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Nikolskaya E. I., Butenko G. M.

State Institute of Genetic and Regenerative Medicine NAMS, Kyiv, Ukraine

e-mail: nakato@bigmir.net

STRUCTURAL-FUNCTIONAL ORGANISATION OF THE BONE MARROW HEMATOPOIETIC STEM CELLS NICHES

ABSTRACT

This article focuses on (1) the analysis of the structural-functional organization of bone marrow niches of the hematopoietic stem cells, (2) the role of the intercellular contact interactions and humoral regulation factors in these niches, in particular CXCL12, SCF and TGFβ, and (3) the intracellular signal pathways: Notch, Wnt and Shh. The two types of niches, switching from one into another: endosteal niches located on the endost surface at the borderline with bone marrow cavity and the vascular niches included into bone marrow parenchyma. It is emphasized that the main role in the formation of the niches of both types is ascribed to the multipotent stromal cells, which serve as a base for differentiation of the osteoblasts, spindle-shaped N-cadherin$^+$CD45$^-$ osteoblasts (SNO-cells), nestin-expressing cells (Nes$^+$ cells), cells with leptin receptor (Lepr$^+$ cells), abundant producing CXCL12 reticular cells (CAR-cells) and NG2-pericytes. The endothelial cells are no less important. Also, the adipocytes, osteoclasts, macrophages and megakaryocytes, regulatory T-cells and neuronal cells are involved in the niche functioning. It is postulated that osteoblasts and CAR-cells play a crucial role in the genesis of immune system cells: common lymphoid precursors, B-lymphocytes, natural killer and dendritic cells.

KEYWORDS: *bone marrow, hematopoietic stem cell niche, multipotent stromal cells, hematopoiesis*

Immune system is the structural-functional and interrelated commonness of the hematopoietic and stromal cells. Different in their origin, structure and functions, the populations and subpopulations of lymphoid and myeloid cells make a complete wholeness in the definite tissues and organs where they closely cooperate with non-hematopoietic elements. Constant and strictly regulated intensity of hematopoiesis with production of various cell types is also conditioned and controlled by cooperation of the hematopoietic and stromal cells. Timely repopulation of the immune system with hematopoietic cells-precursors, lymphocytes, leucocytes and stromal cells, newly maturing in the bone marrow, is the main condition for effective immune system functioning. The number of cells formed per time unit is plentiful. According to the given data, nearly 10^{11} neutrophils (about 100 g of mass) per 24 hours are formed and enter blood stream. Human blood contains $7 \cdot 10^9$ per liter of neutrophils and monocytes making 50-70 % of the total count of blood leucocytes. The remaining great number of blood cells is represented by the lymphocytes and the cells of minor populations. Additionally, an impressive number ($250 \cdot 10^9$) of the erythrocytes is produced daily in human organism, characterizing by its number the intensity of blood formation [1] due to functioning of hematopoietic stem cells (HSCs) in cooperation with stromal elements on the area of bone marrow niches.

HEMATOPOIETIC STEM CELLS – SELF-MAINTENANCE AND MULTIPOTENCY

The exceptional properties of HSCs are self-maintenance and multipotency. As was postulated at the beginning of the 20th century by A. A. Maksimov and finally established at the end of the past century, it is not only the number but also entire known diversity of cell elements of immune and blood systems originate from only one type of the progenitor elements – the HSCs (unitary theory) [2].

Life force and productivity of the HSCs is proved, for example, by the fact that 99.9 % of all blood-generating cells, HSCs included, in the irradiated mice can perish, and the surviving stem cells rapidly renew blood formation, including proper population as well as all types of the committed progenitors [3]. Along same sequence, the fact of non-exhaustion of blood formation after repeated damaging exposures is explained by the presence and functioning of the HSCs [1].

The specialized cells of adult and fetal periods of ontogenesis, residing in the bone marrow (BM) in the quiescent state and capable, together with blood-forming microenvironment, to realize underlying programs for self-maintenance and multipotency allowing them (with maintained amount of HSCs) to release necessary amount of cells into differentiation along various directions that ultimately leads to the formation of all forms of blood elements, including immune system cells. It should be noted that the self-maintenance is not identical to the immortality. Stem cells are characterized by sufficiently long life, commensurable with entire organism's life course. Several genetic regulatory programs have been established which are of great importance in the process of HSCs self-maintenance. It is also known that stem cells in various tissues are controlled by common genetic programs maintaining their nature [4, 5].

It is theorized that stem cells are the clonogenic units which under certain conditions can be increased in their number at the expense

of clonal expansion. The authors also consider that this property is compulsory for the stem cells.

Nevertheless, regarding their self-renewal potency the HSCs show great heterogeneity [6]. In mice, the fraction of $CD34^{-}c\text{-}Kit^{+}Sca\text{-}1^{+}lin^{-}$ bone marrow cells is the highly-enriched HSCs. Cells of minor subpopulation expressing CD150 possess the greatest self-renewal capacity [7-9]. The self-renewal potential is usually inversely proportional to the number of cell divisions [10]. The majority HSCs enter into cell cycle once per month [11-13]. The rest of them, so-called "dormant", switch into it rarely [14], but both subpopulations are initially in the G_0 phase. Studies on the transplantation of individual cells revealed a rare HSCs subpopulation – latent HSCs, showing marked repopulation activity only as a result of their serial transplantation evidencing that latent HSCs have longer dormant periods [9]. Furthermore, the HSCs in dormancy can be influenced by their microenvironment. Thus, being functionally active, the bone marrow and spleen HSCs have different dormant phase by its duration [15].

Investigations on the multipotency are also progressing. There is a belief that it is realized via committing, differentiation and maturation processes. The first stage of differentiation is the committing as a result of which the HSCs capacity for self-maintenance and potency are diminished and there appears a marked «differential» hierarchy in the HSCs pool. There follow the key stages in the final choice of the way of differentiation. Upon their completion the monopotential progenitor cells are formed. The process of further open-way development of these cells with a formation of mature hematopoietic elements (maturation) is frequently designated as «terminal differentiation» and the appearing cells are called «dead-end» [1].

The degree of potency in various types of stem cells differs significantly. Thus, the fertilized oocytes and primordial embryonic cells can give the start to an entire organism (totipotency). The cells of all three germinal layers are formed from the cells of inner mass of blastocyst (pluripotency). The stem cells-parents for 1-2 embryonic leaves are considered to be multi- or polipotent, and the progenitor cells can be limited in their capacity up to oligopotency.

There are essential differences between HSCs and their nearest offspring's in terms of the mechanisms of regulation of their activity: if for HSCs activity is determined by the value and the quality of microenvironment of blood-forming territories with a dominant role of intercellular contacts, their offsprings entering differentiation, are greatly dependent on the humoral, mainly, cytokine regulation [16].

As far back as 80s of the past century a concept was formulated about the HSCs continuum explaining, in large measure, the high heterogeneity of these cells. The continuum incorporates cell elements which differ by duration of self-maintenance and clonogenic, potency and sensitivity to various regulatory influences. It includes the three cell subpopulations: 1) HSCs inside niches possessing maximal capacity for self-maintenance and highest multipotency and insensitive to the humoral regulation; 2) HSCs outside niches with essentially reduced capacity for self-maintenance and limited potency and appearance of noticeable sensitivity to humoral regulation; and 3) non-multipotent committed precursors which completely lost self-maintenance capacity.

According to the authors' opinion [1], this division is conventional enough. However it puts in order our vision of the HSCs system and explains numerous research facts. Thus, for example, next to the class of committed precursors is placed the main body of colony-forming spleen units (CFUs). They produce transient colonies, which can be seen only on days 7-8 following bone marrow cells transplantation to the lethally irradiated mice. Further the CFUs disappear at the expense of complete maturation of the composite blood-forming cells. After 10-11 days these colonies do not reside in the spleen any more. The daughter CFUs are not formed in the transient colonies [17].

In the case of BM transplantation to the irradiated mice, one observes the three waves during hematopoiesis recovery: first – at the expense of 3rd continuum category cells (lineally restricted cells which quickly fill the pool of mature leucocytes as a result of differentiation and proliferation); second – conditioned by differentiation and proliferation of the 2nd category progenitors (multipotent repopulating HSCs – short-term HSCs, ST-HSCs); and 3rd – reflecting HSCs functioning (long-term HSCs, LT-HSCs) [18].

Development of HSCs in the mice and hematopoiesis formation pass several stages beginning from embryonic, depending on the microenvironment. The multipotent mesenchymal stromal cells (MSCs) promote HSCs population. It has been shown that embryonic MSCs, which are capable for linear differentiation, are concentrated at the sites of HSCs dispersion. They first appear in the aorta-gonad-mesonephros region (AGM). On reaching the plateau, the number of MSCs increases considerably in the mature BM. Migration of HSCs is accompanied with MSCs. Moreover, the embryonic MSCs circulate in the blood. Thus concerted localization of MSCs and HSCs on the definite areas in ontogenesis evidences for possible cooperation of these cells during hematopoiesis [19, 20].

Upon passing stage-wise changes in the aorta-gonad-mesonephros region, owing to the known reasons and mechanisms, the HSCs are transferred into the embryonic liver and then in the spleen and bone marrow, where they find a microenvironment required for their maintenance and functioning during an entire life course of the individual. An exception seems to be the BM (yellow) after involution, containing a great number of the adipocytes located perisinusoidally and probably originating from adventitia [1]. Generally, there is an inverse association between adipocyte contents and hematopoiesis intensity [21]. The yellow (fatty) bone marrow can transform into red bone marrow during intensive hematopoietic stress (phenylhydrazine-induced anemia). However upon regeneration completion, the structure of the fatty bone marrow is recovered in the renewed sites. The fat content in the BM cells is not reduced during starvation and therefore they hardly serve a simple fat depot [21].

In humans, blood formation in the BM is terminated in the 2nd trimester [22, 23]. In the long bones it is ceased between 5 and 7 years of life with replacement of the red bone marrow by the yellow one [24]. After birth the spleen is not involved in blood generation, although extramedullary hematopoiesis in it can proceed during hematopoietic stress, presumably, at the expense of proliferation of the committed precursors [25], apparently, of the 3rd and 2nd parts of the continuum. In the rodents the spleen remains hematopoietically active during a whole life course [26].

Still, the main site for blood generation especially in the humans is the BM, the sole specialized organ of hematopoiesis where the HSCs and hematopoietic progenitors generate the hematopoiesis in close interaction with the stromal elements in the niches [27-29].

HEMATOPOIETIC INDUCTIVE MICROENVIRONMENT

The HSCs niches are the anatomical-functional formations. Here the HSCs are located in the stroma, which maintains their viability, self-renewal and differentiation signifying the start of generation of the hematopoietic cells of all lineages, including lymphoid and myeloid elements forming the systems of adaptive and inherent immunity. An active role of stromal cells in HSCs functioning was demonstrated more than forty years ago. Stromal elements involved in this process were designated as the hematopoietic inductive microenvironment (HIM) [30].

Upon bone marrow transplantation into the spleen of the irradiated mice it has been found that colonies in central areas of the injected BM were predominantly granulocytic whereas the BM cells encircled by the spleen stroma generated mainly erythroid colonies. At the same time, mixed colonies settled on the areas of crossing boundaries of both types of stroma [31] that clearly pointed at the great role of stroma in hematopoietic cells function.

Several investigators managed to transfer blood generating microenvironment using the method of heterotopic transplantation of the BM stromal cells [30, 32]. During fragmentary BM transplantation the hematopoietic cells leave the transplant whereas the stromal precursors build a new microenvironment with repopulating hematopoietic recipient cells. Other investigators of such formation in the sites of ectopic blood generation obtained the convincing proofs in the favor of crucial role of the HSCs/stroma interaction [33, 34].

Evidences of donor origin of the stroma in ectopic foci were first obtained using an experimental immunologic approach. The BM of C57BL/6 mice was implanted to the first generation hybrids of this strain and its allogeneic RIII strain. By virtue of co-dominant inheritance of transplant antigens, the F1 hybrid accepted tissues from both parental strains. In turn, the transplant was not rejected and the focus of ectopic blood generation is formed. After 4 weeks it was transferred to the animals of the donor line or the F1 hybrids. In both cases the secondary ectopic sites appeared and functioned for a long time that undoubtedly proves donor origin of the site-generating stroma. During transplantation of the site functioning even as long as 12-14 months it survives well in the parents and is not refilled within this time with recipient's stromal cells potentially capable to form the site of hematopoiesis [35].

Karyotype and antigen analyses have shown that only blood-generating in cells belong to the recipient. The stromal cells, fibroblast-like in particular, in the ectopic sites have donor's genesis. Should the BM be implanted into diffusion chamber impermeable for the cells, there also appears bone and blood-generating microenvironment, suitable for population with blood-generating cells, for example, after chamber wholeness disturbance [36].

Important data concerning stromal regulation of HSCs were obtained in the study carried out in the mice-mutants with genetically-conditioned defect of stromal regulation. Phenotypically, pleiotropic mutation by Sl gene is expressed in severe macrocytic anemia, sterility due to the absence of embryonic cells and white color of animal hair [37]. The HSCs in the mutants were normal that was detected during their transplantation to the wild irradiated mice (+/+), with the following complete recovery of blood formation. However in the reverse scheme of experiment with transplantation of normal HSCs to the mutants no recovery of blood formation took place and anemia in the mutants remained untreated after normal BM transplantation [38].

If the spleen of Sl/Sl^{d} mice is transplanted to the splenectomized siblings, irradiated after some time and injected normal HSCs, no blood generating colonies appear in the transferred spleen. By contrast, in the case of +/+ spleen transplantation to the Sl/Sl^{d} mice an intensive hematopoiesis develops enough sufficient even for anemia correction [39]. Further the +/+ murine spleen was sown to the spleen of the mutants or vice versa. Then these mice were exposed to irradiation and following BM transplantation. In both cases there were more colonies in the +/+ type versus Sl/Sl^{d}type spleen. It is possible to inject the BM stroma of the mutants to +/+ murine spleen or vice versa, then to irradiate them and protect them with syngeneic normal BM. In the first case active erythropoiesis was detected only in the spleen, ambient the implant; and in the second case only in the implanted stroma of the BM [40].

Thus in the mice with genetically abnormal stroma of the blood-forming organs, the absence of any distantly and system-wise factor of HSCs regulation is detected. In one and the same organism there may take place normal blood generation in one sites and pathological in the neighboring ones. All this uniquely evidences about local regulation of HSCs proliferation by stromal hematopoietic microenvironment.

Subsequently, the ideas about HIM were widened to the appearance of a concept explaining the basic laws of HSCs involvement in blood generation, about the niches where the HSCs can be maintained, self-renew and differentiate [41]. The MSCs, osteoblasts, reticular and endothelial cells make the niche base. The osteoclasts, macrophages, pericytes, megakaryocytes, adipocytes and extracellular matrix are added to it.

It is most convenient to study the role of BM stroma in HSCs functioning in the cell cultures beyond complex organism processes. However creation of long-term culture of hematopoietic cells has been a failure during decades. As it appeared, the unsuccessful attempts are explained by the absence in cultures of the stromal elements essentially needed for HSCs viability.

In due course prolonged maintenance of the organ cultures preserving their tissue cytoarchitectonics time has been gained. It has been found that in the organ culture of embryonic liver there takes place maintenance of the HSCs forming *in vitro* various colonies of the blood-generating cells. The cells of these *in vivo* cultures protect the lethally irradiated mice. Of note, only deep-residing HSCs, immediately contact with liver microenvironment, were able to proliferate intensively [42].

The break-through technology was the development of monolayer Dexter-type cultures of HSCs with involvement of stromal elements [43-45]. When seeding bone marrow cells, it is possible to watch the formation of colonies of the fibroblasts, synthesizing type I and type III collagens and fibronectin included into cell microenvironment [46]. In the monolayer cultures of the BM [43] the hematopoietic cells are often placed above the fibroblasts, creating a kind of cobblestone area. It is thought that it is precisely in these areas that the HSCs and MSCs interaction takes place, inducing active blood generation, and the HSCs and proliferating progenitors are stably maintained in culture. This occurs only under conditions of a direct contact of the hematopoietic cells with the sub-layer cells. In dividing them by the filter, impermeable for the cells, hematopoiesis is quickly exhausted [47]. According to some data, the fibroblasts of BM but not of the spleen, skin or bone, considerably improved the survival of the HSCs *in vitro* [48].

The cells maintaining hematopoiesis in the long-term culture (long-term culture initiating cells – LTC-ICs), of the bone marrow were found, called this name since they showed an unprecedented colony formation ability during 5 weeks and more only during co-culturing with stromal fibroblasts. Stromal fibroblasts produced SCF (stem cell factor), IL-3 and granulocyte-colony-stimulating factor (CSF-G). Under such conditions the CD34^{+}CD38^{-} cells showed their self-renewal ability within a 6-week period. Incubation of the HSCs and cells-precursors in the serum-free medium with addition of FLT3-ligand, SCF, IL-3, IL-6, granulocyte colony-stimulating factor (CSF-G) and nerve growth factor (NGF) induced proliferation with formation after 10 days of colonies, including from 4 to 1000 cells, 40 % of which included more than one LTC-IC. During the first 10 days the LTC-IC count increased 30-fold and by the end of the 1st-3rd week 50-fold. In the absence of the soluble stromal factors the effect was decreasing [10].

Better results were obtained during HSCs cultivation in a close contact with the stroma. The authors came to a conclusion that contact of the HSCs with stromal cells plays an indispensable role in increasing the count and improving the properties of HSCs cultured with the cytokines, [49].

Nevertheless, a considerable role of the cytokines in the realization of the effect of stromal cells is obvious. The MSCs constitutively express mRNA IL-6, IL-11, leukemia inhibitory factor (LIF), macrophage colony-stimulating factor (CSF-M) and SCF. In the IL-1-stimulated MSCs it is possible to observe an elevated expression of mRNA of IL-6, IL-11, LIF and the beginning of expression of CSF-G and granulocyte-macrophage colony-stimulating factor (CSF-GM). In the osteogenic condition cultured MSCs, the levels of mRNA of IL-6, IL-11 and LIF decrease whereas expression of CSF-M and SCF remains unchanged; CSF-G and CSF-GM are not detectable, and IL-3 are undetected in the MSCs in no case. However the MSCs pre-incubated in the control or osteogenic medium have similar supporting capacity for LTC-ICs [50].

During co-culture of CD34^{+} cells of the umbilical blood and BM MSCs in the presence of SCF, the flt3-ligand and IL-3, the cells attached to MSCs were located at two levels: on and under the monolayer of MSCs. Predominantly, the erythroid and multipotent progenitors were bound with the MSCs. The free hematopoietic cells had the CD34^{+}CD45^{low}, phenotype being analogous by these markers to the newly-released CD34^{+} cells in fresh-released CD34^{+} cells of the umbilical blood. The relative content of CD34^{+}CD38^{-} HSCs (early, re-populating HSCs) increased from 4-9 % to 53-55 % [51]. A repeated increase of the count of umbilical blood CD34^{+} cells co-cultured with MSCs of the umbilical-placental origin has been demonstrated [52].

Investigations dealing with HSCs *ex vivo* modeling were carried out. One of the backgrounds was found that HSCs need interaction with MSCs for keeping dormancy *in vivo* and *in vitro* [53]. It has been also shown

that intercellular contacts *in vitro* produce a considerable influence on the functional, phenotypic and clonogenic properties of HSCs. A direct contact with MSCs influences on the HSCs migration and gene expression profile of HSCs maintenance during expansion *ex vivo* [54].

During co-culturing of HSCs and MSCs it became possible to single out three different compartments according to the objective criteria: 1) the medium in which the HSCs grow without permanent contact with MSCs; 2) the MSCs surface; and 3) the surrounding surface under the layer of MSCs. The phase-contrast, confocal and electron microscopy identified the non-adhesive cells, adhesive to surface MSCs cells and cells, which migrated under the feeder layer. The merged monolayer of the MSCs can serve as the borderline between two different compartments. These spatial limitations influence on the proliferation and differentiation of the HSCs. Here, the state of the cell cycle of the cultured HSCs became notable. It is worth noting, literature data showed that immediately after release from the peripheral blood the HSCs have the G_0/G_1 cell cycle phase [55] and their status changes in favor to the proliferative cells during growing with stimulating factors [56].

In this work co-culture of HSCs and MSCs led to a considerable increase of G2/M-cells seen over the MSCs layer but not among the non-attached cells or among the cells under MSCs layer. Thus, intercellular contacts on the surface of the MSCs layer promote division of cells, but cells that migrated under MSCs layer preserve phenotype and reduced division rate. Formation of the sub-layer HSCs fraction was slowed during blockade of β1-integrins or CXCR4. The effect was even greater during combined blockade that pointed to the synergic role of β1-integrins and SDF1/CXCR4 axis in the formation of this fraction. It remains unclear whether the environment under MSCs layer actively maintains immature state of HSCs, or whether the MSCs form niche environment which by its mechanism attracts dormant HSCs. Both of the mechanisms can be involved. The authors succeeded showing that predominantly $CD34^+CD38^-$ HSCs migrate across the MSCs layers [57].

ENDOSTEAL NICHE AND OSTEOBLASTS

Self-maintenance, differentiation and proliferation of the HSCs occur in the niches of two types: endosteal and vascular. In each of them, a special contact and humoral interactions between various subpopulations of the stromal cells and HSCs is accomplished, determining further development of the latter.

In the mice blood formation occurs in the bone marrow in the bone cavities. The BM is well vascularized [58]. The central artery passes through BM parenchyma. The smaller-caliber radial arteries branch off from it, gradually becoming smaller in the diameter and passing into the arterioles. The arterioles form a dense vascular network near the endosteum [59]. Here one observes the transfer to the venous vessels cover with the $Sca\text{-}1^-$ endothelium. The venous sinusoids get enlarged and close to the central bone marrow part enter into the central sinus. The sinusoids contain the fenestral areas providing for newly-formed BM cells outlet into the blood [60].

The bone is subject to constant remodeling by way of close cooperation between synthesizing osteoblasts and resorptive osteoclasts, hematopoietic by the origin [61]. When destroying endost components, the osteoclasts induce HSCs mobilization [62]. The osteoblasts are found along the endosteal surface at the borderline between bone and BM. The spindle-shaped $N\text{-}cadherin^+CD45^-$ osteoblastic cells (SNO-cells), expressing a high level of N-cadherin, being immature osteoblasts, are also lined along the bone marrow surface of the endosteal region. Morphologically, the SNO-cells differ significantly from the mature cuboidal osteoblasts [63].

The main function of the osteoblasts during bone remodeling is the secretion of proteins of non-mineralized bone matrix called the osteoid. The extracellular matrix is crucial in niche functioning, particularly in involving HSCs for migration. Type I and IV collagen and fibronectin are found in the endosteum. Type IV collagen and laminin are bound with bone marrow vessels, including arterioles, veins and sinuses. The fibronectin is also distributed in the central part of the BM. All proteins, excluding type IV collagen, are found in the bone. The fibronectin, types III and IV collagen and laminin are also found in the periosteum [64]. Synthesis of matrix proteins is intensified by the osteoblastic lineage cells as they are differentiated from the preosteoblasts to mature osteoblasts. Osteocalcin is practically produced only by the mature cells. They produce large amounts of the type I collagen, osteopontin, sialoprotein and alkaline phosphatase [65-67]. The polysaccharides and, particularly the hyaluronic acid, occupy a definite place in the niche [68]. Hyaluronidase treatment of the mice promotes MSCs mobilization [69].

It is also important that osteoblasts regulate differentiation of the osteoclasts [61, 70]. Similar to the adipocytes, the osteoblasts originate from the differentiating MSCs [71, 72]. The capacity of MSCs for multilinear differentiation and the presence of initially common inter-linear genetic mechanisms in its process is confirmed by temporary expression in the preadipocytes of the products characteristic of the osteoblastic lineage and vice versa [73].

Several investigations demonstrated that primary human osteoblasts maintain proliferation of the primitive hematopoietic progenitors *in vitro* that may evidence for possible participation of the osteoblasts in the hematopoiesis [74-77].

Active role of the osteoblasts as regulators of HSCs in the niches *in vivo* was showed in the two mutually supplementary works [63, 78]. Calvi L. et al. [78] applied influences of the constitutively activated system parathyroid hormone (PTH)/peptide parathormone receptor (PPR) being under the control of osteoblast collagen promoter αI (I) – Colα1(I). In the PPR-transgenic mice the authors observed a significant increase of the HSCs contents in the BM in combination with expansion of the spongiform bone area and increase of the number of the trabecular osteoblasts expressing the high level of Jagged-1, the ligand of signal pathway Notch. PPR activation by the parathyroid hormone increased the number of the osteoblasts in stromal cultures whereas addition of the inhibitor of γ-secretase suppressing Notch activation averted this effect. Finally, during investigation of the stromal cultures of wild type mice stimulated by parathormone the authors noticed reproduction of a situation observable in the culture of cells of the PPR-transgenic mice. Taken together, such observations allowed the authors make a conclusion about participation of the osteoblasts as regulatory component during formation of the bone marrow niches of HSCs.

Another approach was demonstrated by Zhang J. et al. [63] who, using the Mx1-Cre/loxP system, performed conditional knockout of the osteoblast gene receptor of the bone morphogenetic protein Ia (BMPR Ia) and, thus, investigated the role of signal transmission in niche functioning, mediated by an interaction of bone morphogenetic protein (BMP) with its receptor. Within the loxP/Cre system, the target gene is flanked with loxP sequences recognizable by Cre-recombinase, and the Cre gene is placed under gene promoter being activated only under definite conditions. It is known that BMP-BMPR Ia signaling pathway plays a great role in the formation of embryonic and postnatal hematopoiesis. As a result of the experiment in the animals, the authors observed the formation of the ectopic trabecular spongy bone zones containing a considerably increased number of the spindle-shaped $N\text{-}cadherin^+$ cells (SNO-cells). The number of LT-HSCs attached to these cells via N-cadherin was also 2.2-fold increased. A correlation was seen between the number of SNO-cells on the ectopically placed bone surface and an increasing number of HSCs in the tissue.

It was found that the HSCs, resistant to 5-fluorouracil (5-FU) and expressing Tie2 and N-cadherin, are in the contact with the osteocalcin-synthesizing osteoblasts [79] and exhaustion of the osteoblasts expressing thymidine kinase (Col2.3Δtk), owing to the fragment 2.3 kb of the Colα1(I) promoter by ganciclovir treatment disturbed the development of the erythroid cells and B-lymphocytes. Apparently, there took place a compensatory activation of the extramedullary hematopoiesis in the spleen and in the liver [80]. As a result of ablation of endosteal osteoblasts, the process of LT-HSCs self-renewal was disturbed while leukemia development was accelerated [81]. Thus essential role of the osteoblast

lineage cells in the hematopoiesis was again demonstrated and the data were obtained showing the presence of cellular elements beyond bone marrow capable to maintain blood formation.

Many investigators, using labeled HSCs, established their localization after transplantation into endosteal area [43, 58-60, 82-85] that is in conformity with the data showing that LT-HSCs, retaining bromodeoxyuredine (BrdU) over a long time, are predominantly localized close to SNO-cells in the endosteum [60].

Thus expansion of SNO-osteoblasts resultant from elevated functional loading of the PTH/PPR system [78] or BMPR Ia inactivation is associated with increasing number of the HSCs but exhaustion of the osteoblasts in the corresponding transgenic mice by ganciclovir leads to a definite decrease of HSCs contents in the BM and a significant reduction of B-cells and erythroid progenitors [80, 86]. The presented data seem to be sufficient to assign the major role of osteoblasts in the creation of the HSCs niches.

However the PPR, BMPR Ia and Colα1(I) are also expressed by the CAR-cells (CXCL12-abundent reticular cells) being at some distance from the endosteum [87]. Furthermore, the data about contribution of the osteoblasts, especially mature ones, to HSCs maintenance appeared contradictory. Parathormone processing increased the number of ST-HSCs not on the account of osteoblasts expansion but rather owing to Wnt-ligand Wnt10B production by the T-cells [88]. Moreover, increase of the osteoblasts number is not always sufficient for HSCs expansion. Treatment of mice with bone anabolic strontin leads to the expansion of mature osteoblasts but does not affect either the number or the function of HSCs [89]. On the contrary, exhausting osteoblasts count of the HSCs remained normal in the mice with chronic inflammatory arthritis [90]. Earlier studies found that the number of HSCs was not decrease in mice with a reduced number of mature osteoblasts [53, 91]. And, finally, conditional deletion of CXCL12 [92-96] or SCF [94] in the mature osteoblasts did not affect bone marrow HSCs content.

While evaluating the situation, it is important that osteogenic cell lineage be heterogenic and cells had various degree of maturation and were capable to perform various functional activities. Thus, primitive osteogenic cells versus differentiated cells express a higher level of the CXCL12 and SCF and maintain long-term repopulation activity of the HSCs [97]. Investigations into CXCL12-CXCR4 signaling system revealed its necessity for homing and maintenance of HSCs and developing immune cells, including B-lymphocytes, plasmoid dendrite cells (pDC) and NK-cells in the BM [92, 93, 98-107].

At the final stage of osteogenic differentiation, the transcriptional factor Osx (Osterix), also known as Sp7, be expressed mainly by the osteogenic cells [108, 109]. In its absence the osteogenic differentiation does not occur and the bone tissue is missing in the Osx-null mice. In such mice, Runx2 expression is maintained but expression of other osteogenic markers is drastically decreased up to its complete disappearance [110]. In the normal Osx^+ preosteoblasts the markers of extracellular matrix are expressed [110] among which are: collagen I, osteopontin, bone sialoprotein and acid phosphatase. The majority Osx^+ cells in the bone marrow are represented by endosteal osteoblasts and subendostal preosteoblasts. These Osx^+ cells also expressed CXCL12. The CXCL12 is secreted *in vitro* mainly at early stages of differentiation and practically disappear in the mature osteoblasts [111]. If the cxcl12-gene is selectively depressed in the Osx^+ cells, no essential changes occur in the number and function of the HSCs. Alongside, mobilization of hematopoietic progenitors from the BM was intensified so that their number in the blood and in the spleen increased 10- and 8-fold, respectively [93].

The conditional deletion of CXCL12 in the Osx^+ cells [93], Col2,3-Cre osteoblasts [92,95] or BGLAP-Cre osteoblasts [96] does not lead to any defects in the HSCs. At the same time, deletion of CXCL12 in the osteoblasts (Col2,3-Cre) is accompanied with the decrease of common lymphoid progenitors in the BM [92], while deletion in the Osx^+ osteogenic cells leads to the decrease of the number of the committed lymphoid precursors of the B-lymphocytes [93].

There is a feeling that various definite subpopulations of the stromal cells are involved in the development of various subpopulations of HSCs and their progenitors. It seems likely that endosteal region is the microenviroment, including osteogenic cells of the various degree of maturity, fit for maintenance lymphoid progenitors. It is possibly that N-cadherin plays a great role in this process. N-cadherin is calcium-dependent homophil molecule forming adhesive compounds. It is expressed by the subpopulations of osteogenic cells with high activity in immature cells as well as by the HSCs and their progenitors [97]. At least, certain subpopulations of HSCs are localized close to the SNO-cells [60, 84, 85] and N-cadherin is included in HSCs adhesion to the osteogenic progenitors [97]. The osteoblasts are assumed to contact the HSCs by the way of direct interaction via N-cadherin-mediated adhesion [63]. However, with the use of conditional knockout of N-cadherin (Cdh2) in the hematopoietic cells [112], osteoprogenitors [113] and osteoblasts [114] no essential changes in the number of HSCs was seen although the N-cadherin overexpression somewhat increased their number [115,116].

When producing osteopontin, the osteoblasts can decrease the pool of bone marrow HSCs [117]. Expression of angiopoietin and thrombopoietin, which are bound respectively with Tie2 and Mpl, also prevents increase of the number of HSCs [118-120].

During bone resorption by the osteoclasts there takes place release of the calcium which can activate N-cadherin interactions. This can promote HSCs maintenance in the endosteal region [121]. Although other data show that activation of the osteoclasts mobilizes the HSCs [62].

It can be assumed that certain contradiction of some results is determined by the fact that either various specific populations of the osteoblasts are functioning in the niche or that the osteoblasts are not needed for performance of definite functions and they can be forced out by other types of cells to compensate for quantitative or functional instability of the osteoprogenitors.

Besides, Jagged-1 is a positive regulator of the HSCs that acts during constitutively PPR activation [78, 79, 119]. Despite the fact that Jagged-1 shows itself as a decisive factor in PPR-activated increase of the HSCs count, it is not involved in their homeostasis [121]. Negative regulators bound with osteoblasts are thought to be the osteopontin and Dikkopf 1 (inhibitor of Wnt/β-catenin signaling) [117, 123-127].

CXCL12 expressed by the osteoblasts together with CXCR4 play one of the main roles in the chemotaxis, survival and maintenance of HSCs in the BM [105, 128-130]. Very important are also the SCF and IL-8 and less important are some other cytokines activating cells via LFA-1, VLA-4, VLA-5, CD44 and MT1-matrix metalloproteinase [131].

Isolation of HSCs from different zones of bone marrow showed that $CD150^+CD48^-$ LSK ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$) cells from the endosteal niche possess a higher proliferative and homing potential than the HSCs of the same phenotype obtained from the central bone marrow part. It was also established that $CD150^+CD48^+$ LSK cells, earlier defined as the progenitors of B-lymphocytes, are capable for multilineage differentiation only in the case if they are isolated from the endosteal area [29].

After BM transplantation the HSCs migrate inside BM and then out of it during several hours [132]. This property is best expressed in the cord blood $CD34^+$ cells, which faster and more effective engraft after their transplantation to the NOD/SCID mice [133].

On *ex vivo* 3D-model of bidirectional migration the $CD34^+$ cells reached its peak after 24 hours of their culture in simple spheroids, consisting of the non-induced MSCs. These cells did not practically migrate from these spheroids composed of osteo-induced MSCs or mixed cell types. They remained in the central part of the mixed spheroid formed by the osteo-induced cells, evidencing about essential differences in the adhesive activity depending on the properties of the stromal cells. It is worth noting that HSCs, adhering to osteo-induced MSCs, show significant decrease proliferative activity that is also observed in the niches of HSCs *in vivo*. [134].

The osteoblast niche maintains HSCs at dormancy in the G_0 phase, and, as is known, the highest activity in hematopoiesis recovery is conditioned by the HSCs being in dormant state [12, 19, 79, 135, 136].

The dormant HSCs are also involved in the SP (side population) fraction – a small-number fraction of the cells in side positions on the flow cytometer histograms which can rapidly efflux fluorescent dyes Hoechst 33342 [137]. Cells of the SP fraction express ABC (ABCG2) transporter which provide dye efflux from the cell [138].

Analysis of the cell cycle of the LSK cells (in fraction of SP cells) stained by pyronin Y (PY), shows that more than 90 % of LSK-SP cells are in the G_0 phase [79]. The $PY^{low/-}$ and PY^{+} cells reside in the G_0 and G_1 phases of the cell cycle respectively [139]. LSK-SP cells show resistance to 5-fluorouracil (5-FU) whereas the non-SP fraction of the LSK cells is sensitive to it [79]. As 5-FU induces apoptosis of actively dividing cells, it becomes clear that SP-cells are mitotically inactive. It was shown that HSCs of the mice receiving 5-FU, expressing Tie2, N-cadherin and osteocalcin, contacted with the osteoblasts in the endosteum [79].

It is considered that HSCs dormancy is reached via an interaction of the molecules on the surface of the osteoblasts (N-cadherin, angiopoietin-1 and thrombopoietin) with their receptors on the HSCs (N-cadherin, Tie-2 or Mp1, respectively) [53, 63, 79, 119, 120, 140, 141]. An excessive expression of the canonical Wnt inhibitor Dikkopf 1 leads to the loss of HSCs dormancy that is accompanied with the decrease of the serial transplantation potential [126]. The maintaining role of Wnt in the regulation of B-lymphopoiesis by osteoblasts was demonstrated [142].

The long-term repopulating and BrdU-labeled HSCs are located in the endosteal zone [11]. However, only 6 % of these HSCs, being identified by SLAM (signaling lymphocytic activation molecule) markers, were BrdU-positive [135]. The data showing that the number of HSCs did not decrease in some experiments on the mice with a reduced number of the osteoblasts are also disputable [53, 91]. Neither the number of HSCs in mice with deficit of N-cadherin [112] and CXCL12 in the osteoblasts was reduced [92-96]. At the same time, some of the following investigations did not confirm substantial association between the osteoblasts and the HSCs [59, 143]. In addition a definite influence on HSCs of the respective ligands in the experiments studying the role of PPR and BMPR Ia can be linked with expression of these molecules on the CAR-cells.

Nevertheless, it is absolutely clear that osteogenic cells play a definite role in the formation of HSCs microenvironment in the endosteum, forming an endosteal niche. Its influence on the HSCs functioning and hematopoiesis can be determined by the membrane contact interactions and cytokine-receptor interactions, realization of which depends mostly on the cell subpopulation and the degree of maturity of the interacting cells that cannot be always taken properly into account in the experiments.

Still, an exceptional role of the osteogenic cells in bone marrow niche formation is disputable in view of the fact that, hematopoiesis in the ontogenesis is sequentially developing in the yolk sack, AGM region, fetal liver, placenta and in the spleen. However all these areas, from which the HSCs can be isolated, do not contain the osteoblasts. Instead, all of them contain the perivascular cells closely linked with HSCs production. According to the findings of J. Hackney et al., the fetal liver AFT024 endothelial cells maintains HSCs *in vitro* [144].

The above-mentioned contradictory results of studies on the role of the osteoblasts and other stromal cells in the formation of endosteal niche for HSCs and, on the other side, the appearance of data about involvement in the process of the endothelial and perivascular cells have argued investigations studying their importance and formulating the ideas about the vascular niche.

VASCULAR NICHE

Perivascular and endothelial cells

The perivascular and endothelial cells play an important role as niche components. Studies of vascular component in the formation of HSCs niches allowed have a new vision of the endosteal niche as it became clear that the influence of this region is not confined to the endosteal surface, as has been assumed initially [145, 146]. As has already been mentioned, the BM in the endosteal region is well vascularized with arterioles and venous sinusoids with CAR-cells [105]. After syngeneic transplantation to normal mice the HSCs are distributed in the BM in a random way. However being transplanted to the irradiated animals, they get together predominantly on the endosteal surface. This is explained by the immediate proximity of blood vessels and osteoblasts in the layer covering endosteal surface [85,147] and damaging irradiation effect on the vessels, when HSCs leaving the vessels and attached to the osteoblasts [148].

Using the SLAM-markers ($CD150^{+}$, $CD48^{-}$ and $CD41^{-}$), the HSCs were found near the sinusoidal vessels [7]. Using the murine strains expressing green fluorescent protein (GFP) as a result of gene insertion into cxcl12-locus, the CAR-cells were detected in contact with HSCs at the non-endosteal bone marrow region mainly near the sinusoids [105]. The zones with high expression of CXCL12 and E-selectins, important for homing and settling of normal and leukemic HSCs were found in the sinusoidal areas of the trabecular bone [58]. An intimate association of HSCs with the vessels and perivascular cells was demonstrated by the results of activation of the sympathetic nerves the ending of which cover the perivascular and endothelial cells thus allowing to regulate circadian, stress and induced mobilization of the HSCs via noradrenalin release changing vessel permeability and modulating CXCL12 expression [149, 150]. What is more, the sympathetic nervous system plays a key role in BM regeneration, supposedly via realization of similar mechanisms when chemotherapy-caused ablation of adrenergic innervations inhibit there recovery of the HSCs number [151].

CAR-cells

The population of perivascular reticular stromal cells strongly expressing of CXCL12 is known as the CAR-cells. They are mesenchymal progenitors capable for differentiation into adipocytes and osteoblasts [105]. The majority individual CAR-cells express simultaneously the adipogenic and osteogenic genes, including *pparγ*, *runx2* and *Osx* as well as possess potential for differentiation into adipocytes and osteoblasts in culture. In line with this assumption, a brief ablation of CAR-cells *in vivo* disturbs adipogenic and osteogenic differential potential of the bone marrow cells [87]. An exact cell composition of CAR-subpopulation is unknown. Still, it has been established that it represents the cells which express leptin receptor (Lepr), nestin, Mx-1, transcriptional factor Prx-1 which is revealed in 95 % $CD45^{-}TER119^{-}PDGFRa^{+}$ MSCs in bone marrow and transcriptional factor Osx which is needed for differentiation of MSCs into osteoblasts [93, 94, 152-154].

Lepr on the perivascular cells is specific to leptin hormone, which produced by adipocytes and activated of metabolism [155]. $Lepr^{+}$ cells have the phenotype of MSCs progenitors, make 70 % of bone marrow $CD45^{-}TER119^{-}PDGFRa^{+}$ MSCs and produce SCF and CXCL12. During phenotyping they partly overlap with the cells positive to nestin [143,156] and are self-renewing population fit for osteo- and adipogenic regeneration [157, 158].

In contact with CAR-cells are seen the majority $CD150^{+}CD48^{-}CD41^{-}$ HSCs (97 %), early progenitors of the B-cells, plasmatic cells, pDCs and NK-cells thus evidencing that they function as elements of the niche for HSCs and precursors of all immune cells generated by the bone marrow (so-called «reticular niches») [103, 105-107].

CAR-cells are closely fit to sinusoidal endothelium [105]. However they do not express pan-endothelial marker PECAM-1 and marker of smooth muscle cells α-actine (SMαA), evidencing that CAR-cells represent a population different from the endothelial and smooth muscle cells [103]. CAR-cells do not express CD45, Sca-1 either but reveal VCAM-1, CD44, PDGFRα and PDGFRβ, evidencing that they represent a relatively homogenous population of the stromal cells [87].

To assess the role of CAR-cells *in vivo*, the model of diphtheria toxin receptor (DTR) transfection was used. The DTR-GFP complex was transfected into cxcl12-locus (cxcl12-DTR-GFP) that allowed eliminate CXCL12-expressing cells by injecting diphtheria toxin to the mice [159]. In these conditions, the osteoblasts and endothelial cells covering the bone remained undamaged with observing a depletion of the CAR-cells with a 2-fold decrease of the HSCs in the bone marrow. Besides, the number of

proliferating B-cells and erythroid progenitors also decreased. Most of the remaining HSCs were at dormancy with high expression of the genes responsible for myeloid pathway of development [87].

In the sorted CAR-cells of CXCL12-GFP-mice, the CXCL12 and SCF expression was higher in comparison with other bone marrow populations. Therefore a short-term ablation of the CAR-cells *in vivo* markedly depressed SCF and CXCL12 production showing that CAR-cells are the main producer of the CXCL12 and SCF in the BM. Notably, individual CAR-cells express both CXCL12 and SCF [87].

In the whole, the obtained results show that CAR-cells are adipo-osteogenic progenitors generating abundant amount of the cytokines, extremely essential for niche and which are required for proliferation of the B-cell also erythroid progenitors but HSCs maintenance in the undifferentiated state.

In humans, CAR-cells are likely strongly producing CXCL12 bone marrow stromal progenitors, expressing of melanoma cell adhesion molecule MCAM, also called CD146 [160].

Nestin-expressing cells

The protein of intermediate filaments nestin (Nes) is expressed by the neuronal cells [161] as well as it is found in various perivascular and endothelial cells [162]. In the BM of transgenic mice with GFP expression under control of Nes-gene regulatory element, the Nes-GFP$^+$ cells are localized exclusively on the perivascular area. The Nes-GFP$^+$ cells express various levels of GFP depending on their localization. The Nes-GFP^{bright} cells are located periarterially while the Nes-GFP^{dim} cells are located perisinusoidally. The former are associated with the dormant HSCs being maintained after 5-FU injection. The pericyte marker is associated with Nes-GFPbright cells and the Lepr is located predominantly on the Nes-GFPdim cells. NG2-cells deletion leads to transition of HSCs to proliferation and change of their localization [143].

Sixty percent of the CD150$^+$CD48$^-$Lin$^-$ HSCs fit to Nes-GFP$^+$ cells. Expression of mRNA CXCL12 and SCF is much higher in the sorted CD45$^-$ Nes-GFP$^+$ cells versus sorted CD45$^-$ Nes-GFP$^-$ cells [153]. Following *in vivo* depletion of nestin$^+$ cells using DTR-mediated knockout technology, the number of HSCs in the BM reduced 2-fold but it increased in the spleen indicating involvement of nestin-expressing cells in bone marrow HSCs maintenance.

The sorted Nes-GFP$^+$ fraction maintained the cells, forming mesenchymal spheres, colony-forming units of the fibroblasts (CFU-F) and the cells differentiated into adipocytes and osteoblasts. Given the described properties, the cells are classified as MSCs [153]. It is considered that the latter contribute significantly to niche formation [163-165]. Nestin, which is also found in the subpopulation of human CD146+ cells, is expressed by a larger fraction of the perivascular stromal cells of mice together with platelet growth factor receptor PDGFR-α [153, 156]. According to the test of CFU-F formation, these cells make practically all bone marrow MSCs [156]. They are placed near HSCs in the fibers of sympathetic nerves and express genes responsible for HSCs maintenance in the BM, including control of CXCL12 and SCF [156].

Human bone marrow contains the perivascular CD146$^+$CD45$^-$ MSCs. They are capable to form the heterotopic HSCs niches and initiate hematopoiesis [159]. Close to them are the murine CD51$^+$CD105$^+$CD90$^-$CD45$^-$ Tie2$^-$ MSCs, which are also capable to induce HSCs niches by creating bone marrow cavity with vessels and host-origin HSCs in the donor ectopic bone [166]. The importance of bone marrow MSCs in the formation of hematopoietic microenvironment is confirmed by the data concerning the capacity of PDGFRα$^+$Sca-1$^+$CD45$^-$Ter119$^-$ MSCs to differentiate into the osteoblasts, reticulocytes and adipocytes *in vivo* [167].

It is clear that MSCs are one of the main elements of the niche promoting HSCs self-renewal. It is noteworthy that this capacity relative to the primitive CD34$^+$CD38$^-$ is most marked in the MSCs from the BM and cord blood and less marked in the MSCs of the adipose origin that is consistent with high degree of adhesion to the HSCs. Besides, only bone marrow and cord blood MSCs express high level of N-cadherin, VCAM-1, NCAM-1 and integrins. All this, in the authors' opinion, indicates that one of the main mechanisms of maintenance of HSCs self-renewal by means of MSCs is the intercellular contact [168].

Endothelial cells

The endothelial cells paving sinusoidal blood vessels in the BM are also the claimants among those playing one of the main roles in the regulation of HSCs functions. One can guess about the importance of vascular component in niche formation considering close structural association of hematopoiesis with the vascular network.

The BM vascular network is started with the arteries entering via the bone cortex [169] and spreading into typical capillaries which merge into a system of thin-walled sinusoids branching into bone marrow cavity. Some of these capillaries have open «windows» (lumens) and are characterized by the slow blood flow with transport of cells being generated in the BM.

Sinusoidal endothelium is associated with highly-enriched LT-HSCs (CD150$^+$CD48$^-$CD41$^-$Lin$^-$) [7, 170] and Hoxb5$^+$ HSCs, which are closely fit to VE-cadherin$^+$ cells [171] VE-cadherin (CD144) – homophilic adhesive protein being expressed by the vascular endothelial cells, that can be considered as a weighty argument in the favor of endothelial cells as the elements determining the main properties of the niche. An effective involvement of the endothelial cells in the hematopoiesis can be also assumed based on the fact that they express E-selectin [172] and secrete highly active angiogenic factors: FGF2, DLL-1, IGFBP2, ANGPT 1 (angiopoietin 1), DHH and EGF [148, 173-176]. The angiogenic factors of Akt-activated endothelial cells can play the key role in maintenance of balance between the self-renewal and differentiation of the HSCs. Selective activation of AKT1 in the endothelial cells of adult mice following myeloablation increases the number of HSCs and speeds up hematological recovery [174].

It was shown that sinusoidal endothelial cells maintain self-renewal and prevent exhaustion of the HSCs in the serum-free culture and *in vivo* via Notch-signalization. The VEGF2 and VE-cadherin-dependent signaling pathways play an important role in these processes [177]. Thus the fact that endothelial cells promote HSCs self-renewal was also proved in the experiments with Jagged-1 deletion using VE-cadherin-Cre when perivascular cells, including the PDGFRa$^+$CD51$^+$ MSCs, remained unchanged either quantitatively or functionally [178]. At the same time other authors demonstrated that CD31^{hi}endomucin^{hi}-endothelial cells modulate neoangiogenesis indirectly via the Notch-signaling in the perivascular progenitors [179, 180]. A direct involvement of the endothelial cells in HSCs maintenance was demonstrated on the Tie-Cre-mice [92-94].

Production of CXCL12 by vascular endothelium strengthens CD34$^+$ cells adhesion by enhancing expression of the interacting VLA-4 and LFA-1 intergrins with corresponding endothelial ligands VCAM-1 and ICAM-1. As is also known, the endothelial cells obtained from various tissues can maintain the HSCs in cell cultures [181-183]. An assumption was made that the SCF produced by endothelial cells and outside niches influences on the HSCs [94].

Expression of E-selectin (expressed exclusively on the endothelial cells) promotes HSCs proliferation whereas the antagonists of E-selectin promote dormancy and self-renewal [184]. Possibly, the endosteal niche provides hypoxic environment for HSCs maintenance at dormancy, whereas the vascular niche allows the HSCs proliferate and differentiate in the medium with higher oxygen content [14, 185].

Hematopoietic stem cells and hypoxia

Oxygen plays a well-known role in cell breathing. Its intensity significantly influences on the functional state of various cells, tissues, organs and systems. The MSCs and HSCs as well as such structural-functional units of the niches make no exclusion. BM is a unique tissue type with complex hierarchic organization based on an interaction of various types of the stromal and HSCs within definite compartments, some of which need a reduced level of oxygen saturation for their effective functioning [186-188].

It was showed that hematopoiesis is improved *ex vivo* during keeping cell cultures at 1-3 % oxygen saturation. This practice was used as one of the most effective approaches to improving culturing conditions for the stromal and hematopoietic stem cells [189-191]. In the murine bone marrow cells, the balance between LT-HSCs self-renewal and clonogenic expansion of the progenitors is better maintained at 1 % versus 20 % oxygen saturation. These results are interesting for *ex vivo* manipulation with human progenitors, as so low oxygen pressure can suppress excessive proliferative potential of the cells obtained with the use of apheresis method. For example, expansion of CD34+ cells and clonogenic progenitors(CFU-GM, CFU-E and CFU-Mix) was much lower than in the cultures with 1 % versus 20 % of oxygen. On the contrary, human LT-HSCs were maintained and proliferated better at 1 % of oxygen [190].

Using the mathematical modeling of oxygen distribution in the bone marrow, Chow D. et al. [192] expressed an opinion that stem cells stay in the area with very low (close to anoxia) oxygen level protecting them against impacts of oxygen radicals. Using cell markers of hypoxia along with routine technology of blood perfusion measurement, originally used for study of tumor biology, currently provides extensive opportunities for investigating oxygen gradients in the bone marrow. As a result, the data have been obtained showing that HSCs in the bone marrow are isolated in the hypoxic microenvironment. It follows from this that low oxygen level plays a crucial role in the maintenance of normal stem cells functioning.

Many investigators used an intravenous injection of the Hoechst 33342 dye with a following fluorescent microscopy for visualization of the hypoxia zones. According to their obtained data, the hypoxic cells with low fluorescence are localized at more or less constant and relatively large distance from the blood vessels in the hypoxic regions with low Hoechst staining [193-195]. Using the flow cytometry analysis of the disaggregated cells, it became possible to assess quantitative distribution of the gradient of intracellular Hoechst dye concentration, allowing find a correlation between the intensity of staining and the degree of oxygenation [196-199]. Thus, after an intravenous injection of the dye it was possible to detect the wide distribution of fluorescence intensity, from very intensive to less intensive, among bone marrow cells with the formation of clear gradient. This contrasted with the high level of fluorescence in the well-oxygenated blood leucocytes which did not practically efflux the Hoechst dye and a very low staining level in the thymus where most of the cells stay in relatively hypoxic state [199]. In the bone marrow, endosteal regions appeared to be most hypoxic where the HSCs reside at dormancy [14, 198, 200]. Using the flow cytometry and fluorescent microscopy, it has been found that there is a large accordance between oxygen gradient and staining after intravenous injection of the Hoechst dye. The bone marrow cells were sorted to exclude the Hoechst dye and various HSCs subpopulations were isolated [193-196].

For the assessment of bone marrow cells with low oxygen pressure scientists use also chemical hypoxia marker pimonidasole (PIM). After injection *in vivo* it forms stable deposits in the hypoxic regions and can be identified by the anti-PIM antibodies. Processing *in vitro* of PIM-cells isolated from the SP has proved that this marker is really selective for bone marrow cells being under hypoxic condition. The most intensive staining by anti-PIM antibodies was observed in cells residing under anoxia (95 % N2 with 5 % CO_2). In the bone marrow the non-SP cells demonstrated low PIM-staining. In the SP cells low Hoechst dye content was combined with intensive anti-PIM staining. The SP cells, most capable to Hoechst dye efflux had the highest level of PIM-staining [201]. Formation of PIM deposits depends on oxygen pressure in the cells and is an effective marker for the cells with oxygen pressure less than 10 mm Hg [202]. Intensive anti-PIM-staining was observed in the «tip»-SP cells fraction, in which HSCs concentration was the highest [201, 203, 204].

The cytotoxin tirapazamine (TPZ), tropic to hypoxic cells, selectively reduced the HSCs number in the bone marrow. TPZ administration caused a marked decrease (up to 5 % of the initial count) in the number of cells expressing SP phenotype. An explanation of this finding may be that under hypoxic condition the TPZ is reduced into the benzotriazolyl radical and other intermediate compounds. Ultimately this leads to a rupture of the double DNA spiral and cell death. In the presence of oxygen the TPZ-radical is oxidize into non-toxic original compound [205].

The obtained data indicate that bone marrow HSCs niches are organized relative blood supply and oxygenation levels. Moreover, it is likely that a low oxygenation level can serve as the criterion of bone marrow HSCs niche. Probably, the oxygen gradient can accomplish positive effect determining spatial configuration of the hematopoietic system, thus protecting HSCs from toxic and mutagenic effects of free oxygen radicals. As the long-term HSCs are mainly slow-dividing cells with allow effective oxygen demand, they should exist under adequate hypoxic conditions to maintain their dormancy [206]. In addition, some bone marrow cells express the high level of glycolytic enzymes [207] that evidences about their adaptation to anaerobic metabolism [208, 209].

Several studies presented the data about control of CXCL12 chemokine by the HIF-1α factor. HIF-1α lead to the possibility of the homing whereby the HSCs are maintained in the niches and the transplanted HSCs find their niches according to normal inverse relationships between oxygen level and CXCL12. Hypoxia-inducible transcription factors are also involved in the control of genes associated with HSCs self-renewal, including telomerase genes [210, 211], Oct4 [49] and Notch [212]. Inhibition of HIF-1α synthesis leads to the loss of HSCs dormancy and decrease of repopulation activity, whereas stabilization of HIF-1α level induces dormancy and enhances repopulation activity of HSCs [213, 214].

HUMORAL FACTORS IN REGULATION OF THE NICHES

CXCL12 and SCF

CXCL12 and SCF are the factors regulating so essential HSCs properties, as the maintenance in the BM, dormancy and multipotency [93, 215]. SCF is synthesized by the endothelial cells, osteoblasts, bone marrow fibroblasts, CAR-cells, Nestin- and Lepr-expressing MSCs [78, 87, 153, 216-218]. However, an essential decrease in the number of bone marrow HSCs was observed only as a result of conditional Cre-deletion of SCF in the endothelial cells and Lepr-expressing perivascular stromal cells. The highest effect was predetermined by SCF deletion in these both types of cells [94].

CXCL12 is produced by many types of stromal and hematopoietic cells. The importance of this cytokine for realization of HSCs functions is significant. CXCL12 deletion in the endothelial cells with the use of Tie2-Cre led to the decrease of the HSCs number in the BM and their replication capacity [92, 93]. The dormancy termination and decrease of the number of CD34-cKit+Sca1+Lin- HSCs can be reached by deletion of the CXCL12-receptor CXCR4 in the Mx1-Cre-mice [105]. Lepr-Cre deletion or CXCL12 exhaustion in the CAR-cells with the use of Osx-Cre-system was most effective in decreasing of tissue CXCL12 level that led to the mobilization of HSCs into the blood [93]. However CXCL12 concentration is not always the determinant. CXCL12 conditional deletion in the osteoblasts (Col2,3-Cre or BGLAP-Cre) did not influence on the HSCs properties [95, 96], but the development of early lymphoid progenitors and B-lymphocytes was inhibited [92].

TGF-β

TGF-β controls a wide spectrum of the biological processes and restores homeostasis of the immune system after myelosuppressive chemotherapy and promotes maintenance and self-renewal of the HSCs [219]. The non-myelin Schwann cells, covering nerve filaments in the BM, can secrete the activated molecules for TGF-β in the niche and induce TGF-β/SMAD-signaling in the HSCs, contributing to the maintenance and self-renewal via increasing phosphorylation of SMAD2 and SMAD3, causing HSCs dormancy [220]. Under TGF-β influence the myeloid progenitors are stimulated toward proliferation, whereas the lymphoid progenitors are inhibited [221]. TGF-β-blockade in the mice showed that TGF-β inhibition after chemotherapy speeds up hematopoiesis recovery whereas inhibition in the course of homeostasis has no impact on the HSCs [222]. This indicates that TGF-β signaling block can intensify the hematopoietic recovery mainly during regeneration.

Cripto is protein that blocks TGF-β signaling is binding with GRP78, also known as HSPA5, on hypoxic HSCs and activate P13K-Akt–pathway leading to the maintenance of HSCs localized in the endosteal niche. Blockade of Cripto-GRP78-signaling by the blocking antibodies N-20 mobilizes HSCs migration from the endosteal region into central part of BM but cannot alter the HSCs number in the BM, peripheral blood and spleen, showing that local mobilization can take place without into the peripheral blood [223]. The endosteal cells expressing Cripto include mostly ALCAM$^-$Sca-1$^+$ and, to a lesser degree, ALCAM$^-$Sca-1$^-$ cells [223].

INTRACELLULAR SIGNAL PATHWAYS

Sonic hedgehog (Shh)

Shh is the classical way of embryogenesis regulation. In recent years it has been considered as the regulator of HSCs functional activity. Shh and its receptors Patches and Smoothened express on the CD34$^+$Lin-CD38$^-$ HSCs subpopulation. *In vitro* soluble Shh proteins stimulate increasing the HSCs number and enhance their repopulating capacity [224].

Notch

Notch-signaling plays an important role in many processes of the embryonic and postnatal development and regulates the fate of various populations of adult stem cells [222, 226]. Notch favors HSCs self-maintenance. Notch activation is resultant from binding of Jagged-1, Jagged-2 or DLL-1 ligands with Notch receptor [227] and formation of transcriptional activator with the following transcription of Notch genes-targets participating in the self-maintenance process [173, 174, 228]. The angiopoietin-like proteins also act in a similar way [229]. Inhibition of the Notch signal in the HSCs leads to their accelerated differentiation *in vitro* and depletion *in vivo*. From this it follows that Notch-signal is crucially important in HSCs maintenance in the undifferentiated state [230], and that canonical Notch-pathway promotes HSCs self-renewal and maintenance [178].

However, although Notch stimulates HSCs reconstitution after damage in the mice, it remains unclear if the canonical Notch-pathway contributes to homeostatic maintenance of the HSCs [173, 226, 231-233]. Expression of Notch-receptors at early stages of the hematopoiesis is, probably, involved in the choice of differentiation pathway and can be used for identification of specific progenitor cells with predestined fate [233]. As has been found by several investigators, Notch1 facilitates cells committing and directs development of the megakaryocytes, whereas Notch2 marks primary erythroid progenitors [232-234].

Wnt

Similar to Notch, the Wnt is another way of regulation of various tissues development, including hematopoiesis [235, 236]. β-catenin (encoded by CTNNB1 gene) is the most important component of the Wnt pathway, being activated in the HSCs, leads to the expansion of cells and preserving *in vitro* immature state of the HSCs [237, 238]. Deletion of the Wnt3 ligand of canonical Wnt leads to decreasing the number of HSCs and progenitors in the fetal liver and inhibiting self-renewal and long-term repopulating capacity [239] that confirms the role of canonical Wnt in the regulation of HSCs self-renewal.

Although most investigations have dealt with canonical Wnt, the non-canonical Wnt-pathway also influences on the HSCs properties. Thus the non-canonical Wnt-ligand Wnt5a inhibits canonical Wnt-signaling, cell proliferation *in vitro* and increases repopulating HSCs capacity in the murine model [240], acting via the receptor-like thyrosine kinase (Ryk) [241].

It was found that LT-HSCs express the non-canonical Wnt signaling flamingo (Fmi or Celsr) and frizzled 8 (Fzd8), which promote dormancy during homeostatic regulation, thereby preventing nuclear localization of the nuclear factor of activated T-cells, suppress interferon-γ suppression, and act as canonical Wnt signaling antagonists. Frizzled 6 (Fzd6) regulates HSCs expansion. Fzd6 deficit depresses HSCs self-maintenance capacity [242]. Stress-induced activation of the HSCs in mice can lead to the regression of non-canonical Wnt signaling and enhance canonical Wnt signaling leading to HSCs activation [243].

The importance of generation of the gradient of the levels of canonical Wnt signaling is evident. This is confirmed by the findings of differences in the HSCs behavior when in one case the low level of Wnt-signaling is preferable for the HSCs, leading to the maintenance of immature phenotype and enhanced long-term regulating capacity as opposed the medium and high levels when the capacity of HSCs for repopulation is damaged. However complete loss of Wnt-signaling also damages self-renewal, demonstrating high dose-dependence [244].

Thus there exist complex mechanisms of HSCs control via Notch- and Wnt-signaling, demanding the account of canonical and non-canonical Wnt expression via definite types of cells, quantitative relations, combination of various factors, time of their action, initial state of receptors and other effects, occurring in the components of the niches.

Intracellular factors

The universal extracellular factors are no less important in vital activity of the niches and HSCs, such as: anti-apoptotic proteins (Bcl-2, Mcl-1), transcription factors (Tel/Etv 6, c-myc, HoxB4, HoxA4, HoxC4, Gfi1, STAT5, NF-Yα, Hmgb3, SCL), cell cycle inhibitors (p21, p27), proteins of the Polycomb group – PcG (Bmi-1, Mel18, Rae28), as well as proteins involved in the process of chromosome modification (telomerase) [245, 246].

The role of microRNA in the processes under consideration is worth special mentioning. Thus enhancement of miR-132 expression in the BM of mice stimulates the proliferation of HSCs and decreases their number. It is notable that miR-132 effect is mediated by the transcription factor FOXO3 [146].

Taken together, the data concerning niche regulation by the soluble factors and signaling pathways confirm the conception about vascular niche as the regulator of HSCs maintenance and self-renewal.

OTHER CELLS AND MOLECULES INVOLVED IN NICHE FUNCTION

Sympathetic innervations

Owing to the sympathetic innervations and adrenergic signal incoming into bone marrow, HSCs egress into circulation obeys circadian rhythm [150]. This occurs because the sympathetic nerves endings cover around nestin$^+$ MSCs expressing HSCs maintenance genes. These cells respond to the irritants together with sympathetic nervous system via β3-adrenergic receptors that inhibits expression of the some genes: CXCL12, ANGPT 1, c-Kit and VCAM-1. As a result, there takes place HSCs egress from the BM into circulation [149, 153]. The neuropeptide Y (NPY) can play a mobilizing role for the HSCs. The number of HSCs in the BM of the NPY-deficient mice appears to be greatly reduced [149].

Megakaryocytes

The megakaryocytes localized close by HSCs regulate their quiescence through CXCL4 (also known as thrombocyte factor-4) [247] and TGF-β1 secretion [248]. FGF1 production by the megakaryocytes promotes HSCs expansion during stress [248].

Macrophages

The macrophages present the central unit in the erythroblast islets, thereby forming the niche for maintenance of the erythropoietic cells in the norm after hemolytic and myeloablative stresses and during hemoblastosis development [249, 250]. Depletion of the BM by resident macrophages enhances HSCs proliferation and increases the pool of dormant HSCs [134]. The macrophages were attributed to the pool of cells with regulation properties, owing to their humoral effects via non-identified cytokines on nestin$^+$ cells inducing them for CXCL12 secretion and thus maintaining HSCs in the niche [251, 252].

CSF-G treatment in animals, leading to HSCs mobilization and granulocytes production, inhibits both, macrophages and osteoblasts [252, 253], as well as activates noradrenalin secretion by the sympathetic neurons of bone marrow microenvironment [254]. Since the osteoblasts do

not express the receptor to CSF-G, it is believed that suppression of the osteoblasts occurs indirectly, probably, via activation of the macrophages [252, 253] and sympathetic neurons [149, 254]. The macrophages retain HSCs in the spleen via VCAM-1 adhesion molecule [255].

Adipocytes, osteoclasts and regulatory T-cells

Some cell types influence on the HSCs negatively. After MSCs exposure to irradiation or chemotherapy the number of adipocytes increases due to enhanced adipogenic differentiation of the MSCs [256]. A large number of the adipocytes obstruct hematopoietic regeneration and can serve as diagnostic criterion of BM aplasia [256].

The osteoclasts do not influence on HSCs maintenance in the mice that has been demonstrated on the model with a deficit of the cytokines for differentiation of osteoclasts as well as on the c-Fos-deficient and Rankl-deficient mice with the deficit of the osteoclasts [62, 257]. However destroying endosteum, the osteoclasts promote HSCs migration from the BM into circulation. The regulatory T-cells can create privileged zones in the BM to protect donor cells in the niches after allogeneic stem cell transplantation [258].

Pleiotrophin

The growth factor pleiotrophin, also known as heparin binding brain mitogen (HBBM), is coded by PTN gene and expressed by the cells of the ecto- and mesodermal origin. Pleiotrophin secretion by the bone marrow sinusoidal endothelial cells regulates HSCs maintenance via binding and inactivation of the transmembrane protein tyrosine phosphatase receptor type Z (PTRZ) and retention in the BM by CXCR4-CXCL12 axis [175, 259]. Interestingly, the pleiotrophin from various primary stromal cell lineages obtained from the aorta-gonad-mesonephros of the murine embryo also promotes hematopoietic regeneration [259], indicating that various sources of pleiotrophin can maintain the HSCs and their regeneration.

Retinoic acid

The stromal cells, attached to the plastic, secrete the retinoic acid-inactivating enzyme CYP26 for maintenance of the low level of retinoic acid signaling that can promote primitive phenotype of the HSCs and their self-renewal *in vivo* and *in vitro* [260]. Other BM cell types, endothelium and osteoblasts, also secrete CYP26. However their individual role in the maintenance of low level of retinoic acid signaling has not been proved [260, 261].

Thus, it has been convincingly shown that cooperation of the HSCs, MSCs and their progenitors are critical for successful hematopoiesis with generation of all types of the blood cells, despite the fact that prior to the unitary theory, proposed by A. Maksimov, the investigators centered their attention mainly to hematopoietic cells with stemness property. Support for this hypothesis has been the convincing experimental material proving that all blood cells originate from the sole unique cell type – the hematopoietic stem cells.

However it became clear soon that valid cultivation of the HSCs is possible only in the presence of the stromal cells, so-called Dexter cultures. Bone marrow transplantation experiments also showed that normal functioning of the HSCs and hematopoiesis can be accomplished only in the stromal microenvironment. In 1978 year, R. Shophild put forward the theory of bone marrow HSCs niches as unique morphofunctional formations, ensuring the activity of HSCs, their maintenance, self-renewal, multipotency, differentiation, retaining in the bone marrow and migration capacity. The long-term HSCs (most respond the HSCs criteria) go through their life cycle in the niches. They are in the top of side-population fraction, have the phenotype of LSK $CD34^-$ cells, are also characterized according to SLAM-markers as $CD150^+CD49^-CD41^-$, persistent in the G_0 phase and preserve BrdU. The short-term HSCs as more mature $CD34^+$ LSK-cells also function in the niches, residing in G_1 phase as well as the progenitors of the erythroid, lymphoid and stromal lineages.

The niches are subdivided into two types: endosteal and vascular. The former are located close to the endosteal surface. Their main functional units are the osteoblasts and less mature SNO-cells expressing N-cadherin that plays role in the interaction with the HSCs. Owing to this and production of CXCL12, thrombopoietin and some other cytokines, the HSCs are maintained at dormancy in the bone marrow.

However the great part of HSCs at various degree of differentiation reside in the vascular niches formed by the endothelial and perivascular cells: MSCs, CAR-cells, $Lepr^+$ cells, $Nestin^+$ cells and $NG2^+$ pericytes. The stromal cells exert their influence on the HSCs via contact interaction and secretion of the biologically active substances (angiopoietin, CXCL12, SCF and some other cytokines), involving neuronal activation and secretion of TGF-β by the glial cells. Greatly important are the activities of Notch, Wnt and Shh signaling pathways as well as numerous intracellular regulators: polycomb group (PcG), Growth factor independence 1 (Gfi1), High Mobility Group (HMG) proteins, cyclin-dependent kinase (CDK) inhibitors, RAS- and PI3K- signaling pathways, telomerase and other factors.

The endosteal and vascular niches are mostly located side by side following one after another. The results of experiments show that, apart from the bone cells, the perivascular cells, CAR-cells in particular, are involved in the functioning of the endosteal niche. Under certain conditions the HSCs migrate from the endosteal niche into the vascular one (intra-bone marrow mobilization).

The SNO^+ and $Nestin^+$ cells, playing a great role in HSCs functioning are, to some degree, unique and relatively rare. On the contrary, the CAR-cells, forming reticular network owing to their long processes, are numerous. They are essential for the viability of more differentiated progenitors, including immune system cells. 97 % of $CD150^+CD48^-CD41^-$ HSCs, early B-cell precursors, plasma and NK-cells communicate with the CAR-cells. BM depletion by the CAR-cells leads to essential loss of the listed elements, evidencing that all types of immune cells of the bone marrow origin are generated in the reticular network of the vascular niches.

CONCLUSION

The cellular composition of endosteal and vascular bone marrow niches is constructed in such a way that certain types of the stromal cells are localized in such a way that they can provide contact interaction with the HSCs. The specificities of cell populations allow perform humoral regulation in the niches by such stromal chemokine as CXCL12, SCF and TGFβ as well as via intracellular Notch, Wnt, Shh signaling pathways and some other factors. In the HSCs/MSCs cooperation are included SNO-cells, Nes^+ cells, $Lepr^+$ cells, CAR-cells, NG2-pericytes and endothelial cells. The adipocytes, osteoclasts, macrophages, megakaryocytes, neural cells and regulatory T-cells also play a definite support role.

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