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Evolution of the Marrow Adipose Tissue Microenvironment

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Abstract

Adipocytes of the marrow adipose tissue (MAT) are distributed throughout the skeleton, are embedded in extracellular matrix, and are surrounded by cells of the hematopoietic and osteogenic lineages. MAT is a persistent component of the skeletal microenvironment and has the potential to impact local processes including bone accrual and hematopoietic function. In this review, we discuss the initial evolution of MAT in vertebrate lineages while emphasizing comparisons to the development of peripheral adipose, hematopoietic, and skeletal tissues. We then apply these evolutionary clues to define putative functions of MAT. Lastly, we explore the regulation of MAT by two major components of its microenvironment, the extracellular matrix and the nerves embedded within. The extracellular matrix and nerves contribute to both rapid and continuous modification of the MAT niche and may help to explain evolutionary conserved mechanisms underlying the coordinated regulation of blood, bone, and MAT within the skeleton.

Keywords

Evolution; Marrow fat; Bone; Adipose; Marrow adipose tissue; Matrix

Introduction

Marrow adipose tissue (MAT) is a collection of adipocytes, distributed throughout the skeleton. These cells are embedded in extracellular matrix and surrounded by cells of the hematopoietic and osteogenic lineages [1–3]. There is evidence that MAT behaves differently depending on where it is located in the skeleton [4]. For example, MAT in the red, hematopoietic marrow is depleted in response to systemic hemolysis in rabbits [5] or with prolonged cold exposure in mice [4]. The terms regulated and constitutive MAT (rMAT and cMAT) have attempted to capture some of this complexity [4]. Regulated MAT adipocytes are defined as single cells that are interspersed with areas of active hematopoiesis [3]. They form gradually throughout life, are located in the proximal-central regions of the skeleton, and their lipid composition and transcription factor expression mimics peripheral white adipose tissue (WAT) [4]. Constitutive MAT develops shortly after birth in the most distal portions of the skeleton, forming sheets of confluent adipocytes that are relatively

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devoid of hematopoiesis. Relative to rMAT and WAT, cMAT adipocytes have increased lipid unsaturation and higher expression of the transcription factors *Cebpa/Cebpb* [4]. They are refractory to change. It is unknown if differential responses to cold exposure and hemolysis are driven by cell-autonomous differences between rMAT and cMAT adipocytes, or, are instead dictated by their surrounding microenvironment.

There is substantial evidence that MAT has the potential to impact surrounding cells within its local microenvironment, potentially contributing to bone accrual and hematopoietic function [2, 6]. There are also accumulating reports in rodents and humans that MAT expansion is a common feature of metabolic diseases including anorexia, diabetes, obesity, gonadal dysfunction, and estrogen deficiency [7]. MAT can secrete paracrine and endocrine mediators, capable of modifying the action of surrounding cells and distant tissues (reviewed in [7]). It is also becoming increasingly apparent that MAT can undergo pathologic change with age and disease, potentially contributing to fracture risk and metabolic dysfunction [1, 8]. In humans, MAT persists for years after radiation- or bed rest-induced expansion, even after regaining health or resuming physical activity [9, 10]. Thus, identification of the molecular mechanisms underlying MAT expansion and regulation is clearly of high priority with broad implications.

Previous reviews have discussed the function of MAT as it compares to peripheral white and brown adipose tissues (WAT, BAT) [3, 7]. However, the first appearance of MAT in vertebrate evolution, particularly relative to WAT and BAT, has not yet been established. To gain insight into these relationships, the first part of this review examines the presence of MAT in vertebrate lineages with a discussion of how it relates to evolution of WAT, bone, and hematopoiesis. As a component of the microenvironment, MAT has the potential to receive large quantities of information from its surroundings: mechanical forces and chemical signals from the extracellular matrix, paracrine factors from surrounding cells, and endocrine mediators delivered via the circulation. However, we know very little about which of these signals are assimilated by the MAT adipocytes and which are ignored. In addition to its interactions with cells of the hematopoietic and osteogenic lineages, MAT has evolved alongside two additional components of the microenvironment that, up until this point, have been rarely discussed in the context of MAT function. These include the structural and signaling components of the extracellular matrix and the large variety of neural fibers that are embedded within. Thus, we will follow our overview of MAT evolution with a discussion of the microenvironmental determinants of MAT expansion and turnover, with particular emphasis on the extracellular matrix and nerves.

MAT Adipocytes as an Evolutionarily Conserved Component of the Bone Marrow Microenvironment

Placing MAT within the context of vertebrate evolution can inform hypotheses about its function. Indeed, naturally occurring variation between species can be used to test the relationship of a tissue's form and function without the need for experimental modifications [11]. In this section, we will discuss the evolution of MAT with comparisons to the development of bone, adipose, and hematopoietic tissues (Fig. 1).

Vertebrae evolution is thought to trace to a common ancestor that lived 500 to 600 million years ago [11]. The jawed vertebrates include the cartilaginous fishes, bony fishes, amphibians, reptiles, birds, and mammals. Excluding the cartilaginous fish, WAT-like structures are present in the majority of vertebrates with varying degrees of specialization [12–14]. By contrast, BAT has a purely mammalian structure [12, 14]. Though birds, reptiles, and fish are capable of thermogenesis, generally via their muscle, true BAT has not been found despite significant effort [12]. In regards to blood cell production, vertebrate evolution is characterized by an increasing complexity of secondary lymphoid tissues—such as the lymph nodes, bone marrow, and spleen [11]. Ossified ‘skeletal’ tissue first appeared in vertebrate fishes as an exoskeleton. This was followed by specialization of endoskeletal elements, such as the jaw in sharks, that undergo perichondrial ossification and lack a defined marrow space [15]. The pairing of perichondral and endochondral ossification, with subsequent development of a cavity within the bone, did not occur until the evolution of the *Osteichthyes*, also known as bony fishes [15].

Jawless Vertebrates

Jawless vertebrates such as the hagfish lack a defined bone marrow and are supported by a cartilage-like endoskeleton [16, 17]. The sea lamprey, *Petromyzon marinus*, provides an ancestral example of the merging of fat and hematopoiesis [18]. The sea lamprey has an extraskeletal structure known as the supraneural body, also known as the dorsal fat body, that is organized from adipose progenitors of the dorsal connective tissue sheath around the spinal cord and meningeal tissue (Fig. 1a). At the beginning of metamorphosis, this fat body is colonized by hematopoietically active cells—leading to the development of a mixed hematopoietic/adipose organ [18]. This mixed organ is reminiscent of MAT (Fig. 1a), however, since the adipocytes were present prior to hematopoietic infiltration and exist outside of an ossified skeleton, they seem to be more akin to peripheral WAT. Thus, while this is an early example of functional WAT, it does not yet represent MAT.

Cartilaginous Fish

Cartilaginous fish primarily store triacylglycerols in the liver and/or skeletal muscle and lack a defined adipose tissue [12, 14]. The *Elasmobranch* fishes, cartilaginous species such as sharks and rays, also lack lymph nodes and bone marrow. They do, however, have a specialized lymphomyeloid tissue, the epigonal organ, which closely resembles a sinusoidal bone marrow (Fig. 1b). In the *Elasmobranch* species studied to date, the epigonal organ is noted to be devoid of fat [19, 20].

Bony Fish

Bony fish such as zebrafish, rainbow trout, and salmon have defined adipose tissue which contributes to circulating adipokines [12]. Most bony fish do not have a functional bone marrow and instead delegate hematopoiesis to the spleen, kidney, intestinal submucosa, and thymus [21, 22]. In zebrafish, mono- and multi-nucleated osteoclasts contribute to remodeling and allometric growth of the skeleton. Spaces created by resorption of cartilage and/or bone lack hematopoietic activity, but are uniformly filled with adipose tissue [23].

Thus, the MAT in the zebrafish resembles the cMAT found in the distal extremities of mammals [4] (Fig. 1d).

Unlike most bony fishes, some *Osteichthyes* have bone marrow. There is an intriguing report describing the presence of a mature extraskelatal bone marrow organ in the bowfin fish *Amia*, which also extends into the bone-lined cavities of the skull [24, 25] (Fig. 1c). This is thought to represent the first evidence of bone marrow in vertebrates. The marrow organ of this boneless fish is bordered by adipose tissue which fills the space between the cartilage of the skull and the brain [25]. The authors note that this tissue is WAT-like and not comparable to yellow fatty bone marrow, particularly because fatty conversion of marrow in older *Amia* fish or those bred in captivity was not observed [25]. Thus, in these animals, it seems that MAT is not yet present despite evidence of a mature marrow organ, ossified bone, and WAT. By contrast, an image of the jaw of the ray-finned fish *Garra congoensis* clearly demonstrates hematopoietic bone marrow with associated adipocytes of the ‘rMAT’ type [4], thus, the potential for their formation in fish clearly exists [26] (Fig. 1e).

Amphibians and Reptiles

Both reptiles and amphibians have a well-developed adipose tissue; many species also begin to display the depot-like arrangement of fat tissues that is commonly observed in mammals [12]. Unlike the fish, a functional hematopoietic bone marrow becomes the norm rather than the exception. For example, salamanders of the family *Plethodontidae* possess actively hemopoietic bone marrow [27], however, this marrow is unique in that it lacks erythropoietic activity. Unilocular fat-containing cells are frequent in the lymphogranulopoietic bone marrow of the northern slimy salamander *Plethodon glutinosus* and are comparable in number to cells of the granulocyte lineage [27]. These MAT-like cells appear smaller, with a larger rounded nucleus, than traditional mammalian MAT adipocytes [27] (Fig. 1f). In the newt *Notophthalmus viridescens*, an amphibian that lacks a hematopoietic bone marrow, the space within the skeleton is filled with fat cells and fibroblasts—similar to that of the zebrafish [28].

The leopard frog *Rana Pipiens* provides one of the first examples of seasonal variation of MAT [29]. Conversion of fatty yellow marrow to red hemopoietic marrow occurs in frogs to provide space for blood cell formation [29], generally in the early summer. The stroma of the red marrow is packed centrally with fat cells and blood vessels, covered peripherally with a layer of differentiating leukocytes. During seasonal fasting in the frog, the red coloration of the bones has been noted to disappear in the distal extremities, presumably due to the expansion of MAT at the expense of blood cells, concurrent with the loss of peripheral adipose tissue [29, 30]. This is comparable to the recent work in mice and humans showing that MAT increases in states of caloric restriction and anorexia, while peripheral WAT is lost [31, 32].

Unlike the amphibians described above, information concerning the bone marrow of reptiles is relatively sparse, however, images demonstrating a marrow cavity containing both adipocytes and hematopoietic marrow have been documented in the gekkonoid lizard *Ptyodactylus* [33].

Birds

The peripheral WAT in birds closely resembles that of mammals [4]. As in lower vertebrates, erythropoiesis in birds is generally intravascular, meaning that new erythroid cells are formed in the sinusoidal lumen and released directly into circulation [34, 35]. By contrast, mammalian erythropoiesis occurs extravascularly, necessitating trafficking of the new cells into the vascular lumen. Relative to mammals, the bone marrow of lower vertebrates characteristically contains large amounts of lymphatic tissue [35]. The bone marrow of pigeons and chickens, for example, contains numerous centers of lymphatic tissue which blend gradually into the surrounding myeloid tissue [35]. In birds, the lining cells of the vascular sinuses in the bone marrow are frequently closely associated with MAT-like adipocytes [35] (Fig. 1g). As in mammals, chicken MAT progenitors respond to methylprednisone with MAT expansion [36] indicating conserved regulation of MAT between mammalian and avian species [37].

Mammals

The universal presence of MAT in mammals has been reviewed previously [3]. The formation of MAT generally begins shortly after birth in the most distal extremities, such as the fingers, toes, and tail [3]. This early wave of perinatal MAT formation, thought to be cMAT-like, is then followed by a gradual accumulation of rMAT-like adipocytes in areas of hematopoietic marrow with age [3, 4]. Thus far, a consistent finding is that larger species have more MAT that extends farther into their skeleton, displacing the blood-forming bone marrow to central skeletal sites [3].

Based on evidence from rabbits, the distribution of MAT within the marrow is specified embryonically. At birth, prior to MAT formation, sites of fatty marrow are significantly less cellular than central regions of hematopoiesis [38]. The marrow phenotype, fatty versus hematopoietic, is preserved even after transplantation. For example, in rabbits, ectopic implantation of yellow, fatty marrow into the subcutaneous tissue of the abdomen or the capsule of the spleen results in the formation of fatty marrow nodules, while implantation of hematopoietic marrow leads to the formation of hematopoietic nodules that remain active even after 6-months [39, 40]. This occurs despite similar cellular depletion after implantation prior to reconstitution and growth of the nodule [40].

The armadillo provides unique insight into the role of MAT in mammals. The armadillo is a placental mammal that is covered by plates of dermal bone [41]. The dermal plates of the dorsal bands have an irregular marrow cavity, the contents of which fluctuate seasonally. The volume of marrow is greatest and most hematopoietically active in the spring, summer, and fall. In the winter, the dermal plate marrow becomes dull gray-white and filled with fat cells [41]. Bone is deposited simultaneously with MAT expansion, such that the size of the marrow space decreases as it becomes filled with fat. In the spring, fat regression coincides with hematopoietic expansion and osteoclast activation and bone resorption. As in mice, there is a gradient of adiposity with anterior regions of marrow being more hematopoietic and posterior regions more fatty [42]. Interestingly, the armadillo's endoskeleton also has

both hematopoietic and fatty marrow; however, unlike that of the dermal plates, its composition is not acutely regulated by seasonal change [41].

Evolution Summary

From these studies, it appears that in vertebrate species the first evidence of MAT occurs in the bony fishes. This is after the appearance of WAT, which is present in the sea lamprey, and many years earlier than BAT, which is only present in mammals (Fig. 1). In the absence of a hematopoietic marrow, cMAT-like cells fill the space formed by endochondral resorption of the bone. Conversely, in the presence of hematopoiesis within bone, the adipocytes take on the appearance of rMAT-like cells. The presence of both phenotypes in bony fishes suggests that rMAT and cMAT adipocytes evolved at roughly the same time and supports the hypothesis that differences in function are, at least in part, a product of their surrounding microenvironment. Further, the regulation of MAT, for example by glucocorticoids, is conserved between at least some vertebrate species (*e.g.*, chickens and mice).

The Function of Adipose Tissue Within Bone

An assessment of vertebrate evolution establishes that MAT likely appeared ~400 to 500 million years ago in bony fish, both in the presence and absence of hematopoietic bone marrow (Fig. 1). At this point, MAT becomes a ubiquitous, presumably essential, component of the bone marrow. Why? Hypothetically, MAT can respond to its surroundings in several ways. MAT precursors within the marrow can be induced to form new MAT adipocytes [43]. Mature MAT adipocytes can be stimulated to undergo lipid storage through fatty acid uptake or *de novo* lipogenesis. Conversely, stored lipid can be broken down and released by lipolysis [4]. Lastly, MAT adipocytes can be removed from the bone marrow through apoptosis or necrosis [44]. The common theme being dynamic storage and release of energy and, unique to bone, preservation or loss of space. Before discussing the regulation of these functions at a microscopic scale, we will explore several putative functional relationships between MAT, bone, and blood cell production.

MAT and Bone

The relationship between MAT and bone has been reviewed previously [2, 4]. Currently, there is no evidence to suggest that MAT existed prior to the evolution of endochondral bone resorption and subsequent generation of void space within the skeleton. This is in contrast to WAT, which is readily demonstrated in the dorsal fat body of the cartilaginous sea lamprey [18]. This apparent separation in evolution between MAT and WAT supports the current hypothesis that these cells are derived from distinct mesenchymal progenitor populations [7].

In the absence of a hematopoietic bone marrow, such as in the zebrafish [23] or newt [28], MAT fills the skeletal void space and takes on the appearance of cMAT, as defined in mammals [4]. This indicates that filling the bone with MAT, instead of fluid, offers some evolutionary advantage to the bone itself. This could be in the form of mechanical [45] or

biochemical support [46]. For example, MAT adipocytes may protect surrounding cells from the lipotoxic effects of circulating fatty acids and provide a storage reservoir that can be induced to release energy in times of local need [46].

It is also worth noting that more MAT does not always mean less bone. In the dermal plates of the armadillo, increases in bone volume accompany MAT expansion during regression of hematopoietic bone marrow, conversely, MAT loss is accompanied by hematopoietic expansion and bone resorption [41]. The ability of the resulting rMAT-like adipocytes to actively suppress bone formation, perhaps leaving room for blood cell production, remains unknown. However, there have been reports that MAT has the potential to promote osteoclast activity, particularly in the context of cancer metastasis [47].

MAT and Hematopoiesis

With the exception of the bowfin, a basal member of the bony fishes, MAT is easily demonstrated within the hematopoietic bone marrow of most species. One of the evolutionary functions for MAT, as discussed in frogs and armadillos, is the seasonal displacement of hematopoiesis. This likely serves to balance peripheral energy supply with hematopoietic demand and is consistent with work in mice showing that sites of high MAT cause increased quiescence of hematopoietic progenitors, which can be activated in times of need [6].

An intriguing observation in recent years is that MAT is preserved, or even increased, in states of food restriction or anorexia [31]. Conversely, MAT is readily depleted by hematopoietic demand as demonstrated in the context of phenylhydrazine-induced hemolysis [5, 39]. As with cold exposure in rodents [4], loss of MAT with hemolysis occurs primarily in the rMAT-containing regions of the red marrow—not in the yellow, cMAT-enriched areas [5]. This is similar in many ways to the adipose tissue around the lymph nodes, which responds less readily to the lipolytic signals such as norepinephrine than conventional WAT [48]. Perinodal adipocytes are instead stimulated to undergo lipolysis with cytokines, such as TNF α and IL-6, that are released by local immune cells [48, 49]. Most of the lipids in new lymphoid cells are derived from triacylglycerols in perinodal adipocytes [50]. Perinodal adipocytes fuel the local immune response, as needed, without depending on circulating energy reserves. In this way, local paracrine control of adipocyte lipolysis reduces competition with other tissues for essential lipids, enabling immune responses even in times of peripheral energy deficit [51].

There is substantial evidence that the immune system takes priority when it comes to accessing essential lipids. An excellent example of this is illness-induced anorexia. It is well-known that sick animals tend to eat less—despite mounting an aggressive internal defense against disease. This is actually an evolutionarily conserved adaptation that may help to bias physiological pathways to promote immune function. The relationship between illness and anorexia is conserved in species ranging from arthropods [52] to mammals [53, 54]. With the appearance of a functional hematopoietic marrow within the bone, MAT may have become a nutrient source that could be locally conserved, despite peripheral wasting, to

support hematopoiesis in times of systemic demand. Thus, like perinodal WAT, MAT may help to emancipate the immune system from illness, malnutrition, and anorexia [12].

The MAT Microenvironment—Beyond Bone and Blood

The evolution of MAT in vertebrate species implies several possible functional relationships between MAT, bone, and blood. However, the ability of MAT to perform these functions locally within its microenvironment is dictated by several additional layers of complexity. The extracellular matrix is the foundation on which the microenvironment is organized. Embedded within the matrix are the numerous axons of the autonomic and sensory nervous system. These under-discussed components of the MAT microenvironment have the capacity to modify the spatial and temporal interactions between bone, fat, and blood within the skeleton.

Extracellular Matrix

The rise of multicellular organisms necessitated the development of the extracellular matrix scaffolds to organize and stabilize cellular interactions. The major ECM components (collagen fibers, fibronectin, basement membrane proteins...etc.) were all present by the time of the metazoan [55]. Thus, the evolution of vertebrate ECM is defined by its innovation. Through gene duplication, diversification of retained gene products, and domain shuffling, the vertebrate ECM evolved into complex structures that enabled the development of an elaborate central nervous system, a closed circulatory system, and an endoskeleton made of bones, cartilage, ligaments, and tendons. Supramolecular structures also allowed the creation of tissue-specific microenvironments in which the ECM components directly regulate cellular processes. Specifically, the ECM interacts with cellular receptors such as integrins to activate intracellular signaling pathways. The ECM establishes local signaling gradients in both time and space by sequestering (or presenting) molecules like growth factors and cytokines to cell surface receptors. The mechanical rigidity of the matrix mediates cell spreading and motility. Lastly, in bone, the ECM also directs the process of mineralization by providing a scaffold for hydroxyapatite deposition.

The exact composition of the ECM associated with marrow adipocytes has not been thoroughly characterized. However, there have been multiple reports on the spatial localization of ECM components throughout the bone [56–60]. These include fibronectin, laminin, and fibers of collagens-I, III, and IV. Large fibrillar matrix molecules like collagen-I and collagen-III concentrate near the endosteal surface, contributing to the mechanical stiffness of this interface (Fig. 2). Collagen-IV and laminin are basement membrane proteins associated with the marrow sinuses and arteries. Interestingly, collagen-IV has also been shown to localize around marrow adipocytes [56]. The space between the vessels and bone is enriched with mesh-like structures of fibronectin, collagen-V, and collagen-VI [56–58]. Though not discussed by Klein et al. [56], collagen-VI may be enriched around marrow adipocytes; this would be expected as collagen-VI is a major component of the peripheral adipose tissue ECM [61]. Although their spatial localization has not been fully characterized, tenascin-C, fibrillin-1, and alpha-2-HS-glycoprotein (aka. hemonectin) have also been identified within the marrow microenvironment [62–64].

Based on a study performed by Mori et al. [65], adipocytes likely reside in at least two distinct ECM microenvironments. Specifically, in adult rats, visceral (gonadal) WAT is enriched for laminin and fibronectin and expresses very little collagen-I [65]. Conversely, subcutaneous adipose tissue is enriched for collagen-I. When compared to the ECM of the bone marrow, this study suggests that central MAT adipocytes may reside in an ECM niche that is more similar to visceral adipocytes, while the ECM around MAT within the endosteal niche may be more similar to subcutaneous adipose tissue.

Though relatively few reports discuss the consequence of ECM mutations on MAT, Smaldone et al. recently demonstrated that loss of fibrillin-1 by PRX-1-expressing cells caused aberrant activation of the TGF β pathway in the stem cell niche and impeded MAT development [64]. We could not find evidence of collagen's effect on marrow adipocytes specifically, but in general, fibrillar collagens are thought to restrict adipocyte expansion (reviewed in [66]). It is important to note that the majority of the matricellular proteins found in the marrow interact with the collagens [67]; changes in collagen will have a secondary effect on matricellular proteins. Fibronectin is thought to restrain adipocyte cell shape, furthermore, assembly of a fibronectin rich matrix impedes adipocyte differentiation [68–70]. Lastly, laminin is an adipocyte basement membrane protein which in WAT appears to be expressed not by the adipocyte precursor, but the mature adipocyte itself [71].

Matricellular proteins are a second component of the ECM. Unlike the structural ECM proteins, they are dynamically regulated, facilitate proper ECM assembly, and are largely responsible for regulating the biochemical cues cells receive. While bound to structural ECM components, matricellular proteins can also engage cell surface receptors or growth factors. Matricellular protein families associated with the bone marrow include small leucine rich proteoglycans (SLRPs; i.e., decorin, biglycan), small integrin-binding ligand N-linked glycoproteins (SIBLINGs; i.e., osteopontin and BSP-1), osteonectin/SPARC, CCN (i.e., connective tissue growth factor), thrombospondin, and sulfated proteoglycans (heparin and chondroitin sulfate proteoglycans) (reviewed in [72–74]). SPARC (osteonectin) is critical to proper collagen fiber assembly. In the absence of SPARC, collagen fibers appear disorganized and fragmented, and bone volume is severely reduced (reviewed in [75]). Interestingly, during phenotypic characterization of the SPARC-null mouse bones, it was observed that marrow adipocyte volume, but not number, was increased [76]. It is likely that a weakened collagen fiber network facilitated unrestrained growth of the marrow adipocytes. This would be similar to the finding by Khan et al. that loss of collagen-VI allows stress-free expansion of peripheral adipocytes due to a weakened extracellular scaffold [61]. It has been shown, *ex vivo*, that thrombospondin-2 (TSP2) is a negative regulator of adipocytes; bone marrow mesenchymal progenitor cells from TSP2-null mice have increased lipid accumulation and a slight propensity for adipogenesis [77]. Finally, Bi et al. [78] demonstrated that the SIBLINGs biglycan and decorin support maturation of bone marrow stromal cells (BMSC) into osteoblasts, and using a BMSC transplant model, they found loss of the two proteins impairs bone formation and appears to be associated with adipocyte accumulation.

The following are examples of matricellular proteins that have been shown to regulate peripheral adipose tissue biology, but have not yet been linked to MAT. Osteopontin

facilitates the attachment of cells (monocytes, osteoclasts) to the matrix thereby supporting adhesion and motility. Interestingly, osteopontin-deficient mice are equally susceptible to diet-induced obesity (relative to wild-type mice), but due to impaired macrophage recruitment these obese mice are metabolically healthy [79]. Thrombospondin-1 (TSP-1) promotes adipocyte proliferation, and through its interaction with CD36 facilitates fatty acid uptake. TSP-1's capacity to activate transforming growth factor beta (TGF β) would also support excess adiposity. Indeed, TSP-1 deficient mice are protected from fat gain despite normal caloric intake or energy expenditure [80]. Heparin sulfate proteoglycans (HSPGs) have been shown to facilitate lipid accumulation in adipocytes. The proposed mechanism is that HSPGs help anchor apoE-enriched VLDL (apoE-VLDL) and lipoprotein lipase (LPL) near the adipocyte cell surface facilitating fatty acid release from triglycerides and subsequent uptake by fatty acid transporters on the cell surface [81].

Our knowledge of the dynamic relationship between adipocytes and the ECM in peripheral adipose tissue (reviewed in [65, 82, 83]) leads us to predict that marrow ECM will undergo remodeling with MAT expansion or depletion, influence MAT cellular processes, and contribute to the differentiation of MAT adipocytes from progenitor cells. Further defining the ECM-MAT microenvironment will provide insight to the biochemical and biophysical factors influencing the adipocytes.

Innervation of the Skeleton—Overview

The motor neural networks were the first to evolve, followed by the sensory and autonomic [84]. All vertebrates have a well-developed motor network. Sensory systems and their neuropeptides are also highly conserved, though their function has been adapted to suit the needs of each particular species [85]. The autonomic nervous system, consisting of the parasympathetic and sympathetic divisions, has shifted with respect to the balance between adrenergic and cholinergic fibers during vertebrate evolution [86]. The relevance of these findings to nerve function within the skeleton and hematopoietic organs, particularly in an evolutionary context, is poorly defined. Thus, this section will discuss autonomic and sensory nerves as a component of the MAT microenvironment primarily based on what has been reported in the mammalian literature.

Nerves are classified based on their diameter, conduction velocity, and function. The 'efferent' nerves are those that send an output from the central nervous system to the periphery—generally motor neurons and autonomic. Conversely, the 'afferent' sensory nerves are those that receive an input from the periphery and send it to the central nervous system. Another important distinction is whether the peripheral nerve axons are myelinated or unmyelinated. This is directly related to the conduction velocity, with more myelination leading to faster nerve conduction. Skeletal innervation has been reported in reptiles, birds, and mammals [87–91]. The mammalian skeleton is highly innervated by sympathetic efferent and somatosensory afferent nerves of the thinly myelinated A- δ and unmyelinated C fiber types [92]. Within the bone marrow, unmyelinated fibers greatly outnumber myelinated axons, as much as 24-fold in one study [93]; they have an average diameter of 0.4 and 1.4 μm , respectively [93]. The periosteum contains the highest density of nerve fibers; however,

given its small total volume, there are actually more axons in the mineralized bone and marrow [92].

Incorporation of nerve endings within the skeletal microenvironment allows for rapid modulation of surrounding cells by neurotransmitters. These short-acting molecules and peptides can provide selective stimulation to regions of cells, or an entire tissue, depending on upstream signal integration. Neurotransmitters are also unique in that they often invoke a quantitative response that is 'tunable' in terms of magnitude.

Autonomic Innervation

In mammals, autonomic fibers within bone are primarily of the sympathetic division, though a recent report also supports the parasympathetic innervation of the skeleton (Fig. 3a) [91]. Before reaching the skeleton, presynaptic autonomic nerves travel from the spinal cord to the sympathetic ganglia, postsynaptic nerves then extend to the bone. In the case of the tibia, sympathetic efferent fibers descend in the sciatic nerve, alongside the sensory and motor neurons, then pass through the medial popliteal nerve to enter the bone alongside nutrient vessels [94]. Within the bone marrow, most sympathetic fibers form dense networks around the arteriolar blood vessels [94], however, sympathetic nerve endings are also present in the mineralized bone and hematopoietic marrow space (Fig. 3b) [92].

Sympathetic postsynaptic nerves release norepinephrine, a potent vasoregulator [94], osteoclast activator [95], and inducer of adipocyte lipolysis [96]. Production of norepinephrine is mediated by tyrosine hydroxylase, the rate-limiting enzyme of catecholamine biosynthesis that catalyzes the conversion of L-tyrosine to L-DOPA. L-DOPA can be sequentially converted to dopamine, norepinephrine, and epinephrine depending on which enzymes are present in the cell. In addition to sympathetic neurons, several types of immune cells can express tyrosine hydroxylase and make catecholamines [97–99]. Norepinephrine acts on receptors of the α 1-, α 2-, and β -adrenergic families, each with three subtypes. Lymphoid organs including the thymus, spleen, lymph nodes, gut-associated lymphoid tissue (GALT), and bone marrow are innervated by sympathetic fibers [100], and sympathoadrenergic regulation of hematopoiesis is a well-established phenomenon [101]. In healthy mice, β 2-adrenergic activation enhances mobilization of hematopoietic progenitor cells [102]. In myeloproliferative neoplasms, sympathetic fibers, schwann cells, and nestin + progenitor cells are reduced; however, treatment with β 3-adrenergic agonists can delay progression of disease [103]. Loss of sympathetic innervation can also promote leukemic bone marrow infiltration in a model of acute myeloid leukemia [104]. Thus, the sympathetic efferent fibers have the potential to function within the hematopoietic marrow, presumably releasing neurotransmitters in the vicinity of MAT adipocytes.

Based on evidence in WAT [96], we would predict that norepinephrine can act directly on MAT adipocytes, stimulating breakdown of lipid and release of fatty acids by lipolysis. This hypothesis is supported by rodent models of increased sympathetic tone. For example, in mice, cold exposure for 21 days leads to loss of rMAT adipocytes within the red bone marrow due to decreases in cell size and number [4]. Stimulation of sympathetic outflow with leptin, injected directly into the brain, also results in rapid loss of MAT within the red

marrow [105]. Conversely, the *ob/ob* mouse has low sympathetic tone and high MAT volume [106, 107]. Many genetic rodent models have been used to model the action of norepinephrine on adrenergic receptors (reviewed in [107]). Unfortunately, the MAT phenotypes of these mice have not yet been reported; this includes several adrenergic-receptor mutants [95, 108–112], dopamine β -hydroxylase knock-out [113], and mice with downstream changes in intracellular mediators such as G_s [114], PDE4 [115], or GRK2 [116]. Future work is needed to define the expression of adrenergic receptors on MAT adipocytes and establish the regulation of rMAT and cMAT by norepinephrine.

Sympathetic drive to WAT, as measured by norepinephrine turnover, is not uniform [96]. This may also be true in the skeleton. In WAT adipocytes, norepinephrine-induced lipolysis depends on the number, type, and affinity of adrenergic receptors on the cell membrane [96]. The β_1 -, β_2 -, and β_3 -adrenergic receptors promote lipolysis, while the α_2 -receptor is inhibitory [96]. Current evidence supports a hypothetical model, whereby release of norepinephrine into the local microenvironment stimulates mobilization of hematopoietic progenitors in addition to lipid breakdown and fatty acid release by MAT adipocytes. In this model, the sympathetic nervous system would help to maintain blood cell production while utilizing MAT as a local fuel source. Though this is an attractive hypothesis, there are likely many surrounding factors, such as the ECM, with the potential to modify these relationships. Clearly, more work is needed to understand the complex interplay between sympathetic efferent neurotransmission and coordination of the MAT microenvironment.

Sensory Innervation

Sensory afferent nerves can be divided into categories based on expression of neuropeptides and are classified as peptidergic or nonpeptidergic (Fig. 3a). The skeleton is highly innervated by peptidergic nerve fibers [92, 117]. Nonpeptidergic fibers, based on expression of isolectin B4, have not been detected [92]. Based on studies in developing rats, the appearance of peptidergic fibers generally occurs within the skeleton around the same time as the initiation of mineralization [87]. Peptidergic sensory fibers release neuropeptides including calcitonin gene-related peptide (CGRP) and substance P which are potent vasodilators [118, 119] and osteoanabolic [120, 121]. There are also reports that CGRP and substance P can stimulate adipocyte lipolysis [122, 123]. There is evidence that vasoactive intestinal peptide (VIP +) and neuropeptide Y positive (NPY +) fibers are also present in bone, though their distribution does not appear to be as extensive and their role remains unclear [120, 124].

Neuropeptides are derived from large precursor proteins, generated through alternative splicing and/or precursor processing. Packaged neuropeptides are stored in secretory granules and released from axon terminals into the local microenvironment. Nonsynaptic neurotransmitter release, as occurs within the bone marrow, is much slower than synaptic transmission (minutes vs seconds) and results in diffusion of neuropeptides through a greater volume of space [121]. Thus, though they are classified as ‘sensory afferent’ nerves, peptidergic fibers can also release signaling mediators that can modulate local cellular functions in an efferent manner.

CGRP is a member of the calcitonin family of peptides and has two isoforms, CGRP α and CGRP β , of which the α -isoform is released from sensory nerve terminals. CGRP-containing nerve fibers in the bone marrow are unmyelinated [117]. They enter the bone with nutrient vessels, extend away from the vasculature, and terminate near the osteochondral junction (Fig. 3b) [121]. They maintain their extension along (and in contact) with the trabecular bone, despite skeletal growth and remodeling, suggesting that CGRP-containing nerve fibers can adapt to alterations in their environment [117, 121]. CGRP is generally osteoanabolic, thought to promote bone formation by its positive actions on osteoblasts [121, 125] and inhibition of osteoclasts [121]. In vivo, in mice, CGRP α can stimulate fatty acid β -oxidation and mobilization of lipid from muscle, but not epididymal adipose tissue [126]. However, in vitro, CGRP α is a potent inducer of adipocyte lipolysis [126]. CGRP α knock-out mice are osteopenic; the MAT phenotype has not been reported [125]. Thus, the regulation of MAT by CGRP remains unclear.

Substance P is a member of the tachykinin family and has been isolated from all major vertebrate groups [85]. Substance P-containing fibers do not distribute as widely throughout the bone marrow, and terminate as free nerve endings shortly after entering the marrow space (Fig. 3b) [121]. The function of substance P likely evolved throughout vertebrate evolution, as there is only 64 % sequence identity between goldfish and mammals [127]. Even within the fish there are functional differences; it decreases blood flow to the gut in dogfish, but increases blood flow to gut in rainbow trout and lungfish [85]. There is in vitro evidence that substance P can support hematopoiesis [128] and both substance P and CGRP are known to have a variety of pro-inflammatory actions [129]. For example, elimination of capsaicin-sensitive peptidergic sensory fibers can selectively reduce intramedullary inflammation in adjuvant arthritic rats [129]. Interestingly, this also prevents MAT loss in this model [129], providing evidence of a relationship between sensory innervation and MAT regulation. Similarly, knock-out of receptors for substance P or CGRP reduces inflammation and allodynia in a mouse tibia fracture model [130]. In addition to regulating hematopoietic function, there is in vitro evidence that substance P can stimulate lipolysis [122]. However, its actions on MAT adipocytes remain entirely unknown.

Sensory and sympathetic nerves within the bone are tightly coupled. Ablation of sympathetic nerves by chemical treatment with guanethidine increases substance P and CGRP-positive sensory fibers in the bone by 23–54 % [131] and enhances CGRP and substance P expression in the dorsal root ganglion [119]. There is evidence in WAT that sensory neurons respond to leptin, provoking dose-dependent increases in the firing of sensory nerves from the fat pad, and eliciting increases in sympathetic nerve activity in the contralateral fat pad, potentially via a sensory-sympathetic reflex arc [132, 133]. Similar to the sympathetic neurons, peptidergic sensory fibers have the potential to regulate hematopoietic function through neuropeptides such as CGRP and substance P. Peptidergic sensory fibers may also contribute to lipolytic responses of adipocytes, providing another mechanism by which MAT and hematopoiesis could be coupled. Future work is needed to define the actions of sensory neuropeptides on MAT adipocytes and to study their relevance in an in vivo setting.

Conclusions

As demonstrated by the bony fishes—MAT evolved in two settings, both with and without adjacent hematopoiesis. MAT then became a persistent component of the local environment in all higher vertebrate species studied to date, implying that MAT has important, context-specific functions depending on its surrounding microenvironment. For example, MAT within the marrow niche may help to regulate hematopoietic function, whereas bone-lining adipocytes may influence bone formation and remodeling. Central to the balance between MAT, blood, and bone are two understudied components of the MAT microenvironment—the extracellular matrix and the nervous system. These structures are highly conserved throughout vertebrate evolution and there exists a significant basis, particularly in the context of WAT, for their role as essential modifiers of adipose tissue function. They have the ability to build upon and modulate local cellular functions and may explain coordinated changes in MAT, hematopoiesis, and bone. It is important to note that in some pathologic states MAT may become detrimental to bone homeostasis (e.g., cancer metastasis, obesity/diabetes, inflammatory arthritis), however, its evolutionary conservation across vertebrate species suggests that MAT likely has positive effects on skeletal health.

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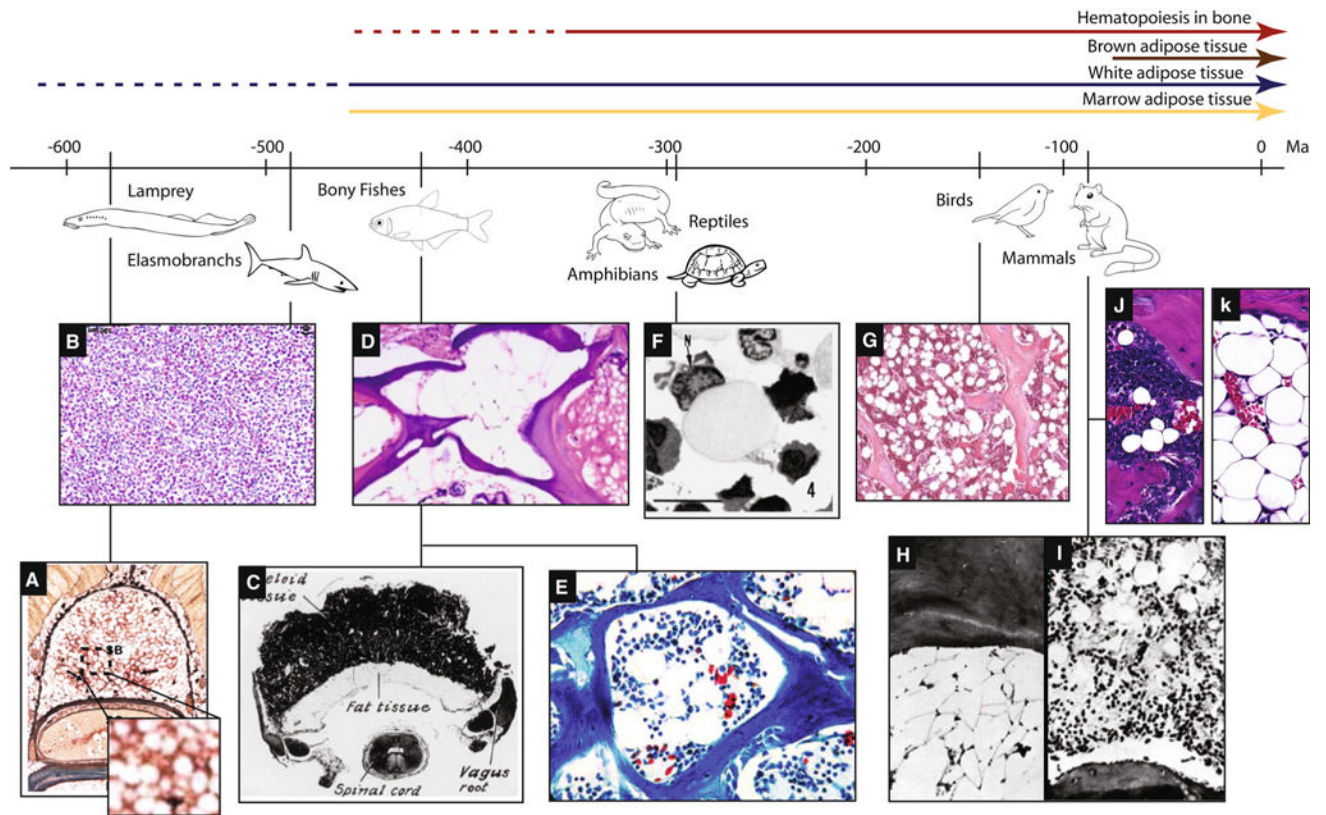


Fig. 1. Evolution of MAT in vertebrates relative to bone marrow and peripheral adipose tissue. The first evidence of WAT in vertebrates occurs in the sea lamprey, prior to the evolution of an ossified skeleton. The *Elasmobranchs*, a group of cartilaginous fishes, have a partially ossified skeleton but no bone marrow, MAT, or WAT. MAT first becomes apparent in the bony fishes, after the evolution of endochondral bone resorption, both in the presence and absence of hematopoietic marrow. By comparison, brown adipose tissue is only present in mammals. **a** Groups of hematopoietic cells in the adipose tissue of the supraneural body of a postmetamorphic sea lamprey [18]. **b** Normal epigonal organ composed of a relatively uniform sheet of mature and developing granulocytes without adipocytes [134]. **c** Cross-section through the myeloid organ of *Amia* bony fish with adjacent fat tissue [25]. **d** Cross-section of zebrafish bone showing fatty filling of the skeletal space (*middle*) and adjacent cartilage (*right*) [135]. **e** Cancellous bone of the lower jaw in the bony fish *Garra congoensis* with numerous marrow adipocytes (Image credit: Franck Genten) [26]. **f** Slimy salamander *Plethodon glutinosus*. Semi-thin plastic section of a fat cell in marrow. The densely stained nucleus (N) is located eccentrically in the cell, and the cytoplasm is filled with lightly stained lipid material [27]. **g** Bone marrow of a female Leghorn chicken showing trabeculae, hematopoietic marrow, and abundant adipocytes [36]. **h** Armadillo dermal plate marrow in December. Few hematopoietic cells are present but fat cells are abundant [41]. **i** Armadillo dermal plate marrow in October. Active hematopoietic cells interspersed with adipocytes in a typical dermal plate marrow [41]. **j** *Red*, hematopoietic bone marrow of a C3H/HeJ mouse femur containing rMAT adipocytes (Image by E.L.S.). **k** *Yellow*, fatty bone marrow of a

C3H/HeJ mouse caudal vertebrae containing cMAT adipocytes (Image by E.L.S.). Adapted from [11] (Color figure online)

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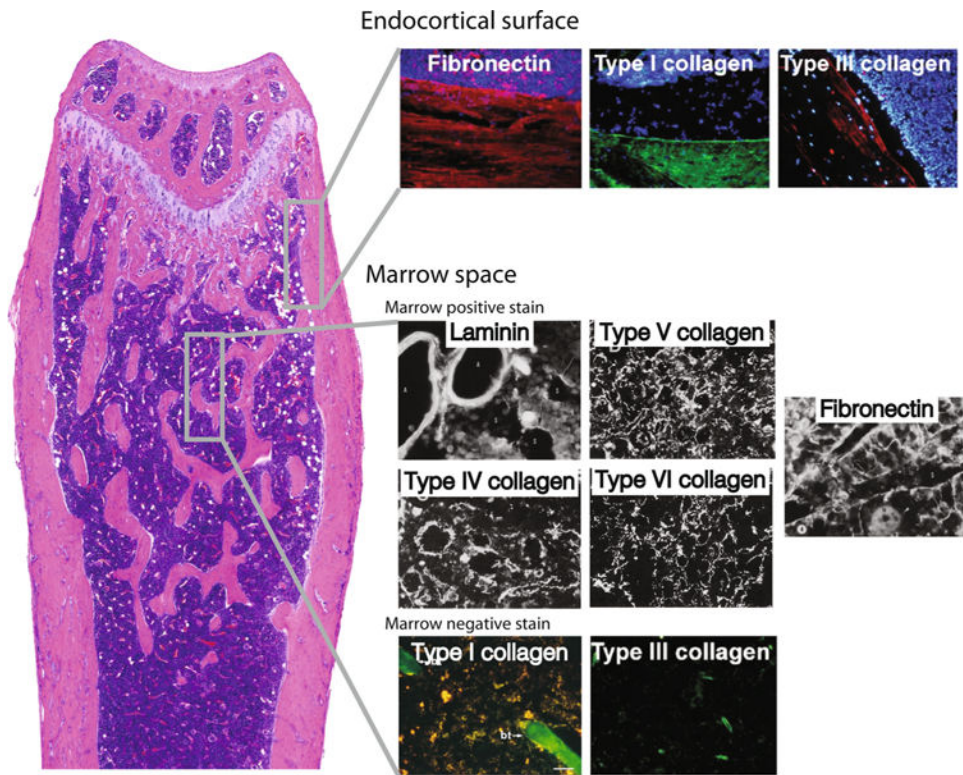


Fig. 2. Distribution of extracellular matrix (ECM) structures within the bone. ECM composition varies across the bone; marrow adipocytes likely reside in distinct ECM microenvironments depending on their location in the marrow space. This figure is a compilation of immunofluorescence images from multiple studies, providing a basic overview of skeletal ECM organization. The endocortical surface is enriched with fibronectin and large fiber collagens (collagen-I, collagen-III). Shown is fibronectin in *red*, collagen-I in *green*, and collagen-III in *red*. *Blue* staining is nuclear staining of marrow cells. Collagen-I and collagen-III staining in the marrow space images (*bottom*) is limited to trabecular bone (collagen-I, *green*) or vessels (collagen-III, *green*). The remaining marrow space pictures are *black-and-white* immunofluorescence images. Laminin and collagen-IV (*white*) are basement membrane proteins associated with the marrow sinuses and arteries. Collagen-IV is also shown to localize around marrow adipocytes. The space between the vessels and bone is enriched with collagen-V and collagen-VI fibers, as well as mesh-like structures of fibronectin. Based on these images, collagen-VI may be concentrated around marrow adipocytes, similar to collagen-IV. Images reproduced with permission from the following sources: [56–58, 60] (Color figure online)

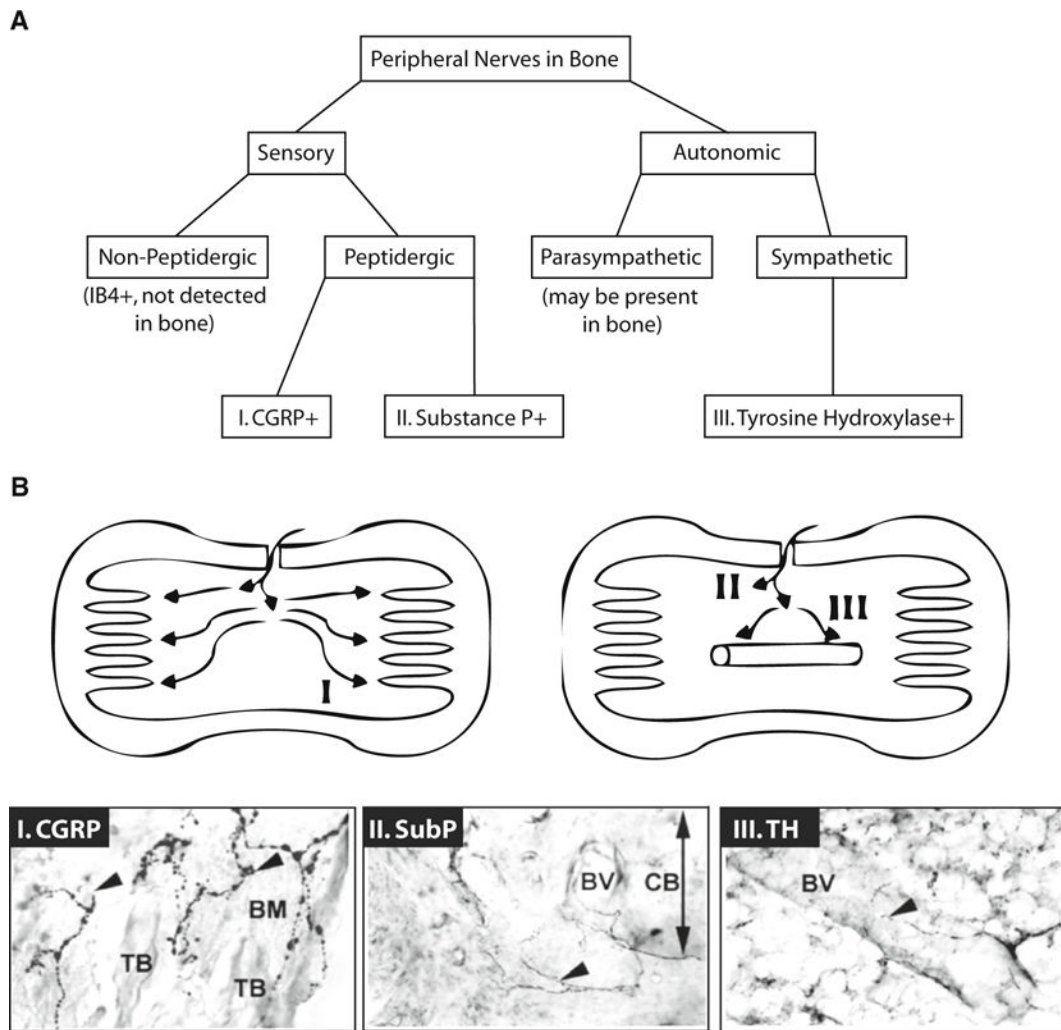


Fig. 3. Peripheral nerve distribution in bone. **a** Sensory and autonomic nerves enter the skeleton with nutrient vessels and are widely distributed around the vasculature, throughout the medullary cavity, and within the ossified matrix. Nonpeptidergic sensory nerve fibers, as evidenced by isolectin B4 (IB4) staining, have not been detected in bone [92]. However, unmyelinated peptidergic sensory fibers containing CGRP, substance P, or both neuropeptides are common [121]. There is limited evidence that the bone may be innervated by parasympathetic fibers [91]. By contrast, tyrosine hydroxylase expressing sympathetic efferent neurons are readily demonstrated [92, 121]. **b** Within the bone, three main patterns of neuron distribution have been identified. Pattern I, which corresponds to CGRP + sensory fibers, shows axons extending apart from the vessels, subsequently terminating in the vicinity of the osteochondral junction. Pattern II represents the substance P + sensory fibers with terminate shortly after entering through the cortical bone. Pattern III shows the tyrosine hydroxylase-positive (TH +) sympathetic axons which spiral around blood vessels with some extension into the marrow space. Corresponding immunohistochemical stains are also

presented. TB: trabecular bone, BM: bone marrow, BV: blood vessel, CB: cortical bone.
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