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Crosstalk between endothelial cells and bone in development, homeostasis and inflammatory disease

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Abstract

Blood vessels form a versatile transport network that is best known for its critical roles in processes such tissue oxygenation, metabolism, and immune surveillance. In addition, the vasculature provides local, often organ-specific molecular signals that control the behaviour of other cell types in their vicinity during development, homeostasis, and regeneration but also in disease processes. In the skeletal system, the local vasculature is an active player both in bone formation and resorption. In addition, blood vessels participate in inflammatory processes and contribute to the pathogenesis of rheumatoid arthritis and osteoarthritis. This review summarizes the current understanding of the architecture, angiogenic growth and functional properties of the bone vasculature. We also discuss the impact of ageing and several pathological conditions.

Introduction

Blood vessels represent an extensively branched and hierarchically organized system of endothelial tubules, which, with a few exceptions such as cartilage and the lens of the eye, extends into every tissue in the body. The transport of a wide range of different cargoes, including hormones, gases, nutrients, waste products, and circulating cells, is the main function of this vascular network. Meeting physiological demands in the majority of organs requires cooperation with a second endothelial system, the lymphatic vasculature, which mediates liquid homeostasis, nutrient uptake, and immune surveillance [1,2] . In the skeletal system, lymphatic vessels are normally absent and the emergence of ectopic lymphatics is associated with massive osteolysis and progressive bone loss in human disorders such as Gorham–Stout disease [3,4] . Likewise, changes affecting the blood vessel network, which is the focus of this article, are associated with the progression of bone diseases including cancer and osteoporosis [5,6] . Disruption of the vascular supply to bone, which can be caused by a variety of conditions including bone fracture, joint dislocation, accidentally during

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surgery, or high-dose corticosteroid treatment, will trigger osteonecrosis characterized by massive local cell death [7–9] . Conversely, ectopic angiogenic blood vessel growth and inflammation of the synovial membrane, a layer of connective tissue lining the inner surface of articular joint capsules, are closely integrated processes in the pathogenesis of osteoarthritis (OA) [10,11] . These few examples illustrate why it is critical to understand fundamental features of the vasculature in the skeletal system, its crosstalk with other cell types, and the molecular signals controlling bone homeostasis and repair but also pathobiological processes.

Endothelial cell (EC) networks in different organs exhibit specialized morphological features and gene expression profiles, which reflect different functional roles [12–15] . While the vasculature in lung, for example, is specialized for gas exchange, it participates in blood ultrafiltration in kidney, supports metabolic processes in liver, or is part of the blood-brain barrier protecting the central nervous system against potentially toxic substances and immune cells from the circulation. In addition, ECs are frequently a source of paracrine (so-called “angiocrine”) acting molecular signals, which control the behaviour of other cell types in the surrounding tissue [16,17] . EC-derived instructive signals have been shown to regulate endodermal cells during the liver and pancreas development in the early mouse embryo [18,19] . In addition to crucial roles in directing hepatic and pulmonary development, angiocrine signals control the regeneration of these organs after tissue injury [20–22] . Moreover, vascular endothelium provides protective and nurturing niches for multiple adult stem cell populations such as neural stem cells [23,24] , spermatogonial stem cells [25] , muscle stem cells [26] , and hepatic progenitors [27] . In the skeletal system, ECs and vessel-associated reticular cells provide niche microenvironments for hematopoietic stem cells with great implications for lifelong blood formation in the healthy organism but also hematologic diseases like leukaemia [28–31] . Similarly, ECs communicate with osteoprogenitor cells during bone development and fracture healing [32–34] . The identification of different capillary subtypes with distinct locations and functional roles in long bone has further enhanced our understanding of the heterogeneity and specialization of the bone vasculature. These findings also shed new light on bone development and homeostasis but also on osteoporosis, osteoarthritis, ageing and fracture healing, which we will discuss in this review article.

Vessels in skeletal development

Blood vessels in endochondral ossification

The generation of skeletal elements during development can involve two distinct modes of ossification. Flat bones such as cranium and ilium are generated through the direct conversion of mesenchymal cells into bone forming cells (osteoblasts) in a process termed intramembranous ossification. In contrast, endochondral ossification, which involves the formation of an intermediate cartilage template that is subsequently converted into calcified tissue, generates the majority of the skeletal structures including the appendicular skeleton and vertebrae [35,36] . These processes have been predominantly studied in animal models and this article refers to findings in mice unless mentioned otherwise. The invasion of growing blood vessels is an important step in all modes of osteogenesis and is triggered by extracellular matrix and signals such as vascular endothelial growth factor A (VEGF-A).

VEGF-A is a known master regulator of angiogenesis, which activates signalling through VEGFR2, a receptor tyrosine kinase expressed by endothelial cells (ECs) but also by osteoprogenitors and other cell populations [37–39] . Conversely, hypertrophic chondrocytes and osteogenic progenitors are major sources of VEGF-A and thereby regulate angiogenesis in bone [38,40–42] . Angiogenesis involves EC proliferation and, in most developing and regenerating organs, the emergence of endothelial sprouts from pre-existing vessels [43] . Pointed, filopodia-extending endothelial protrusions extend from the periosteal vasculature during the vascularization of the femoral cartilage shaft in the embryo and lead to formation of a first vessel plexus. This process is coupled to ossification and formation of the primary ossification centre (POC) [38,40,44,45] and, later, the secondary ossification centre in the epiphysis (Fig. 1). In contrast, extension of the POC in postnatal femur or tibia involves a different mode of angiogenesis, namely the extension of blunt vessel buds from vessel loops (arches) in close proximity of hypertrophic growth plate chondrocytes [33,46] (Fig. 1). Historic experiments using ink injection or corrosion casting in combination with electron microscopy had already indicated the existence of bulb-shaped terminal vessel structures near the growth plate but lacked insight into the organization and behaviour of the ECs surrounding the vessel lumen [47–49] . Modern static and dynamic microscopic imaging confirm that distal vessel buds are fully lumenized and show that they are formed by multiple ECs, which interact with the surrounding chondrocyte matrix through short filopodia. Vessel buds protrude into the space released by the apoptosis of growth plate chondrocytes and new vessel arches are generated by the anastomosis of two adjoining buds [46] (Fig. 2). At their proximal end, the distal arches are connected to relatively straight, column-shaped capillaries that are strongly associated with perivascular bone mesenchymal stromal cells (BMSCs) and osteoprogenitors.

EC subpopulations in the skeletal system

The ECs of all three substructures, buds, arches and columns, share high expression of the cell adhesion molecule CD31/PECAM1 (platelet and endothelial cell adhesion molecule 1) and the sialoglycoprotein Endomucin (Emcn). High expression of these two markers and association with osteoprogenitors are also defining features of capillaries in the endosteum lining the inner surface of compact bone. Accordingly, we have previously summarised these capillaries and their ECs under the term $CD31^{hi}$ $Emcn^{hi}$ or type H [32,33] . Endosteal type H vessels connect to the highly branched and relatively irregular sinusoidal vasculature of the bone marrow cavity, which is formed by ECs expressing comparably low levels of CD31 and Emcn ($CD31^{lo}$ $Emcn^{lo}$ or type L) (Fig. 1) [32] . The base of the type H capillary columns in the metaphysis is also connected to the BM vasculature at the metaphyseal-diaphyseal interface (Fig. 2). Another, transiently existing EC population with strong osteoprogenitor association and high expression of CD31 and Emcn, termed type E (for embryonic), was discovered in embryonic and early postnatal long bone [50] . Type E ECs show particularly high expression of angiogenic and pro-osteogenic genes, induce osteogenic differentiation of mesenchymal cells in 3D spheroid cultures, and, as genetic lineage tracing shows, give rise to type H and type L ECs in postnatal life (Fig. 2) [50] . While this review does not cover the function of the bone vasculature in blood formation, it is important to mention that sinusoidal (type L) ECs of the BM play important roles in the trafficking of haematopoietic cells [51–53] and include specialized vessels serving as vascular niches for myelopoiesis [54] .

Molecular pathways driving bone angiogenesis

Apart from VEGF-A signalling, several pathways have found to control bone angiogenesis and type H vessel formation (Textbox 1) with strong implications for osteogenesis. Endothelial Notch signalling is known to inhibit EC proliferation, sprouting and vessel growth in many different organs and experimental conditions. In bone, however, Notch activation in ECs promotes angiogenesis, type H vessel formation and osteogenesis [33] . The basis for these organ-specific differences in endothelial Notch function remain unknown. Hypoxia-inducible factor (HIF) signalling upregulates VEGF-A expression in hypoxic tissues many different cell types including chondrocytes. In bone ECs, HIF-1α controls type H vessel formation and increases endochondral angiogenesis and osteogenesis [32,55] . Osteoblasts regulate angiogenesis and type H vessels in a paracrine fashion through the secretion of soluble SLIT3 and activation of Robo receptors in ECs [56–58] . Bone morphogenetic proteins (BMPs) are well known for their ability to promote osteogenesis, but some of the ligands can also activate ECs and stimulate blood vessel growth, providing another molecular link between angiogenesis and osteogenesis [59,60] . The transcriptional co-regulators Yap1 and Wwtr1/Taz, components of the Hippo signalling pathway, were found to suppress bone angiogenesis. Postnatal EC-specific loss-of-function mutant mice show augmented angiogenesis, higher expression of HIF pathway target genes and increased bone formation [61] .

Vascular architecture in bone

Blood vessel organisation and hydrodynamics

Insight into the heterogeneity of ECs in the skeletal system is critical for understanding functional roles and regional specialisation. In recent years, single cell RNA sequencing data has been used to establish cell atlases for many different species, organs and conditions. The analysis of adult bone stromal cells, however, has so far uncovered a surprisingly limited number of EC subpopulations, which correspond to arterial and arteriolar, sinusoidal and mitotic cells [70–72] . An independent approach, namely single cell protein expression mapping by CyTOF mass cytometry, uncovered 28 distinct stromal cell subsets with 3 endothelial populations including arterial and sinusoidal ECs. A third and CD31+ population was found in the bone fraction [73] and might represent type H ECs or arterioles located in the proximity of osteoblast lineage cells.

Morphologically, the vasculature of long bone displays the classical hierarchical arrangement of arteries, veins and interconnecting capillaries. In femur, which has been studied most extensively, multiple different sources of blood supply have been described [74–76] . A so-called nutrient artery enters the diaphysis through the cortex, extends over considerable distance through the marrow cavity, and branches out in the metaphysis. The epiphysis and associated cartilage are supplied by epiphyseal arteries and vessels of the ring of La Croix, a perichondral structure that surrounds the growth plate laterally [77–80] . Small periosteal vessels, recently redescribed as transcortical vessels, cross the cortex at numerous locations along the bone shaft and contribute substantially to both afferent and efferent blood flow [76,81,82] (Fig. 1; Fig. 3). Periosteal vessels may facilitate the direct access

of cells from the BM to nearby tissues, as has been shown for the migration of skull bone marrow-derived myeloid cells towards the surface of the adjacent brain [83] .

In long bone, both periosteal vessels and arteriolar branches emerging from the nutrient artery feed into the type H capillaries of the endosteum, which, in turn, drains into the sinusoidal vasculature of bone marrow [84] . Similarly, the distalmost arterioles in the metaphysis connect to type H capillaries near the growth plate and thereby provide flow that will reach the BM through the metaphyseal-diaphyseal interface (Fig. 1; Fig. 3). The type L sinusoidal capillaries in the femoral BM drain into a large central vein, which is a major route for outbound flow. While arterioles have few side branches and are relatively narrow with a diameter of about 10 μm or less, capillaries in the metaphysis and diaphysis are much wider and have numerous interconnections. Accordingly, arterial laminar flow becomes turbulent and slows down rapidly after entry into the capillary network [46,51] (Fig. 3). These features together with the spatial distribution of arterial-capillary connections also generate distinct metabolic zones characterized by high hypoxia in BM and higher levels of oxygenation in the metaphysis and endosteum [32,46,84] . Slow flow in sinusoidal vessels may also facilitate the transendothelial migration of blood cells and, as mentioned above, leukocyte trafficking is indeed confined to sinusoidal vessels in adult mice [51–53] (Fig. 3).

Role of mechanical forces

Mechanical forces and, in particular, increased loading can promote bone formation in the adult organism. Chondrocytes and osteocytes, fully differentiated osteoblast lineage cells that are embedded in calcified bone, play important roles in mechanosensing [85,86] . The osteocyte lacuno-canalicular network is connected to adjacent blood vessels and, similar to osteoblasts, osteocytes may be a source of VEGF to control bone angiogenesis and EC behaviour [87,88] . Interestingly, hindlimb unloading-induced bone loss is accompanied by reduction of type H capillaries, whereas mechanical loading stimulates bone angiogenesis and type H vessel formation [89,90] .

Osteoporosis and osteoarthritis

Special relationship between ECs and osteoclasts

Bone is a surprisingly dynamic tissue that undergoes livelong renewal and remodelling, which involve the balanced activity of bone-forming osteoblasts and bone-degrading osteoclasts. Impairment of this balance results in reduced bone mineral density (osteopenia) or osteoporosis, a disease characterized by bone weakness, increased risk of fracturing, loss of mobility, and chronic pain. Osteoporosis is very common in aged people and especially in postmenopausal women. Bone loss also occurs during rheumatoid arthritis (RA) and osteoarthritis and is based in part on higher activity of bone resorbing osteoclasts. Whereas interaction of bone forming osteoblasts and vasculature during bone loss during aging is discussed below, we focus in this section here on the unique role of bone resorbing osteoclasts in vascular growth and its implication in bone remodelling and osteoporosis.

Osteoclasts are unique myeloid cells derived from monocyte precursors that fuse and thereby generate polynuclear and strongly polarized cells. Cytoskeletal protrusions enable

osteoclasts to build a sealing zone and generate an acidic compartment for bone resorption. A landmark study discovered that osteoclasts in bone emerge from tissue-resident erythro-myeloid progenitors (EMPs) but undergo fusion with circulation monocytes throughout life and in response to pathological challenges [91] (Fig. 4). Apart from monocyte precursors, dendritic cells (DCs) were also shown to fuse to osteoclasts or contribute to their generation in pathological bone conditions, including arthritis, periodontitis and osteopetrosis, but also in other inflammatory diseases including inflammatory bowel disease [92] . Depending on whether osteoclasts were derived from monocytes or from DCs, they were found for either immunologically tolerogenic or pro-inflammatory, respectively [92] .

Osteoclast lineage cells in the regulation of bone angiogenesis

Osteoclasts and their progenitors have been implicated in the regulation of vascular growth in bone by providing various pro-angiogenic factors (summarized in ref. [93]). In addition, osteoclasts were identified as a source of the matrix metallopeptidase 9 (MMP9), which is important for angiogenesis both in explants and *in vivo* [94] (Fig. 4). The conclusion that osteoclasts stimulate angiogenesis through MMP9 was challenged by another study describing a subgroup of vessel-associated osteoclasts (VAOs) [95] . VAOs are reportedly involved in the anastomoses of type H vessels but not in the resorption of the hypertrophic cartilage. The same study used genetic experiments to show that MMP9 provided by ECs, but not by osteoclasts, is essential for essential for cartilage resorption and directional bone growth [95] (Fig. 4).

Preosteoclasts, but not monocytes or mature osteoclasts, were found to induce angiogenesis, type H vessel formation and osteogenesis via the secretion of platelet-derived growth factor B (PDGF-B) [96] . Ovariectomy-induced osteoporosis in mice leads to a reduction in serum and BM levels of PDGF-B with a concomitant decrease of type H vessels in long bone. Treatment with exogenous PDGF-B or administration of cathepsin K, which increases the number of preosteoclasts and thereby the endogenous levels of PDGF-B, stimulates type H vessel formation and osteogenesis in ovariectomized mice [96] .

$CD31^{hi}$ $Emcn^{hi}$ vessels have been implicated in OA development by several studies [97–99] . Preosteoclast-mediated release of PDGF-B contributes to OA development and $CD31^{hi}$ $Emcn^{hi}$ vessels are induced in the DMM model (destabilization of the medial meniscus) in subchondral bone and start to invade the joint cartilage. Conditional ablation of PDGF-B expression in preosteoclasts attenuates aberrant subchondral bone angiogenesis and joint damage, whereas transgenic overexpression of PDGF-B in preosteoclasts results in spontaneous osteoarthritis [99] . In all the studies above, the exact mechanism of PDGF-B function remains to be elucidated. Expression of the corresponding receptor, the tyrosine kinase PDGFRβ, is absent in ECs but found in various mesenchymal stromal cell populations, including skeletal stem and progenitor cells, as well as committed osteoblast lineage cells and synoviocytes [100–102] .

Effect of glucocorticoids and anti-resorptive drugs

Glucocorticoids (GCs) are used for the treatment of inflammatory diseases such as RA, asthma or skin conditions, but adverse side effects include GC-induced osteoporosis (GIO),

which is very frequent due to the abundant use of steroids [103–105] . Conditional mutations impairing glucocorticoid signalling revealed a pivotal role for GC action in osteoblasts [106] , osteoclasts [107–109] and osteocytes [110,111] .

Only recently, the impact of the pharmacological effects of GCs on the bone vasculature was considered. In the femoral head, but less in distal femur, GC administration decreases the local vascularization, accompanied by decreased HIF-1α and VEGF expression [112] . In juvenile mice, bone angiogenesis and type H vessel were disrupted by GC administration, which was linked to reduced PDGF-B expression in preosteoclasts [113] . This effect of GC relies, in part, on cathepsin K. Inhibition of cathepsin K blocked Prednisolone-induced effects in the secondary spongiosa, the region where newly formed bony trabeculae are remodelled into mature trabeculae, and even enhanced H type vessels in the primary spongiosa, the site near the growth plate where trabecular bone formation is initiated [114] . Direct regulation of *Pdgfb*, the gene encoding PDGF-B, via transrepression of p65-NFkB was suggested, but effects were only observed at very high doses and the putative NF-kB binding site was not functionally evaluated [113] .

While numerous studies have linked preosteoclasts to the regulation of vessels, mature resorbing osteoclasts seem less important in this context. In tail vertebrae of mice treated with clodronate, an anti-osteoporotic drug belonging to the group of bisphosphonates, vessels are present despite blocked osteoclast-mediated bone resorption [115] . Similarly, lack of osteoclasts in osteopetrotic knockout mice lacking the transcription factor c-Fos does not lead to the absence of blood vessels. On the other hand, improved bone sample processing and imaging revealed that the treatment of mice with another bisphosphonate, namely Alendronate, leads to an increase of type H vessels in long bone [46] .

Bone inflammation

Interactions between vessels and fibroblast-like synoviocytes

During inflammatory bone disease bone destruction is a consequence of chronic inflammation. For this inflammatory process, the cross-talk between vessels, leukocytes, but also stromal cells are major driving forces. One major example is rheumatoid arthritis. RA is a complex bona fide autoimmune disease that affects bone and joint integrity with unclear aetiology. Chronic inflammation, synovial swelling and pannus formation with subsequent bone and cartilage degradation are hallmarks of RA. Aberrant angiogenesis is observed in the subchondral area and in the pannus itself (reviewed in ref. [116]) (Fig. 5). Tissue-resident macrophages and fibroblast-like synoviocytes (FLS) are presumably the first trigger of inflammation-induced angiogenesis. This is likely to occur in concert with hypoxia-controlled signalling pathways (via HIF), pro-inflammatory mediators and pro-angiogenic factors (such as VEGF-A or angipoietin-1) to induce vessel sprouting (reviewed in ref. [116]). On the other hand, the presence of vessels appears to define the degree of inflammatory potential of FLS by inducing a position-dependent gene expression program [117] (Fig. 5). This spans from FLS lining the synovial membrane up to those that are in close vicinity of blood vessels. The closer FLS are located to vessels the more they express the pro-inflammatory marker Thy1 (CD90) and resemble active pro-inflammatory cells. The “lining” Thy1-negative FLS at the synovial luminal side are supposed to play a larger

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part in tissue destruction, as revealed by cell ablation and transplantation experiments [118] (Fig. 5). Instructive signals, such as expression of the Notch ligand Dll4 by ECs, leading to the activation of Notch3 on FLS promotes the Thy1+ pro-inflammatory signature [117]. Thus, vessels support the pro-inflammatory phenotype of FLS during the arthritic process and, accordingly, genetic and pharmacological inhibition of Notch signalling ameliorates inflammation.

Crosstalk between bone vessels and macrophages

Apart from the interactions between FLS and the endothelium in RA models, there are also important roles of macrophages. In the context of joint inflammation, macrophages can be subdivided by their functional phenotype. Recently identified subsets of resident macrophages are providing a barrier in the synovium, thus protecting against excessive inflammation, while recruited monocyte-derived macrophages in the synovial cavity actively contribute to joint inflammation [119] (Fig. 5). Epithelial cell-like macrophages at the synovial lining may be derived from self-renewing resident macrophages located in the synovial tissue. During inflammation, this barrier becomes disrupted and allows infiltration of inflammatory cells, including poly-morphonuclear granulocytes (PMNs) and macrophages, from the circulation, involving trans-endothelial migration.

In addition, it is very likely that vessels communicate with macrophages also indirectly during arthritis. Vessels polarize FLS towards Thy1+ pro-inflammatory phenotype via DLL4/Notch signalling [117]. In turn, these pro-inflammatory FLS could lead to pro-inflammatory polarization of macrophages. This scenario is supported by a study that demonstrate that immune suppressive glucocorticoids decrease inflammation via FLS leading to anti-inflammatory polarization of macrophages [120] (Fig. 5). Thus, an EC-FLS-macrophage interaction axis is driving inflammation in arthritis, with different FLS subpopulations driving inflammation and bone destruction, respectively. This axis might also promote the resolution of inflammation and could therefore provide an unexploited therapeutic target.

Bone repair and ageing

Role of vessels in regenerative osteogenesis

Bone development and fracture repair share many features and both processes rely on angiogenesis [40,121]. The entry of osteoblast precursors correlates with blood vessel ingrowth into cartilage during the developmental formation of the POC, but simultaneous entry of vessels and osteoblastic cells is also observed during fracture healing [34]. Treatment of mice with a soluble, neutralizing VEGF receptor not only decreases angiogenesis during repair of femoral fractures but also impairs osteogenesis, callus mineralization and bone healing. Conversely, exogenous VEGF-A can enhance blood vessel formation, ossification and callus remodelling [122]. Osteoblast lineage cells are an important source VEGF-A and thereby contribute to different phases of bone repair. During the repair of drilled lesions in tibia, VEGF-A provided by osteoblasts was found to promote macrophage recruitment and angiogenesis in the inflammation phase, which initiates the repair process [39]. Later in regeneration, in the endochondral ossification stage, osteoblast- and hypertrophic

chondrocyte–derived VEGF-A stimulates vessel growth, osteoclast recruitment and cartilage resorption at the repair site. The role of osteoblasts as source of VEGF-A extends into the final remodelling phase of the repair process [39] . Perivascular bone mesenchymal stromal cells expressing Gli1 (glioma-associated oncogene homolog 1) were reported to interact with type H capillaries during bone development and defect healing. While defect healing involves the expansion of type H ECs, this increase and bone repair are impaired by genetic ablation of Gli1+ cells [123] . It was also shown that both osteoprogenitors and macrophages show VEGF-A immunosignal and are closely associated with type H vessels in the forming and maturing callus in a mouse osteotomy model [124] . In addition to the well-established function of VEGF-A, other molecular signals will mediate the crosstalk between different cell populations in growing and regenerating bone. In a mouse model of augmented postnatal bone formation, an increase in type H vessels precedes the appearance of the high bone mass phenotype. Effects on the vasculature were mediated osteoblast-derived SLIT3, which activates the receptor Robo1 on ECs. Remarkably, administration of recombinant SLIT3 improves bone fracture healing and suppresses OVX-induced bone loss [56] .

Vessels as a potential target in anti-osteoporotic treatments

Ageing is associated with a loss of mineralised bone and increased fracture risk [125] , which are further enhanced in osteoporosis (Fig. 6). These conditions are associated with reduced skeletal blood flow both in human patients [126,127] and in animal models [46,128,129] , which might affect a range of physiological features including nutrient delivery, tissue metabolism, or the influx of calcium and phosphate [130,131] . Surgical or pharmacological interference with normal blood flow alters EC behaviour and reduces the abundance of type H vessels in murine femur [46] . Like in the OVX-induced osteoporosis model, normal ageing results in a profound diminishment of type H vessels and associated osteoprogenitors but also of arteries and arterioles in femur (Fig. 6), which is likely to contribute to reduced perfusion and impaired bone homeostasis [32,46,55] . In mice, enhanced HIF activity in ECs via tissue-specific inactivation of VHL leads to increases in type H vasculature and perivascular osteoprogenitors, resulting in augmented trabecular bone formation. Treatment of aged mice with deferoxamine mesylate (DFM), which enhances HIF1α stability and activity, also increases type H vasculature and mineralized trabecular bone [32] . Likewise, EC-specific genetic approaches enhancing Notch signalling lead to the growth of type H vessels, increase in osteoprogenitor and trabecular bone formation in ageing animals [46,55] . These proof-of-principle experiments indicate skeletal blood vessels are not only responding to ageing processes in the surrounding tissue but might also potentially represent a therapeutic target for the treatment of osteoporosis either alone or in combination with anabolic or anti-resorptive drugs.

Relevance for human ageing and osteoporosis

While future research will undoubtedly enhance our understanding of the roles of the bone vasculature in health and disease further, several reports already suggest that certain key findings might be relevant for human subjects. Human bone ECs (termed hRECs) expressing the cell surface protein Endoglin/CD105, are associated with skeletal development and regeneration, share critical features with murine type H endothelium [132] . Human type H vessels have been reported to be a sensitive biomarker of bone mass in ageing subjects and

osteoporotic patients [133] . Likewise, CD31^{hi} Emcn^{hi} EC abundance is positively associated with bone mineral density in human femur neck and total hip BMD but not lumbar vertebra. Moreover, CD31^{hi} Emcn^{hi} EC percentage in postmenopausal subjects was found to be significantly lower relative to premenopausal women [134] . Taken together, the existing evidence is encouraging and indicates that at least some of the fundamental findings made in mice are of broader relevance and might be translatable to humans. At the same time, it has to be considered that the number of published reports is still rather limited and more research is needed to get a better understanding of the processes in the healthy and diseased human skeletal system.

Conclusions

Even though it is unquestionable that future work will have to provide more insight into the role of the vasculature and capillary subpopulations in bone development, homeostasis, regeneration, healthy ageing, and disease, it is increasingly evident that vascular cells are more than just building blocks of a transport network and, instead, actively control critical processes through communication with a variety of other cell types. Such interactions include the crosstalk with chondrocytes and perivascular osteoblasts lineage cells, which is bidirectional and results in a coupling of angiogenesis and osteogenesis. Osteoclasts are also associated with bone vessels and the abundance of type H ECs and bone angiogenesis are controlled by signals provided by preosteoclasts. In contrast, there is relatively limited evidence for a potential direct regulation of osteoclasts by EC-derived signals [95,135,136] . Thus, it remains to be addressed whether bone ECs directly control osteoclastogenesis and thereby bone turnover and fracture healing but also conditions such as osteopenia and osteoporosis. Furthermore, vessels are likely to play important roles in OA and other inflammatory conditions not just through their role in immune cell signalling but also through interaction with synoviocytes and other cell populations in the joint. The identification of relevant molecular signals and potential therapeutic relevance require investigation.

Taken together, it is clear that the vasculature in the skeletal system is much more than a passive conduit system and learning more about its function, dynamic modulation, the molecular crosstalk with other cell types the local microenvironment, offers great opportunities. Insight into the role of bone vessels is highly relevant for fundamental physiological processes in healthy bone but might also reveal new potential targets for the treatment of diseases such as osteoporosis or OA, which currently impose huge burdens on our ageing population.

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Box 1

Signalling pathways controlling bone angiogenesis

VEGF-A

The growth factor is member of the VEGF family and generated in different isoforms, some of which critical lack sequence motifs required for retention at the cell surface and matrix binding. Several cell surface receptors and co-receptors for VEGF-A (VEGFR1, VEGFR2, Neuropilin-1) are known. VEGF-A is also an important regulator of vascular permeability. Apart from its important function in ECs, VEGF-A is known to control osteoblast lineage cells and inflammatory cells [39,62] .

Hypoxia-inducible factor

HIF heterodimers are transcription factors composed of one α (HIF-1α, HIF-2α and HIF-3α) and a common β subunit (HIF-1β). HIF proteins are unstable under high oxygen conditions, which involves HIF hydroxylation by prolyl hydroxylases (PHDs), ubiquitination by the von-Hippel Lindau (VHL) ubiquitin ligase and proteasomal degradation.

Notch

ECs predominantly express the Notch receptors 1 and 4 as well as the ligand Dll4. Dll4 levels are increased by Notch signalling and in response to VEGF-A, which generates feedback loops because Notch also suppresses VEGFR2 signalling. Notch has important cell-autonomous roles in many different cell types and is also critically required for the maintenance of osteoprogenitors [63,64] .

Slit-Robo

Slit ligands are secreted proteins that were originally identified as regulators of axon guidance in invertebrates. In mammals, three known family members (Slit1, Slit2, and Slit3) and their Robo (Roundabout) transmembrane receptors (Robo1-4) are involved in numerous processes including neuronal wiring and angiogenesis [65,66] .

Hippo pathway

Yap1 and Taz are transcriptional co-activators that promote gene expression and growth through interactions with DNA-binding TEAD (Transcriptional Enhanced Associate Domain) transcription factors (TEAD1-4) but also other transcriptional regulators. In response to phosphorylation by an upstream signalling cascade involving the Stk3/4 (Hippo; Mst1/2) and Lats1/2 protein kinases, Yap1 and Taz are retained in the cytoplasm and subjected to proteasomal degradation [67] .

Bone Morphogenetic Proteins (BMPs)

Large family of ligands belonging to the Transforming Growth Factor β (TGFβ) superfamily. BMP signalling involves binding to type I and type II heterotetrameric TGFβ family serine/threonine kinase receptor complexes followed by phosphorylation of

SMAD proteins (Smad1/5), which are transferred from the cytoplasm into the nucleus to control gene expression [68,69] .

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Key points

- The vascular system is essential for bone development and growth.
- Capillary endothelial cells represent multiple subpopulations with distinct molecular and functional properties.
- The type H endothelial subpopulation communicates with chondrocytes and perivascular osteoblast lineage cells.
- The endothelium is closely intertwined with inflammatory cells during bone inflammation.
- Preosteoclasts secret factors affecting bone angiogenesis and the abundance of type H endothelial cells.
- Interdependent cross-talk of endothelial cells and other cell populations in bone might provide novel entry points for therapy of bone erosion.

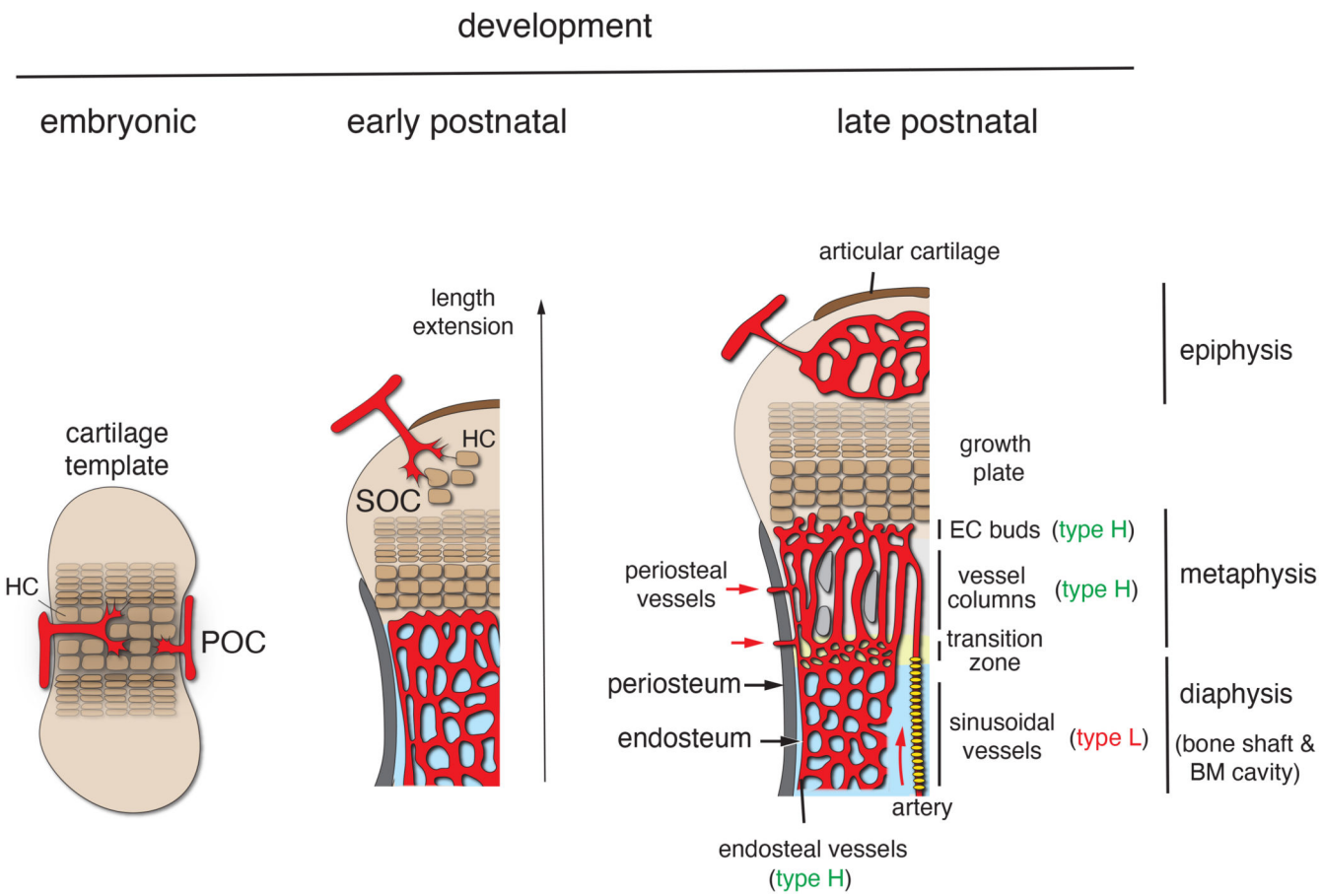

Fig. 1. Organisation of the bone vasculature during development.

During endochondral osteogenesis in the developing embryo (left), signals provided by hypertrophic chondrocytes (HC) trigger the invasion of blood vessels into an initially avascular cartilage template (left). This process coincides with the onset of osteogenesis and the formation of the primary ossification centre (POC). Similarly, vessel ingrowth into hypertrophic cartilage of the distal end of long bone, which occurs postnatally in mice (centre), triggers secondary ossification centre (SOC) formation (centre). Postnatal growth and bone length extension in late postnatal and adolescent mice (right) is accompanied by the establishment of morphologically and molecularly distinct capillary subpopulations. $CD31^{hi}$ $Emcn^{hi}$ (type H) ECs include vessel buds in direct proximity of the growth plate, metaphyseal vessel columns and endosteal capillaries, whereas sinusoidal (type L) ECs in bone marrow (BM) show comparably low expression of CD31 and Emcn. In the transition zone at the metaphyseal-diaphyseal interface, type H columns are progressively remodelled into sinusoidal vessels during expansion of the BM cavity. Red arrows indicate perfusion through arteries and periosteal vessels.

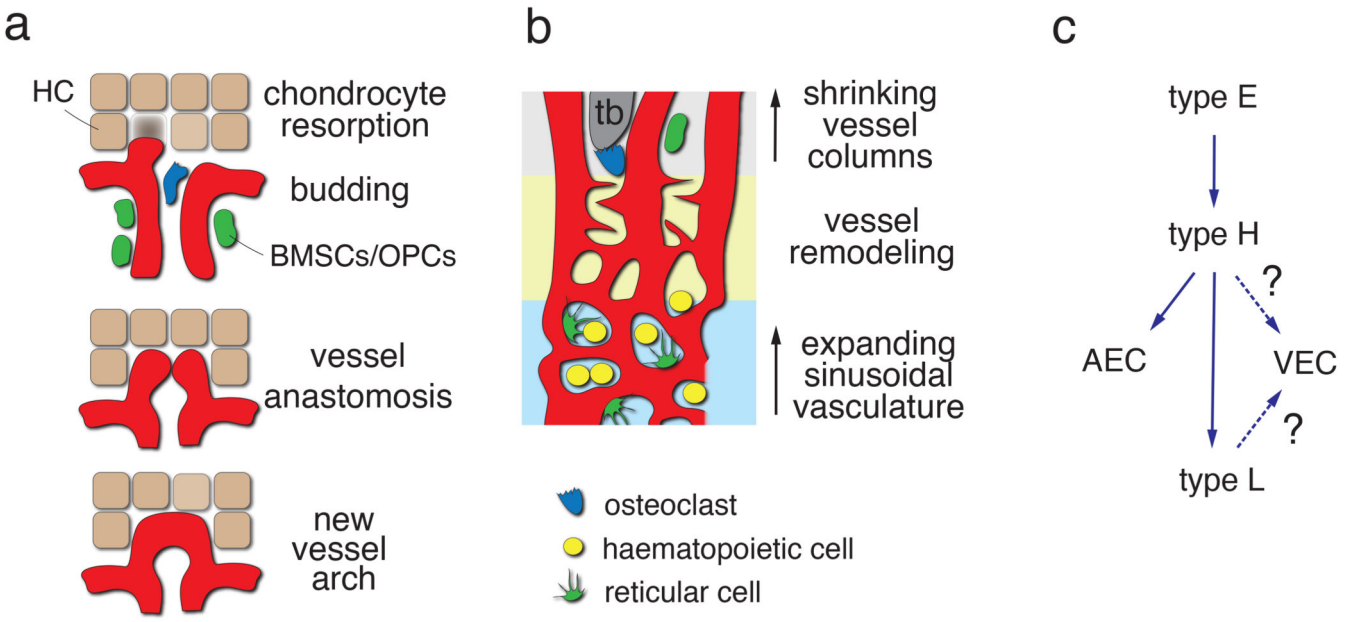

Fig. 2. Vessel formation, remodelling and EC heterogeneity in bone.

(**a**) Resorption of hypertrophic chondrocytes (HC) in the growth plate enables the invasion of type H vessel buds, which emerge from distal vessel arches (top). Anastomotic fusion of contiguous buds (centre) leads to the formation of new arch-shaped vessels (bottom) from which new buds can emerge subsequently. Osteoclasts (blue), bone mesenchymal stromal cells (BMSCs; green) and osteoprogenitor cells (OPCs; green) are associated with metaphyseal type H vessels. (**b**) Reduction of the metaphysis after the decline of developmental growth is accompanied by expansion of the BM cavity. BM contains haematopoietic cells and reticular cells, whereas immature bone mesenchymal cells are mostly confined to the metaphysis and endosteum. BM expansion involves remodelling of type H vessel columns (top) into sinusoidal (type L) vessels (bottom) at the metaphyseal-diaphyseal interface (yellow area) through endothelial sprouting. (**c**) Hierarchy of EC subpopulations in bone. Type E ECs are abundant in embryonic long bone and give rise to type H cells, which, subsequently, can generate arterial and venous ECs but also type L sinusoidal ECs. While sinusoidal vessels directly connect to the large central vein, the lineage relationship between the $CD31^{lo}$ $Emcn^{lo}$ subpopulation and venous ECs is unclear.

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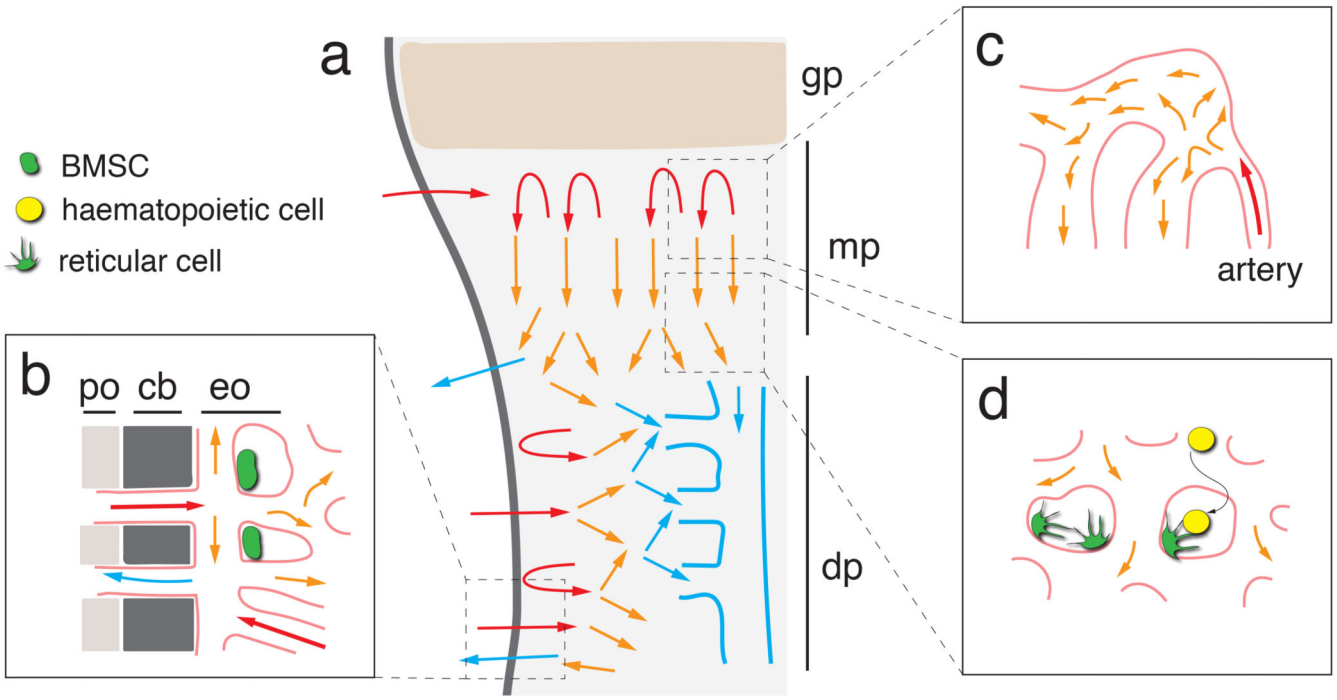

Fig. 3. Blood flow in long bone.

(**a**) Overview image showing arterial (red arrows) flow in the metaphysis and endosteum. Perfused blood flow at low speed through capillaries and sinusoidal vessels (orangearrows) before entering draining veins (blue). Growth plate (gp), metaphysis (mp) and diaphysis (dp) are indicated. (**b**) Periosteal (po) vessels penetrate through cortical bone (cb) and supply the endosteum, which harbours a fraction immature bone mesenchymal stromal cells (green). (**c**) While the relatively narrow arteries and arterioles in bone permit laminar perfusion, flow slows down substantially and becomes turbulent after entry into capillaries. (**d**) Flow is very slow in the sinusoidal vasculature, which facilitates transendothelial migration of homing leukocytes (yellow). Reticular cells associated with sinusoidal ECs are shown in green.

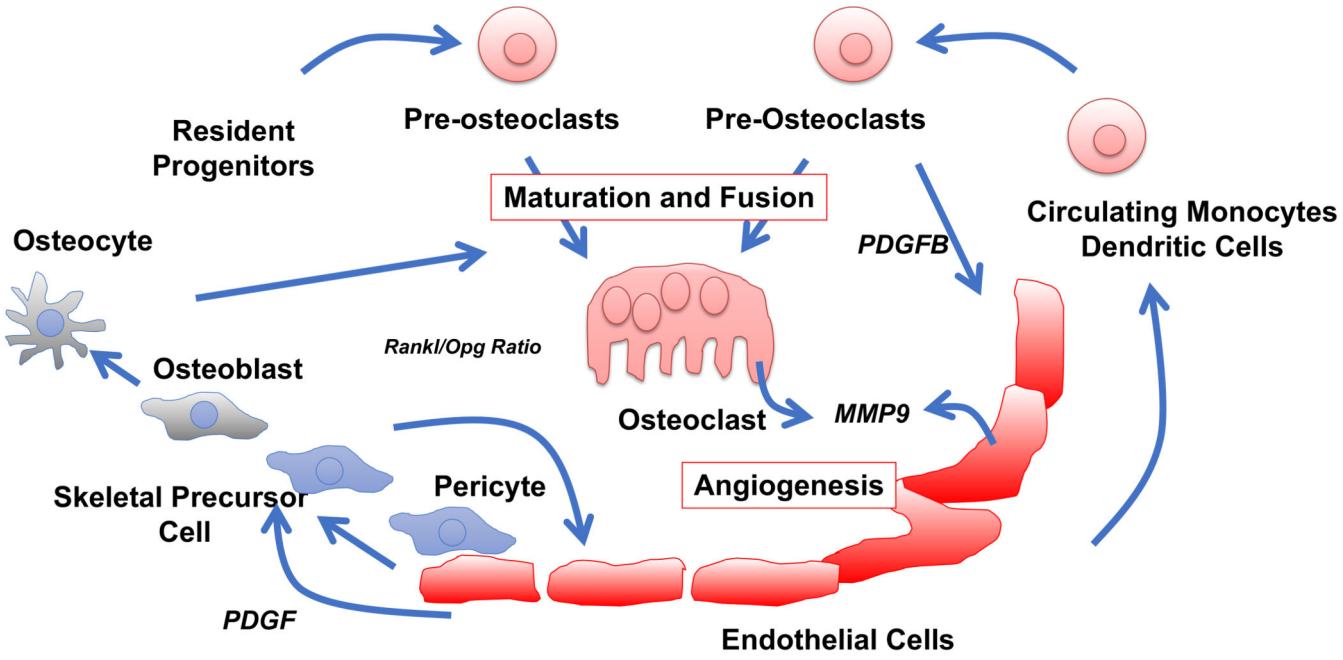

Fig. 4. Osteoclast-EC crosstalk.

Osteoclasts are generated by maturation and fusion of myeloid resident and circulating precursor cells, the latter derived from the circulation. The rate-limiting signalling molecule important for fusion is RANKL released mainly by osteocytes and osteoblasts, derived from skeletal precursor cells that are often perivascular. RANKL has to exceed the antagonistic acting osteoprotegerin (Opg). Non-fused pre-osteoclasts are essential triggers of angiogenesis via PDGF-B, whereas mature osteoclasts were suggested to facilitate angiogenesis by degradation of extracellular matrix. Recent data challenge this concept by demonstrating EC-derived MMP9 is required for angiogenic processes in areas with non-degradative osteoclasts.

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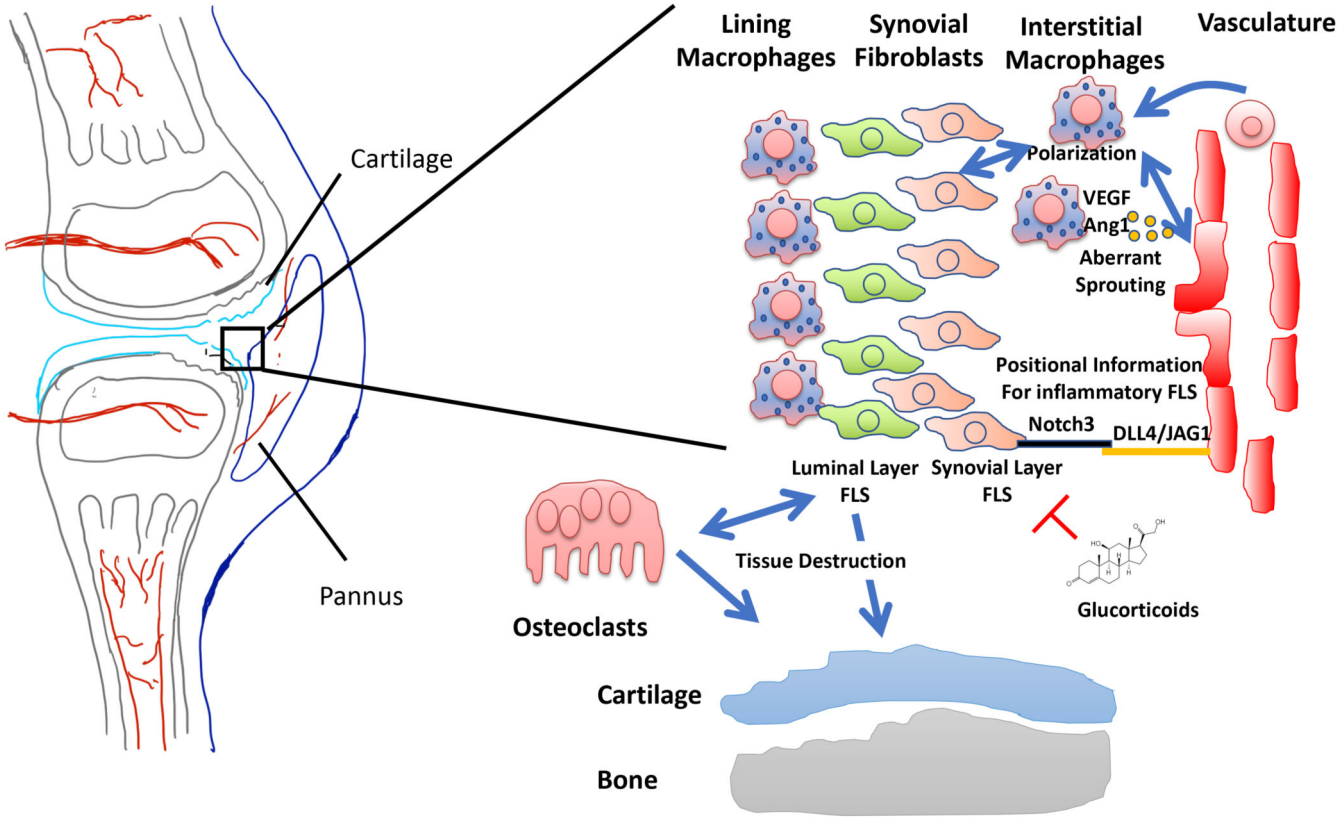

Fig. 5. Interplay of vasculature, fibroblast like synoviocytes (FLS) and macrophages during inflammation of rheumatoid arthritis.

The pannus consists of recently discovered lining macrophages, luminal FLS located more distant from vasculature and synovial layer FLS close to the vasculature, interstitial resident and BM monocyte derived macrophages and freshly recruited macrophages and other immune cells (not shown). Tissue resident macrophages and FLS trigger aberrant angiogenesis. The vasculature itself provides positional information for FLS, whether they belong to the inflammatory active synovial layer FLS or the more tissue destructive luminal layer FLS. Luminal layer FLS promote tissue destruction presumably via inducing osteoclastogenesis and degradative enzyme release. Synovial layer FLS promote pro-inflammatory macrophage polarization which in turn triggers angiogenesis. Anti-inflammatory acting glucocorticoids suppress inflammation via action on FLS in arthritis.

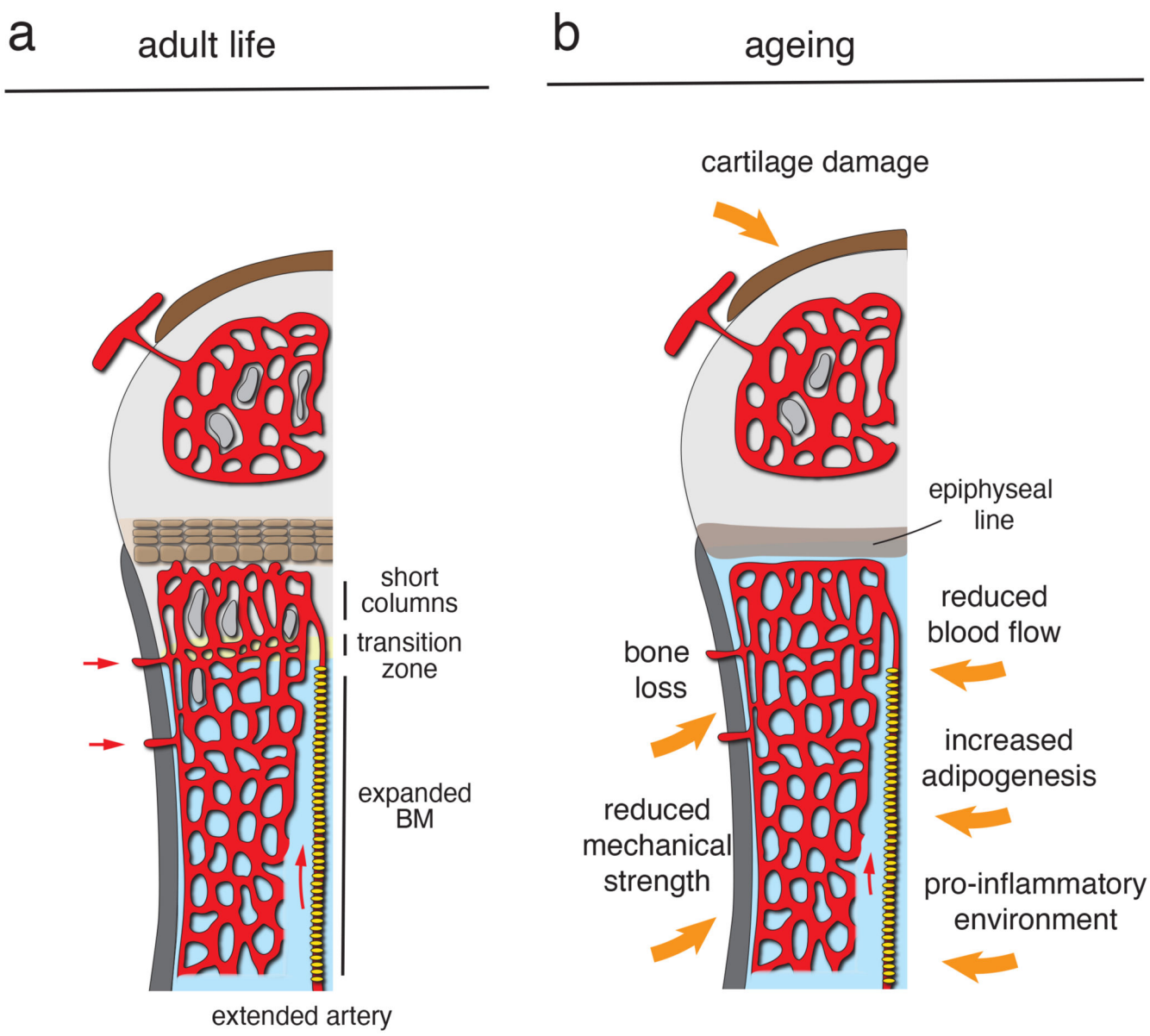

Fig. 6. Remodelling of the bone vasculature in adult life and ageing.

(**a**) Type H columns are progressively remodelled into sinusoidal vessels in the transition zone at the metaphyseal-diaphyseal interface during development, resulting in a substantial expansion of the BM cavity in adult mice. Accordingly, the abundance of type H ECs and length of vessel columns declines with age. (**b**) In aged mice, type H ECs are scarce and the bone shaft is largely remodelled into a large marrow cavity. Features of bone ageing include the gradual loss of mineralized bone, reduced mechanical strength, increased adipogenesis, increased baseline inflammation, and damage in the articular cartilage. The number of arteries and blood flow are also reduced.