NUCLEAR RECEPTORS IN BONE PHYSIOLOGY AND DISEASES

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Imai Y, Youn M-Y, Inoue K, Takada I, Kouzmenko A, Kato S. Nuclear Receptors in Bone Physiology and Diseases. Physiol Rev 93: 481–523, 2013; doi:10.1152/physrev.00008.2012.—During the last decade, our view on the skeleton as a mere solid physical support structure has been transformed, as bone emerged as a dynamic, constantly remodeling tissue with systemic regulatory functions including those of an endocrine organ. Reflecting this remarkable functional complexity, distinct classes of humoral and intracellular regulatory factors have been shown to control vital processes in the bone. Among these regulators, nuclear receptors (NRs) play fundamental roles in bone development, growth, and maintenance. NRs are DNA-binding transcription factors that act as intracellular transducers of the respective ligand signaling pathways through modulation of expression of specific sets of cognate target genes. Aberrant NR signaling caused by receptor or ligand deficiency may profoundly affect bone health and compromise skeletal functions. Ligand dependency of NR action underlies a major strategy of therapeutic intervention to correct aberrant NR signaling, and significant efforts have been made to design novel synthetic NR ligands with enhanced beneficial properties and reduced potential negative side effects. As an example, estrogen deficiency causes bone loss and leads to development of osteoporosis, the most prevalent skeletal disorder in postmenopausal women. Since administration of natural estrogens for the treatment of osteoporosis often associates with undesirable side effects, several synthetic estrogen receptor ligands have been developed with higher therapeutic efficacy and specificity. This review presents current progress in our understanding of the roles of various nuclear receptor-mediated signaling pathways in bone physiology and disease, and in development of advanced NR ligands for treatment of common skeletal disorders.

I. INTRODUCTION

Bone is a central element in skeletal tissues supporting the body and is a place of mineral deposit in vertebrate organisms throughout the life (152, 405). During the morphogenesis and growth stages, bone patterns and structures are formed and developed in a spatiotemporal manner under complex and highly coordinated mechanisms (6). Growing bone supports and limits overall growth of the organism. Once the skeleton is developmentally matured, the major role of bone is shifted to an endocrine function, primarily controlling mineral homeostasis through bone metabolism (130).
NRs are DNA-binding transcription factors and belong to the gene superfamily composed of 48 members in humans (299). NRs bind to specific DNA elements in the promoters and other regulatory regions of target genes (299). Activated by binding specific ligands, NRs control expression of the target genes at the transcriptional level. NR ligands include steroid/thyroid hormones, fat-soluble vitamins A and D, some endogenous metabolites, as well as synthetic chemical compounds.

Synthetic ligands for NRs may be applicable to attenuate initiation and development of some common bone diseases. It has been clinically proven, for example, that synthetic estrogen analogs called selective estrogen receptor modulators (SERMs) (221, 405) effectively ameliorate conditions of patients with osteoporosis, the most prevalent bone disease in the developed countries (343). Moreover, recent studies have revealed that the bone functions as an endocrine organ, producing several secreted proteins important for the homeostasis (130). Physiology of the bone tissue is controlled by a wide range of regulatory factors, including steroid hormones, vitamin D, parathyroid hormone (PTH), various cytokines, and growth factors (39, 391).

A. Bone Cell Differentiation

1. Osteoblasts

The osteoblast functions are crucial for the bone formation, producing extracellular bone matrix proteins, type I collagen, and noncollagenous proteins such as osteocalcin and osteopontin. Osteoblast-secreted noncollagenous proteins contribute to mineralization of the bone matrix with hydroxyapatite by binding calcium and phosphate, thereby regulating depositions of minerals. Osteoblasts are differentiated from mesenchymal stem cells. It has been reported that their differentiation is tightly regulated by several transcription factors such as Runx2, Osterix, ATF4, and some others (209).

Runx2 was identified as the first osteoblast-specific transcription factor and is a member of the Runt-containing transcription factor family (100, 236). The family members bind to DNA through their Runt domain and can also interact with various nuclear proteins through other structural domains. The significance of Runx2 function in osteoblast differentiation was confirmed by the phenotypes of mouse mutants lacking Runx2 that lacked osteoblasts and bone formation (236). Moreover, the analyses of human genetic studies revealed that Runx2 is the dominant gene in cleidocranial dysplasia (257, 333).

Runx2 exerts its regulatory action as a transcription factor, the function of which can be enhanced by interaction with other nuclear proteins. For example, Schnurri-3 is a zinc finger-containing protein involved in the recombination of immunoglobulin genes. Schnurri-3-deficient mice exhibited increased bone mass attributed to accelerated bone formation due to accumulation of Runx2 proteins in osteoblasts (198).

Osterix is another essential zinc finger-containing transcription factor that is important in osteoblast differentiation.
The transcription of Osterix appears to be regulated by Runx2, since Osterix is not expressed in Runx2-deficient cells. Moreover, Osterix is a key factor in the differentiation from osteoblast progenitor cells to osteoblasts. Indeed, Osterix-deficient mice lack mature osteoblasts and exhibit severe impairment in bone formation (340). In addition, the nuclear factor of activated T cells cytoplasmic 1 (Nfatc1), a pivotal regulator in differentiation of bone resorbing osteoclasts, and Osterix form a complex that binds to DNA, and this association is crucial for the transcriptional activity of Osterix. Thus Nfatc1 and Osterix appear to coordinately regulate osteoblastic bone formation. (235). Furthermore, NO66, a Junomji C domain-containing protein, directly interacts with Osterix and inhibits Osterix-mediated transcription by modulating the status of histone modifications in promoter regions (439). This shows that transcriptional activity of Osterix can be regulated by transcriptional co-factors including epigenetic regulators.

ATF4 is a transcription factor that belongs to the CREB family of B ZIP proteins. Apparently, ATF4 is important for the maintenance of osteoblastic functions (549), since impairments of ATF4 function can cause human genetic skeletal diseases such as Coffin-Lowry syndrome and neurofibromatosis type I (107, 549).

2. Osteocytes

Osteocytes are located within the bone matrix (31). Osteocytes differentiate from osteoblasts. However, in contrast to osteoblast differentiation from mesenchymal stem cells, the precise molecular mechanisms of “osteocytogenesis” are essentially unknown, especially in regard to key regulatory transcription factors. Although osteocytes constitute more than 90% of the cells in bone, their function is not fully understood, nor is the mechanism of their differentiation. Some reports have suggested that osteocytes can differentiate directly from osteoblasts in the absence of positive regulation (301). Osteocytes differ from osteoblasts in their morphology and array of expressed proteins, as well as in their location. Bone matrix embedded osteocytes form dendritic processes that are extended to the mineralization front and are important for the extensive osteocyte-osteocyte interaction. Osteocytes appear to function as mechanosensors cells communicating through this complex dendritic network. Bone mass can be increased by appropriate intensity, frequency, and amount of loading (404, 411, 496). At the same time, mechanical loading or unloading affects gene expression profiles of osteocytes in vivo (143, 442), supporting the conclusion that osteocytes may act as mechanosensors in addition to their various functions in the maintenance of bone and mineral metabolism (32, 342, 488). For example, mineralizing and mature osteocytes can produce dentin matrix protein 1 (Dmp1), which controls fibroblast growth factor 23 (FGF23) expression in osteocytes (119, 280). FGF23 plays an important role in phosphate metabolism (64, 440, 559) through its binding with Klotho as a coreceptor (498). Patients with Dmp1 loss-of-function mutations exhibited autosomal recessive hypophosphatemia rickets (ARHR) with an increased serum level of FGF23 (119, 280). Consistently, Dmp1 null mice recapitulate the ARHR phenotypes (119, 280). Furthermore, osteocytes can express the sclerostin protein, a product of the SOST gene. Sclerostin acts as an extracellular Wnt signal antagonist by binding with LRP5/6, a Wnt co-receptor in osteoblasts (77, 541). In this way, osteocytes can negatively regulate bone formation through inhibition of Wnt signaling. It has been recently reported that osteocytes express receptor activator of NF-κB ligand (RANKL), an essential cytokine for osteoclastogenesis, and facilitate osteoclast formation (341, 538). Thus osteocytes might regulate bone and mineral metabolisms by both systemic and local influences.

3. Osteoclasts

Osteoclasts are multinucleated cells that can resorb bone matrix. Osteoclasts are derived from hematopoietic stem cells, specifically the monocyte/macrophage lineage of cells. Macrophage-colony stimulating factor (M-CSF) is indispensable for osteoclast differentiation and function (234, 555). M-CSF can induce receptor activator NF-κB (RANK) expression in osteoclast progenitor cells. RANKL is a key factor in stimulation of osteoclast differentiation, while osteoprotegerin (OPG) inhibits osteoclastogenesis acting as a soluble decoy receptor of RANK (475). RANKL and OPG were originally identified as the osteoclast differentiation factors expressed by osteoblasts (466, 551). However, recent studies have revealed that RANKL can be expressed by various types of cells including osteoblasts, osteocytes, and T-cells (237, 238) in both physiological and pathological conditions. The binding of RANKL with RANK induces trimerization of RANK followed by the recruitment of TRAF (TNF receptor-associated factor) family adaptor molecules, with particular TRAF6 known as the major adaptor protein (277, 338). Next, activation of TRAF6 by RANKL-RANK stimulation leads to activation of NF-κB and mitogen-activated protein kinases (MAPKs), such as p38 and JNK (JUN NH2-terminal kinase). These RANKL-RANK-induced signaling pathways stimulate complex formation of transcription factors, such as activator protein 1 (AP1) by inducing one of a member of AP1 complex, c-Fos (315, 477). The induction of c-Fos is regulated by CaMK IV, CREB (cAMP response element binding protein), and NF-κB activation (417). RANKL stimulates the expression of Nfatc1, a master transcription factor for osteoclast differentiation, through both the TRAF and c-Fos pathways. NFATc1 can induce the expression of a number of osteoclast differentiation marker genes such as those encoding tartrate resistant acid phosphatase (TRAP) and cathepsin K (Ctsk), enzymes critical for the osteoclastic bone resorbing functions, as well as NFATc1 itself (475). NFATc1 was originally identified in T cells, as suggested by its name. However, it has been shown that NFATc1 has an important
and essential role in osteoclastogenesis (476). The overlap of molecules and signaling pathways between two research fields, i.e., bone biology and immunology, generated a new research field, “osteoimmunology” (475). The concept of osteoimmunology is helpful to understand both the complex immunological mechanisms in bone biology as well as involvement of bone cells in immunological reactions and processes.

Multinucleated mature osteoclasts are formed by differentiation of fused mononuclear osteoclast progenitor cells. This multinucleation step is essential for the bone resorbing function of osteoclasts. Yagi et al. (542) identified a key molecule of osteoclastic fusion and named it dendritic cell-specific transmembrane protein (DC-STAMP). Monocytes/macrophages derived from DC-STAMP null mice can be differentiated into osteoclast progenitors, but cannot undergo fusion and cannot differentiate into mature multinucleated osteoclasts even in the presence of RANKL. Interestingly, DC-STAMP null mice exhibit high bone mass similar to those observed in patients with osteopetrosis (542).

**B. Bone Remodeling**

During the embryogenesis, skeletal formation involves bone modeling and growth. In a mature skeleton, bones continue to undergo remodeling by osteoclastic bone resorption and osteoblastic bone formation (FIGURE 1) (208). Osteoclasts resorb old bone matrix and make resorbed cavities, termed “Howship lacunae.” In this concave surface, osteoblasts produce bone matrix composed of type I collagen, osteocalcin, osteopontin, and some other proteins to fill the lacunae. This unmineralized bone matrix is called an osteoid. On the surface of the osteoid, osteoblasts mineralize the bone matrix with hydroxypatite to complete the remodeling of the bone. In this remodeling process, it is critical that bone resorption and bone formation are balanced to maintain the homeostasis of bone tissue. If this balance equilibrium is altered, a pathological condition may develop.

If osteoblastic bone formation overtakes osteoclastic bone resorption, it would lead to an increase in the bone mass. Elevated bone mass can be attributed either to a decrease in the bone resorption [due to the failures of osteoclastogenesis and/or osteoclast functions (390)] or an increase in the bone formation due to abnormally high activation of osteoblasts (77, 502). Deficiencies of osteoclastogenesis and/or osteoclast functions cause osteopetrosis or pycnodysostosis that may develop as a result of functional deficiency of some of the key molecules, such as M-CSF (555), RANKL (449), RANK (148, 269), c-Fos (315), NF-κB (180), Ctsk (133, 268, 414), carbonic anhydrase (445), and H+ -ATPase (127, 239, 450), as observed in humans with genetic diseases and knockout mice. A hyperactivation of bone formation causes osteosclerosis or hyperostosis due to constitutively active mutations in mediators of the TGF-β and Wnt signaling, as it has been revealed by genetic analyses in patients (41, 502, 533) and genetically engineered mutant mice (77).

![FIGURE 1. Bone cellular structure. Three major types of cells constitute bone tissue: osteoclasts are bone resorbing cells derived from a monocyte lineage of the hematopoietic stem cells, osteoblasts are bone forming cells descended from the mesenchymal stem cells, and osteocytes are bone matrix embedded cells originated from osteoblasts. Mature quiescent osteoblasts on the bone surfaces are distinguished as bone lining cells.](image-url)
A prevalence of osteoclastic bone resorption over osteoblastic bone formation increases a risk of development of osteopenia or osteoporosis. Osteoporosis is well known as a bone metabolic disease associated with high risks for bone fractures that often lead to prolonged incapacitation, although patients may be unaware of the disease because of a lack of clear symptoms (408). A large population of postmenopausal women suffer from high turnover osteoporosis due to estrogen deficiency (451). Although more prevalent among women, elderly individuals of both sexes are at risk of developing osteoporosis (222). Osteoporosis may be also caused by a side effect of glucocorticoid treatment of autoimmune diseases, such as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA) (520). Furthermore, astronauts often suffer from osteoporosis after prolonged space flight (220, 250, 433). Long-term bed rest also reduces bone mass, and malnutrition may worsen long-term immobility-induced osteoporosis (518). Osteoporosis is a more significant and frequent clinical problem than high bone mass pathologies. To treat osteoporosis, many kinds of drugs have been tested and applied in clinical practice. Almost all currently used antiresorptive pharmaceuticals, such as bisphosphonates (116), selective estrogen receptor modulators (SERMs) (400), vitamin D analogs (310, 312) and anti-RANKL antibody (78, 448) act as anticatabolic agents (408). In contrast, anabolic therapeutic agents enhance bone formation. Currently, PTH is the only anabolic agent approved for the clinical use. However, several kinds of anabolic compounds, such as Ca sensing receptor antagonists, anti-sclerostin antibody and anti-Dkk1 antibody are undergoing preclinical or clinical trials (86). In addition, strontium ranelate may potentially produce a dual therapeutic effect owing to its anti-resorptive and anabolic properties (121).

As described above, the relationship between osteoblasts and osteoclasts, termed “coupling,” is particularly important in the maintenance of bone homeostasis in remodeling. Differentiation of osteoclasts from monocytes/macrophages is regulated by M-CSF, RANKL, and OPG. These pivotal factors for osteoclast differentiation are mainly derived from osteoblastic lineage cells (42). In studies on the osteoblastic-osteoclastic coupling, regulatory signals from osteoblasts control osteoclasts as well as signals from osteoclasts to osteoblasts have been investigated. There are at least three possible mechanisms of the coupling. First, the factors released from the bone matrix by osteoclastic resorption might stimulate osteoblastic bone formation. Second, secreted factors from osteoclasts might control osteoblastic bone formation. Third, direct contact between osteoclast and osteoblast might regulate the differentiation and/or functions of both cell lineages.

Recent studies have clarified some aspects of the coupling. Active TGF-β1 released from bone matrices by osteoclastic bone resorption facilitates bone formation by recruitment of bone marrow mesenchymal stem cells to the bone resorptive sites. This recruitment is mediated by the SMAD signaling pathway (481) and can be modulated by PTH signaling (385, 534). It has been reported that PTH signals through G(α)s require LRP6, Wnt ligand coreceptor, to accumulate intracellular cAMP (508). Thus the factors released from the bone matrix may regulate the coupling. On the other hand, osteoclast conditioned medium can enhance mesenchymal cell migration and differentiation, suggesting that osteoclasts may secrete some factors to recruit osteoprogenitors to the site of bone remodeling, possibly through S1P and BMP6, and stimulate bone formation by activation of Wnt/BMP pathways (364). In addition, Zhao et al. (565) revealed that signaling through Ephrin B2 (Efnb2, an NFATc1 target gene) in osteoclast precursors represses osteoclastogenesis by inhibiting the c-Fos-NFATc1 cascade, whereas signaling through Eph receptor B4 (Ephb4) in osteoblasts facilitates osteoblastic differentiation. Overexpression of Ephb4 in osteoblasts increases bone mass in transgenic mice (314, 565). These and similar recent reports represent significant progress in our understanding of the mechanisms of tightly coupled processes of osteoclast-mediated bone resorption and osteoblast-mediated bone formation.

III. NUCLEAR RECEPTORS

A. Nuclear Receptor Gene Superfamily

NRs are DNA-binding transcription factors and form the NR gene superfamily that is well conserved among metazoans from C. elegans to human (299). In human, 48 members of the superfamily have been identified, and each of the known NR gene loci is precisely mapped on the chromosomes (224). NR family members are classified into two groups. One group consists of NRs for which the endogenous ligands have been identified. NRs of the other group are referred to as orphan NRs, as their endogenous ligands remain unknown, if exist at all. Certain NRs were originally designated as orphan NRs, but later were renamed as “adopted” NRs when their potential endogenous ligands had emerged (137). It is still unclear if all of the known mammalian orphan NRs require an endogenous ligand for the activation. Since most of the known insect NRs appear to be “orphan,” and NRs in metazoan are assumed to evolutionarily derive from the same ancestral orphan NR, the presence of orphan NRs without functional endogenous ligands seems very likely in humans. However, synthetic ligands capable to modulate the function of some of these “ligandless” human orphan NRs have been recently identified (172, 452, 453). These findings raise a possibility that all NRs may be potential pharmacological targets, irrespective of the absence of endogenous ligands.
B. NR as a Gene Regulator

As DNA-binding transcription factors, NRs recognize and bind specific DNA element sequences in the target gene loci to transcriptionally control expression of particular sets of target genes (FIGURE 3) (225, 299). However, presence of cis response elements might be insufficient for NR binding to occur, since recent ChIP-sequencing analysis revealed that the numbers of consensus or close to consensus NR binding genomic sequences were consistently greater than the numbers of actual NR binding sites. Significantly, most of the NR binding sites have been mapped in the vicinity of DNA binding sites for other transcription factors (46, 286, 510). Thus it appears that recruitment of NRs to chromatin for gene regulation may also require their interaction with other classes of DNA binding transcription factors that presumably serve as anchors for NR association with chromatin. NR bound to chromatin may regulate gene expression as a transcriptional activator or repressor, depending on the presence of ligand and/or other transcription factors, and context of a given target gene promoter and/or enhancer elements (215, 553). Consistently, the mode of NR action may diverge in a promoter-, enhancer-, and/or cell-context-dependent manner. In general, NRs mediate most of the biological actions of their cognate ligands through positive and negative transcriptional regulation of target genes in response to the ligand binding to NRs. On the other hand, orphan NRs act as constitutive transcriptional regulators, either activators or repressors (215, 553). DNA-binding transcription factors bound to chromatin alongside of NR may be effective to modulate transcriptional activity of this NR. The products (mRNA or miRNA) of NR target genes

FIGURE 2. Nuclear receptor (NR) superfamily. NR superfamily members are divided into two main groups depending on the identification of endogenous ligands. NRs for which specific cognate ligands have been identified are known as endocrine NRs (top panels). NRs of this group bind to specific DNA elements as homodimers (top left) or heterodimers with RXR (top right). The other group is referred to as orphan NRs, for which endogenous ligands remain unknown (bottom panels). The orphan NRs bind to specific DNA elements as monomers or homo- and heterodimers. ER, estrogen receptor; AR, androgen receptor; PR, progesterone receptor; GR, glucocorticoid receptor; MR, mineralocorticoid receptor; VDR, vitamin D receptor; RAR, retinoic acid receptor; PPAR, peroxisome proliferator-activated receptor; LXR, liver X receptor; FXR, farnesoid X receptor; SXR/PXR, steroid and xenobiotic receptor/pregnane X receptor; RXR, retinoid X receptor; CAR, constitutive androstane receptor; TR, thyroid hormone receptor; Nurr1, nuclear receptor related 1; Nor1, neuron-derived orphan receptor 1; ERR, estrogen-related receptor; COUP-TF, chicken ovalbumin upstream promoter-transcription factor; SHP, small heterodimer partner; HNF4, hepatocyte nuclear factor-4; TLX, homolog of the Drosophila tailless gene; PNR, photoreceptor cell-specific nuclear receptor; SF-1, steroidogenic factor 1; LRH, liver receptor homolog; GCNF, germ cell nuclear factor; TR2/4, testicular receptor 2/4; DAX-1, dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1; EAR-2, V-erbA-related protein 2.
exert biological action as downstream factors of the NR-mediated signaling pathway (20, 74, 544) (FIGURE 3).

C. NR Structure and Function

As the members of the same gene superfamily, all NRs bear significant structural and functional similarities. In NR protein primary structure, several distinct functional domains have been identified and designated A to E from the NH₂-terminal end (FIGURE 4) (299). The hallmark of the NR gene superfamily members from different species is a presence in the protein middle region, or C domain of two zinc fingers formed by four cysteine residues. This C domain is evolutionary highly conserved and serves as a DNA binding domain (DBD). Ligand binding domain (LBD) localizes in a less conserved E domain that is composed of 12 α-helices forming a hydrophobic cave in the center to accommodate the ligand. Based on X-ray structural analysis, the LBD domain is depicted as a globular structure. It has been established that the ligand binding triggers drastic shift in the positioning of the most COOH-terminal α-helix 12 (H12), resulting in the co-repressor switching (106, 205, 428). Ligand binding also induces dimerization followed by nuclear translocation of NR dimers. Both the E and A/B domains function as docking sites for transcriptional coregulators, and thereby the activities of these domains in transcriptional controls are cell type specific, presumably owing to a variety of coregulator species expressed in a given cell type (482, 492). The transcriptional activity of E and A/B domains can be also modulated through posttranslational protein modifications (PTMs) (usually acetylation, phosphorylation or ubiquitination) in response to signals from activated cell membrane receptors (FIGURE 3) (166, 213, 279).
D. Mechanism of Transcriptional Controls by NRs

Expression of NR target genes is regulated at the transcriptional level. The principles of transcriptional activation of target genes by NRs are comparatively well established in terms of transcriptional events with the characterization of transcriptional NR coregulators (215, 553), whereas the molecular basis underlying NR-mediated transcriptional repression is far from being clear (334, 373).

Ligand binding to NR causes dissociation of transcriptional corepressors and association of coactivators through ligand binding-induced conformational alterations of NR, including drastic shifting of the α-helix 12 position in the LBD (106, 428). NR corepressors form multisubunit complexes, in which histone deacetylases (HDACs) represent functional key subunits (156, 215, 553). Since HDACs catalyze histone deacetylation, HDAC-associated unliganded NRs are potent to inactivate the chromatin in the vicinity of NR binding sites. Consistently, histone acetyltransferases (HATs) are known to coactivate NR-induced transcriptions through histone acetylation and also to form multisubunit complexes (206, 215, 553). In addition, general NR coactivators represent physical mediators bridging NR with basic transcription machinery for promoting efficient transcription by RNA polymerase II (389, 558).

IV. NUCLEAR RECEPTORS AND BONE METABOLISM: STEROID HORMONE RECEPTORS

A. Estrogen Receptors

Two subtypes of Estrogen Receptors (ERs), α and β, exert biological effects of estrogens (74, 243, 299). Nuclear estrogen-bound ERs mediate the genomic actions of estrogens through estrogen response element (ERE)-dependent transcription of target genes (46, 47, 213, 214). In contrast, immediate estrogen responses, so-called nongenomic actions, probably involve cytoplasmic ERs and/or uncharacterized estrogen receptors on the cell membrane (329).
Ligand-activated ERα and ERβ specifically bind to ERs in the regulatory regions of the target genes as homo- (α-α, β-β) and/or hetero- (α-β) dimers (243, 299, 377). There is no significant difference between ERs and ERβ in the binding affinity for endogenous estrogens. However, ER subtypes seem to have different affinities for SERMs (149). Compared with ERα, ERβ displays a significantly lower ability of hormone-induced transcriptional activation when bound with SERMs (275). Therefore, ERβ can be considered as a possible dominant negative equivalent of ERα that modifies the induction of endogenous estrogen target genes. The molar or quantitative ratio of ERβ to ERα in a cell may indicate a cell’s sensitivity and the extent of its biological responses to estrogens (177). Expression patterns of ERα and ERβ overlap in various types of cells, organs, and tissues.

1. Estrogen functions in bone

Estrogens are female steroid hormones and crucial regulators in various biological processes such as the development and maintenance of female reproductive organs and the maintenance of bone. Postmenopausal osteoporosis caused by estrogen deficiency is one of the most widely recognized locomotive diseases, especially in developed countries. Osteoporosis in both genders is now considered a serious pathological condition of middle age and elderly people because of the high risk of fracture, often leading to high morbidity and mortality (105, 343).

In women with impaired estrogen signaling or estrogen deficiency, there is a noticeable reduction of bone mass due to increased or imbalanced bone resorption versus bone formation. The postmenopausal osteoporotic bone phenotype can be experimentally induced in female rodents by ovariectomy (OVX) followed by estrogen depletion (152, 153). Clinically available estrogens and SERMs are effective in normalizing increased bone resorption and can rescue bone loss in osteoporotic patients and ovariectomized animals (96, 153, 344). Multiple genetic studies and clinical observations indicate that male patients lacking either estrogen biosynthesis or ERα function exhibit the symptoms of osteoporosis (200, 447). Thus it is obvious that estrogens exert osteoprotective effects and play a pivotal role in the maintenance of skeletal homeostasis in both genders.

To clarify the physiological functions of ERs in vivo, the first mice lacking ERs were generated in the early 1990s (74). However, neither male nor female of these ER-deficient mice displayed typical osteoporotic phenotypes (74, 469, 531). Consequently, the roles of ERs in bone health remained unclear, and indirect mechanisms through extraskeletal tissues have been assumed to account for the osteoprotective actions of estrogens (357, 560).

2. Conventional ER-deficient mice

The first generated ERα knockout mice (ERαKO) displayed various phenotypic abnormalities. In accord with previous in vitro and in vivo evidences, ERαKO exhibited poorly developed female reproductive organs, and their phenotypes were similar to ovariectomized mice, except bone phenotypes (74). However, the first ERαKO expressed shortened ER transcripts, and residual ERα activity was postulated to exert the endothelial effects of estrogens (368). Later, complete ERα knockout mice (ERα−/−) were generated without detectable ERα transcripts (103). Phenotypes of these ERα−/− mice substantiated the significance of ERα for female reproductive organs reported earlier in the ERαKO mice (103). Several groups have generated mice with a disrupted ERβ gene. However, the reported phenotypes of differently generated ERβ-deficient mice have been inconsistent with the abnormalities of estrogen target tissues (177, 531). The inconsistencies between these initial studies might be due to incomplete knockouts of the ERβ (ERβKO), which would permit expression of partial transcripts. Although the phenotypic differences reported by these studies could be based on residual ERβ activity, the molecular mechanisms of the differences in observed phenotypes remain unclear. Interestingly, recently generated complete ERβ knockout (ERβ−/−) mice exhibited significantly fewer defects (11, 103). Since ERα−/− mice display much more severe abnormalities in estrogen target tissues than ERβ−/− mice (11, 74, 90, 469, 531), it has been postulated that the major physiological actions of estrogens are mediated through ERα, which appears to be the primary estrogen receptor.

Unlike other estrogen target tissues or organs, the osteophenotype of female mice was not significantly altered by disruption of ERα (ERα−/−), ERβ (ERβ−/−), or both (ERαβ−/−) ER genes (274, 437, 532). These results were unexpected, because OVX in the same mouse strains decreased bone mass due to higher bone turnover, resembling that seen in estrogen deficiency-induced postmenopausal osteoporotic women (152, 343, 401, 560).

Unexpectedly, female ERα−/− mice displayed increased bone mass with low bone turnover, while ovariectomized females in the same strain exhibited decreased bone mass due to increased bone resorption (274, 437). The absence of the negative feedback loop in estrogen synthesis due to the lack of ERα in the hypothalamus in ERα−/− mice leads to a remarkably high level of serum testosterone, a major estrogen precursor. The absence of osteoporotic phenotypes in mice lacking ERα might be due to an excessive amount of circulating testosterone that results in enhanced androgen signaling and osteoprotective effects (401). Supporting this possibility, OVX decreases bone mass in ERα−/− mice, whereas androgen administration increases bone mass in ovariectomized ERα−/− (73, 436).
**ERβ**-/- female mice exhibit slightly decreased bone resorption and increased trabecular bone mass without changes of serum levels of sex steroid hormones (437). Since bone loss in ovariectomized **ERα**-/- females can be partially rescued by estrogen treatment (436), it is possible that **ERβ** partially mediates the osteoprotective actions of estrogens (436, 531). The levels of circulating sex steroid hormones were also elevated in double knockout (**ERαβ**-/-) mice, similar to that observed in **ERα**-/. In contrast to **ERα**-/- or **ERβ**-/- mice, **ERαβ**-/- mice exhibited a significant reduction in the bone mass, but it was less severe than that observed in OVX wild-type females (73, 274, 437). Although these findings suggest coordination between **ERα** and **ERβ** in the osteoprotective action of estrogens, female mice lacking both **ERα** and **ERβ** fail to recapitulate the bone phenotype of estrogen-deficient postmenopausal osteoporosis. It can also be postulated that androgen receptor (AR) may partially compensate for the absence of ERs in these mice at high levels of serum sex steroid hormones.

Interestingly, male mice disrupted in one (**ERα**-/- or **ERβ**-/-) or both (**ERαβ**-/-) of the ER receptors displayed no clear bone mass alteration. Furthermore, male **ERα**-/- mice exhibited increased trabecular bone mass due to decreased bone resorption with elevated serum level of testosterone (73, 274, 437). This indicated that the high level of serum testosterone in male **ERα**-/- mice counteracts diminished estrogen action through facilitated AR signaling in the bone (217, 436). However, these observations are incompatible with severe osteoporosis and unfused epiphyses observed in male patients harboring nonfunctional **ERα** due to hereditary mutations in spite of high level of circulating testosterone (74, 200, 503). Estrogen treatment could not rescue the phenotypes of these male patients.

Aromatase (CYP19) is an enzyme that catalyzes conversion of androgens into estrogens. Clinical observations of reduced bone mass in aromatase-deficient male patients support the idea that **ERα**-mediated estrogen signaling plays a pivotal role in the maintenance of the male skeleton (38, 294, 447). As serum estrogen levels are apparently lower in males than in females, the local synthesis of estrogens from testosterone by aromatase appears to maintain physiological osteoprotective estrogen action in male bones. Aromatase-deficient male patients displayed an osteoporotic bone phenotype and unclosed epiphyses as do **ERα**-deficient men (200). As expected, such skeletal defects could be compensated by estrogen treatment only in aromatase-deficient, but not in **ERα**-deficient patients (356). Bone loss phenotype was experimentally recapitulated in male aromatase-deficient mice (18, 124); however, increased bone length due to unfused epiphyses in human male patients was not observed in male aromatase-deficient mice. This contradiction is presumably explained by the innate absence of epiphyseal plate closure in mice (18, 503). Thus the osteoporotic bone phenotypes observed in inherited aromatase-deficiency male patients and in experimentally generated aromatase-deficient male mice indicate that aromatase-mediated locally synthesized estrogens are critical for the maintenance of bone health in males.

### 3. Osteoclast-specific ERα-deficient mice

As described above, studies of systemic estrogen deficiency and impaired estrogen signaling in experimental animal models have not identified the osteoprotective mechanisms of estrogens. In fact, systemic disruption of estrogen signaling in whole animals by conventional knockout of the ER genes disturbs the endocrine system and can lead to potential pathological outcomes such as inflammatory cytokine production (357, 560). Thus it appeared that a bone cellspecific knockout of the ER genes was essential to directly evaluate the role of estrogen signaling in the bone without influences from its systemic actions or possible secondary effects.

The osteoprotective action of estrogens is associated with a regulation of bone resorption and maintenance of normal bone remodeling. Therefore, we selectively disrupted the **ERα** gene in mature osteoclasts (339). As cathepsin K is abundantly expressed only in the mature osteoclasts (268), the Cre recombinase gene was knocked into the gene locus of cathepsin K, and the resulting Cre knock-in mouse line (Ctsk-Cre) was shown to express Cre recombinase at detectable and effective levels only in sufficiently developed osteoclasts, thus Ctsk-Cre/+ mice seem to be heterozygous Ctsk knockout mice. Osteoclast-specific **ERα** knockout mice (**ERα**D0/D0) were generated by crossing the Ctsk-Cre mice with floxed **ERα**-deficient mice that were previously used to obtain complete **ERα** knockout mice (**ERα**-/-) (103). As expected, **ERα**D0/D0 mice displayed neither clear alterations of circulating sex steroids, nor follicle stimulating hormone (FSH), nor phenotypic abnormalities in growth and reproduction regardless of gender. Significant bone mass reduction in trabecular bone with high turnover bone metabolism was observed only in the **ERα**D0/D0 females (and not males) at 8 wk of age. OVX induced only a slight bone loss in the **ERα**D0/D0 mice when compared with the bone loss in the ovariectomized control mice. Moreover, 17βestradiol failed to recover bone mass in the ovariectomized **ERα**D0/D0 females.

Note that bone mass reduction is evident primarily in trabecular areas in estrogen-deficient women and female rodents. Thus the osteoporotic phenotypes observed in **ERα**D0/D0 females seem to substantiate the idea that the osteoprotective action of estrogens is mediated, at least in part, by osteoclastic **ERα** in females (339). We found that the pro-apoptotic actions of estrogens in mature osteoclasts underlie the antiresorptive effects of estrogens in bone since estrogens can initiate osteoclastic apoptosis through induction of the Fas ligand gene (Fasl) (339). Furthermore, bone phenotypes of osteoclastic **ERα**-deficient mice produced by...
using different Cre expression mice lines have confirmed that estrogens regulate the life span of osteoclasts and exert their osteoprotective action through the osteoclastic ER\(\alpha\) (305). These observations agree with previous evidences that estrogen deficiency in mice induced by OVX leads to reduced apoptosis and extended life span of the osteoclasts (171).

Osteoporotic phenotypes observed in male patients harboring loss-of-function ER\(\alpha\) mutation (18, 124, 447) would suggest that the osteoprotective action of estrogens should be also crucial in male bone physiology. However, no detectable abnormality has been observed in bone tissue in ER\(\alpha^{Ox/Ox}\) male mice (339). It is not clear why ER\(\alpha^{Ox/Ox}\) males do not display abnormal bone phenotypes, and we can only speculate about possible mechanisms of osteoprotective estrogen actions in males. First, high levels of circulating androgens and activated AR may play pivotal roles in male bone formation and resorption, even in the absence of ER\(\alpha\). Second, in Ctsk-Cre mice, ER\(\alpha\) is likely disrupted only in mature osteoclasts, whereas activated ER\(\alpha\) may fulfill crucial functions at earlier stages of osteoclastogenesis regardless of sex. There may be stage-specific roles of ER\(\alpha\) action during osteoclast differentiation and maturation that differ between males and females. Besides controlling the life span of osteoclasts, estrogens may also have inhibitory effects on osteoclastogenesis and/or osteoclastic function in vitro (426, 460). Generation and analyses of differentiation stage-specific osteoclastic ER\(\alpha\) knockout mice may resolve these issues. Third, ER\(\alpha\) in osteoblasts might mediate the osteoprotective actions of estrogens, and the osteoblastic ER\(\alpha\) might have a dominant function in estrogen osteoprotective action in males. Estrogens have been shown to stimulate osteoclastogenesis in vitro (351). Furthermore, Krum et al. (246) reported strong evidence that Fasl is a direct ER target gene in cultured osteoblasts, as determined by genome wide ChIP on chip analysis. Thus it is possible that estrogens could facilitate apoptosis of mature osteoclasts via induction of Fasl expression in both osteoclasts and osteoblasts (245). Previous reports demonstrating ant apoptotic action of estrogens on osteoblasts (240, 241) suggest the existence of different pathways or mechanisms of estrogen action in these types of bone cells. The existence of cell and tissue-specific regulation of ER transcriptional activity has been recently supported by demonstration that different pioneer factors facilitate ER chromatin binding in different types of cells. While FOXA1 has been shown to be critical for ER\(\alpha\) transcriptional action in breast cancer and liver cells (173, 202), GATA4 appears to act as a primary pioneer factor for ER recruitment to target chromatin loci in osteoblasts (327). Generation of osteoblast-specific ER\(\alpha\) knockout mice is, therefore, essential to elucidate the impact of osteoblastic ER on bone metabolism and cell specificity of ER signaling pathways in osteoblasts and osteoclasts. In addition, there are other types of cells in bone, such as bone marrow cells and osteocytes that remain to be explored for the involvement of their ERs in the osteoprotective action of estrogens, especially in males.

Thus estrogens exert osteoprotective actions in both sexes. Important features observed in postmenopausal osteoporosis, such as bone loss with high bone turnover, have been recapitulated in osteoclast-specific ER\(\alpha\) knockout female mice (339). This observation is in apparent contrast to the phenotypes of mice with conventional or systemic ER knockouts that displayed increased bone mass. In addition, it seems that osteoclastic ER\(\alpha\) mediates estrogen-dependent reduction of bone resorption through induction of osteoclastic apoptosis in females. Primary cultured osteoclasts derived from bone marrow cells of males and females respond in the same way to estrogen. However, osteoclast-selective ablation of ER\(\alpha\) in male mice failed to recapitulate bone defects or elongated long bones due to unfused epiphyses (339) that were observed in male patients with dysfunctional estrogen signaling (200, 447). This contradiction indicates gender-specific mechanisms in the osteoprotective actions of estrogens in vivo. For example, in male bones, positive effects of estrogens on bone mass might be preferentially mediated by ER\(\alpha\) in other types of cells rather than through the antiresorptive action of osteoclastic ER\(\alpha\), which is pivotal in females. This hypothesis can be examined by comparison between various cell type-specific ER\(\alpha\) knockout phenotypes in males and females to clarify sex-specific molecular and cellular mechanisms of anabolic (not antinablic) action of estrogens in the skeleton. The compensatory effects of AR with high concentrations of circulating androgens may elucidate different physiological results of osteoclast-specific ER\(\alpha\) knockouts in male mice. This possibility may be clarified with generation of double osteoclast-specific AR and ER\(\alpha\) knockout.

4. Indirect osteoprotective action of estrogen

Systemically circulating estrogens affect the function of many types of cells and tissues. Thus it is possible that bone mass regulation by estrogens could be influenced by secondary effects through other estrogen target organs that in response to estrogens may secrete bone remodeling factors in an endocrine or paracrine manner. It has been postulated that the osteoprotective effects of estrogens might be mediated by such estrogen-induced factors. For example, insulin-like growth factors (IGFs) that are known to stimulate osteoblastogenesis, are secreted mainly by the liver. Their effects on the skeletal development are influenced by the growth hormone (GH), especially during longitudinal growth stages (435, 441, 503). Thus, IGFs production induced by estrogens might be a part of the osteoprotective effects of estrogen. However, the molecular mechanism of estrogen-induced IGF regulation in bone is largely unknown.
FSH can mediate indirect estrogen action on maintenance of bone mass (560). FSH is an upstream pituitary hormone that stimulates estrogen synthesis in the ovary. FSH secretion is under negative-feedback regulation of circulating estrogens that activate ERs in the hypothalamus, consequently suppressing FSH production. Estrogen deficiency in postmenopausal women is usually associated with increased levels of circulating FSH. It has been reported by Sun et al. (468) that FSH signaling-defective female mice displayed increased bone mass with low bone turnover and without significant alteration of serum estrogen levels. This group also showed that FSH positively affected osteoclastogenesis. Thus they hypothesized that increased FSH levels caused by estrogen deficiency could reduce bone mass via acceleration of the osteoclastogenesis (468). However, there is some inconsistency in clinical reports on the relationship between levels of FSH and bone mass and mineral density in postmenopausal women (92, 147, 457). Therefore, it is necessary to determine the effects of FSH on the bone metabolism under normal physiological concentrations of circulating sex steroids.

Estrogen deficiency-induced osteoporosis is associated with persistently high turnover bone metabolism, which causes progressive bone loss. This indicates that osteoclastogenesis and/or osteoclastic activities are enhanced under decreased estrogen concentrations. Since it is well known that inflammatory cytokines are able to induce osteoclastogenesis in vitro, various cytokines expressed by cells other than osteoblasts or osteoclasts have been examined for a possible contribution to osteoclastogenesis induced by estrogen deficiency (357, 405). From this point of view, immune cells in the bone marrow could mediate bone remodeling via paracrine secretion of various pro- and/or antiosteoclastogenic cytokines. Among immune cells, T cells are considered as the most likely regulators of bone resorption. Adaptive immune responses are activated by estrogen deficiency, leading to stimulated production of interleukin-7 and IGF-1 by activated T cells residing in bone followed by increased secretion of interferon-γ (IFN-γ) (406, 412). IFN-γ is one of the factors related to osteoclastogenesis in concert with locally produced RANKL and TNF (477, 483). In cell culture systems, it was shown that these cytokines activated transcription factors, such as AP-1 and NF-κB, known to promote osteoclastogenesis. Recent study suggests that RANKL expressed in B cells may play a role in inflammation-induced bone loss. Thus loss of bone mass under conditions of estrogen deficiency can be ascribed, at least in part, to a resultant increase of pro-osteoclastogenic inflammatory cytokine production (357). However, this cytokine-dependent acceleration of differentiation and increased activity of osteoclasts is secondary to the estrogen deficiency and, therefore, cannot clearly explain the molecular mechanisms of beneficial effects of estrogens on bone metabolisms.

B. Androgen Receptor

Androgen receptor (AR) acts as a ligand-dependent transcription factor by homodimerizing and binding to a specific DNA sequence, the androgen response element (ARE). Males have a higher bone mineral density and lower risk for fracture and osteoporosis than females (503). Reduction of circulating androgen is closely related to various symptoms and features of aging, such as a cognitive decline, reduction of muscle mass and muscle strength, decrease in bone mass, and increase in abdominal fat mass (108). The elevated strength of male bone is likely caused by the anabolic effects of androgens. Supporting that view, AR knockout (ARKO) mice display high bone turnover with increased bone resorption and associated reduction in trabecular and cortical bone mass, but without change in the bone morphology. Bone mass reduction induced by orchiectomy in male ARKO mice was partially reversed by treatment with testosterone, which can be converted into estradiol. In primary cultured osteoblasts and osteoclasts of ARKO mice, it was shown that AR function was needed for androgen-mediated suppression of osteoclastogenesis (217). However, it has not been determined whether androgen-AR signaling directly targets bone forming osteoblasts or bone resorptive osteoclasts.

It remains unclear, however, whether bone loss in ARKO mice is caused by a systemic endocrine disturbance or represents a direct consequence of the lack of AR in the bone tissue. Notini et al. (349) analyzed the role of AR in osteoblasts using osteoblast-specific AR deficient mice (ObARKO) generated by mating 2.3 kb-Col1a1-1-Cre with AR flox mice with targeted exon 3 (DNA-binding domain). They concluded that disruption of the DNA binding-dependent functions of the AR, specifically in osteoblasts in male mice, led to increased bone resorption and decreased structural strength of the long bones, resulting in a reduction in trabecular bone volume at 32 wk of age. This group also described bone phenotypes of another osteoblast-specific ARKO, using osteocalcin promoter-driven Cre recombinase. They concluded that androgens exert their functions via AR in osteoblasts to preserve bone mass by controlling bone resorption and bone formation coordinated with matrix synthesis and mineralization (60). Taking these observations together, osteoblastic AR has direct functions in the maintenance of bone metabolism as well as influence on secondary effects of endocrine systems. More detailed study is required to clarify the molecular basis of androgen anabolic action in bone.

C. Progesterone Receptors

The progesterone receptor (PR) exists in two isoforms, A (PR-A) and B (PR-B), that are generated from the same gene through transcription from alternative promoters. The expressions of both isoforms are regulated by the estrogen/ER signaling (211). Progesterone reportedly exhibits anti-estro-
Gen effects in uterine epithelium in mice so that ligand-bounded PR-A suppresses the transcriptional activities of ER and PR-B. PR-A knockout (PRAKO) mice generated by selective ablation of the PR-A isoform exhibited enhanced responsiveness of uterine epithelial growth to progesterone (331).

The function of PR in the bone tissue is less understood than that in reproductive organs. Osteoblasts (292) and osteoclasts (369) express both PR-A and PR-B (550), suggesting that progesterone may exert its actions on both types of bone cells. Some in vitro studies showed that progesterone can be metabolized by osteoblasts (388) and that progesterone bounded PR exerts its anti-apoptotic action on osteoblast through inhibiting the activations of caspase-9 and -3 (514). Recent studies using conventional PR knockout (PRKO) mice (289) reported that PRKO mutants exhibited high bone mass with increased bone formation and reduced osteoclast surfaces (398). These findings agree with clinical observations that oral contraceptives containing progesterone can modestly reduce bone mineral density (76, 79). An in vitro study indicated that osteogenic differentiation is accelerated in primary cultured bone marrow cells derived from PRKO mice compared with that of WT mice (550). Taken together, these findings suggested that progesterone might act negatively on bone homeostasis by inhibiting osteoblastic differentiation and bone formation. However, a number of important issues remain unanswered, including whether progesterone directly affects bone tissue? Interestingly, recent studies of progesterone effects on breast cancer have shown that progesterone/PR stimulation can induce RANKL expression in mammary gland epithelial cells that is believed to associate with an onset and development of breast cancers (14, 146, 201, 288, 421). These reports suggest that progesterone/PR signaling might play a regulatory role in bone metabolism through effects on the RANK/ RANKL-induced osteoclastogenesis.

D. Glucocorticoid Receptors

Similar to other NRs, the glucocorticoid receptor (GR) acts as a ligand-dependent transcription factor to mediate genomic actions of glucocorticoids. GR forms homodimers or heterodimers with the other transcription factor to regulate transcription in targeted cells. Glucocorticoids (GCs) can accelerate bone resorption and reduce bone formation (44, 181). Excessive exposure to glucocorticoids (resulting from Cushing’s syndrome or prolonged steroid therapy) decreases bone mineral density (BMD) and may lead to development of glucocorticoid-induced osteoporosis (GIO) (157, 283, 284, 355, 363). In GIO, bone mass reduction, which is more apparent in trabecular than in cortical bones, appears to be closely related to both the dose and duration of the treatment with glucocorticoids (44).

GCs suppress bone-forming osteoblasts in vivo through facilitating the apoptosis (520–522) and suppression of osteoblast-related differentiation marker genes such as Runx2 and type I collagen (372). Reportedly, these steroids have the potential to induce the expression of adipocytic genes in the bone marrow mesenchymal stem cells (371). Inconsistent with these observations, however, the ability of cultured osteoprogenitor cells to form mineralized bone nodules was apparently accelerated by treatment with GCs (15, 384). This paradox suggests that suppressive effects of GCs on bone formation in vivo may reflect GCs action in nonosteoblastic bone cells that then release secondary paracrine factors directly affecting the osteoblastic bone formation.

GR function is critical at least during the postnatal development, as GR knockout (GRKO) mice die within two hours of delivery due to respiratory failure (67). Due to the neonatal lethality, the physiological functions of GR in the tissue and organs of mature adults have not been clarified. However, Kim et al. (223) reported the phenotypes of conditional GR knockout mice in osteoclastic lineage cells (OcGRKO) using mice with the lysozyme promoter-driven Cre recombinase expression. Using this mouse line, they compared the effects of dexamethasone (DEX) in primary cultured osteoclasts derived from bone marrow cells of the control and OcGRKO mice to determine direct impact of GR action in the bone-resorptive cells. Although DEX enhanced the life span of osteoclasts, their bone resorptive activity in vitro was suppressed by DEX. OcGRKO mice are resistant to the inhibitory effects of GCs on bone formation. Consistently, GCs fail to decrease bone formation in osteoblast-specific GR knockout mice produced by using the Runx2-Cre mouse line (393). These results may indicate that the negative effects of GCs on bone formation are mediated through osteoblastic GR. However, in contrast to the effects of GCs in OcGRKO mice, in mice with mutation disrupting GR homodimerization treatment with GCs resulted in reduction of bone mass in vivo, or enhanced apoptosis, and suppressed osteoblast differentiation in vitro. Interestingly, in this mouse line, the expression levels of AP-1-inducible inflammatory cytokines is increased, and GRs retain the ability to form heterdimers with Jun. These results suggest that some of GC effects on osteoblasts may be due to suppression of cytokines, such as interleukin-11, through interaction between monomeric GR and AP-1 (393). Taken together, direct glucocorticoid-GR signaling in bone can reduce bone mass though complex intra- and intercellular regulatory interactions between different types of bone cells.

E. Mineralocorticoid Receptor

Mineralocorticoid receptor (MR) mediates genomic actions of mineralocorticoid hormones, such as aldosterone. It is well known that MR is expressed in distal renal tubules, blood vessels, and the heart and has been shown to control renal functions and blood pressure. The structure of MR is similar to GR, and MR shows functional homology to GR.
including binding to identical response elements (13). Moreover, in vitro binding studies show that glucocorticoids and aldosterone bind to MR with similar affinities and induce gene transcription of the MR target genes (12). Consequently, MR is also able to mediate the action of glucocorticoids, and effects of glucocorticoids should be considered when analyzing MR function. Although patients with high concentration of mineralocorticoids exhibited osteopenia, high risk of fractures, and short stature (326), the function of MR in bone tissue is largely unknown.

One study reported that MR is expressed in bone-resorbing osteoclasts and bone-forming osteoblasts (25). Another study showed that vascular smooth muscle cells express functional MR and that ligand-activated MR facilitated osteoblastic differentiation and mineralization (187). These reports, however, cannot explain clinical observations that high concentration of mineralocorticoids may induce osteopenia, since it remains unclear whether this osteopenia is caused by the increase of osteoclastic bone resorption or reduction of osteoblastic bone formation. Obviously, further analysis of the biological effects of mineralocorticoids and glucocorticoids through MR is required to clarify MR functions in vivo.

V. NUCLEAR RECEPTORS AND BONE METABOLISM: FAT-SOLUBLE VITAMIN RECEPTORS

Fat-soluble vitamins A and D are considered as pro-hormones. Strictly speaking however, both are neither vitamins nor hormones. They are normally obtained from dietary sources or, in the case of vitamin D, synthesized in the skin in response to ultraviolet light exposure and require further enzymatic modifications in specialized cells and tissues to produce physiologically active derivatives capable of activating corresponding intracellular receptors (101, 380).

A. Vitamin D Receptor

The vitamin D receptor (VDR) is a member of the thyroid hormone and retinoic acid receptor subfamily of nuclear hormone receptors that mediates physiological actions of the biologically active form of vitamin D, 1,25-dihydroxyvitamin D$_3$, or 1,25(OH)$_2$D$_3$. Following activation by the ligand, VDR heterodimerizes with retinoid X receptor (RXR), and VDR/RXR complexes bind to specific genomic DNA sequences (vitamin D response elements, VDREs) in the proximal promoter and distal regulatory regions of target genes in a wide variety of cells to regulate proliferation, differentiation, apoptosis, mineral homeostasis, immune response, metabolism, and some other functions (39, 155, 212, 321). Ligand-bound VDR/RXR heterodimers trans-activate target genes through binding at two direct hexameric repeats (A/G)G(G/T)TCA spaced by three nonspecific nucleotides, or so-called DR3 (direct repeat with a 3 bp spacer) VDRE. VDR also mediates 1,25(OH)$_2$D$_3$-dependent trans-repression through binding to negative VDREs (nVDREs) that contain a single copy of a consensus DNA repeat of the positive DR3 VDRE. Mechanisms of negative regulation by VDR appear to be more diverse and are less understood. They involve VDR action as a cofactor that represses gene expression via interaction with other transcription factors and without direct binding to DNA (128, 334).

It has been long established that VDR is an important regulator of bone and cartilage functions. The significance of VDR in human bone physiology can be illustrated by the abnormalities observed in patients with hereditary hypocalcemic vitamin D-resistant rickets, also known as vitamin D-dependent hereditary rickets type II (VDDR-II). VDDR-II is an autosomal recessive disease that develops as a consequence of loss-of-function mutations in the human VDR gene. Clinical manifestations of VDDR-II include low bone mineral density, rachitic malformation, growth retardation and short stature, hypocalcemia, hypophosphatemia, hyperparathyroidism, and increased serum levels of alkaline phosphatase and 1,25(OH)$_2$D$_3$ (128, 297, 334). Similar defects, except elevated serum 1,25(OH)$_2$D$_3$, are typically observed in patients with pseudovitamin D deficiency, or vitamin D-dependent hereditary rickets type I (VDDR-I) that develop as a result of inactivating mutations in the 25-hydroxyvitamin D 1a-hydroxylase (CYP27B1) gene affecting the biosynthesis of 1,25(OH)$_2$D$_3$ (216, 226).

Several research groups generated independent lines of VDR knockout mice (272, 500, 556), and phenotypes of the VDR$^{-/-}$ animals displayed arrays of abnormalities commonly observed in VDDR-II patients (TABLE 1). Considering that classical functions of the VDR had been long associated with bone growth and maintenance, and that important VDR target genes were known to express in bone cells, it was rather surprising that the bone phenotype of homozygous VDR-deficient mice was rescued when the blood calcium and phosphate levels in the mice were normalized by a special diet (271, 306, 307). The successful rescue of bone phenotype in VDR$^{-/-}$ mice implies that the fundamental biological role of VDR is to promote intestinal calcium and phosphate absorption, particularly under conditions of low calcium availability. Therefore, these and earlier studies on vitamin D-deficient rats (150, 523) suggest that vitamin D and its biologically active metabolites are not required for reproduction and development, and that VDR signaling is redundant for the promotion and sustainment of bone growth and mineralization if serum calcium and phosphate are maintained at physiological levels. However, further studies on VDR$^{-/-}$ animals identified novel functions of VDR in the bone that could not be deduced as merely consequential effects of the systemic VDR.
action of maintaining serum calcium and phosphate homeostasis.

1. Role of VDR signaling in bone cell differentiation

Prior to ossification, the expression of VDR in mammalian embryonic development is already detectable during formation of the mesenchymal condensations in bone primordia and then throughout the osteoblast/osteocyte and chondrocyte lineages (196). Such an early expression of VDR in the developing fetal skeleton suggests that it may regulate the differentiation of mesenchymal progenitors into bone cells. Although VDR-deficient mice display normal embryonic bone formation and the rescue diet is able to normalize postnatal bone growth and mineralization, more detailed examination of VDR- and 1,25(OH)2D3-deficient animals have identified direct effects of VDR signaling on bone formation and osteoblast differentiation (for review, see Refs. 144, 158). While osteoclast numbers appeared to be normal in the VDR−/− mice (9), osteoblast numbers and osteoblast colony forming units were reduced in bones and bone marrow stromal cells, respectively, from singly, VDR−/− or CYP27B1−/− and doubly, VDR−/−CYP27B1−/− knockout animals regardless of the type of diet (358). Osteocalcin promoter-driven transgenic overexpression of VDR in mature osteoblasts (cuboidal osteoblasts, osteocytes, and lining cells) increased both trabecular and cortical bone mass that was associated with decreased bone resorption through suppression of osteoclastogenesis (131). Unexpectedly, heterozygous VDR+/− mice displayed higher bone mineral density and formation compared with the wild-type littermates, whereas ablation of the VDR in differentiating osteoblasts in VDRf/f mice by the 2.3kb 1(I) collagen promoter-driven Cre recombinase led to increased mass and mineral density in bones of the transgenic animals associated with decreased rates of the osteoclastogenesis and, consequently, bone resorption (Yamamoto et al. Endocrinology. In press). This is consistent with observation that the volume and mineral density of VDR−/− bone engrafts transplanted into wild-type recipients were higher than those of wild-type bone engrafts under the same conditions (479). These data suggest that mechanisms and effects of the osteoblastic VDR signaling on osteoclastogenesis are differentiation stage-specific: stimulatory from the immature osteoblasts (478) and inhibitory from the mature osteoblasts and osteocytes (19). Interestingly, chondrocyte-specific VDR knockout did not affect chondrocyte development, but resulted in increased trabecular bone mass due to reduced osteoclasts numbers (308), implying that, in osteoblasts and chondrocytes, VDR acts as a regulator of osteoclastogenesis.

Thus VDR signaling exerts direct physiological effects in cells of the chondrocytic and osteoblastic lineages, thereby affecting bone mass and mineral density independently from its systemic effects through regulation of calcium and phosphate homeostasis.
2. VDR target genes in bone cells

It has been estimated in various studies on gene expression profiling that VDR signaling is involved directly or indirectly in the regulation of up to 5% of human and mouse genes (reviewed in Ref. 39), implying a wide range of VDR biological actions and effects. In the skeleton, VDR is expressed in the bone osteoblasts and osteocytes and in cartilage chondrocytes (461).

The osteocalcin gene was the first gene shown to be directly controlled by the VDR and became a classical model for studies on 1,25(OH)2D3-dependent transactivation. It is induced during late stages of osteoblastic differentiation with the onset of mineralization and remains expressed at high levels in surviving osteocytes and lining cells (463). Although osteocalcin represents the most abundant noncollagenous osteoblast-specific protein and may contribute to the density and structural integrity of bone tissue, its exact role in the skeleton is not completely understood. Osteocalcin-deficient mice develop bones of higher mass and improved functional quality owing to an increase in bone formation without apparent defects in bone resorption (99). It was noticed, however, that osteocalcin-deficient mice were glucose intolerant and accumulated high amounts of visceral fat. This was consistent with earlier observations that 1,25(OH)2D3 deficiency impaired both insulin secretion and glucose tolerance in humans and experimental animals (59, 132, 348). Similar defects in insulin production, insulin sensitivity in target tissues, and glucose tolerance have been observed in VDR−/− mice (561). Further studies have identified bone-secrected osteocalcin as a hormone that stimulates both proliferation of pancreatic β-cells and insulin production, and improves insulin metabolic responsiveness in target tissues (260). Thus VDR signaling in osteoblasts can influence systemic energy metabolism through regulation of the osteocalcin gene expression in the bone (130, 259).

Besides osteocalcin, VDR controls bone expression of several genes encoding important effectors of both bone and systemic homeostasis, such as osteopontin (OPN), OPG, RANKL, Runx-related transcription factor X2 (Runx2), and fibroblast growth factor 23 (FGF23).

OPN, also known as early T-cell activation gene-1 (ETA-1) or secreted phosphoprotein 1 (SPP1), is a multifunctional secreted phosphoglycoprotein that was initially isolated from the bone extracellular matrix (353, 395). OPN is expressed in various types of cells, including osteoclasts, osteoblasts, chondrocytes, macrophages, activated T cells, smooth muscle cells, and epithelial cells. Outside the skeleton, comparatively high levels of OPN have been found in kidneys, placenta, smooth muscles, and secretory epithelia (317, 512). Osteoblastic OPN is highly responsive to 1,25(OH)2D3 and is thought to control bone mineralization and remodeling. OPN was proposed to anchor bone-resorbing osteoclasts to the mineral matrix of bone surfaces (395). OPN-deficient bones are hypermineralized and more fragile than normal bones, largely due to impaired bone resorption (182, 330). Nonskeletal OPN appears to regulate ectopic calcification acting as a natural inhibitor of soft tissue mineralization (458, 464).

RANKL represents one of the genes that is most responsive to induction by 1,25(OH)2D3 and is highly expressed in osteoblasts and stromal cells, especially in areas undergoing active bone remodeling or inflammatory osteolysis (154). RANKL, a cytokine from the tumor necrosis factor (TNF) superfamily, acts through activation of its receptor RANK. RANKL/RANK signaling is indispensable for the development of multinucleated bone-resorptive osteoclasts from monocyte progenitors and promotes survival and activity of mature osteoclasts (219). OPG is a soluble glycoprotein widely expressed in most tissues, including bone osteoblasts. OPG is a decoy receptor that protects bone from excessive resorption by binding to RANKL, thereby inhibiting RANKL/RANK signaling and preventing differentiation of osteoclasts. Therefore, the ratio of RANKL and OPG concentrations in the bone represents a major determinant of bone mass and strength (110, 219). Thus, through direct regulation of skeletal production of RANKL and OPG, osteoblastic VDR signaling couples bone formation to bone resorption and controls normal adult bone remodeling (154).

Runx2 is a key regulator of hypertrophic chondrocyte and osteoblast differentiation (129, 554). It acts as both a DNA binding transcription factor and a coregulatory component of nuclear complexes with other factors, including VDR, to regulate the stage-specific expression of genes controlling chondrogenesis and osteoclastogenesis (83, 303, 359, 425). VDR suppresses Runx2 gene transcription through interaction with VDREs in the proximal promoter and distal regulatory regions of the gene (98, 456).

1,25(OH)2D3/VDR signaling induces production of FGF23 in osteoblasts and osteocytes (23, 416, 546). FGF23 is a circulating glycosylated peptide that suppresses renal phosphate reabsorption and production of 1,25(OH)2D3, thus functioning as a phosphaturic factor and counter-regulatory hormone to 1,25(OH)2D3 (28, 115, 165, 179, 557). Excess of circulating 1,25(OH)2D3 can cause symptoms similar to the phenotype of FGF23 knockout mice that is characterized by ectopic calcification, osteoporosis, skin atrophy, arteriosclerosis, and chronic obstructive pulmonary disease (429, 430). Disruption of the vitamin D signaling by ablation of either VDR or CYP27B1 rescues the FGF23−/− phenotype (160, 397), supporting a view that FGF23 functions as a negative feedback regulator that restrains the mineralotropic and osteotrophic actions of 1,25(OH)2D3 (28, 165).
3. **Osteogenic VDR agonists**

Besides its fundamental physiological function of regulating calcium homeostasis, VDR signaling is known to exert non-calcemic effects on immunity, cell growth, and differentiation. Hence, VDR agonists are thought to be potentially beneficial in treatment of VDDR-I, renal failure, psoriasis, autoimmune disorders, and certain cancers (39, 336). Calcitriol and its synthetic analog alfalcacidol have been used in combination with other osteogenic compounds for the treatment of osteoporosis and osteomalacia (85, 117, 186). However, the therapeutic application of 1,25(OH)2D3 is limited due to deleterious side effects such as hypercalcemia and heterotropic ossification. Therefore, significant efforts have been made to develop novel non-calcemic VDR ligands with improved therapeutic properties (48, 85, 178, 291, 443, 444).

A number of vitamin D analogs have been synthesized that exhibit a separation between bone efficacy and hypercalcemia. Several VDR ligands with prominent osteogenic effects are currently undergoing various stages of preclinical development or clinical trials. These include 2-methylene-19-nor-(20R)-1a,25(OH)2D3, designated 2MD (218, 427) and 2MD derivatives (24, 432); 1a-fluoro-16-ene-20-epi-23-ene-26,27-bishomo-25(OH)D3, or Ro-26–9228 (183, 367); and 1a-fluoro-16-ene-20-epi-23-ene-26,27-bishomo-25(OH)D3, or ED-71 (310, 312).

The phase III of clinical trials has been completed for ED-71, also known as eldecalcitol. Longitudinal clinical studies have shown that ED-71 can effectively increase lumbar spine bone mineral density, decrease bone turnover, and lower risk of vertebral and wrist fractures in osteoporotic patients regardless on their vitamin D status. ED-71 at a clinically effective dose was well tolerated in these patients without causing sustained hypercalcemia or other apparent side effects (185, 309).

**B. Retinoid Acid Receptors**

Retinoic acid (RA), a biologically active form of the vitamin A or retinol, plays a crucial role in regulation of vertebrate embryonic development, postnatal growth, and physiology of adult organs and tissues. Retinoids may exert both beneficial and detrimental effects. RA deficiency dramatically alters fundamental physiological and developmental processes. It is also well documented that RA is teratogenic and treatments with retinoids are contraindicated during pregnancy (524, 526). Thus appropriate physiological effects of RA require a very narrow range of RA concentrations to avoid negative consequences of both deficiency and toxicity (101). Biological effects of RA are mediated by the action of two types of retinoic acid receptors: RA receptors (RAR) and retinoid X receptors (RXR). Each of these receptor types consists of three subtypes (RARα, RARβ, RARγ and RXRα, RXRβ, RXRγ, respectively) that originate from distinct genes. Alternative promoter usage and splicing generate several isoforms of each of the receptor subtypes, further adding to the complexity of RA signaling (52).

All-trans-RA (ATRA), the abundant and most potent biologically active derivate of vitamin A, is a high-affinity ligand for RARs, whereas 9-cis-RA can activate both types of the receptors, RARs and RXRs. The physiological role of 9-cis-RA remains unconfirmed, however, since this isomer is detectable in vivo only under conditions of excess of vitamin A or ATRA (101, 323).

RARs and RXRs transduce RA signaling as RAR/RXR heterodimers. Loss-of-function studies have demonstrated requirements for distinct RAR and, probably RXR subtype combinations at different stages of mouse embryonic development and postnatal growth (304). In addition, RXRs function as common heterodimerization partners for several members of the nuclear receptor superfamily, such as VDR, TRs, PPARs, LXR, FXR, and other so-called subfamily 1 nuclear receptors (251). The RXR heterodimerization partners do not exhibit a marked preference for any of the three RXR subtypes. It has been suggested that RXR primarily functions as a scaffold protein to facilitate DNA binding for these receptors (55).

RAR/RXR heterodimers act as transcription factors through binding to the specific RA response element (RARE) DNA sequences that correspond to direct repeats of the canonical hexameric motif (A/G)(G/C)TCA separated by five, one, or two nucleotides and referred to as DR5, DR1, or DR2 elements, respectively (298). In the absence of the ligand, RAR/RXR heterodimers act as transcriptional suppressors via recruitment to the RAREs of corepressor complexes. Binding of RAR agonist triggers the dismissal of corepressors and recruitment of coactivator complexes at DR5 and DR2 RAREs to stimulate the transcription (141, 254, 373, 374). In contrast, RXR agonist binding is unable to induce corepressor-coactivator complex exchange and activate the RARE-dependent transcription in the absence of RAR agonist (135). This phenomenon is referred to as RXR subordination (52, 304). Although formation of RXR homodimers and their binding to DR1 elements has been demonstrated in vitro, the existence of autonomous physiological function of RXR/RXR homodimers remains elusive (176, 506). Interestingly, RAR ligand binding does not cause the dissociation of corepressors from DR1-bound RAR/RXR heterodimers, suggesting that the DR1 RARE might be universally repressive (247).

1. **Role of RARs in the skeleton**

It has been established that in early embryonic development, RA/RARs provide instructive signaling for posterior neuroectoderm and posterior foregut endoderm, and permissive signaling for trunk mesoderm. At later stages, RA/RARs are involved in the development of the heart, eye, lung, pancreas, head structures and skeleton, genitourinary tract, and other organs (reviewed in Refs. 94, 101, 347).
Such a profound influence of RA signaling on a wide range of the critical events in the morphogenesis and organogenesis creates significant methodological difficulties for dissecting its specific functions in the physiology of adult organs and tissues, including the skeleton.

High alimentary intake and serum levels of retinol have been associated with increased risk of bone fractures in humans (122, 324) and in laboratory animals (195, 273). However, RA appears to stimulate the regeneration of deer antlers (8), whereas vitamin A deficiency was reported to delay bone healing/repair in mice (480). These and similar observations might reflect both a wide spectrum of RA biological effects in bone and narrow range of physiologically optimal RA concentrations (101).

Studies on cultured bone cells and tissues have shown that effects of retinoids depend on the cell type and context. RA has been shown to stimulate bone resorption through activation of differentiated osteoclasts (233, 407, 499). However, RA suppressed the resorption in the presence of RA in the cells of osteoblastic lineage: blocking the recruitment of precursor cells to the osteoblastic lineage and progenitors. RA has been shown to stimulate osteoclast precursor proliferation and inhibit RANKL-induced osteoclast differentiation in both human and murine cells (70, 71, 168).

RA has been reported to promote the differentiation of primary osteoblasts (402, 454, 547) and to synergize with the BMP signaling to promote the osteogenic differentiation of mesenchymal cells (93, 188, 442) and adipose-derived adult stromal cells (266, 352, 507) at the expense of adipogenesis. In other experimental settings, RA produced inhibitory effects on the expression of osteoblastogenesis marker genes in vitro (2, 63).

Studies on developing zebrafish identified distinct effects of RA in the cells of osteoblastic lineage: blocking the recruitment of precursor cells to the osteoblastic lineage and promotion of bone matrix synthesis in mature osteoblasts (270). This dual action of RA results in inhibitory effects on the induction of ossification in early development, and in the stimulation of bone formation after skeleton ossification has been initiated (252, 270, 459, 566). These findings supported previous reports on the crucial role of unliganded RAR-mediated gene repression in skeletal progenitor cell differentiation (95, 162, 524). Aberrant localized RA signaling has been implicated in the development of multiple congenital skeletal abnormalities in humans and experimental animals (253).

Although a large body of evidence has been accumulated on the effects of RA in differentiating and mature bone cells, our understanding of the roles of specific RAR subtypes in mediation of these effects, particularly in vivo, remains fragmented. Earlier experiments with knockout of individual RARs in mice suggested a high degree of functional redundancy among the RAR subtypes, since single RAR-KO mice were found to be viable and displaying few abnormalities (reviewed in Ref. 134). However, double RAR subtype KO is lethal owing to numerous developmental abnormalities (276, 320). Compound ablation of the RARγ (RARα+/RARγ− or RARβ+/RARγ−) has been shown to cause severe skeletal defects including homeotic transformations, growth retardation, and limb malformations (276). Further studies confirmed the pivotal role of RARγ in the skeletal development and maintenance. RARγ was found to be the highest expressed RAR subtype in mouse growth plates (528). Conditional double KO of RARγ with either RARα or RARβ in the cartilage has been shown to lead to significant skeletal growth retardation and growth plate abnormalities, whereas cartilage deficiency in both RARα and RARβ had no apparent effect on the bone formation and growth (528). Furthermore, cartilage is an avascular tissue, and the upper zones of mouse limb growth plates are devoid of active retinoids (527, 528), suggesting that in the growth plate chondrocytes, RARγ operates as an unliganded transcriptional repressor. Recent report on inhibition of heterotopic ossification by selective RARγ agonists (431) further supported conclusion on the central role of the RARγ in the skeletal development, growth, and maintenance.

VI. NUCLEAR RECEPTORS AND BONE METABOLISM: FATTY ACID-RELATED RECEPTORS

A. Peroxisome Proliferator-Activated Receptors

The first peroxisome proliferator-activated receptor (PPAR) was originally identified as an orphan receptor (later designated PPARα) activated by various synthetic compounds including hypolipidemic drugs known to cause proliferation of peroxisomes and activation of the peroxisomal β-oxidation of fatty acids (184). Subsequently, three PPAR subtypes have been identified: α, β/δ, and γ (97, 325). PPARs of all the subtypes form heterodimers with RXRs and bind to consensus peroxisome proliferator response element (PPRE), composed of two core motifs separated by a single base pair (DR1 element: 5′-AGGTCA-N-AGGTCA-3′) (325).

PPARs are pivotal regulators of the systemic energy metabolism and are highly expressed in liver, adipose tissue, and skeletal muscles. Activated PPARα and PPARβ/δ promote energy expenditure, whereas energy storage is associated with the activation of PPARγ. Although the profound roles of PPARs in energy metabolisms have been intensively illustrated over the last two decades in adipose, liver, and muscle tissues, bone was not considered as a significant target for the PPAR action.
However, recently, bone has emerged as an endocrine organ that is actively involved in systemic regulation of the energy metabolism, and physiological roles of PPARs in bone cells are now drawing much attention.

PPARα is expressed predominantly in the liver, and to a lesser extent in the heart, skeletal muscles, and bone. Hepatic PPARα plays a crucial role in regulation of fatty acid oxidation, which provides energy for peripheral tissues. Expression of PPARδ (also known as PPARβ) is detectable in many tissues including bone (325), and genetic mutations of PPARδ have revealed its primary function as a fat burner. PPARγ has two major isoforms (γ1 and γ2) generated by alternative promoter usage and primary transcript splicing. PPARγ1 is expressed at low levels in many cell types and tissues including macrophages, kidney, uterus, breast, prostate, and adipocyte (265). PPARγ2 is a predominant isoform expressing in adipose tissue (491). The expressions of both PPARγ1 and PPARγ2 are significantly induced during the adipogenesis, and both isoforms are potent adipogenic factors that shift the differentiation of bone marrow mesenchymal stem cells (MSCs) into adipocytes and inhibit MSCs differentiation into osteoblasts, chondrocytes, or muscle cells. A balanced MSC differentiation into osteoblasts and adipocytes is an important factor in the maintenance of bone mass and strength. As patients with osteoporosis often display increased fat content in the bone marrow, regulation of pro-adipogenic function of PPARγ represents an important factor supporting bone homeostasis.

Arachidonic acid metabolites, such as leukotriene B4 and 1-palmitoyl-2-oleoyl-sn-glycerol-3-phosphocholine (16:0/18:1-GPC), are thought to be endogenous ligands for PPARα (51, 91). Prostaglandin I2 has been reported as an endogenous ligand for PPARδ (159), while 15-deoxy-Δ12,14-PGJ2 (15d-PGJ2), 9-HODE, 13-HODE, and nitrolinoleic-acid are known as PPARγ ligands (228, 337, 419). In addition to eicosanoid derivatives, metabolites of polyunsaturated fatty acids have been found to activate PPARγ function. A number of synthetic compounds are registered as selective activators of a particular PPAR subtype or as pan-ligands. Fibrate derivatives (Wy14643 (184), bezafibrate, GW409544 (539)) are PPARα-specific agonists, whereas GW6471 is a PPARα-specific antagonist (540). L165041 (27) and GW501516 (354) are specific agonists for PPARδ. A class of thiazolidinediones (TZDs) (264), GW9662 (170), THO921 (489), and CLX-09717 (467), are known to act as specific PPARγ agonists. Two TZD derivatives, rosiglitazone and pioglitazone, have been clinically successful as anti-diabetic agents with antihyperglycemic profile.

1. **Role of PPARα in bone cells**

PPARα−/− mice are impaired to meet energy demands during fasting and suffer from hypoglycemia, hyperlipidemia, and hypoketonemia (261). In the fasting state, whole body use of glucose was aberrantly increased in adipose tissues of PPARα−/− mice, apparently due to upregulated expression of the neuropeptide Y (NPY) and proopiomelanocortin (POMC) in the hypothalamus (232). Conversely, PPARα activation with specific agonists reduces adiposity and improves hepatic and muscle steatosis, leading to improvement in insulin sensitivity (263). Fibrates are currently on the market and are clinically used to lower plasma triglyceride levels in patients with hypertriglyceridemia (396).

### A) **ROLES OF PPARα IN OSTEOLASTIC CELLS**

Although expression of PPARα is detectable in the bone cells as well as in the cells of hematopoietic and mesenchymal lineages (136), PPARα−/− mice do not exhibit an obvious bone phenotype (535). Bone mass of PPARα−/− mice was not altered, and bone cortical area did not differ from that in control animals. In addition, the differentiation potential of the bone marrow MSCs toward osteoblasts and adipocytes appeared normal in PPARα−/− mice (535). Nevertheless, PPARα deficiency may affect bone morphology, as a significantly larger bone marrow space was observed in male PPARα−/− mice (535).

In a cell culture system, PPARα was shown to shift bone marrow myeloid cell commitment toward the B cell lineage (548), suggesting a regulatory role of PPARα in the bone marrow environment.

In vitro studies demonstrated that bezafibrate [PPARα-specific agonists (474)] stimulated osteoblast differentiation (463) and inhibited osteoclastogenesis (53). In intact rats, bezafibrate stimulated periosteal bone formation (465) and increased femoral BMD, while decreasing the bone marrow space (471). These studies suggest that bezafibrate may have anabolic effects in bone; however, clinical assessment of bone health showed that there was no significant reduction in the incidence of bone fractures in the patients treated with fibrates (319).

### B) **ROLES OF PPARα IN CARTILAGE**

Recent studies have shown that activated PPARs are potent to attenuate TGF-β-dependent proteoglycan synthesis in rat chondrocytes, whereas the matrix metalloproteinase (MMP) activity was suppressed only by PPARα agonists (381). In cartilage explants from osteoarthritic patients, treatment with PPARα agonist (Wy14643) was effective to suppress mRNA expression levels of MMP1, MMP3, and MMP13 (65). Since activation of TGF-β signaling and elevated MMP activities have been established to associate with the development of osteoarthritis (OA), these results imply that PPARα agonists may be beneficial for the treatment of OA.

2. **PPARδ (β)**

The body sizes of **PPARδ−/−** mice were significantly smaller than those of control animals at every stage of the development, from fetus to adults, but no phenotypic abnormality was observed in their tissues including bone (376). Animal models with genetically altered PPARδ expression and application of selective PPARδ agonists in intact animals re-
vealed a critical role of this nuclear receptor in the regulation of fat-burning processes. Transgenic expression of PPARδ in adipose tissue made the transformed mice resistant to high-fat diet-induced obesity, hyperlipidemia, and tissue steatosis. In contrast, when fed with high-fat diet, PPARδ−/− mice displayed reduced expression of the uncoupling protein 1 (Ucp1) and were prone to obesity (516). In the skeletal muscles, activated PPARα upregulated fatty acid oxidation and energy expenditure to a far greater extent than activated PPARα (21). Thus PPARδ appears to perform a unique function as a fat burner, as opposite to a fat-storing function of PPARγ.

**A) PPARδ FUNCTION IN OSTEOCLASTS.** In rabbits, PPARδ is highly expressed in mature osteoclasts, and its agonist was potent to induce bone-resorbing activity and upregulation of cathespin K gene expression in isolated mature rabbit osteoclasts (300). Likewise, in cultured human osteoclasts derived from peripheral blood mononuclear cell, selective PPARδ agonist L165041 (27) stimulated the resorptive activity of mature osteoclasts. However, this compound was inhibitory for human osteoclast maturation (53). Thus, at the present stage, adverse actions of activated PPARδ(β) obscure our understanding of the physiological roles of osteoclastic PPARδ(β).

**3. PPARγ**

Initially, PPARγ was identified as a transcription factor regulating adipocyte differentiation (491), and this adipogenic function of PPARγ was further confirmed by various experimental approaches. Synthetic agonists of PPARγ have been successfully used for the treatment of type II diabetes mellitus (DM), although the molecular basis of PPARγ beneficial actions in type II DM development and treatment still remains to be determined. Recently, clinical studies of the type II DM patients treated with TDZs showed an increased risk of bone fracture incidence with decreased BMD compared with patients treated with other classes of glucose-lowering medications (203, 204, 423, 552). It remains unclear whether such side effects can be attributed to a generally higher risk of bone fracture incidence in type II DM patients compared with healthy individuals irrespective of the BMD (1, 346, 422). However, it is likely that induced by activated PPARγ adipogenesis in the bone marrow is causal for the skeletal side effects. Thus there is a caveat that synthetic PPARγ ligands may exhibit side effects detrimental to the bone health.

**A) EFFECTS OF PPARγ IN BONE MARROW MSC.** PPARγ is commonly accepted as a master adipogenic factor, since activation of PPARγ in precursors of nonadipogenic lineage cells triggers their transdifferentiation into adipocytes (167, 491). For example, endogenous and synthetic PPARγ agonists promote adipogenesis and inhibit osteoblastogenesis in primary bone marrow MSC culture (256). Moreover, treatment of mice with rosiglitazone resulted in increased bone marrow adiposity and decreased BMD (or bone mass) apparently through suppression of the pro-osteoblastic transcription factors, Runx2, Osterix, and Dlx5 (7, 413). Consistently, haplo-insufficiency of PPARγ in mice resulted in enhanced osteoblastogenesis and decreased bone marrow adipogenesis with increased trabecular bone volume (4). Similarly, an abnormally increased bone formation leading to insufficient space in the marrow cavity to maintain normal hematopoiesis has been observed in PPARγ<sup>−/−</sup> mice that carry a hypomorphic mutation in the PPARγ locus and show reduced expression of both PPARγ isoforms in the white adipose (66). Collectively, these in vivo and in vitro studies suggest that in the bone marrow, PPARγ functions as a differentiation switch between osteoblastogenesis and adipogenesis, acting as an inhibitor of osteoblastogenesis. However, the molecular basis of such inhibitory action of PPARγ in osteoblastogenesis is still poorly understood.

In addition to the ligand-dependent activation, various signaling pathways are known to modulate the transactivation function of PPARγ in the MSCs. For example, several cytokines (TGF-β1, and BMP2) inhibited adipocyte differentiation from bone marrow MSCs through suppression of the PPARγ transactivation function (163, 345).

Wnt ligands regulate differentiation of MSCs into osteoblasts and adipocytes (410). The canonical Wnt ligands (Wnt1, Wnt3a, Wnt5b, Wnt7a, Wnt10b, etc.) bind to cell membrane heterodimers between frizzled receptor (Fzd) and low-density lipoprotein-related protein (LRP5/6) and transmit their signals by inducing intracellular translocation of β-catenin into the nucleus and subsequent activation the T-cell factor/lymphoid-enhancing factor (TCF/LEF) family of transcription factors. The canonical Wnt signal promotes bone formation, as it was initially illustrated in clinical studies that gain-of-function mutation in the Wnt receptor subunit gene, LRP5 stimulated osteoblastogenesis in humans (145). Further in vitro studies showed that bone formation is stimulated by canonical Wnt signaling at the stages of maturation of osteoblastic precursors into mature osteoblasts rather than by determining osteoblastic cell fate in the bone marrow MSCs (22, 142, 244, 302). The other group of Wnt ligands (Wnt4, Wnt5a, and Wnt11, etc.) activates the noncanonical Wnt signaling through cell membrane heterodimers of Fzd and pathway-specific coreceptors such as knypek associated with membrane receptor-type tyrosine kinases Ror1 and Ror2 (504). As mice deficient of noncanonical Wnt signal transducers exhibit severe developmental defects including impaired bone formation (350, 545), it is likely that the noncanonical Wnt signaling supports osteoblastogenesis.
Cross-talks between Wnt signals and various nuclear receptor-mediated signaling pathways have been described in many cell types (332). A noncanonical Wnt ligand, Wnt5a, has been shown to induce osteoblastogenesis and suppress PPARγ-dependent adipogenesis in cultured MSCs. Consistently, Wnts5a+/− mice exhibited an accumulation of adipocytes in the bone marrow and reduced BMD (473). Cyto genetic and biochemical characterization of the downstream factors in the signaling has revealed that Wnt5a activates a MAP kinase-like, Nemo-like kinase (NLK). Next, activated NLK forms a complex with PPARγ and SET domain, bifurcated 1 (SETDB1). Since SETDB1 is known as a histone H3 lysine 9 (H3K9), methyltransferase that renders chromatin transcriptionally inactive, Wnt5a, appears to serve as a switcher of adipogenic potential (473). Setdb1−/− mice exhibited an accumulation of adipocytes in the bone marrow and reduced BMD (473). Cytogenetic and biochemical characterization of the downstream factors in the signaling has revealed that Wnt5a activates a MAP kinase-like, Nemo-like kinase (NLK). Next, activated NLK forms a complex with PPARγ and SET domain, bifurcated 1 (SETDB1). Since SETDB1 is known as a histone H3 lysine 9 (H3K9), methyltransferase that renders chromatin transcriptionally inactive, Wnt5a, appears to serve as a switcher of MSC differentiation fate from adipogenesis into osteoblastogenesis via suppression of PPARγ function (473).

B. Liver X Receptor α and β

Liver X receptors (LXRs) α and β are thought to function as cholesterol sensors and regulate the expression of genes involved in cholesterol metabolism. Oxidized derivatives of cholesterol and oxysterols are known as endogenous ligands of the LXRs (190, 529). Δ-Glucose and Δ-glucose-6-phosphate are also reported to activate LXRs and, expectedly, both of them were effective to regulate LXR-mediated biological events in hepatic glucose metabolism and fatty acid synthesis (328). LXRs heterodimerize with RXRs and recognize DR4-type DNA elements (5′-AGGTCA-NNNN -AGGTCA-3′) as binding sites in the target genes (529).

When fed a cholesterol-rich diet, LXRα−/− mice develop hepatic steatosis associated with cholesteryl-ester accumulation. This has been attributed to the lack of LXRx-mediated upregulation of the Cyp7a1 that encodes a rate-limiting enzyme in the conversion of cholesterol into bile acid (363). In contrast to LXRα−/− mice, LXRβ−/− mice maintained their resistance to dietary cholesterol and exhibited no apparent aberrant phenotype (5).

1. Roles of LXRs in bone

A detailed analysis of bone phenotypes of LXR-deficient mice has been reported (403). A significant increase in BMD attributed to cortical bone areas with less bone resorptive activity was observed in female LXRα−/− mice at the age of 4 mo, but not in male LXRα−/− mice. The LXRα−/− mice displayed no difference in trabecular areas, whereas osteoclastic differentiation in trabecular areas was affected in female LXRα−/− mice without alteration in BMD. Although it remains to be studied if LXRs are activated by endogenous ligands or are constitutively functional without ligand binding in osteoclasts, these findings imply that LXRs are regulators of osteoclastic differentiation and function. Interestingly, LXRα deficiency affected bone phenotype only in the mutant females, suggesting an interaction between LXR and sex steroid signaling pathways.
2. Effects of LXR agonists in bone cells

A short-term (48 h) treatment with LXR agonist reduced mRNA levels of osteocalcin and alkaline phosphatase in primary cultured murine osteoblasts, while oral administration of LXR agonists for 6 days to mice did not affect bone morphology (383).

The expressions of both LXRs and cholesterol efflux-related genes (APOA1, ABCA1) were significantly lower in osteoarthritic cartilages compared with normal human cartilage samples. Consistently, aberrant intracellular lipid accumulation was found in primary cultured chondrocytes from OA patients, whereas treatment with LXR agonist induced APOA1 and ABCA1 genes and decreased lipid accumulation in chondrocytes (495). Likewise, in human articular cartilage explants, an LXR agonist was shown to inhibit cytokine-induced proteoglycan degradation (68). Thus these findings imply that LXR-activating compounds may be effective in prevention of pathological development of OA through stimulation in chondrocytes of cholesterol efflux.

C. Farnesoid X Receptor

Farnesoid X receptor (FXR) was originally identified as an orphan receptor that could be activated by farnesoids (125). However, after the identification of bile acids (BAs) as its endogenous ligands, FXR was also referred to as BAR (296, 361, 511). BAs are amphipathic molecules with a steroid backbone that are synthesized from cholesterol exclusively in parenchymal cells of the liver. Chenodeoxycholic acid (CDCA), cholic acid (CA), deoxycholic acid (DCA), and lithocholic acid (LCA) are potent to activate FXR at their physiological concentrations (~100 μM). FXR heterodimerizes with RXR subtypes, and FXR/RXR heterodimers bind to multiple direct repeat elements (DR2, DR4, and DR5). Inverted repeats, IR0 (5′-AGGTCA-TGACCT-3′) and IR1 (5′-AGGTCA-N-TGACCT-3′), were also found to bind FXR/RXR (126).

FXR−/− mice show elevated serum and hepatic levels of bile acid, cholesterol, and triglycerides, with a pro-atherogenic serum lipoprotein profile. Under standard housing and dietary conditions, FXR−/− mice had no overt bone abnormality and appeared indistinguishable from their wild-type littermates (438). However, in human osteosarcoma cell line (Saos-2) and bone marrow stroma cells, agonist-activated FXR was shown to bind to the Runx2 promoter, and stimulated the expression of osteoblastic marker genes, such as bone sialoprotein (BSP), osteocalcin (OC), osteopontin (OPN), and alkaline phosphatase (ALP) (175). Together with the report characterizing FXR endogenous ligands in human MG63 cells and bone tissues (434), these data suggest that activated FXR might potentiate bone formation.

D. Steroid and Xenobiotic Receptor/Pregnane X Receptor

The human steroid and xenobiotic receptor (SXR) and its murine ortholog, pregnane X receptor (PXR) (also known as PAR and NR1I2), were identified as orphan receptors regulating gene expression of cytochrome P-450 monoxygenase (CYP3A), a pivotal enzyme in the initial catabolism of a wide range of xenobiotics (29, 30, 229). Besides the regulation of CYP enzymes such as CYP3A4, CYP2B6, and CYP2C8, SXR was shown to regulate the expression of conjugation enzymes such as UDP-glucuronosyltransferase (UGT1A1) and sulfotransferase (SULT1A1), and ABC family transporters, ABCB1 and ABCC2 (104, 470). SXR can be activated by various pharmaceutical agents such as taxol, rifampicin (RIF), SR12813, clotrimazole, phenobarbital, an herbal antidepressant St. John’s wort (Hypericum perforatum), as well as peptide mimetic HIV protease inhibitors such as ritonavir (227). However, owing to species specificity in ligand recognition, some human SXR agonists (RIF, clotrimazole, and phenobarbital) were reported unable to activate the murine SXR ortholog PXR (537). DNA binding sites for SXR in the target genes consist of directly repeated two 5′-AGGTCA-3′ core motifs separated by 3 bps, or so called DR3 elements.

PXR−/− mice are viable, fertile, breed with normal Mendelian distribution, and do not exhibit any overt phenotypic abnormality including serum parameters (462, 537). However, PXR-deficient mice do not respond with Cyp3a gene induction to the administration of its prototypic inducers such as progrenolone-16a-carbonitrile (PCN) and dexamethasone, confirming the key role of PXR/SXR in activation of response to xenobiotics.

1. SXR function in bone

Reported action of the vitamin K₂, or menaquinone-4 (MK4), as an SXR agonist suggested a possibility of SXR function in the bone homeostasis (472), since MK4 has been clinically demonstrated to be effective as anti-osteoporotic agent reducing the incidence of bone fractures (5, 6). Supporting the osteoprotective activity of MK4 in humans, SXR-mediated MK4 signaling has been shown to induce the expression of key osteoblastic marker genes like ALP, OPG, OPN, and OC in human osteosarcoma cells (472). In addition, several genes of secreted extracellular matrix proteins, such as Tsukushi and matrilin-2, have been identified as SXR/MK4 target genes in human osteoblastic cells (174). As Tsukushi has been shown to mediate the MK4-induced collagen accumulation (174), MK4-activated SXR is likely to stimulate bone formation and maintenance. These
suggest that SXR signaling might promote the osteoblastogenesis.

Data from in vitro cell culture systems have been further experimentally recapitulated in vivo, by disruption of the PXR gene in mice (16). Four-month-old PXR<sup>−/−</sup> female mice displayed osteopenia with low bone turnover, reduced stiffness in cortical bones, and bone formation. These implicate PXR/SXR as a significant factor that supports bone remodeling and formation. In this respect, an identification of endogenous SXR ligands in bone would be quite a promising project.

**VII. NUCLEAR RECEPTORS AND BONE METABOLISM: OTHER RECEPTORS**

**A. NR4A Receptors**

The NR4A subgroup of orphan nuclear receptors consists of NR4A1 (also called Nur77 or NGFI-B), NR4A2 (also called Nurr1), and NR4A3 (also called NOR1) (69). Because of the absence of structural cavities for ligand binding in these nuclear receptors, they can be considered as ligand-independent NRs (17, 394, 517). Generally, these receptors exert their transcriptional activities by binding as monomers or homodimers to NGFI-B response element (NBRE; AAAGGTCA) and Nur-response element (NuRE), respectively (335, 530). Additionally, NR4A1 and NR4A2 form transcriptionally active heterodimers with RXR that bind DR5 motifs (direct repeats separated by five nucleotides), and these heterodimers are efficiently activated by RXR ligands (126, 375).

NR4A receptors are abundantly expressed in the brain and neuroendocrine tissues (137). NR4A2 is expressed at high levels particularly in dopaminergic neurons during development (536, 564). Therefore, most studies of NR4A receptors have focused on the nervous system. However, it was recently reported that NR4A receptors are also expressed in other tissues including the liver, arterial wall smooth muscle cells, and macrophages (295, 316, 366, 563). The expression of NR4A receptors in these tissues can be induced in response to various stimuli, whereas NR4A receptors are constitutively expressed in the brain (316).

NR4A receptors are also expressed in bone tissue, especially in osteoblasts (249, 484), implying their functional activity in bone formation. Three groups have reported that NR4A receptors regulate the expression of several osteoblastic marker genes such as osteopontin (OPN), osteocalcin (OCN), collagen type I alpha 1 (COL1A1), and alkaline phosphatase (ALP), suggesting the presence of potential NBREs in the promoter region of these genes (249, 258, 379). NR4A2 overexpression led to increased expression of ALP, COL1A1, and OCN genes. In contrast, the expression of these genes was decreased in MC3T3-E1 cells with NR4A2-knockdown and in primary cultured mouse calvarial osteoblasts derived from Nurr1 null mice (249, 258, 379). These reports suggested that NR4A2 might play a regulatory role in both early (COL1A1 and ALP) and late (OCN) stages of osteoblast differentiation.

In the bone, the expression of NR4A receptors can be induced by two key regulators, PTH and fibroblast growth factor 8b (FGF-8b) (248, 378, 484, 485). Tetradis and coworkers (378, 484, 485) reported that PTH can induce the expression of NR4A receptors in primary cultured osteoblasts and in organ cultured calvariae. Similarly, Lammi et al. (248) showed that the expression of NR4A receptors was increased in MC3T3-E1 cells or mouse bone marrow mesenchymal stem cells treated with FGF-8b, resulting in increased proliferation of preosteoblasts.

Although the functions of NR4A receptors in osteoblasts have been demonstrated, the role of NR4A receptors in other types of bone cells is unclear. Similarly, while NR4A receptors are expressed in the bone marrow mesenchymal stem cells (242), their functions in chondrocytes and adipocytes differentiated from mesenchymal stem cells remain to be elucidated.

NR4A2 is essential for differentiation of dopaminergic neurons because NR4A2 knockout mice die soon after birth due to their failure to generate midbrain dopaminergic neurons (49, 50, 562). Moreover, mutations of NR4A2 may predispose to the development of Parkinson’s disease (255). Interestingly, Sato et al. (418) reported that Parkinson’s disease patients showed a high incidence of hip fractures (418). A decreased activity of NR4A2 during osteoblast differentiation might underlie a high incidence of bone fractures in the patients with Parkinson’s disease. However, because of neonatal lethality of conventional NR4A2 knockout mice, the physiological roles of NR4A2 in skeletal modeling during growth and bone remodeling after skeletal maturation has not been examined. Generation and analyses of bone specific NR4A2 conditional knockout mice would be essential to clarify this question.

**B. Estrogen Receptor-Related Receptors**

The estrogen receptor-related receptors (ERRs), a subgroup of the orphan nuclear receptors, include three subtypes: ERR<sub>α</sub>, ERR<sub>β</sub>, and ERR<sub>γ</sub> (493). These receptors are closely related to the ERs, sharing high homology in their DNA binding domains. However, they do not bind the classical ER ligands, and no natural ERR ligand has been identified so far (138, 164, 493). Additionally, ERRs activate transcription of the target genes through
binding to a specific ERRE (TNAAGGTCA), distinct from estrogen response element (ERE) DNA sequences (57, 87, 102).

ERRs are expressed in several tissues during embryonic development and in the adult organism (37). Consistent with their expression patterns, a number of physiological functions for ERRs have been suggested. For example, ERRβ appears to regulate placental formation (285), maintenance of pluripotency in embryonic stem cells (501), and the fate of certain epithelial cells of the inner ear (56). ERRα and -β are involved in several aspects of metabolic control with PPARγ and peroxisome proliferator-activated receptor gamma coactivator 1 (PGC-1) (139, 505). ERRα transcripts have been identified in the ossification zones of the long and short bones during mouse embryonic development (36). Similarly, ERRα is continuously expressed during ex vivo differentiation of rat osteoblast progenitors originating from calvaria or bone marrow (33, 35). Therefore, it is expected that ERRα play an important role in bone metabolism.

The functional importance of ERRs in bone was first investigated using ex vivo approaches with rat calvarial cells. ERRα knockdown by anti-sense oligonucleotides reduced the proliferation and differentiation of calvarial cells and decreased the number of mineralized colonies, reduced the proliferation and differentiation of calvarial cells (34). Therefore, it was suggested that ERRα function resulted in an increased number of adipocyte colonies (35). Additionally, when calvarial cells were induced to differentiate into adipocytes, the inhibition of ERRα function resulted in a higher number of adipocyte colonies (34). Therefore, it was suggested that ERRα could act as a switching factor, inducing differentiation of precursor cells into the osteoblastic pathway and repressing the adipocytic pathway.

However, recent in vivo studies using ERRα knockout (ERRα−/−) mice suggested the opposite conclusion against the results mentioned above. Two independently established lines of ERRα−/− mice showed increased trabecular BMD compared with those in wild-type mice (89, 486). In both cases, increased BMD appeared to be caused by enhanced osteoblast activities. Osteoblast surface and bone formation rates were increased in ERRα−/−, while the volume of marrow fat cells was reduced (89). Thus, in vivo, ERRα can be considered as a switching factor in mesenchymal cell fate decision, inducing commitment to the adipocytic lineage. Moreover, the function of ERRα in osteoclasts was suggested from the phenotype of aged ERRα−/− mice that displayed a significantly reduced number of osteoclasts and reduced bone resorption (519), although it remains elusive whether this phenotype was caused by the osteoclastic ERRα or the secondary effects from other cell types in bone.

In addition to ERRα, it was reported that ERRγ has an important role in regulation of bone metabolism. Jeong et al. (192) showed that ERRγ negatively affected BMP2-mediated osteoblast differentiation of MC3T3 and C2C12 cell lines. ERRγ interacted with Runx2, a master transcription factor for osteoblast differentiation, thereby preventing physical interaction between Runx2 and p300 coactivator and impairing transcriptional activity of Runx2. This idea was further confirmed by the BMP2-induced ectopic bone formation assay (192).

Thus there are divergent interpretations of the roles of ERRs in bone. This might result from differences between in vitro cell culture and in vivo animal models. Although the clinical significance of ERRs in human bone tissue remains to be uncovered, ERRs therapeutic targeting may offer a possibility of increasing bone formation by enhancing osteoblast differentiation and activity.

C. Retinoid-Related Orphan Receptors

Retinoid-related orphan receptors (RORs) constitute a subfamily that includes three members: RORα that is present in four splicing isoforms (RORα1, RORα2, RORα3, and RORα4), RORβ, and RORγ (109, 193, 420). Natural ligands for these receptors remain unknown. Each of these receptors appears to act as a constitutive transcriptional activator by binding to a RORE [(A/T)₆AGGTCA] as a monomer (140, 318).

RORα is expressed in various tissues, including the brain, skeletal muscles, kidney, testis, and hair follicles (45, 140, 193, 420). Among the four known splicing isoforms, the expression of RORα2 and RORα3 is limited to immune cells and testis, respectively (193, 382). Additionally, RORβ is brain specific and is highly expressed in the pineal gland. RORγ is expressed most highly in the thymus (137, 193).

RORs are also expressed in the osteoblastic lineage cells, suggesting their functions in the bone. Specifically, RORα and RORγ (but not RORβ) are expressed in mesenchymal stem cells derived from bone marrow (322). Furthermore, mRNA level of RORα is significantly increased during osteoblast differentiation of mesenchymal stem cells, implying its positive regulatory functions in osteoblasts.

The role of RORs in bone metabolism was analyzed in vitro experiments. Benderdour et al. (26) showed that RORα1 modulates the expression of osteoblast marker genes. Overexpressed RORα1 increased the expression of early- and late-stage osteoblastic differentiation marker genes (ALP, COL1A1, and OCN), and consistently, knockdown of RORα1 inhibited the expression of...
these genes. Additionally, RORα1 has the inhibitory effect for TNF-α-induced NF-κB activation (26, 88). NF-κB signaling is critical in bone resorption through stimulation of osteoclast differentiation (3, 194) and induction of inflammatory cytokines and matrix metalloproteinases (MMPs) that are involved in extracellular matrix degradation (80, 207, 281, 392). Therefore, it is suggested that RORα1 may play an important protective role in bone metabolism.

In vivo evidence for the function of RORα in bone metabolism was first obtained by studying the staggerer (sg) mutant mice, a mouse line that carries a deletion within the RORα gene (151). Homozygote sg mutants (sg/sg) have thin long bones compared with the heterozygote and wild-type animals (322). Also, the bones of these mice are osteopenic. RORα-deficient mice consistently exhibit abnormalities in bone formation and maintenance (287), suggesting that RORα functions as a positive regulator of the osteoblastogenesis.

D. Chick Ovalbumin Upstream Promoter-Transcription Factors

Chick ovalbumin upstream promoter-transcription factors (COUP-TFs) include two members: COUP-TFI and COUP-TFII (513). COUP-TFs exert their transcriptional activities by binding to an imperfect repeat element (AGGTC)A (362, 415, 494). COUP-TFs bind as monomers to its target promoters but also may bind as heterodimers with RXRs. Because RXRs also form a heterodimer with various nuclear receptors including VDR, TR, and RAR, it is anticipated that COUP-TFs can inhibit the ligand-dependent activities of VDR, TR, and RAR by competition for heterodimerization with RXRs (72, 230, 231, 375). Thus the ratio of COUP-TFs levels to RXR levels in a given cell may allow the balancing of diverse hormonal and growth signals (282).

Although there is some overlapping expression patterns of COUP-TFs during mouse embryogenesis, COUP-TFI is more highly expressed in nervous tissues, while COUP-TFII is preferentially expressed in mesenchymal stem cells in developing tissues and organs, such as stomach, salivary gland, pancreas primordium, lung, kidney, and prostate (370, 386). Expression pattern of COUP-TFI correlates with that of BMP-4, implying potential significance in bone metabolism. In E10.5 embryos, there is a distal-to-proximal direction gradient of BMP-4 expression, while COUP-TFI expression shows an opposite proximal-to-distal gradient (197, 282). In the limbs of E14.5 embryos, localization of BMP-4 expression overlaps with that of COUP-TFI expression in the apoptotic regions between the developing digits and in the mesenchymal cells of perichondrium (197, 282). Feng et al. (118) reported that COUP-TFI could modulate BMP-4 gene transcription in osteoblasts. They found a potential regulatory response region of COUP-TFI in the BMP-4 promoter and that COUP-TFI was specifically bound to this region DNA. Additionally, they showed that COUP-TFI inhibited BMP-4 promoter activity in fetal rat calvarial osteoblasts by overexpression of COUP-TFI. These results suggest that COUP-TFI controls the BMP expression level.

Targeted disruption of mouse COUP-TFI resulted in perinatal lethality owing to a defective nervous system development (387). Thus it is necessary to generate a conditional, bone-cell type specific COUP-TFI knockout mouse line to examine its functions in the bone metabolism.

E. Small Heterodimer Partner

Small heterodimer partner (SHP) is an atypical orphan nuclear receptor that has only a putative LBD and two LXXLL-like motifs (424). Because SHP does not possess a DNA-binding domain, it exerts its activity through interaction with various nuclear receptors and transcription factors including ERs, PPARs, RARs, LXRrs, c-Jun, Smads, and HDACs. Through these interactions, SHP negatively regulates transcriptional activities of these transcription factors, with exception of PPARs (54, 210, 262).

SHP participates in various cellular functions such as cell proliferation, differentiation, and metabolism depending on the cell types (161, 169, 360, 455). For example, using SHP null or transgenic mice, it was found that hepatic SHP has multiple regulatory roles in metabolic pathways including hepatic bile acids, lipids, and glucose homeostasis (54, 262).

In bone tissue, SHP has been shown to function as a positive regulator of osteoblastic bone formation (191). SHP expression is significantly increased during the osteoblastogenesis, and it is also induced by BMP-2, a strong positive regulator of bone formation. SHP physically interacts with Runx2 on the promoter region of osteocalcin gene. Overexpression of SHP increases the transcriptional activity of Runx2 via a competition for association with HDAC4 (191). These results indicate that SHP is involved in the regulation of osteoblastogenesis. Consistent with the in vitro results, trabecular BMDs of long bones were significantly decreased in SHP knockout mice with decreased osteoblast and osteoclast numbers (191). Since loss of SHP expression had no significant effect on the expression of osteoclast-specific marker genes, it was thought that osteoclast numbers were reduced by the indirect effects mediated by osteoblasts. Furthermore, SHP knockout mice showed considerably fewer chondrocytes than WT mice (191).
Natural ligands of SHP have not been identified. However, for therapeutic drug development, the efforts are underway to discover the compounds that may regulate activities of SHP (114, 189). For example, Farnaha et al. (115) reported that adamantyl-substituted retinoid-related compounds (CD437/AHPN and 3-Cl-AHPC/MM002) promote interaction between SHP and a corepressor complex (115). Jang et al. (188) showed that metformin that is already used as an anti-diabetic drug induces osteoblast differentiation via SHP-mediated activation of Runx2 (188). Therefore, therapies based on the manipulation of SHP activity might offer new possibilities for treating bone diseases.

VIII. CONCLUSION AND FUTURE PERSPECTIVES

NRs mediate systemic action of lipophilic hormones and play a crucial role in the bone physiology and pathophysiology. Recent studies delineated many functions of NRs in the bone development and maintenance, and uncov-
ered molecular mechanisms of NR specific actions in bone cells, still leaving many questions to be further addressed. Particularly, clinical applications of NR synthetic ligands for treatment of skeletal disorders remain largely at initial stages and require comprehensive understanding of bone cell-type specific function of NRs as well as NR coregulators.

A. Spatiotemporal Functions of NRs in Bone Tissue

The cellular functions of osteoblasts and osteoclasts have been well documented, leading us to understand how these cells support bone growth and remodeling (32, 152, 208, 391, 475), whereas the role of other bone cells remained not as clear. Recently, using a variety of modern experimental approaches, unexpected roles of osteocytes as endocrine cells and conductor cells for bone remodeling have been uncovered (217, 339, 556). Although the significance of osteocytes in bone maintenance and serum mineral control has become evident, little information of NR function in osteocytes is still available. Nevertheless, detectable levels of the expression of certain NRs in these cells indicate that NRs are likely to play an important regulatory role in osteocytes, and possible actions of vitamin D and sex hormones in osteocytes have already been suggested in some recent studies (217, 339, 556). In this respect, characterization of effects of osteocytic VDR, ERs, and AR in the bone by a cell type-specific conditional gene disruption in osteocytes would verify such an idea.

Osteocytes represent the terminal stage of osteoblastic cell lineage differentiation. It appears that the roles of osteoblasts in bone remodeling vary at different stages of their differentiation (31, 208, 236, 340, 549). This suggests that functions of NRs in the osteoblastic lineage cells may change during the cell differentiation. Although

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**FIGURE 6.** Ligand-specific conformational alterations of nuclear receptor protein define ligand type-specific coregulator recruitment and subsequent physiological effects. Ligand binding triggers a shift in the positioning of the most C-CDH-terminal α-helix 12 (top panel). Certain synthetic ligands may induce NR protein conformation different from that of a natural ligand-activated NR, resulting in the coregulator switching (middle panel). As a result of different complex association, a different pattern of NR target gene expression is induced, potentially exerting tissue-specific and desirable therapeutic effects (bottom panel).
osteoblast-specific KO of NR genes has successfully illustrated the involvement of NRs in regulation of bone physiology (60, 349), we cannot exclude a possibility that NRs may exert different regulatory action at different stages of the osteoblastic differentiation (FIGURE 1, 5). Similarly, cytodifferentiation stage-specific KO of NRs (305, 339) in osteoclasts (FIGURE 5) would be of a great interest, since the osteoclastic differentiation includes several distinct stages leading to formation of mature multi-nucleated bone-resorptive cells.

B. Synthetic NR Ligands for Treatment of Skeletal Disorders

A group of synthetic ER ligands, named as SERMs, have been successfully used to ameliorate osteoporotic bone loss or aberrantly enhanced bone resorption and without increased incidence of estrogen-dependent tumor development in female reproductive organs (343, 344, 408). Thus SERMs represent NR synthetic ligands with beneficial properties for treatment of skeletal disorders. Owing to its anabolic actions in bone and muscle tissues, androgens are expected to be a promising lead hormone for elderly male patients suffering from age-related bone loss and sarcopenia (43, 123). Synthetic AR ligands are now under intensive development to generate SARMs that would be effective in skeletal organs, but without side effects, such as development of prostate cancer. Vitamin D and its synthetic analog ED-71 are approved and widely used in Japan to potentiate bone strength and prevent bone fractures, presumably by inducing mineral accumulation in bones (185, 309, 313). As beneficial effects of vitamin D are still debated by North American and European practitioners (409), vitamin D supplements are shown to be beneficial for bone health in Japanese population under conditions of deficient dietary calcium intake. In contrast, synthetic glucocorticoids are drawing clinical concerns when chronically administered as anti-inflammatory agents, since GR ligands are causative for bone loss, known as secondary osteoporosis (10). Therefore, significant efforts have been made to develop novel anti-inflammatory GR ligands lacking negative side effects on bone mass and mineral density, and some promising lead compounds are currently undergoing clinical trials.

Therapeutically, NRs are considered as important therapeutic targets for treatment of various skeletal diseases. These might also include joint disorders, even though NR involvement at least in some of them still remains to be addressed. To accelerate NR-targeted drug development, a variety of modern approaches have been applied, such as structural analysis of liganded NR proteins and pharmacological tests using genetically engineered animals. In this respect, pharmacological lessons from SERMs are particularly instructive, since protein conformation of the raloxifene-bound ERα was shown to be markedly different from that of the estrogen-bound ERα (428), and resulted in association with different sets of transcriptional coregulators (446). These observations were further supported by DNA microarray studies which showed that estrogen- and raloxifene-induced gene expression patterns were not identical. Thus these finding have formulated a concept that some synthetic ligands may induce NR protein conformation different from that of a natural ligand-activated NR to trigger atypical co- regulator complex association and, consequently, different pattern of NR target gene expression, thereby exerting tissue-specific and desirable therapeutic effects (61, 62, 84). Since ligand-induced interactions of coregulators with NR reflect ligand-specific conformational alteration of NR protein structure (FIGURE 6), identification of coregulator complexes bound by a synthetic compound-activated NR may provide a promising strategy for design and screening of synthetic NR ligands with desirable tissue specificity and/or pharmacological effects (278, 290). This approach may also link specific coregulator-NR interactions to particular desirable therapeutic outcomes and, thus, identify transcriptional coregulators as novel pharmaceutical targets in treatment of skeletal disorders.

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NUCLEAR RECEPTORS IN BONE PHYSIOLOGY AND DISEASES


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