Peter Pietschmann
Editor

Principles of Osteoimmunology

Molecular Mechanisms and Clinical Applications

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Title image legend
Epifluorescence image of multinucleated osteoclasts and precursor cells derived from murine bone marrow cells after eight days of culture. Osteoclast differentiation was induced by medium supplementation with receptor activator of NF-κB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF). Cells were stained for nuclei, the calcitonin receptor, α-tubulin and the precursor cell specific F4/80 macrophage marker. The colours of the image were artistically enhanced. The image was captured by M. Schepelmann as part of the project discussed in the chapter "Towards the automated detection and characterization of osteoclasts in microscopic images", Heindl et al., in this book.

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Preface

Osteoimmunology is a rapidly developing research field on the crosstalk between bone and the immune system. Examples of such immune-bone interactions are pathogenic mechanisms of bone diseases that are caused by or related to altered immune reactions. The English term "osteoimmunology" was first used in 2000 by Arron and Choi in a comment in Nature (430: 535). Nevertheless, the concept that osteoclasts, multinucleated bone resorbing cells, develop from the monocyte-macrophage lineage dates back to the 1920s. In the 1980s proinflammatory cytokines such as interleukin-1 or TNF-alpha were shown to stimulate bone degradation. The discovery of the RANK/RANKL/osteoprotegerin system and the development of an antibody-based targeted therapy for osteoporosis and other bone diseases have significantly increased the momentum of osteoimmunology.

The purpose of this book is to give an introduction to the emerging field of osteoimmunology to scientists and clinicians working in immunology, pathophysiology and osteology. The book is organized into 11 chapters. The first chapters give an introduction to cell and molecular biology of bone and the immune system, including methodological issues such as automated cell detection and bone markers. Dedicated chapters also describe effects of vitamin D on the immune system and immunological aspects of biomechanics. The next chapters deal with molecular mechanisms and the clinical presentation of osteoimmune diseases such as osteoporosis and rheumatoid arthritis as well as preclinical and clinical data on the treatment of bone diseases by RANKL inhibition. The final chapter describes osteoimmunological aspects of periodontal diseases.

I am very thankful to all authors who contributed to this book for their valuable time, expertise and effort. Moreover, I would like to acknowledge the great help and dedication of the staff from SpringerWienNewYork, in particular Mag. Angelika Heller and Dr. Amrei Strehl. Special thanks also to Maria Steiner and Birgit Schwarz for their continuous support of this book project.

I am convinced that our readers will enjoy the book as much as I enjoyed editing it.

Peter Pietschmann August 2011

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Basics of Bone Biology

1

Martina Rauner, Nicola Stein, Lorenz C. Hofbauer

1.1 Introduction to Bone

1.1.1 Bone Function and Structure

Bone is the major constituent of the skeleton which is a hallmark of all higher vertebrates. Besides the protection of internal organs and the support of body structures, the most important functions of bone are to serve as an attachment site for muscles allowing locomotion and provide a cavity for hematopoiesis in the bone marrow (Mendez-Ferrer et al. 2010; Zaidi 2007). Moreover, bone has a central role in mineral homeostasis as it functions as a reservoir for inorganic ions that can be mobilized rapidly on metabolic demand.

Although bone is often considered an inert, static material, it is a highly organized, living tissue that undergoes constant remodeling. Different cell lineages have emerged to serve distinct skeletal functions. While cells from the hematopoietic lineage, such as osteoclasts, break down bone tissue to remove old and damaged bone, or release calcium to maintain calcium homeostasis, cells from the mesenchymal lineage, including chondroblasts, fibroblasts and osteoblasts construct and later remodel bone tissue (Jiang et al. 2002). Osteoblasts produce the organic components of the extracellular matrix, which mainly includes type I collagen (approximately 95 %), but also non-collagenous proteins (i. e. osteocalcin, osteopontin, osteonectin, bone sialoprotein) and proteoglycans. The inorganic matrix predominantly contains calcium and phosphorus, appearing as hydroxyapatite crystals ($[3Ca_3(PO_4)_2](OH)_2$), and is deposited into the collagenous matrix. This complex organization confers rigidity and strength to the skeleton while maintaining a high degree of elasticity.

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Two types of osseous tissues are found in all bones: cortical or compact bone and trabecular or cancellous bone, sometimes also referred to as spongy bone (Fig. 1). Cortical bone is mainly found in the shafts of long bones (diaphyses) and is made of numerous overlapping cylindrical units termed Haversian systems or osteons. The central Haversian canal, containing the blood vessel and nerves, is surrounded by densely packed collagen fibrils which are formed into concentric lamellae. Osteocytes, terminally differentiated osteoblasts, are located between concentric lamellae and are connected to each other via canaliculi, allowing the exchange of nutrients and metabolic waste and the sensation of mechanical stress. Volkmann's canals are responsible for the conjunction of blood vessels from the inner and outer bone surfaces to the vessels of the Haversian canals. The dense organization of cortical bone thus provides maximum strength and load-bearing capacity by being highly resistant to bending and torsion. Cancellous bone, on the other side, is predominantly found at the ends of long bones (epiphyses) as well as in flat bones and in vertebral bodies where force may be applied at variable angles. It is composed of a meshwork of trabeculae, thereby reducing skeletal weight without compromising strength. This particular construction also establishes a vast surface area. Considering that bone remodeling only takes place at bone surfaces, cancellous bone is quick to render metabolic activities but also disproportionally susceptible to damage when net bone loss occurs.

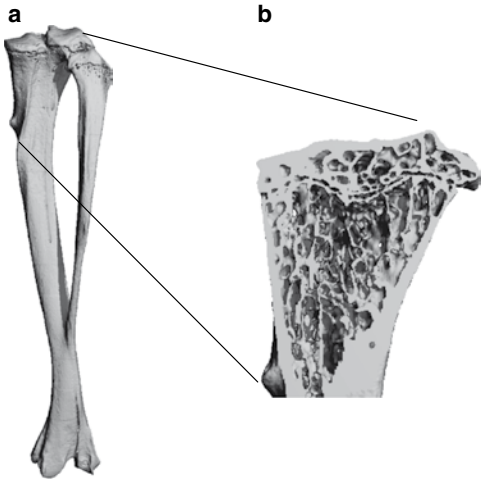

Fig. 1 Illustration of compact and cancellous bone. (**a**) Whole tibial structure obtained by micro-computed tomography. 12 microns isotropic spatial resolution. (**b**) Lateral view of the tibia. The trabecular meshwork structure is clearly visible at the epiphysis. Cortical bone is found at the shaft. Source: Peter Varga and Phillipe Zysset, Technical University of Vienna, Austria

1.1.2 Ossification Processes

Ossification occurs either intramembranously or endochondrally. During skeletal development, flat bones (e. g. calvariae) and some irregular bones are formed by intramembranous ossification where bony tissue directly forms from the connective tissue without an intermediate cartilage stage (Blair et al. 2008). Within this process, mesenchymal stem cells (MSCs) condense into highly vascularized sheets of primitive connective tissue at sites of eventual bone formation. Certain MSCs group together and differentiate into osteoblasts that deposit extracellular matrix (osteoid) which is subsequently mineralized to form the bone matrix. These small aggregates of bone tissue, termed bone spicules, continuously expand with new MSCs lining on the surface, differentiating into osteoblasts and secreting extracellular matrix. Once they become embedded by the secreted mineralized matrix, osteoblasts terminally differentiate into osteocytes. As the bone spicules grow and interconnect with others, a trabecular network of woven bone – also referred to as primary spongiosa – is formed. Although woven bone forms quickly with the collagen fibres being randomly organized, it is structurally weak. Thus, it is soon replaced by a more solid lamellar bone, which is composed of a highly organized collagen structure (Frost and Jee, 1994). Several collagen fibers align in the same layer, and several such concentric layers stacked in alternating orientations finally constitute a bone unit called osteon (Parfitt 1988). This highly sophisticated organization confers strength and resistance to torsion forces to lamellar bone. However, the complex architecture and orderly deposition of collagen fibers requires more time and restricts the formation of osteoid to 1–2 μm per day. Besides the creation of woven bone in fetal bone development, it may also occur in adults after fractures or in patients with Paget's disease (Parfitt 1994).

In contrast to flat and irregular bones, bones of the vertebral column, pelvis, and extremities develop by endochondral ossification. Thereby, hyaline cartilage devoid of blood vessels is first formed and then replaced by bone matrix starting at the primary ossification center. During embryonic development chondrocytes congregate to a cartilaginous model that alleges the shape of the future bone and after the local enlargement of chondrocytes (hypertrophy) endochondral bone formation is initiated in the middle of the shaft at the primary ossification center. The perichondrium, which surrounds the cartilage model, becomes invaded with blood vessels and then is called periosteum (Stanka et al. 1991; Streeten and Brandi 1990; Trueta and Buhr 1963). The periosteum contains layers of MSCs that differentiate into osteoblasts during development, when the bone increases its width (appositional growth), or after fractures, when new bone formation is required. In addition to its important function to supply nutrients via the blood vessels, the periosteum contains nociceptor nerve endings that allow the sensation of pain (Fortier and Nixon 1997; Grubb, 2004; Jimenez-Andrade et al. 2010).

The growth plates are characterized by the orderly proliferation and maturation of chondrocytes in longitudinal columns, forming stratified zones of reserve, proliferative, maturing, and hypertrophic cartilage (Poole et al. 1991). Hypertrophic chondrocytes secrete large amounts of a specialized extracellular matrix rich in col-

lagen type X and alkaline phosphatase, which becomes calcified. After the calcification of the collagenous matrix, hypertrophic chondrocytes start producing matrix metalloproteinase 13, which is crucial for the subsequent degradation of the cartilage matrix, and undergo apoptosis (Stickens et al. 2004). By doing so, transverse septa of cartilage matrix surrounding them are broken down, leaving vertical septa largely intact, but allowing the entry of capillaries and invading cells of the ossification front. These cells mainly include cells of the mesenchymal (osteoblast precursors and stromal cells) and hematopoietic lineages (osteoclast precursors and other hematopoietic lineages that constitute the bone marrow). After osteoblast precursor cells have migrated to the surface of remnant cartilage spicules, they differentiate into fully mature osteoblasts and deposit a predominantly type I collagen-containing extracellular matrix (osteoid), which subsequently becomes mineralized into the mature bone matrix. The ossification continues towards the ends of the bones, where the further elongation of long bones occurs in the growth plates of the metaphysis. Finally, the trabecular bone in the diaphysis is broken down by osteoclasts to open up the medullary cavity.

The same process applies to the secondary ossification center, located in the epiphysis, except that the trabecular bone is retained (Alini et al. 1996). The length of bones increases until the early twenties through a process similar to endochondral ossification (Riggs et al. 1999). The cartilage in the epiphyseal plate proliferates constantly and is continuously replaced by bone matrix until the skeleton has reached maturity and the epiphyseal plate has become almost completely ossified. The articular cartilage remains uncalcified and covers the ends of the long bones. Due to its incredibly low coefficient of friction, coupled with its ability to bear very large compressive loads, articular cartilage is ideally suited for placement in joints, such as the knee and hip.

1.2 Bone Remodeling

During a person's life-time, continuously changing functional demands require permanent adaptation of the bone structure and microarchitecture. Wolff has observed this principle of functional adaptation already over 100 years ago (Wolff 1892). The process of where "form follows function" occurs in conditions of disuse (as during immobility, space flights, or long-term bed rest), overloading (weight gain), growth, and after fracture healing, and consists of two activities, namely, bone formation and bone resorption (Sommerfeldt and Rubin 2001; Frost 1990). While these processes are locally separated in modeling (Frost 1990), bone remodeling is characterized by the spatial and temporal coupling of bone formation by osteoblasts and bone resorption by osteoclasts (Rodan and Martin 1981). The so called basic multicellular unit (BMU) is covered by a canopy of cells that creates a bone remodeling compartment (BRC). While the nature of the canopy cells remains under debate, evidence in humans suggests that it is bone-lining cells, generating a unique microenvironment to facilitate coupled osteoclastic bone resorption and osteoblastic synthesis

(Andersen et al. 2009). Interestingly, the action of BMUs slightly differs in cortical (endocortical as well as intracortical surfaces) and trabecular bone. While in cortical bone the BMU forms a cylindrical tunnel of about 2,000 µm long and 200 µm wide and the BMU burrows through the bone with a speed of 20–40 µm/day, the remodeling process in trabecular bone is mainly a surface event reaching a depth of approximately 50 µm. With a speed of 25 µm/day, active remodeling sites of BMUs in trabecular bone cover areas of varying sizes ranging from about 100 – 1,000 µm². In general, approximately 5–25 % of bone surface is undergoing bone remodeling (Parfitt 1994; Raisz 1988), thereby restoring microdamages and ensuring mechanical integrity as well as regulating the release of calcium and phosphorus, while maintaining the global bone morphology.

An active BMU performs one bone remodeling cycle that occurs over several weeks and includes four main processes: activation, resorption, reversal and formation (Parfitt 1988). While the process of bone resorption is usually accomplished within 2–3 weeks, the new synthesis of bone requires around 2–3 months. The remodeling cycle is initiated by the detection of signals that induce the activation of the quiescent bone surface, which is covered with bone lining cells. These signals may be provided through osteocytes that sense mechanical strain or are affected by structural damage, which severs the processes of osteocytes in their canaliculi and leads to osteocyte apoptosis (Aguirre et al. 2006; Bonewald 2007; Hazenberg et al. 2006; Verborgt et al. 2002). Alternatively, hormone actions (e. g. estrogen or parathyroid hormone (PTH)) due to more systemic changes in homeostasis or effects of corticosteroids on bone cells may negatively alter osteocyte biology. Current research points towards an intricate communication between osteocytes, which sense bone damage deep within the osteon or hemiosteons, and lining cells on the bone surface, which receive signals through the long processes of osteocytes, and communicate the health status of the bone to the marrow environment to initiate the establishment of a BRC (Hauge et al. 2001). Osteocyte apoptosis may also contribute to the recruitment of osteoclast precursor cells by diminishing the osteocytic secretion of factors that usually inhibit osteoclast formation, such as transforming growth factor-β (TGF-β) (Heino et al. 2002). *In vivo* evidence indicates that osteocyte apoptosis precedes osteoclast formation as osteocyte apoptosis occurs within three days of immobilization and is followed within two weeks by osteoclastogenesis (Aguirre et al. 2006). Although the process of osteoclast precursor attraction is not fully understood yet, osteoblast-secreted products including monocyte chemoattractant protein-1 (MCP-1) and the osteoclast differentiating factor receptor activator of NF-kB ligand (RANKL) may play an important role (Li et al. 2007). After osteoclast precursor cells are recruited to the activated surface they fuse to form mature, bone resorbing osteoclasts (Vaananen and Horton 1995). The osteoclasts attach to the surface and form a ruffled border at the bone/osteoclast surface that is completely surrounded by a sealing zone. Thereby, osteoclasts create an isolated acidic microenvironment in order to dissolve the inorganic matrix and degrade the organic matrix with specific enzymes (Teitelbaum 2000). As bone resorption subsides and a resorption pit with a demineralized collagen matrix remains, osteoclasts disappear and mononuclear cells of undetermined lineage remove the colla-

gen remnants and prepare the surface for bone formation. This phase is called reversal. Currently, there is a debate about whether the reversal cell is of hematopoietic or mesenchymal origin. Recent evidence suggests that this cell type may be a resident macrophage of the bone termed osteomacs (Pettit et al. 2008). These cells are positive for the macrophage markers F4/80+ and CD68, but negative for the osteoclast marker tartrate-resistant acid phosphatase (TRAP), and are found throughout the periosteum and endosteum. Moreover, these cells have been shown to produce MMPs, which are required for matrix degradation, as well as TGF-β and ephrin B2, which may promote osteoblast recruitment, differentiation, and/or activation of bone lining cells (Chang et al. 2008; Compagni et al. 2003). Thus, these cells would be the ideal coupling agents of bone resorption and formation. However, further research is needed to clarify the nature of the reversal cells. After the reversal phase, the bone remodeling cycle is finished with the synthesis and deposition of bone matrix by osteoblasts until an equal amount of bone is reproduced. Also in this case, the mechanisms that terminate bone formation are not known, but may be mediated by signals from osteocytes that have become embedded in the mature bone matrix. Finally, bone lining cells build a canopy covering the surface, keeping the material dormant until the next cycle (Fig. 2).

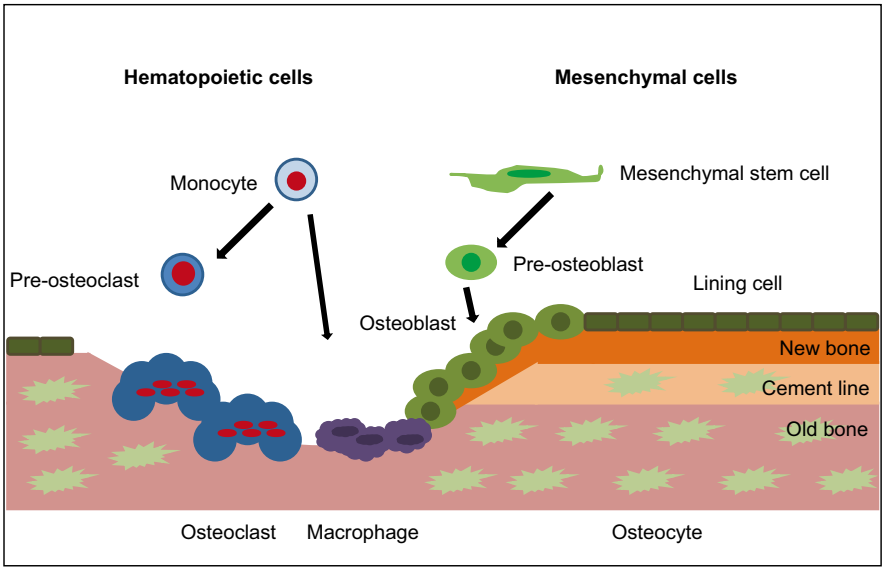

Fig. 2 Bone remodeling. Monocytes from the hematopoietic lineage differentiate into osteoclasts, which resorb old and damaged bone tissue. Macrophages, which also originate from the hematopoietic lineage, contribute to the initiation of bone remodeling and attract osteoblast precursors that mature to bone-forming osteoblasts at the bone surface. After filling the resorption lacunae, osteoblasts become embedded by the bone matrix and turn into osteocytes. Quiescent lining cells remain at the bone surface

1.3 Key Players of Bone Remodeling

1.3.1 Cells of the Osteoblast Lineage – Osteoblasts, Osteocytes, Bone Lining Cells

Osteoblasts are derived from MSCs and their primary function is to synthesize the organic collagenous matrix and orchestrate its mineralization by producing bone matrix proteins including osteocalcin, osteopontin and bone sialoprotein, and providing optimal environmental conditions for crystal formation (Ducy et al. 2000). Due to their active protein machinery, osteoblasts have a prominent golgi apparatus and endoplasmatic reticulum. As mentioned earlier, osteoblasts are also the main producers of RANKL and its decoy receptor osteoprotegerin (OPG), and are therefore critically involved in regulating osteoclastogenesis (see also 1.4.2). Fully differentiated osteoblasts that are surrounded by mineralized bone tissue are called osteocytes and act as mechanosensors in bone tissue (Paic et al. 2009). They are the most numerous cells within the bone tissue and scattered evenly through the matrix. With their flattened morphology and long processes, they form a sensory network which allows the detection of abnormal strain situations such as generated by microcracks (Hirao et al. 2007; Martin and Seeman 2008). By communicating these signals to bone lining cells (the second terminally differentiated osteoblast cell type) or secrete factors that recruit osteoclasts, osteocytes initiate the repair of damaged bone. Other emerging roles of osteoblast lineage cells include the maintenance of hematopoietic stem cell (HSC) niches and HSC homing, as well as acting as non-professional antigen-presenting cells in conditions of inflammation (Fleming et al. 2008; Jung et al. 2007; Mendez-Ferrer et al. 2010; Ruiz et al. 2003; Schrum et al. 2003; Skjodt et al. 1989). While the capacity to stimulate effector cells of the immune system may only be relevant under pathophysiological conditions, the osteoblast-driven maintenance of the stem cell niche is of critical importance for the homeostasis of hematopoiesis. Experiments in mice have shown that the number of long-term repopulating HSCs increases or decreases in parallel with *in vivo* osteoblast stimulation by PTH or osteoblast ablation using a mouse genetic approach (Visnjic et al. 2004). Although the underlying signaling events are not fully understood yet, several mechanisms such as the selective expression of signaling molecules (i. e. jagged, G-protein Gsα), adhesion molecules (i. e. integrins, N-cadherin), and components of the ECM (i. e. proteoglycans) may determine the long-term repopulating ability of HSCs and their ability to home into the bone marrow.

MSCs give rise to a variety of cells including osteoblasts, adipocytes, chondrocytes, and myoblasts (Pittenger et al. 1999). The MSC goes through several progressive steps in generating progeny with progressively more limited differentiation capacities until the differentiated end-stage cell is able to express distinct functional markers and morphological traits. Typical osteoblast markers include alkaline phosphatase (ALP) and type I collagen, as well as various non-collagenous proteins such as osteocalcin, osteopontin or bone sialoprotein. However, cells of the osteoblastic lineage also selectively express proteins at distinct differentiation stages, such as

RANKL in immature osteoblasts or osteocalcin and sclerostin in fully mature osteoblasts or osteocytes.

Osteoblasts express receptors for various hormones including PTH, 1,25-dihydroxyvitamin D_3, estrogen, glucocorticoids, and leptin, which are involved in the regulation of osteoblast differentiation (see 1.4.1). Furthermore, osteoblasts are regulated by multiple local factors including bone morphogenetic proteins (2, 4, 6, and 7) (Shore et al. 2006; Storm and Kingsley 1999; Wu et al. 2003; Wutzl et al. 2010), growth factors (transforming growth factor-β, epidermal growth factor, insulin-like growth factor) (Canalis 2009), Sonic and Indian hedgehogs (Guan et al. 2009; Maeda et al. 2007), as well as members of the Wnt family in a paracrine and autocrine fashion (Bodine and Komm 2006). Because the Wnt signaling pathway is of such critical importance for bone mass maintenance, it will be discussed here in more detail.

Wnt signaling is highly conserved throughout evolution among a variety of species and plays an important role in regulating cellular processes, such as proliferation, differentiation, cell survival and motility (van Amerongen and Nusse 2009). Wnt signaling further plays a key role in embryonic development and maintenance of tissue homeostasis, including bone. Wnt proteins are cystein-rich glycoproteins that act on target cells by binding to the seven-span transmembrane receptor protein Frizzled (FZD), and low-density lipoprotein receptor-related proteins 5 and 6 (LRP 5/6). In bone, various components of this pathway have been shown to positively or negatively regulate osteoblast differentiation (Bodine and Komm 2006). Evidence that the Wnt/β-catenin pathway is involved in bone mass homeostasis has been provided by observations of mutations in the LRP5 gene, in which gain-of-function mutations led to a high bone mass phenotype in humans and mice, and loss-of-function mutations led to low bone mass phenotypes (Boyden et al. 2002; Gong et al. 2001; Van Wesenbeeck et al. 2003).

Wnt signaling comprises several pathways, that are usually divided into the canonical or β-catenin-dependent pathway and non-canonical or β-catenin-independent pathways. As the canonical Wnt pathway seems to be critical for bone mass maintenance, only this pathway will be presented in this chapter. In the absence of Wnt ligands, cytoplasmic levels of β-catenin are kept low through the continuous ubiquitin-proteasome-mediated degradation of β-catenin, which is regulated by a multiprotein complex containing axin, adenomatous polyposis coli, glycogen synthase kinase 3β (GSK3β), and casein kinase 1α (CK1α). The canonical pathway is activated upon binding of Wnt proteins to a receptor complex consisting of FZD and its co-receptor, LRP5 or LRP6. Disheveled (Dvl) is then phosphorylated by CK1α and in turn induces the formation of another protein complex consisting of Dvl, Frat1, axin as well as LRP5/6 and FZD. This interaction ultimately leads to the inhibition of GSK3β and results in the stabilization of β-catenin, which then translocates into the nucleus to join T cell factor (TCF)/lymphoid enhancer binding factor (LEF) and other factors to induce the transcription of Wnt target genes (Clevers 2006). Wnt signaling is regulated at various levels such as through the presence or absence of multiple Wnt ligands, co-receptors, intracellular signaling molecules, and transcription factors. Furthermore, it is tightly regulated by a series of extracellular inhibitors including members of the secreted frizzled-related protein (sFRP) family and Wnt inhibitory

factor that bind to Wnt ligands, as well as dickkopfs (Dkks) and sclerostin, both binding LRP5/6 (Semenov et al. 2005; Tian et al. 2003). In both cases, these interactions lead to the blockade of Wnt ligands binding to FZD receptors. Many Wnt inhibitors have been proposed as therapeutic targets for increasing bone mass by applying neutralizing antibodies, whereas sclerostin may be of particular interest due to its specific expression solely in osteocytes (Keller and Kneissel 2005; Paszty et al. 2010).

At a transcriptional level, osteoblast differentiation is induced by the master transcription factor runx2 (runt-related transcription factor 2, also called core binding factor 1, Cbfa1) and several signaling pathways converge to increase runx2 expression. Runx2-deficient mice have no osteoblasts and thus only contain a cartilage-like skeleton (Harada et al. 1999; Miller et al. 2002). Intriguingly, although runx2 supports osteogenic differentiation, it inhibits osteoblast maturation into osteocytes, keeping osteoblasts in an immature state (Lian et al. 2006). Runx2 expression is induced by BMPs, TGFβ1, Indian hedgehog, members of the Wnt pathway and is tightly regulated by various post-translational modifications as well as co-repressors, such as Twist and menin-1, and co-activations, such as TAZ. Even though runx2 is regarded as the master transcription factor for osteoblasts, other transcription factors also participate in the regulation of osteoblast differentiation, including osterix (also called specificity protein 7, sp7) (Kim et al. 2006a), β-catenin (Krishnan et al. 2006), dlx3 and dlx5 (distal-less homeobox) (Harris et al. 2003), msx2 (homeobox factor) (Liu et al. 1999; Satokata et al. 2000), ATF4 (activating transcription factor 4) (Tozum et al. 2004), as well as NFATc1 (nuclear factor of activated T cells c1) (Koga et al. 2005). Many of these factors control osteoblast differentiation at very specific locations such as dlx proteins in the skull.

After osteoblasts have fully matured and deposited a mineralized matrix surrounding them, they become osteocytes, which serve functions different from matrix deposition. As mentioned above, osteocytes are evenly located throughout the bone tissue and produce a dense network by connecting each other via gap junctions on their processes. In this respect, connexion-43 seems to play a critical role in the formation of hemichannels which allow an extensive communication between two osteocytes (Plotkin et al. 2002, 2008). Mice made osteocyte-depleted exhibit enhanced bone fragility, intracortical porosity, and microfractures, indicating the crucial function of osteocytes to maintain bone integrity (Tatsumi et al. 2007). Also several other studies have shown that loss of osteocyte viability is related to bone loss (Aguirre et al. 2006; Teti and Zallone 2009; Weinstein et al. 2000). Besides mechanosensation, osteocytes express several mineralization inhibitors including fetuin-A, dentin matrix protein-1, phex, and the Wnt inhibitor sclerostin, which allows them to control the amount and quality of the bone matrix (Coen et al. 2009; Liu et al. 2009; Poole et al. 2005). Dentin matrix protein-1-deficient mice, for example, show impaired osteocyte maturation, increased fibroblast-growth factor-23 expression, and severe abnormalities of bone mineralization (Feng et al. 2006). Of note, also glucocorticoids have the potential to increase the expression of mineralization inhibitors, thereby compromising bone quality and bone strength (Yao et al. 2008).

1.3.2 Cells of the Osteoclast Lineage – Osteomacs and Osteoclasts

Tissue-resident macrophages, also referred to as osteomacs, and osteoclasts both derive from the hematopoietic monocytic lineage. The concept of osteomacs has only recently been developed due to thorough observations of periosteal and endosteal tissues and the bone remodeling compartment (Pettit et al. 2008). Pettit et al. determined that osteomacs constitute about one sixth of the total cells within osteal tissues and span a network along bone surfaces with their stellate morphology. Due to their abundance and widespread location, it is likely that osteomacs contribute to immune surveillance in the bone marrow compartment and react quickly to inflammatory stimuli. Osteomacs are distinguishable from osteoclasts by the expression of the murine macrophage marker F4/80, which is not present on osteoclasts, and by being negative for osteoclast-specific markers such as TRAP (Chang et al. 2008). As they are also located at the bone remodeling compartment, it has been suggested that osteomacs participate in the reversal phase of bone remodeling and closely interact with osteoblasts through the production of osteoblast-stimulating factors, such as bone morphogenetic protein-2 or transforming growth factor-β.

Osteoclasts are tissue-specific giant polykaryons (up to 100 μm in diameter) derived from the monocyte/macrophage hematopoietic lineage and are the only cells capable of breaking down large amounts of mineralized bone, dentine and calcified cartilage (Teitelbaum 2000). Bone resorption is a crucial step in bone remodeling which is necessary for healthy bone homeostasis, thereby, repairing microdamages and adapting to new mechanical loads and altered metabolic conditions. Bone remodeling starts with the retraction of bone lining cells uncovering bone tissue and attracting mononuclear precursors to the bone surface. The earliest step in osteoclastogenesis is the determination of the stem cell precursor to the osteoclastic lineage following the induction of PU.1 (Tondravi et al. 1997). Soon thereafter, precursors express the M-CSF receptor, *c-fms*, and after activation with the ligand, proliferation is induced. The next determination step towards a mature osteoclast is the expression of receptor activator of NFkB (RANK). The presence of its ligand, RANKL, is essential for the formation and fusion of multinucleated cells. Mice lacking either RANKL or RANK have no osteoclasts and suffer severe osteopetrosis (for more detail see 1.4.2) (Anderson et al. 1997; Dougall et al. 1999; Kong et al. 1999b; Lacey et al., 1998; Yasuda et al. 1998). RANK signaling activates several transcription factors that are essential for osteoclastogenesis including activated protein-1, NFkB, or nuclear factor of activated T cells (NFAT). In the osteoclast, most signals converge to induce the activity of NFATc1. This is also proven genetically, as embryonic precursors lacking NFATc1 fail to become osteoclasts (Takayanagi et al. 2002). Importantly, NFATc1 is indispensible and sufficient for osteoclastogenesis, as its overexpression yields osteoclasts even in the absence of RANK signaling (Matsuo et al. 2004).

Although several down-stream effectors of RANK signaling induce NFATc1 expression, the mechanisms that induce NFATc1 in a calcium-dependent way have only recently been identified. Therein, immunoreceptor tyrosine-based activation

motifs (ITAMs)-containing adaptor molecules, such as DAP (DNAX-activating protein) 12 and Fc common receptor γ chain (FcRγ) have been shown to be indispensable for osteoclastogenesis as mice deficient for both receptors are severely osteopetrotic (Koga et al. 2004; Mocsai et al. 2004). The activation of phospholipase-Cγ, Syk, and Tec kinases has been shown to be required for the activation of calcineurin-dependent calcium (Faccio et al. 2005; Mocsai et al. 2004; Wada et al. 2005). Paired immunoglobulin-like receptor-A (PIR-A) and osteoclast-associated receptor (OSCAR) have been found to associate with FcRγ (Kim et al. 2002), whereas triggering receptor expressed on myeloid cells-2 (TREM-2) and signal-regulatory protein-b1 (SIRP-β1) bind to DAP12. These signals are considered to act as co-stimulatory signals for RANKL in osteoclast precursors, since those signals alone are not able to induce osteoclastogenesis (Koga et al. 2004).

Mature osteoclasts express several specific proteins including TRAP, cathepsin K, calcitonin receptor (CTR), and integrin receptors (Teitelbaum 2000, 2003). Via integrins, osteoclasts attach very tightly to the matrix (sealing zone), thereby, creating an isolated lacuna (Howship's lacuna) able to maintain an acidic environment necessary for matrix dissolution (Mimura et al. 1994; Miyauchi et al. 1991). At least four integrin receptors are expressed in osteoclasts, including $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_2\beta_1$ and $\alpha_v\beta_1$ binding to various extracellular matrix proteins such as vitronectin, collagen, osteopontin and BSP. After attachment, intracellular rearrangements lead to the polarization of the cell borders, whereas the sealing zone is adjacent to the baso-lateral domain and the ruffled border, respectively. At the opposite side of the ruffled border emerges the functional secretory domain. The ruffled border and the functional secretory domain are connected to each other via microtubules on which exocytotic vesicle traffic has been observed, suggesting the secretion of resorbed material into the extracellular space (Vaananen and Horton 1995). In addition to the development of distinct membrane domains, the cytoskeleton undergoes organizational changes, creating a dense actin-ring in osteoclasts preparing for resorption (Silver et al., 1988). This process has been shown to be greatly dependent on Rho-GTPases, which require the mevalonate pathway for isoprenylation and activation (Chellaiah 2006). Of note, bisphosphonates have been shown to block osteoclast activity by inhibiting farnesyl diphosphate synthase, a critical enzyme in the mevalonate pathway.

The resorption of bone matrix takes place in the resorption lacuna. The ruffled border is formed by the fusion of cytoplasmic acidic vacuoles, thereby releasing acid into the resorption lacuna and initiating rapid dissolution of the hydroxyapatite crystals (Blair et al.,1989; Teti et al. 1989). Furthermore, ATPases, located in the ruffled border, additionally transport protons into the Howship's lacuna (Li et al. 1999; Mattsson et al. 1994). The protons are supplied by the reaction of water and carbon dioxide catalyzed by the enzyme carbonic anhydrase II resulting in the formation of protons and HCO_3^-. Whereas H^+ is pumped into the resorption lacuna, HCO_3^- is transported into the extracellular space via HCO_3/Cl exchangers. The imported chloride ions are also pumped into the resorption lacuna to form hydrochloric acid with a pH as low as 4, which is capable of dissolving the mineralized matrix (Silver et al. 1988). The organic matrix is degraded by various enzymes, including

TRAP, cathepsin K and matrix MMP-9. Cathepsin K is a lysosomal cysteine proteinase capable of degrading type I collagen (Gelb et al. 1996). Although osteoclasts form in cathepsin K-deficient mice, build a ruffled border and are able to mobilize bone mineral, they are unable to efficiently degrade the collagen matrix and thus resorb bone (Saftig et al. 1998). Furthermore, active osteoclasts express high levels of matrix metalloproteinases such as TRAP and MMP-9 (Okada et al. 1995; Wucherpfennig et al. 1994). Using electron microscopy Okada and colleagues were able to show that MMP-9 degraded collagen into fragments, suggesting the involvement of MMP-9 in the resorption process. Stronger evidence is provided by mice lacking MMP-9, which are severely osteopetrotic and have difficulties in the endochondral ossification process, as the collagen matrix is only insufficiently being broken down (Engsig et al. 2000).

After the resorption of bone tissue, osteoclasts die by apoptosis and are quickly removed by phagocytes (Teitelbaum and Ross 2003). At present, little is known about the molecular mechanisms that terminate osteoclast resorption and initiate osteoclast apoptosis *in vivo*. Nevertheless, targeting osteoclasts for apoptosis, such as by using bisphosphonates or also the more obsolete therapy with estrogen and progestin, has, until recently, been the predominant approach to prevent bone destruction in conditions of bone loss such as post-menopausal osteoporosis, therapy-induced or cancer-related bone loss.

1.4 Regulation of Bone Remodeling

1.4.1 Hormones

Bone formation and resorption, as well as the cell machinery that performs those tasks, are under the subtle control of various hormones, whereas the most extensively studies ones are estrogens and androgens, parathyroid hormone (PTH), 1,25-dihydroxyvitamin D_3, and glucocorticoids, due to their common use as anti-inflammatory drugs. These major endocrine regulators will be discussed in more detail. However, it should be noted that bone homeostasis is also regulated by other hormones such as calcitonin (Huebner et al. 2008), leptin (Karsenty and Ducy 2006), and hormones of the anterior pituitary gland (follicle-stimulating hormone, thyroid-stimulating hormone, and adrenocorticotropic hormones) (Imam et al. 2009).

PTH is a peptide hormone and one of the most important regulators of calcium ion homeostasis (Kronenberg 2006; Lanske et al. 1999). PTH is produced and secreted by C cells in the parathyroid gland in response to low blood calcium levels and acts on the kidney, bone and intestine to maintain blood calcium concentrations. In bone, PTH stimulates the production of interleukin-6 and RANKL by osteoblasts and stromal cells, thereby promoting the differentiation, activation and survival of osteoclasts (Dai et al. 2006; Greenfield et al. 1993). Thus, PTH as well as PTHrP (PTH-related protein) promote bone resorption and consequently

the release of calcium (Lanske et al. 1999; Pollock et al. 1996). However, it should be noted that an intermittent exposure to PTH has bone anabolic effects mainly by increasing osteoblast functions, and is thus currently the only approved anabolic treatment option in the treatment of postmenopausal osteoporosis (Bilezikian and Kurland 2001).

Calcitriol (1α,25-dihydroxyvitamin D_3), the active hormonal form of vitamin D, is a steroid hormone either ingested from the diet or synthesized in the skin from 7-dehydrocholesterol through exposure to sunlight (Webb and Holick 1988). Its importance for the development and maintenance of the mineralized skeleton was demonstrated in studies using vitamin D receptor or 1α(OH)ase knock-out mice (Dardenne et al. 2001; Panda et al. 2004). The mineralization defect was normalized after a high-calcium, high-phosphate and high-lactose diet (rescue diet) was administered. However, the administration of only $1,25(OH)_2D_3$ to 1α(OH)ase knock-out mice was not sufficient to normalize the impaired mineralization if hypocalcemia was not corrected (Panda et al. 2004). Moreover, vitamin D-deficient mice showed an increase in osteoblast number, bone formation and bone volume as well as increased serum ALP levels. Additionally, osteoclast numbers were decreased due to a decreased production of RANKL and an enhanced production of OPG (Kitazawa et al. 2003).

Besides PTH and calcitriol, which mainly regulate calcium homeostasis, estrogens and androgens are sex steroids with profound effects on bone. In contrast to PTH and 1,25-dihydroxyvitamin D_3, they enhance bone formation and inhibit bone resorption (Carani et al. 1997; Khosla et al. 2001; Leder et al. 2003). Lack of estrogen as well as testosterone inevitably leads to an increased bone turn-over rate with a simultaneous increase in osteoclastic bone resorption as well as osteoblastic bone formation (Eghbali-Fatourechi et al. 2003; Khosla and Riggs 2003; Weitzmann et al. 2002). However, the net effect of estrogen deficiency is bone loss as a result of an increased production of RANKL and a decreased production of OPG in osteoblastic cells as well as an increase in the secretion of pro-inflammatory and pro-resorptive cytokines in lymphocytes such as IL-1, IL-6 and tumor necrosis factor-alpha (TNF-α) (Jilka et al. 1992, 1995; Tanaka et al. 1993). Although clinical trials have shown that hormone replacement therapy decreased the incidence of major osteoporotic fractures (Cauley et al. 1995; Orwoll et al. 1996), serious side effects including cardiovascular disease and cancer have occurred and therefore other medications are now used in the treatment of osteoporosis (e. g. selective estrogen receptor modulators such as raloxifene) (Riggs and Hartmann 2003).

Bone cells also contain glucocorticoid receptors that confer responsiveness to endogenously produced and exogenously administered glucocorticoids. While active forms of endogenous glucocorticoids such as cortisol are necessary for bone development, glucocorticoid excess is detrimental to many metabolic systems including bone. Studies in mice lacking 11β-hydroxysteroid dehydrogenase-2, an enzyme that inactivates active glucocorticoids, showed that endogenous glucocorticoids are necessary to prime MSCs to the osteoblastic lineage (Eijken et al. 2005; Hamidouche et al. 2008; Sher et al. 2004; Zhang et al. 2008) and support osteoblastogenesis. This is also recapitulated in human osteoblast cultures, which require

physiological amounts of glucocorticoids to differentiate into fully mature, mineralizing osteoblasts (Rauner et al. 2010). Moreover, when the glucocorticoid receptor was specifically deleted in osteoclasts glucocorticoids enhanced the lifetime of osteoclasts, but at the same time inhibited bone resorption by disrupting the osteoclastic cytoskeleton (Kim et al. 2006a). In contrast to these bone-anabolic physiological effects of glucocorticoids, the prolonged exposure to synthetic glucocorticoids, such as required to treat inflammation or organ rejection, results in severe bone loss already within the first months of administration. The pathophysiology of glucocorticoid-induced bone loss includes the transient hyperactivation of osteoclasts due to an increased RANKL/OPG ratio in osteoblasts (Hofbauer et al. 1999, 2009) and a severely inhibited osteoblast function, mediated by the suppression of critical pro-osteoblastic factors such as runx2, Wnt, and BMP signaling, as well as the induction of mineralization inhibitors such as dentin matrix protein-1 or phex (O'Brien et al. 2004; Rauner et al. 2010; Wang et al. 2005, 2008; Yao et al. 2008). Animal studies suggest that glucocorticoid-induced osteoporosis may be successfully prevented administering bisphosphonates, PTH, or denosumab (Hofbauer et al. 2009; Yao et al. 2008). Analogous to selective estrogen receptor modulators, selective glucocorticoid receptor modulators have also been developed that display an improved benefit/risk ratio, but need to be verified in human studies.

1.4.2 RANKL/OPG

Although interactions between osteoblasts and osteoclasts have already been observed in the 1980s by Rodan and colleagues, it took another 15 years to identify the two main negotiators in osteoblast-osteoclast communication, RANKL and OPG (Anderson et al. 1997; Kong et al. 1999b; Lacey et al. 1998; Yasuda et al. 1998) (Fig. 3). Today, the high efforts invested in understanding and characterizing the RANKL/RANK/OPG system have led to detailed knowledge of the pathogenesis of metabolic bone diseases and have already contributed to the development of inno-

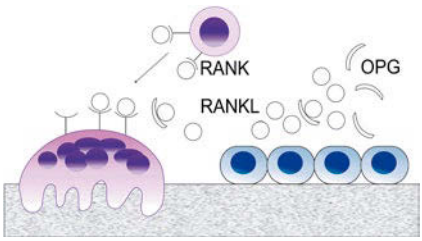

Fig. 3 RANK/RANKL/OPG system. RANK is expressed on mononuclear osteoclast precursor cells (*pink*). Upon binding of RANKL (*circles*), produced by osteoblasts (*blue*), osteoclast differentiation is induced. RANKL/RANK signaling is also active in mature osteoclasts (*pink*) to promote resorption activity and prolong survival. OPG (half circle), which is also produced by osteoblasts, is a soluble decoy receptor for RANKL and can thereby prevent binding of RANKL to RANK and thus the induction of osteoclastogenesis

vative therapeutic drugs that are now in clinical use (human anti-RANKL antibody, denosumab, Prolia) (Cummings et al. 2009; Smith et al. 2009).

As mentioned earlier, bone formation and bone resorption are coupled processes in remodeling. Often, dysregulations favoring osteoclastogenesis are responsible for the development of metabolic bone diseases, such as osteoporosis, Paget's disease, rheumatoid arthritis or osteoarthritis. The discovery of RANKL and its receptors RANK and OPG has finally highlighted the molecular processes in osteoclastogenesis, raising the possibility to inhibit the development of osteoclasts, rescuing bone from exorbitant resorption.

In 1997 Simonet et al. discovered a protein which exposed an osteopetrotic phenotype when overexpressed in transgenic mice (Simonet et al. 1997). Investigating even further, they found that this protein was secreted by preosteoblasts/stromal cells and was capable to inhibit osteoclast development and activation. Due to its bone-protective effects they named it osteoprotegerin (OPG). OPG belongs to the TNF receptor superfamily, although it lacks a transmembrane and cytoplasmic domain. OPG is expressed on a variety of tissues, including lung, heart, kidney, liver, stomach, intestine, brain, spinal cord, thyroid gland and bone, indicating multiple possible functions. The most prominent role of OPG has been assigned to bone protection. However, recent investigations have also proposed important functions of OPG in endothelial cell survival (Holen et al. 2005; Malyankar et al. 2000) and vascular calcification (Bucay et al. 1998; Al-Fakhri et al. 2005; Rasmussen et al. 2006).

After the identification of OPG followed the discovery of RANKL, which does not only have a huge repertoire of names (TRANCE: TNF-related activation-induced cytokine; ODF: osteoclast differentiating factor; OPGL: osteoprotegerin ligand; TNFSF11: TNF superfamily member 11), but also many facets regarding its structure, function and appearance in tissues. The names originated from the four discoverers, each one having used different approaches to identify the protein. They either searched for a ligand for OPG (Yasuda et al. 1998), screened for apoptosis-regulating genes in T cell hybridomas (Kong et al. 1999b), or found RANKL to induce osteoclastogenesis (Lacey et al. 1998) and enhance the life span of dendritic cells (Anderson et al. 1997). Kartsogiannis and colleagues detected RANKL protein and mRNA expression in a variety of tissues, including bone, brain, heart, kidney, liver, lung, intestine, skeletal muscle, mammary tissue, placenta, spleen, thymus and testis (Kartsogiannis et al. 1999). This extensive distribution of RANKL throughout the body already indicates its multiple functions, whereas the most important one is dedicated to the regulation of bone remodeling. RANKL knock-out mice reveal a severe osteopetrotic phenotype due to the absence of osteoclasts. Furthermore, defects in tooth eruption, lymph node genesis, mammary gland and lymphocyte development were reported, as well as disturbances in T cell/dendritic cell interactions and thermoregulation (Kong et al. 1999a; Martin and Gillespie 2001). RANKL is a member of the TNF superfamily and is mainly expressed in preosteoblasts/stromal cells as well as activated T cells. It exists in three isoforms: RANKL1 and RANKL2 are type II transmembrane proteins, whereas RANKL2 encodes for a shorter intracellular domain. RANKL3 is a soluble protein, supposed to be cleaved by TACE (TNFα-converting enzyme, a metalloprotease) from the transmembrane