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# Bone: a key aspect to understand phenomena in clinical hematology

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Abstract

The bone marrow (BM) is located inside the bone. Now, it appears that bone tissue functionally communicates with the BM hematopoietic system. Osteoblast lineage cells serve as a part of the microenvironment for immature hematopoietic (stem/progenitor) cells. In contrast, mature hematopoietic cells such as neutrophils and macrophages play a critical role to regulate osteoblast activity. A progressive distortion of this precise inter-organ communication between hematopoietic and skeletal systems may lead to hematologic disorders. Recent studies have revealed that vitamin D receptor is a pivotal bridging molecule for this network and for the pathogenesis of myelofibrosis.

Introduction

From the evolutional aspect, osteogenesis and hematopoiesis take place in different organs. For example, fish do not have a bone marrow (BM) cavity in the bone tissue and hematopoiesis is carried out in the kidney. However, even for studies of mammals in which the BM is located inside the bone tissue with direct contact, bone metabolism and hematology have been researched separately. Since the identification of osteoblasts as a part of the microenvironment for hematopoietic stem/progenitor cells (HSCs/HPCs) [1-5], research interests in both fields are becoming closer. In this review, the mechanisms of the two phenomena observed in clinical hematology are introduced based on the basic research associated with bone metabolism, (A) HSC/HPC mobilization from the BM to circulation by cytokine granulocyte colony-stimulating factor (G-CSF) and (B) pathogenesis of myelofibrosis, as examples for transient and irreversible distortions of inter-organ communication between hematopoietic and skeletal systems, respectively.

Multiple players in hematopoiesis and HSC/HPC mobilization

HSC transplantation (HSCT) is an important therapeutic option for the cure of intractable hematologic disorders such as leukemia, lymphoma, myelodysplastic syndrome, myelofibrosis, and aplastic anemia. In the case of allogeneic transplantation, healthy volunteers provide hematopoietic cell sources that contain HSCs/HPCs. The BM was a major HSCT graft in the 20th century; however, the vast majority has been replaced by peripheral blood stem cells (PBSCs) in the past two decades. The standard method to harvest PBSCs is the usual procedure of apheresis after the mobilization of HSCs/HPCs from the BM to circulation by the administration of G-CSF for 4 to 5 consecutive days [6]. The clear advantage of this method over BM harvest is the no need for general anesthesia and operating room.

Despite the worldwide consensus that G-CSF is a standard mobilizing agent in the clinic, it is not clear yet how G-CSF induces the change of HSC/HPC location from the BM to peripheral blood. Studies have revealed that G-CSF, in addition to the expansion of neutrophils as its original pharmacological effect, triggers many changes in different types of cells in the BM microenvironment, many of which are deeply associated with bone metabolism. Among supportive cells for HSC/HPC maintenance in the BM, we examined the change of osteoblasts during G-CSF treatment and found that G-CSF strongly suppresses osteoblast activity [7]. This was an indirect effect because osteoblasts do not express the G-CSF receptor. We finally found that G-CSF stimulates the sympathetic nervous system (SNS), which leads to the high catecholaminergic tone in the BM with an organ specificity. The stimulation of β2-adrenergic receptor (AR) on osteoblasts by this sympathetic signal strongly suppresses the number and function of osteoblasts, which results in the release of HSCs/HPCs from the BM microenvironment [7]. Subsequently, it was shown that the sympathetic nerve expresses the functional G-CSF receptor and its stimulation leads to the suppression of the reuptake, but not the facilitation of release, of catecholamine at the synapse [8]. We also reported that bone

matrix-embedded osteocytes, the end-terminal differentiation stage of osteoblast lineage cells, significantly contribute to HSC/HPC trafficking as supportive modulators of osteoblasts [9]. In addition, we proposed the concept that the bone tissue equipped with osteocytes contributes to immunity and energy metabolism through the regulation of lymphopoietic microenvironment and fat storage in the body [10]. Thus, hematopoiesis appears to be closely regulated by several different machineries such as nervous and skeletal systems.

Vitamin D receptor (VDR) as a bridging molecule for multiple systems

In a downstream signaling pathway of β2-AR in osteoblasts during G-CSF-induced HSC/HPC mobilization, we identified VDR as an indispensable molecule in this phenomenon. VDR has been extensively studied as a critical regulator of bone metabolism and its deficiency (VDR$^{-/-}$ mice) results in characteristic features of rickets type II [11]. We found that G-CSF fails to mobilize HSCs/HPCs in VDR$^{-/-}$ mice even after the rescue of rickets phenotype by high calcium diet [12]. Sympathetic signal through β2-AR induces rapid (2 h) and strong (10 times increase in both mRNA and protein levels) up-regulation of VDR in osteoblasts. This enhanced VDR, by forming a complex with an active vitamin D [1,25(OH)$_2$D$_3$], keeps the downstream signal to facilitate HSC/HPC mobilization, such as receptor activator of nuclear factor-κB ligand (RANKL), at high level for a long time (several hours) [12]. It is reported that RANKL activates bone-resorbing osteoclasts, which degrade anchoring proteins for HSCs/HPCs in the BM such as CXCL12 by the production of proteolytic enzymes [13]. Thus, we identified VDR as a critical bridging molecule for nervous, skeletal, and hematopoietic systems (Fig. 1).

The unclear points in VDR-mediated HSC/HPC mobilization are as follows: (1) factors that induce the up-regulation of VDR other than β2-AR signaling in an osteoblastic microenvironment, (2) downstream signals of VDR other than RANKL, and (3) identification of cell specificity among mesenchymal lineage cells (mesenchymal progenitors, osteoblasts, and osteocytes) for the regulation of VDR signaling, all of which are important issues that need to be addressed in the future.

There is a time lag between HSC/HPC mobilization and the initiation of G-CSF administration (4–5 days for prominent mobilization). It has been reported that the down-regulation of serum osteocalcin after the initiation of G-CSF takes a few days [14]. In addition, the effect of G-CSF on the nerve is not the stimulation of the release but the suppression of the re-uptake of catecholamine at the synapse [8]. Thus, it may take a while to accumulate neurotransmitters in BM.

Poor mobilizers exist in 10–20% of human healthy donors, which is one of the unsolved clinical problems. It is well known that even mice with identical genetic background such as littermate males can produce a wide range of mobilization efficiency, including poor mobilizers, after the administration of the same dose of G-CSF. We have recently shown that BM neutrophils express β3-AR, and sympathetic stimulation activates the arachidonic acid cascade in BM neutrophils to produce prostaglandin E2 (PGE2) via stress-inducible prostaglandin synthase mPGES-1, which supports osteoblast activity [15]. Mobilization efficiency may be determined by the balance between the mobilization-promoting signal relay such as SNS and the mobilization-suppressing feedback machinery such as PGE2 from BM neutrophils, which may make it difficult to predict poor mobilizers or to avoid insufficient mobilization.

Contribution of the hematopoietic system to bone metabolism

In addition to the contribution of bone cells (osteoblast lineage cells) to the hematopoietic system as mentioned above, the contribution of hematopoietic cells to bone formation has been emerging. As mentioned above, BM neutrophils promote osteoblast activity under SNS control [15]. In addition to bone-resorbing osteoclasts, the classical myeloid cell contribution to bone metabolism, recent studies have shed light on a new class of bone-specific macrophages, OsteoMacs, as a strong supporter for osteoblasts [16]. It has been shown that OsteoMacs exist as a single layer and cover the bone-lining osteoblasts, forming a canopy-like structure [17]. The deletion of macrophages,

including OsteoMacs by the macrophage Fas-induced apoptosis (MaFIA) transgenic (Tg) system, results in the disappearance of osteoblasts from the bone surface and also in HSC/HPC mobilization [17-19]. Even in vitro, common knowledge about osteoblast functions obtained by the primary calvarial osteoblast culture was reported to be at least partially mediated by the contaminated OsteoMacs [17]. For example, the depletion of macrophages from this culture resulted in tremendous impairments of tumor necrosis factor (TNF) production, osteoblast differentiation, and mineralization after an appropriate stimulation for each osteoblast activity [17]. The importance of OsteoMacs are also demonstrated in vivo for the osteoblast differentiation of mesenchymal progenitors and parathyroid hormone-induced facilitation of bone formation [20, 21]. In vivo depletion of OsteoMacs does not affect the number and function of osteoclasts [20, 21], which suggests that osteoclasts and OsteoMacs are the different classes of macrophages. It has been suggested that the candidates for the signals from OsteoMacs to support osteoblast activity are oncostatin M and TNF-α [16, 22]; however, no direct proof by an in vivo study has been demonstrated.

Collectively, hematopoietic myeloid (innate immune) cells regulate bone metabolism in several ways. Two different (hematopoietic and mesenchymal) stem cell systems in the BM are both important players to precisely form bone tissue through their differentiation toward neutrophils/OsteoMacs/osteoclasts and osteoblasts/osteocytes, respectively, many of which are governed by the SNS (Fig. 2). The bone is an artistic work of inter-organ communication.

(B) Myelofibrosis and bone metabolism

Classical theory for the pathogenesis of myelofibrosis
Myeloproliferative neoplasms (MPNs) include polycythemia vera (PV), essential

thrombocythemia (ET), and primary myelofibrosis (PMF). Most PV patients and more than half of the patients with ET and PMF carry a somatic mutation of JAK2V617F in HSCs and their progeny [23, 24]. Myelofibrosis appears during the clinical course of MPNs, which is an unfavorable condition for disease control such as subsequent progression to massive splenomegaly and increased incidence of leukemic transformation. Myelofibrosis is characterized by the occupation of marrow cavity with spindle-shaped α-smooth muscle actin (α-SMA)-positive stromal cells (known as myofibroblasts) and with collagen fibers visualized by silver staining, together with a thickening and irregularity of the trabecular bone, so-called osteosclerosis [25]. The current common understanding of the pathogenesis of myelofibrosis is the strong stimulation of fibrosis-causing myofibroblasts, derived from their certain mesenchymal precursors such as Gli1-positive and leptin receptor-positive cells, by megakaryocyte-derived factors such as transforming growth factor-β1 (TGF-β1) and platelet-derived growth factor (PDGF) [26-28]. As a result of long-term stimulation, myofibroblasts may be thought to be a part of malignancy.

Some part of this theory is supported by the accumulation of knowledge from clinical observations of increased megakaryocytes in the BM and experimental results of mouse models. However, in many basic studies, thrombopoietin (TPO)-overexpressing mice were used as a model of myelofibrosis, which leads to the possibility that this theory is somehow biased toward the megakaryocyte-dependent story for the explanation of myelofibrosis [29, 30]. In addition, it does not explain the cause of osteosclerosis. Although some studies have proposed that osteoprotegerin (OPG) may take part in the formation of osteosclerosis by the transplantation of TPO-overexpressing marrow into OPG-deficient mice [31], it does not reasonably explain how MPNs cause osteosclerosis because OPG-deficient mice are originally osteoporotic due to high osteoclast activity. Furthermore, it has been clinically proven that myelofibrosis can be cured by the replacement of the hematopoietic system by allogeneic BM transplantation, which suggests that myofibroblasts are not a part of malignancy.

Thus, these irreconcilable factors prompted us to challenge a thought

that myelofibrosis in MPNs might develop depending on a novel pathway other than the megakaryocyte story. Following our previous study about HSC/HPC mobilization [12], we again identified VDR as a key molecule for this abnormal status of the BM in clinical hematology [32].

A basic model of myelofibrosis in association with VDR

During our previous study about the function of VDR in HSC/HPC mobilization, we found that chimeric mice generated by the transplantation of wild-type (WT) BM cells into lethally irradiated VDR$^{-/-}$ mice developed severe myelofibrosis with osteosclerosis in the trabecular bone area with a prior exhaustion of HSC activity in a few months after transplantation (referred to as the basic model hereafter). Transplantation of the BM from CAG-enhanced green fluorescent protein (EGFP) Tg mice into VDR$^{-/-}$ recipients in combination with immunohistochemical/immunofluorescence staining for F4/80 or osterix revealed that marrow fibrosis was composed of two different cell types, GFP$^{+}$F4/80$^{+}$ donor-derived macrophages and GFP$^{-}$osterix$^{+}$ recipient-derived pre-osteoblasts. Importantly, these two different cell types were distributed mutually with identical spindle-shaped morphology in hematoxylin-eosin staining. GFP$^{-}$osterix$^{+}$ pre-osteoblasts and bone-lining osteoblasts were positive for α-SMA and a collagen-specific molecular chaperone HSP47, which suggested that these cells cause myelofibrosis and osteosclerosis. In other words, so-called myofibroblasts in myelofibrosis were most likely pre-osteoblasts stimulated by donor-derived macrophages.

It is well known that the plasma level of active vitamin D is extremely high in VDR$^{-/-}$ mice [11]. In addition, transplantation of VDR$^{-/-}$ BM, instead of WT BM, into VDR$^{-/-}$ recipients resulted in no myelofibrosis with normal hematopoietic recovery, and low vitamin D diet prevented the development of myelofibrosis in the basic model. Thus, we hypothesized that exposure of VDR-positive hematopoietic cells, perhaps immature HSCs/HPCs, in high vitamin D microenvironment triggered myelofibrosis. Indeed, we confirmed a high expression of VDR mRNA in HSC/HPC fraction (lineage$^{-}$Sca-1$^{+}$c-kit$^{+}$) and also

found that a certain level of active vitamin D equivalent to the one observed in VDR$^{-/-}$ mice was potent enough for a skewed in vitro differentiation of the human progenitor cell line HL-60 toward macrophages. Consistently, macrophage depletion in the basic model with clodronate liposome effectively prevented myelofibrosis.

Collectively, it was shown that VDR-positive HSCs/HPCs stimulated by a high level of vitamin D differentiated toward pathogenic macrophages, which strongly drove host osteoblast lineage cells. This is a novel theory for the pathogenesis of myelofibrosis with osteosclerosis based on the study with our original basic model.


JAK2V617F-driven myelofibrosis in association with VDR

We next tried to apply this VDR-macrophage platform to human-type myelofibrosis. JAK2V617F Tg mice nicely recapitulate the characteristic features of human MPNs such as myeloproliferation and myelofibrosis with osteosclerosis [33]. We confirmed that marrow fibrotic tissue was composed of CD169$^{+}$ macrophages and osterix$^{+}$ pre-osteoblasts. Low vitamin D diet prevented myelofibrosis also in JAK2V617F Tg mice. Transplantation of JAK2V617F Tg/VDR$^{+/+}$ BM into WT mice resulted in severe myelofibrosis (JAK BM chimera), whereas it was significantly ameliorated by transplantation with JAK2V617F Tg/VDR$^{-/-}$ BM. Furthermore, macrophage depletion from JAK BM chimera using the MaFIA Tg system almost completely blocked myelofibrosis formation without affecting the number and morphology of megakaryocytes. Because MaFIA Tg can effectively deplete macrophages, OsteoMacs in particular, myelofibrosis may be due to an abnormal proliferation of activated OsteoMacs, which promote osteoblast lineage cells for collagen production. VDR signal in HSCs/HPCs is a driver for OsteoMacs in this pathogenesis (Fig. 3).

Several issues in VDR-mediated myelofibrosis remain unclear. First, unlike our basic model, the level of active vitamin D was not high in JAK2V617F Tg mice [32] and also in human MPN patients [34]. It is likely that activated

macrophages, which have the enzyme responsible for the final hydroxylation step of 25-hydroxyvitamin D [35], may self-produce $1,25(OH)_2D_3$ for the stimulation of themselves as well as HSCs/HPCs. Second, the signals from pathogenic OsteoMacs to stimulate pre-osteoblasts/osteoblasts remain identified. Perhaps the factors with which normal OsteoMacs support osteoblasts, such as oncostatin M and TNF-α as mentioned before, may be strong candidates. However, OsteoMacs differentiated from JAK2V617F+ HSCs/HPCs by the stimulation via VDR might additionally acquire different ways to extremely drive osteoblast activity.

Conclusive remarks

Thus, bone metabolism is a key aspect to understand phenomena in clinical hematology. The mechanism of HSC/HPC mobilization and the true pathogenesis of myelofibrosis are indeed transient and irreversible/progressive distortion of inter-organ communication between skeletal and hematopoietic systems, respectively. In both cases, VDR plays critical roles as a bridging molecule for multiple organ network.

A simple fact is that the BM is a part of the bone in mammals. Although it is not common to deeply learn bone metabolism for hematologists, bone biology may be an unavoidable field for clinicians in hematology.

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Figure legends

Figure 1. VDR leads the sympathetic nerve signal to HSC/HPC mobilization. Catecholaminergic signal via β2-AR strongly induces the up-regulation of VDR in osteoblasts. Then, the vitamin D/VDR complex maintains the long-lasting signals for HSC/HPC mobilization. The nervous system can regulate signal transduction by the dynamic control of the receptor expression despite the stable level of the ligand.

Figure 2. Two stem cell systems in the BM cooperate to form bone tissue. Myeloid (innate immune cell) differentiation toward neutrophils/OsteoMacs/osteoclasts from hematopoietic stem cells (HSCs) and osteoblast lineage differentiation toward osteoblasts/osteocytes from mesenchymal stem cells (MSCs) precisely cooperate to form bone tissue. Many of these cells are functionally modulated by the sympathetic nervous system (SNS).

Figure 3. A novel pathogenesis of myelofibrosis.
As a classical theory, megakaryocyte-derived growth factors such as TGF-β and PDGF have been thought to be critical stimulators for α-SMA-positive myofibroblasts. Our study added a novel pathway that macrophages, whose differentiation is skewed by the VDR signal in HSCs/HPCs, strongly stimulate the proliferation and activation of pre-osteoblasts and mature osteoblasts, which lead to myelofibrosis and osteosclerosis. In other words, abnormal OsteoMacs are the central pathogenesis of myelofibrosis. VDR is a key molecule to mediate this progressive distortion of inter-organ communication between skeletal and hematopoietic systems.
HSP47: a collagen-specific molecular chaperone to show the actual producers of collagen fibers in the marrow cavity and at the endosteal bone surface for myelofibrosis and osteosclerosis, respectively.
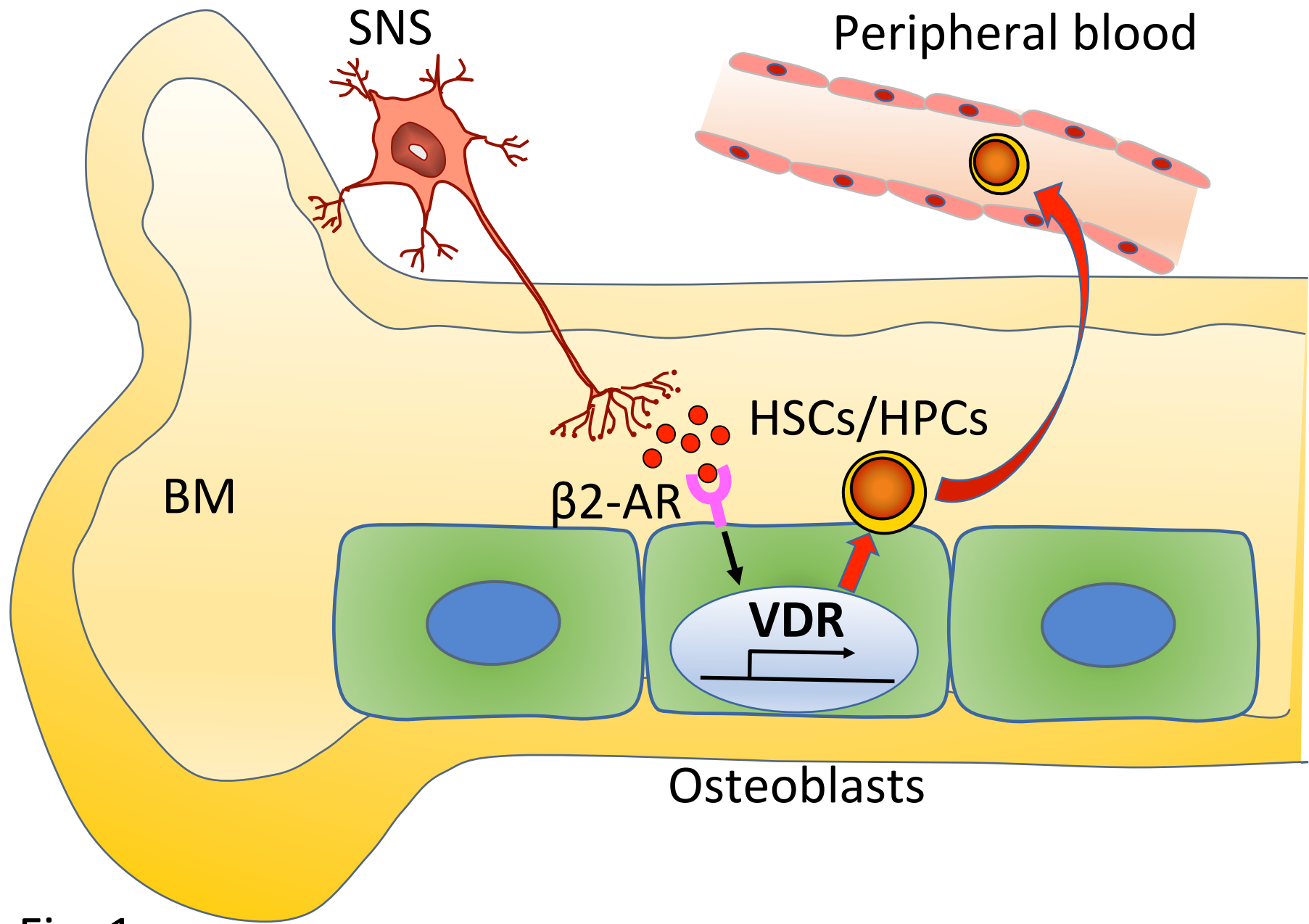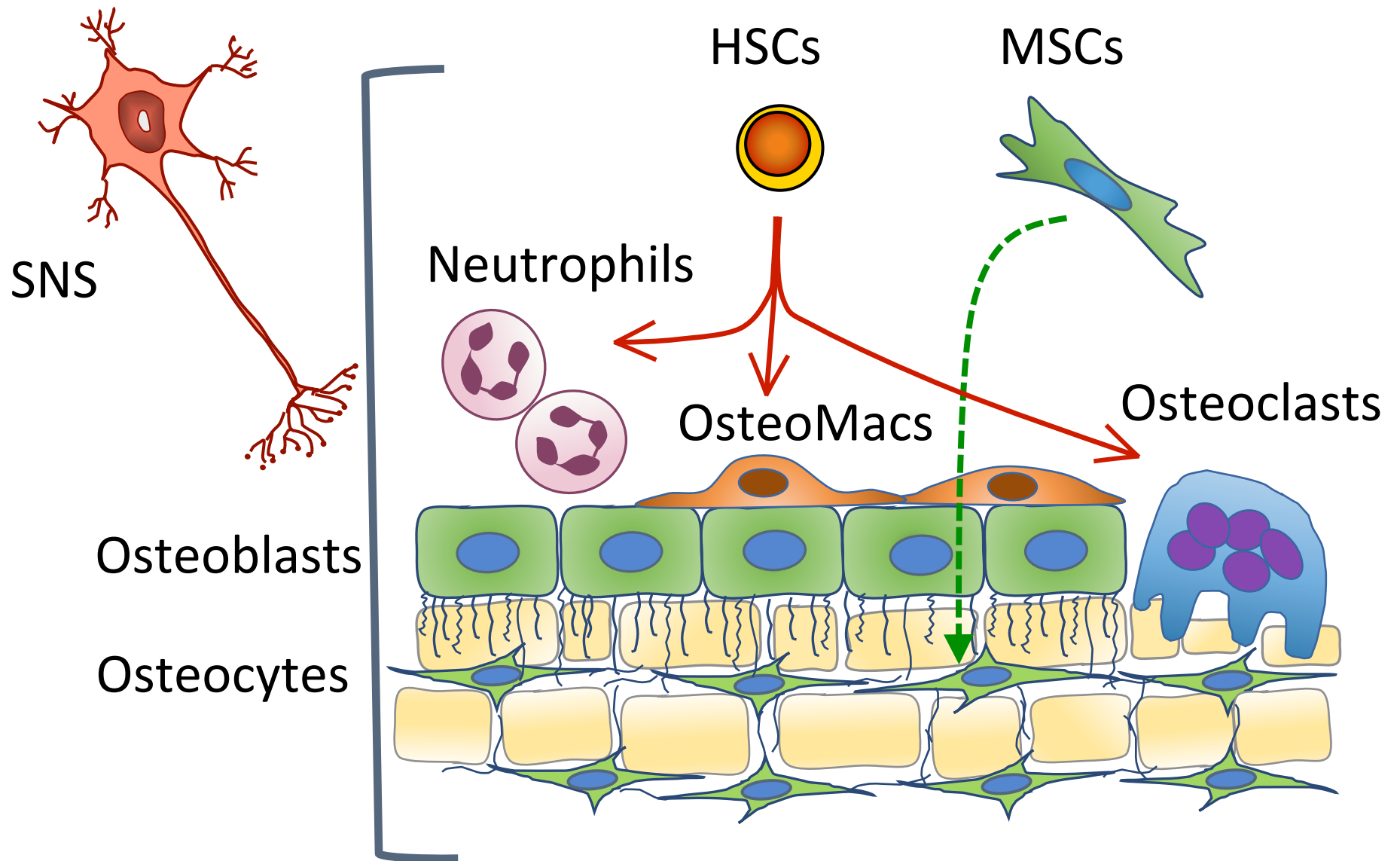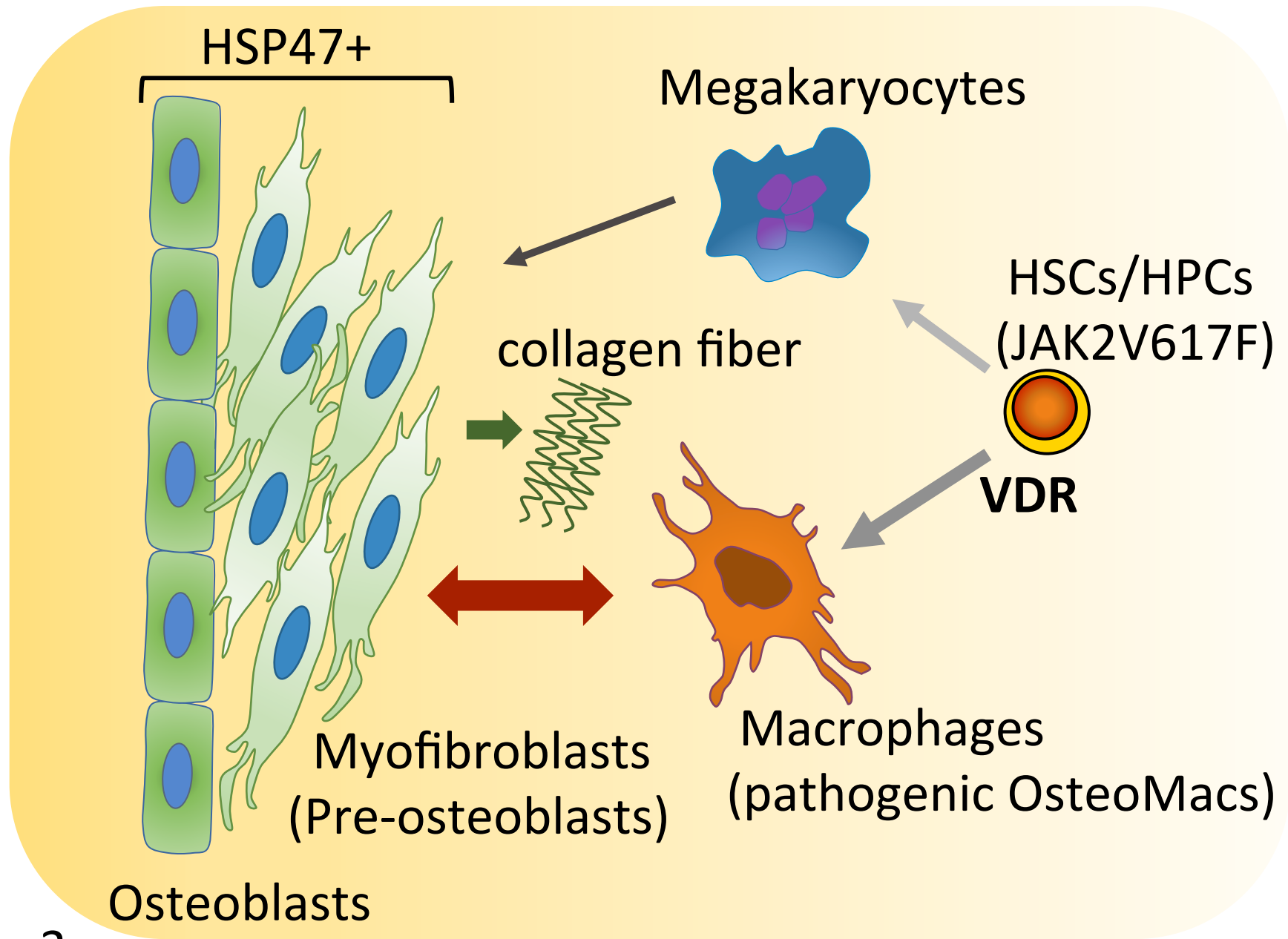
16

SNS

Peripheral blood

BM

β2-AR

HSCs/HPCs

VDR

Osteoblasts

Fig. 1

SNS

HSCs

MSCs

Neutrophils

OsteoMacs

Osteoclasts

Osteoblasts

Osteocytes

Fig. 2

Fig. 3