Review Article

Availability of Haematopoietic Niches for Transplanted Stem Cells

(mouse / haematopoiesis / haematopoietic stem cell / progenitors / niche / bone marrow transplantation / irradiation / cytostatics / engraftment / stem cell mutations)

K. FORGÁČOVÁ, E. NEČAS

Centre of Experimental Haematology and Institute of Pathological Physiology, First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

Abstract. Following transplantation, donor haematopoietic stem cells (HSCs) must reach specific parts of haematopoietic stroma tissue known as stem cell niches to become engrafted and to start blood cell production. Regularly, they have to compete with the host's HSCs for a limited number of niches. The exact mechanisms of HSC engraftment as well as of niche "opening" to incoming HSCs by conditioning treatments are not well-known yet. Significant and stable engraftment of syngeneic donor HSCs can be achieved in untreated mice only after transplantation of very large numbers of marrow cells. Engraftment can be largely facilitated by the stem cell mutations reducing numbers of the host HSCs. Pre-transplantation manipulations of the host haematopoietic tissue enhance engraftment depending on how much they damage HSCs. Ionizing radiation appears to be the most effective in this respect despite proliferative quiescence of a majority of HSCs. The review summarizes major achievements in deciphering biological principles of the HSCs and their engraftment after transplantation obtained in experimental research studying murine haematopoiesis.

Received July 2, 2012. Accepted July 9, 2012.

Corresponding author: Katarína Forgáčová, Institute of Pathological Physiology, First Faculty of Medicine, Charles University in Prague, U Nemocnice 5, 128 53 Prague 2, Czech Republic. Phone: (+420) 224 965 913; Fax: (+420) 224 912 834; e-mail: kforg@lf1.cuni.cz

This work was supported by the projects PRVOUK-P24/LF1/3, UNCE204021 and SVV-2012-264507 from Charles University in Prague.

Abbreviations: 5-FU – 5-fluorouracil, AGM – aorto-gonad-mesonephros, BMCs – bone marrow cells, CAR cells – CXCL12--abundant reticular cells, CFU-S – colony-forming unit-spleen, CXCL12 – stromal cell-derived factor 1 (SDF-1), CXCR4 – CXCL12 receptor, CY – cyclophosphamide, ECM – extracellular matrix, FACS – fluorescence-activated cell sorting, G-CSF – granulocyte colony-stimulating factor, GFP – green fluorescent protein, HA – hyaluronic acid, HSCs – haematopoietic stem cells, LFA-1 – lymphocyte function-associated antigen 1, LSK – Lin^{low} $Sca\text{-}1^{+}c\text{-}kit^{+}$ cells, LTRCs – long-term repopulating cells, Mdm2 – murine double minute oncogene, MMP – matrix metalloproteinase, Puma – p53 up-regulated modifier of apoptosis, ROS – radical oxygen species, SCF – stem cell factor, SDF-1 – stromal-derived factor 1, SLAM – signalling lymphocyte activation molecule, Slug – Slug/Snail2 zinc-finger transcription factor, SP – side population, STAT5 – signal transducer and activator of transcription 5, VLA-4 – very late antigen 4.

1. Introduction

Haematopoietic tissue is formed by haematopoietic cells and haematopoietic stroma and is engaged in blood cell production. In adult mammals, it is found in the bone marrow, lymphatic organs, spleen and thymus. During embryonic and foetal development some other tissues are active in blood cell production: the yolk sac, placenta, aorto-gonad-mesonephros (AGM) region and foetal liver. The spleen and the liver may also harbour haematopoiesis in adulthood under some pathological conditions and during some compensatory reactions, a condition called extramedullary haematopoiesis. Haematopoietic tissue is distributed in various parts of the body and precisely responds to requirements for a particular type of blood cells. The total number of blood cells produced by haematopoietic tissue during the lifetime of the organism is several times greater than the total number of all cells present in the body. Fig. 1 provides an overview of the hierarchical organization of murine haematopoietic tissue and indicates the numbers of blood cells produced daily. It is well known from transplantation experiments that the immense capacity of haematopoietic tissue to produce blood cells is not limited to the lifespan of the organism and, theoretically, the tissue may be viewed as an inexhaustible source of blood cells. This longevity of the haematopoietic tissue is based on the presence of haematopoietic stem cells (HSCs), which enable its long-term maintenance and continuous blood cell production, as well as its capacity to regenerate from damage (Huang et al., 2007). HSCs possess a large proliferative potential and are capable of differentiating into all blood cell types. Understandably,

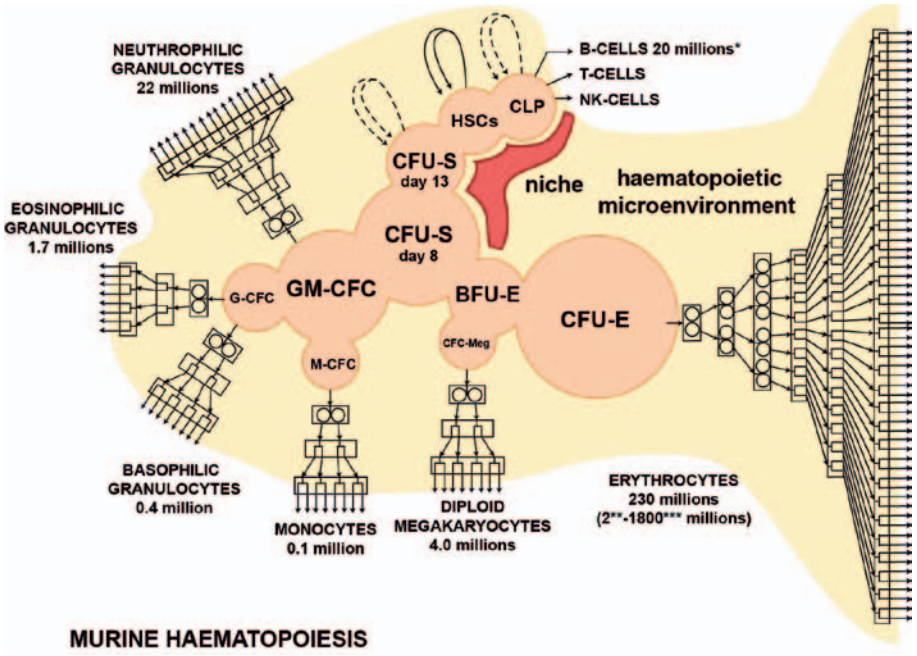

Fig. 1. Cellular hierarchy of murine haematopoiesis and the rates of blood cell production. CLP – common lymphoid progenitor, CFU-S, BFU-E, CFU-E, CFU-Meg, CFU-GM, CFU-G, CFU-M – progenitors with variable capacity for repeated cell divisions and limited or absent capacity for self-renewal. B cells, T cells and NK cells represent the lymphopoiesis. Dendritic cell and mast cell lineages are not represented. Precursors of blood cells are depicted as binomically multiplying and maturing cells. The estimates of the daily production of mature blood cells and megakaryocytes in the mouse are from the paper by Novak and Necas (1994). The large range of the possible production of erythrocytes corresponds to conditions of suppressed or enhanced erythropoietin stimulation.

HSCs have been a subject of intensive research for decades. Transplantation-based assays of HSCs have been developed, providing the most rigorous ways of determining the stem cell features of a cell. Last but not least, this research is an important part of the scientific basis underlying clinical bone marrow and other stem cell transplantations. This review focuses on conditions enabling engraftment of transplanted HSCs in syngeneic/ /congeneic mice models.

2. Haematopoietic stem and progenitor cells

2.1. Haematopoietic stem cell

The haematopoietic stem cell is by far the best characterized adult stem cell. It served to establish the concept of stem cells more than a hundred years ago (Maximov, 1909), to provide direct evidence for the presence of stem cells in the bone marrow (Brecher and Cronkite, 1951; Ford et al., 1956; Till and McCulloch, 1961), to establish the basic features of stem cells such as their self-renewal and multiple differentiation options. It also resulted in clinical HSC transplantations (Appelbaum, 2007), the most successful achievement in regenerative medicine to date.

HSCs represent only a very tiny fraction of all bone marrow cells and do not possess adequately unique phenotypic markers that would enable their microscopic detection. The lack of an explicit hierarchical anatomical structure of the marrow does not allow HSCs to be traced and located in a way that was possible e.g. in the intestinal epithelium. (*) On the other hand, the possibility of subjecting suspensions of haematopoietic cells to analysis by flow cytometry, allowing detection and separation of very rare and even single cells, the availability of monoclonal antibodies against various lineage-specific and clonogenic cell antigens, the availability of *in vitro* and *in vivo* clonal assays, and most importantly the transplantation assay utilizing syngeneic and congeneic mice have enabled sophisticated experimental research into haematopoietic stem cells, particularly those of mice.

The specific phenotype of HSCs and progenitor cells is characterized by antigenic cell surface markers and some other features that can be determined by flow cytometry. Murine HSCs are positive for Sca-1 (an adhesion molecule) and c-kit (receptor of stem cell factor) surface antigenic markers and lack the lineage markers of blood precursor cells (B220, CD4, CD8, Gr-1, Mac-1, Ter-119). Although cells with the $Lin^{-}Sca\text{-}1^{+}c\text{-}kit^{+}$ (LSK) phenotype greatly contribute to haematopoietic reconstituting activity, this bone marrow fraction contains progenitor cells as well as long-term HSCs (Challen et al., 2009). Many strategies, using several additional markers, have been found to enrich bone marrow in HSCs, including the identification of HSCs as being $LSK\text{-}CD34^{-}$, $Flk\text{-}2^{-}$, $CD150^{+}CD48^{-}$ cells (Adolfsson et al., 2001; Kiel et al., 2005) and the Hoechst-effluxing

*The challenge represented by studies into HSCs can be metaphorically likened to a task of identifying and studying approximately 3,500 to 9,000 specific individuals randomly distributed among 350 millions of the residents of the North America continent.

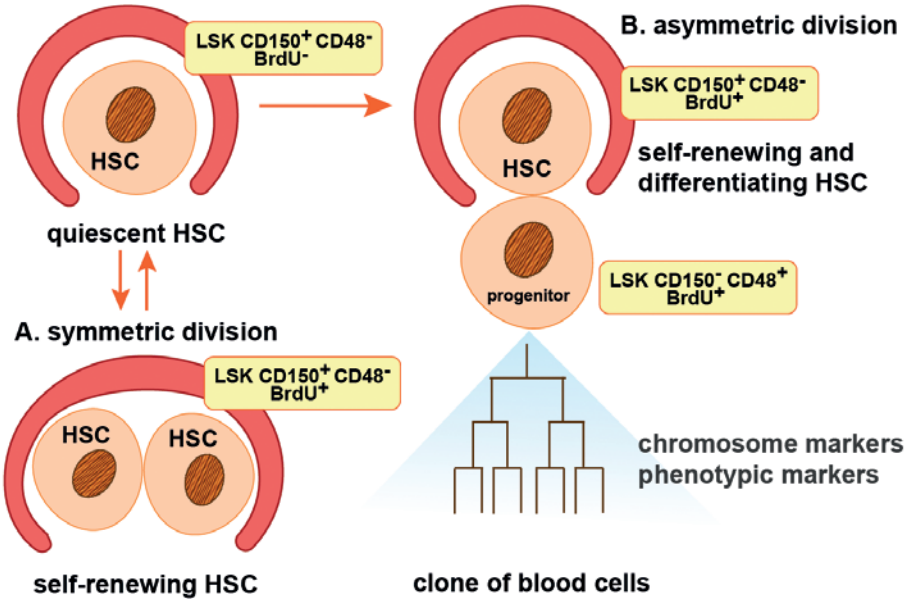

Fig. 2. Three possible states of HSCs: quiescent HSC (LSK CD150$^+$CD48$^-$BrdU$^-$), self-renewing HSC (LSK CD150$^+$CD48$^+$BrdU$^+$) and self-renewing and differentiating HSC (LSK CD150$^-$CD48$^+$). Cells in all these states have the phenotype of HSCs or progenitors when analysed by flow cytometry. However, only HSCs which undergo asymmetric division and generate progenitors manifest themselves by clonal expansion and are detected by indirect methods utilizing chromosome markers or differences in bone marrow and blood cells.

side population (SP) (Goodell et al., 1996). The incidence of HSCs is approximately 0.0025 % (estimated from Kiel et al., 2005), i.e. there are approximately 9,000 HSCs in the whole marrow of the mouse (349 million cells on average, Novak and Necas, 1994). This corresponds well with the previous estimate of one HSC per 40,000 bone marrow cells (Micklem et al., 1987) and the estimate of McCarthy (1997) of 8,000 to 11,000 of HSCs in the mouse. Harrison et al. (1990) concluded that there was approximately one HSC in 100,000 marrow cells, i.e. approximately 3,500 HSCs in the mouse. They are normally slowly proliferating cells in adult bone marrow, but can switch to a more intensive proliferation, particularly after damage to the haematopoietic tissue (Wilson et al., 2008). Their proliferation may result in self-renewal (symmetric or asymmetric cell division providing two or one new HSC) or in the generation of a progenitor (asymmetric cell division, or symmetric division with both daughter cells differentiating into progenitors). Both quiescent and proliferating stem cells can be detected by flow cytometry analysis of haematopoietic tissue (Barbier et al., 2012) with limits given by their low numbers, intrinsic developmental heterogeneity (Sieburg et al., 2006; Dykstra et al., 2007; Morita et al., 2010; Lu et al., 2011) and possible phenotypic fluctuations/oscillations (Quesenberry et al., 2002; Quesenberry and Aliotta, 2008). Assays based on the longevity of blood cell production derived from HSCs are highly sensitive, with the capacity of detecting a single HSC, but they reflect only those stem cells or bone marrow differentiated cells which generate progenitors and blood cells (Fig. 2). In principle, a HSC can stay dormant for a very long time (Wilson et al., 2008) and may manifest itself by the production of blood cells only after being transplanted (Morita et al., 2010). Also, after transplantation, HSCs are not equally active in blood cell production (Capel et al., 1990; Lu et al., 2011).

A very specific functional feature of HSCs is their ability to reproduce themselves (self-renewal) and to recover their number in case it is reduced. Due to this unique property, a single HSC can re-establish and maintain complete haematopoiesis, as it has been presented in Fig. 1 (Osawa et al., 1996; Benveniste et al., 2003; Morita et al., 2010). Due to generation of its copies in self-renewing cell divisions, HSC may colonize distant parts of the haematopoietic tissue (Micklem et al., 1987), and the haematopoiesis can be transplanted to secondary or tertiary recipients. HSCs are source of both myeloid and lymphoid cells. However, they can intrinsically differ in the ratio with which they generate the myeloid and the lymphoid progeny (Sieburg et al., 2006; Dykstra et al., 2007; Cho et al, 2008; Challen et al., 2010; Beerman et al., 2010). HSCs that provide stronger myeloid repopulation over that of the lymphoid one express a high level of the CD150 (SLAM F1) antigen. HSCs with low to negative CD150 marker serve mainly for lymphoid repopulation and more weakly for the myeloid one. The latter appear to be developmental descendants of CD150^{high} HSCs (Morita et al., 2010). When these HSCs engraft in recipients, they begin to proliferate, self-reproduce, differentiate into progenitors, and produce blood cell myeloid and lymphoid progeny. Studies with single-cell transplantations have revealed another type of HSCs which remain silent after transplantation and only manifest themselves when the first recipient's marrow is re-transplanted to secondary recipients (Morita et al., 2010).

Although HSCs have a very high proliferative potential, they rarely divide under physiological conditions. Several studies (Spangrude and Johnson, 1990; Goodell et al., 1996; Cheshier et al., 1999) have shown that at any particular time, most HSCs are in the G_0 state. Some HSCs divide only five times within the lifespan of the mouse (Wilson et al., 2008). The proliferation of HSCs apparently takes place "on demand" *in vivo*, when the body needs more blood cells, and particularly when the number of HSCs has been reduced after damage to the haematopoietic tissue.

2.2. Progenitors

Haematopoietic progenitors are proliferating cells (Nečas and Znojil, 1987; Nečas et al., 1990) with a large potential for cell production (Nečas et al., 1995). They provide a large functional reserve, enabling a relatively rapid increase in the production of a specific type of blood cells (Nečas et al., 1998). In contrast to HSCs, they have a limited potential for self-reproduction and restricted differentiation options. The probability of self-reproduction compared to their losses due to differentiation/apoptosis is biased towards the latter. They are thus destined to clonal extinction after various periods of functioning and production of blood cells (Capel et al., 1990; Drize et al., 1996). They have a larger volume than stem cells (Lanzkron et al., 1999). Using flow cytometry analysis, they can be distinguished from HSCs by the expression of CD34 and Flt3 (Adolfsson et al., 2001) and the SLAM pattern of $CD150^{-}CD48^{+}$ (Kiel et al., 2005). They encompass a large spectrum of cells with a highly variable predestination for further differentiation and a limited self-renewal and proliferation potential. Major representatives of the various progenitors are depicted in Fig. 1. Their differences from HSCs and among themselves are determined by a specific gene expression profile projected into the presence, amount and mutual ratio of specific transcription factors (Forsberg et al., 2005; Petriv et al., 2010; Wang and Zhang, 2010; Nakajima, 2011).

3. Haematopoietic stem cell niche

The maintenance of HSCs and the control of their self-renewal, differentiation, and eventually apoptosis and migration depend on the specific microenvironment in which they reside – the haematopoietic stem cell niche.

The haematopoietic stem cell niche is a specialized stroma supporting and controlling HSCs. Together they form a principal functional unit of haematopoietic tissue. HSCs can be physically separated from their niches, handled *in vitro*, and transplanted to conditioned recipients either directly into the haematopoietic tissue or via the circulation. They engraft with a very high efficiency (Benveniste et al., 2003; Morita et al., 2010), which assumes guidance and strong attraction of transplanted HSCs and specific recognition of the niche structures.

The niche is assumed to exert control over the fate of HSCs and progenitors within limits given by the gene expression status of the particular cell. The options are therefore slightly different for stem cells and progenitors. Apart from their inner differences in the activity of critical genes, stem cells and progenitors also use different niches. This is understandable, since while progenitors should actively proliferate and differentiate into more mature cells, stem cells should self-renew and, in the case of haematopoietic stem cells, remain quiescent and metabolically inactive. The various possible fates of a haematopoietic stem cell or a progenitor are depicted in Fig. 3.

Although the very first concept of the niche was established more than 30 years ago (Schofield, 1978), this microenvironment was inaccessible for direct observations for a long time. Askenasy et al. (2003) developed a technique that enabled optical tracking of cells labelled with fluorescent markers in recipient bone marrow *in vivo*. They defined the haematopoietic niche as a three-dimensional functional unit composed of several stromal cells, extracellular matrix, and bone surface, which hosts a cluster of transplanted cells. Lo Celso et al. (2009) visualized haematopoietic stem cells *in vivo* using advanced light microscopy techniques. The transplantation of bone marrow cells or cells highly enriched in HSCs into the circulation of non-irradiated or irradiated recipients resulted in the migration of donor cells to the vicinity of the endosteal bone of the calvaria or epiphyseal trabecular bone of the tibia (Lo Celso et al., 2009; Xie et al., 2009; Bengtsson et al., 2011). However, transplanted cells only started to proliferate in irradiated recipients (Lo Celso et al., 2009). Yoshimoto et al. (2003), using transplanted cells isolated from transgeneic GFP mice, demonstrated that HSCs preferentially engraft at the epiphysis of the femurs or short flat bones.

3.1. Structural components of niches

Structurally, the haematopoietic stem cell niche unit can be divided into two main parts: haematopoietic stem or progenitor cells and stromal components. Stromal components contain blood vessels and include endothelial cells, adipocytes and other stromal cells, as well as specialized osteoblasts and macrophages, These supporting cells express specific membrane-bound molecules and secrete paracrine factors, as well as various extracellular matrix components contributing to the chemical signature of the niche that also include the long-range signals that reach the niche and HSCs through the circulation. The nervous system is represented by sympathetic nerve endings (reviewed e.g. in Can, 2008; Ehninger and Trumpp, 2011).

3.2. Established interactions between the niche and HSCs

As indicated above, the essential role of the niche is to keep HSCs in a quiescent state, to control their self-renewal and precisely replace it by differentiation into progenitor cells for recovery of their number after damage to the haematopoietic tissue. These functions are

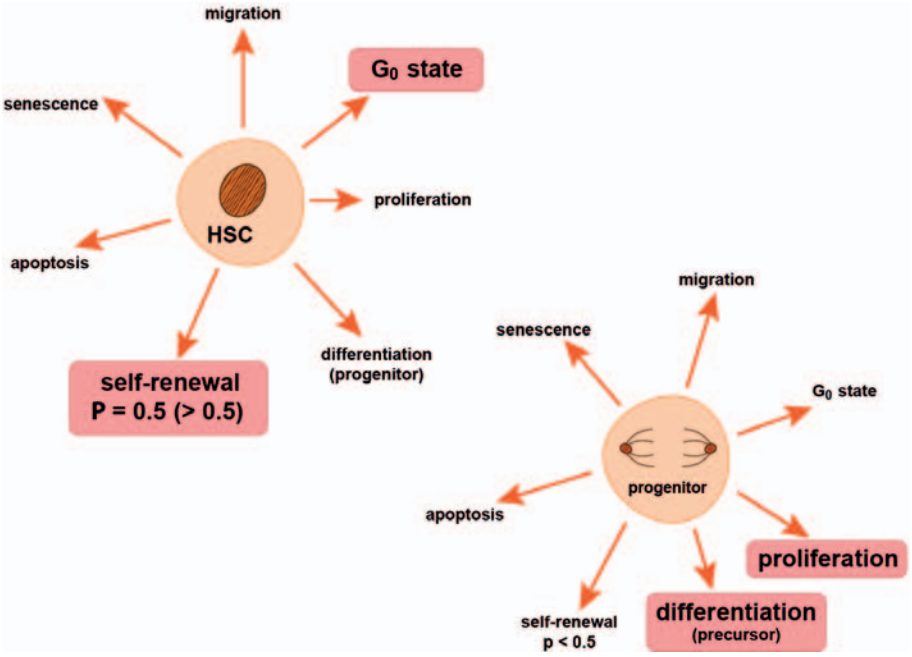

Fig. 3. Possible fates of haematopoietic stem and progenitor cells. When affected by various stimuli, the cells may undergo self-renewal division, proliferation and differentiation, remain in the G_0 state, migrate into the blood, or succumb to apoptosis or proliferative senescence. Under normal circumstances, HSCs remain predominantly in the G_0 state and can repeatedly self-renew. Their long-term probability (P) to self-renew is 0.5 and can be transiently > 0.5 after transplantation or after partial loss of HSCs. In contrast, progenitors are actively proliferating cells biased to differentiation into haematopoietic precursors, and consequently their long-term probability to self-renew is always < 0.5. This makes them dependent on the supply of cells from the stem cell compartment.

ensured by specific interactions between the niche and HSCs. Specialized osteoblasts have been shown to be principal cellular components of the HSC niche in the bone marrow. Together with other stroma cells (reviewed by Ehninger and Trumpp, 2011), these osteoblasts produce factors maintaining stem cells quiescent (angiopoietin 1, thrombopoietin), inducing their proliferation in instances of tissue damage or stress (Wnt, interferon γ), control their migration and localization in the bone marrow (CXCL12) and maintain their vitality (SCF, thrombopoietin, interleukins). Also, high concentrations of extracellular calcium seem to take part in the stem cell-niche interactions. Mice with deficient calcium-sensing receptor in HSCs failed to engraft (Adams et al., 2006). The "osteoblastic niche" harbours multipotent HSCs with unrestricted self-renewal capability and is responsible for the lifelong maintenance of haematopoiesis. When transplanted and accepted by the niches, HSCs can reproduce themselves, colonize available niches, and support and maintain haematopoiesis for the rest of the organism lifetime, and hence they are designated as long-term repopulating cells (LTRCs) in a rigorous syngeneic mouse-based transplantation assay.

3.3. A niche for a single HSC or for several HSCs? An inducible niche?

The number of HSCs is constant in adult bone marrow, which is thought to be due to a limited and fixed number of niches. At steady state, the majority of HSCs are probably single cells (Wilson et al, 2008; Lo Celso et al., 2009) located near the bone surface, the endosteum, which is lined with osteoblasts. The niches for HSCs are rare inside haematopoietic tissue, as is indicated by the low numbers of HSCs. The number of HSC niches should approach the total number of HSCs, i.e. 9,000 in the whole of the haematopoietic tissue or approximately 600 in the femoral bone marrow, often employed in analyses as a representative unit of total bone marrow. The numbers of niches should be even lower if there is one HSC among 100,000 marrow cells (Harrison et al., 1990) or if a niche can harbour more than one HSC. Maloney et al. (1978) estimated that the bone marrow of W/W^v mice is compartmentalized into approximately 2,600 stem cell regulatory units. The low and limited number of HSC niches likely determines the maximum number of HSCs. This is also an important function of the niches with respect to the intrinsically unrestricted capacity of HSCs to self-renew (Yin and Li, 2006; Arai and Suda, 2007; Wilson et al., 2007; Kiel at al., 2008).

The niche concept includes several assumptions which are supported by mostly indirect evidence. One of the assumptions is that niches determine the maximum number of HSCs. The evidence for this consists in a relatively constant number of HSCs throughout the life of the organism, and in no significant overshoot during regeneration from damage based on enhanced symmetric self-renewing cell divisions. By inducing large ectopic bones in otherwise normal mice, it was possible

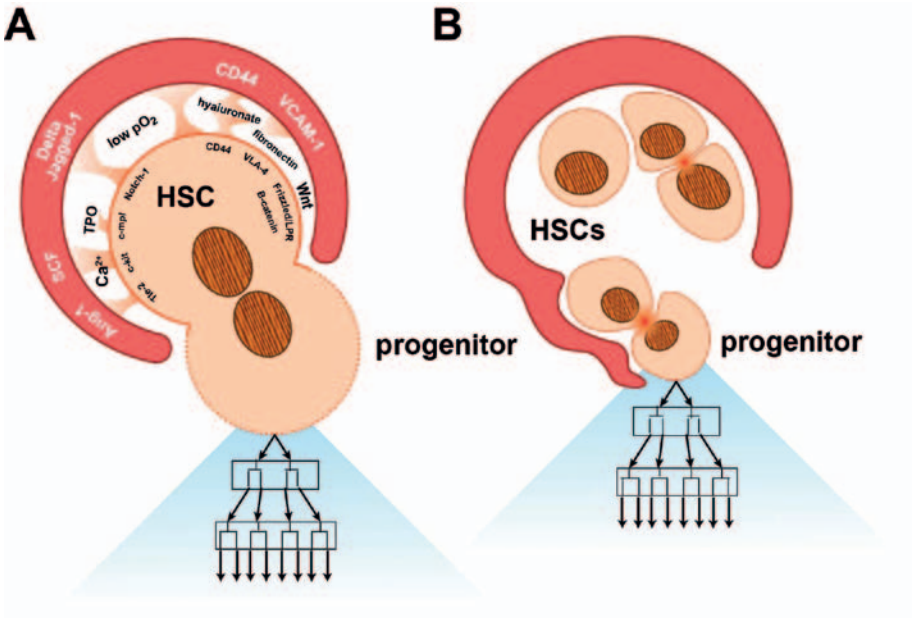

Fig. 4. Haematopoietic stem cell niche unit. A – Niche harbouring a single stem cell favouring asymmetric division due to extrinsic and potentially intrinsic (unequal distribution of transcription factors and other cellular components) factors. Examples of established interactions are depicted (see the text). B – Niche containing a group of HSCs and potentially progenitors.

to increase the total number of colony-forming unit--spleen (CFU-S) progenitor cells two- to four-fold (Chertkov et al., 1980). This result can also be regarded as a piece of supporting evidence that the niches determine the number of early progenitor CFUs. The stimulation of bone formation through the action of the parathyroid hormone-related peptide also increased the number of HSCs two-fold (Calvi et al., 2003) and this was attributed to an increased number of niches. The most slowly proliferating haematopoietic cells, probably haematopoietic stem cells, were shown to exist as single cells (Wilson et al., 2008). A niche that could accommodate only a single cell could be predetermined for the asymmetric divisions, which provide progenitor cells for blood cell production and keep the pool of stem cells constant (Fig. 4A).

Mice deficient in the Lnk signalling adaptor protein had approximately ten-fold increased numbers of HSCs due to their increased self-renewal and compromized apoptotic machinery (Takaki et al., 2002; Ema et al, 2005; Suzuki et al, 2012). This raises the questions of whether self-renewing haematopoietic stem cells could accumulate in the niche (Fig. 4B) or whether self-renewing stem cells could induce new niches. Lnk-deficient mice thus represent a significant challenge to the current thinking on the niche-HSCs relationships.

4. Homing and engraftment of donor HSCs introduced into circulation

Trafficking of haematopoietic stem cells after their intravenous application into blood circulation, locating and identifying the bone marrow and overcoming the endothelial barrier of bone marrow sinusoids, requires active navigation, a process termed homing. Homing is the first and essential step in clinical or experimental stem cell transplantation. It is a rapid process – transplanted cells home to femoral bone marrow within minutes after intravenous injection (Askenasy et al., 2003). Homing is thought to be a coordinated, multistep process which involves signalling, chemoattraction by stromal-derived factor 1 (SDF-1; CXCL12 chemokine) and stem cell factor (SCF), activation of the lymphocyte function-associated antigen 1 (LFA-1), very late antigen 4 (VLA-4) and CD44 cytoskeleton rearrangement, membrane type 1-matrix metalloproteinase (MMP) activation and secretion of MMP2/9. SCF is the product of the murine Sl locus. It can be produced in both membrane-bound and soluble forms and is a ligand for the receptor encoded by the c-*kit* protooncogene, a member of the type III receptor tyrosine kinase family. The membrane-bound form of SCF stimulates the adherence of stem cells to the stroma.

Rolling and firm adhesion of progenitors to endothelial cells in small marrow sinusoids under blood flow is followed by trans-endothelial migration across the physical endothelium/extracellular matrix (ECM) barrier. HSCs synthesize and express glycosaminoglycan hyaluronic acid (HA), whose presence on HSCs is critical for their trans-marrow migration to the endosteal region after transplantation (Nilsson et al., 2003). HA is the first molecule identified to have a significant impact on the lodgement of engrafting HSCs. Stem cells finalize their homing by selective access and anchorage to their specialized niches in the extravascular space of the endosteum region and in periarterial sites (Lapidot et al., 2005). Using *in vivo* tracking of HSCs, Askenasy et al. (2002) observed that successful homing and lodgement occur when several cells adhere to form a primary clus-

ter in the subendosteal areas of the femoral epiphysis, close to the endosteal surface. The clustering pattern of donor cells and formation of early clusters were observed not only in recipients conditioned by radio- or chemotherapy (busulphan), but also in non-conditioned recipients. The location of the clusters in the epiphysis and the size of the early clusters are independent of the number of donor cells.

Many cell types, including long-term repopulating HSCs, short-term repopulating progenitors as well as some specialized mature cells can home to the bone marrow, but only HSCs initiate long-term repopulation.

Labelled donor marrow cells highly enriched in HSCs were detected in the calvarium bone marrow after 20 min both in non-irradiated and lethally irradiated recipients. In non-irradiated mice, the cells were localized to the vicinity of blood vessel sinusoids, but more than 30 μm from the endosteum. They were still present two weeks after transplantation. In irradiated recipients and also in non-irradiated W/W^v mice (see chapter 5.1.2.) injected cells localized significantly closer to the endosteum. In irradiated recipients, donor cells started to divide within 24 hours, forming clusters comprised of two and more cells (Lo Celso et al., 2009).

5. Availability of stem cell niches for transplanted stem cells

Haematopoietic stem cell transplantation is a procedure when intravenously administered HSCs restore haematopoiesis after its entire or partial destruction by radio- or chemotherapy-based conditioning. All interactions between HSCs, progenitor cells, and their niches are temporarily interrupted to be re-established in the host haematopoietic tissue. Without conditioning, transplantation results in a low level of chimerism, even in a syngeneic setting (see chapter 5.1.1), mostly due to an unfavourable quantitative ratio between the host and donor haematopoietic tissues (Colvin et al., 2004). The ratio can be ameliorated by conditioning the recipients with treatments resulting in cytoreduction of the host haematopoiesis, but this is very inefficient if HSCs are not targeted (see chapter 5.2). Ideally, the conditioning treatment should deprive stem cell niches of HSCs while preserving the niches functionally intact. Preferably, progenitor and blood precursor cells should also be spared to avoid bone marrow aplasia and transient pancytopoenia in the blood. No such ideal conditioning treatment has been developed to date. Also, relatively little is known regarding the damage done to stem and progenitor cells by different conditioning treatments.

5.1. Transplantation without prior haematopoiesis suppression

5.1.1. Transplantation to normal unconditioned (non-myeloablated) syngeneic mice

In the absence of conditioning, only a very limited space is assumed to be available for transplanted HSCs to settle and engraft. This assumption was derived from the early observation of Micklem et al. (1968) showing that the transplantation of 20 million bone marrow cells to normal CBA mice resulted in approximately only 2.5% chimerism among the dividing precursors of blood cells in the bone marrow. However, such a conclusion may not be correct since normal mice have on average 349 million bone marrow cells (Novak and Necas, 1994) and 20 million such cells thus represent only 5–6 % of the total marrow cells present in non-ablated recipient mice. If the chimerism of 2.5% reflected the ratio between the numbers of the donor and the host functional HSCs, 40–50 % of donor HSCs had engrafted and produced blood cell precursors in the experiments of Micklem et al. (1968). Brecher et al. (1982), also using CBA mice, arrived at a similar estimate (30% efficiency) after the transplantation of 20–200 million donor bone marrow cells to non-ablated normal recipients. In another study, transplantation of 100 million marrow cells to non-ablated Balb/c recipients resulted in 24.9% chimerism (Nilsson et al., 1997), suggesting the engraftment and activity of up to 80 % donor HSCs. The engraftment and functional efficiency of bone marrow cells, intravenously transplanted to unconditioned normal recipients, was probably most thoroughly dealt with in the experiments of Rao et al. (1997) and Colvin et al. (2004), who used Balb/c mice. Their results suggest that practically all intravenously transplanted HSCs, contained in unfractionated marrow cells, had engrafted and efficiently replaced an adequate part of the host HSCs. The high efficiency of bone marrow transplantation to unconditioned recipients demonstrated in CBA and Balb/c mice was not fully confirmed when C57Bl/6 mice were used (Brecher et al., 1982). Results published by Saxe et al. (1984) suggested an efficiency of about 20 % for C57Bl/6 mice, those of Voralia et al. (1987) suggested efficiency close to 50 %. We have not observed significant donor chimerism either in the blood or in the marrow after transplanting marrow from a half or two femurs to normal C57Bl/6 recipients using congenic mice differing in the CD45 allele (Ly5.1/Ly5.2 experimental system; unpublished results). Using Balb/c mice, Wu and Keating (1993) established chimerism resulting from transplantation of 20 million marrow cells to completely untreated recipients at different levels of the haematopoietic cell hierarchy from day-12 CFU-S, through GM-CFC and marrow precursors to circulating blood cells (the hierarchy see in Fig. 1). There was a steady decline in the chimerism level measured eight weeks after the transplantation, from 26 % in the CFU-S population to zero in the blood cells. The presence of 26 % of donor CFU-S progenitors suggests a highly selective capture and maintenance of intravenously injected haematopoietic progenitors (day-12 CFU-S) or HSCs, since the chimerism of only 6 % could be anticipated from the ratio of injected marrow cells with those present in the recipients (20 millions/349 millions).

The majority of captured donor early progenitors (day-12 CFU-S) must have remained functionally dor-

mant in non-ablated recipients during the eight weeks that elapsed between transplantation and marrow examination. In the above-mentioned experiments, chimerism was determined between two to 13 weeks after transplantation. Hence, the results reflected the engraftment of progenitor cells as well as that of HSCs. However, Stewart et al. (1993) extended these measurements to long-acting HSCs by demonstrating donor chimerism in non-myeloablated mice persisting for six months and even for more than two years. The transplantation of bone marrow to non-ablated syngenic recipients leads to several important conclusions: (1) the capture and engraftment of HSCs introduced into the circulation by normal haematopoietic tissue is highly effective; (2) the HSCs of the host do not appear to benefit from being settled in the niches, since they are efficiently replaced by transplanted HSCs; (3) the ratio between the numbers of host and donor HSCs is a major determinant of the resulting chimerism level; (4) transplanted HSCs may remain functionally dormant but can survive in the marrow for extended periods of time; (5) the observation that a subpopulation of marrow cells enriched in HSCs by a FACS-based purification procedure engrafted into non-myeloablated recipients with a significantly lower efficiency than HSCs contained in unseparated marrow remains unexplained (Nilsson et al, 1997).

5.1.2. Availability of niches in *W/W^v* mice, *Lig4^{Y288C}* mice and STAT5 knock-out mice

W/W^v mice are double mutants of c-*kit* (*kit* oncogene), the gene that encodes the transmembrane protein tyrosin kinase receptor c-kit for SCF, also known as the KIT ligand. Apart from common lymphoid progenitors, which express low levels of c-kit, all other haematopoietic progenitor and HSCs are phenotypically characterized by c-kit expression on their surface. In *W/W^v* mice one allele (*W*) has a deletion and the receptor does not bind the ligand, while the second allele (*W^v*) carries a hypomorphic mutation affecting the ATP binding domain, which causes a decreased activity of the encoded c-kit receptor. As a consequence, cells from this mouse mutant have an impaired capacity to respond to SCF. The haematopoietic phenotype of this mouse is characterized by significantly decreased numbers of CFU-S progenitors and erythroblasts. These mice have hypoplastic marrow accompanied by severe macrocytic anaemia and less severe deficiencies of granulocyte precursors and megakaryocytes (Chabot et al., 1988; Geissler et al., 1988). *W/W^v* mice can be readily transplanted with HSCs from syngeneic donors without any preconditioning (Harrison, 1972; Maloney et al., 1982). Donor CFU-S numbers increase exponentially during the first week; afterwards their growth rate decreases (Maloney at al., 1982). This transplantation corrects macrocytic anaemia (Boggs et al., 1982) and the host haematopoiesis is steadily replaced by that derived from transplanted HSCs, which occurs over a number of months (Maloney et al., 1982). It is of interest that granulocytic cells are replaced by those derived from donor HSCs at a much slower rate than the erythroid lineage (Barker et al., 1988). The c-kit defect (*W/W^v*) has been recently utilized for construction of a mouse combining this defect with severe defects in immune responses, enabling transplantation of not only syngeneic but also allogeneic and xenogeneic (human) HSCs without preconditioning (Waskow et al., 2009). There are many other mutations of c-*kit*; some of them are gain-of-function and related to a wide variety of cancers. Various loss-of-function mutations, including that of *W^v*, reduce the competitiveness of HSCs when challenged with normal HSCs, and the affected mice engraft syngeneic normal HSCs without preconditioning, although the mutations may only exert a very mild impact on steady-state haematopoiesis (Barker and McFarland, 1988; Sharma et al., 2007).

The ability of *W/W^v* mice and other *W* mutants to engraft transplanted normal HSCs from syngeneic donors could be either caused by the presence of niches lacking HSCs or by niches occupied by "weak" c-kit function-low HSCs, which are readily out-competed by transplanted "higher fitness" normal c-kit function HSCs. The stem cell fitness defect mediated by various mutations appears to be graded. This is indicated by the ability of HSCs carrying the *W^{41}/W^{41}* mutation of c-*kit* to engraft into the haematopoietic tissue of *W/W^v* mice without preconditioning, while the *W^{41}/W^{41}* mice also engraft normal +/+ HSCs without preconditioning (Geissler and Russel, 1983).

In *Lig4^{Y288C}* mice the numbers of HSCs progressively decrease with age due to the defects in the repair of DNA damage caused by deficiency in non-homologous end-joining DNA ligase IV (Nijnik et al., 2007). They engraft congeneic HSCs without prior myeloablative treatment at the age of 10 to 14 weeks.

The conditional deletion of signal transducer and activator of transcription 5 (STAT5) enabled significant engraftment of transplanted congeneic HSCs (Wang et al., 2009). The deletion caused increased proliferation and depletion in the HSC pool.

5.1.3. Conditioning by targeting c-kit signalling

A c-kit blocking antibody was used by Czechowicz et al. (2007) and Xue et al. (2010) to enhance the engraftment of transplanted HSCs. The procedures were partially effective, but in the experiments of Xue et al. (2010) only when combined with a low dose of irradiation.

Fewkes et al. (2010) administered sunitinib, a potent tyrosin kinase inhibitor that also targets c-kit, to recipient mice. This treatment mildly increased the engraftment of donor cells in normal recipients, but not in *W/W^v* mice with suppressed c-kit functionality.

5.1.4. Conditioning by targeting SDF-1/CXCR4 signalling

The signalling by stromal cell-derived factor-1 (SDF-1, CXCL12 chemokine), expressed by endothelial cells, osteoblasts and other stromal cells (CAR cells), and its CXCR4 receptor found on HSCs is part of the link between these two structural units. Kang et al. (2010)

reported that the post-transplant administration of AMD3100 (Plerixafor), a CXCR4 receptor antagonist, increases niche availability through the mobilization of the recipient's residual stem cells and enhances donor cell engraftment.

5.2. Conditioning by cytostatics

Cytostatic drugs target proliferating cells and may induce significant hypoplasia of the haematopoietic tissue. As most HSCs do not engage in proliferation, the engraftment of donor HSCs may be potentiated disproportionately less compared to the degree of marrow hypoplasia. There is a plethora of experimental studies aimed at conditioning mice for the transplantation of HSCs by cytostatic drugs. Collectively, they support the idea that the engraftment of donor cells is potentiated by reduction in the numbers of HSCs and not those of progenitors and maturing blood cell precursors. We present only examples of the drugs and their effects.

5.2.1. 5-fluorouracil (5-FU)

5-fluorouracil (5-FU) is a pyrimidine analogue whose metabolites incorporate into DNA and RNA and induce cell cycle arrest and apoptosis. HSCs are little affected directly, but their proliferation rate is secondarily increased, which makes them more sensitive to a second dose of 5-FU delivered at the right time. Goebel et al. (2004) achieved significantly higher engraftment of donor HSCs in mice treated with two doses of 5-FU (150 mg/kg) given five days apart compared to a single dose. The potentiation roughly correlated with the degree to which the long-term reconstituting potential of the recipient's bone marrow was diminished.

5.2.2. Cyclophosphamide (CY)

Cyclophosphamide is an alkylating agent that damages proliferating cells irrespective of the cell cycle phase. A dose of 135 mg/kg causes severe, though transient bone marrow hypoplasia with cellularity decreasing to ~ 15 % (Šefc et al., 2003); 400 mg/kg is close to its lethal dose in mice. Yoshikawa et al. (2000) transplanted 10^7 BMCs (corresponding to ~ 3.5 % of the total mouse bone marrow cells; Novak and Necas, 1994) to congenic mice that earlier received from 50 to 400 mg/kg of CY. There was only 3–4% donor chimerism in mice pre-treated with 400 mg/kg despite very severe bone marrow hypoplasia. Robinson et al. (2000) transplanted 3×10^7 BMCs to mice pre-treated with a protocol consisting of a single dose of 200 mg/kg of CY followed by four daily injections of G-CSF. The treatment with CY and G-CSF resulted in significant reduction of progenitors in the marrow, but it hardly increased the engraftment of transplanted cells compared to the controls.

5.2.3. Busulphan

Busulphan is an alkylating agent that also damages HSCs. Mauch et al. (1988) achieved 50% donor chimerism after the transplantation of 10^7 bone marrow cells to mice conditioned with doses of busulphan that had similar effects on the survival of non-transplanted mice and on the number of CFU-S progenitors as ionizing radiation. Down and Ploemacher (1993) demonstrated significant and stable engraftment of donor cells in mice treated with 50 mg/kg busulphan, similar to that achieved in mice irradiated with 6 Gy.

5.3. Conditioning with ionizing irradiation

Irradiation damages cells by inducing double-strand DNA breaks and causing other damage mediated predominantly by oxygen radical species (ROS), which also damage other cellular components by causing lipid peroxidation and protein alkylation. Generally, proliferating cells are more sensitive to radiation-induced damage compared to differentiated, functionally specialized and non-proliferating cells. Consequently, the small intestinal epithelium, haematopoietic tissue and the epidermis of the skin are among the most sensitive tissues. Exceptions to this rule are non-proliferating (G_0) lymphocytes, which are highly sensitive (Vokurková et al., 2006). Depending on the extent of damage, the outcome for an affected cell can be full recovery, cellular senescence, or death. All haematopoietic cells, including proliferatively quiescent (G_0) stem cells, are highly sensitive to radiation damage.

5.3.1. Damage to the haematopoietic tissue after irradiation

The irradiation of mouse haematopoieic tissue with doses from 1 to 10 Gy causes transient or permanent bone marrow hypoplasia, depending on the dose. The minimum bone marrow cellularity is reached approximately12 h after submyeloablative doses (Nečas and Znojil, 1988). The haematopoietic stroma, including its cellular part, is more resistant to irradiation. However, it is also subject to damage and the bone marrow undergoes extensive remodelling following irradiation (Bengtsson et al., 2011). Li et al. (2008) and Lo Celso et al. (2009) demonstrated significant damage to sinusoidal endothelial cells and to the circulation. Dominici et al. (2009) demonstrated damage to the osteoblasts in myeloablatively irradiated mice followed by their rapid regeneration. Also the trabecular bone, a predominant location to which transplanted HSCs home and engraft, is damaged even by submyeloablative irradiation (Green et al., 2012). Surprisingly, this does not seem to compromize the homing and engraftment of transplanted HSCs, which is highly efficient (Matsuzaki et al., 2004).

5.3.2. Making niches available for transplanted HSCs after irradiation

Haematopoietic stem cells appear to be present in the haematopoietic tissue as single cells (Wilson et al., 2008). Therefore, a niche containing a HSC may not to be ready to engraft another such cell. Although this assumption is speculative, it corresponds to the current knowledge and the idea of the space that has to be created to enable engraftment of the transplanted HSCs.

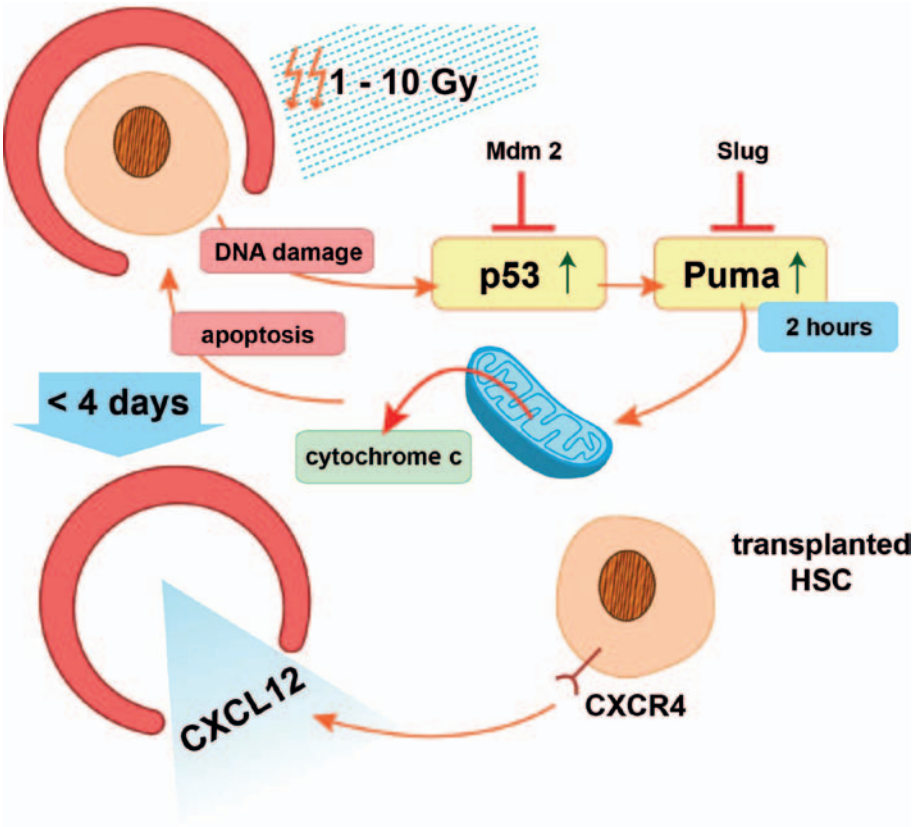

Fig. 5. Significant role of p53 in emptying haematopoietic stem cell niches after irradiation. p53, as well as its target p53 up-regulated modifier of apoptosis (Puma), are rapidly up-regulated by DNA damage in HSCs after irradiation. This causes release of mitochondrial cytochrome c leading to apoptosis through activation of caspases. The potent mechanism of apoptosis induction in HSCs is inhibited by the Mdm2 and Slug proteins. CXCL12 chemokine concentration gradient is assumed to navigate HSCs to niches by means of the CXCR4 receptor expressed in HSCs.

5.3.2.1. Little evidence for a role of cellular senescence affecting HSCs in creating space for transplanted cells

Cellular senescence irreversibly ceases proliferation in aging or damaged cells through the inhibition of cyclin-dependent kinases (Sabin and Anderson, 2011). The cells affected by senescence remain in the tissue but suffer from irreversible growth arrest. They are increasingly resistant to apoptosis, have elevated levels of p16, p19 and p21 inhibitors of cyclin-dependent kinases. Other phenotypic markers are increased senescence-associated β galactosidase activity, presence of detectable foci of DNA damage and a senescence-associated secretory phenotype. HSCs affected by proliferative senescence would remain physically present in their niches, but they would not contribute to blood cell production. As such, they would become silent parts of the haematopoietic tissue presumably still occupying niches and blocking engraftment of transplanted HSCs.

Submyeloablatively or myeloablatively irradiated mice readily engraft syngeneic donor HSCs administered immediately or during the first few hours after irradiation, which would be unlikely if HSCs of the recipient affected by the senescence occupied the niches. There is also very little evidence for radiation-induced partial damage to HSCs surviving in mice exposed to submyeloablative irradiation since they compete well with non-irradiated donor cells in the primary and even secondary recipients (Pelichovská et al., 2008; Michalova et al., 2011; Hlobeňová et al., 2012). The outcome of submyeloablative irradiation thus appears to be a surviving fraction of fully functional HSCs and the apoptotic death of the second fraction of HSCs.

5.3.2.2. Evidence for HSC apoptosis induced by p53 and Puma in creating space for transplanted cells

There is persuasive evidence that the intracellular signalling initiated by up-regulation of the p53 protein has a dominant role in the death of HSCs in irradiated haematopoietic tissue. p53 is up-regulated by DNA damage shortly after irradiation. Of the several p53 target genes, the one for the BH3-only protein Puma (p53 up-regulated modifier of apoptosis) is critical. Similarly to mice lacking functional *Trp53* gene encoding the p53 protein, mice lacking functional gene for Puma are increasingly resistant to the irradiation-induced haematological tissue failure (Shao et al., 2010; Yu et al., 2010). On the other hand, the haematopoietic tissue of mice lacking the gene for Mdm2, coding an inhibitor of p53 and thus increasing p53 signalling, as well as of the mice lacking the gene *Slug* encoding inhibitor of Puma, are more sensitive to irradiation. The role of p53 in the apoptosis induced by irradiation is summarized in Fig. 5.

Marusyk et al. (2010) observed no significant loss of phenotypic HSCs within 48 h of irradiation, but a functional assay indicated significantly reduced numbers of them. Due to a low proliferation rate, HSCs can physically remain in their niches after irradiation before they succumb to apoptosis. Submyeloablative doses of irradiation induced apoptosis in bone marrow cells after several hours and apototic cells were present up to four days after irradiation (Christophorou et al., 2005).

High doses of irradiation, which are uniformly lethal without the transplantation of donor HSCs, result in the conversion of haematopoiesis purely to the donor type due to the absolute elimination of the host HSCs. Lower doses of irradiation result in partial chimerism, the degree of which is quantitatively related to the radiation dose (Down et al., 1991). The efficiency with which ionizing radiation increases engraftment of donor HSCs is a major argument for the necessity of creating space for donor HSCs by emptying niches.

6. Conclusions

Niche and a stem cell represent a basic functional unit of haematopoietic tissue. They are very rare structures distributed in the bone marrow among more than 200 bones of the mouse. Their total number can be estimated to be between 3,000 and 9,000. Although stem cells can survive separated from the niche for a limited time, their long-term survival and control of their function fully depend on mutual interactions with their niche. While the HSCs and progenitor cells of the mouse are well defined, their niches are still vaguely defined both structurally and functionally. They appear to attract HSCs introduced into the circulation efficiently. How much this depends on the niches being deprived of HSCs ("empty") is uncertain, since non-myeloablated haematopoietic tissue also engrafts syngeneic donor HSCs. Reduction in the numbers of HSCs, but not in the total number of bone marrow cells, enhances the engraftment of donor cells significantly. The exposure of mice to ionizing radiation is highly effective in enhancing the engraftment of donor cells. Although irradiation may induce proliferation senescence in the exposed cells, there is little evidence that this contributes to the enhanced engraftment of transplanted HSCs. The apoptotic death of HSCs mediated by the expression and activation of the protein p53 and its target gene *Puma* appears to be the main mechanism by which niches are made available to transplanted donor cells after irradiation.

Acknowledgement

The authors thank Dis. Veronika Sýkorová for graphical design of the figures.

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