Chapter 8
Semaphorins in Bone Homeostasis

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Abstract Intercellular communication between cells within bone is essential for the regulation of bone homeostasis. Growing evidence reveals that semaphorins have crucial roles in this process, including osteoclastic bone resorption and osteoblastic bone formation. Semaphorin 4D (Sema4D), derived from osteoclasts, has a potent inhibitory effect on osteoblast differentiation without hampering osteoclastic bone resorption. Sema3A, which is highly expressed in osteoblast lineage cells, maintains bone homeostasis by simultaneously inhibiting osteoclast differentiation and promoting osteoblast differentiation. Sema3A also has a role in the regulation of innervation, indicating the importance of future studies on the interactions among bone cells and neurons. Other semaphorins and their receptors have also been implicated in bone metabolism. These studies provide a scientific basis for future therapeutic approaches to bone diseases.

Keywords Osteoclast • Osteoblast • Bone remodeling

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8.1 Introduction

Bone has multiple functions, including locomotive activity, the storage of minerals, and the harboring of hematopoietic stem cells. Because of the nature of bone as a rigid supporting tissue, bone can appear to be unchangeable and inert. However, the bony skeleton is in fact an organ that is dynamically changing over the human lifespan, characterized predominantly by skeletal growth during childhood and subsequently continuous turnover, termed bone remodeling, throughout life. Bone remodeling is a finely balanced activity that is carried out by specialized groups of cells, including osteoclasts, which are multinucleated cells that resorb bone, and osteoblasts, which refill the resorption cavities created by osteoclasts (Zaidi 2007; Henriksen et al. 2011). An imbalance between bone resorption and formation results in metabolic bone disorders such as osteoporosis, a disease of low bone mass with increased susceptibility to bone fractures (Seeman and Delmas 2006; Takayanagi 2007). Antiresorptive agents, including bisphosphonates, are currently most commonly used for the treatment of osteoporosis (Rachner et al. 2011). However, treatment with these antiresorptive agents has certain limitations, for example, a low turnover state in which bone resorption decreases along with a simultaneous decrease in bone formation, with the result that efficacy is compromised (Lewiecki 2011; Kawai et al. 2011). Each antiresorptive agent approach is based on a different molecular mechanism, but a number of reports suggest that the level of formation is coupled to that of resorption through "coupling factors" (Sims and Martin 2014). Thus, understanding the mechanisms underlying bone remodeling and coupling is important for a better understanding of both the skeletal system and the development of novel osteoprotective agents.

Semaphorins, initially identified as an evolutionally conserved guidance for developing axons, are a family of secreted and cell membrane-anchored proteins (Tran et al. 2007; Pasterkamp 2012). The predominant receptors for semaphorins are the two groups of proteins known as the plexins and neuropilins, although some semaphorins do independently function through other receptors. Recently, it has been demonstrated that semaphorins play an important role in diverse biological processes outside the nervous system, including cardiovascular development, immune response, and tumor progression, as had been suggested by their diverse expression profiles in a wide variety of tissues (Gu and Giraudo 2013; Kumanogoh and Kikutani 2013). It has also recently been suggested that the semaphorins are involved in bone homeostasis through the regulation of cell–cell communication between osteoclasts and osteoblasts during bone remodeling (Negishi-Koga and Takayanagi 2012; Kang and Kumanogoh 2013). This review summarizes the current knowledge of semaphorin-mediated regulation of bone metabolism based on recent studies.

8.2 Bone Cells and Bone Remodeling

Osteoclasts are derived from monocyte/macrophage lineage precursor cells, and their differentiation is under the control of mesenchymal cells, mainly osteoblast lineage cells, that express receptor activator of nuclear factor-κB (NF-κB) ligand (RANKL) (Fig. 8.1) (Takayanagi 2007; Henriksen et al. 2011). Osteoprotegerin (Opg), a decoy receptor for RANKL, is also expressed by osteoblast lineage cells to counterbalance the action of RANKL (Nakashima et al. 2012). Active osteoclasts enclose resorption pits on the bone surface (Teitelbaum 2011). The ring-shaped structure referred to as the sealing zone is essential for the development of resorption pits, into which osteoclasts secrete hydrochloric acid for the purpose of creating an acidic environment to decalcify the bone matrix (Sobacchi et al. 2013). After the bone mineral content has been dissolved, metalloproteinases and cathepsins, which are secreted by osteoclasts, remove the collagenous bone matrix to complete the process of resorption (Henriksen et al. 2011). Osteoclasts undergo apoptosis after bone resorption is complete (Manolagas 2000; Tanaka et al. 2006).

Following resorption, pre-osteoblasts of the mesenchymal lineage move into the resorbed area and start to proliferate and differentiate into mature osteoblasts (Fig. 8.1) (Dirckx et al. 2013; Crane and Cao 2014). These cells produce and deposit organic matrix, called osteoid, a substance predominantly composed of collagen, to fill in the resorbed cavities. Osteoid forms a scaffold in which minerals, including calcium and phosphate, begin to crystallize. During this process, a large amount of various growth factors, such as transforming growth factor (TGF)-β and insulin-like growth factor 1 (IGF-1), are synthesized and stored in the bone matrix (Sims and Martin 2014; Crane and Cao 2014). When osteoclasts resorb the bone matrix, growth factors embedded in the bone are released and activated in the resorption pits and are thus able to induce the recruitment and differentiation of osteoblast precursor cells (Fig. 8.1). After the resorbed areas are replenished with newly synthesized bone, some of the osteoblasts undergo apoptosis, whereas others become quiescent as lining cells, which cover the surface of bone (Manolagas 2000). Some of the active osteoblasts are trapped within the matrix they secreted, thereby becoming osteocytes (Fig. 8.1). Osteocytes communicate with themselves and other cells in the bone marrow, including osteoclasts and osteoblasts, via long cytoplasmic extensions (Dallas et al. 2013). The extensive connectivity of the lacuno-canalicular system allows osteocytes to transmit signals to and from tissues throughout the body. Osteocytes play an important role in sensing local changes in mechanical strain or microdamage, leading to the recruitment of osteoclast precursor cells to the bone surface (Dallas et al. 2013). Recent studies have demonstrated that osteocytes are an essential source of RANKL to control osteoclast differentiation in adult bone remodeling, whereas osteoblasts or hypertrophic chondrocytes are an important source during skeletal development (O'Brien et al. 2013). It has also been shown that osteocytes also have a key role in mineral homeostasis, functioning as the endocrine organ secreting fibroblast growth factor (FGF)-23 (Fukumoto and Martin 2009; DiGirolamo et al. 2012).

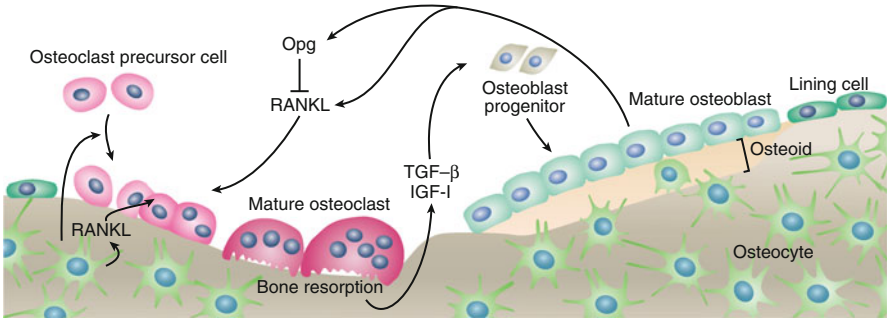

Fig. 8.1 Bone remodeling is a process that ensures the turnover of old bone and the repair of damaged bone, while keeping bone mass intact and allowing for maintenance of mechanical integrity by the requirements of calcium and phosphate metabolism in adults. New bone formation occurs at the site of resorbed bone, suggesting a tight coupling between osteoclasts and osteoblasts. The remodeling cycle can be triggered from an accumulation of microdamage or changes in mechanical loading sensed by osteocytes under the influence of hormones, cytokines, and other factors. Osteocytes communicate with surface or marrow cells to promote the recruitment of osteoclast precursor cells and the differentiation into mature osteoclasts, which is induced by RANKL derived from osteoblast lineage cells, especially osteocytes, and bone resorption starts. Opg, a soluble decoy receptor for RANKL, is produced by osteoblast lineage cells to inhibit the action of RANKL, thereby suppressing any excess active osteoclasts. In response to osteoclastic bone resorption, primary coupling factors, including IGF-1 and TGF-β, are released and stimulated to initiate the migration of osteoblast progenitors to the resorbed sites, coupling new bone formation to bone resorption spatiotemporally. Active osteoblasts replenish the resorbed area with new bone. Some of the osteoblasts become embedded within the matrix and differentiate into osteocytes

Recently, it has been made clear that the bone remodeling process is modulated by numerous humoral and coupling factors in the endocrine, immune, and nervous systems (Sims and Martin 2014). These factors are expressed or secreted from osteoclasts or osteoblasts in the bone matrix. The communication between osteoclasts and osteoblasts is regulated by a wide variety of local and systemic factors to maintain bone homeostasis adequately. As bone remodeling is a multicellular process, a better understanding of the cellular crosstalk that takes place between and among the cells in bone is important for ultimately modulating this process. Recently, there have been significant advances in our understanding of the role of semaphorins in the regulation of bone homeostasis (Negishi-Koga and Takayanagi 2012; Kang and Kumanogoh 2013), which is discussed in this review.

8.3 The Regulation of Osteoclastogenesis by Plexin-A1

Plexin-A1, a receptor for class 5 and 6 semaphorins,is also known to form a receptor complex with neuropilins for class 3 semaphorins (Pasterkamp 2012). It has been suggested that Plexin-A1 has important functions in the immune system, as shown by its high expression in dendritic cells (DCs), in addition to its role in the nervous system. *Plxna1*$^{-/-}$ mice have abnormalities in antigen-specific T-cell responses and

DC trafficking into draining lymph nodes (Takegahara et al. 2006; Takamatsu et al. 2010). Interestingly, these mice also display a high bone mass phenotype as a result of impaired osteoclast differentiation (Takegahara et al. 2006). It has been reported that the Plexin-A1 expressed on osteoclast precursor cells binds a complex composed of the triggering receptor expressed on myeloid cells 2 (TREM2) and DNAX activation protein 12 (DAP12), in response to Sema6D ligation (Fig. 8.2a).

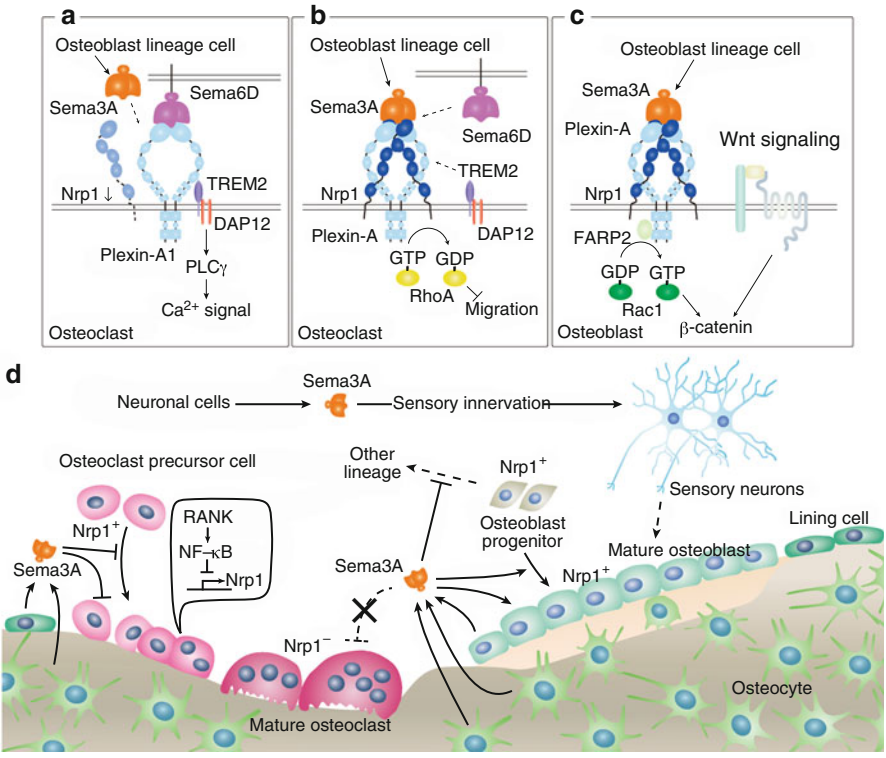

Fig. 8.2 Sema3A–Nrp1–Plexin-A1 regulates bone metabolism. (**a**) After RANKL stimulation, Nrp1 expression is blocked by NF-κB signaling, with the result that the Sema3A-induced inhibition is no longer effective and allowing Plexin-A1 to associate with Sema6D. The binding of Sema6D to Plexin-A1 facilitates the formation of a complex with TREM2 and DAP12, which activates osteoclast differentiation through the ITAM-mediated calcium signaling pathway. (**b**) In the absence of RANKL, Sema3A inhibits RhoA activation via the Nrp1–Plexin-A receptor complex and therefore suppresses the migration of osteoclast precursor cells. Furthermore, the Sema3A–Nrp1–Plexin-A1 complex inhibits osteoclast differentiation by sequestering Plexin-A1 from TREM2 and Sema6D. (**c**) During bone formation, Sema3A activates Rac1 through RacGEF FARP2 downstream of the Nrp1–Plexin-A1 complex. Rac1 activation enhances the nuclear localization of the β-catenin induced by canonical Wnt signaling, which is essential for osteoblast differentiation. (**d**) Osteoblast lineage cells produce and secrete Sema3A to sequester osteoclast precursor cells away from osteoblastic cells and inhibit the differentiation into osteoclasts during bone formation. Concomitantly, Sema3A promotes osteoblast differentiation and bone formation in an autocrine/paracrine manner, thereby shifting the balance of bone turnover from resorption to formation. The Sema3A produced in neurons is implicated in bone metabolism through the regulation of sensory innervation, not a direct effect on osteoblasts

The immunoglobulin-like receptor TREM2 and DAP12 activate the immunoreceptor tyrosine-based activation motif (ITAM) signaling pathway, which is critical for the subsequent activation of Ca^{2+} signaling and osteoclast differentiation (Fig. 8.2a) (Koga et al. 2004). In addition, FERM, RhoGEF, and pleckstrin domain protein 2 (FARP2), a Rac-specific guanyl nucleotide exchange factor (GEF) (Toyofuku et al. 2005), were shown to be involved in mature osteoclasts in vitro in actin rearrangement and podosome formation, in response to the class 6 semaphorins, including Sema6B and Sema6D (Takegahara et al. 2010). As the growing evidence suggested that immune and nervous systems share molecules that are implicated in the maintenance of bone metabolism, the discovery of Plexin-A1 as a regulator of osteoclast differentiation highlights the start of semaphorin studies in bone biology.

8.4 Sema4D Regulates Osteoblastic Bone Formation

During bone resorption, various coupling factors are secreted that recruit osteoblast precursor cells to the site of future bone formation. However, osteoblast recruitment and differentiation need to be suppressed to complete the resorption of damaged or aged bone adequately. Axon-guidance molecules, such as semaphorins and ephrins, are involved in cell–cell communication outside the nervous system. From the transcriptomic analysis of genes encoding axon-guidance molecules during osteoclast differentiation, Sema4D was found to be highly and selectively induced after RANKL stimulation (Negishi-Koga et al. 2011). Sema4D, a membrane protein, which is also functional in the soluble form after proteolytic cleavage, regulates the activation and survival of B cells and DCs and inhibits monocyte migration, mainly through CD72 (Kumanogoh and Kikutani 2013). Recent studies have demonstrated that the binding of Sema4D to class B plexins induces tumor angiogenesis, progression, and metastasis (Gu and Giraudo 2013). *Sema4d*$^{-/-}$ mice have increased bone mass caused by the activation of osteoblast differentiation and bone formation without any change in bone resorption. Among the bone cells examined, Sema4D is exclusively expressed in premature and mature osteoclasts, whereas Plexin-B1, a receptor for class 4 semaphorins, is expressed in osteoblasts, indicating that the Sema4D expressed on osteoclasts directly acts on the Plexin-B1 expressed on osteoblasts (Fig. 8.3a). Indeed, *Plxnb1*$^{-/-}$ mice recapitulate the high bone phenotype observed in *Sema4d*$^{-/-}$ mice (Negishi-Koga et al. 2011).

The binding of Sema4D to Plexin-B1 leads to the recruitment of PDZ-RhoGEF and leukemia-associated Rho guanine nucleotide exchange factor (LARG), both of which are GEF for RhoA GTPases (Fig. 8.3b). Activated RhoA and its effector, Rho-associated coiled-coil-containing protein kinase (ROCK), negatively regulate osteoblast differentiation through the inhibition of the phosphorylation of insulin receptor substrate 1 (IRS-1), which is downstream of the IGF-1 receptor (Fig. 8.3b).

Semaphorins are known to be involved in the regulation of cellular motility via the modulation of Rho family GTPases (Tran et al. 2007). Indeed, Sema4D-induced

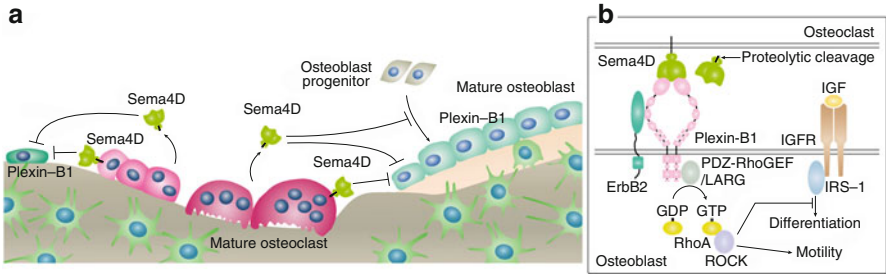

Fig. 8.3 Inhibition of osteoblastic bone formation by osteoclast-derived Sema4D. (**a**) Osteoclastic expression of Sema4D is amplified in response to RANKL stimulation, which is supplied by osteoblastic cells. Sema4D inhibits osteoblast differentiation in the proximity of bone-resorbing osteoclasts and repels osteoblast progenitors by increasing their motility, thus acting as a negative feedback loop during bone resorption. (**b**) Binding of Sema4D to the Plexin-B1–ErbB2 receptor complex activates RhoA via PDZ-RhoGEF and LARG. RhoA activates ROCK so as to inhibit IRS-1 downstream of IGF-1 signaling, which is essential for osteoblast differentiation. Activated RhoA-ROCK also promotes the motility of osteoblasts, thereby repulsing bone-forming osteoblasts

RhoA activation promotes osteoblast motility. In normal bone, it is frequently observed that bone-forming osteoblasts are sequestered from bone-resorbing osteoclasts. In *Sema4d*$^{-/-}$ and *Plxnb1*$^{-/-}$ mice, as well as RhoA DN^{OB} (an osteoblast-specific dominant-negative form of RhoA transgenic) mice, osteoblasts were observed to be in close proximity to osteoclasts. These findings suggest that osteoclast-derived Sema4D suppresses RhoA activation in osteoblasts, thereby inhibiting the differentiation of osteoblasts and keeping osteoblasts away from osteoclasts to enable efficient osteoclastic bone resorption (Fig. 8.3a, b).

8.5 Sema3A Synchronously Promotes Osteoblastogenesis and Inhibits Osteoclastogenesis

During osteoblastic bone formation, the recruitment and differentiation of osteoclasts must be tightly restricted. It is likely that osteoblasts contribute to the regulation of osteoclasts by producing pro- and anti-osteoclastogenic factors. However, an inhibitory factor of osteoclast differentiation derived from osteoblasts has not been identified except for Opg (Takayanagi 2007; Nakashima et al. 2012). Opg-deficient osteoblastic cell-conditioned medium inhibited RANKL-induced osteoclast differentiation, suggesting the presence of other osteoblast-secreted protein(s) involved in the negative regulation of osteoclast differentiation. Based on the anti-osteoclastic activity of Opg-deficient osteoblastic cell-conditioned medium, an axon-guidance molecule, Sema3A, was determined to be a novel negative regulator of osteoclast differentiation (Hayashi et al. 2012). Among the various cells tested, Sema3A mRNA and protein were predominantly expressed in osteoblast lineage cells but were not detected in osteoclasts. The receptor complex for Sema3A is

composed of the ligand-binding subunit neuropilin-1 (Nrp1) and one of the class A plexins. These proteins are expressed on osteoblast lineage and osteoclast precursor cells, respectively.

As already discussed, Sema6D–Plexin-A1 promotes osteoclast differentiation through the activation of ITAM signaling through the formation of the Plexin-A1–TREM2–DAP12 complex (Fig. 8.2a). In the absence of RANKL, the binding of Sema3A to the Nrp1–Plexin-A1 complex on osteoclast precursor cells blocked the interaction between Plexin-A1 and the TREM2–DAP12 complex so as to suppress ITAM signaling as well as RANKL and Sema6D-induced osteoclast differentiation (Fig. 8.2b). On the other hand, the expression of Nrp1 was downregulated by stimulation with RANKL (Fig. 8.2a). Therefore, RANKL-induced downregulation of Nrp1 expression is important for proper osteoclast differentiation to overcome the inhibitory effect of Sema3A. This RANKL-induced inhibition of Nrp1 expression is dependent on the NF-κB transcription factor, but not NFATc1 or c-Fos. The NF-κB-mediated inhibition of Nrp1 expression was also observed in tumor-associated macrophages via the Hif2-mediated activation of NF-κB in a hypoxic condition (Casazza et al. 2013).

It is well established that semaphorin–plexin signaling regulates cell migration via the modulation of the Rho family of small GTPases (Tran et al. 2007). The migration of osteoclast precursor cells toward M-CSF was suppressed by the presence of Sema3A via the abrogation of RhoA activation in response to M-CSF, but not Rac activation (Fig. 8.2b). Taken together, in the absence of RANKL, Sema3A binding to Nrp1–Plexin-A receptor inhibits RhoA activation and therefore suppresses migration of osteoclast precursor cells. In addition, the Sema3A–Nrp1 axis inhibits osteoclast differentiation by sequestering Plexin-A1 from TREM2. After the RANKL stimulation by which Nrp1 is rapidly downregulated, Plexin-A1 forms a complex with TREM2 and DAP12 that facilitates osteoclast differentiation (Fig. 8.2a, b).

Sema3a$^{-/-}$ mice, as well as *Nrp1*$^{\text{Sema}-}$ mice, in which the semaphorin-binding site of Nrp1 is genetically disrupted, are severely osteopenic because of both enhanced osteoclastic bone resorption and reduced osteoblastic bone formation. In both *Sema3a*$^{-/-}$ and *Nrp1*$^{\text{Sema}-}$ mice, a massive increase in adipocytes in the bone marrow was observed. These results suggest that Sema3A is also important for the differentiation of mesenchymal progenitors into osteoblasts in bone marrow and inhibits the commitment of mesenchymal cells to adipocytes.

What are the mechanisms by which Sema3A promotes bone formation and inhibits adipocyte differentiation? Canonical Wnt/β-catenin signaling has been considered to have osteogenic and anti-adipogenic effects and to control skeletal development and integrity (Monroe et al. 2012; Baron and Kneissel 2013). Sema3A induces Rac1 activation, which was reported to control nuclear localization of β-catenin during canonical Wnt signaling (Wu et al. 2008), through FARP2 (Fig. 8.2c). The activation of Rac, the nuclear localization of β-catenin and the mRNA expression of transcriptional targets of β-catenin in response to the canonical Wnt ligand were considerably reduced in *Sema3a*$^{-/-}$ osteoblasts. Taken together, Sema3A not only inhibits RANKL-induced osteoclast differentiation, but also activates osteoblast differentiation (Fig. 8.2c, d).

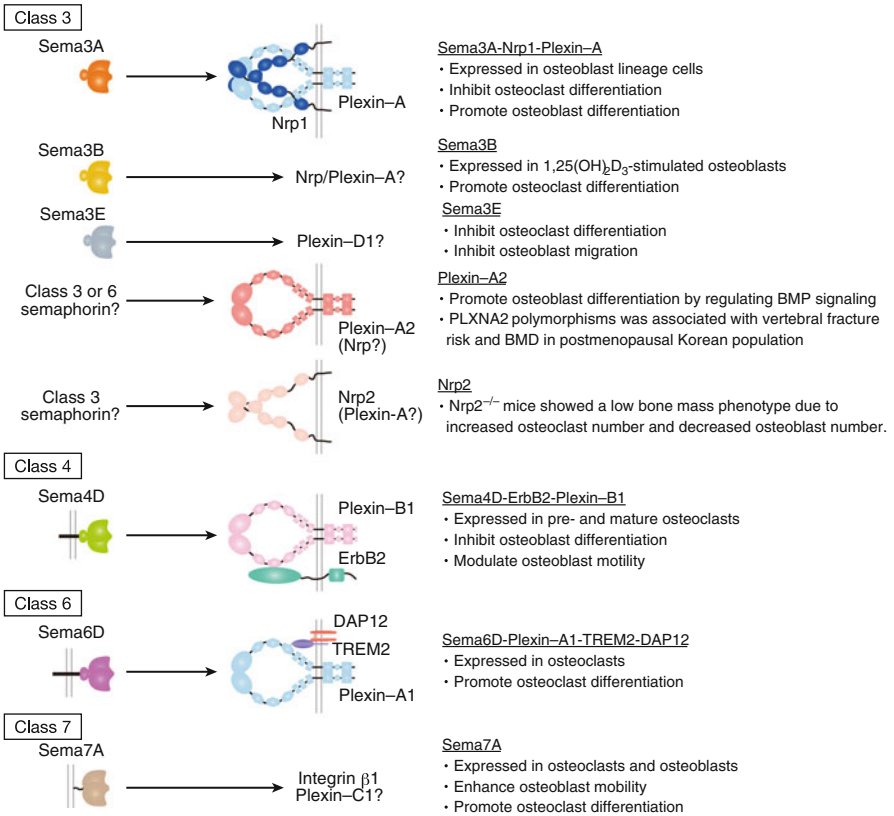

Fig. 8.4 Semaphorins in bone remodeling. A summary of the function of the semaphorin family and its receptors in bone remodeling. Various semaphorins and their receptors are involved in the regulation of osteoclasts and osteoblasts

The importance of Sema3A signaling in bone is supported by the finding that polymorphisms in *PLXNA2* (encoding Plexin-A2, which is a member of the type A plexins and a component of the receptor complex for Sema3A), are associated with low bone mineral density and an increased risk of bone fracture in postmenopausal women of a Korean cohort (Hwang et al. 2006). Furthermore, another group showed that Plexin-A2 expression in osteoblastic cells was induced by an osteogenic medium, whereas BMP-2 treatment and Plexin-A2 increased osteoblastic differentiation through the regulation of Smad and Akt signaling downstream of BMP2 (Fig. 8.4) (Oh et al. 2012).

Hofmann et al. recently reported that the biallelic mutations in the *SEMA3A* gene (a combination of a nonsense mutation in exon 1 and a splice site mutation in exon 9) resulted in a novel disorder characterized by skeletal abnormalities, including postnatal short stature, barrel thorax, funnel chest, and flattened vertebrae of the upper thoracic region, along with multiple minor congenital anomalies (Hofmann

et al. 2013). It is interesting that some of the phenotypes in *Sema3a*$^{-/-}$ mice are recapitulated in the patient, but intellectual development and heart abnormalities were not prominent, possibly because the approximately 20 % remaining *SEMA3A* expression is sufficient to maintain some organs and physiological processes.

8.6 Sema3A-Mediated Regulation of Sensory Innervation of Bone

After the first report of Sema3A function in bone homeostasis, another source of Sema3A in the regulation of bone mass accrual was reported (Fukuda et al. 2013). They confirmed that osteoblastic differentiation from calvarial cells derived from *Sema3a*$^{-/-}$ mice was impaired in a cell-autonomous manner and that Sema3A inhibits osteoclast differentiation in vitro, in agreement with the earlier study.

However, osteoblast lineage cell-specific *Sema3a* conditional knockout mice generated by crossing *Col1a1-Cre*$^{+}$ mice with *Sema3a*$^{flox/flox}$ mice had normal bone mass, bone formation rate, and osteoblast/osteoclast numbers in the lumbar vertebrae, although the *Sema3a* level in bone was significantly decreased and osteoblastic differentiation from calvarial cells derived from *Col1a1-Cre*$^{+}$ *Sema3a*$^{flox/flox}$ mice was impaired in vitro (Fukuda et al. 2013). The *Sp7* (encoding Osterix)-*Cre*$^{+}$ *Sema3a*$^{flox/flox}$ mice were shown to have a normal bone volume, at least in the lumbar vertebra. These results suggest that the mechanism by which Sema3A controls bone homeostasis is more complex than originally hypothesized, and Sema3A expressed in cells other than osteoblasts might be involved.

The neuron-specific *Sema3a* knockout mice generated by crossing *Nes* (encoding Nestin)-*Cre*$^{+}$ *Sema3a*$^{flox/flox}$ and *Syn1* (encoding synapsin I)-*Cre*$^{+}$ *Sema3a*$^{flox/flox}$ mice developed a 25 % reduction in bone mass phenotype because of decreased osteoblastic bone formation without any change in osteoblast number. Sensory innervation into bone, but not sympathetic innervation, was significantly reduced in mice lacking *Sema3a* in the neurons. In comparison, sensory innervation into bone in mice lacking *Sema3a* in osteoblasts was normal. It was proposed that sensory innervation was involved in the regulation of bone mass accrual in mice during development (Fig. 8.2d).

There findings give rise to interesting questions: How do sensory nerves promote bone formation? Why does the loss of Sema3A, a chemorepulsive factor, selectively affect sensory innervation into bone? Why is sympathetic innervation into bone unaffected in *Sema3a*$^{-/-}$ mice, although sympathetic nerves normally express Sema3A receptors and sympathetic innervation into other organs, become impaired in *Sema3a*$^{-/-}$ mice? (Elefteriou 2013). In addition, recent reports showed that Sema3A is involved in the development of gonadotropin-releasing hormone neurons in the hypothalamus (Cariboni et al. 2011; Giacobini et al. 2014). Therefore, the effect on the production of pituitary hormones and sex steroids should be considered in both global and neuron-specific Sema3A knockout mice.

8.7 Other Semaphorins and Their Receptors in Bone Cell Regulation

Several other semaphorins and their receptors are involved in the regulation of bone homeostasis (Fig. 8.4). It is reported that the expression of Sema3B, a member of the class 3 semaphorins, was induced in osteoblastic cells by treatment with $1,25(OH)_2D_3$, a key regulator of bone and mineral homeostasis (Sutton et al. 2008). Osteoclast differentiation supported by osteoblastic cells derived from *Sema3b* transgenic mice under the control of *Col1a1* promoter was significantly increased compared with a culture with osteoblastic cells derived from wild-type (WT) mice. Therefore, osteoblast-derived Sema3B may promote osteoclast differentiation. These transgenic mice display an osteopenic phenotype as a result of increased osteoclast differentiation along with normal osteoblastic parameters. However, a detailed analysis of $Sema3b^{-/-}$ mice is required to further determine the role of Sema3B in vivo.

The mRNA and protein expression of Nrp2, which is another family member of the neuropilins and a receptor for the class 3 semaphorins except for Sema3A, Sema3D, and Sema3E, were significantly induced during RANKL-induced osteoclast differentiation under the control of NF-κB (Verlinden et al. 2013). Nrp2 expression in osteoblasts and osteoclasts, but not in growth plate chondrocytes or osteocytes, was confirmed by immunofluorescence of the murine long bones. $Nrp2^{-/-}$ mice exhibited a mild low bone mass phenotype accompanied by an increased osteoclast number and a decreased osteoblast number, without any significant changes in the parameters of the function of osteoclasts and osteoblasts. Sema3B, one of the ligands of Nrp2, is a positive regulator of osteoclast differentiation, as previously stated, and the addition of recombinant Sema3C or Sema3F had no effect on osteoclast differentiation. Therefore, other class 3 semaphorins or the vascular endothelial growth factor (VEGF) family of ligands may have an influence on osteoclast and osteoblast differentiation via Nrp2.

Sema3E is expressed in osteoblasts and its receptor, Plexin-D1, is expressed in both osteoblasts and osteoclasts (Hughes et al. 2012). RANKL-induced osteoclast differentiation was shown to be decreased by treatment with the recombinant Sema3E protein. It was also shown that osteoblast migration, but not differentiation, was inhibited by Sema3E in a wound-healing assay. Thus, Sema3E derived from osteoblasts may have a role in the regulation of bone homeostasis through an effect exerted on osteoblast migration in an autocrine/paracrine manner and the inhibition of osteoclast differentiation in a paracrine manner, although the in vivo importance of Sema3E should be thoroughly investigated in the future. It was also reported that the class 3 semaphorins, except for Sema3F, are expressed in osteoblasts and that their expression is differentially regulated by differentiation and diverse signaling pathways, including $1,25(OH)_2D_3$ and canonical Wnt/β-catenin signaling.

Sema7A, a glycosyl phosphatidyl inositol-anchored semaphorin that is a ligand for Plexin-C1 and integrins, is expressed in osteoblasts and osteoclasts (Delorme et al. 2005). In contrast to Sema3E, Sema7A promotes osteoblast migration through integrin β1 and osteoclast differentiation.

Collectively, these reports suggest that the semaphorins are pivotal in the regulation of bone homeostasis through several distinct mechanisms (Fig. 8.4).

8.8 Potential for Therapeutic Targeting of Semaphorin

Bisphosphonates are currently the drugs most widely used for the treatment of osteoporosis. However, the prolonged use of antiresorptive agents often leads to the suppression of bone formation coupled with the target of inhibited bone resorption, resulting in the accumulation of low-quality bone (Lewiecki 2011; Kawai et al. 2011). Consequently, there is a strong need for anabolic therapies that positively increase bone mass by stimulating new bone formation instead of antiresorptive treatments, but such agents have been essentially unavailable except for parathyroid hormone or an anti-sclerotin antibody. Therefore, it would be desirable to develop new therapeutic approaches that effectively increase bone mass independently of a coupling mechanism.

To test whether the inhibition of Sema4D signaling would be a useful approach to the treatment of osteoporosis, the effect of anti-Sema4D antibody administration on bone loss in ovariectomized mice, a model of postmenopausal osteoporosis, was examined (Negishi-Koga et al. 2011). The administration of anti-Sema4D antibody prevented bone loss after ovariectomy through the activation of bone formation without affecting osteoclastic bone resorption. Importantly, the anti-Sema4D antibody stimulated bone formation and increased bone mass, even after the osteoporotic condition had developed in ovariectomized mice. Thus, the inhibition of the Sema4D–Plexin-B1 pathway is a potentially valuable therapeutic strategy for the treatment of osteopenic diseases.

Sema3A functions as a potent osteoprotective factor by decreasing bone resorption and increasing bone formation. To investigate the in vivo effect of recombinant Sema3A administration on bone metabolism, wild-type mice were injected with recombinant Sema3A once a week (Hayashi et al. 2012). The trabecular bone volume and osteoblastic parameters were significantly increased and osteoclastic parameters were decreased, indicating a combined bone-increasing effect of recombinant Sema3A through both the stimulation of osteoblastic bone formation and the inhibition of osteoclastic bone resorption. The therapeutic potential of Sema3A treatment was further investigated in a bone regeneration model of mouse cortical bone defects and a mouse model of postmenopausal osteoporosis. Sema3A treatment promoted the regeneration of cortical bone after drill-hole injury and prevented bone loss after ovariectomy, accompanied by significant increases in osteoblast number and bone formation and decreases in osteoclast number and bone resorption. Remarkably, the action of Sema3A as an anti-resorptive agent is not

accompanied by reduction in bone formation. Therefore, the activity of Sema3A, which uncouples the process of bone remodeling, is exceptional in that almost all the drugs that inhibit bone degradation also decrease bone formation.

Other semaphorin family members may also be therapeutic targets in bone diseases, although more detailed analyses are required to develop such strategies.

8.9 Concluding Remarks

Remarkable progress has been made in our understanding of the molecular basis underlying cell–cell communication among bone cells. Since the function of Plexin-A1 in bone metabolism was reported, the semaphorin–plexin system has been shown to have various important roles in the regulation of bone metabolism and is emerging as a target for future bone and joint disease therapeutics. Other axon-guidance molecules, including ephrins, are reported to be important in the regulation of bone metabolism, raising the question of why a number of axon-guidance molecules play key roles in bone. Further studies are required to establish safe, efficient, and (hopefully) groundbreaking therapies for bone diseases through a rebalancing of bone remodeling by modulating semaphorin–plexin signaling.

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