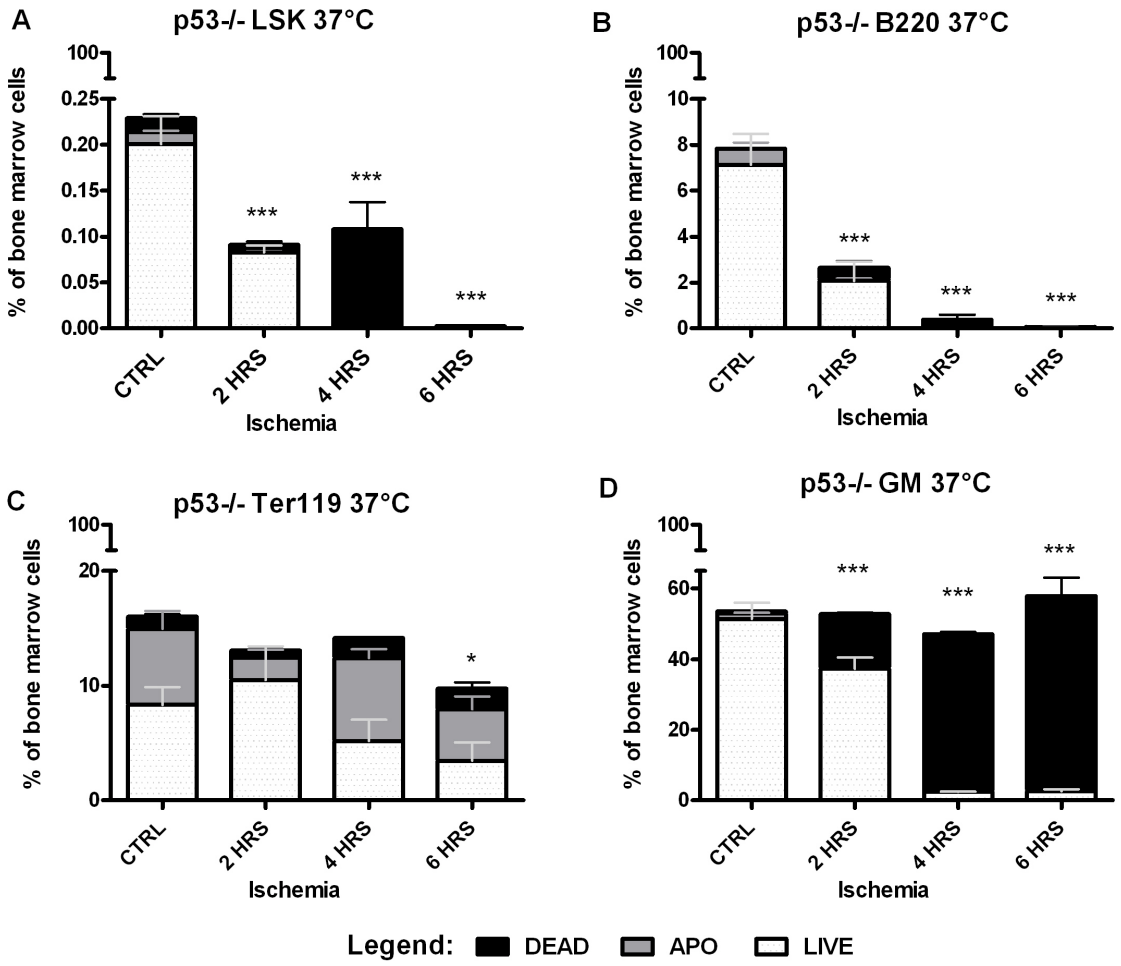

Figure 23 – Viability of p53-/- bone marrow subpopulations during ischemia at 37°C. p53-/- BM was exposed to ischemia for up to 6 hours at 37°C. Ratio of live, apoptotic and dead cells was determined in LSK population **(A)**, in B220+ (B220) population **(B)**, in Ter119+ (Ter119) population **(C)**, and in Gr-1+Mac-1+ (GM) population **(D)**. Data are presented as mean ± SEM (n=3). Significance of difference from the controls (for live cell fraction): ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.3 Effects of metabolic inhibition on bone marrow cells

Bone marrow cells were flushed to the medium with/without KCN, 2-deoxy-D-glucose (2-DG), KCN and 2-DG, or D-glucose (glc). D-glucose was used as a comparative treatment for 2-DG. After 2 and 20 hours of incubation at 37°C, repopulating ability of the hematopoietic stem cells, frequencies of LSK SLAM and LSK SP cells, and viability of bone marrow were determined.

4.3.1 Transplantability of hematopoietic stem cells after metabolic inhibition

Figure 24, p. 55 and Table S9, Appendix 2, p. IX show the repopulating ability of short-term and long-term repopulating hematopoietic stem cells after the incubation in medium with/without metabolic inhibitors, transplanted to sublethally irradiated (6 Gy) congenic recipients. After the incubation without inhibitors, the engraftment of HSC was not decreased, as compared to control (Figure 24A). KCN influenced the long-term repopulating ability of HSC only after 20 hours when the 10 mM concentration of KCN was used. Short-term repopulating ability (14 days after transplantation) was declined also after twenty-hour inhibition by 1 mM KCN (Figure 24B). 2-deoxy-D-glucose caused a decline in the engraftment already after 2 hours of incubation (Figure 24C). The combined inhibition by 10 mM KCN and 50 mM 2-deoxy-D-glucose completely destroyed the short-term as well as the long-term repopulating ability of HSC after 2 hours as well as after 20 hours (Figure 24D).

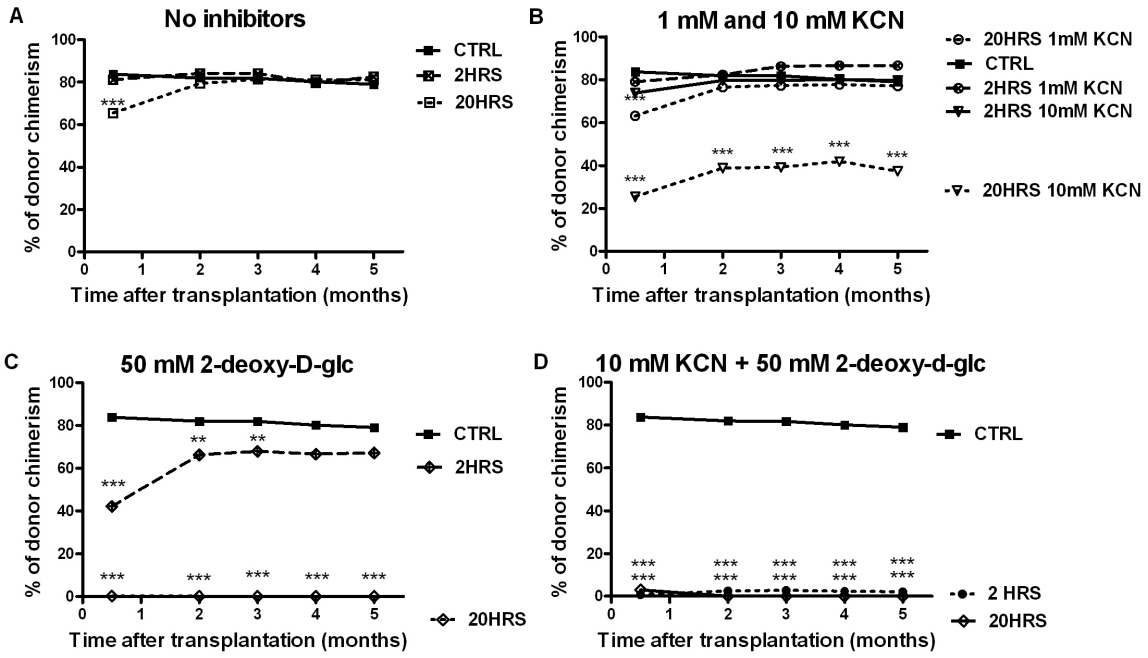

Figure 24 - Engraftment of hematopoietic stem cells after metabolic inhibition in sublethally irradiated recipients. Donor cells (Ly5.1) were transplanted after 0 (CTRL), 2 and 20 hours of incubation in medium without inhibitors **(A)**, with 1mM or 10 mM KCN **(B)**, with 50 mM 2-deoxy-D-glucose (2-DG) **(C)**, or with 10 mM KCN and 50 mM 2-DG **(D)**. Ly5.2 recipients (n=4-14) were irradiated with 6 Gy and transplanted with half of the donor femur. The engraftment is presented as chimerism of Ly5.1+ cells (mean) detected in peripheral blood of Ly5.2 recipients. Significance of difference from the controls at individual time points: ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.3.2 Representation of LSK SLAM and LSK SP cells after metabolic inhibition

Frequency of the HSC identified as LSK SLAM and LSK SP cells in the bone marrow after inhibition of metabolism is shown in Figure 25, p. 56. The incubation of bone marrow cells in medium without metabolic inhibitors influenced neither the LSK SLAM nor the LSK SP population (Figure 25A). After two-hour or twenty-hour inhibition by 1 mM or 10 mM KCN, there was no decline in the LSK SLAM population. Frequency of the LSK SP cells decreased significantly after 20 hours of the inhibition by 10 mM KCN (Figure 25B). Two-hour incubation with 50 mM D-glucose (glc) or 50 mM 2-deoxy-D-glucose (2-DG) decreased neither the LSK SLAM nor the LSK SP numbers. The LSK SP population declined after twenty-hour incubation with D-glucose, whereas the LSK SLAM population remained unchanged. Twenty-hour inhibition by 2-deoxy-D-glucose caused a significant decline in both LSK SLAM and LSK SP populations (Figure 25C). Two-hour as well as twenty-hour inhibition by the combination of 10 mM KCN and 50 mM 2-deoxy-D-glucose resulted in complete depletion of both LSK SLAM and LSK SP cells (Figure 25D).

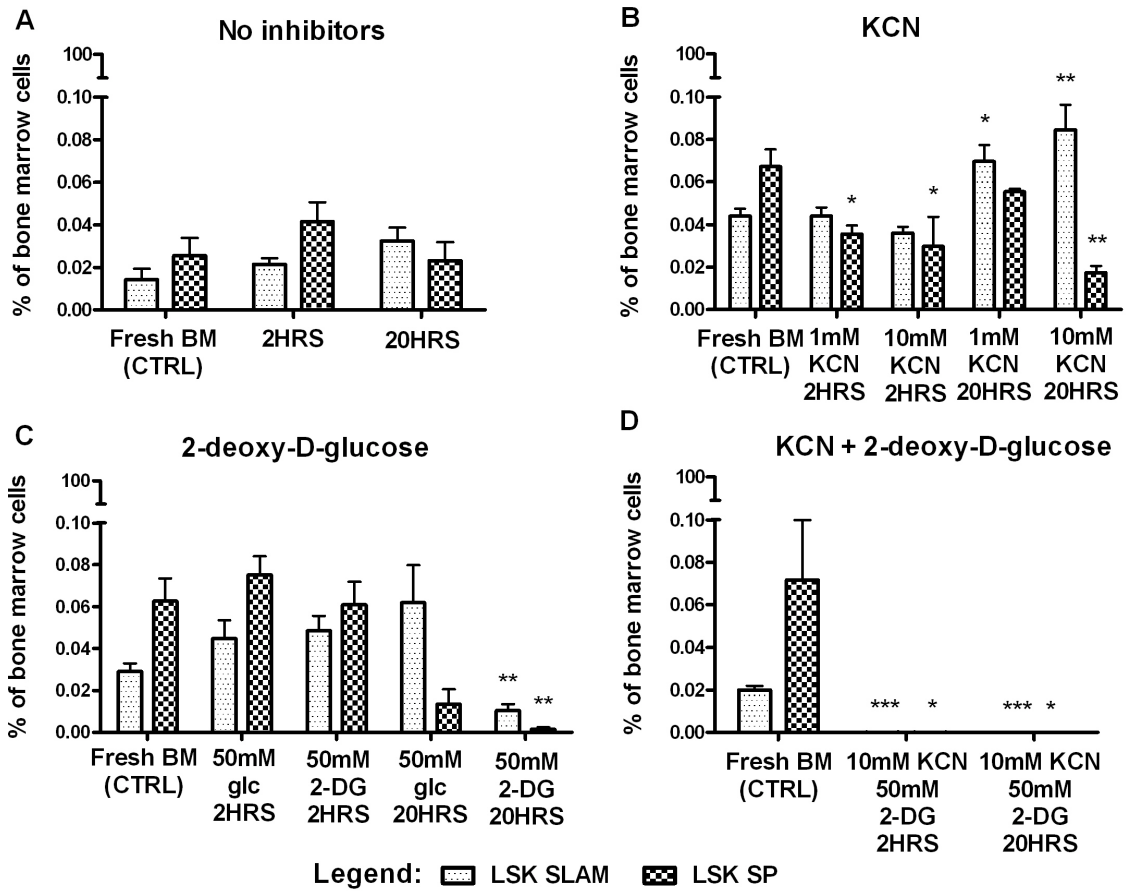

Figure 25 – Representation of LSK SLAM and LSK SP cells in bone marrow after metabolic inhibition. Frequencies of LSK SLAM and LSK SP cells in BM were detected after 0 (CTRL), 2 and 20 hours of incubation in medium without inhibitors **(A)**, with 1mM or 10 mM KCN **(B)** with 50 mM glucose (glc) or 50 mM 2-deoxy-D-glucose **(C)** or with 10 mM KCN and 50 mM 2-deoxy-D-glucose **(D)**. Data are presented as mean ± SEM (n=3). Significance of difference from the controls: ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.3.3 Viability of bone marrow cells exposed to metabolic inhibition

Ratio of live, apoptotic and dead cells in the bone marrow after the inhibition of electron transport chain by KCN and/or after the inhibition of glycolysis by 2-deoxy-D-glucose was determined in the whole BM, in LSK population, B-lymphopoietic population ($B220^+$), granulo-monopoietic population ($Gr\text{-}1^+Mac\text{-}1^+$) and erythropoietic population ($Ter119^+$).

The average cellularity of the femurs before incubations was (27.07 ± 0.72) $x10^6$ (mean ± SEM). The incubation without metabolic inhibitors did not cause any significant changes neither in the number of cells nor in the ratio of live, apoptotic and dead bone marrow cells (Figure 26A, B, p. 58). After two-hour and twenty-hour incubation with either 1mM or 10 mM KCN, the number of cells was significantly decreased only after 20 hours (Figure 26C). An increase in the apoptotic and dead fraction was observed in all samples incubated with KCN (Figure 26D). After incubation with 50 mM 2-deoxy-D-glucose (2-DG) as well as with 50 mM D-glucose (glc), the cellularity was decreased similarly in both two-hour and twenty-hour intervals (Figure 26E). The fraction of apoptotic and dead cells was significantly increased in all samples, as compared to control; the highest increase in apoptotic/dead cell fraction was observed after 20 hours with 50 mM 2-deoxy-D-glucose (Figure 26F). After the combined inhibition with 10 mM KCN and 50 mM 2-deoxy-D-glucose, the cellularity of bone marrow as well as the number of live cells were significantly decreased already after 2 hours (Figure 26G, H). After 20 hours, just the apoptotic and dead cells were detected in the bone marrow.

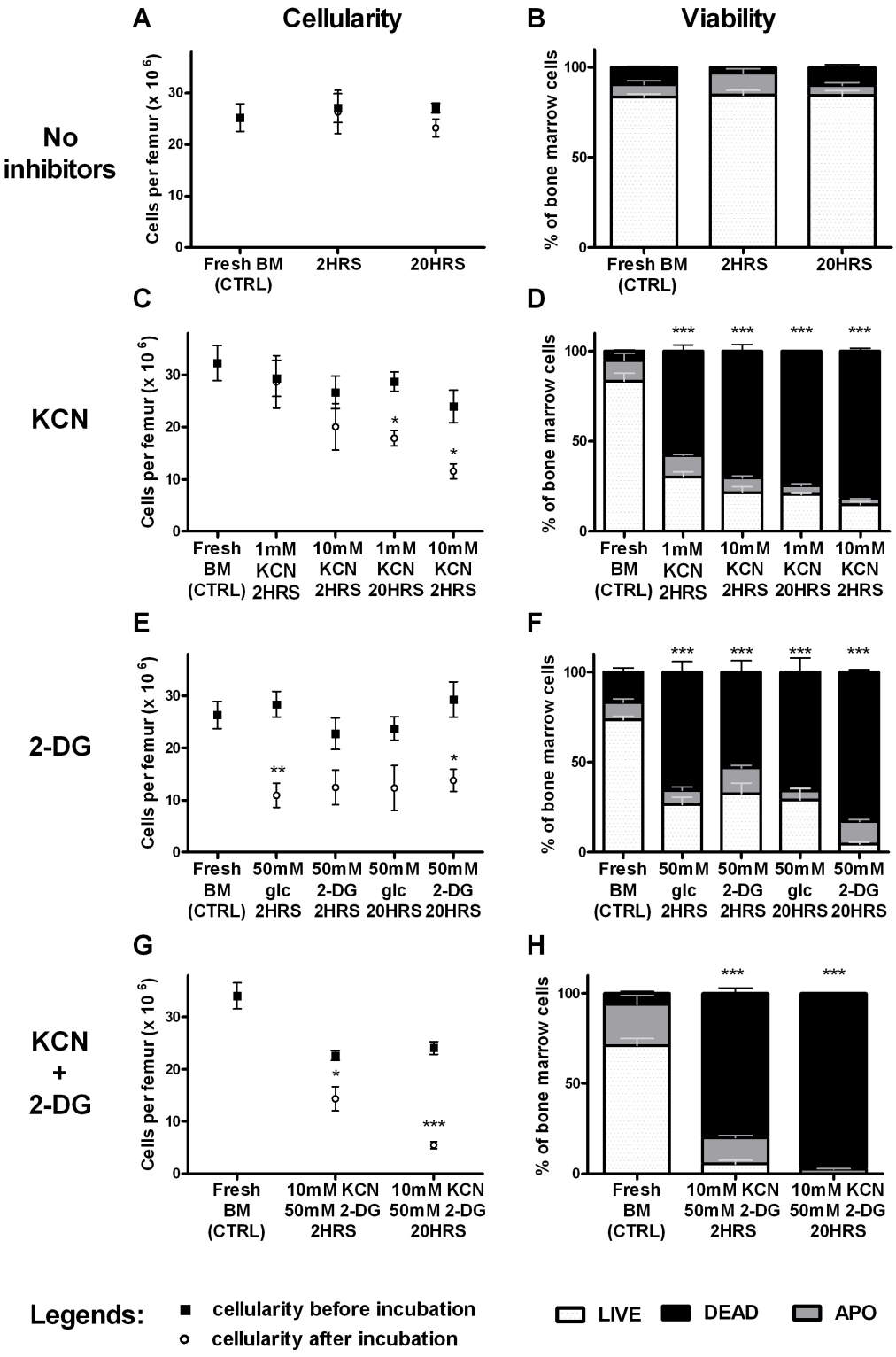

Figure 26 – Cellularity and viability of bone marrow cells after metabolic inhibition. Cellularity of bone marrow before and after incubation, and ratio of live, apoptotic and dead cells after 0 (CTRL), 2 and 20 hours of incubation in medium without inhibitors **(A, B)**, with 1mM or 10 mM KCN **(C, D)** with 50 mM glucose (glc) or 50 mM 2-deoxy-D-glucose (2-DG) **(E, F)**, or with 10 mM KCN and 50 mM 2-DG **(G, H)**. Data are presented as mean ± SEM (n=3). Cellularity declines are included in dead cell fractions. Significance of difference from the controls (for the cellularity decline and for live cell fraction): ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.3.3.1 Viability of bone marrow subpopulations during incubation in medium without metabolic inhibitors

Frequencies of LSK population, $B220^+$ population, $Ter119^+$ population and $Gr\text{-}1^+Mac\text{-}1^+$ population, and viability of these populations after the incubation in medium without metabolic inhibitors are presented in Figure 27. There were no significant changes in any population after two-hour or twenty-hour incubation, apart from the $Ter119^+$ population. The number of bone marrow cells with Ter119 phenotype decreased sharply after 20 hours of the incubation (Figure 27C).

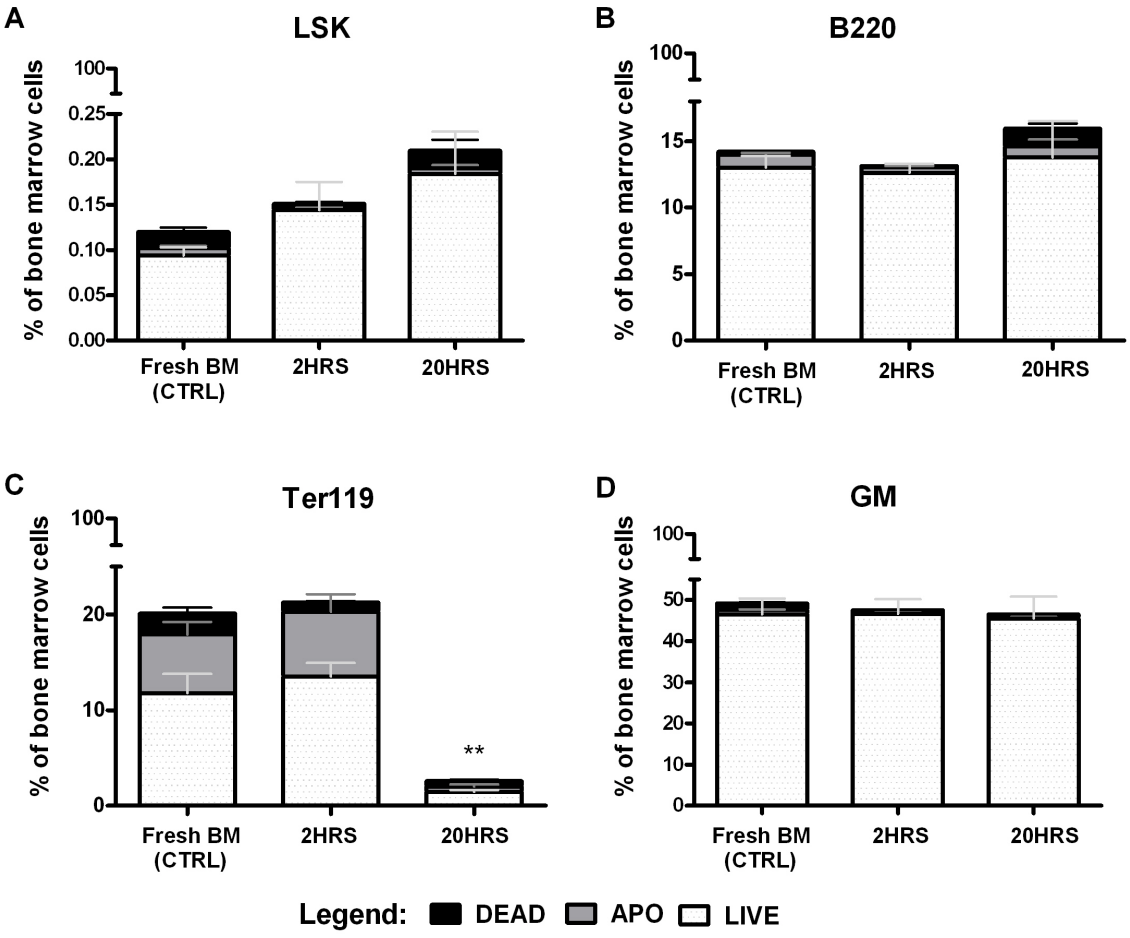

Figure 27 – Viability of bone marrow subpopulations after incubation in medium without metabolic inhibitors. Ratio of live, apoptotic and dead cells after 0 (CTRL), 2 and 20 hours of incubation in LSK population **(A)**, in B220+ (B220) population **(B)**, in Ter119+ (Ter119) population **(C)**, and in Gr-1+Mac-1+ (GM) population **(D)**. Data are presented as mean ± SEM (n=3). Significance of difference from the controls (for live cell fraction): ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.3.3.2 Viability of bone marrow subpopulations during KCN inhibition

Frequencies of bone marrow subpopulations and the ratio of live, apoptotic and dead cells within these populations after 2 hours and 20 hours of the inhibition with 1 mM KCN or 10 mM KCN are shown in Figure 28. In the LSK population (Figure 28A) and in the Gr-1$^+$Mac-1$^+$ population (Figure 28D), no significant changes were detected. In the B220$^+$ population, fractions of apoptotic and dead cells were increased to almost 90 % after 10 mM KCN (Figure 28B). Approximately 50 % of the Ter119$^+$ cells were apoptotic/dead in the control sample and this fraction rose to about 90 % after 20 hours of the incubation with 1 mM KCN or 10 mM KCN (Figure 28C).

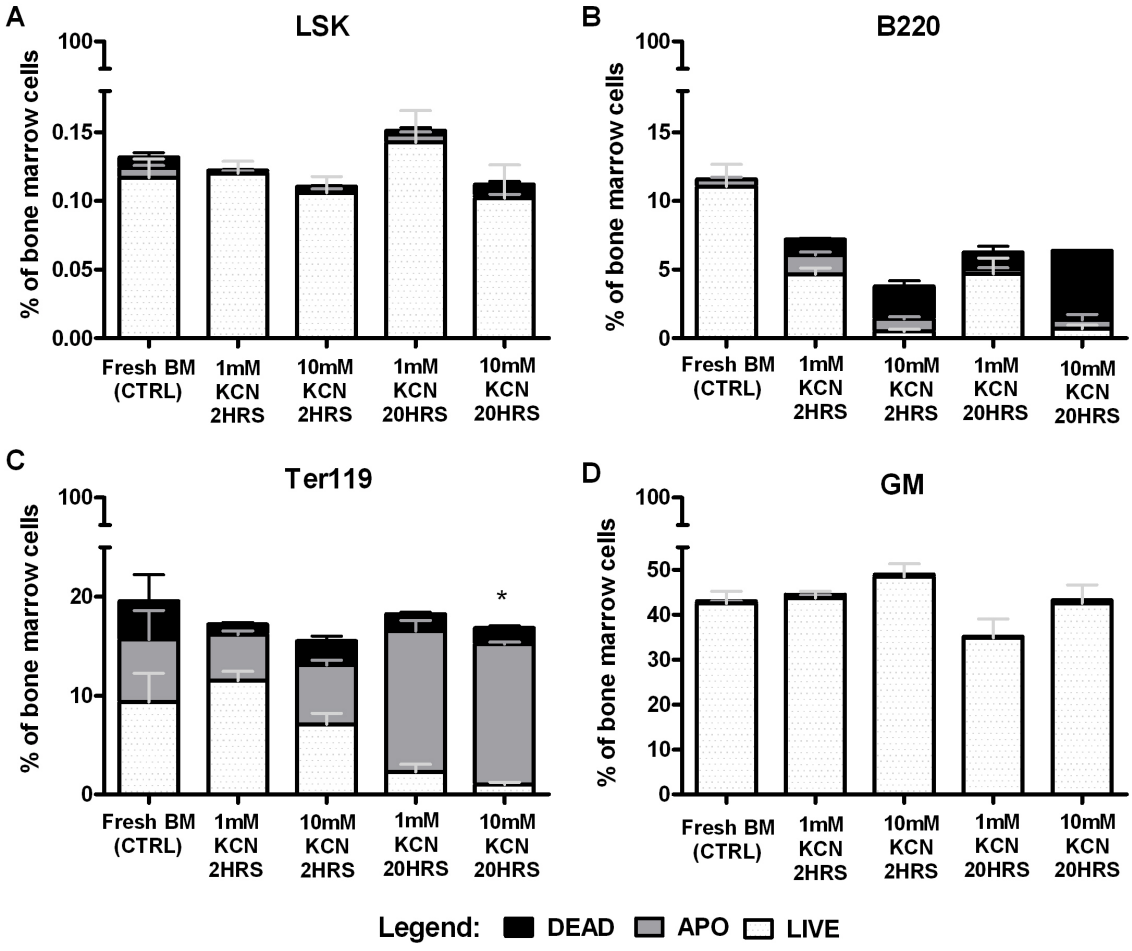

Figure 28 – Viability of bone marrow subpopulations during KCN inhibition. Ratio of live, apoptotic and dead cells after 0 (CTRL), 2 and 20 hours of KCN inhibition in LSK population **(A)**, in B220+ (B220) population **(B)**, in Ter119+ (Ter119) population **(C)**, and in Gr-1+Mac-1+ (GM) population **(D)**. Data are presented as mean ± SEM (n=3). Significance of difference from the controls (for live cell fraction): ***P < 0.005, **P < 0.01, *P < 0.05.

4.3.3.3 Viability of bone marrow subpopulations during 2-deoxy-D-glucose inhibition

Viability of bone marrow subpopulations after two-hour and twenty-hour incubation in medium with 50 mM D-glucose or with 50 mM 2-deoxy-D-glucose (2-DG) is presented in Figure 29. Frequencies of all the populations as well as viability of the cells were significantly decreased after 20 hours of the incubation with 2-deoxy-D-glucose. Moreover, the LSK phenotype was no more detectable at this time point (Figure 29A). The $B220^+$, $Ter119^+$ and $Gr\text{-}1^+Mac\text{-}1^+$ cells were detectable but mostly apoptotic or dead (Figure 29B, C, D). In the $Ter119^+$ population, the decline was registered also after twenty-hour incubation with D-glucose.

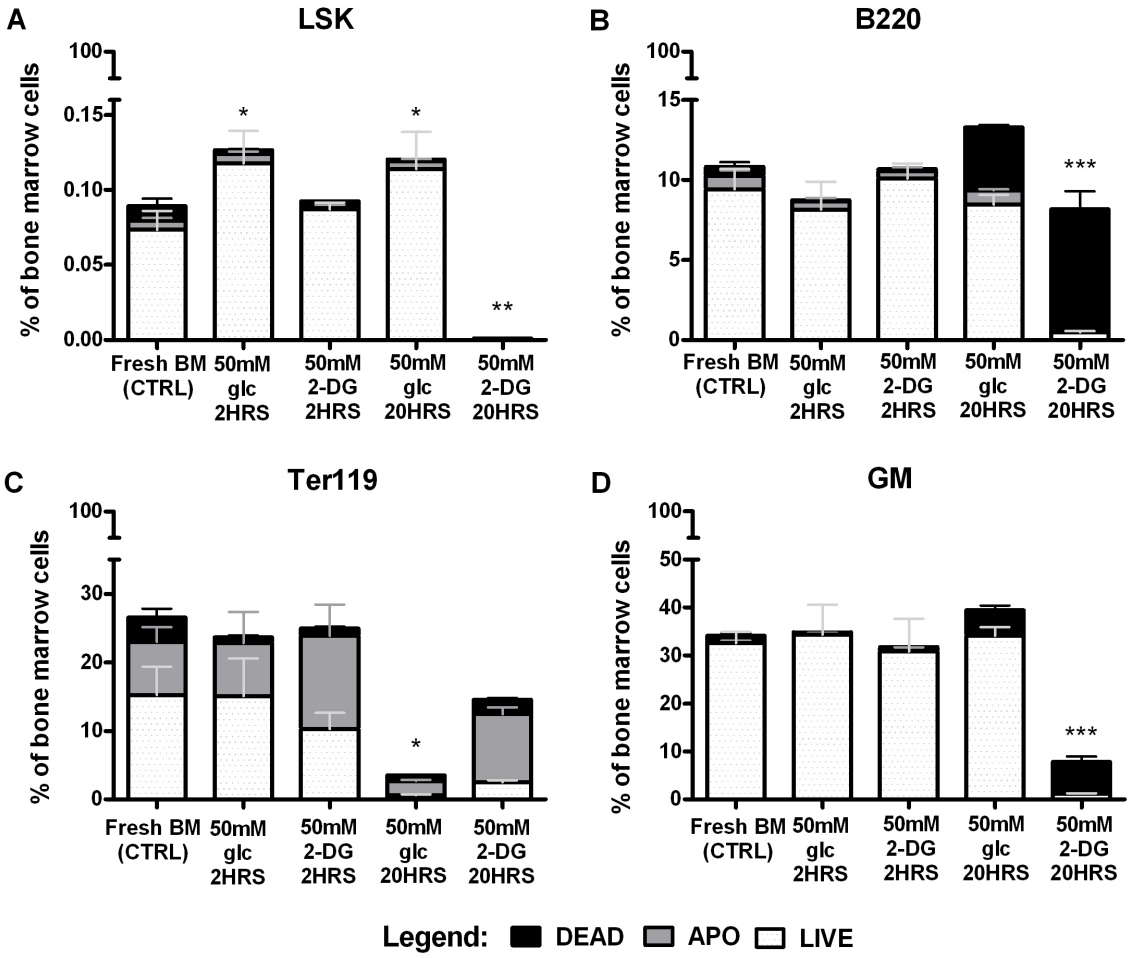

Figure 29 – Viability of bone marrow subpopulations during 2-deoxy-D-glucose inhibition. Ratio of live, apoptotic and dead cells after 0 (CTRL), 2 and 20 hours of incubation with D-glucose or 2-deoxy-D-glucose in LSK population **(A)**, in B220+ (B220) population **(B)**, in Ter119+ (Ter119) population **(C)**, and in Gr-1+Mac-1+ (GM) population **(D)**. Data are presented as mean ± SEM (n=3). Significance of difference from the controls (for the live cell fraction): ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.3.3.4 Viability of bone marrow subpopulations during combined inhibition by KCN and 2-DG

Frequencies of bone marrow subpopulations as well as viability of these populations are shown in Figure 30. Both two-hour and twenty-hour intervals caused depletion of live cells in the LSK, $B220^{+}$ and $Ter119^{+}$ populations (Figure 30A, B, C). The $Gr\text{-}1^{+}Mac\text{-}1^{+}$ population declined gradually (Figure 30D).

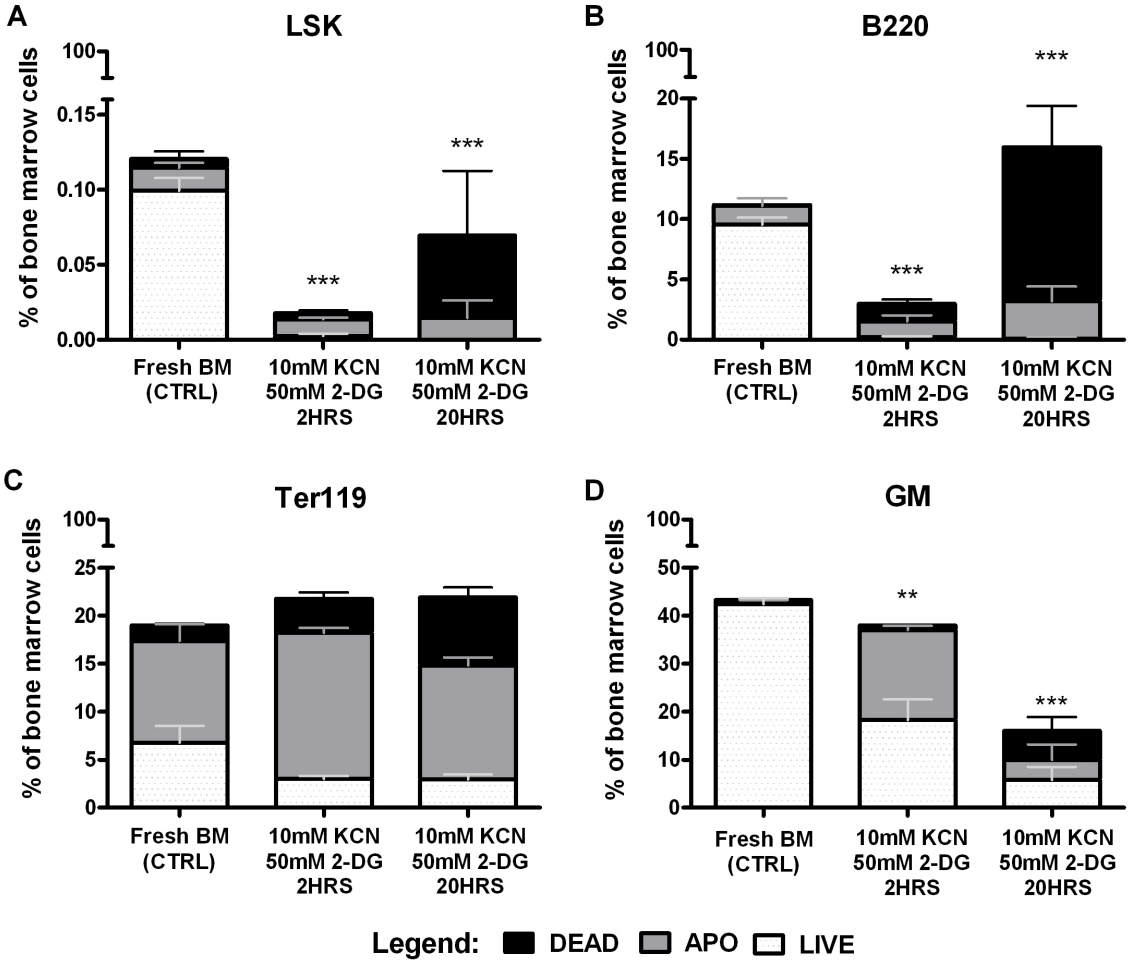

Figure 30 – Viability of bone marrow subpopulations during combined KCN and 2-DG inhibition. Ratio of live, apoptotic and dead cells after 0 (CTRL), 2 and 20 hours of KCN and 2-deoxy-D-glucose inhibition in LSK population **(A)**, in B220+ (B220) population **(B)**, in Ter119+ (Ter119) population **(C)**, and in Gr-1+Mac-1+ (GM) population **(D)**. Data are presented as mean ± SEM (n=3). Significance of difference from the controls (for the live cell fraction): ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

4.4 Effects of i*n vitro* storage on transplantability of hematopoietic stem cells

Bone marrow cells were harvested from femurs and stored in medium with 10 % FBS (v/v) without specific hematopoietic growth factors for 1 and 2 days at 37°C and for 2 and 4 days at 4°C, respectively. Afterwards, the bone marrow cells in the amount equivalent to half of the femur were transplanted to sublethally irradiated (6 Gy) recipients and the engraftment of the donor cells was followed up in recipient's peripheral blood for 6 months after transplantation. The engraftment is presented in Figure 31 and in Table S10, Appendix 2, p. X. There was no change in the repopulating ability of HSC caused by in vitro storage.

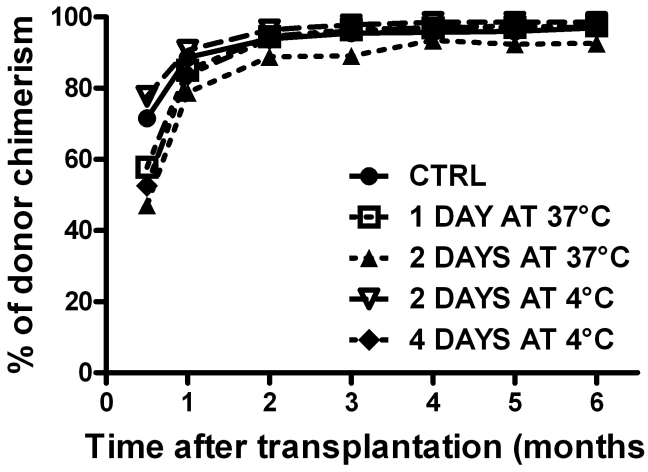

Figure 31 - Engraftment of *in vitro* stored HSC in sublethally irradiated recipients. Donor cells (Ly5.1) were stored *in vitro* for 1 and 2 days at 37°C, and for 2 and 4 days at 4°C. Ly5.2 recipients (n=4-5) were irradiated with 6 Gy and transplanted with half of the donor femur. The engraftment is presented as chimerism of Ly5.1+ cells (mean) detected in peripheral blood of Ly5.2 recipients. Significance of difference from the controls at individual time points: ***$P < 0.005$, **$P < 0.01$, *$P < 0.05$.

5 DISCUSSION

Hematopoietic stem cells have been widely studied for more than half a century. During this time, the HSC were thoroughly functionally described and phenotypically characterized, which made it possible to explore their unique features up to the molecular level. Simultaneously, a method of hematopoietic stem cell transplantation (HSCT) was developed and has become a routine part of clinical practice in treatment of various hematological disorders. Although HSCT saves every year many lives worldwide, some of the patients lack a suitable donor. Therefore, finding new ways how to enlarge the number of donors still remains a challenging task.

In this study, the response of mouse bone marrow cells to ischemia and metabolic inhibition was investigated to verify a possibility of using cadaveric bone marrow as a source of hematopoietic stem and progenitor cells for HSCT. In addition, an impact of *in vitro* storage on HSC transplantability was examined.

5.1 Effects of ischemia on bone marrow

Stem cells can be a source of tissue regeneration after various kinds of tissue damage. As such, they should be among the most resistant cells when the tissue is exposed to unfavorable conditions. Hematopoietic stem cells are slow-cycling (mostly quiescent) cells residing in hypoxic regions of bone marrow, which utilize anaerobic glycolysis to gain energy [3, 9, 10]. Because of their oxygen independent metabolism and proliferative quiescence, significant tolerance to a critical shortage of oxygen and energy supply (e.g. *post mortem*) might be assumed. Several studies have been published to these days, exploring the cadaveric BM as a possible source of hematopoietic stem and progenitor cells for transplantation; however bone marrow cells were always harvested immediately after the cessation of circulation, and their functioning has been studied just *in vitro* [122, 123, 135, 136]. The possibility of delayed harvesting of BM for transplantation has not been explored to our knowledge. Our study is the first one testing the functional properties of HSC by transplantation and engraftment to submyeloablated (sublethally irradiated) and/or to myeloablated (lethally irradiated) recipient. A competitive repopulation assay was used to compare the transplantability of cadaveric HSC with fresh HSC, co-transplanted to myeloablated recipient.

In detail, the influence of extended ischemia at various temperatures on the phenotypic and functional characteristics of bone marrow cells and hematopoietic stem cells was investigated, using mouse experimental model. Bone marrow ischemia was modeled in intact femurs removed from the body and stored in PBS to avoid drying.

Our results demonstrate that mouse HSC survive ischemia for a considerable length of time, even when the temperature of bone marrow is maintained at 37°C. The repopulating ability of hematopoietic stem and progenitor cells kept in femurs at 37°C and harvested 2 hours *post mortem* was fully preserved. At 20°C and 4°C, this period was significantly extended (Figure 11, p. 41, Figure 12, p. 42). These results are in agreement with the cytometric determination of LSK SLAM and LSK SP cell numbers. Representation of LSK SLAM cells was not significantly decreased in cadaveric BM as long as its repopulating ability was maintained (Figure 13, p. 43). This was also true of LSK SP population, except for at 37°C, when the number of LSK SP cells decreased rapidly. SP phenotype is based on active efflux of Hoechst 33342 from HSC via the ATP-dependent ABCG2 transporter [31]. The decline of cells with LSK SP phenotype could be explained by a lack of energy for the dye efflux, whereas the functional properties of HSC are maintained for a prolonged period of time. These results show that most of the stem cell phenotype used for their detection by flow cytometry is preserved in cadaveric BM in parallel with their repopulating ability.

The short-term repopulating cells (producing blood cells for up to 1 month after bone marrow transplantation) were more susceptible to damage by ischemia than the long-term repopulating cells (Figure 11, p. 41), which is in agreement with their higher proliferation and metabolic activity [137, 138]. The shorter time of STRC survival in ischemia should be considered during harvesting of the cells for transplantation, because the STRC restore recipient's hematopoiesis until the LTRC start to proliferate.

Furthermore, the viability of bone marrow subpopulations during ischemia was explored. As expected, bone marrow cells survived longer at lower temperatures and the longer period of ischemia the more cells were presented in dead fraction of bone marrow (Figure 14, p. 44). This increase is probably a consequence of necrosis rather than apoptosis, because ischemia is a known causative factor of necrosis [139]. The same trend as in the whole BM was also observed in BM subpopulations: LSK, $B220^+$, $Ter119^+$, and $Gr\text{-}1^+Mac\text{-}1^+$ (Figure 15, p. 45, Figure 16, p. 46, Figure 17, p. 47). At 37°C, fewer cells were included in the apoptotic fraction than at 20°C and at 4°C. This can be explained by

high energy demands of apoptotic process [140], which probably cannot be covered under hypoxic conditions at 37°C. At lower temperatures, cell metabolism is restricted and the energy is utilized more slowly.

Moreover, specific differences were discovered among individual populations. In the $Ter119^+$ population, more apoptotic cells were detected at all temperatures than in other populations tested. About 30 % of $Ter119^+$ cells were apoptotic or dead also in fresh bone marrow (control), which indicates high sensitivity of $Ter119^+$ cells to the manipulation during staining. The LSK population (enriched in both hematopoietic stem and progenitor cells) survived ischemia at 37°C better than the differentiated precursors of blood cells present in BM (Figure 15, p. 45). Because HSC are slow-cycling cells [3] and more than 50 % of differentiated blood precursor cells proliferate actively [138], the result appears to reflect a particular proliferation rate.

We collected femurs from the body immediately after sacrificing and stored them in PBS at defined temperatures. Such experimental design is easier to perform. In order to confirm that this experimental design is equivalent to the situation when bone marrow is collected after *post mortem* ischemia inside the body, we compared both approaches after 3 hours at 37°C. As expected, there was no significant difference in the frequency and functional properties of HSC (Figure 18, p. 48), in the bone marrow cellularity, or in the ratio of live and dead fraction (Figure 19, p. 49).

5.2 Effects of ischemia on $p53^{-/-}$ bone marrow

Role of tumor suppressor p53 in regulation of hematopoietic stem cells has been previously described in multiple studies [61-64]. It has been demonstrated that $p53^{-/-}$ HSC escape from quiescence and their repopulating ability is decreased as compared to the wild-type [63]. Due to the increased proliferation, $p53^{-/-}$ HSC should be more sensitive to the lack of oxygen and metabolic substrates than their wild-type counterparts. On the other hand, p53 plays also an important role in apoptosis [141]. If HSC during ischemia died by p53-dependent apoptosis, deletion of *p53* could lead to prolonged survival of the HSC. To test these two hypotheses, influence of ischemia at 37°C on $p53^{-/-}$ bone marrow subpopulations and $p53^{-/-}$ HSC was investigated in this study.

Nevertheless, our findings did not confirm any of the expectations. The repopulating ability of $p53^{-/-}$ HSC (Figure 20, p. 50) as well as the frequencies of LSK SLAM and LSK

SP cells (Figure 21, p. 51) were decreased by ischemia; however the engraftment was not lower than that of the wild-type HSC after ischemia at 37°C (Figure 11, p. 41). Because any differences between the $p53^{-/-}$ and wild-type bone marrow cells were not observed even in control groups, we conclude that although the $p53^{-/-}$ HSC are less quiescent [62], a not negligible fraction of primitive HSC still remains in bone marrow, capable of long-term engraftment and resistant to the lack of oxygen and energy.

Increase in fractions of apoptotic and dead cells in $p53^{-/-}$ bone marrow (Figure 22, p. 52) and in $p53^{-/-}$ bone marrow subpopulations (Figure 23, p. 53) after ischemia at 37°C was also similar as in wild-type BM (Figure 14A, p. 44, Figure 15, p. 45,). A minor apoptotic fraction was observed in the wild-type as well as in $p53^{-/-}$ BM. These results indicate that p53-independent apoptosis is induced by ischemia.

5.3 Metabolic inhibition *in vitro*

Cyanide anion is a known inhibitor of cytochrome c oxidase in the fourth complex of the electron transport chain. It binds near to the active site of the enzyme and prevents the electron transport to oxygen, which results in interruption of aerobic respiration and in reduced production of ATP [142, 143]. 2-deoxy-D-glucose (2-DG) is a glucose analog that inhibits glucose utilization by the cell. 2-DG enters the cell through the glucose transporters and is phosphorylated by hexokinase. However, 2-deoxy-D-glucose-6-phosphate is unable to undergo further metabolism, accumulates in the cell, inhibits glucose metabolism, and causes cell death [144, 145].

We have studied influence of 1 mM KCN, 10 mM KCN, 50 mM 2-DG and 10 mM KCN together with 50 mM 2-DG on functional properties and phenotype of bone marrow cells of C57Bl/6 mice.

KCN concentration of 1 mM was chosen according to our oxygraphic measurement of bone marrow cell respiration, kindly performed by Dr. Drahota (Institute of Physiology and Center for Applied Genomics, Academy of Science of the Czech Republic). Figure 32, p. 68 shows dependence of bone marrow respiration on KCN concentration. At the concentration of 880 μM, the respiration was almost completely inhibited. Because hematopoietic stem cells prefer cytoplasmic glycolysis to aerobic respiration [10], we decided to use also ten times higher concentration (10 mM) in our experiments.

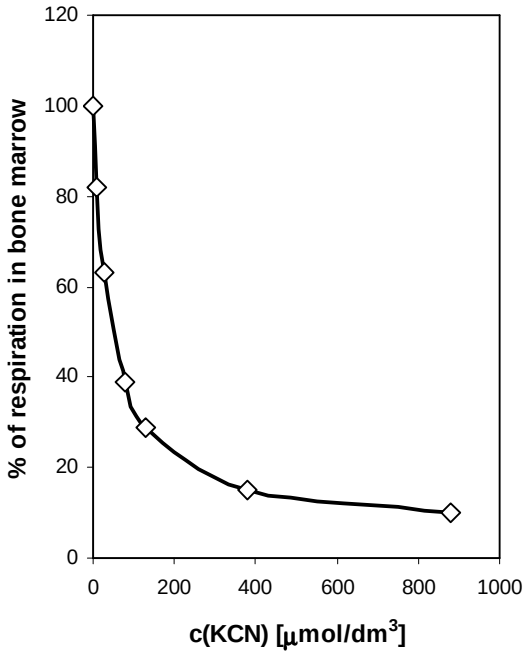

Figure 32 – Inhibition of aerobic respiration in bone marrow by KCN.

To reach an effective inhibition, we use 50 mM 2-DG in our experiments. Because this concentration is high and could influence osmolarity of the cultivation medium, we used incubation with 50 mM D-glucose as a control.

At first, we tested the repopulating ability of HSC after metabolic inhibition. (Figure 24, p. 55) The long-term repopulating cells were shown to be relatively resistant to the KCN inhibition; KCN decreased the long-term repopulating ability just after twenty-hour incubation when it was used at 10 mM concentration. The short-term repopulating cells were influenced also by 1mM KCN after 20 hours of incubation. Unlike cyanide, 2-deoxy-D-glucose reduced the transplantability of HSC already after two-hour inhibition. Combination of both inhibitors was shown to be fatal for the HSC. Frequencies of primitive hematopoietic stem cells in bone marrow (identified as LSK SLAM and LSK SP) after the incubations with inhibitors (Figure 25, p. 56) corresponded with their repopulating ability (Figure 24, p. 55). Increase in the frequency of LSK SLAM cells after 20 hours of KCN inhibition is probably only apparent, caused by the depletion of more differentiated cells from the live fraction of BM. Relative resistance of the HSC to KCN inhibition and their susceptibility to 2-DG inhibition gives us an additional evidence of their resistance to the oxygen shortage due to the anaerobic metabolism.

Subsequently, the cellularity and viability of bone marrow as well as the viability of bone marrow subpopulations after metabolic inhibition were explored (Figure 26, p. 58).

1 mM KCN did not significantly decrease the number of nucleated cells in bone marrow, though it decreased the number of live cells in the samples after both two-hour and twenty-hour inhibition, as compared to the fresh bone marrow and to the samples incubated without inhibitors. These findings are consistent with previous studies, which have demonstrated induction of apoptosis by electron transport chain inhibitors in various cell types [146, 147]. However, considering lack of energy due to the KCN inhibition, necrosis could also participate on cell death; unlike apoptosis, necrosis is an ATP-independent process [139]. Incubation of bone marrow cells with 10 mM KCN resulted in a significant decline in the number of nucleated cells, which was reflected in viability of bone marrow cells. Nevertheless, there were still live cells after the incubation, which indicates a presence of subpopulation/subpopulations in the bone marrow, resistant to KCN inhibition, i.e. with restricted aerobic metabolism.

The inhibition by 50 mM 2-DG also decreased BM cellularity and viability of bone marrow cells in both intervals tested (Figure 26C, p. 58). However, the decline in cellularity as well as in fraction of live cells was observed also after incubation with 50 mM D-glucose. It has been described, that high glucose concentration can lead to apoptosis through the oxidative stress and mitochondrial proteins [148]. Because 2-DG cannot be metabolized in the same way, its effect is probably really caused by inhibition of energy metabolism. The effect of 2-DG was shown to be enhanced by addition of 10 mM KCN (Figure 26D, p. 58) and the combination of inhibitors seems to be lethal for bone marrow cells. Since glucose was also present in the medium used in the experiments (DMEM low glucose), limited glycolysis and subsequent aerobic respiration could occur in the cells even in the presence of 2-DG. Addition of KCN to 2-DG resulted in complete inhibition of cell metabolism.

Analysis of apoptosis and necrosis in individual subpopulations of bone marrow after metabolic inhibition was performed to find the differences between LSK cells and more differentiated bone marrow cells. LSK population, enriched in hematopoietic stem cells that prefer anaerobic metabolism [10], should be the most protected population from the KCN inhibition. All of the subpopulations were expected to be sensitive to 2-DG inhibition, because all cells need either aerobic or anaerobic glycolysis to cover their energy demands.

After two-hour or twenty-hour incubation in medium without inhibitors, frequencies and viability of all subpopulations were preserved, with the exception of $Ter119^{+}$

subpopulation after 20 hours (Figure 27, p. 59). As expected, LSK population was resistant to the KCN inhibition (Figure 28, p. 60); however also granulo-monopoietic population survived the KCN treatment better then the populations of B-lymphopoietic and erythropoietic cells. $Ter119^+$ population was again the most sensitive one. Viability after 2-DG inhibition was similar in all populations; the live fraction of the cells was not influenced after 2 hours, whereas after 20 hours the live cells were almost completely depleted (Figure 29, p. 61). As well as in the whole bone marrow, the effect of 2-DG inhibition was enhanced by addition of 10 mM KCN (Figure 30, p. 62).

5.4 *In vitro* storage of bone marrow cells

The impact of short-term liquid storage (up to 4 days) on bone marrow cells has already been described in previous studies, focused on viability and stem cell recovery [149] clonogenic potential [122, 135], kinetics of apoptosis and production of reactive oxygen species [123] and also on the engraftment potential of HSC [150, 151]. These studies confirm that the storage of bone marrow cells at 4°C preserves engraftment potential of HSC from overnight storage up to one week. In our study, we examined the tolerance of HSC to unfavorable conditions *in vitro*, when bone marrow cells were stored in a medium with fetal bovine serum and without specific growth factors, which are assumed to be essential for maintenance of HSC viability. Transplantation of these cells to sublethally irradiated recipients demonstrates a preserved transplantability of both STRC and LTRC for at least 2 days of storage at 37°C and 4 days of storage at 4°C (Figure 31, p. 63). Thus, the HSC stored in a suspension of bone marrow cells survived for a significantly longer period than those left *in situ* under ischemic conditions.

6 CONCLUSIONS

- The repopulating ability of hematopoietic stem cells exposed to ischemia at 37°C was maintained for up to 2 hours, when the HSC were transplanted to sublethally (6 Gy) irradiated recipients. This time was extended to 6 hours and 12 hours after ischemia at 20°C and 4°C, respectively.

- In lethally irradiated recipients, HSC exposed to ischemia successfully competed with HSC from fresh bone marrow for up to 2 hours, 12 hours, and 24 hours at 37°C, 20°C, and 4°C, respectively.

- Frequencies of LSK SLAM and LSK SP cells (highly enriched in HSC) in bone marrow exposed to ischemia at 37°C, 20°C, or 4°C were preserved in compliance with the repopulating ability. LSK population (enriched in hematopoietic stem cells and progenitors) was significantly more resistant to ischemia at 37°C than differentiated populations of bone marrow when fractions of live, apoptotic and dead cells were compared.

- HSC with targeted deletion of apoptotic and quiescence regulator p53 did not differ from the wild-type HSC in repopulating ability or in frequencies of LSK SLAM and LSK SP cells in BM, fresh or exposed to ischemia at 37°C.

- HSC were shown to be relatively resistant to *in vitro* inhibition by electron transport chain inhibitor potassium cyanide, which indicates an important role of their anaerobic metabolism in their survival of ischemia.

- Repopulating ability of HSC was fully preserved for at least 2 days of *in vitro* storage at 37°C and for at least 4 days of *in vitro* storage at 4°C, despite the absence of specific hematopoietic growth factors.

Based on our findings, we can conclude that **cadaveric bone marrow** could be used as an alternative **source of HSC for transplantation**. However, regarding the prolonged survival of HSC *in vitro,* cells for transplantation should be harvested as soon as possible.

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8 LIST OF PUBLICATIONS

Hlobenova T, Sefc L, Chang KT, Savvulidi F, **Michalova J,** Necas E. B-lymphopoiesis gains sensitivity to subsequent inhibition by estrogens during final phase of fetal development. Dev Comp Immunol. 2012; 36(2):385-389. **IF = 3.293**

Michalova J, Savvulidi F, Sefc L, Faltusova K, Forgacova K, Necas E. Hematopoietic stem cells survive circulation arrest and reconstitute hematopoiesis in myeloablated mice. Biol Blood Marrow Transplant. 2011; 17(9):1273-1281. **IF = 3.275**

Vojtova L, Zima T, Tesar V, **Michalova J,** Prikryl P, Dostalova G, Linhart A. Study of urinary proteomes in Anderson-Fabry disease. Ren Fail. 2010; 32(10):1202-1209. **IF = 0.84**

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Manuscripts in preparation

Forgacova K, Savvulidi F, **Michalova J,** Sefc L, Necas E. Transplantation window induced by submyeloablative irradiation depends on fitness of hematopoietic stem cells.

Savvulidi F, Forgacova K, **Michalova J,** Paral P, Necas E, Sefc L. Two novel strategies for detecting murine hematopoietic stem cells by flow cytometry ignoring lineage markers.

9 APPENDICES

Appendix 1

Supplementary figures

Appendix 2

Supplementary tables

Appendix 3

Hlobenova T, Sefc L, Chang KT, Savvulidi F, **Michalova J,** Necas E. B-lymphopoiesis gains sensitivity to subsequent inhibition by estrogens during final phase of fetal development. Dev Comp Immunol. 2012; 36(2):385-389. **IF = 3.293**

Appendix 4

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Appendix 5

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Appendix 6

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